# INVESTIGATIONS INTO THE PROTEOLYTIC SUSCEPTIBILITY OF NON-ENZYMICALLY GLYCATED CONNECTIVE TISSUE COMPONENTS OF PERIODONTAL AND ENDODONTIC IMPORTANCE


By

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# Abstract: Investigations into the proteolytic susceptibility of non-enzymically glycated connective tissue components of periodontal and endodontic importance.

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**Purpose of the Study:** Non-enzymic glycation of proteins is a ubiquitous pathophysiological consequence of diabetes and is also known to increase with age, its affects are mainly seen on longstanding circulatory and tissue proteins such as collagen. However, the effects of glycation on the degradation of collagen, a feature of tissue remodelling, infection and invasion, has surprisingly received little research attention. The present investigation attempts to remedy this via the development of an *in vitro* model to produce glycated collagen and examine its susceptibility to proteolytic breakdown by host and bacterial enzymes.

**Methods:** Native type I skin and tendon collagen, and porcine skin gelatin, were glycated *in vitro* via exposure to glucose. The effectiveness and extent of the glycation process were measured by assaying for hydroxymethylfurfural (HMF). Type I soluble and insoluble collagens were found to be modified by up to 125 and 30 nmol HMF per mg protein respectively; gelatin could be glycated to varying degrees in a dose-dependent manner by exposure to a range of glucose concentrations, resulting in maximal glycation up to around 60 nmol HMF per mg protein. The glycated proteins were tested for susceptibility towards mammalian trypsin, an extracellular protease fraction of *Enterococcus faecalis* OG1RF, and *Clostridium histolyticum* collagenase.

**Summary of Results:** Gelatin digestion by trypsin was reduced with increased levels of glycation. In contrast, digestion of glycated type I soluble and insoluble collagens by the secreted *E. faecalis* protease fraction was increased compared to the non-glycated controls.

**Conclusions:** The results indicate that the susceptibility of the glycated native collagen to bacterial proteases is increased and that this may render tropocollagen molecules less functional. In addition, glycated collagen peptides in the form of gelatin are rendered less susceptible to degradation by the host enzyme. A hypothesis to explain these effects of glycation on the susceptibility of gelatin and collagen is discussed. These findings may help explain the effects that non-enzymic glycation of collagen has during endodontic infection, connective tissue remodelling and repair.

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# Abbreviations

| | |
|---|---|
| AGEs | Advanced Glycation Endproducts |
| BCA | Bicinchoninic acid |
| BCG | Bromocresol green |
| BHI | Brain heart infusion |
| ECM | Extracellular matrix |
| HbA1c | Glycated haemoglobin |
| HMF | Hydroxymethylfurfural |
| NF-kβ | Nuclear factor kappa beta |
| OD | optical density |
| RAGEs | Receptors for AGEs |
| TBA | Thiobarbituric |
| UV | Ultra violet |

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# CHAPTER 1: INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder characterised by hyperglycaemia. The global prevalence of diabetes is estimated to be around 8.5 % of the adult population, and is on the rise (Cho et al., 2017). Diabetes is strongly associated with certain medical and dental complications for example neuropathy, nephropathy, retinopathy, and periodontal disease, for which the putative pathogenic mechanisms are continually being researched. One of these mechanisms is related to the formation of Advanced Glycation Endproducts (AGEs). These AGEs arise through non-enzymic glycation as a result of chronic hyperglycaemia. AGEs may act upon certain receptors for AGEs (RAGEs) inducing and exacerbating inflammation, furthermore the (non-enzymatic) covalent attachment of glucose to proteins may lead to changes in the molecular configuration and consequently affecting the structural and functional role of proteins. However, little attention has been given to the potential effects of glycation of extracellular matrix (ECM) proteins of oral tissues, particularly in periodontal and endodontic infections, and their impact on the disease and severity inflammation.

Collagen, mainly type I, is an important structural protein of the ECM of dental and periodontal tissues including the pulp, dentine, cementum, periodontal ligament and alveolar bone. Collagen, as a longstanding protein, has the potential to become glycated in states of hyperglycaemia. Microorganisms invading endodontic and periodontal tissues adhere to collagen initiating the formation of biofilms, and may degrade collagen which serves to both provide nutrients and aid invasion. The breakdown of collagen is also an

important process during remodelling and tissue repair in wound healing. Therefore glycation of collagen in states of long standing hyperglycaemia, for example in diabetic patients may, lead to altered structural and functional roles of collagen. Consequently this may have implications on disease severity and progression, wound healing and repair in endodontic and periodontal tissues.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Diabetes mellitus

Diabetes is a chronic metabolic disorder which is characterised by hyperglycaemia, as a consequence of defects in insulin secretion, action, or both. Type 2 diabetes encompasses patients who have insulin resistance, and relative rather than absolute deficiency of insulin. Whilst type 1 diabetes arises due to insulin deficiency. The pathological hallmarks of diabetes arise as a consequence of chronic hyperglycaemia. These include retinopathy, neuropathy, nephropathy and cardiovascular disease (Chawla et al., 2016). Furthermore diabetes has been strongly linked to a variety of oral complications including periodontal disease, poor wound healing and increased risk of infection.

According to Diabetes UK State Of The Nation (England) Report 2016 there are over three million people suffering from type 1 and 2 diabetes in the UK, furthermore there are still many patients living with diabetes that are yet to be diagnosed (Askew, 2016).

The severity of symptoms and complications of diabetes correlate with the degree of hyperglycaemia, which maybe more elevated in undiagnosed and poorly controlled conditions of diabetes. As well as improving awareness of symptoms, assistance in seeking help, improvement in diagnosis and management, it is important to study the putative mechanisms that link diabetes to its complications. This will not only help inform patients of the possible complications but also aid in the development of strategies to help manage diabetes and its complications.

## 2.2 Endodontic Infections

Endodontic infections are is initiated by bacteria resulting in inflammation, infection and necrosis of the dental pulp and subsequently inflammation of the periradicular tissues. The severity of infection is mediated by the interaction between the invading microorganisms and the host response, examples of the symptoms include; pain, swelling, tooth mobility. Research of the literature demonstrates that most of the evidence on the incidence of endodontic infection within Europe is based on a small subpopulation in multiple countries, more importantly it is a very common disease affecting millions of people worldwide.

Microorganisms are essential for the development and progression of endodontic infections (Kakehashi et al., 1965). The management is root canal treatment, the rationale being is to prevent the development of or cure existing periapical infection by eradicating microorganisms and preventing their entry into the root canal system and periapical tissues. Alternatively the tooth can be extracted. Root canal treatment has gained popularity because of increased and predictable success rates (Ng et al., 2011b). However, there are multiple factors which have an impact on the prognosis of the involved tooth. This include; the presence and size of a radiographic lesion indicating presence and severity of a periapical infection, the technical quality of the root canal treatment including aseptic techniques, and also the presence of diabetes mellitus has been shown to reduced survival rate (Ng et al., 2011a). This is possibly due to implications on the host's ability to heal and repair (Segura-Egea et al., 2015). Furthermore there are challenges associated with access of instruments and antimicrobial solutions to the complex anatomy of the root canal system. Additionally, some microorganisms are resistant to antimicrobial treatment and can survive in the root canal after biomechanical preparation, for example *Enterococcus faecalis,* which has consistently been recovered from root canals undergoing retreatment

due to failed endodontic treatment with persistent infection (Love, 2001, Gopikrishna et al., 2006).

Endodontic infections are polymicrobial in nature, the main microorganisms consistently recovered from infected root canals are bacteria. These include for example black pigmented gram-negative anaerobic rods (*Prevotella spp., Porphyromonas gingivalis, Porphyromonas endodontalis), Tannerella forsythia, Fusobacterium spp., Treponema spp*., gram-positive anaerobic rods (*Actinomyces spp.*), gram-positive cocci (*Streptococcus spp., Enterococcus faecalis*) and fungi (*Candida albicans*) (Narayanan and Vaishnavi, 2010). These microorganisms are usually contained within the root canal system due to the defensive barriers of the host, however in circumstances where microorganisms overcome this neutrophil barrier, they progress towards and through the apex into the periapical tissues, thereby establishing an extra-radicular infection which is mainly dominated by anaerobic bacterial species which include for example *Actinomyces spp., Porphyromonas gingivalis, Porphyromonas endodontalis, Prevotella spp. and Fusobacterium nucleatum* (Narayanan and Vaishnavi, 2010).

## 2.3 Diabetes and Endodontic Infection

### 2.3.1 Diabetes and periodontal infection

The relationship between diabetes and oral health has been extensively studied, particularly with regards to periodontal disease. This relationship appears to be bidirectional in that poor glycaemic control exacerbates the severity of periodontal disease, and reciprocally poorly controlled periodontal disease is associated with compromised glycaemic control (Casanova et al., 2014). The management of periodontal condition has also been shown to correlate with reduction in glycated haemoglobin level (termed HbA1c) (Casanova et al., 2014). Non-enzymically glycated haemoglobin $HBA_{1c}$ is used as a clinical indicator for diagnosing diabetes and for monitoring glycaemic control.

Albumin, the major plasma protein is also vulnerable to glycation. Elevated levels of glycated albumin have been detected in the gingival crevicular fluid of diabetics, and this correlates with the severity of periodontitis (Kajiura et al., 2014). Although epidemiologic evidence exists, there is scarcity of the literature regarding the pathogenesis and healing of endodontic infections in diabetics. Both periodontal disease and endodontic infections are chronic oral inflammatory conditions, caused predominantly by gram negative anaerobes, and are associated with elevated levels of systemic cytokines, and much of the research has focused on this potential pathophysiological relationship with regards to association with diabetes (Segura-Egea et al., 2015).

### 2.3.2 Diabetes and endodontic infection

Cross-sectional epidemiological studies have demonstrated an association between diabetes and increased prevalence of radiographic lesion of apical periodontitis (Falk et al., 1989, Marotta et al., 2012). Furthermore, a systematic review of seven epidemiological studies has concluded that the available scientific literature indicates a significant association between; the presence of endodontically treated teeth, and the presence of apical lesions associated with endodontically treated teeth with diabetes (Segura-Egea et al., 2016). Additionally, evidence of increased incidence periodontal disease of teeth with endodontic involvement in diabetics has been reported, as well as increased risk of pain and endodontic flare-ups (Fouad and Burleson, 2003). A prospective clinical study demonstrated that teeth in patients suffering from diabetes had a higher chance of being extracted after endodontic treatment (Ng et al., 2011b). This negative influence of diabetes on the survival rate of endodontically treated teeth was consistent with previous findings (Mindiola et al., 2006), who showed that tooth survival in diabetic patients was 89.7%, compared to 96.1% of non-diabetics, which was a small but statistically significant difference. This suggests that diabetes could be a disease modifier.

Although some evidence exists, the literature is limited regarding the association between diabetes and the severity of inflammation of endodontic infections. However such an association has been repeatedly shown in animal experiments. For example in streptozotocin induced diabetic rats there was an increased risk of alveolar bone resorption and consequently increased lesion size of periapical tissues (Kohsaka et al., 1996, Fouad et al., 2002, Iwama et al., 2003), more severe pulpal inflammation (Kohsaka et al., 1996, Garber et al., 2009), furthermore compromised dentine bridge formation following pulp capping (Garber et al., 2009), compared to non-diabetic controls. Based on

these findings, it would appear that hyperglycemia may adversely affect pulpal healing in rats.

## 2.4 Pathophysiological Features of Diabetes Mellitus

The putative links between diabetes and endodontic disease are related to three main pathophysiological features; these are impaired immunity, hyperglycaemia and production of irreversible AGEs, Figure 2.1.

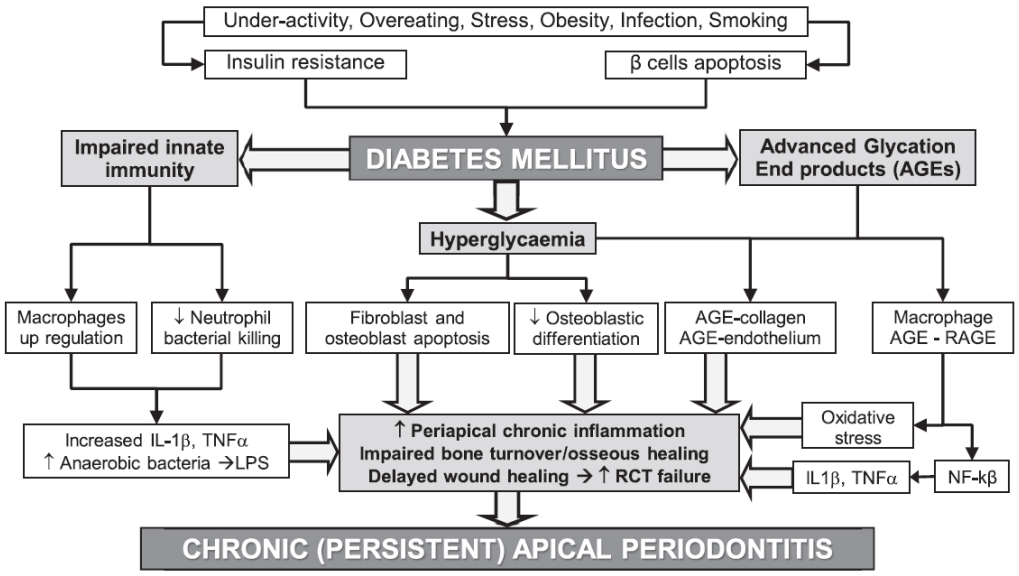


**Figure 2. 1: The three main biological mechanisms by which diabetes may influence periapical status; impaired immunity (left), hyperglycaemia (middle) and the formation of AGEs (Right).** Firstly, the function of the innate immunity is altered as neutrophil phagocytosis is decreased, and macrophages are up-regulated, with increased production of pro-inflammatory cytokines. Secondly, hyperglycaemia induces osteoblast apoptosis, inhibits their differentiation and proliferation leading to impairment in collagen formation. Thirdly, AGEs bind to collagen leading to alteration in bone metabolism, reducing bone formation and osteoblastic differentiation. AGEs also bind to RAGEs on macrophages activating nuclear factor kappa beta (KF-Kβ), thereby increasing cellular oxidative stress and up regulating pro-inflammatory cytokines. Therefore diabetes predisposes to chronic inflammation, diminishes tissue repair capacity, and may increase risk of infection and delayed of wound healing (Segura-Egea et al., 2015).

In diabetes, the function of the innate immune response which is the first line of defence against pathogens is altered. This includes decrease in neutrophil phagocytosis with increased production of pro-inflammatory cytokines (Lima et al., 2013). Furthermore, hyperglycaemia can also inhibit macrophage function, leading to an inflammatory state that impairs the host cellular proliferation with subsequent impact on wound healing of both the dental pulp and periapical tissues as demonstrated in experimentally induced diabetes in rats (Garber et al., 2009). Secondly, hyperglycaemia has been reported to impair collateral circulation leading to structural alterations in the dental pulp and periapical tissues (Bender and Bender, 2003). Thirdly, the formation of AGEs, which bind to RAGEs on macrophages leads to the activation of NF-kβ with subsequent increase in cellular oxidative stress and up-regulation of pro-inflammatory cytokines (Cai et al., 2012). A positive linear correlation has been found between the expression of RAGE and NF-kβ in periradicular tissues obtained from 38 diabetic patients (Crabtree et al., 2008). Immuno-histochemical examination of surgically removed human periapical granulomas has demonstrated a marked expression of RAGE, and AGE by endothelial cells, suggesting the binding of AGEs to RAGEs may potentially trigger cellular activation and mediate tissue injury and destruction during the process of periapical granuloma formation (Takeichi et al., 2011). Furthermore, the binding of AGEs to collagen has been suggested to alter bone metabolism, osteoblastic cell proliferation and differentiation and subsequent bone formation (Tanaka et al., 2013). A study demonstrated that treating cultured periodontal ligament fibroblasts with AGE-modified bovine serum albumin resulted in apoptosis, suggesting a possible involvement of AGEs and RAGEs in inducing apoptosis and their participation in and exacerbation of periodontal destruction, with possible implications for impaired repair after root canal treatment (Li et al., 2014). AGEs have also been detected in the pulp of human teeth and their level is significantly associated with plasma AGE (Reece, 2015a).

The above observations suggest that diabetes predisposes to chronic inflammation with greater susceptibility to infections, compromised tissue repair and immune response, aggravating periapical inflammation, impairing bone turnover and subsequently delaying wound healing. These effects may account for the observed increased prevalence of endodontic infections in diabetic patients.

The three main potential pathophysiological links of diabetes to endodontic infection have been discussed. One of those includes the effects of AGEs on the host cellular response. However, according to our knowledge at the time of writing, the effects of glycation on the structural and functional roles of collagen, the main component of the dental and periodontal ECM, has not been looked into as potential pathological association between diabetes and endodontic infection. The next section will discuss the glycation process and its presence in tissues, the molecular structure and importance of collagen in dental tissues, and the potential impact of glycation on collagen.

## 2.5 Glycation of Proteins and the Formation of Advanced Glycation Endproducts

### 2.5.1 Glycation - the Maillard reaction

The Maillard reaction is a non-enzymatic glycation reaction between the carbonyl group of a reducing sugar and proteins, preferentially lysine or arginine (Hadley et al., 1998, Paul and Bailey, 1996). This multi-step chemical reaction leads to a series of intermediate compounds, for example a Schiff's base and Amadori products, and finally resulting in the formation of AGEs in which the molecular configuration of the proteins maybe altered with subsequent impact on their structural and functional properties, (Figures 2.2 and 2.3).

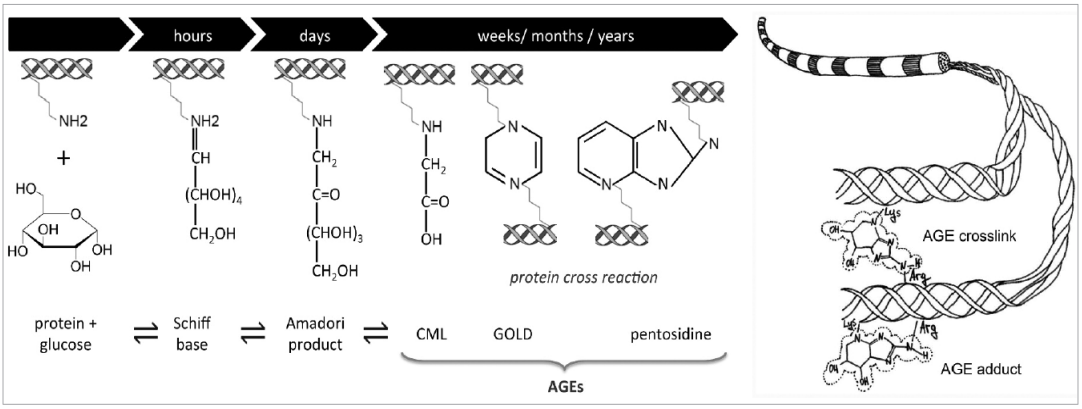


**Figure 2. 2: Series of reactions during the glycation process leading to the formation of AGEs.** The glycation reaction initially begins with the formation of a reversible Schiff base between a carbohydrate, typically glucose and a protein amino group for example a collagen lysine side-chain. The unstable Schiff base becomes a stable intermediate keto amine also known as an Amadori product. Following this, a series of complex reactions over the course of months or years take place, leading to the formation of irreversible AGEs. Taken from (Snedeker and Gautieri, 2014b).

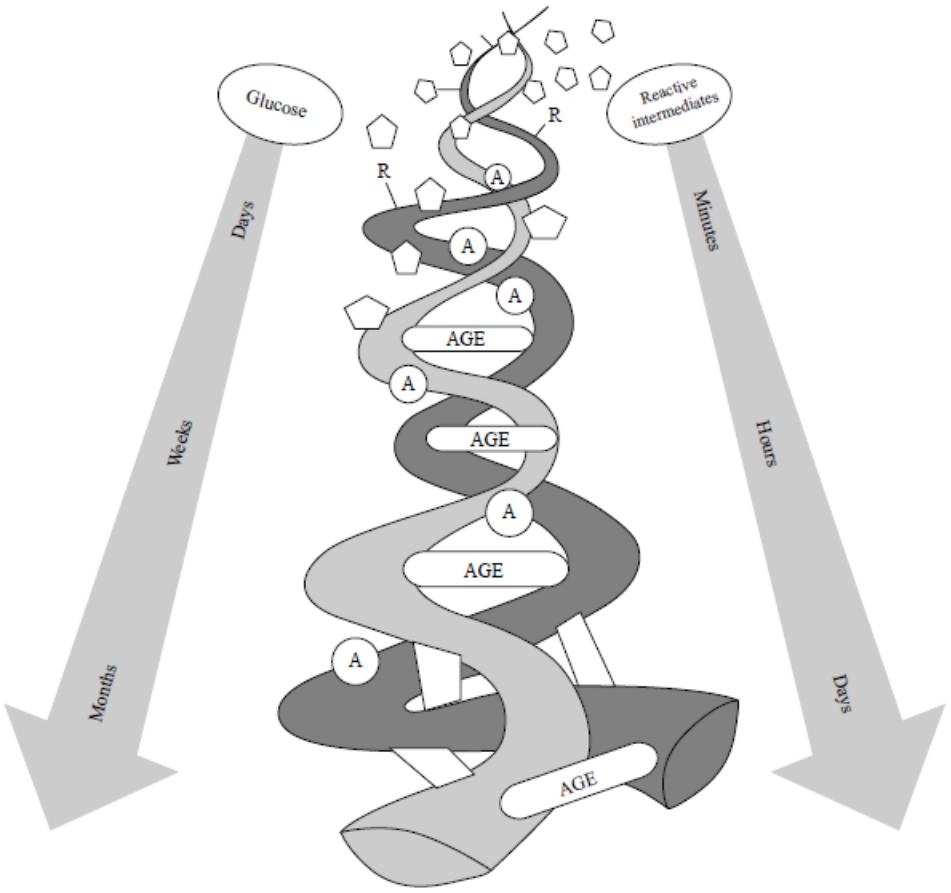


**Figure 2. 3: Schematic diagram illustrating the course of AGE formation on a hypothetical fibrillary protein.** Open chains of sugar molecules or glycolytic intermediates (pentagonal shapes) react with amino groups to form Schiff bases and Amadori products, which eventually form AGEs. This process may take several weeks during which the formation of irreversible cross-links between protein fibrils can have a serious influence on protein structure and function. Taken from (Stitt, 2001a).

The Maillard reaction was first described in 1912 at the University of Nancy by the French scientist Luis Camille Maillard, who was working on heated reactions between glucose and amino acids (Maillard, 1912). However, it did not draw immediate attention from researchers until 50 years later, when its significance was first recognised by food scientists, since it was observed that exposure of foods containing carbohydrates and proteins to heat, resulted in colour changes with enhanced aroma and flavour (Van Boekel, 2006). For that reason, glycation is a process that is highly desirable for the food industry. Although limited in relation to endodontics, in more recent years the pathophysiological

significance of this ubiquitous reaction has emerged as a field of study in medicine in its own right.

### 2.5.2 Conditions that promote the Maillard reaction

It is well established that non-enzymatic glycation is an age related process, however it is accelerated in diabetes and particularly in poorly controlled or undiagnosed patients where long standing elevated glucose levels in plasma is a common feature. Glycation of proteins is well documented in diabetics. For example, the use of $HbA_{1c}$ as a diagnostic test was introduced in the mid-1980s and since then has become a cornerstone of clinical diagnostic practice where it reflects the average plasma glucose levels over the previous 8 to 12 week period (Nathan et al., 2007).

AGEs may also be acquired through food, and introduced into the circulation with nutrients that have been processed by certain methods including for example dry heat (Koschinsky et al., 1997). Similarly, animal studies have demonstrated the increased accumulation of AGEs in multiple organs of mice exposed to dietary AGEs (Tessier et al., 2016). Not surprisingly reactive glycation products “glycotoxins” have also been detected in aqueous extracts of tobacco and tobacco smoke since the curing of tobacco is essentially a Maillard reaction. The combustion of these adducts during smoking releases these glycotoxins which may enter the blood stream and react with proteins to form AGEs (Cerami et al., 1997).

From a chemical perspective, it is well established that non-enzymic glycation reaction takes place in acidic conditions. The optimal pH approaching 5 (although this drops at both at lower and higher pH). This is because the first chemical step requires the protonation of the C=O group. This is then then attacked by the nucleophilic amine group (usually of the lysine side chain) to form the Schiff’s base. However, from a biological

perspective it is unlikely that optimal glycation occurs in tissues where such low pH conditions may never be encountered. Nevertheless, it is known that the pH of inflamed tissues can dip to between 6 and 7, (Schneider et al., 2007), and this would be relevant in terms of both chronic periodontal and endodontic infections. For this reason experiments carried out to produce glycated gelatin and collagen employed slightly acid conditions (pH 6.8).

### 2.5.3 Tissues affected by glycation

The literature demonstrates a remarkable increase in scientific research on the potential impact of AGEs as a putative pathophysiological mechanism for both age and diabetes related complications. For example the detection of increased AGEs in the lens and the cornea of patients with retinopathy and cataracts (Stitt, 2001b). In an animal study, an association has been reported between increased consumption of AGEs through dietary fat and increased incidence of chronic kidney disease (Ejtahed et al., 2016).

### 2.5.4 Therapeutic measures targeting glycation

Current research into the application of therapeutic measures in preventing or limiting the extent of diseases associated with AGEs are investigating the effects of preventing glucose uptake though Anti-AGEs. Examples of this include competitive inhibitors which prevents sugars covalently attaching to susceptible molecules like albumin, alternatively the use of de-glycating enzymes which involves the breakdown of such covalent bonds after they have formed between sugars and molecules (Cai et al., 2002). These have shown some promising results in pre-clinical models, however except for the diagnosis of diabetes through assessment of $HbA_{1c}$, most of this research is at an early stage and is it yet to inform any clinical decision making.

# 2.6 Collagen

## 2.6.1 Introduction

Collagen, because it is a long-lived protein within the connective tissues, is a prime target for non-enzymic glycation. Since this project will investigate the effects of glycation, it is appropriate to discuss in some detail the biochemical structure and function of collagen, furthermore the potential impact of glycation on collagen. The term collagen comes from the Greek words "κολλα", meaning "glue", and which originates from the use of animal skin as a source of glue (Chamberlain et al., 2011). Collagen is a protein found in every multicellular organism within the animal kingdom. It is the most abundant protein in mammals, constituting over a quarter of the total amount of protein present, therefore both quantitatively and functionally collagen is the most important protein (Kavitha and Thampan, 2008). It is found in diverse tissues for example tendons and ligaments, bone, skin, basement membranes, cartilage and blood vessel walls (Canty and Kadler, 2005). In the ECM it provides strength, fulfilling connective, structural, force transmission and other specialist roles throughout the body, including cell migration, angiogenesis and tissue repair (Kadler et al., 2007).

## 2.6.2 Molecular organisation of collagen

The tropocollagen molecule is made up of three polypeptide chains, each chain comprises just over a thousand amino acids (UniProt, 2012). The primary structure of each triple helical region of each of these polypeptides consists of a repeating triplet amino acid sequences Gly-X-Y, in which glycine occupies every third position in the polypeptide chain, and X and Y are amino acids other than glycine (Snedeker and Gautieri, 2014a, Gelse et al., 2003). Proline occupies approximately 28 % of X positions, and hydroxyproline approximately 38% of Y positions (Ramshaw et al., 1998). The polypeptide is also abundant

in lysine. Gly-Pro-Hyp is the most common triplet in the chain, having a relative frequency of 10.5 % in the α-chain. This sequence determines the spatial configuration and stability of the tropocollagen molecule.

Each alpha chain is coiled in a left-handed sense forming a helix, each complete turn being 0.9 nm in length or slightly over three amino acids since the length of each amino acid is 0.29 nm. However the extreme ends that flank the polypeptide chain do not have a Gly-X-Y sequence and are therefore are non-helical. These are termed telopeptides. The $NH_2$ terminus contains 10-16 and the COOH terminus contains 25-27 amino acids that have non-helical conformation. The three minor polypeptide helices are twisted about each other in a right-handed sense with each complete turn approximately 9 nm (30 amino acids) in length, giving rise to the triple-helical tropocollagen molecule that is approximately 300 nm long and 1.5 nm wide (Miller and Wray, 1971, Shoulders and Raines, 2009), that has non-helical ends which constitute approximately 3 % of the molecule's amino acid content (UniProt, 2012), Figure 2.4.

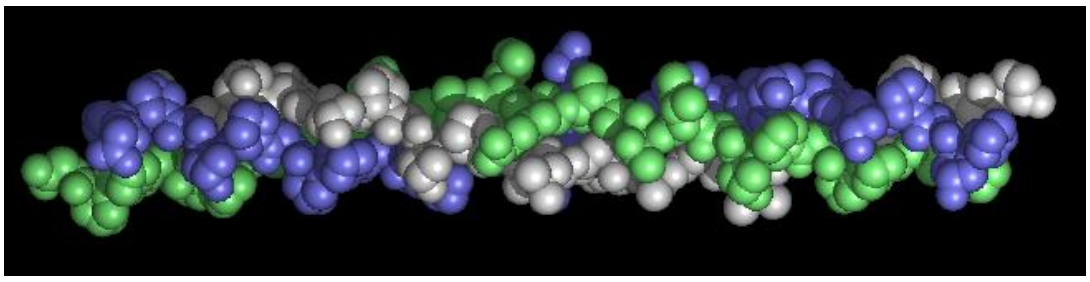

**Figure 2. 4: A space filling representation of a tropocollagen molecule. Three polypeptide chains arranged in a triple helical configuration, taken from (Zhuang and Khan, 2011).**

Hydrogen, the side-chain of glycine, which is the smallest of all amino acids, projects towards the inner aspect of the major triple helix, Figure 2.5, therefore not sterically disrupting the close association that exists between the three polypeptide chains in forming the collagen molecule (van der Rest and Garrone, 1991, Myllyharju and Kivirikko, 2004). This is the significance of glycine being every third amino acid in the sequence. However other amino acids with bulky side chains for example those with ring structures of proline and hydroxyproline projects outwards and so will not sterically impair the formation of the triple helical structure. Therefore, occurrence of large amounts of glycine, proline and hydroxyproline in the chain is the most important feature in determining this spatial conformation referred to as the "coiled coil". The chains assembled into this coil are closely packed together, leaving very little room in the interior of the collagen molecule. The triple helical structure of the molecule is stabilised by transverse inter-chain hydrogen bonds between sharing the hydrogen atom of the NH groups of glycines in one minor helix with the oxygen atom of the CO groups on the other two strands, as well as Van der Waal's interactions between amino acid residues on adjacent strands (Shoulders and Raines, 2009).

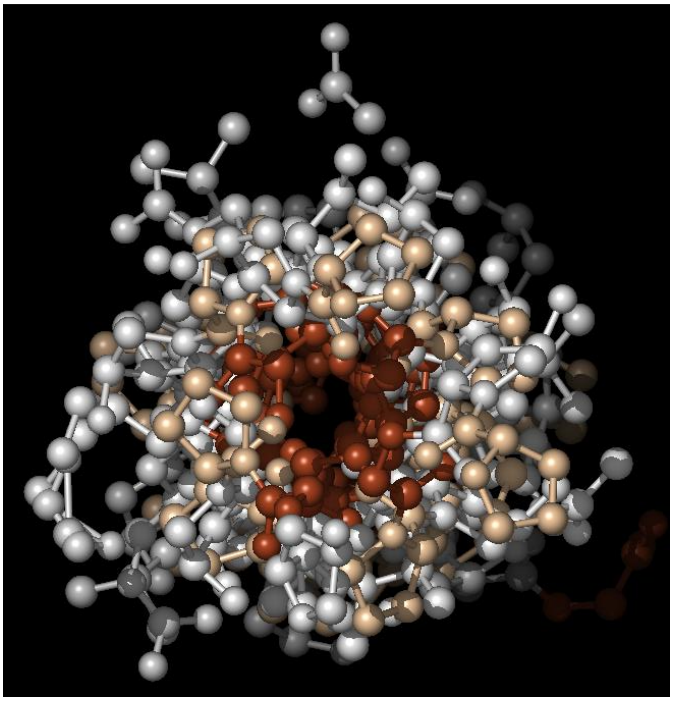

**Figure 2. 5: A space filling representation of the tropocollagen molecule end-on.** Glycines (brown) project towards the inner aspect of the triple helix, proline (wheat) and hydroxyproline (grey/white), project outwards. Taken from (Zhuang and Khan, 2011).

Collagen molecules interact with each other through projections of amino acid side chains from the central axis of the triple helix, and cross-liking of the non-helical ends, forming a continuum of elongated molecules resulting in a fibrillar structure that is analogous to a rope of with enormous tensile strength, with potential for flexibility and elasticity. Following the assembly of the molecules into fibrils, cross-linking takes place, and this serves to further stabilise the structure, increase fibrillar strength and prevents excessive slippage between collagen molecules (Avery and Bailey, 2005, Sasaki and Odajima, 1996). This chemical cross-linking is an important phase of maturation. It is first initiated by the conversion of a telopeptide lysine and hydroxylysine to allysine and hydroxyallysine by lysyl oxidase, through oxidative deamination of the amino group creating amino acids containing reactive aldehyde groups (Myllyla et al., 2007, Bailey et al., 1998). These react with $NH_2$ groups of hydroxylysine in the helical portion of the adjacent molecule forming aldimine cross-linking covalent bonds which tie the ends of the collagen

molecules together (Bailey et al., 1998, Bailey, 2001). In newly formed collagen, immature cross-links predominate, with maturation these divalent cross-links spontaneously react further with species on other molecules, to give more stable trivalent cross-links (Willett et al., 2007). The types of cross-links formed are also dependent on the type of tissue. Furthermore the interaction of collagen with other extracellular matrix proteins for example elastin, and water binding proteoglycans which then results in the formation of a highly sophisticated architectural arrangement.

This background information allows us to understand the biological properties written into these molecules that lead to the close packing of the polypeptide chains and strong inter and intra molecular interaction, which gives native collagen the resilience and high resistance to attack by most proteases (unless denatured into gelatine).

### 2.6.3 Types of collagen

There are now known to be 28 different types of collagen, which have been reviewed comprehensively (Gordon and Hahn, 2010). Of these, types I, II and III collagens make up 80 – 90% of the collagen in the body (Kavitha and Thampan, 2008). They are distinguished by the length and continuity of their triple helical domains, the ratios of hydroxylated to non-hydroxylated residues, and the degree of hydroxylysine glycosylation. The different collagen types are associated with particular tissues and this heterogeneity may reflect the different physiological function of each type.

Quantitatively, the most prevalent form of collagen in the body is type I, which was the only type recognised until 1969, and is found virtually in all connective tissues including; tendon, bone, skin (Canty and Kadler, 2005) and cornea (Meek and Fullwood, 2001). The type I collagen molecule is known as a heterotrimer, assembled from two

different polypeptide chains, with molecular composition (α1)2 (α2), in which two of the alpha chains are identical, and the third is distinct.

### 2.6.4 The role of collagen within dental tissues

Dentine is made up of a large number of small tubules in a mineralised organic matrix (30 % by volume). Approximately 90% of the organic matrix of dentine and predentine are type I collagen fibrils, with traces of type III and V (Berkovitz et al., 2002 -a). This organic matrix and the tubular structure of dentine provides it with greater compressive, tensile and flexural strength than enamel. Flexural strength provides a degree of deformability to the overlying enamel reducing the risk of fracture under masticatory loads. Unlike in most soft tissues, the collagen in dentine being similar to that of bone, is relatively insoluble in acid or in neutral solutions (Gage et al., 1989b).

The dental pulp is a highly specialised connective tissue which is responsible for the formation and eruption of the tooth. Following completion of eruption, odontoblasts within the dental pulp continue the formation of secondary and tertiary dentine throughout life. The plexus of Raschkow, a highly innervated area is responsible for sensation of noxious stimuli. Collagen is the major component of the dental pulp and constitutes by dry weight of approximately 34 % of its total protein content (Gage et al., 1989c). On a wet weight basis, the content is approximately 3-4%. Collagen is predominantly of type I, however type III constitutes approximately 25-45 % of total collagen content (Gage et al., 1989c). This large amount of type III collagen are thought to be associated with rapid remodelling. Collagen, along with other ground substances of the ECM provide a scaffold that stabilises the structure of the tissues. Soluble collagen being newly synthesised is immature and not cross-linked, this contributes to its relative ease of extractability. Investigations into the extractability of pulpal collagen found that the

amount of soluble collagen within the dental pulp that is extractable with either 0.5 M acetic acid or neutral salt solution was found to be small, indicating that the majority of collagen within the dental pulp is cross-linked and thus cannot be considered immature (van Amerongen et al., 1983). Since collagens become gradually cross linked with time (Zerlotti, 1964), it is not surprising that Amerongen found large percentage of insoluble collagens in dental pulps extracted from teeth. In their investigations, the collagen content of the dental pulp (dry weight) in premolars and third molars was found to be 25.7 % and 31.9 % respectively.

Cementum is a mineralised tooth tissue of mesoderm origin, which is laid down throughout life to help maintain the tooth in its functional position. By volume, it constitutes 33 % of organic content, virtually all of which is type I collagen fibres (Berkovitz et al., 2002 -b). These fibres are embedded within the cementum as Sharpey's fibres, providing stability and anchoring the cementum to the surrounding bone.

The periodontal ligament occupies a space between cementum covering the roots and the alveolar bone and is composed of epithelial-covered soft connective tissue. The fibres of the periodontal ligament are embedded in the alveolar bone and cementum providing a soft tissue continuity between the two mineralised tissues. These fibres are predominantly composed of collagen. The presence of covalent cross-links between collagen molecules stabilises the ligament fibres and increases the tensile strength providing resilience. Approximately 90 % of the connective tissue is collagenous, 70 % type I collagen, and 20 % type III, the latter thought to be associated with rapid turnover of the periodontal ligament (Berkovitz et al., 2002 -d).

The organic matrix of alveolar bone which houses the sockets is made up of 90 % collagen, which is predominantly if not solely type I (Berkovitz et al., 2002 -c). Although type III can also be found particularly in immature or healing bone, the amount of which

peaks around 5-7 days of healing. The collagen in bone is virtually only slightly soluble in dilute acid solutions and virtually insoluble in neutral salts solutions, less than 0.5 % of the total collagen being solubilised (Gage et al., 1989a). This insolubility is due to its cross-links which are necessary for the structural stability and normal functioning of the bone collagen fibrils.

## 2.7 Glycation of Collagen

The rate of collagen turnover within the body varies depending on the type of tissue. Long lived collagen proteins in tissues that have a slow turnover are more likely to undergo glycation and accumulate AGEs with time (Verzijl et al., 2000). Since collagen is the most widespread protein in the body, and present in many different types of tissues, glycation of collagen is likely to contribute to a variety of pathological effects. This may be consequential to the following; changes in structure, susceptibility to degradation, or interaction with cells. These various effects of glycation have been studies in isolation, and will be discussed in the following sections.

### 2.7.1 Glycation of collagen within dental tissues

AGEs have been detected in dental and periodontal tissues and were found to be associated with the presence and duration of diabetes. For example, investigations into the accumulation of AGEs in diabetic patients with periodontal disease found that the gingival levels of AGEs were increased (Zizzi et al., 2013). Furthermore, there was a significant correlation between gingival expression of AGEs and the duration of both type 1 and type 2 diabetes. Type 1 diabetes did however present a significantly higher percentage of AGE positive cells in the epithelium, vessels and fibroblasts of gingival tissues than type 2 diabetics (Zizzi et al. 2013). As well as the association between the prevalence of diabetes and increased levels of AGEs, Takeda et al found that serum AGEs were significantly

associated with the severity of periodontitis in type 2 diabetic patients, were increased levels of AGEs positively correlated with more severe periodontal disease (Takeda et al., 2006). Although there is scarcity in the literature regarding glycation and endodontic infections, AGEs within the dental pulp have also been reported, Reece (2015) developed a model for detecting AGEs within the dental pulp, and after studying 23 diabetic and non-diabetic patients it was found that levels of AGEs within the dental pulp positively correlate with that of the plasma (Reece, 2015a). RAGE expression levels in the clinically inflamed dental pulp were found to be higher than those in healthy pulps (Tancharoen et al., 2014).

### 2.7.2 Stage of collagen glycation

During glycation, glucose reacts with the side chains of the positively charged amino acid lysine. This reaction results in the loss of positive charge of lysine (Hadley et al., 1998). Since lysine resides are abundant in the α-chains of the collage molecule, approximately 106 per chain (Bairoch et al., 2005), glycation has the potential to alter the charge property of the molecule. However, there are only few lysines that are available for glycation (Reiser et al., 1992). Programs using proteomic data, for example NetGlycate 1.0 Server, have predicted that glycation results in approximately 13 modifications per type I tropocollagen molecule (Johansen et al., 2006). Further exposure to glucose may lead to an increased number of lysine amino acids being modified than that of the predicted 13, moreover other amino acids including arginine and terminal valine may also become modified, for example the terminal amino groups of arginine residues are also susceptible to modification (Paul and Bailey, 1996). In the next section, some changes which may also have implications in terms of endodontic and periodontal tissues during both homeostasis and disease will be discussed.

### 2.7.3 Structural changes occurring to collagen upon glycation

Intermolecular cross-links may occur between two adjacent molecules and involve lysine to lysine or lysine to arginine between the triple helical domains of adjacent molecules, with reported consequences including increases stiffness, reduces solubility, and enzyme resistance (Avery and Bailey, 2006), as well as reduced cell spreading through the matrix of glycated ECM proteins (Boonkaew et al., 2014).

### 2.7.4 Susceptibility of glycated collagen to digestion

Degradation of collagen within the body occurs during remodelling, inflammation and wound healing by host enzymes for example serine proteases of macrophages and neutrophils (Chen et al., 2003). Furthermore, in endodontic and periodontal infection, microorganisms such as *Enterococcus. faecalis* secretes extracellular gelatinase and trypsin-like enzymes which may participate in collagen peptide breakdown and may aid in both invasion, and acquisition of nutrients. Therefore changes in the susceptibility of collagen to digestion following glycation is an important factor to consider.

Cross-linking due to glycation has been shown to reduce the solubility of collagen in dilute acid (Tian et al., 1996, Rathi and Chandrakasan, 1990). Although the literature is equivocal on the effect of Maillard reaction on subsequent enzymatic hydrolysis of collagen, the majority of findings in the literature indicate that glycated collagen becomes less susceptible to degradation. For example *in vitro* glycation of bovine dentin resulted in reduced degradation by pepsin (Kleter et al., 1997) . However, digestion of collagen may also depend on the nature of the collagen substrate, type of enzymes and conditions used. Tian and co-workers found that glycated fibrous collagen was much less stable than those of the non-glycated form against proteolytic digestion by pepsin at 30°C and 35°C (Tian et al., 1996), in which glycation increased the proteolytic digestion of the triple helical regions

of collagen by pepsin rather than making them resistance to digestion. In their discussion, it was suggested that intermolecular cross-links in glycated fibrous collagen increase its proteolytic digestion by inducing local unfolding of the triple-helical strands at 30°C, thereby making them more accessible to pepsin (Tian et al., 1996).

For enzymes that are specific for lysine and arginine residues (for example trypsin), it is commonly assumed that the hydrolysis is negatively influenced by glycation because the glycated cleavage site cannot be utilized by trypsin. The extent of trypsin hydrolysis is expected to decrease as a consequence of glycation. This was demonstrated on other proteins, for example α-lactalbumin glycated with D-glucose demonstrated reduced hydrolysis by lysine/arginine specific proteases (bovine and procine trypsin) (Chen et al., 2003). This implies that trypsin cannot hydrolyse glycated cleavage sites. Similarly, α-chemotrypsin which has cleavage site in close proximity to lysine residues, its activity may also be hindered. (Deng et al., 2017).

Investigations on experimentally induced diabetes in rats found a significant relationship between elevated blood glucose concentration and increased glycation og newly synthesized collagen, that is associated with increased collagenase activity and decreased wound collagen content. (Hennessey et al., 1990). This indicates that extracellular glycosylation of proteins in diabetics may contribute to observed impairments in wound healing.

The inconsistent reports on the effect of Maillard reaction on collagen hydrolysis may be related to enzymes recognising their binding sites on the polypeptide chain differently, increased cross-linking, or alteration in the folding of the molecule.

### 2.7.5 Interactions of glycated collagen with human cells

Collagen-cell interactions, through cell surface receptors known as integrins are important, and collagen has now been shown to provide a dynamic matrix for cells to interact with and take part in the regulation of cell differentiation, cell migration, stimulating and controlling development and growth. However interaction with cells also takes place during infection and invasion. Adhesion mediated binding to collagen is a crucial step in the pathogenic process of many bacterial infections. For example *E. faecalis* expresses, a binding protein termed Ace, which is involved in the attachment to collagen and other ECM proteins (Nallapareddy et al., 2000), and this thought to be important for both colonisation and biofilm formation.

Methylglyoxal is an intermediate in the glycation process, which reacts predominantly with arginine residues to form imidazolone compounds, and this has been shown to reduce both adhesion and spreading of cancerous cells in type I collagen, indicating that glycation of arginine residues would inhibit cell-mediated interaction (Avery and Bailey, 2006, Paul and Bailey, 1999).

Glycation of type A porcine skin gelatin (a denatured form of collagen) by incubation with a range for ribose concentrations between 10-250 mM for seven days, resulted in increased cross-linking, and glycation dose dependent decrease in the attachment and spreading of human periodontal ligament cells to gelatin (Boonkaew et al., 2014).

## 2.8 Conclusion

In this chapter, the epidemiologic link between diabetes and endodontic infections has been outline. Three main pathophysiological links between diabetes and endodontic infection have been discussed, including impaired immunity, hyperglycaemia and AGEs. The effects of AGEs on cellular response has been outlined. To the best of the author's knowledge at the time of writing, the effects of the structural and functional roles of collagen, has not been investigated in relation to diabetes and endodontic or periodontal infection. The importance of collagen in the ECM of dental and periodontal tissues, the potential for glycation and the possible implications in terms of susceptibility to digestion, cross-linking and cell interactions has been outlined above. The next chapter will discuss the purpose of this project in investigating the effects of glycation of collagen on its susceptibility to digestion in relation to important endodontic and host enzymes.

# CHAPTER 3: THESIS AIM AND OBJECTIVES

## 3.1 Introduction

So far the following topics have been outlined; the association of diabetes with endodontic and periodontal infections, the potential pathophysiological links between diabetes with dental infection, (including the detection of AGEs within dental tissues for example the pulp), and the association of AGEs with the severity of periodontal disease and reduced pulpal response to electronic tests (Reece, 2015b).

The importance of collagen within the ECM in terms of both structure and function, along with an appraisal of the literature on the impact of glycation in terms structural changes, resistance to proteases and binding to human cells.

The relevance of collagen-cell binding and collagen degradation-during bacterial infections for example in the formation of biofilms, bacterial invasion and acquisition of nutrients has been discussed along with the importance of collagen degradation during remodelling of the ECM.

Section 2.7.4 highlighted the conflicting findings in the literature regarding the impact of glycation on protease degradation of the susceptibility of collagen to proteases, a factor which may not only depend on the enzyme and its ability to recognise its cleavage site, but on the experimental conditions in such assays.

## 3.2 Project aims

The aim of this thesis was to investigate the impact of *in vitro* glucose-mediated non-enzymic glycation on the susceptibility of collagen and gelatin to digestion by bacterial and host proteases.

More specifically the objectives were to;

- develop protocols for *in vitro* preparation of glycated proteins of importance to endodontic and periodontal tissues i.e., collagen and peptides derived from it i.e. gelatin,
- develop methods for quantitation of the extent of collagen and gelatin glycation,
- develop suitable proteolytic assays for testing the susceptibility of glycated proteins to host and bacterial enzymes,
- and to determine whether glycation impacts on protease breakdown of glycated proteins.

The following sections in this chapter will investigate the relevance of host and bacterial enzymes used in this investigation, specifically trypsin *and E. faecalis* proteases.

## 3.3 The relevance of *Enterococcus faecalis* in endodontic infections

### 3.3.1 The association of *E. faecalis* with endodontic infection

*Enterococcus faecalis* is gram-positive, facultative anaerobic bacteria, which usually grows in pairs or short chains of cocci. It exists as normal inhabitant of the alimentary tract of humans and other animals (Hubble et al., 2003).

Researchers began investigating the association of *E. faecalis* with intracanal infections in the 1960s. Since then *E. faecalis* has been frequently isolated from both primary endodontic infections with a prevalence of 4-40 % (Rocas et al., 2004), but more so from persistent endodontic infections with a prevalence of 29-77 % (Rocas et al., 2004, Stuart et al., 2006). A systematic review (Zhang et al., 2015) on 10 studies including an overall 972 teeth concluded that the rate of detection of *E. faecalis* was higher in persistent infections compared with untreated chronic periapical periodontits as primary infections; the difference being significant. *E. faecalis* cells are usually found in pure culture or as a major component of cultures isolated from root canals of previously root filled teeth with apical periodontitis requiring retreatment (Peciuliene et al., 2000). One logical explanation for the transient existence of enterococci in the oral cavity is that they are introduced there via food ingestion (Zehnder and Guggenheim, 2009). Many milk and meat products, vegetables and olives commonly contain enterococci (Franz et al., 2003) and it is consumption of these foods that may be responsible for their appearance in oral cavity. Once these bacteria have entered the oral cavity, they may have access to enter the root canal at any point during or after treatment if the coronal seal is compromised.

### 3.3.2 Virulence factors of *E. faecalis*

*E. faecalis* expresses numerous virulence factors including gelatinase, cytolysin, serine proteases and adhesins, which are recognised to play important roles in bacterial colonization, biofilm formation, and persistence in the root canal system (Stuart et al., 2006, Kayaoglu and Orstavik, 2004).

Some strains of *E. faecalis* has the ability to bind to collagen in dentine through its collagen binding protein Ace (Hubble et al., 2003). Adhesion to root canal walls allows the organism to form a biofilm which may increase its ability to colonize and persist at infection sites (Mohamed and Huang, 2007).

*E. faecalis* has the ability to survive in environments that would otherwise be toxic to many other bacteria. For example *E. faecalis* can still be recovered from canals after preparation and irrigation with hypochlorite and EDTA (Peciuliene et al., 2000).

*E. faecalis* is also capable of maintaining intracellular pH homeostasis through the action of proton pumps, which enhance its survival potential in the presence of medications such as calcium hydroxide aimed at creating a high pH environment leading to denaturation and damage to bacterial DNA (Kayaoglu and Orstavik, 2004). Several studies have demonstrated the survival of *E. faecalis* in the alkaline environment brought about by the use of calcium hydroxide (Bystrom et al., 1985, Distel et al., 2002).

Furthermore, *E. faecalis* also has the ability to enter a viable but non-cultivable state in adverse conditions, in which a number of mechanisms exist. This includes an increase in the resistance of its cell wall to mechanical disruption enhances its ability to survive (Signoretto et al., 2000).

### 3.3.3 Extracellular secreted proteases of *E. faecalis*

The *E. faecalis* proteases, a gelatinase and a serine trypsin-like protease are considered important virulence factors. These are co-transcribed through regulation by the *fsr* regulatory system (Qin et al., 2001, Qin et al., 2000). The contribution of these two enzymes to disease caused by *E. faecalis* disease has been demonstrated in a number of animal models (Engelbert et al., 2004, Gutschik et al., 1979, Singh et al., 2005). For example, several of these studies involve the use of insertion mutants of *fsr*, the encoding gene for gelatinase and serine protease, creating a mutant mutant *E. faecalis* that is unable to produce these enzymes. When compared to the wild-type bacteria this mutation has been shown to reduce the pathogenicity of *E. faecalis,* indicating the importance of these enzymes in contributing to its virulence factors (Sifri et al., 2002).

There are a number of mechanisms by which gelatinase and serine protease contribute to the pathogenesis of *E. faecalis*. For example; they can manipulate and subvert the host's innate immune responses (Potempa and Pike, 2009), promote the adhesion to dentine collagen by Ace binding protein (Hubble et al., 2003), contribute to biofilm production (Hancock and Perego, 2004, Thomas et al., 2008), and aid in the degradation of collagen (Makinen, 1989).

### 3.3.4 Degradation of extracellular matrix proteins by *E. faecalis*

The gelatinase of *E. faecalis* has a broard substrate specificity and a pronounced ability to attack collagenous materials and certain bioactive peptides (Makinen et al., 1989b). The enzyme was especially active against Azocoll and gelatin, and was also able to hydrolyse soluble and insoluble collagens at a lower rate. Gelatinase has been shown to hydrolysed peptide bonds by attacking a variety of amino acid residues including; Ser, Thr,

Tyr, Gly, Gln, Phe, His, Val and in particular, cleave Gly-Leu bonds (Makinen et al., 1989b), which are abundant in collagen alpha chains. The activity of *E. faecalis* OG1RF gelatinase has been demonstrated on agar culture plates containing gelatin. Following growth of the bacteria, overnight at 37°C, zones of digestion develop around the colonies (Qin et al., 2000). Digestion of the extracellular matrix proteins may not only allow invasion of the microorganism through break down of tissues, but the peptides and amino acids produced in the process as result of activity of both gelatinase and serine proteases could be an important nutrient source for *E. faecalis* itself and for other co-invading bacteria growing in the root canal system.

The susceptibility of glycated collagen to digestion by the secreted extracellular gelatinase and serine proteases enzymes of *E. faecalis* is therefore of relevance. This is because *E. faecalis* has consistently been linked to endodontic infection and failure of root canal treatment. As discussed above, studies have also demonstrated an association between diabetes and endodontic infections. Since glycation of collagen, the major component of the ECM of dental and periodontal tissues, leads to a number of changes including cross-linking and charge alteration, with the potential of local unfolding, this may have implications in reducing the resistance of it to enzymatic degradation by *E. faecalis* and might play a role in exacerbating endodontic infections. Therefore these enzymes were chosen for investigating the effect of glycation on the susceptibility of type I collagen to enzymatic digestion.

The binding of *e. faecalis* to collagen through its collagen binding protein (ace), which is enhanced by the serine protease, is an important step in colonisation and the formation of a biofilm (Hubble et al., 2003). Some studies have been carried out on the influence of glycation on attachment to and spreading of host derived cells through glycated gelatin (Boonkaew et al., 2014), and so it was considered relevant to investigate

what effects glycation might have on the binding of collagen to *E. faecalis* since it is possible that this might influence invasion of the connective tissue by the organism.

## 3.4 Trypsin – as a representative host enzyme for proteolytic digestion of glycated proteins

Trypsin belongs to the serine-protease family of proteases, and is widely used a model enzyme to examine the protease susceptibility and to probe the structural features of a wide variety of proteins. In addition, in some pathological conditions including periodontitis, host immune cells such as macrophages and neutrophils express trypsin-like proteases (Chen et al., 2003). Trypsin was found to be predominantly expressed by macrophages, and was upregulated when these cells became activated. Serine proteases are also expressed by neutrophils (cathepsin G) and play a role during inflammation (Meyer-Hoffert and Wiedow, 2011, Pham, 2006). Since macrophages and neutrophils have important roles in inflammatory conditions including endodontic and periodontal pathological conditions (Wahlgren et al., 2002), and in wound healing by degrading ECM components during biological debridement (Koh and DiPietro, 2011), the activity of closely related enzymes like trypsin on glycated proteins are of interest and relevance.

Trypsin-like proteases cleave peptide bonds following a positively charged amino acid, i.e. as lysine or arginine (Evnin et al., 1990). Since glycation targets the side chains of lysine amino acids, this may affect the enzyme's ability to recognise its binding site. This may be due to the following reasons; 1- reaction with a reducing glucose results in the loss of a positive charge in lysine residues consequently altering the charge property of the peptide chain (Hadley et al., 1998), 2- binding of glucose may physically block the enzyme from recognizing and binding to its cleavage site.

For these reasons trypsin may be considered as a suitable representative host enzyme of those found in inflammation sites.

## 3.5 Conclusion

This chapter has briefly reviewed the association between diabetes and dental infection, including the potential pathological link. However, according to our knowledge at the time of writing, the effect of glycation on the susceptibility of collagen to degradation by host and bacterial enzymes has not been investigated from an endodontic point of view. Therefore the aims are to develop protocols for glycation of collagen and gelatin, and assess their susceptibility to digestion by relevant enzymes including *E. faecalis* proteases and trypsin.

# CHAPTER 4: INVESTIGATIONS INTO THE USE OF AZOCOLL AS A POTENTIAL *IN VITRO* NON-ENZYMICALLY GLYCATABLE PROTEASE SUBSTRATE

## 4.1 Introduction

Non-enzymic Glycation of tissues occurring with age and as a result of diabetes has been established in the literature. In addition, the association between diabetes and endodontic infection has been discussed above. In order to fulfil the objective set out in chapter 3, including preparation of glycation proteins for assessment of susceptibility to digestion, it was necessary to initially investigate the use of both glycation and digestion protocols using a suitable collagen substrate (for example Azocoll), and an enzyme as a positive control, in this case *C. histolyticum* collagenase. The objectives were to determine A) if glycation Could be achieved by incubation of Azocoll with glucose, B) to determine the extent of glycation using the HMF assay as described by (Fluckiger and Winterhalter, 1976), C) the minimum incubation period, and 4) glucose concentrations required to obtain glycation levels similar to those found in tissues of diabetic humans and other animal species, D) to determine whether glycated Azocoll could be used as a model test substrate with which to probe protease susceptibility employing C. *histolyticum* collagenase as a positive control protease.

## 4.2 Investigations into the glycation of Azocoll

### 4.2.1 Azocoll as a glycatable protease substrate

Azocoll is an insoluble ground or fibrous collagen-rich proteins preparation usually made from bovine skin, to which a bright red Azo-dye is covalently attached. Because it is used widely as a substrate for assaying proteolytic enzymes (Moore, 1969, Chavira et al., 1984), and because it is collagen-rich, it presented a potentially useful substrate to glycate and then to employ as a test substrate. It is available commercially for example though Sigma-Aldrich Ltd. (EG, product number A4341). Upon proteolytic degradation, the Azo dye along with Azo-bound soluble peptides are released which have a spectrophotometric absorbance peak at 520 nm. This $A_{520}$ of released dye / dye peptides can be used to assess the degradation of the substrate, and therefore enzyme activity (Chavira et al., 1984). If such a substrate could be also glycated, then it might be a useful and convenient chromogenic substrate.

Although the use of Azocoll has disadvantages including; clumping of the substrate, uneven distribution of equal amounts of substrates to assay tubes, and difficulties in rapid sampling of a liquid suspension, is nevertheless still used to evaluate bacterial protease activity (Ksiazek et al., 2015). Therefore to test the feasibility of the glycation and digestion protocols, Azocoll was firstly evaluated as a substrate for glycation and digestion by *Clostridium histolyticum* collagenase.

### 4.2.2 Glycation of Azocoll by incubation with glucose

Sixteen 25 mg replicates of fibrous Azocoll were weighed into lock-top microfuge tubes. The Azocoll was washed by end-over-end mixing with 0.05 M Tris-HCl, 1.0 mM $CaCl_2$, pH 7.8 for 2 h, to remove unbound dye and dye-bound soluble peptides, as described in previous studies (Chavira et al., 1984).

These samples were then transferred into petri-dishes (9 cm in diameter). To eight experimental samples was added 0.5 ml of 2.5 M glucose giving a final concentration of 0.25 M, dissolved in 4.5 ml of 20mM sodium phosphate buffer $Na_2HPO_4$. To eight non-glycated tubes, instead of glucose, 5 ml of the above buffer was added. To all sixteen tubes, sodium azide (final concentration 0.065 % w/v), and 50 µl of penicillin/streptomycin (10,000 U per ml and 100 µg per ml, respectively) was added to act as antimicrobial agents. These were then incubated at 37°C for 30 days. Incubation with similar concentration of glucose has been used previously to achieve glycation (Reiser et al., 1992, Hori et al., 2012, Bailey et al., 1995). For example, "Reiser et al simply incubated the collagen fibres *ex vivo* from rat tail tendons with glucose (500 mg per ml or 2.78 M) in 20 mM sodium phosphate buffer containing 3 mM sodium azide at 37°C for 24 h. Bailey et al, incubated the collagen with either glucose (24 mg per ml or 0.13 M) or ribose (2 mg per ml), in phosphate-buffered saline solution with incubation times ranging from 2-30 days. "

Throughout the 30 day incubation period, the Azocoll was sampled to assess the extent of glycation at 1, 4, 7, 10, 14, 21, 25 and 30 days. This was carried out in the following way, at each time point a 5 ml of water buffer was added to samples to suspend the Azocoll particles and then transferred into a 20 ml universal screw-top tubes, and votex mixed and then re-centrifuged at 5000 g for 5 mins to pellet the Azocoll, and supernatants decanted thus removing any unbound glucose. This washing step was repeated twice more.

The glycated and non-glycated samples were then distributed into pre-weighed lock-top microfuge tubes, and allowed for evaporation of any residual buffer solution at room temperature. Once a constant weight was reached, the amount of Azocoll in each tube was calculated by subtracting the mass of the tube.

### 4.2.3 Assessment of the extent of glycation of Aocoll using the hydroxymethylfurfural (HMF) assay method

A HMF assay was used to assess the extent of glycation, as described by (Fluckiger and Winterhalter, 1976). To each Azocoll containing microfuge tube was added 0.75 ml of 0.5 M oxalic acid in distilled water, and hydrolysis was allowed to take place at 100°C for 4 h using the heat block. Control tubes containing no collagen were included.

Following the hydrolysis step, the solutions were allowed to cool and then filtered through 30 KDa micro-filters, to separate the peptides (100 KDa) which were retained in the filter, from the glycation products (162 Da).

Following this, to 0.8 ml of each reactant solution was then added 0.2 ml of 0.05 M thiobarbituric acid (TBA) and allowed to react with the glycation products at 40°C for 1 h, producing a chromogen with a characteristic absorbance at 443 nm wavelength. The reactant solutions (1 ml) were then transferred to cuvettes and the absorbance spectra was read using Pharmacia Biotech Ultrospec 2000, and Wavescan software. Following subtraction of the background reading, the $A_{433}$ was calculated. Through reference to the standard HMF calibration curve (Figure 4.1), and the amount of collagen per tube as determined gravimetrically, the mean value of HMF (nmol per mg protein) was then calculated. The results are presented in Figure 4.2.

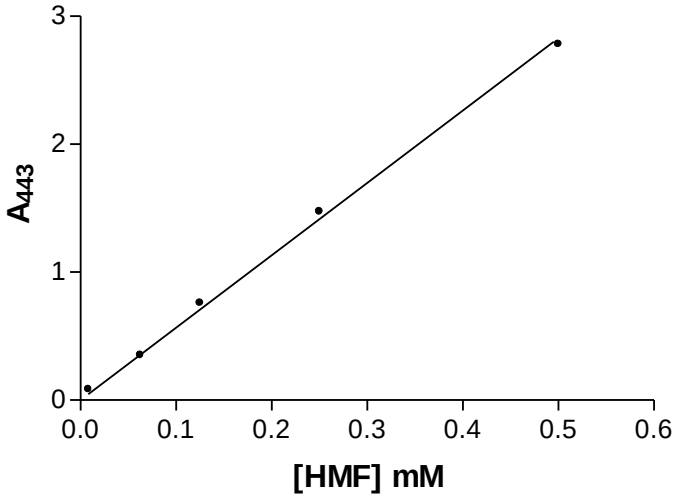


**Figure 4. 1**: **Standard hydroxymethylfurfural (HMF) calibration curve.**

The calibration curve for the 5-(hydroxymethyl)-furfural 99% (HMF) standard was carried out in the following way. HMF (Sigma-Aldrich Ltd. product number W501808) was weighed out and dissolved in water to produce standard molarities ranging 4 to 0.25 mM. To 0.5 ml of each was then added 0.25 ml 1.5 M oxalic acid and they were heated for 4 h at 100°C, followed by addition of 0.25 ml 40 % trichloroacetic acid (this step when used for test samples is to precipitate protein leaving the hydrolysis products in solution). Then to 0.8 ml of the sample was added 0.2 ml of 0.05 M thiobarbituric acid (TBA) which was allowed to react at 40°C for 1 h, producing a chromogen with maximum absorbance at 433 nm. The samples were then transferred to cuvettes and the absorbance spectra were recorded using the Pharmacia Biotech Ultrospec 2000 instrument and Wavescan software. The absorbance at 433 nm was then measured and this was corrected by subtraction of the absorbance for water blank sample which had been processed the same as the HMF standards. This experiment allowed for construction of a standard HMF calibration curve, Figure 4.3, for which direct measurements of the HMF molarity could be read for any 0.5 ml test protein sample solutions which had been processed similarly.

## 4.2.4 Results

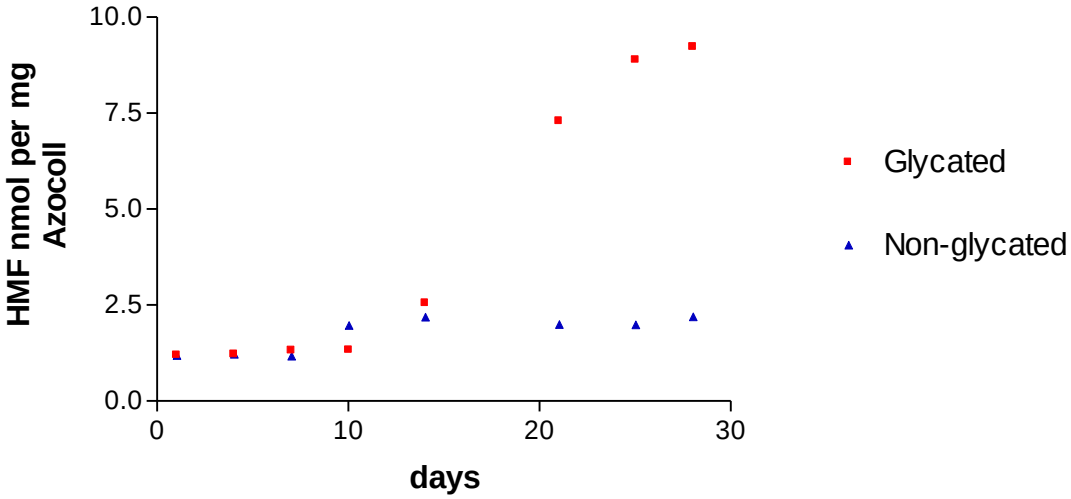


**Figure 4. 2**: **Time course of glycation expressed as nmol HMF per mg for glycated and non-glycated Azocoll.** *In vitro* non-enzymic glycation was carried out over a period of 30 days. Glycation was mediated through incubation with 0.25 M glucose at 37°C.

HMF assays revealed that the "non-glycated" Azocoll possessed a very small amount of glycation. This is most likely a result of the normal low level of glycation occurring during the lifespan of the tissues from which the Azocoll was derived. Glycation of Azocoll by incubation with glucose resulted in an increased level of HMF per mg which was particularly noticeable after 20 days. From then on the extent of glycation increased up to the 30 day period.

The results presented in Figure 4.2 indicate that this glycation protocol was successful. Consequently, a further 200 mg of Azocoll was prepared for glycation by incubation with 0.25 M glucose for 30 days, as described above. This resulted in a preparation in which the extent of glycation were calculated as 10.2 (± 0.33 n=3) HMF nmol per mg for the glycated and 1.53 (± 0.04 n=3) HMF nmol per mg for the non-glycated samples. These preparations were then used in the following digestion experiments with *C. histolyticum* collagenase.

### 4.2.5 Experimental challenges in the use of Azocoll as a substrate for glycation

A maximum value for glycation of 8.35 nmol Hmf per mg was obtained for the procedure described above. This value was of the order of that obtained for a number of normal collagen extracts from connective tissue of man and other species (Miksik and Deyl, 1991). However, whilst the above experiments demonstrated that in vitro glycation of Azocoll was achievable after 30 days, the data obtained appeared to suggest that using the present conditions, a maximum effect had been obtained. This implied that extending the time of incubation may not have brought about greater glycation. In addition, using fibrous Azocoll also has proved to be challenging, firstly in terms of handling since the fibres are very sticky and this result in loss of substrate throughout the preparation stage for example weighing and transferring samples. Secondly, the fibres clump together, and this has an impact on the protease assay itself, in terms of ensuring maximum interaction between the enzyme and the substrate. It was found that other batches of Azocoll purchased from sigma were of a more granular form. This was even more challenging to handle as it became electrostatically charged - adhering to the spatula, weighing boats and plastic and glassware etc. Approximate 30 % of collagen was lost between the initial weighing and following washing. Furthermore, dye was lost during the glycation incubation step, which interfered with the HMF assay. The Azo dye may also adversely affect the extent of glycation achieved as it may hinder access of the glucose to their lysing targets.

## 4.3 Digestion of glycated and non-glycated Azocoll by *Clostridium Histolyticum* collagenase

Following 30 days of glycation of fibrous Azocoll, a tube assay using *Clostridium histolyticum* collagenase was prepared to assess its relative susceptibility to digestion. This enzyme was chosen as a model positive control protease because it is a potent protease, which in terms of collagen peptides is related to the specificity for gly-X bonds which occur at every third amino acid in the polypeptide chain.

Since the Pharmacia Biotech Ultrospec 2000 spectrophotometer is only accurate to an absorbance reading of 3, and it has previously been reported that for typical Azocoll preparation 1 mg per ml of Azocoll will yield an $A_{520}$ of 0.593 (Chavira et al., 1984), it was decided to set up digestion tubes containing up to 5 mg per ml of Azocoll to keep the maximum absorbance readings (of fully hydrolysed Azocoll) within the accuracy of the instrument.

From the stock samples of glycated and non-glycated Azocoll, three 5 mg replicates of each was transferred into round-end 3.0 ml tubes. To each tube was added 1 ml of 0.1M Tris 0.14M NaCl 36mM $CaCl_2$ buffer, pH 7.5. *C. histolyticum* collagenase has activity at a broad pH range of 6.3 to 8.5, and the enzyme is assayed at pH 7.5 (Schomberg et al., 1991). Followed by 0.1 ml of 1 mg per ml of the *C. histolyticum* collagenase Sigma-Aldrich Ltd. (product number C5138). A control of 5 mg per ml Azocoll from each glycated and non-glycated samples containing no enzyme was also included to account for any dye released Azocoll that was not related to digestion. The tubes were then incubated at 37°C until all the Azocoll had been digested, during which they were end-over-end mixed.

The enzyme activity was assessed by measuring the $A_{520}$ which relates to the absorbance maximum of the azo dye released upon fragmentation of the collagen by the enzyme. This was carried out at time points throughout the incubation period of 5 h, during which the collagen was pelleted by centrifuging at 14000 g for 5 minutes, and 1 ml of the supernatants sampled into cuvettes for spectrophotometric absorbance readings. These samples were then transferred back into the assay tubes to maintain the enzyme to substrate ratio. Following subtraction of the background control, the mean $A_{520}$ of three replicates was calculated and the results presented in Figure 4.3.

### 4.4.1 Results

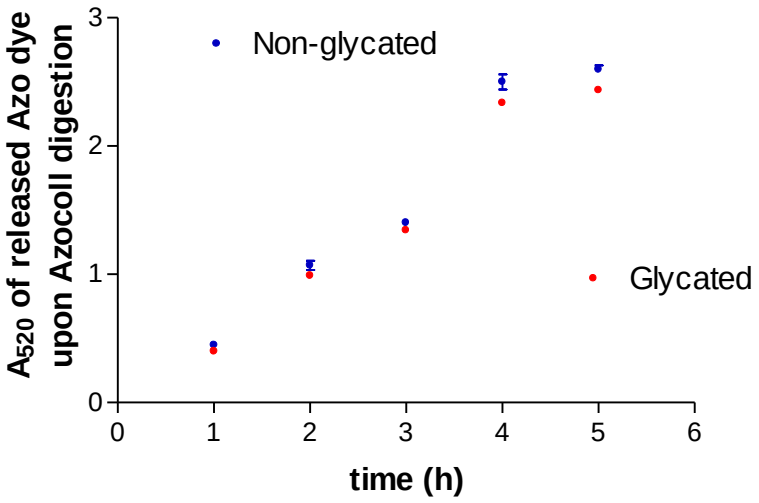


**Figure 4. 3**: **Hydrolysis of glycated and non-glycated Azocoll by *C. histolyticum* collagenase.** The digestion was performed in, in 0.1M Tris 0.14M NaCl 36mM $CaCl_2$ buffer, pH 7.5, at 37°C. Digestion of Azocoll increases steadily up to 3 h, from then there is a greater increase in digestion, which starts to plateaue around 5 h.

The absorbance of azo dye released measured at $A_{520}$ upon digestion of glycated and non-glycated Azocoll was very similar throughout the digestion period of 5 h. This indicated that there was no difference in the susceptibility of the glycated Azocoll to digestion compared to its non-glycated counterpart.

The results presented in Figure 4.3 demonstrate that this method, using a tube assay, with agitation to maintain constant mixing and access of the enzyme to the substrate indicated that it was a suitable method to assess collagenase digestion of the glycated and non-glycated Azocoll. However, there appeared to be no difference between the two Azocoll substrates in terms of azo dye released, and hence extent of digestion. This is not surprising since *C. histolyticum* collagenase can attack the polypeptide alpha chains of the collagen molecule at every third amino acid (glycine) in the chain i.e. approximately three hundred sites of action. It is therefore likely that any differences in susceptibility between the glycated and non-glycated substrates (should they exist) may not be manifest during digestion with *C. histolyticum* collagenase.

## 4.4 Discussion

In the search for a collagen preparation as a suitable model protein for glycation and as a subsequent substrate to test for protease susceptibility, Azocoll was initially evaluated. Azocoll is a widely used collagen-rich substrate for such assays because it is pre-stained and can be used in a tube assay. This was despite the recognised disadvantages for the need to pre-wash Azocoll prior use due to release of both unbound dye and dye-bound soluble peptides (Chavira et al., 1984). Preliminary experiments demonstrated that it was possible to glycated Azocoll *in vitro*. The extent of *in vitro* glycation under the conditions used here showed an increase after 20 days and continued to rise for the remainder of the incubation period of 30 days. However, Azocoll was found to be glycated to a low degree following incubation with 0.25 M glucose even after 30 days giving a value 9.24 nmol HMF per mg, which was similar to the level of glycation of collagens analysed from a variety normal tissues (Miksik and Deyl, 1991). The reason for this low level of glycation despite the fact that a much larger glucose concentration was used than that which may ever be encountered in vivo, is not known. However, one may speculate that the glycation reaction

may be may be slowed down or impaired by the presence of the covalently bound Azo-dye, this might precluding access of the glucose to their target lysine sites. Furthermore Azocoll is an insoluble product and the fibrous, and cross-linked nature of this colalgen containing substare may also impede access of the glucose to their target sites.

Digestion experiments were carried out using *C. histolyticum* collagenase. It was found that both the glycated and non-glycated Azocoll were digested to a similar extent. However, given the fibrous nature of the preparation and the low level of glycation that was achieved after 30 days (as reasoned above which were lower than those found in found in number of normal human and animal tissues (Miksik and Deyl, 1991), it was decided to experiment with an alternative granular form of Azocoll. However, it was found that this granular form had the tendency to be easily electrostatically charged, and adhered to glass and plastic ware. Moreover, the Azo dye was released during the incubation period with glucose, even following pre-washing of the Azocoll, and this was also found to interfere with the HMF assay by imparting a slight pink colour to the hmf assay solutions post oxalic acid hydrolysis. These issues were considered major drawbacks in the use of Azocoll and so an alternative preparation of collagen was sought which will be discussed in chapter 5.

## 4.5 Conclusion

Although Azocoll was found to be a suitable collagen substrate which could be glycated, it was demonstrated that incubation with 0.25 M glucose for 30 days achieved a low level of glycation, similar to levels found in collagens from normal tissues. Given the time limitations, instead of increasing the incubation time of glycation, it was considered that one way forward to achieve a greater degree of glycation was to increase the glucose

concentration. Nevertheless, the tube protease assay was found to be reliable method of assessment of protease activity for glycated Azocoll.

# CHAPTER 5: *IN VITRO* GLYCATION OF TYPE A GELATIN, TYPE I SOLUBLE AND INSOLUBLE COLLAGENS

## 5.1 INTRODUCTION

This chapter will describe experiments undertaken to prepare glycated proteins of relevance to dental tissues including, soluble and insoluble type I collagens and type A porcine skin gelatin which has been used by others (Boonkaew et al., 2014) as an alternative glycatable substrate model for collagen. These substrates will then be used (as described below in chapter 6) for assessment of their susceptibility to digestion by host and bacterial enzymes of relevance to dental infection including trypsin which is expressed by neutrophils and macrophages in tissues at foci of inflammation, and *E. faecalis*. A protocol was described for glycation in chapter 4, in which Azocoll was incubated with 0.25 M glucose for 30 days. It was found that the extent of glycation achieved was lower than that found in tissues extracted from non-diabetic humans. The Possible reasons for this include too low a glucose concentration; impaired access of the glucose to the glycatable lysine residues of the Azo-bound collagen due to cross linking, and lastly too short an incubation period. However, with regards to the latter, it was found that the extent of glycation appeared to plateau around 30 days. Therefore it was decided to determine whether this low glycation level might be improved upon by 1) increasing the glucose concentration to 1.25 M, 2) using non-chemically modified collagen substrates, and 4) to investigate the possibility of obtaining a substrate (gelatin) with variable degrees of cross-linking. It was envisaged that the use of unmodified, native collagen substrates would get around the problem of interference which occurred through release of the azo dye during assessment of the extent of glycation using the HMF assay. Since release of Azo-dye from Azocoll during the process interfered with the absorption spectral readings of the HMF assay.

## 5.2 *In vitro* glycation of gelatin

### 5.2.1 Type A porcine skin gelatin- as a glycatable protease substrate

Gelatin is heterogeneous mixture of water-soluble proteins which is obtained from connective tissues and contains a large proportion of collagen-derived peptides of high molecular weight. Its biochemistry and properties have long been established (Eastoe, 1955). Gelatin is used widely in the food industry because it imparts the "melt-in-the-mouth characteristics" and is able to form thermo-reversible gels which add to the texture and consistency of foods (GMIA, 2012). Type I collagen constitutes the major component of the extracellular matrix of dental and periodontal tissues (Gage et al., 1989a). Because it is a long-lived protein and is remodelled during homeostasis, wound healing, or after bacterial degradation it is susceptible to glycation throughout life (Miksik and Deyl, 1991). The rate of such glycation may increase in chronic states of hyperglycaemia as seen in diabetics (Lyons and Kennedy, 1985). For that reason, it is an important structural component to investigate as its resistance to degradation may have potential complications associated with bacterial invasion or altered remodelling and impaired wound healing.

Type A peptide gelatin from porcine skin is inexpensive and available from manufacturers world-wide. The substrate used here was purchased from Sigma-Aldrich Ltd. (product number G1890). It is obtained from denatured (acid-cured) porcine skin, extracted via boiling. Gelatin is almost all collagen derived as determined by its hydroxyproline content. For example type I collagen contains approximately 13.5 % hydroxyproline (Neuman and Logan 1950) and porcine skin gelatin comprises around 13.1 % (Eastoe, 1955). Giving it a collagen content of about 13.1 ÷13.5 x 100 % = 97 %.

In the previous experiments using Azocoll as a potential substrate for glycation by incubation with 0.25 M glucose, it was found that glycation could be progressively achieved through an incubation period of up to only approximately 30 days (see, Figure 4.2). This resulted in a glycation level of only 8.3 nmol HMF per mg protein. Taking this finding into account, it was decided to incubate gelatin with glucose for 35 days. In the subsequent experiments using gelatin, the concentration of glucose was increased to 1.25 M, but instead of using a fixed level of glucose, a range of concentrations from 0.125 - 1.25 M were used. This would allow for the determination whether a dose-dependent increase in glycation might be achievable. This might also give an insight into whether there was a limit to the extent of glycation attained i.e., would the reaction plateau which might indicate that all the available glycatable residues had been reached.

In these experiments, the gelatin was glycated by incubation with glucose in the following way. Seven replicates of 2 g of type A porcine skin gelatin (Sigma-Aldrich ltd., product number G1890) were initially dissolved in 20 ml of 0.02 M phosphate buffer, pH 6.8, in glass tubes by heating at 60°C for 30 min, to give a 10 % w/v solution. Previously researches have used gelatin at 5 % (Boonkaew et al., 2014). By increasing the gelatin concentration here to 10 %, this would theoretically achieve a two-fold greater level of glycation on the basis that the reactant (gelatin) is increased in concentration. Following this, the gelatin solutions were then transferred into sterile plastic specimen containers, and to six of these, D-glucose (Sigma-Aldrich Ltd. product number G5500) was added and stirred to dissolve giving final glucose concentrations in the range of 0.125 M to 1.25 M. The seventh container of gelatin solution did not receive any glucose and was used as a non-glycated control for the experiment. The weight of D-glucose added and the concentrations in the corresponding solutions is presented in Table 5.1. Following this, antimicrobial agents were added including; sodium azide (final concentration 0.065 % w/v), and a penicillin/streptomycin solution (Sigma-Aldrich ltd product number P4333) to give a

penicillin final concentration of 100 International Units per ml, and streptomycin at 100 µg per ml final concentration. The gelatin solutions were then incubated for 35 days at 37°C with stirring to maintain the gelatin samples in a liquid state.

| Solid glucose (g) added to each 20 ml of gelatin solution | Concentration (M) of glucose in each gelatin solution |
|---|---|
| 4.5 | 1.25 |
| 3.6 | 1 |
| 2.7 | 0.75 |
| 1.8 | 0.5 |
| 0.9 | 0.25 |
| 0.45 | 0.125 |
| 0 (control) | 0 |

**Table 5. 1: Amounts of D-glucose added to each gelatin sample and the glucose concentration achieved in the corresponding solution.**

Following the incubation step, excess non-bound glucose was removed in the following way. Gelatin solutions were transferred into pre-boiled dialysis tubes (Visking tubing, The Scientific Instrument Company, Eastleigh, Hampshire, UK), and dialysed against three replicates of 10 L volumes of deionised water for at 37°C, over a 24 h period, to remove the glucose and at the same time to maintain the gelatin in a liquid state. The dialysis step at 37°C was essential as this; 1- aided the diffusion and dialysis of the glucose which would have been less efficient had this been done at 4°C or room temperature and 2- ensured that the gelatin (at these concentrations, 10 %) would remain in a liquid state. To confirm that the dialysis step had successfully removed unbound excess glucose, the gelatin solutions were tested for the presence of any residual glucose. This was carried out using the glucose oxidase assay kit (Sigma-Aldrich Ltd. GAGO20-1KT) as per manufacturer's

instructions. No residual glucose was detected in any of the gelatin solutions, which were then stored at -20°C until further required.

### 5.2.2 Assessment of protein concentration of gelatin

To easily assess the amount of gelatin in solution following the dialysis step, $A_{273}$ ultra violet (UV) readings were measured after 1:4 dilutions of samples from each gelatin solution was scanned over the UV-visible range as described by (Makharza et al., 2015), using Pharmacia Biotech Ultrospec 2000 and the data acquired using Wavescan software. This effectively is a measure of the absorbance contributions of the aromatic amino acids, tryptophan, tyrosine and phenylalanine in the peptides. The protein concentration in each gelatin solution was then calculated by referencing to a standard UV calibration curve (Figure 5.1), which was constructed from a non-glycated gelatin stock solution, taking into account the fact that the samples had been diluted 1 in 4. The results are presented in Table 5.2.

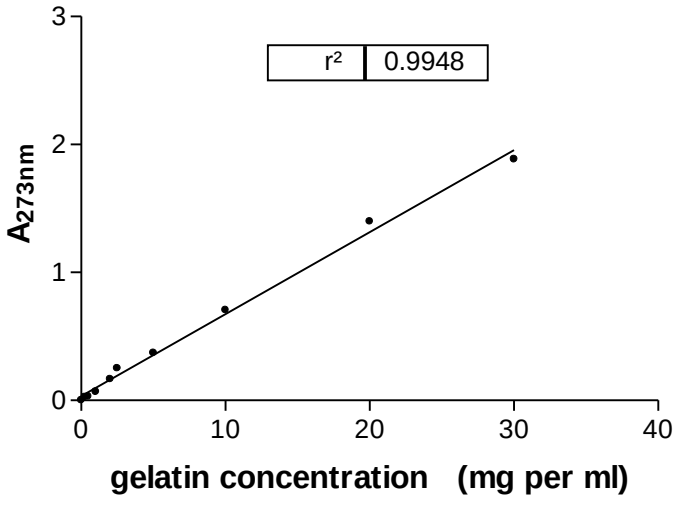


**Figure 5. 1: UV absorbance calibration curve of porcine skin gelatin.** The gelatin stock solution was prepared by heating at 60°C in water for 30 min and immediately diluted to give samples in the concentration range 0 - 30 mg per ml.

| sample # | Glucose concentrations (M) | Gelatin concentration (mg per ml) |
|---|---|---|
| 1 | 1.25 | 71.92 |
| 2 | 1 | 74.4 |
| 3 | 0.75 | 64.8 |
| 4 | 0.5 | 68.39 |
| 5 | 0.25 | 76.3 |
| 6 | 0.125 | 68.88 |
| 7 | Buffer control | 66.24 |

**Table 5. 2: Concentrations of gelatin in the sacs post dialysis, as determined by UV absorbance at 273 nm.**

From the A273 UV readings, the gelatin concentrations post-dialysis were determined and presented in Table 5.2. It was seen that all the solutions appeared to contain similar levels of protein despite the fact that they obviously different in volume which increased (due to taking in more water by osmosis) as the glucose concentrations increased. This may be explained by the observation of an increased absorbance in the 300 - 310 UV region of the gelatin samples incubated in the highest concentrations of glucose. This was seen to be the case when the spectroscopic scans were examined (Figure 5.2).

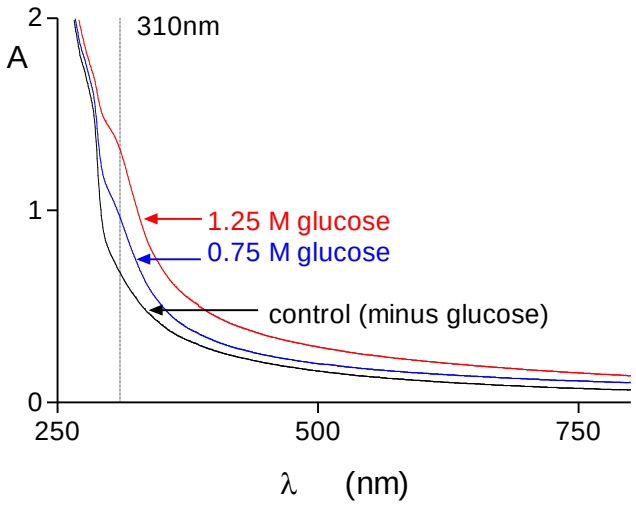


**Figure 5. 2: UV-visible spectra of gelatin samples incubated with 1.25 M and 0.75 M glucose, and the non-glycated (control).** An increase in the absorbance readings at approximately 310 nm was observed in the glycated gelatin samples compared to the non-glycated control.

The increase in the UV readings around 310 nm wavelength in the glycated gelatin samples was most likely attributed to the presence of Schiff base and Amadori products with double bonds which would absorb in the UV range (Deng et al., 2017), and which may have contributed to the overall UV absorbance. This would affect the quantitation of protein concentration as determined by the $A_{273}$. As a consequence it was necessary to standardise the protein concentration of the gelatin samples using an alternative protein assay. For this, the Bicinchoninic acid (BCA) method was used employing the TPRO 562 Protein Assay kit (Sigma-Aldrich Ltd.), which was carried out as per manufacturer's protocol with gelatin standards in the range of 0.4 – 25 mg per ml, a calibration curve was constructed using these data (Figure 5.3).

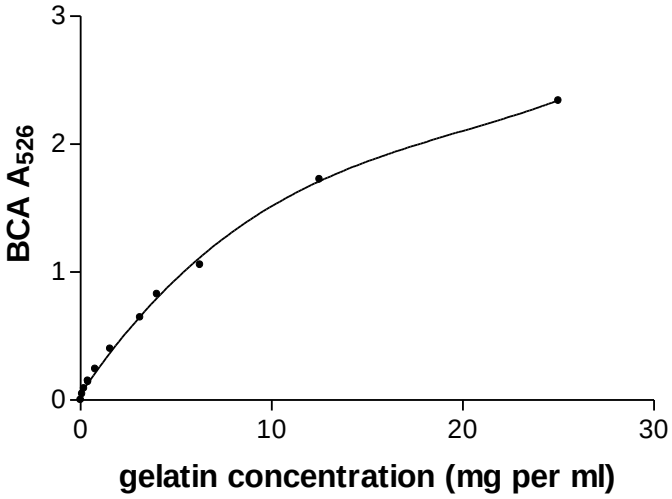


**Figure 5. 3: BCA standard calibration curve of net absorbance versus protein concentration for determination of gelatin protein concentration.**

Samples from the gelatin solutions following dialysis were diluted (1:5) to fall into the linear range of the BCA calibration curve (Figure 5.3), and duplicate 50 µl gelatin samples were mixed with 950 µl BCA working reagent, vortexed to give thorough mixing, and heated at 37°C for 30 minutes. A 50 µl deionised water sample was included as a control. The reaction solutions were then transferred to cuvettes and the $A_{562}$ was read on the spectrophotometer. The control was then subtracted from the absorbance readings to correct for the background. The absorbance values were computed and used to determine the protein concentration with reference to the calibration curve (Figure 5.3), taking into account the dilution factor to determine the protein concentration. The results are presented in Table 5.3.

| [Glucose] (M) the sample was glycated with | Average gelatin concentration of stock (mg per ml) n=2 |
|---|---|
| 1.25 | 24.375 |
| 1 | 27.53 |
| 0.75 | 30.44 |
| 0.5 | 36.39 |
| 0.25 | 38.91 |
| 0.125 | 42.71 |
| Buffer control | 47.83 |

**Table 5. 3: Protein concentration (mg per ml) of gelatin following glycation and after the dialysis step.** Protein was assayed using the BCA method.

From the results presented in Table 5.3 it was evident that the gelatin preparations originally containing the highest glucose concentrations in the glycation incubations were more dilute. This was predictable given that the presence of glucose would have resulted in a greater uptake of water into the dialysis sacs due to osmosis, resulting in larger volumes and consequently lower concentrations of protein.

### 5.2.3 Assessment of the extent of glycation of gelatin

The measurements to determine the extent of gelatin glycation were carried out using the assay for HMF. Three replicates of 0.5 ml of each gelatin solution was transferred into lock-top microfuge tubes. Then, 0.25 ml of 1.5 M oxalic acid (final concentration 0.5 M) was added to each gelatin containing tube, including 0.5 ml of water as a control, and allowed to hydrolyse at 100°C for 4 h, to release the covalently bound glucose in the form of HMF. The tubes containing the reaction mixture were then allowed to cool at room temperature. Following this, 0.25 ml of 40 % trichloroacetic acid (TCA) was added and

allowed to stand at room temperature for 30 min to precipitate the denatured protein, then centrifuged at 14000 g for 5 min at room temperature to sediment the ptorein. To 0.8 ml of each reaction solution was then added 0.2 ml of 0.05 M thiobarbituric acid (TBA), and allowed to react at 40°C for 1 h. The solutions were then transferred to cuvettes and the $A_{433}$ measured. Following subtraction of the control background absorbance, a mean of the replicates was calculated to give the molar amount of the HMF referencing to the standard calibration curve (Figure 4.1). The results are presented in Table 5.4.

| [Glucose] Molarity | Mean gelatin concentration of stock (mg per ml) n=2 | Mean level of HMF (nmol) per mg protein (n=3) |
|---|---|---|
| 1.25 | 24.375 | 67.05 |
| 1 | 27.53 | 56.908 |
| 0.75 | 30.44 | 40.834 |
| 0.5 | 36.39 | 25.199 |
| 0.25 | 38.91 | 14.700 |
| 0.125 | 42.71 | 9.029 |
| 0 | 47.83 | 3.429 |

**Table 5. 4: Extent of glycation of porcine skin gelatin based on assay for HMF.**

The results in Table 5.4 are presented in Figure 5.4. It was evident from this, that a positive correlation between the molarity of glucose used in the incubation and the HMF per mg of protein. This indicated that incubation with increasingly higher concentrations of glucose achieved a greater extent of glycation of the protein. Usefully, this eliminated the need for lengthy incubation periods to achieve glycation.

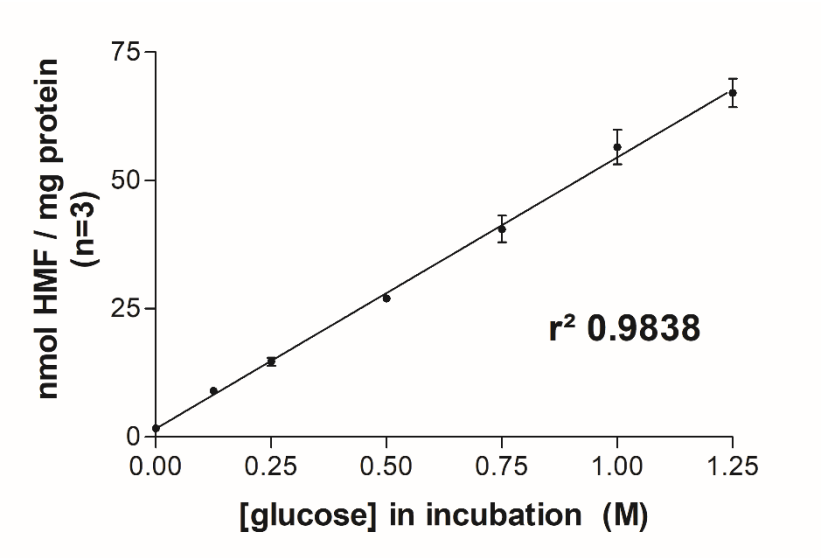


**Figure 5. 4: The relationship between glucose concentration in the incubation mixture and the extent of glycation of porcine skin gelatin.** The glycation level was measured by assaying for HMF and the protein concentration determined using the BCA method.

## 5.2.4 Calculation of the theoretical extent of glucose modification of gelatin peptides

Using the value of 67 nmol HMF per mg protein as the maximum extent of glycation of the gelatin achieved, it is possible to theoretically calculate approximately the number of glucose modifications to the peptides. This makes two assumptions; the first is that type A porcine gelatin is composed almost entirely of collagen; the second being that the peptides in this preparation were minimally degraded and are of a similar molecular size to collagen alpha chains from which they are derived. Collagen comprises of approximately 13.5 % (w/v) hydroxyproline (Neuman and Logan, 1950). Similar levels have been quoted for porcine skin gelatin, 13.1 % (w/v) (Eastoe, 1955). This indicates that the porcine skin gelatin used in these investigations is almost all collagen (in this case, 13.1/13.5 = 97 %).

The peptides in the type A gelatin preparation used here are quoted in the data sheet for this Sigma-Aldrich Ltd. product to be in the range of 50,000 - 100,000 Da. It must be noted that some gelatin peptides may be larger than individual collagen alpha chains

should there be any cross links to partial peptides of other alpha chains and which do not become cleaved during the mild acid extraction procedures used for this type of preparation. Therefore, using the upper molecular mass value of 100,000 Da (as quoted above) and assuming that all the peptides in the gelatin are derived from collagen chains, this indicates that each peptide has approximately 6 glucose modifications. This was calculated in the following way, 1 mg of gelatin is equivalent to 1 ÷ 100 kDA = 1 mmol x 10-5 (i.e. 10 nmol). Given the upper HMF level of 67 nmol per mg, this gives a maximum of 67 ÷ 10 = 6.7 modifications (7 to the nearest whole number).

### 5.2.5 Glycation of bulk preparation of gelatin

For the subsequent set of experiments using 1 % gelatin / 1 % agar co-cast for a plate protease diffusion assays, it was decided to produce a bulk preparation of glycated gelatin substrate, in the following way; two replicates of 10 g of the type A porcine skin as used above were dissolved in 100 ml of 0.02 M phosphate buffer, pH 6.8, in glass tubes by heating at 60°C for 30 min, to give a 10 % w/v solution. The gelatin solutions were then transferred to plastic specimen containers. To one of these was added 22.5 g D-glucose (Sigma-Aldrich Ltd. G8270) and stirred to dissolve giving a final concentration of 1.25 M (equivalent to the maximum glycated gelatin in the series as described above). To both gelatin solutions was then added sodium azide, and penicillin/streptomycin solution as antimicrobial agents. The gelatin solutions were then incubated at 37°C with stirring to maintain the gelatin samples in a liquid state, for 35 days.

Following the incubation period, the glucose incubation sample appeared with a slight straw colouration indicating that glycation had occurred, Figure 5.5, similar to previous findings in which slides of dentine that were exposed to glucose turned pale yellow (Kleter et al., 1997).

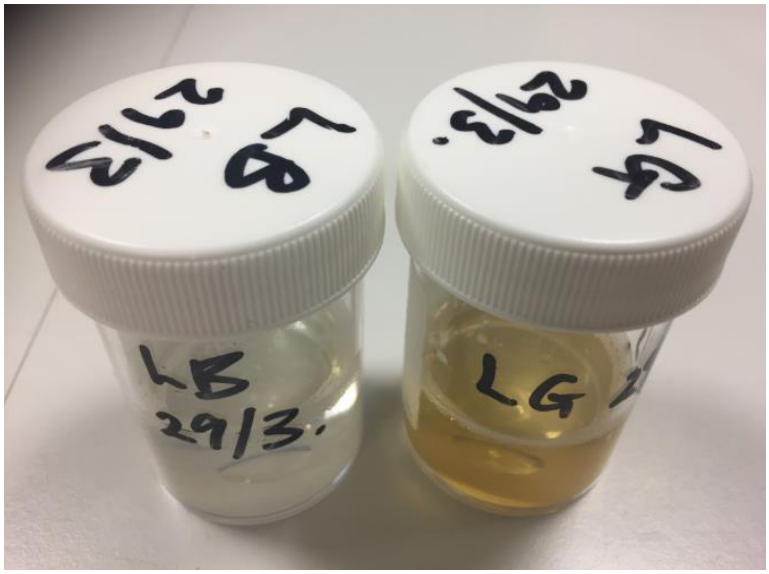


**Figure 5. 5: Type I soluble collagen incubated with glucose turned straw coloured compared to the non-glycated counterpart.**

As for the series of glycated gelatin, the same protocols were followed to remove excess glucose through dialysis, quantify the protein concentration through the BCA assay and the amount of glycation through the HMF assay, with reference to the relevant standard calibration curves in Figures 4.1 and 5.3 respectively. The extent of glycation was determined to be 41.44 (± 0.38 n=3) HMF nmol per mg protein for glycated, and 0.90 (± 0.12 n=3) HMF nmol per mg protein for the non-glycated gelatin solutions. The results are presented in Figure 5.6.

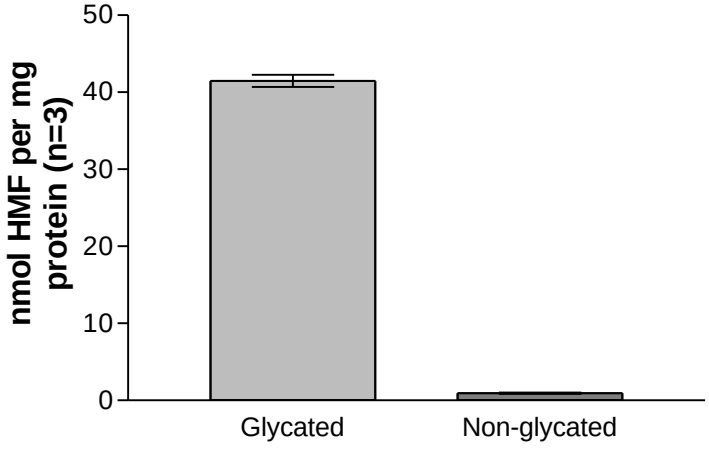


**Figure 5. 6: The extent of glycation for the bulk preparations of glycated and non-glycated gelatin.** The gelatin was incubated with 1.25 M D-glucose, at 37°C for 35 days.

The results in Figure 5.6 show that the gelatin sample incubated with glucose was forty five-fold more glycated than the control. Calculations showed there were approximately four glucose modifications per peptide chain.

### 5.2.6 Discussion

There are no set methods in the literature for producing glycated proteins *in vitro*. The reaction depends on a number of factors including the nature and concentration of the substrate, concentration of glucose, pH, and time. For example, as demonstrated (Figure 5.4) the greater the glucose concentration, the greater the extent of glycation achieved. Furthermore, greater levels of glycation were achieved for gelatin (14.7 nmol HMF per mg protein) compared to Azocoll (8.35 nmol HMF per mg protein) using the same glucose concentration (0.25 M). This can be explained on the basis that glucose most likely has easier access to lysine in the gelatin peptides as compared to Azocoll since in the liquid state the former will be largely in a random coil. Furthermore, the Azo dye may impeded access of the glucose to the glycation target site in Azocoll. Overall this section describes a method in which gelatin was glycated to variable extents, and this can be used to assess a pattern in its susceptibility to digestion with glycation.

## 5.3 *In vitro* glycation of type I soluble collagen

Collagen is the major component of the dental pulp and constitutes by dry weight of approximately 34 % of its total protein content (Gage et al., 1989c). The main collagen of the dental pulp is type I, which makes up 56 % of the total collagen content (Berkovitz et al., 2002 -a). Soluble collagen represents a newly synthesised collagen that is not yet cross linked (Harris and Griffin, 1967). Both Types I and III are found in the dental pulp which is considered to be a loose connective tissue which also contains other main organic components proteoglycans and glycoproteins (Seltzer and Bender, 1975). Type III collagens

are difficult to source and so type I soluble collagen which is more readily available was used.

The type I soluble collagen preparations, like gelatin, are easy to handle and so was chosen as a representative model to glycated. The preparation used in these experiments was obtained from Sigma-Aldrich Ltd. (product number C9791). This purified collagen preparation which is extracted from calf skin using dilute acid as described in the method of (Gallop and Seifter, 1955), in which the skin is washed thoroughly with cold water and cut into pieces then ground with an electrical tissue grinder at 0°C to 2°C. The collagen is then extracted with 0.5 M sodium acetate, followed by treatment with 0.075 M citrate buffer of pH 3.7. Following this, the extracted collagen is clarified by centrifuging and dialysed against 0.02 M sodium hydrogen phosphate solution, and the resulting collagen fibrils are harvested, washed with distilled water, and then lyophilised.

### 5.3.1 Preparation of type I soluble collagen substrate for glycation

Collagens of this kind are insoluble in aqueous buffers at neutral pH, but it is well known that they can be solubilised in dilute organic acids, such as acetic acid (Gilmcher and Katz, 1965). To achieve solubilisation, a 100 mg batch of collagen was mixed with 20 ml 0.05 M acetic acid and stirred gently at 4°C for 48 h until dissolved. This produced a slightly cloudy and viscous solution. The solution of collagen was divided equally to give two 10 ml aliquots, one for glycation and the other to serve as non-glycated control.

### 5.3.2 Glycation procedure for the type I soluble collagen

The glycation procedure used here was one adapted from that used for *in vitro* glycation of haemoglobin (Ch'ng and Marinah, 1988), in which the glycation mixture of the protein and glucose was allowed to progressively dry, thus elevating the level of both reactants. It was decided to take this approach in the expectation that a greater extent of glycation might be achieved without the need of increasing the incubation time period. To carry out this, to each of the above collagen solutions was added 1 ml of 1 M $Na_2HPO_4/Na_2HPO_4$ buffer, pH 6.8, to neutralise the acetic acid and poise the pH at 6.8, but to maintain the collagen in solution. Then, sodium azide, penicillin / streptomycin were added to both collagen solutions (as described above). For the glycation sample 2.47 g of solid D-glucose powder was gradually added with stirring until fully dissolved, to give a concentration of 1.25 M (this being equivalent to the maximum concentration as used for the gelation glycation experiments). Both samples were then incubated at 37°C for 35 days in 9 cm diameter sterile plastic petri-dishes.

Following the incubation periods, the dried collagen films were solubilised from the surface of the petri-dishes by the addition of 20 ml 0.1 M acetic acid, and the gel-like collagen mass was gently stirred at 4°C until it was again fully soluble. The glucose and buffer salts were then removed from the above collagen solutions by dialysing them against four changes each of 2 L of deionised water at 4°C, and the dialysis water being constantly stirred. The dialysis retentate of the samples was then tested for the presence of any residual glucose using the glucose oxidase assay kit (Sigma-Aldrich Ltd) as for the glycated gelatin. No residual glucose was detected and so it was deemed suitable for further use and for assay of extent of glucose glycation.

### 5.3.3 Assessment of the extent of glycation of type I soluble collagen

The collagen samples after the above treatments were assayed for HMF as a measure of the extent of glycation in the following way. Three replicates of 1 ml samples were transferred to pre-weighed, lock-top microfuge tubes, and dried to constant weight by heating at 60°C. The amount of collagen was calculated gravimetrically, by subtracting the weight of the tubes. These measurements showed that the original collagen solutions were 1.35mg (± 0.12 n=3) mg per ml for the glycated sample, and 1.8mg (± 0.1 n=3) mg per ml for non-glycated (control) collagen.

Next, 0.5 ml of deionised water was added to the tubes containing the dried soluble collagen samples, followed by 0.25 ml of 1.5 M oxalic acid was then added to each tube (giving a final molarity of 0.5 M), and heated for 4 h at 100°C. The samples were then allowed to cool at room temperature prior to the addition of 0.25 ml of 40 % TCA acid and allowed to stand for 30 min. To 0.8 ml of each sample was added 0.2 ml of 0.05 M TBA acid, and this was allowed to react at 40°C for 1 h. The samples, including a control (water), were transferred into cuvettes and the $A_{433}$ was measured spectrophotometrically. Following subtraction of the control, the values were used to calculate the amount of HMF by referring to the HMF standard calibration curve Figure 4.1. These values were then divided by the protein concentrations as calculated gravimetrically (see above), to give the extent of glycation per mg of protein. The results are presented in Figure 5.7.

## 5.3.4 Results

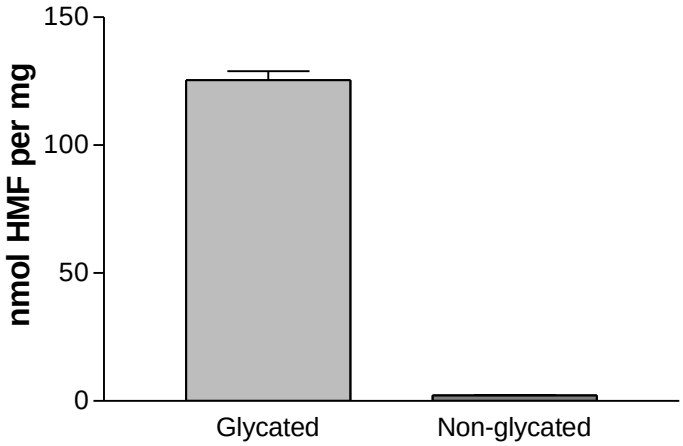


**Figure 5. 7: The extent of glycation of type I soluble collagen after *in vitro* incubation for 35 days with 1.25 M glucose, at 37°C.**

The results of the extent of soluble collagen glycation (Figure 5.7) are 125.41 (± 6.10 n=3) nmol HMF per mg for the glycated collagen 30 day incubation, 2.42 (± 0.10 n=3) nmol HMF per mg for the non-glycated (control). The control sample of collagen where no glucose was added during incubation, has a very low intrinsic level of glycation. The extent of glycation achieved by incubation with glucose was found to be approximately 60-fold that found in the control. Using the above values the extent of glycation of the collagen was calculated on a molar basis. The molecular weight of type 1 tropocollagen structure is 283 kDa (Agrawal CM et al., 2014 ). Hence, 1 mg of collagen is equivalent to $3.53 \times 10^{-6}$ mmol (i.e., 3.53 nmol). Thus, the molar glycation ratio of HMF to collagen was as follows. For the glycated collagen sample, the molar glycation ration of HMF to collagen is 125.41/ 3.53 = 35.5. This indicates that each tropocollagen molecule had been modified (to the nearest whole number) by 36 glucose additions (i.e., 12 per collagen α chain).

### 5.3.5 Discussion

The low level of intrinsic glycation of soluble collagen found in the control sample is presumed to be a consequence of the exposure of the collagen to glucose *in vivo*, and which would have occurred during the lifespan of the tissues (in this case, skin).

The level of achieved for glycation of the soluble collagens following 35 day incubation was quadruple those seen in vivo (Miksik and Deyl, 1991). This is almost certainly attributable to the fact that they were exposed to a higher level of glucose than found *in vivo*.

It is not known whether there is a maximum level of glycation achievable since theoretically collagens that are turned over quickly may not be exposed indefinitely to sugars. There are 106 lysine residues per tropocollagen molecule, 38 in α-1 and 30 in α-2, see Figures 5.8 and 5.9. However, it is noteworthy that not all lysines in the alpha chains may be glycated. In terms of future work it would be interesting to determine whether an upper limit to the extent of glycation might be reached experimentally. Regarding the possible number of glucose additions to proteins it is possible to gain some insight into which lysines might be modified. The NetGlycate 1.0 Server of non-enzymic glycation prediction can be used to gauge this. This predicts the glycation of lysine residues in mammalian proteins. The data used to develop this prediction program was obtained from the amino acid sequences extracted from the UniProt database for a number of proteins (Bairoch et al., 2005), in which only experimentally verified glycation sites were used. For the type I tropocollagen a prediction of 13 glycations is possible (Johansen et al., 2006). Inserting the amino acid sequence of the individual type I collagen alpha chains into NetGlycate 1.0 Server glycation prediction program, gives a prediction of 13 glycations sites

per tropocollagen molecule. A total of 5 glycation sites in α-1 and 3 glycation sites in α-1 chains, appendix 3 and 4.

Clearly however, here, the soluble collagen samples incubated 35 days was found to have 36 modifications per tropocollagen molecule. This would indicate that more than the predicted number of glycations is achievable. Overall the values of glycation levels achieved are comparable with observations of glycation published (Miksik and Deyl, 1991) of 29.13 ± 3.15 nmol HMF per mg of for normal human skin collagen.

QLSYGYDEK STGGISVPGP MGPSGPRGLP GPPGAPGPQG
FQGPPGEPGE PGASGPMGPR GPPGPPGKNG DDGEAGKPGR PGERGPPGPQ
GARGLPGTAGLPGMKGHRGF SGLDGAKGDA GPAGPKGEPG SPGENGAPGQ
MGPRGLPGER GRPGAPGPAG ARGNDGATGA AGPPGPTGPA GPPGFPGAVG
AKGEAGPQGP RGSEGPQGVR GEPGPPGPAG AAGPAGNPGA DGQPGAKGAN
GAPGIAGAPG FPGARGPSGP QGPGGPPGPK GNSGEPGAPG SKGDTGAKGE
PGPVGVQGPP GPAGEEGKRG ARGEPGPTGL PGPPGERGGP GSRGFPGADG
VAGPKGPAGE RGSPGPAGPK GSPGEAGRPG EAGLPGAKGL TGSPGSPGPD
GKTGPPGPAG QDGRPGPPGP PGARGQAGVM GFPGPKGAAG EPGKAGERGV
PGPPGAVGPA GKDGEAGAQG PPGPAGPAGE RGEQGPAGSP GFQGLPGPAG
PPGEAGKPGE QGVPGDLGAP GPSGARGERG FPGERGVQGP PGPAGPRGAN
GAPGNDGAKG DAGAPGAPGS QGAPGLQGMP GERGAAGLPG PKGDRGDAGP
KGADGSPGKD GVRGLTGPIG PPGPAGAPGD KGESGPSGPA GPTGARGAPG
DRGEPGPPGP AGFAGPPGAD GQPGAKGEPG DAGAKGDAGP PGPAGPAGPP
GPIGNVGAPG AKGARGSAGP PGATGFPGAA GRVGPPGPSG NAGPPGPPGP
AGKEGGKGPR GETGPAGRPG EVGPPGPPGP AGEKGSPGAD GPAGAPGTPG
PQGIAGQRGV VGLPGQRGER GFPGLPGPSG EPGKQGPSGA SGERGPPGPM
GPPGLAGPPG ESGREGAPGA EGSPGRDGSP GAKGDRGETG PAGPPGAPGA
PGAPGPVGPA GKSGDRGETG PAGPTGPVGP VGARGPAGPQ GPRGDKGETG
EQGDRGIKGH RGFSGLQGPP GPPGSPGEQG PSGASGPAGP RGPPGSAGAP
GKDGLNGLPG PIGPPGPRGR TGDAGPVGPP GPPGPPGPPG PPSAGFDFSF
LPQPPQEKAH DGGRYYRA

**Figure 5. 8: The amino acid sequence of alpha 1 chain of the collagen molecule.** Lysine residues which are potential sites for glycation (highlighted red).


**Figure 5. 9: The amino acid sequence of alpha 2 chain of the collagen molecule.** Lysine residues which are potential sites for glycation (highlighted red).

## 5.4 *In vitro* glycation of type I insoluble collagen

Type I collagen from bovine Achilles tendon was also chosen as a representative substrate for glycation and for investigations into the susceptibility to proteolysis by the *E. faecalis* OG1RF secreted protease fraction. This was chosen because type I collagen makes up 70 % of the total collagen in the dense connective tissue of the periodontal ligament (Berkovitz et al., 2002).

Some workers, have used type I insoluble collagen which was extracted from rat tail tendons as a glycatable substrate (Reiser et al., 1992, Bailey et al., 1995). Reiser et al simply incubated the collagen fibres *ex vivo* from rat tail tendons with glucose (500 mg per ml or 2.78 M) in 20 mM sodium phosphate buffer containing 3 mM sodium azide at 37°C for 24 h. Bailey et al, incubated the collagen with either glucose (24 mg per ml or 0.13 M) or ribose (2 mg per ml), in phosphate-buffered saline solution with incubation times ranging from 2-30 days. Since access to collagen preparations from either animal or human tissues was not possible in this project, freeze dried preparations of insoluble bovine Achilles tendon were used because of the ease of availability commercially and was obtained from Sigma-Aldrich Ltd (product number C9879). These products are usually prepared by the method described by Einbinder and co-workers (Einbinder and Schubert, 1951), this involves cleaning the tendons of non-collagenous tendon tissues, cutting into small pieces, and then washing with 2 % sodium phosphate buffer to remove any soluble proteins. The insoluble tendon fragments are then extracted for 6 days at 0°C with 25 % potassium chloride solution which removes the glycosaminoglycan / proteoglycan fraction. However, compared to collagen fibres obtained from tissues, commercial insoluble freeze dried collagen substrates like this present a number of technical difficulties, both in terms of *in vitro* glycation and in their use as a protease substrate. Physically, this type of collagen preparation is usually in the form of freeze dried, finely divided fibres or as fine

granules. In this lyophilised state it presents a problem at the molecular level in terms of the access to which the glucose in the incubating solution has to the individual fibrils and subsequently to the tropocollagen molecules. Also, native insoluble collagen preparations are, by definition, highly cross linked, and so the number of free lysine side chains (which are normally involved in such cross links) may be reduced in number, and subsequently less would be available for glucose modification. As a consequence this may influence the overall degree of *in vitro* glycation. Additionally, some of these insoluble preparations are finely divided to different degrees and therefore would have differences in the total surface area in suspension during the glycation procedure. To get round this problem it was decided to take advantage of the observation that the highly cross linked collagen fibre preparations, although not being solubilised by acid treatment (as was the case of the dried type I soluble collagen), can become hydrated by exposure to dilute organic acids, for example 0.05 M acetic acid (Gilmcher and Katz, 1965).

### 5.4.1 Preparation of type I insoluble collagen substrate for glycation

For these *in vitro* experiments 1 g of the bovine Achilles tendon collagen was suspended at a concentration of 1 % (w/v) in 0.05 M acetic acid and then allowed to swell at 4°C for 48 h. The suspension was periodically stirred to ensure even mixing. After this time, the hydrated swollen collagen had taken on the consistency of a thick wall paper paste. To render this suitable for glycation, the acetic acid in the suspension was neutralised by the addition of 1 tenth volume of 1M $Na_2HPO4/Na_2HPO4$ buffer, pH 6.8. At this point, following the addition of the neutralising buffer solution, the consistency of the sample went from being a paste-like to that of a flocculent suspension. To achieve an even dispersion of the individual hydrated swollen particles, the collagen suspension was then homogenised using an Utra Turrax (IKA LABORTECH) tissue homogeniser for 5 min. This

preparation was then divided into two equal aliquots, one for the glycation reaction and the other for the non-glycated control.

### 5.4.2 Glycation procedure for the type I insoluble collagen

To each of the above collagen samples was then added sodium azide and penicillin/streptomycin solution to act as antimicrobial agents as described above. For the glycation preparation powdered D-glucose (Sigma-Aldrich Ltd., product number G5500) was added slowly until dissolved to bring the concentration to 1.25 M (this being equivalent to the maximum concentration as used for the gelation glycation experiments). Both samples were then incubated at 37°C for 35 days, and solution allowed to dry, thereby increasing the concentration of the reactants.

Following the incubation step, the samples were dialysed to remove the glucose and buffer salts. For this, the collagen was transferred to pre-boiled dialysis sacs as used above, and dialysed against running tap water for 8 h, followed by dialysis against two-replicate 2 litre changes of deionised water at 4°C, during which the dialysis water was stirred. The samples were then assayed for the possible presence of any residual unbound glucose using the glucose oxidase assay kit (as described above for the gelatin and soluble type 1 collagen glycation experiments). This is an important step to ensure that all glucose was removed from the samples through the dialysis step since any contaminating glucose would react on heating to form HMF (Penín et al., 2017), which would interfere with an accurate assessment of the glycation. These samples were found not to contain any residual glucose and so were then stored frozen as aqueous suspensions at -20°C until required.

### 5.4.3 Assessment of the extent of glycation of type I insoluble collagen

The type I insoluble collagen samples after the dialysis treatment were then assayed for the extent of glycation using the HMF assay as described by (Fluckiger and Winterhalter, 1976). For this, three 1 ml aliquots of the collagen suspensions were pipetted into pre-weighed lockable snap-top microfuge tubes, and then heated to constant weight by evaporation of the water on a heating block at 60°C. Once these had achieved a reproducible constant weight, the weight of collagen in each tube was determined gravimetrically by subtracting the starting from the final weight. To each of the dried samples, was next added 0.5 ml of deionised water, followed by 0.25 ml of 1.5 M of oxalic acid to achieve the correct molarity of oxalic acid for the hydrolysis step (i.e., 0.5 M). These were then capped and heated at 100°C for 4 h. After this time, they were cooled to room temperature and 0.25 ml of 40% (w/v) TCA was added and these were left at room temperature for 30 min to precipitate the denatured protein, and then centrifuged at 14000 g for 5 min at room temperature to sediment the protein. To 0.8 ml of each sample was added 0.2 ml of 0.05 M TBA, and this was allowed to react by heating at 40°C for 1 h. The oxalic acid hydrolysis step released the covalently bound glucose in the form of HMF, which was then reacted with TBA to produce the yellow-orange coloured chromogen. Once cooled, the samples were then transferred into cuvettes and the absorbance of the chromogen read at 433 nm spectrophotometrically. The molarity of the HMF in each of the original suspensions of collagen was then determined by reference to the HMF standard curve (Figure 4.1) as described above, and from this the level of HMF per mg collagen calculated (Figure 5.1).

## 5.4.4 Results

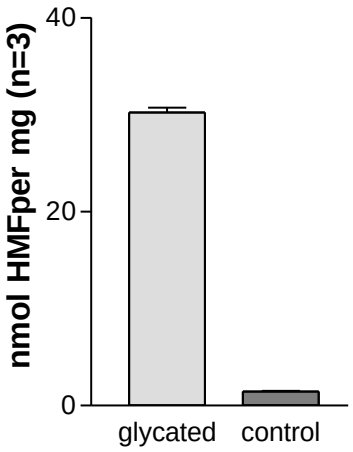


**Figure 5. 10: The extent of glycation achieved for insoluble type I bovine Achilles tendon collagen following incubation with glucose at 37°C for 35 days.** A 10 % w/v suspension of collagen was incubated with 1.25 m glucose.

The results obtained after 35 day incubation procedure using a glucose concentration of 1.25 M resulted in a glycation level of 30.23 (± 0.48; n=3) nmol HMF per mg collagen. For the non-glycated collagen sample this was 1.45 (± 0.09; n=3) nmol HMF per mg collagen. The sample incubated with 1.25 M glucose was twenty-fold more glycated than the control. These values are within the ranges of HMF levels found in the insoluble collagens extracted from both human and animal species (Miksik and Deyl, 1991). For example, human aortic collagen glycation values of between 22.82 and 29.13 nmol HMF per mg collagen were found.

Using the current data it is possible to theoretically calculate the extent of glycation of the collagen on a molar basis i.e. as a ratio of the amount of hmf to the molar amount of collagen. The molecular weight of type I tropocollagen structure (including the N- and C-telopeptides and the helical segments) is 283 kDa (Agrawal CM, 2014). Hence, 1 mg of collagen is equivalent to 1 ÷283,000 = 3.53 x $10^{-6}$ mmol. This is $10^{-3}$ micromol which is 3.53 nmol. Thus, the molar glycation ratio of HMF to collagen is 30.23 divided by 3.53 =

8.6. This indicates that each tropocollagen molecule had been modified by 9 glucose additions (to the nearest whole number) (i.e., 3 per collagen α chain). This is a lower value compared to that achieved during glycation of the soluble type I calf skin collagen and porcine skin-derived gelatin, under the same experimental glycation conditions, where values of approximately 60 nmol HMF per mg were found.

### 5.4.5 Discussion

The *in vitro* glycation methods used here achieved levels of glucose modifications which were higher than those obtained for collagens from tissues of man and other species (Miksik and Deyl, 1991). However, the lower level of glycation achieved with insoluble collagen compared to gelatin or soluble collagen is not surprising given the fact that bovine Achilles tendon used here would, by virtue of its insolubility, have a high degree of cross-linking, and consequently a reduced number of free lysine sidechains available for glycation. Furthermore, the tightly packed cross linked structure of collagen fibres may reduce access of glucose molecules (Slatter et al., 2008). Therefore, it may be argued that overall low extent of glycation for the insoluble collagen (despite the large number of lysine side chains potentially available for modification) may largely be determined by the cross-linked nature of the fibrils. Additionally, other factors may be contributing to this reduced level of glycation achieved as compared to the soluble type I collagen and gelatin. Irrespective of the complication of cross links in decreasing the number of free lysine side chains available for glycation, the susceptibility of lysine residues in any one peptide depends upon the proximity to Asp, Glu and other lysine residues (Johansen et al., 2006). Overall the number of glycation modification per polypeptide chain was 8, and tropocollagen molecule 24, which is higher than that predicted by NetGlycate as discussed above.

## 5.4 Conclusions of *in vitro* glycation of gelatin, soluble and insoluble type I collagen.

In this chapter, an *in vitro* glycation model involving incubation with glucose was used to induce non-enzymatic glycation of gelatin, soluble and insoluble type I collagen. Preliminary time course experiments using Azocoll glycated with a fixed concentration of glucose (250 mM) indicated that a maximal level of glycation might be achieved (in this case after 30 days incubation). Considering this, experiments were devised to investigate the possibility to improve upon this level of glycation in the subsequent experiments which used gelatin, calf skin soluble collagen and bovine Achilles tendon insoluble collagens as substrates, by using a higher concentration of glucose (1.25 M), and drying the solution/suspension of the reaction mixtures in the case of collagen, in which much higher levels of glycations were obtained than predicted by NetGlycate particularly for the soluble collagen. Furthermore, it was found feasible to prepare a series of gelatins that were glycated to a variable degree by incubation with a range of concentrations of glucose from 0.125 M to 1.25 M. This will make it possible to investigate any possible relationship between extent of glycation and susceptibility to protease.

# CHAPTER 6: INVESTIGATIONS INTO THE PROEASE SUSCEPTIBILITY OF GLYCATED PROTEINS

## 6.1 Introduction

The association between diabetes and endodontic infection has been discussed in chapter 2, along with the potential pathophysiological mechanisms, one of which is the formation of AGEs through glycation and their effects on the cellular response of the immune system. Glycation modifies the structure of collagen, for example through cross linking, as discussed in chapter 2. It was discussed that previous studies on the effects of hyperglycaemia on collagen degradation have yielded inconsistent results. This chapter describes the investigations into the digestion of glycated proteins of relevance to the extracellular matrix of dental tissues, including; type I soluble and insoluble collagens that were prepared as described in chapter 5. Additionally, type A porcine skin gelatin, will be used as a model substrate for collagen since its peptides are derived mainly from collagen. Their susceptibility to both host enzymes for example trypsin, and bacterial enzymes for example *E. faecalis* and *C. histolyticum* will be investigated. The results may give clues regarding some potential contributing factors of the association between diabetes and endodontic infection.

## 6.2 Growth of *E. faecalis OG1RF and* preparation of the extracellular soluble secreted protease fraction

The OG1RF strain (previously named 2Sa) was originally isolated from human saliva (Gold et al., 1975). The *E. faecalis* OG1RF secreted protease fraction contains a gelatinase and a trypsin-like serine protease. The Gelatinase which has specificity for Gly-Leu bonds, is active against gelatin and Azocoll (Makinen et al., 1989b). The trypsin-like serine protease has specificity for lysine and arginine residues. The contribution of these proteases produced by *E. faecalis* to disease has been demonstrated in a number of animal models, for example, further to the discussions above the importance of collagen degradation of *E. faecalis* in contributing to its pathogenicity has been demonstrated in rats where it was found that leaking anastomotic tissue were colonised by *E. faecalis* strains that showed an increased collagen-degrading activity (Shogan et al., 2015).

Furthermore, because an association between *E. faecalis* and endodontic disease has been demonstrated. It is relevant to investigate the susceptibility of glycated and non-glycated type I soluble collagen to degradation by these extracellular proteases.

The *E. faecalis* strain (OGRF1) from which the secreted protease fraction was obtained was kindly provided by Dr Malcolm Horsburgh, Institute of Integrative Biology, University of Liverpool. Experiments described below to prepare the extracellular secreted protease fraction from the organism were performed by Dr Smalley.

The *E. faecalis* strain OGRF1 was cultured from frozen stocks at 37°C on 5 % (v/v) horse blood (TCS Biologicals, Wirral, Cheshire) agar plates (using Blood Agar Base Oxoid Ltd.) to check for purity. Once pure colonies of the cells were obtained these were sub-

cultured into 200 ml of Brain Heart Infusion Broth (Oxoid Ltd) and grown overnight as a static culture at 37°C as described by (Yuan and Hayashi, 2009).

The cells were centrifuged at 50000 g for 30 min and the supernatant brain heart infusion (BHI) broth containing the secreted protease and other peptides was removed. The supernatant liquor was cooled to 4°C and the soluble proteins were precipitated by the addition of solid ammonium sulphate to a saturation of 60 % (Yuan and Hayashi, 2009). The amount of ammonium sulphate needed for the volume of growth supernatant to achieve 60 % saturation was calculated from data for biochemical research (Dawson et al., 1968). This allows for separation of the larger proteins from small peptides derived from both the organism and those in the BHI broth growth medium which are left in solution. In this case starting with 220ml, 79.42g of ammonium sulphate was added slowly with continuous stirring to prevent local over concentration of ammonium sulphate. The precipitation was allowed to complete by standing the supernatant overnight at 4°C.

The cloudy suspension of precipitated proteins was then centrifuged at 5000 g for 30 min at 4°C and the clear supernatant carefully decanted from the pellet. The pellet was then washed with 2 x 20 ml of 60 % saturated ammonium sulphate to remove any residual small molecular weight peptides and then it was dissolved in 2 ml of 10mM Tris, 1 mM $CaCl_2$, buffer pH 7.5 containing 14 mM NaCl. It was then filtered through a 0.2 µm filter (Pall Corporation product number 4192) to remove any residual bacterial cells. The solution was then spun through a 10 kD molecular weight cut-off filter (PALL Nanospin) to concentrate the secreted proteins. The concentrate was further washed with the above Tris/Ca buffer as above to remove residual ammonium sulphate. The volume of the secreted protease fraction was reduced to 200 µl and stored at -20 °C until required.

The concentration of the protease extract was determined using the BCA assay as described in section 5.2.1, the results was 16.7mg/ml.

## 6.3 Investigations into the susceptibility of glycated porcine skin gelatin to proteolytic digestion by trypsin, *C. histolyticum* collagenase and the secreted protease fraction of *E. faecalis* OG1RF

### 6.3.1 Introduction

The choice of gelatin as a glycatable protease substrate of relevance to the ECM of dental tissues has been discussed above. Furthermore, this product has many advantages in terms of its application as a protease substrate, since it is easy to solubilise and handle, and can be used to create gels, films or co-cast with agar to be used for analysis in a gel diffusion assay for protease activity (Vijayaraghavan and Vincent, 2013, Montville, 1983). Furthermore it has been used by some workers as an alternative to the glycated collagen model to study cell behaviour in relationship to diabetes and aging (Boonkaew et al., 2014). For these reasons, it was decided to use this for non-enzymic glycation studies.

### 6.3.2 Gelatin / agar co-cast plate diffusion assays

#### 6.3.2.1 Protease assay employing co-cast gelatin / agar as a substrate

A simple, and both qualitative and quantitative method for determining protease activity based on a plate diffusion assay has been described (Montville, 1983). In these assays gelatin (1 % w/v) is co-cast with agar (1 % w/v) from the liquid state (by heating the agar and gelatin to 100°C) in petri-dishes or square plates to create a solidified substrate. Uniform circular holes are punched in the agar gel to form wells which can then be filled with the protease of interest. The proteases diffuse through this gel to digest the protein (the agar being inert to proteolytic activity). In the experiments carried out by Montville

(Montville, 1983), the gelatin-agar plates demonstrated distinct and well-delineated zones of clearing upon digestion by the proteases of *Bacillus cereus* and *Clostridium botulinum*. The diameters of these digestion zones were found to be linearly proportional to the $\log_{10}$ of enzyme concentration and so it is useful for assaying the activity and concentration of any given protease preparation.

Vijayaraghavan and co-workers (Vijayaraghavan and Vincent, 2013) also co-cast 1 % gelatin with 1 % agar in their plate diffusion protease assays. However, in their experiments, following the incubation with proteases (from *Bacillus subtilis* strain VV) for a predetermined period, the gelatin-agar plates were stained with 0.0015 % (w/v) Bromocresol Green (BCG) for 20 – 30 min at room temperature. The proteolytic activity was determined as a clear / colourless zone whereas the undigested regions of the plates were greenish-blue in colour. This is because BCG only binds to unhydrolysed protein in the gels. This inexpensive, sensitive and rapid method in which the gelatin substrate is co-cast with agar and stained with BCG is convenient in outlining the digestion zones for analysis to determine the proteolytic activity on a plate diffusion assay.

Based on these methods which were designed to assess the effect of enzyme concentration, it was decided to use a co-cast of gelatin / agar gels in a plate diffusion assay to evaluate the possible protease susceptibility of both glycated and non-glycated gelatins. Furthermore, since BCG does not interfere with the enzyme activity (Vijayaraghavan and Vincent, 2013), it was decided to pre-stain the gelatin-agar solutions prior to casting them in the plates. Pre-staining the gelatin-agar in this way has two advantages; it creates a uniformly stained substrate, and it allows ongoing assessment of the protease activity without terminating the experiment.

### 6.3.2.2 Gelatin / agar plate diffusion assay for determination of protease activity using *C. histolyticum* collagenase

A preliminary experiment was set up using *C. histolyticum* collagenase as positive control enzyme to test the suitability of the plate diffusion method to assay the susceptibilities of glycated and non-glycated gelatin substrates.

In this assay 1 % gelatin (Sigma-Aldrich Ltd. product number G1890) was co-cast with 1 % agar (FLUKA Analytical, product number 43409250). For this, four 5 ml replicates of 1 % gelatin-1 % agar liquid solutions prepared by heating through autoclaving (at 120°C for 20 min), were stained by adding 30 µl BCG (Sigma-Aldrich Ltd. Product number 114359) to give a final concentration of 0.0015 % w/v. The gelatin-agar samples were then pipetted onto 5.5 cm diameter petri-dishes, and allowed to cool at room temperature on a flat bench to solidify. During curing, the plates were partially covered with lids to allow excess water to evaporate to prevent condensation dripping back onto the surface of the plates. Following curing of the gelatin / agar into a solid state, four 7 mm diameter wells were punched equidistantly on each plate using the wide ends of sterilised glass Pasteur pipettes. The wells on each plate were loaded with 20 µl of *C. histolyticum* collagenase (Sigma-Aldrich Ltd. Product number C5138) ranging from 0.1 to 0.0001 mg per ml in (0.14M NaCL, 0.1M Tris, pH 7.5) and incubated at 37°C. Following 7 h of incubation, clear zones of digestion were observed, example presented in Figure 6.1. The petri-dishes were scanned on HP Scanjet G4050 and images acquired via HP Solution Centre Program (settings: Large format film to HP PSE, colour, *.jpg, 300ppi, 40% scaling). The images were imported into ImageJ, the scale of the images was set using the diameter (55 mm) of the petri-dish as a reference point. The boundaries of the clear digestion zones were outlined, and from this, the areas were obtained. The area of the digestion zones was plotted versus the log of the

enzyme concentration on GraphPad Prism software version 3.03. The results are presented in Figure 6.2.

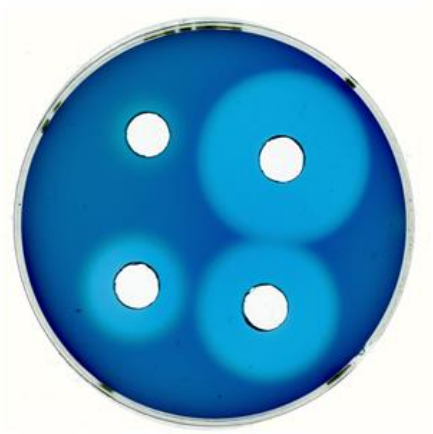

**Figure 6. 1: Example of gelatin-agar co-cast plate diffusion assay**. Digestion was carried out using BCG (0.0015 %) stained glycated and non-glycated which was co-cast at a concentration of 1 % (w/v) with 1 % agar. Incubation was carried out at 37°C for 2 days.

## Results

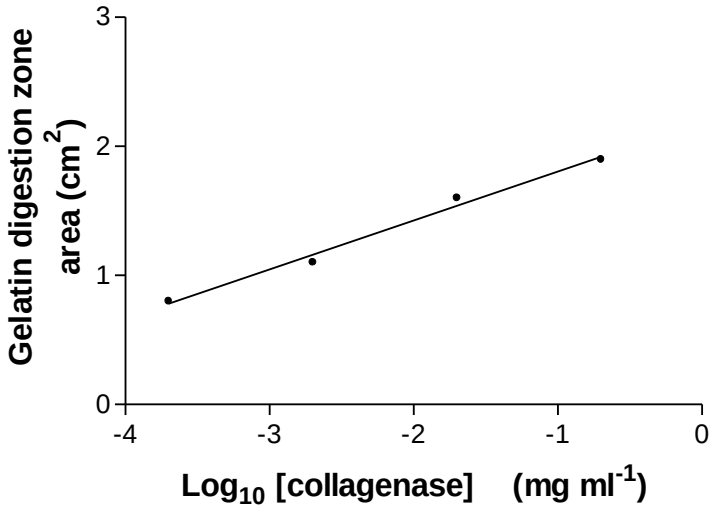


**Figure 6. 2: Relationship between digestion zone area and $log_{10}$ concentration of *C. histolyticum* collagenase for 1 % type A non-glycated gelatin / 1 % agar gel.** The gels were incubated at 37°C for 7h.

The results in Figure 6.2 show that the area of digestion zone was linearly proportional to $\log_{10}$ of C. *histolyticum* collagenase activity over the concentration range 0.1 - 0.0001 mg per ml.

## Conclusions

Although limited, there are data in the literature regarding the use of gelatin as a collagen model to study the effects of non-enzymic glycation. For example, (Boonkaew et al., 2014) incubated gelatin with ribose or methylglyoxal and found a dosed-dependent increase in cross-linking. These glycated gelatins were then used to assess the behaviour of human periodontal ligament attachment and cell spreading, in which both were reduced with increased cross-linking associated with glycation (Boonkaew et al., 2014). Consequently it was decided to employ a 1 % gelatin co-cast with 1 % agar in a plate diffusion assay at 37°C.

A method for the detection of protease activity on agar plates using 0.0015 % BCG previously described (Vijayaraghavan and Vincent, 2013), was used. However, some modifications to these methods included the pre-staining of gelatin / agar co-cast to achieve a uniform stained gel layer to allow ongoing assessment of digestion without terminating the experiment. Digestion experiments using a non-glycated gelatin preparation in this plate format with *C. histolyticum*, were carried out and it was found that it is easy to measure areas of digestion zones which could be analysed using ImageJ. The co-casting of 1 % w/v gelatin and agar was found to be very conservative in terms of substrate and for this these reason it was evaluated for studying breakdown of both glycated and non-glycation gelatin substrates.

### 6.3.2.3 Gelatin / agar plate diffusion assay for determination of protease activity using trypsin

The substrate used for these experiments was a bulk preparation of the glycated type A gelatin. This degree of glycation of this was 41.44 nmol HMF per mg. The non-glycated control for this series of investigations was 0.906 nmol HMF per mg. They were used as 1 % gelatin / 1 % agar co-cast gels. Three replicates of each were set up in 5.5 cm petri-dishes as described above. The wells in the gels in each petri-dish were then loaded with 20 µl of trypsin solutions ranging from 0.2 to 0.0002 mg per ml in 0.14M NaCl, 0.1M Tris buffer, pH 7.5. They were then incubated at 37°C. During the incubation period, expanding colourless zones of digestion in the BCG stained gelatin were observed. The gels were then scanned in batches and using the HP Scanjet G4050 and HP Solution Centre Program (settings: Large format film to HP PSE, colour, *.jpg, 300ppi, 40% scaling). The images were imported onto ImageJ and the scale of the images were set to that of the diameter of the petri dish (55 mm) as a reference point. The boundaries of the clear digestion zones were outlined, and from this, the areas were obtained. The areas of the replicate zones were then plotted versus the $\log_{10}$ of the enzyme concentration. The results are presented in Figure 6.3. The zones produced by the lowest concentration of trypsin were too small and indistinct to accurately quantify. However, measurable zone diameters could be easily determined for the zones produced by the trypsin over the concentration range 0.2-0.002 mg per ml.

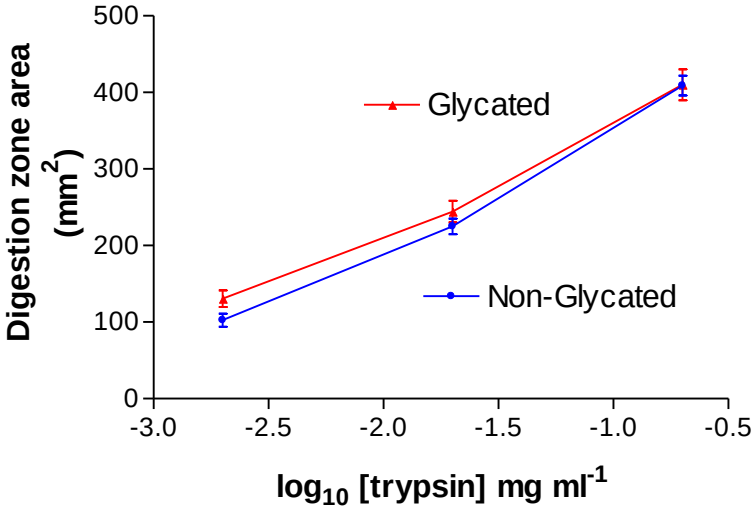


**Figure 6. 3: Plots of digestion zone area versus $log_{10}$ trypsin concentration.** Digestion was carried out using BCG (0.0015 %) stained glycated and non-glycated which was co-cast at a concentration of 1 % (w/v) with 1 % agar. Incubation was carried out at 37°C for 2 days.

It can be see that the area of digestion zones was proportional to the $log_{10}$ of trypsin concentrations over 0.2-0.002 mg per ml. However, no difference was seen between the susceptibilities of the two substrates in terms of total digestion over a 7 day period at 37°C.

### 6.3.2.6 Conclusions of gelatin / agar co-cast plate diffusion assays

The plate diffusion assay using 5.5 cm diameter petri-dishes was demanding in terms of the amount of substrates needed but more so in terms of the enzyme used, especially for proposed studies with the *E. faecalis* protease. For example, for a 5 ml 1 % gel solution- 0.5 g of gelatin is required. Given that multiple-sample as replicates are required, this would be heavy on resources. Furthermore the gelatin-agar gel formed a meniscus at the edge of the plate, producing a thicker gel-agar depth around the periphery. This was found to distort the digestion zones. Since larger diameter plates (9 cm) were even more costly in terms of substrates it was decided to search for an alternative method of producing thinner gels on a reduced scale. The method which was chosen was that

based upon the work of Thirst, in which microscope slides were used to carry the gel (Thirst, 1957). Moreover, using these methods, it was possible to avoid the use of agar as a gelling agent. This is discussed in the following section.

## 6.3.3 Gelatin diffusion assays

### 6.3.3.1 Microscope slide format for producing gelatin gels for protease assay

A method for investigated bacterial enzyme activity on a gelatin substrate that was quick and required smaller amounts of both substrate and enzyme has been used by (Thirst, 1957). In these experiments, Thirst used clean dry microscope slides which were dipped in a molten 5 % (w/v) aqueous solution of gelatin at 37-40°C. The excess liquid was then drained and the slides stood on-end to dry in the air, leaving a thin coating of gelatin on the slides. These were then stored in petri-dishes in the refrigerator to maintain the gelatin in a gel-like state. Instead of punching holes into the gelatin substrates, drops of bacterial culture containing proteolytic enzymes were spotted directly onto the slides. These were then incubated at 20°C for 2 – 20 h (and sometimes for up to 30 days), in petri-dishes containing a strip of moist filter paper to minimise evaporation from the drops and to keep the semi-dry gelatin layer moist. The slides were then stained with carbol fuchsin at the end of the incubation period. Where hydrolysis of the gelatin had occurred, clear areas around the enzyme spots indicating zones digestion of the gelatin films were seen. This method was also used to study the gelatinase of Helminth larvae, in which liquefaction of gelatin presented as clear zones on stained slides (Lewert and Lee, 1954).

This method of applying the gelatin substrate directly on microscope slides was used in the subsequent set of investigations using this substrate, but with some modifications. This included; instead of dipping the slides into an aqueous solution of

gelatin, a precise volume of the molten gelatin solution was pipetted onto each microscope slide. This produced a more precise concentration of gelatin across the whole area of the slide. Frosted-end histological slides were used but the frosted portion was not used, except for labelling.

In addition, the concentration of gelatin used was explored further because gelatins are by definition liquid rather than gel at 37°C, and it is known that as the temperature decreases and gelatins become gels they take on a helical rather than a random coil form (Djabourov et al., 1988, Gornalla and Terentjeva, 2008), and so might be considered to be structurally more similar in nature to tropocollagen molecules. Moreover for most gelatin preparations it is also known that as the gel strength *i.e.*, the protein concentration, is increased, the proportion of peptides which are able to form triple helical structures increases forming gel which resembles more a "collagen-like" matrix. Investigations to determine what gelatin percentage would remain as a solid gel at room temperature were carried out using solutions of 1 – 10 % w/v gelatin preparations on microscope slides.

#### 6.3.3.2 Investigations to determine the stability of gelatin gels in the absence of agar

To be able to examine gelatin in a "gel" rather than a liquid state, it was important to initially determine the appropriate concentration of gelatin that formed a stable gel at room temperature. This was determined using gelatin that was not pre-treated. A series of gelatin solutions ranging in concentration between 1 and 10 % (w/v) were prepared by suspending the gelatin in deionised water and then heating at 60°C for 20 min. Following this, three 1 ml replicate volumes of each solution were pipetted onto microscope slides, and allowed to evenly cover the non-frosted part of the slide (Fisherbrand FB58628). These were allowed to set on a flat and level bench at room temperature. The following

observations were made regarding the state of the gelatin; the solutions of gelatin at concentrations of 1 to 5 % did not solidify even at 4°C. The 7% concentration gelatin solution partially solidified after 20 minutes at room temperature and became more stable after a further period of 60 min at 4°C. In contrast to concentrations of 8 and 10 % which set within 10 minutes and remained stable at room temperature for over 24 h. However, concentrations of 8 to 10 % w/v were found to be too viscous to pipette even when molten whereas the 7 % solutions did not have this problem. It was concluded that the 7% gels were more convenient to handle and therefore used in subsequent experiments.

## Discussion

Investigations into the ability of 1 -10 % (w/v) type A gelatin solutions to set indicated that a 1 % solution would not remain in the form of a gel even at 4°C. Therefore when co-cast with 1 % agar one would expect it to be a in a liquid form and hence the peptides would more likely to be as a random coils rather than taking on a helical configuration. If this is the case then diffusion of the protease through the agar / gelatin gel matrix is limited by the presence of the agar (which acts as the gelling agent). It is noteworthy that (Boonkaew et al., 2014) who examined the spreading of periodontal ligament cells through glycated gelatin used microtitre plate wells which were coated with an adherent layer of 5% w/v gelatin. So, it may be argued that the use of gelatin gels of a higher percentage and hence a greater helical content might be more representative of the tropocollagen structures found within the extracellular matrix. So, it may be reasoned that the protease diffusion assays in the agar-gelatin form (Montville, 1983), whilst being suitable for determining enzyme concentrations, are less suited determination of the susceptibility of the substrate, which in a liquid form would present no resistance to protease which may have easy access to attack the peptides. Given that glycation is known

to retard collagen peptide proteolysis (Paul and Bailey, 1996), the question arises as to whether the use of a 1% gelatin/1% agar diffusion assay it is likely any differences in digestion observed between the glycated and non-glycated forms may not be observable. In hindsight the 1 % gelatin / 1 % agar co-cast lends itself better for qualitative assessment of enzymes and enzyme concentration. Because of this it was decided that the glycated and non-glycated gels should be examined in the absence of agar, but at a higher concentration.

The minimum concentration of gelatin alone that remained solid at room temperature was found to be 7 %. It is noteworthy that on wet basis, collagen forms 3-5 % of the pulp (Kaur and Kakar, 2014). Therefore concentrations of gelatin in the range of 1-7 % are appropriate, where the gelatin can be prepared in a gel like state at a higher percentage i.e., 7 %.

#### 6.3.3.3 Trypsin digestion of gelatin with different degrees of glycation

Trypsin, a ubiquitous host enzyme closely related to other serine proteases for example those expressed by macrophages (Chen et al., 2003), was used as a representative of host enzymes of those that maybe encountered during inflammation and remodelling. This was to assess the impact of glycation of these proteins to degradation. This may have potential consequences on remodelling and repair.

Glucose modifications to lysine residues may have the predicted effect of reducing susceptibility to trypsin, and so this was tested using the series of gelatin samples which had been glycated to different degrees. It was therefore decide to firstly examine the gelatin susceptibility using the microscope slide format as described by Thirst but with some slight modifications. For this 3 ml aliquots of each of the gelatins described above were stained with BCG but at 0.0075 % (w/v) to increase the contrast. Then, 1 ml of each

was pipetted onto a microscope slide and allowed to set at room temperature for 10 min, and then dried overnight at 4°C giving uniform films.

Following this, 5 µl volumes of trypsin (at 0.005, 0.05 and 0.5 mg per ml) in 50mM Tris - 5mM $CaCl_2$, pH 7.5 buffer, were spotted onto each slide, and then placed in petri-dishes on top of circles of dampened Whatman filter paper to prevent complete evaporation of the enzyme solution (Thirst, 1957), and incubated at 37°C. Clear zones of digestion were observed over a period after 4 days. The gelatin films were scanned batch-wise to ensure equal lighting conditions using the HP Scanjet as above using Large format film to HP PSE, colour, *.jpg, 300ppi, 40% scaling settings. This time, instead of measuring the area, the integrated densities of these digestion zones were computed as these represent a combination of both the area (zone) which has been digested and the degree to which the protein within the zone has been degraded. The image was then imported into imageJ, the scale was set using the width of the slide (25 mm), the digestion zones were outlined using the versatile magic wand setting (Figure 6.4), which identifies contiguous pixels of equal intensities which identifies the boundaries, and the integrated density computed. The integrated density is the average pixel intensity over the whole zone multiplied by the total area of the zone. This is both a measure of how far the protease has diffused and the extent to which the gelatin has been broken down (clearer zones are brighter in colour and give a higher intensity). Only digestion zones produced by the 0.5 mg per ml trypsin were analysed because the digestion zone boundaries of lower trypsin concentrations were less distinct. The integrated density was then plotted against the extent of glycation of the gelatin. The results are presented in Figure 6.5.

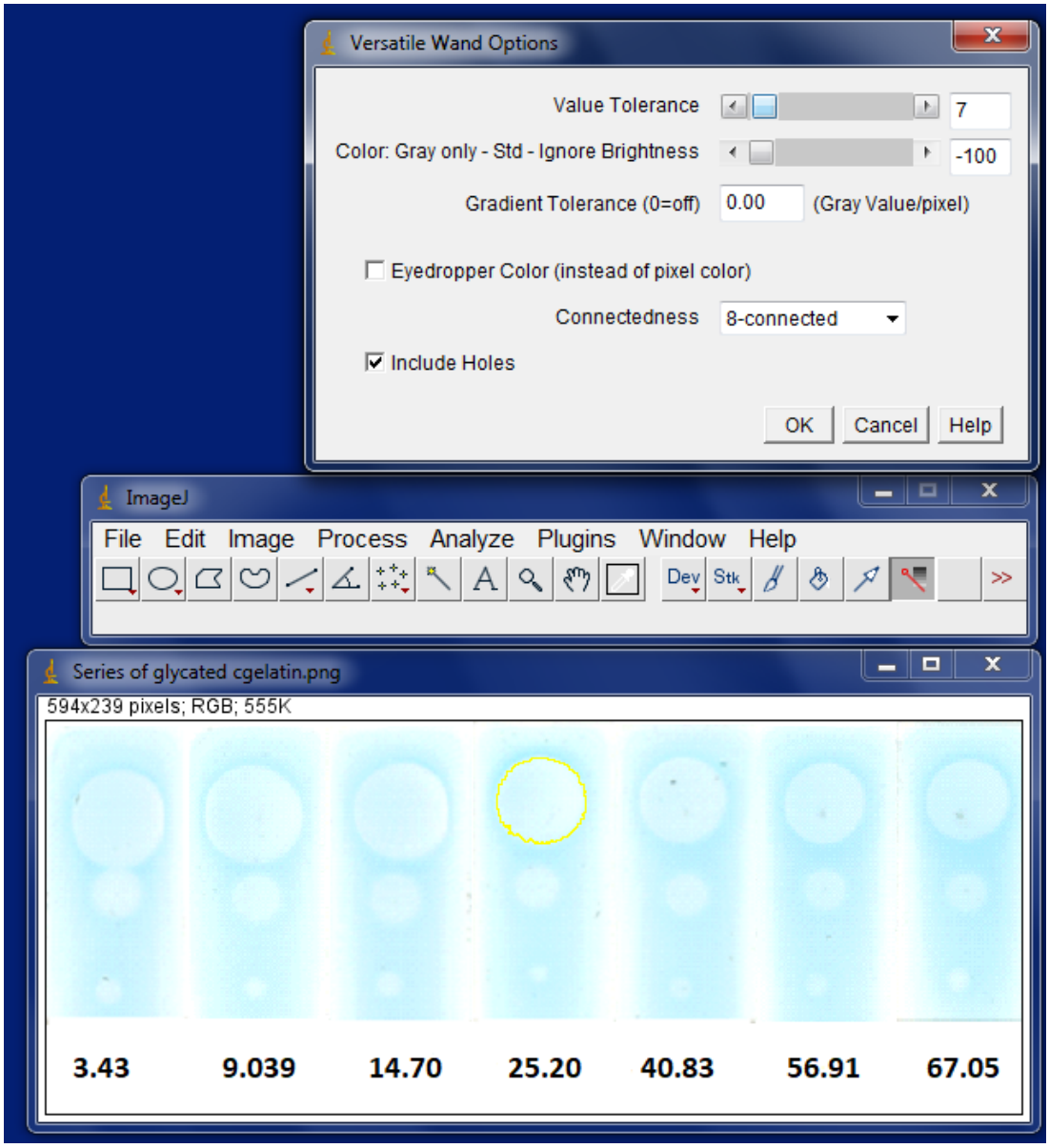


**Figure 6. 4: Example of a digestion zone selected using the versatile magic wand tool in Imagej from which the integrated densities of the digestion zones were computed.** The tolerance level was set (in this case 7) to delineate the edge of the digestion zone

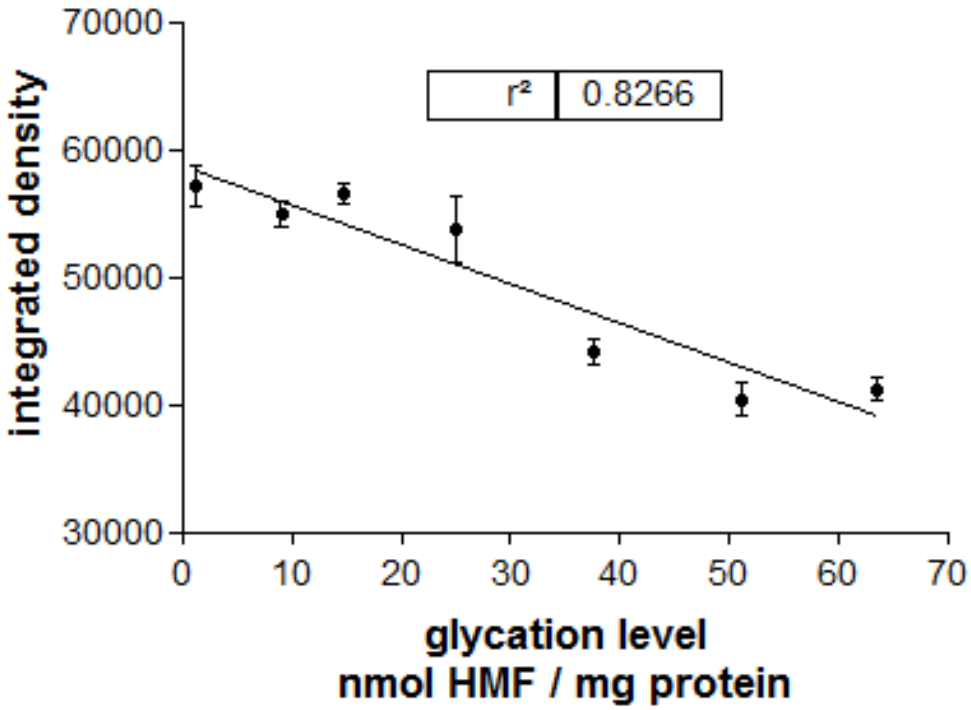


**Figure 6. 5: Trypsin digestion of dried 2 % gelatin films with variable glycation.** Digestion after 4 days at 37°C by 0.5 mg per ml trypsin was assessed as a function of the loss of BCG staining as measured by integrated density using ImageJ.

These data showed that there was an inverse relationship between the extent of gelatin glycation and degree of trypsin digestion.

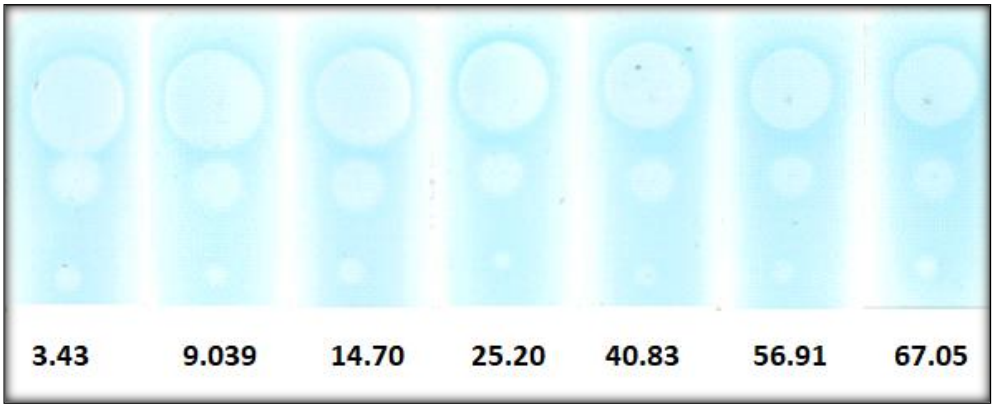


**Figure 6. 6: Examples of trypsin digestion of BCG stained 2 % films of gelatin glycated to different degrees.** Incubation was carried out at 37°C for 4 days with trypsin at concentrations of 0.005, 0.05 and 0.5mg per ml. The extent of glycation of each gelatin is noted on each slide.

## Discussion

Using a modification of the method described by Thirst (1957) a microscope slide format was used to produce dried films of gelatin which had been glycated to varying degrees by exposure to glucose. Integrated denisty measurements of the digestion zones allowed assessment of both the extent to which the trypsin had diffused through the film, and the degree to which that digestion zone had been cleared by the enzyme.

The results obtained in Figure 6.5 indicate the glycation reduces the susceptibility of type A gelatin to degradation by trypsin. The extent of breakdown of gelatin by trypsin was negatively correlated with the extent of glycation. This decreased level of digestion can be explained on the basis that lysine recognition sites for trypsin become blocked by glucose modifications. This result was in agreement with findings reported by (Deng et al., 2017), in which the extent of hydrolysis of α-lactalbumin by bovine and porcine trypsin decreased with increasing levels of glycation. This supports the hypothesis that the ability of trypsin to attack glycated cleavage sites was reduced. Importantly, this may have implications for wound healing. It is known that trypsin-like serine proteases are expressed by neutrophils and macrophages at sites of inflammation (Chen et al., 2003). If

tropocollagen molecules become unwound as a result of other protease activities (such as matrix metalloproteinases) in the course of normal tissue remodelling then peptides thus released may not be as easily degraded and may retard the tissue reparative process within the extracellular matrix.

#### 6.3.3.4 *C. histolyticum* collagenase digestion of glycated gelatin gels

Although the use of dried gelatin films lends itself well to quantitative investigations into the enzyme activities (thirst, 1957), it is difficult to know what concentration of gelatin is achieved as the film dries and when the spots of enzyme solution are applied. In view of this it was also decided to assess degradation of the glycated gelatins in the form of 7% hydrated gels. To provide sufficient substrates for this series of experiments, a bulk preparation of glycated and non-glycated gelatin was made using 1.25 M glucose which was incubated as described above. This yielded a glycated gelatin which was 41.44 nmol HMF per mg. The control non-glycated sample was 0.90 nmol HMF per mg put in these values 41.44 nmol HMF mg per ml and non-glycated/control 0.906 (± 0.12 n=3) nmol MF mg per ml. The preparations were standardised by firstly concentrating them by boiling to reduce the volume by evaporation. Following this, accurate quantitation of the protein concentrations was determined using the BCA assay and the gelatin solutions were diluted appropriately to give final concentrations of 7 % w/v.

Initial experiments were set up to test this system using *Clostridium histolyticum* collagenase as a positive control. In this model where the concentration is increased to 7 %, the peptides are more likely to be in the helical form (which accounts for the gelling properties (Djabourov et al., 1988, Gornalla and Terentjeva, 2008)). In this form it may be argued that the gel-like state is more akin to a collagen matrix that may be found within tissue.

Whilst still molten, they were stained with BCG final concentration 0.0015 % (w/v), and three 1 ml replicates were applied to microscope slides and allowed to set at room temperature for 10 min, prior incubating at 4°C for 24 h in petri-dishes (on dampened absorbent paper with dishes covered with lids to prevent drying) to allow the gels to set uniformly. Following this, 5 µl aliquots of 0.5, 0.05, and 0.005 mg per ml solutions of *C. histolyticum* collagenase in 0.14M NaCL, 0.1M Tris, pH 7.5, were pipetted onto each slide, and incubated at room temperature during which the gels remained stable.

Following the appearance of digestion zones, the slides were scanned as above and the digestion zones produced by 0.5 mg per ml *C. histolyticum* collagenase after 10 h were the most distinct and analysed as above. The results are presented in Figures 6.7 and 6.8.

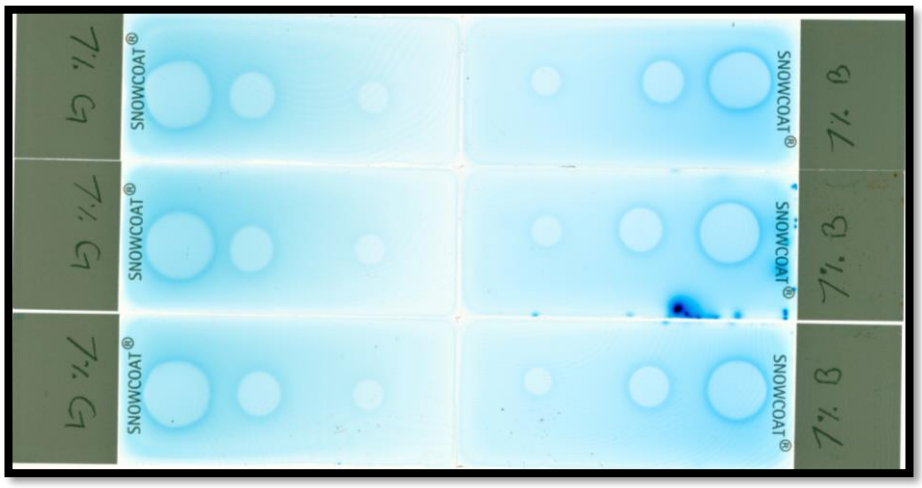


**Figure 6. 7: *C. histolyticum* collagenase digestion of 7% gelatin gels.** Replicate Slides carrying gels composed of glycated (left) and non-glycated (right) gelatins were incubated with 0.5, 0.05 and 0.005 mg per ml *C. histolyticum* collagenase in 0.14M NaCL, 0.1M Tris, pH 7.5, at 4°C for 10 h.

Digestion zones of 0.5 mg per ml collagenase were well defined and were used to calculate the integrated densities, and it observable that the area of these zones are greater with the glycated (left) as compared to the non-glycated (right) 7 % gelatin slides. These zones were analysed using imageJ, results presented in Figure 6.8.

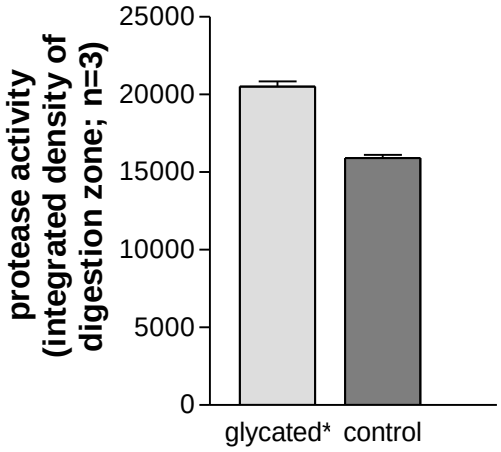


**Figure 6. 8: Digestion of glycated and non-glycated 7 % gelatin gels by C. *histolyticum* collagenase (n=3).**

The results in, Figure 6.8, show that *C. histolyticum* collagenase was able to digest both glycated and non-glycated gelatin following incubation at 4°C for 10 h. However, there was an increase in the integrated density of the digestion zones of the glycated gelatin compared to the non-glycated counterpart, indicating that the glycated gelatin was more susceptible to breakdown compared to the non-glycated counterpart.

## Discussion

Following the 10 h of incubation, it was found that the digestion of the glycated gelatin by *C. histolyticum* collagenase in the form of 7 % gels was greater than the non-glycated counterpart. This difference was observed at the highest concentration of *C. histolyticum* - 0.5 mg per ml. The reasons for this are not entirely clear. However, a possibility that the enzyme more easily diffused into the glycated gel as it was noticed that the spots appeared to spread more easily when applied to gel surface when initially applied; it may also be likely that the enzyme gains easier access through the glycated gel matrix by diffusion compared; and the glycated gelatin may intrinsically be more susceptible to attack by the enzyme through some change in its physical properties; one such possibility being a decrease in the number of positive charges which would otherwise

be imparted by lysine residues. However, although the extent of glycation was 45-fold greater than that of the control, it is debatable as to how much glycation modification of a relatively small number of lysine residues might influence the enzyme activity. *C. histolyticum* collagenase attacks the tropocollagen molecule at Gly-X bonds and so potentially could cleave at approximately 300 sites along peptides derived from individual alpha chains. Therefore it is felt unlikely that modification of lysines or arginines by glycation had a major impact on the binding of these enzymes to their target side. However, modification of lysine residues by glycation may alter the interaction between the alpha chains of the tropocollagen molecule, altering the configuration of the molecules (Tian et al., 1996). This may explain that the increased susceptibility to breakdown may be attributed to the fact that glycation affects the shift from more of a helical to a random coil arrangement resulting in greater diffusion of the enzyme through its matrix and subsequently enhanced access for digestion of the peptides.

### 6.3.3.5 Trypsin digestion of glycated gelatin gels

A similar experimental setup as above was used to examine the susceptibility of 7 % gels to trypsin digestion. Trypsin was applied as 5 µL spots in 0.14M NaCl, 0.1M Tris buffer, pH 7.5, at concentrations of; 0.005, 0.05, and 0.5 mg per ml and digestion carried out at 4°C. However, for these experiments, the slides were examined and rescanned repeatedly for an extended time period. A mean of the integrated densities of the digestion zones of 0.5 mg per ml trypsin on the five-replicate slides were calculated and the results are presented in Figure 6.9.

## Results

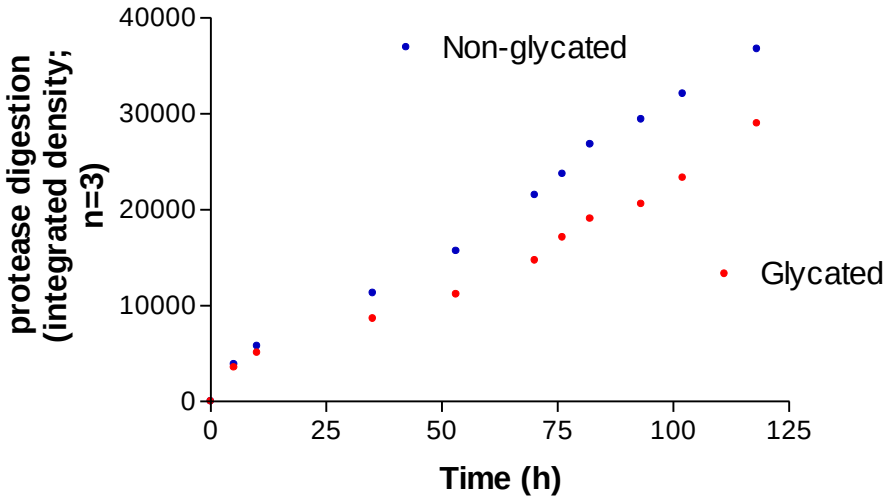


**Figure 6. 9: Time course of trypsin (0.5 mg per ml in 0.14M NaCl, 0.1M Tris buffer, pH 7.5) digestion of glycated and non-glycated gelatin in the form of 7 %.**

Digestion of the 7 % glycated and non-glycated gelatin films by 0.5 mg per ml trypsin was detectable after 5 h. The digestion progressively increased up to 118 h and the glycated gelatin was less digested than the non-glycated counterpart.

### Discussion

The above results showed that glycation of gelatin reduced its susceptibility to digestion by trypsin. This is not surprising, since glycation results in modification to lysine which are trypsin cleavage sites. This is in agreement with previous findings on trypsin digestion of glycated α-lactalbumin (Deng et al., 2017), where it was found that the maximum degree of hydrolysis of glycated α-lactalbumin by porcine and bovine trypsin reduced with greater degree of glycation. It cannot be ruled out also that arginine residues which are also glycation sites (Paul and Bailey, 1996), and which are trypsin attack sites, may have been concomitantly modified, resulting in reduced gelatin breakdown.

## 6.3.4 Conclusion

Initially gelatin was used as a substrate by co-casting with agar, which initially seemed to be a method conservative with regards to the amount of gelatin used. However it was found that the 5.5cm petri-dishes were heavy on both the substrate and also the enzymes. Furthermore, from the results obtained, the use of a concentration of 1 % gelatin that was co-cast with agar proved to be a useful diffusion assay for investigating enzyme concentration. However, this was not suitable for assessment of the susceptibility of glycated collagen to degradation, particularly at 1 % as the gelatin remained liquid within the agar matrix.

It was then decided to use an alternative method, in which gelatin substrate was used without agar. Since agar is not found in the pulp, this was more representative of the ECM of dental tissues. Preliminary experiments to determine the stability of gelatin gels in the absence of agar demonstrated that the lowest concentration of gelatin that remained as a stable gel at room temperature was 7 %. Percentages of 2 % as a film and 7 % as a gel

were used, and these were within similar percentages of collagen found within the wet weight of the pulp 3-5 % (Kaur and Kakar, 2014). Therefore concentrations of gelatin in the range of 1-7 % were appropriate to use in these assays.

Since trypsin will not attack native triple helical tropocollagen molecules, the individual peptide alpha chains may be degraded by this enzyme once the triple helical structure has been disrupted. Therefore although trypsin may not play an active role in any initial attack on the native collagen, it should be capable of participating in tropocollagen breakdown as a secondary effect. A negative correlation was found between the extent of glycation and degree of digestion of gelatin films by trypsin. This was not surprising since glycation modifies both lysine and arginine residues, the cleavage binding sites of trypsin. Therefore it is likely that other serine-proteases such as trypsin-like enzymes of macrophages will have a reduced activity against glycated proteins of the ECM. Thus, even though native tropocollagens might be attacked by mammalian collagenases, the resultant unwound peptides may be retarded in their breakdown. This may have potential consequences in delaying wound repair and healing, in diabetic patients.

It is noteworthy that whilst digestion of glycated 7% gels by trypsin was decreased as the degree of glycation increased, the *C. histolyticum* collagenase digestion was greater than that of the control non-glycated gelatin. Since it may be expected that the gelatin peptides are in more of a helical arrangement in the higher percentage gels (7 %) it is possible that any helical form of gelatin peptides may suffer disruption as a result of glycation in the same manner as proposed by (Stultz and Edelman, 2003b) for native tropocollagens.

## 6.4 Sirius Red F3B as a stain for collagen quantitation

### 6.4.1 Introduction

In these experiments to assess breakdown of the glycated collagens, use was made of Sirius Red F3B (SR) which is a specific stain for collagen (Junqueira et al., 1979). At the molecular level, SR which is an elongated sulphonated azo dye binds in a linear fashion to intact tropocollagen molecules (Junqueira et al., 1979). It has been shown that this dye binds to collagen through a strong interaction of its acid sulphonic groups with the basic groups of collagen molecules, for example lysine, hydroxylysine and guanidine groups of arginine (Junqueira et al., 1979). As determined experimentally, it is known that the molar stoichiometric ratio of SR binding to tropocollagen is 126 to 1 (Junqueira et al., 1979). This means that SR will bind maximally 126 dye molecules to one tropocollagen molecule leading to precipitation of the collagen (Junqueira et al., 1979, Marotta and Martino, 1985). Because of this, SR has been employed as a basis for assaying both soluble and insoluble collagens both in solution and in whole tissues (Kliment et al., 2011, Junqueira et al., 1979, Constantine and Mowry, 1968).

However, since glycation modifies the side chains of lysine (Robins and Bailey, 1972), alteration to this part of the molecule may adversely impact the binding of SR. This modification of positively charged lysine by glycation has been demonstrated by (Hadley et al., 1998), who incubated human scleral collagen *in vitro* with fructose. It was found that upon glycation of the collagen, there was a reduced uptake of phosphotungstic acid (PTA) stain. PTA binds to positively charged amino acid side-chains and leads to characteristic banding pattern of collagen seen on electron microscope. It was found that it was found that the surface charge of the fibrils was altered following glycation, demonstrated by a

reduced uptake of phosphotungstic acid stain by the glycated collagen. For this reason, it was important to investigate the impact of glycation of collagen on the binding to SR.

### 6.4.2 Sirius Red F3B binding to glycated and non-glycated soluble collagen

Samples from the glycated (1.35 mg per ml) and non-glycated (1.8 mg per ml) stock solutions of type I soluble collagen were standardised to 1 mg per ml. From these, a series of concentrations in the range of 0.025 – 1 mg per ml of collagen solutions of 1 ml each were set up by addition of deionised water. This series of collagen solutions was then stained by the addition of 0.5 ml 0.05 % (w/v) Sirius Red F3B (in 0.1 M acetic acid) and the tubes mixed end-over-end for 30 min at room temperature. This produced small precipitated particles of Sirius Red-stained collagen which were then pelleted by centrifugation at 14,000 g for 5 min. The pellets were then washed (with 0.1 M acetic acid) and re-pelleted by centrifugation twice more as above to remove all the non-bound SR dye. All the residual acetic acid was removed and then the SR dye was released from the collagen by addition of 0.6 ml 0.2 M NaOH. To aid this, the pellets were vortex mixed followed by 5 min of sonication. The solutions of the desorbed dye were then scanned spectrophotometrically in semi-micro cuvettes. The scans were background corrected for the absorbance of NaOH and the absorbance peak at 540 nm was recorded. The $A_{540}$ associated with SR released were plotted versus the series of collagen concentrations using Graphpad Prism version 3.03 software, and the results are presented in Figure 6.10.

## Results

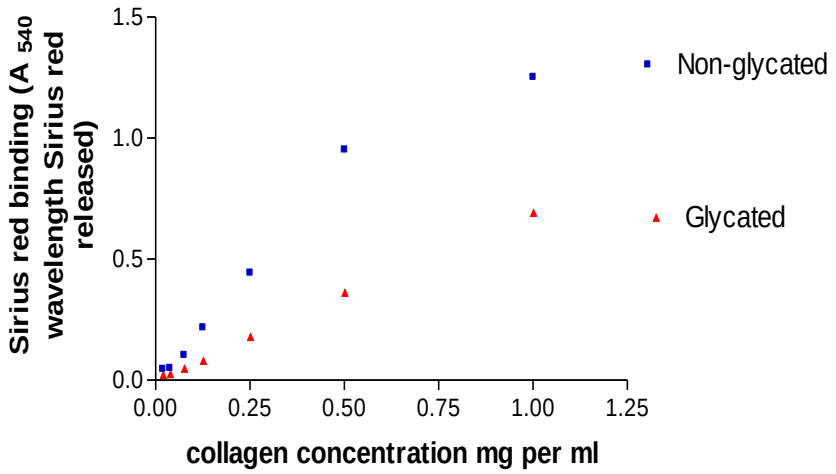


**Figure 6. 10: Calibration curve for Sirius Red F3B stained type I soluble glycated and non-glycated collagen.** The $A_{540}$ of SR released from a series of glycated and non-glycated collagen standard concentrations ranging from 0.025 to 1 mg per ml were measured after addition of NaOH.

The results in Figure 6.10 show an increase in the $A_{540}$ of SR released with increasing concentration of type I soluble collagen. Furthermore, a greater $A_{540}$ associated with SR dye released from non-glycated compared to the same concentration of glycated collagen. This indicates that glycation negatively impacts the binding of SR to collagen.

## Discussion

The calibration curve obtained from the SR-stained glycated and non-glycated type I soluble collagen preparations (Figure 6.10) revealed that that the glycated collagen bound less SR dye than the non-glycated control. This is not surprising, since glycation modifies the side chains of lysines (Robins and Bailey, 1972), this also being the target for binding of the SR sulphonic groups (Marotta and Martino, 1985). This is in agreement with previous findings using PTA to stain glycated collagen (Hadley et al., 1998). This finding is important,

because when using the SR binding as an assay method, direct comparisons of glycated and non-glycated collagens cannot be assumed using the $A_{540}$ of SR. It is however possible to quantify the amount of collagen in glycated and non-glycated collagen samples by referring to their corresponding calibration curves and this was used in the investigations of binding of *E. faecalis* cells to glycated collagen described in chapter 7. However, when designing the digestion experiments for the type I soluble collagen, it was decided to use SR to quantify the amount of collagen remaining following digestion as a percentage of the starting amount. For assays using the insoluble collagen, the samples were stained prior digestion and this method is described in the next section.

### 6.4.3 Sirius Red F3B staining of insoluble collagen

Assays for degradation of insoluble collagen are technically difficult. A commercially available assay, the Sircoll Assay (Chow and Zhang, 2011), is used for collagen quantitation and lends itself more to determination of the collagen content of tissues rather than to the handling of multiple samples such as a time course o to measure enzyme activity. It was therefore decided to experiment with a format similar to that used for the Azocoll assay, in which the collagen was stained with Sirius red prior to its use in the enzyme assay.

Collagen staining has conventionally been carried out using SR under acid conditions, however SR has also been found to stain native collagen at neutral pH and in water (Lee et al., 1998b). This staining behaviour allowed for a modification of the SR-collagen binding assay where the insoluble collagen was pre-stained before being exposed to the proteolytic enzyme fraction and thus avoided any possibility that there may be residual acid in the stained sample which might have affected enzyme activity. Thus,

during proteolytic breakup of the SR-stained collagen, SR and small SR-stained soluble peptides would be released and could be measured spectrophotometrically at $A_{540}$ (Lee et al., 1998b).

For assays involving the insoluble collagen substrates, the ability to stain with SR at neutral pH was taken advantage of. Given the molar binding ratio of SR to tropocollagen of 126:1 as stated above, the concentration of SR needed to achieve maximal staining was calculated for a 1 mg collagen per ml suspension (equivalent to 3.53 µmol). With the molecular weight of SR being 1373 Da, this represents 126 x 3.5 ~ 420 µmol per ml, which equates to 420 x 1373 = 0.615 mg per ml (i.e., 0.0615 % w/v). Based upon this, initial experiments were performed to evaluate the feasibility of pre-staining the collagen with the 0.05 % (w/v) solution of SR. However in these experiments it was found that this dye concentration was too great and unbound dye was continuously released from the sample even after extensive washing. This is not unexpected as the highly cross-linked fibrous nature of the collagen might not allow complete dye access to the whole of the fibrils and therefore individual tropocollagen structures (unlike soluble tropocollagen molecules). It is also known that SR can self-aggregate (Puchtler et al., 1988), and that this may have occurred if the SR dye did not have complete access to stain all the tropocollagens. To get round this, a 10-fold reduction in SR concentration (to 0.005 % v/v) was used in subsequent experiments and it was found that only two washes were required to remove excess SR dye.

## 6.5 Investigations into the susceptibility of glycated type I soluble calf skin collagen to proteolytic digestion by the secreted protease fraction of *E. faecalis* OG1RF

### 6.5.1 Introduction

The choice of soluble collagen as a glycatable protease substrate was discussed in section 5.3. This substrate represents immature collagen within the ECM that is not yet cross-linked. The soluble collagen substrates used in this experiment, were prepared as described in section 5.3. The extent of glycation achieved for the glycated sample was 125.41 mg per ml. The non-glycated contained 2.42 nmol HMF mg per ml which is thought to have occurred throughout the lifetime of the tissues from the animals they were obtained from.

The protein concentrations following dialysis were determined gravimetrically as discussed in section 1.35 mg per ml for the glycated, and 1.8 mg per ml for the non-glycated. The concentrations were standardised to 1 mg per ml.

### 6.5.2 Protease assay to investigate the susceptibility of glycated and non-glycate soluble collagen with *E. faecalis* protease fraction

The breakdown of the soluble collagen substrates by the secreted protease fraction was carried out using a tube assay rather than the gel diffusion method to conserve the reagent. In these assays, three 1 ml replicates of glycated and non-glycated samples were transferred to lock-top microfuge tubes. Following this, 15 µl of *E. faecalis* protease fraction (stock protein concentration of 2.5mg per ml in 10 mM Tris, 1 mM $CaCl_2$,

pH 7.5) was added to each tube bringing the protein concentration to 37 µg per ml. The tubes were then incubated at 37°C.

The amount of collagen digested, was determined indirectly by measuring the amount of collagen undigested. At set time points between, a 50 µl aliquots were taken from each tube, to which was then added 10 µl of 1 M acetic acid to arrest the protease activity. These samples were used to determine the amount of undigested collagen at the specific time-point, in the following way. The collagen samples were stained by the addition of 0.005 % (w/v) Sirius Red F3B in 0.1 M acetic acid. The tubes were then mixed end-over-end at room temperature for 30 min. The undigested soluble collagen is precipitated at this stage as the SR dye binds to it. The SR-collagen complex thus formed was then pelleted by centrifuging at 14,000 g for 5 min, and the supernatant carefully pipetted off to remove unbound SR dye. The pellets were washed in 0.1 M acetic acid twice to remove any excess unbound SR dye, and the bound dye in the collagen pellets was then released by the addition of 0.6 ml of 0.2 M sodium hydroxide. This was aided by vortex mixing for 20 s, followed by sonication for 5 min to ensure complete the dissolution of the dye. The spectral readings of $A_{540}$ of the released SR dye in the alkaline solution were then taken. This assay is based on the principle that only intact tropocollagen molecules will bind the dye and become precipitated (Lee et al., 1998a). As breakdown proceeds, The $A_{540}$ values would thus be expected to decrease with time from the initial starting level. The results are presented in Figure 6.11 and are shown as percentage decrease in collagen from the time zero point.

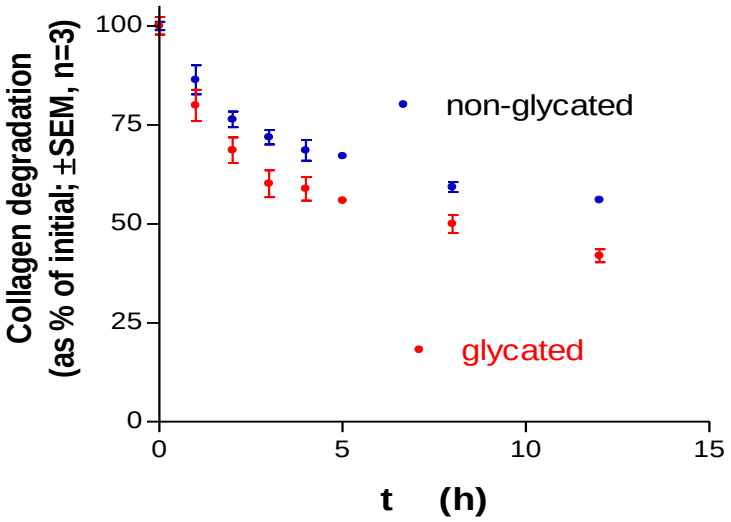


**Figure 6. 11: Glycated and non-glycated type I soluble collagen degradation, by the *E. faecalis* secreted protease fraction.** 1 mg per ml type I soluble collagen was incubated with *E. faecalis* protease fraction (37 µg per ml), in 10 mM Tris, 1 mM $CaCl_2$, pH 7.5 at 37°C. Following digestion, the remaining collagen in solution was quantified after staining with SR.

The results shown in Figure 6.11 indicate that both the glycated and non-glycated type I soluble collagen was susceptible to breakdown by the *E. faecalis* secreted protease fraction. However, there was a greater digestion of the glycated type I collagen compared to the non-glycated counterpart.

## 6.6 Investigations into the susceptibility of glycated type I insoluble bovine achilles tendon collagen to proteolytic digestion by the secreted protease fraction of *E. faecalis* OG1RF

The insoluble collagen substrates used in this experiment, were prepared as described in section 5.4. The extent of glycation achieved for the glycated sample was 30.23 nmol HMF mg per ml. The non-glycated contained 1.45 nmol HMF mg per ml.

The protein concentrations following dialysis were determined gravimetrically and standardised to 1 mg per ml. Following standardization of protein concentration, six replicate aliquots of 1 mg per ml suspensions of the glycated and non-glycated insoluble collagen samples were pipetted into microfuge tubes and the collagen was pelleted by centrifuging at 14,000 g for 5 minutes at room temperature, the supernatant decanted. Then 1 ml of SR (0.005 % w/v in $H_2O$) was pipetted into each of the collagen containing microfuge tubes, which were then vortex mixed for 5 seconds to re-suspend the collagen and then mixed end-over-end for 30 minutes at room temperature to allow staining of the collagen. The collagen was then pelleted by centrifugation at 14,000 g for 5 minutes, and the excess SR dye decanted. A 1 ml of deionised water was pipetted into each tube, which were then vortex mixed for 10 seconds to wash the collagen, centrifuged for 5 minutes and the supernatant decanted to remove the excess dye. This step was repeated to ensure all unbound excess dye was washed off.

Following staining of the collagen, 0.994 ml of de-ionised water was added to each collagen-containing tube, and vortex mixed for 10 seconds to re-suspend the collagen fibres. To five replicates of each of the glycated and non-glycated collagens was added 6 µl of the *E. faecalis* OG1RF protease extract in Tris/$CaCl_2$, pH 7.5 (giving a final buffer molarity of 10 mM Tris, 1 mM CaCl2) and a protein concentration of the protease fraction in the

digestion mixture of 0.1 mg per ml. This buffer was chosen because the optimum activity of *E. faecalis* proteases was found to be in the range of pH 7.4-7.6 (Makinen, 1989). Into the remaining one of each tubes of the glycated and non-glycated collagen, 6 µl of the same buffer was added to control for any SR dye release from the stained collagen during the experiment that was unrelated to enzyme activity. All the tubes were then incubated at 37°C for 28 h.

The enzyme activity was assessed by measuring the $A_{540}$ at time points throughout the incubation period, during which the stained collagen was pelleted by centrifuging at 14000 g for 5 minutes, and the supernatants sampled into cuvettes and the absorbance readings taken. To maintain the same concentration of the protease fraction to the substrate, the supernatant (after the absorbance reading) was then transferred back into the collagen-containing tubes, and vortex mixed for 10 seconds to re-suspend the collagen fibres and to allow the enzyme fraction re-access to the fibres. These absorbance readings were corrected by subtraction of the background control values and the results are presented in Figure 6.12.

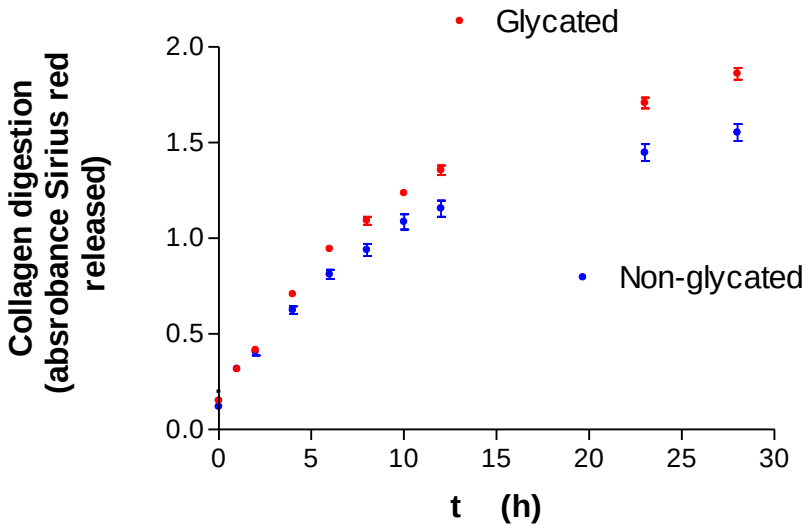


**Figure 6. 12: Degradation of glycated and non-glycated type I insoluble bovine achilles tendon collagen by the extracellular secreted protease fraction of *E. faecalis* OG1RF.**

Degradation of collagen was measured as a function of SR dye released from the pre-stained collagen substrates following correction of any loss of SR from the collagen in the absence of enzyme. Substrate concentrations were 1 mg per ml, and the reaction carried out at 37°C, in 10 mM Tris, 1 mM $CaCl_2$, and the *E. faecalis* OG1RF protease fraction concentration was 0.1 mg per ml. Statistical analysis at the end point revealed a statistically significant differences were observed between the degradation of the glycated and non-glycated insoluble collagen substrates at 28 h (n=5 p=0.009, repeated measures ANOVA).

During the incubation of type I insoluble collagen with *E. faecalis* OG1RF protease fraction, there was an increase in the $A_{540}$ associated with the release of SR dye with time. The release of SR dye was observed from the first time point measurement of 1 h, and continued through the experiment. From 5 h into the experimental period, an increase in the SR dye release became apparent for the glycated collagen compared to the non-glycated counterpart. These differences in absorbance were found to be statistically significant (p=0.009) using a repeated measures analysis of variance. This indicated an

increased susceptibility of the glycated type I insoluble collagen to digestion by *E. faecalis* OG1RF protease fraction. This differential breakdown of the glycated collagen was observed throughout the remainder of the incubation period.

## Discussion

The above results indicate that glycated type I insoluble bovine achilles tendon collagen was more susceptible to digestion by the *E. faecalis* OG1RF secreted protease fraction compared to the non-glycated counterpart. *E. faecalis* OG1RF is a known protease-positive strain which produces two proteases, one a "gelatinase" and the other a trypsin-like serine protease (Gold et al., 1975). The gelatinase has been shown to have activity against gelatin and Azocoll, and has specificity for Glycine-Leucine bonds (Makinen et al., 1989a) which are abundant in collagen. On the other hand, the trypsin-like serine protease has specificity for both arginine and lysine residues (Makinen et al., 1989a). The purification of the individual extracellular proteases from the extracellular secreted protein fraction was beyond the scope of the project. Thus, at present it is unclear which of the two proteases was the main contributor to the glycated collagen breakdown, although it may be argued that these proteases may be expressed and secreted together and if so may function in tandem to degrade extracellular matrix proteins.

However, one possibility was that from a biochemical perspective, glycation of the tropocollagen molecules would result in loss of the positive charges of the side chains of lysine which may in turn enhance the enzyme-substrate interaction (Avery and Bailey, 2006). Indeed, this modification of the positively charged lysine by glycation has been demonstrated by (Hadley et al., 1998), who incubated human scleral collagen in vitro with fructose. It was found that upon glycation of the collagen, there was a reduced uptake of phosphotungstic acid (PTA) stain which normally binds to positively charged amino acid

side-chains and leads to characteristic banding pattern of collagen seen on electron microscope.

Studies using bovine dentine, found that degradation of dentine collagen by pepsin was greatly depressed following glucose pre-treatment (Kleter et al., 1997). The reason for this is not clear as pepsin attacks Phe and Leu and to a lesser extent Glu linkages, which may be unaffected by glycation. It is thought to be cause by by acid denaturation (pH 2) of collagen during the incubation at 37°C

The reason why glycated type I soluble and insoluble collagen were more degraded by the extracellular protease fraction of *E. faecalis* OG1RF are not known. However, if the trypsin-like serine protease was involved in the degradation of the alpha chains one would expect that that the glycated collagens would be less degraded, because the lysine are modified by glycation and that would hinder the serine protease from recognising and binding to its cleavage site. On the other hand, Gly-Leu bonds which the gelatinase is specific for are numerous in the alpha chains (18 in the α-1 and 28 in the α-2 chains, a total of 64 in the tropocollagen molecule) and may present as potential attack sites in both glycated and non-glycated collagens.

Although the above speculations are possible, there is at present no experimental data on the *E. faecalis* to support this. However, a theory for increased susceptibility of collagen to breakdown during hyperglycaemia has been proposed (Stultz and Edelman, 2003a). Although lysine residues are the major sites for glycation, the terminal amino groups of arginine residues are also susceptible to modification (Paul and Bailey, 1996). Furthermore, the AGEs resulting from these modifications can be considered as either ones that form intermolecular cross-links or those that do not. Although prolonged incubation of collagen using high glucose concentrations yields collagen fibres that are resistant to breakdown (Paul and Bailey, 1996), it is important to point out that collagen extracted

from achilles tendons of rats which have been kept in short term hyperglycaemic states (28 days) is more susceptible to *in vitro* cleavage compared to collagen from normal rats (Leung et al., 1986). In addition, collagen isolated from chick calvarias that have been exposed to high concentrations of extracellular glucose for 24 h is more readily degraded relative to collagen isolated from calvarias incubated in media free of glucose (Lien et al., 1992). These important findings have been explained on the basis that arginine residues, located in areas of the alpha chains that are poor in Proline and Hydroxyproline residues, can become glycated. This destabilises the triple helical structure at certain Gly-Leu residues, leading to the cleavage of these bonds by mammalian collagenase (Stultz and Edelman, 2003b). Since the gelatinase of *E. faecalis* protease fraction has specificity for Gly-Leu bonds (Makinen et al., 1989b), this may explain the results obtained in which the glycated collagen was more susceptible to digestion. Furthermore, it was found that glycated 7 % gelatin was more digested by *C. histolyticum* compared to its non-glycated counterpart. Since *C. histolyticum* attacks the chain at Gly-X bonds, which are numerous in the chain (approx. 300), glycation of arginine residues nearby may destabilise the helical structure as discussed above, making these bonds within the glycated gelatin more susceptible to digestion.

# CHAPTER 7: DISCUSSIONS AND CONCLUSION

## 7.1 Discussion

This project was set to and achieved *in vitro* protocols for non-enzymic glycation of collagen and gelatin through incubation with glucose. It was observed that an increased degree of glycation could be achieved by incubation with increased levels of glucose over a short time period (35 days). It was possible to determine the extent of glycation of gelatin and collagen substrates by employing the widely used chemical assay (for HMF). Similar levels and above of those found in non-diabetic human tissues were achieved (Miksik and Deyl, 1991). The levels of glycation for the insoluble collagen were similar to those found in collagen of aortic tissue, 27.15 nmol HMF per mg protein, obtained from rats with experimentally induced diabetes (Deyl et al., 1990).

Regarding the methodologies used here, it was possible to produce variably glycated gelatin fractions which could be used as a stable gel to examine their susceptibility to protease enzymes. This enabled the observation that increased gelatin glycation correlated negatively with susceptibility to trypsin. Type I soluble and insoluble collagens were not amendable to this because of the quantities of substrates needed and the fact that the insoluble collagen could not be employed in this way to form gels. Nevertheless, the use of higher percentage gelatin gels might be considered as a model for the collagen matrix. Had time and resources permitted, it would have been informative to determine what effects glycation to variable degrees may have had on breakdown of native collagens. In addition, it would also be useful to make comparisons between other batches of glycated gelatin and collagen to enable more repeat measures of their protease

susceptibilities. Furthermore, increasing the number or replicates would improve the reliability of the experiment.

Following preparation of the glycated substrates, assays were established in which glycated and non-glycated gelatin could be used in the form of dried films or gels, and soluble and insoluble collagens were incubated in tube assays to test their susceptibilities to host and bacterial proteases. As a result, it was found that the glycated collagen was more susceptible to digestion by the secreted protease fraction of *E. faecalis*, compared to its non-glycated counterpart. With regards to gelatin, a correlation between increasing extent of glycation and resistance to digestion by trypsin was observed. In contrast to the retardation of trypsin digestion of glycated gelatin, collagenase of *C. histolyticum* was able to digest glycated gelatin to a greater degree.

In the last phase of the project, attention was concentrated on the protease fraction of *E. faecalis* (strain OG1RF). *E. faecalis* was examined because it is an organism associated with failed endodontic treatment, with virulence factors including the expression of a gelatinase and a trypsin-like protease. The breakdown by the secreted protease fraction of *E. faecalis* of both type I soluble and insoluble collagens was increased after glycation. It has been shown that glycation decreases protein breakdown because of the formation of cross links. The results obtained for the glycated collagen is therefore somewhat paradoxical. However, a theoretical model explaining the increased susceptibility of glycated collagen to proteolysis has been described in the literature (Stultz and Edelman, 2003b), and was discussed in chapter 6.

This model proposes that non-cross linking modifications occurring to arginine residues in the regions of the tropocollagen which are poor in proline and hydroxyproline leads to instability. As a consequence Gly-Leu bonds in the vicinity of these modified arginine residues are rendered more susceptible to mammalian collagenase. Gly-Leu

bonds are numerous in the alpha chain, and importantly in terms of collagen breakdown, the gelatinase of *E. faecalis* has specificity for these. Similarly, the collagenase of *C. histolyticum* has specificity for Gly-X bonds (approximately 300 per alpha chain) which explains its ability to extensively digest collagen.

So, in line with the protease model described by (Stultz and Edelman, 2003b), it is possible, based on the experimental findings obtained here, to hypothesise that long term glycation modifications of collagens resulting in cross link formation might have the effect of retarding collagen breakdown when tissue remodelling is required. This in itself may compromise wound healing and the resolution of endodontic infections. However, it is equally likely that short term periods of hyperglycaemia (which do not result in cross linking) may also have a detrimental impact on the tissues in that they may result in destabilisation of tropocollagen structures which become primed for attack by proteases such as the *E faecalis* gelatinase which has specificity for Gly-Leu bonds.

Proteomic analysis of the glycation patterns of the collagens and gelatin peptides employed here was beyond the scope of the project. In addition, it was not possible to analyse the fragmentation patters of the collagen peptides after digestion with the *E. faecalis* protease fraction or the collagenase of *C. histolyticum*. Consequently it cannot be determined whether the non-cross-linking modifications to arginine residues described above were influential in bringing about the increased proteolysis. In addition without further purifying the individual proteases from *E. faecalis* it was not possible to examine their individual contributions to the breakdown of collagen. Nevertheless, based on the experimental findings of this project and in light of the theoretical protease model described above, one might propose the following in the context of the pathology of endodontic infection, relatively short periods of hyperglycaemia may alter the equilibrium distribution of states to favour vulnerable states of collagen. Thus one could envisage a

scenario where glycation of tropocollagen molecules are rendered more susceptible to proteolytic attack by mammalian collagenases and by enzymes secreted by *E. faecalis* (in the context of endodontic infection). For example in streptozotocin induced diabetes rats there was an increased risk of alveolar bone resorption and consequently increased lesion size of periapical tissues (Kohsaka et al., 1996, Fouad et al., 2002, Iwama et al., 2003) Similarly, this may explain the increased susceptibility of gelatin peptides to *C. histolyticm*.

Gelatin peptides were found to be more resistant to digestion by trypsin following glycation. In agreement with previous results on α-lactalbumin, this is not surprising since glycation modifications to lysine alters the charge and may physical block trypsin from recognising its cleavage site (trypsin). This may have potential implications on delaying wound healing. Proteolytic scission of glycated collagen, for example during bacterial invasion, may render the tropocollagen molecules non- or less functional from a structural point of view, due to unwinding of the molecules and release of peptides. The subsequent digestion of these peptides by for example trypsin-like enzymes produced by macrophages (Chen et al., 2003) and neutrophils (Meyer-Hoffert and Wiedow, 2011) at sites of inflammation , which may be needed for biological debridement for complete remodelling (Polimeni et al., 2006), may be then be impaired. Consequently this may have a detrimental impact on the process of remodelling of collagen matrices, tissue repair and wound healing.

Whilst it was possible to determine the extent of glycation of the collagen and gelatin substrates following *in vitro* incubation with glucose employing the widely used chemical assay (for HMF), the determination of the glycation patterns were beyond the scope of the project. Such investigations would have provided a valuable insight into the reasons why the glycated peptides were rendered more or less susceptible to proteolytic breakdown. This is an area for future research.

*E. faecalis* is not the only pathogenic species involved in endodontic infection, nor is it the only cause of endodontic treatment failure. There are many other proteolytic species which are worth of study, including for example *Porphyromonas. gingivalis, Prevotella. Intermedia, and Tannerella. forsythia*, which are producers of potent proteases (gingipain, interpain and karilysin, respectively).

A number of studies have demonstrated that glycation modification of alpha chains adversely impacts their interaction with the matrix, for example increased cross-linking, and glycation dose dependent decrease in the attachment and spreading of human periodontal ligament cells to gelatin (Boonkaew et al., 2014). In contrast, AGE accumulation in experimentally induced diabetes in mice was demonstrated to enhance the binding of *E. coli* to AGE modified urothelial surface proteins (Ozer et al., 2015), suggesting a role for increased bacterial adherence during urinary tract infections (de Man et al., 1988).

Despite the above findings, it is surprising that little or no attention has been paid to the potential influence of host protein glycation on bacterial adhesion. In this context it is known that *E. faecalis* expresses a cell-surface collagen-binding protein (designated Ace) which is important in biofilm formation. In terms of interactions of glycated collagens with bacterial cells is one area of work which deserves attention.

Finally, the biochemical findings of this project cannot be directly extrapolated to the clinical scenario. However, another area for future research would be to investigate ECM proteins derived from pulps and the periodontium of diabetic patients with endodontic and periodontal disease. Analysis of the patterns of glycation of collagens, determination of their susceptibility to host and bacterial proteases, and proteomic analyses of peptide breakdown would be informative of the potential pathophysiological

link between glycation in diabetes and endodontic / periodontal infection, at the molecular level.

## 7.2 Conclusion

The main findings of this project were;

1. Glycation of gelatin and collagen was achieved by incubation with glucose
2. Increased degree of glycation was achieved by incubation with increased levels of glucose
3. Glycated gelatin was more resistant to digestion by trypsin
4. Glycated type I soluble and insoluble collagens were more susceptible to digestion by the secreted protease fraction of *E. faecalis* OG1RF strain (expresses gelatinase and a trypsin-like protease)

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# CHAPTER 9: APPENDICES

## Appendix 1 – Type I collagen α-1 amino acid sequence

QLSYGYDEK STGGISVPGP MGPSGPRGLP GPPGAPGPQG
FQGPPGEPGE PGASGPMGPR GPPGPPGKNG DDGEAGKPGR PGERGPPGPQ
GARGLPGTAGLPGMKGHRGF SGLDGAKGDA GPAGPKGEPG SPGENGAPGQ
MGPRGLPGER GRPGAPGPAG ARGNDGATGA AGPPGPTGPA GPPGFPGAVG
AKGEAGPQGP RGSEGPQGVR GEPGPPGPAG AAGPAGNPGA DGQPGAKGAN
GAPGIAGAPG FPGARGPSGP QGPGGPPGPK GNSGEPGAPG SKGDTGAKGE
PGPVGVQGPP GPAGEEGKRG ARGEPGPTGL PGPPGERGGP GSRGFPGADG
VAGPKGPAGE RGSPGPAGPK GSPGEAGRPG EAGLPGAKGL TGSPGSPGPD
GKTGPPGPAG QDGRPGPPGP PGARGQAGVM GFPGPKGAAG EPGKAGERGV
PGPPGAVGPA GKDGEAGAQG PPGPAGPAGE RGEQGPAGSP GFQGLPGPAG
PPGEAGKPGE QGVPGDLGAP GPSGARGERG FPGERGVQGP PGPAGPRGAN
GAPGNDGAKG DAGAPGAPGS QGAPGLQGMP GERGAAGLPG PKGDRGDAGP
KGADGSPGKD GVRGLTGPIG PPGPAGAPGD KGESGPSGPA GPTGARGAPG
DRGEPGPPGP AGFAGPPGAD GQPGAKGEPG DAGAKGDAGP PGPAGPAGPP
GPIGNVGAPG AKGARGSAGP PGATGFPGAA GRVGPPGPSG NAGPPGPPGP
AGKEGGKGPR GETGPAGRPG EVGPPGPPGP AGEKGSPGAD GPAGAPGTPG
PQGIAGQRGV VGLPGQRGER GFPGLPGPSG EPGKQGPSGA SGERGPPGPM
GPPGLAGPPG ESGREGAPGA EGSPGRDGSP GAKGDRGETG PAGPPGAPGA
PGAPGPVGPA GKSGDRGETG PAGPTGPVGP VGARGPAGPQ GPRGDKGETG
EQGDRGIKGH RGFSGLQGPP GPPGSPGEQG PSGASGPAGP RGPPGSAGAP
GKDGLNGLPG PIGPPGPRGR TGDAGPVGPP GPPGPPGPPG PPSAGFDFSF
LPQPPQEKAH DGGRYYRA

**The amino acid sequence of alpha 1 chain of the collagen molecule.** Lysine residues which are potential sites for glycation (highlighted red). Arginine which are potential sites for glycation (highlighted in green). Glycine-Leucine sequences sites for *E. faecalis* gelatinase binding (highlighted green).

# Appendix 2 – Type I collagen α-2 amino acid sequence

Q YDGKGVGLGP GPMGLMGPRG
PPGAAGAPGP QGFQGPAGEP GEPGQTGPAG ARGPAGPPGK AGEDGHPGKP
GRPGERGVVG PQGARGFPGT PGLPGFKGIR GHNGLDGLKG QPGAPGVKGE
PGAPGENGTP GQTGARGLPG ERGRVGAPGP AGARGSDGSV GPVGPAGPIG
SAGPPGFPGA PGPKGEIGAV GNAGPAGPAG PRGEVGLPGL SGPVGPPGNP
GANGLTGAKG AAGLPGVAGA PGLPGPRGIP GPVGAAGATG ARGLVGEPGP
AGSKGESGNK GEPGSAGPQG PPGPSGEEGK RGPNGEAGSA GPPGPPGLRG
SPGSRGLPGA DGRAGVMGPP GSRGASGPAG VRGPNGDAGR PGEPGLMGPR
GLPGSPGNIG PAGKEGPVGL PGIDGRPGPI GPAGARGEPG NIGFPGPKGP
TGDPGKNGDK GHAGLAGARG APGPDGNNGA QGPPGPQGVQ GGKGEQGPPG
PPGFQGLPGP SGPAGEVGKP GERGLHGEFG LPGPAGPRGE RGPPGESGAA
GPTGPIGSRG PSGPPGPDGN KGEPGVVGAV GTAGPSGPSG LPGERGAAGI
PGGKGEKGEP GLRGEIGNPG RDGARGAPGA VGAPGPAGAT GDRGEAGAAG
PAGPAGPRGS PGERGEVGPA GPNGFAGPAG AAGQPGAKGE RGAKGPKGEN
GVVGPTGPVG AAGPAGPNGP PGPAGSRGDG GPPGMTGFPG AAGRTGPPGP
SGISGPPGPP GPAGKEGLRG PRGDQGPVGR TGEVGAVGPP GFAGEKGPSG
EAGTAGPPGT PGPQGLLGAP GILGLPGSRG ERGLPGVAGA VGEPGPLGIA
GPPGARGPPG AVGSPGVNGA PGEAGRDGNP GNDGPPGRDG QPGHKGERGY
PGNIGPVGAA GAPGPHGPVG PAGKHGNRGE TGPSGPVGPA GAVGPRGPSG
PQGIRGDKGE PGEKGPRGLP GLKGHNGLQG LPGIAGHHGD QGAPGSVGPA
GPRGPAGPSG PAGKDGRTGH PGTVGPAGIR GPQGHQGPAG PPGPPGPPGP
PGVSGGGYDF GYDGDFYR

**The amino acid sequence of alpha 2 chain of the collagen molecule.** Lysine residues which are potential sites for glycation (highlighted red). Arginine which are potential sites for

glycation (highlighted in green). Glycine-Leucine sequences sites for *E. faecalis* gelatinase binding (highlighted green).

# Appendix 3 – NetGlycate prediction results for α-1 chain

```
Alpha 1 primary sequence       1057 amino acids
#
# netglycate-1.0 prediction results
#
# Sequence                      #    Score            Answer
# ----------------------------------------------------------
# Sequence                      9    0.960   glycate    YES
# Sequence                     67   -0.872   glycate     .
# Sequence                     76    0.802   glycate    YES
# Sequence                    104   -0.779   glycate     .
# Sequence                    116   -0.879   glycate     .
# Sequence                    125   -0.945   glycate     .
# Sequence                    191   -0.898   glycate     .
# Sequence                    236   -0.749   glycate     .
# Sequence                    269   -0.924   glycate     .
# Sequence                    281   -0.886   glycate     .
# Sequence                    287   -0.876   glycate     .
# Sequence                    307   -0.540   glycate     .
# Sequence                    344   -0.663   glycate     .
# Sequence                    359   -0.950   glycate     .
# Sequence                    377   -0.855   glycate     .
# Sequence                    391   -0.843   glycate     .
# Sequence                    425   -0.809   glycate     .
# Sequence                    433   -0.797   glycate     .
# Sequence                    451   -0.755   glycate     .
# Sequence                    496   -0.878   glycate     .
# Sequence                    548   -0.773   glycate     .
# Sequence                    581   -0.913   glycate     .
# Sequence                    590   -0.964   glycate     .
# Sequence                    598    0.739   glycate    YES
# Sequence                    620    0.574   glycate    YES
# Sequence                    665   -0.852   glycate     .
# Sequence                    674   -0.932   glycate     .
# Sequence                    701   -0.850   glycate     .
# Sequence                    742   -0.863   glycate     .
# Sequence                    746   -0.784   glycate     .
# Sequence                    773   -0.881   glycate     .
# Sequence                    823   -0.914   glycate     .
# Sequence                    872   -0.791   glycate     .
# Sequence                    901   -0.909   glycate     .
# Sequence                    935   -0.970   glycate     .
# Sequence                    947   -0.978   glycate     .
# Sequence                    991    0.855   glycate    YES
# Sequence                   1047   -0.409   glycate     .
#
```

```
QLSYGYDEKS TGGISVPGPM GPSGPRGLPG PPGAPGPQGF QGPPGEPGEP   #     50
GASGPMGPRG PPGPPGKNGD DGEAGKPGRP GERGPPGPQG ARGLPGTAGL   #    100
PGMKGHRGFS GLDGAKGDAG PAGPKGEPGS PGENGAPGQM GPRGLPGERG   #    150
RPGAPGPAGA RGNDGATGAA GPPGPTGPAG PPGFPGAVGA KGEAGPQGPR   #    200
GSEGPQGVRG EPGPPGPAGA AGPAGNPGAD GQPGAKGANG APGIAGAPGF   #    250
PGARGPSGPQ GPGGPPGPKG NSGEPGAPGS KGDTGAKGEP GPVGVQGPPG   #    300
PAGEEGKRGA RGEPGPTGLP GPPGERGGPG SRGFPGADGV AGPKGPAGER   #    350
GSPGPAGPKG SPGEAGRPGE AGLPGAKGLT GSPGSPGPDG KTGPPGPAGQ   #    400
DGRPGPPGPP GARGQAGVMG FPGPKGAAGE PGKAGERGVP GPPGAVGPAG   #    450
KDGEAGAQGP PGPAGPAGER GEQGPAGSPG FQGLPGPAGP PGEAGKPGEQ   #    500
GVPGDLGAPG PSGARGERGF PGERGVQGPP GPAGPRGANG APGNDGAKGD   #    550
AGAPGAPGSQ GAPGLQGMPG ERGAAGLPGP KGDRGDAGPK GADGSPGKDG   #    600
VRGLTGPIGP PGPAGAPGDK GESGPSGPAG PTGARGAPGD RGEPGPPGPA   #    650
GFAGPPGADG QPGAKGEPGD AGAKGDAGPP GPAGPAGPPG PIGNVGAPGA   #    700
KGARGSAGPP GATGFPGAAG RVGPPGPSGN AGPPGPPGPA GKEGGKGPRG   #    750
ETGPAGRPGE VGPPGPPGPA GEKGSPGADG PAGAPGTPGP QGIAGQRGVV   #    800
GLPGQRGERG FPGLPGPSGE PGKQGPSGAS GERGPPGPMG PPGLAGPPGE   #    850
SGREGAPGAE GSPGRDGSPG AKGDRGETGP AGPPGAPGAP GAPGPVGPAG   #    900
KSGDRGETGP AGPTGPVGPV GARGPAGPQG PRGDKGETGE QGDRGIKGHR   #    950
GFSGLQGPPG PPGSPGEQGP SGASGPAGPR GPPGSAGAPG KDGLNGLPGP   #   1000
IGPPGPRGRT GDAGPVGPPG PPGPPGPPGP PSAGFDFSFL PQPPQEKAHD   #   1050
GGRYYRA                                                  #   1100
```

```
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%1  ..................................................   #    250
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%1  ..................................................   #   1050
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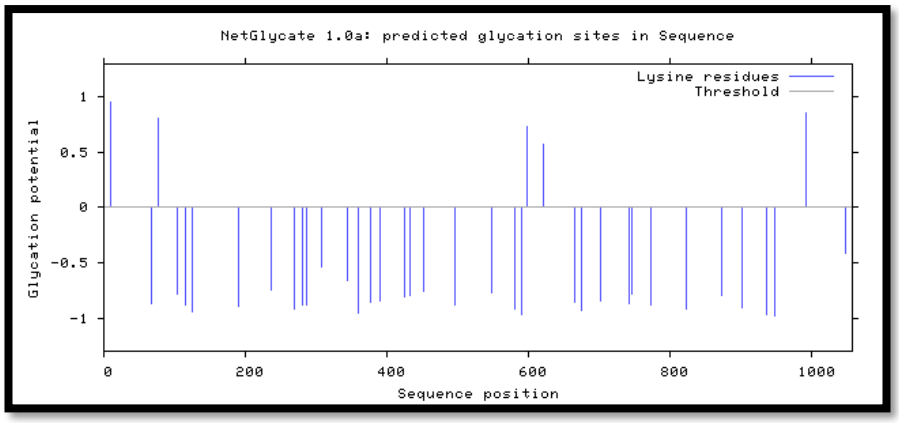


**Predicted positions of glycated lysine residues in the type I collagen alpha 1 chain obtained by the NetGlycate 1.0 prediction program.** This shows a potential of 5 lysine glycation sites per α 1 chain, and their positions in the chain.

# Appendix 4 – NetGlycate prediction results for α-2 chain

```
Alpha 2 primary sequence       1039 amino acids
#
# netglycate-1.0 prediction results
#
# Sequence                      #    Score            Answer
# ----------------------------------------------------------
# Sequence                      5    0.904   glycate    YES
# Sequence                     61   -0.867   glycate     .
# Sequence                     70    0.560   glycate    YES
# Sequence                     98   -0.898   glycate     .
# Sequence                    110   -0.706   glycate     .
# Sequence                    119   -0.889   glycate     .
# Sequence                    185   -0.813   glycate     .
# Sequence                    230   -0.527   glycate     .
# Sequence                    275   -0.903   glycate     .
# Sequence                    281   -0.934   glycate     .
# Sequence                    301    0.719   glycate    YES
# Sequence                    385   -0.893   glycate     .
# Sequence                    419   -0.775   glycate     .
# Sequence                    427   -0.894   glycate     .
# Sequence                    431   -0.972   glycate     .
# Sequence                    464   -0.589   glycate     .
# Sequence                    490   -0.782   glycate     .
# Sequence                    542   -0.936   glycate     .
# Sequence                    575   -0.866   glycate     .
# Sequence                    578   -0.970   glycate     .
# Sequence                    659   -0.718   glycate     .
# Sequence                    665   -0.816   glycate     .
# Sequence                    668   -0.938   glycate     .
# Sequence                    736   -0.829   glycate     .
# Sequence                    767   -0.906   glycate     .
# Sequence                    866   -0.924   glycate     .
# Sequence                    895   -0.882   glycate     .
# Sequence                    929   -0.920   glycate     .
# Sequence                    935   -0.952   glycate     .
# Sequence                    944   -0.968   glycate     .
# Sequence                    985   -0.915   glycate     .
#
```

```
QYDGKGVGLG PGPMGLMGPR GPPGAAGAPGP QGFQGPAGEP GEPGQTGPA   #     50
GARGPAGPPG KAGEDGHPGK PGRPGERGVVG PQGARGFPGT PGLPGFKGI   #    100
RGHNGLDGLK GQPGAPGVKG EPGAPGENGTP GQTGARGLPG ERGRVGAPG   #    150
PAGARGSDGS VGPVGPAGPI GSAGPPGFPGA PGPKGEIGAV GNAGPAGPA   #    200
GPRGEVGLPG LSGPVGPPGN PGANGLTGAKG AAGLPGVAGA PGLPGPRGI   #    250
PGPVGAAGAT GARGLVGEPG PAGSKGESGNK GEPGSAGPQG PPGPSGEEG   #    300
KRGPNGEAGS AGPPGPPGLR GSPGSRGLPGA DGRAGVMGPP GSRGASGPA   #    350
GVRGPNGDAG RPGEPGLMGP RGLPGSPGNIG PAGKEGPVGL PGIDGRPGP   #    400
IGPAGARGEP GNIGFPGPKG PTGDPGKNGDK GHAGLAGARG APGPDGNNG   #    450
AQGPPGPQGV QGGKGEQGPP GPPGFQGLPGP SGPAGEVGKP GERGLHGEF   #    500
GLPGPAGPRG ERGPPGESGA AGPTGPIGSRG PSGPPGPDGN KGEPGVVGA   #    550
VGTAGPSGPS GLPGERGAAG IPGGKGEKGEP GLRGEIGNPG RDGARGAPG   #    600
AVGAPGPAGA TGDRGEAGAA GPAGPAGPRGS PGERGEVGPA GPNGFAGPA   #    650
GAAGQPGAKG ERGAKGPKGE NGVVGPTGPVG AAGPAGPNGP PGPAGSRGD   #    700
GGPPGMTGFP GAAGRTGPPG PSGISGPPGPP GPAGKEGLRG PRGDQGPVG   #    750
RTGEVGAVGP PGFAGEKGPS GEAGTAGPPGT PGPQGLLGAP GILGLPGSR   #    800
GERGLPGVAG AVGEPGPLGI AGPPGARGPPG AVGSPGVNGA PGEAGRDGN   #    850
PGNDGPPGRD GQPGHKGERG YPGNIGPVGAA GAPGPHGPVG PAGKHGNRG   #    900
ETGPSGPVGP AGAVGPRGPS GPQGIRGDKGE PGEKGPRGLP GLKGHNGLQ   #    950
GLPGIAGHHG DQGAPGSVGP AGPRGPAGPSG PAGKDGRTGH PGTVGPAGI   #   1000
RGPQGHQGPA GPPGPPGPPG PPGVSGGGYDF GYDGDFYR               #   1050
```

```
%1  ....G.....................................................   #     50
%1  ..................G.....................................   #    100
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%1  .....................................................     #    250
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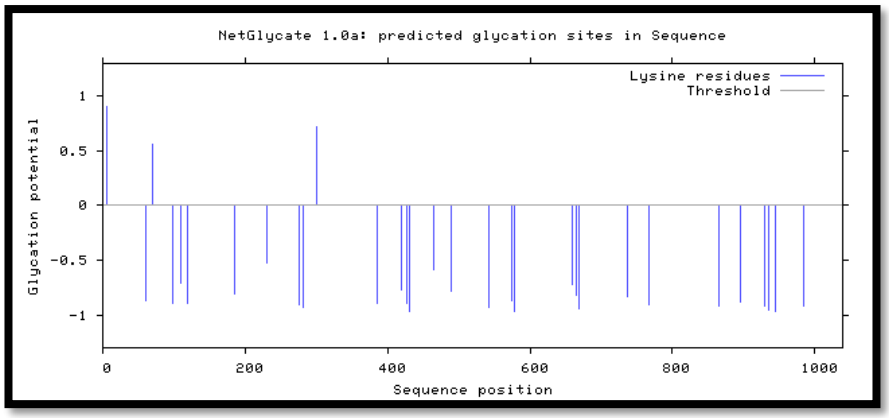


**Predicted positions of glycated lysine residues in the type I collagen alpha 2 chain obtained by the NetGylcate 1.0 prediction program.** This shows a potential of 3 lysine glycation sites per α 2 chain, and their positions in the chain.