Bioconversion of specified risk materials-derived peptides for tackifier and torrefied wood binder applications

by

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Abstract

Due to the safety concerns relating to the spread of bovine spongiform encephalopathy (BSE) or 'mad cow disease' in North America, strict bans for specified risk materials (SRMs) have been introduced to the rendering process. SRMs are characterized as the tissues in cattle where misfolded proteins, the possible infection source for BSE, are concentrated. According to those bans, SRMs are required to be segregated and disposed strictly, resulting in significant additional costs to companies. To relieve the economic pressure on rendering industries, an approved SRM disposal method, thermal hydrolysis, is followed to convert these proteinaceous waste materials into non-infective peptides. Due to the binding property, water solubility and bio-degradability displayed in the obtained SRM-derived peptides, these peptides have been examined as a promising feedstock for the development of value-added bio-based products. Here, the purpose of this research was to valorize SRM-derived peptides for commercially competitive binders, such as tackifiers and torrefied wood binder.

As a binding agent in hydro-mulch mixtures, tackifiers are employed to bind seed and mulch fibres to the soil to restore the vegetation in eroded areas. In addition to the binding property, tackifiers must also achieve a great balance between water resistance and water-holding capacity: water resistance or hydrophobicity is required to prevent diffusion and loss of function caused by rain-wash; water-holding capacity helps to maintain the necessary moisture for the rooting and growth of plants. To first increase the hydrophobicity,

a glutaraldehyde cross-linking strategy was introduced to the SRM-derived peptides. Moreover, both the binding property and water-holding capacity of the resulting products were assessed to compare with unmodified peptides, showing no sacrifice in these performance measures.

To improve the binding property and water-holding capacity of SRM-derived peptides, epoxidized poly (vinyl alcohol) (PVA) was synthesized and employed as a cross-linking agent for peptides-based tackifier development. The resulting cross-linking products displayed higher binding strength with adjustable water-holding capacity than unmodified peptides. The performance of the cross-linked products was even comparable to commercially available tackifiers. Hence, the two cross-linking strategies examined in this research demonstrated a novel valorization pathway of converting SRM-derived peptides into sustainable tackifiers.

As a renewable source of fuels, torrefied wood pellets are produced to handle growing energy demands and the environmental concerns relating to the utilization of conventional fossil fuel. To improve the competitiveness of torrefied wood pellets against other source of fuels, i.e., coals, torrefied wood binder was studied as an extra pellet additive, and was expected to increase energy density, durability, and storage life of torrefied wood pellets.

Based on the binding property shown in the tackifiers study, I started with SRM-derived peptides to develop a novel binder for pellet industries, but found that unmodified peptides didn't help to improve the strength of pellets. This finding made the cross-linking strategy necessary for SRM-derived peptides, leading to the concept of using PVA-EPC-Peptides as a binder for pellets production. PVA-EPC-Peptides (EPC stands for epicholohydrin) is a cross-linked product obtained from the reaction between SRM-derived peptides and epoxidized PVA, which displayed higher binding strength than unmodified peptides in tackifier study. It was observed in this research that the pellets produced with 3.0 wt.% of PVA-EPC-Peptides exhibited higher compression strength than that of the pellets produced without any binder, via the following densification conditions: a die temperature of 150℃, 250 MPa holding pressure, 1 min holding time, and a 3.0 wt.% moisture content of the binder/wood mixture. The density of the pellets produced with 3.0 wt.% of PVA-EPC-Peptides was also found higher than that of the pellets produced without any binder. Therefore, this research demonstrates another feasible valorization pathway of converting SRM-derived peptides into a torrefied wood binder.

In summary, this research has provided a technology platform by which the otherwise hazardous proteinaceous waste, SRMs, can be valorized into tackifiers and torrefied wood binders. This technology platform is developed not only to produce an alternative income for the rendering companies, but also to create sustainable products that satisfy the related industries.

Preface

This thesis is an original work by Tao Shui under the supervision of Prof. David C. Bressler, including the development of methodology, validation of results, data analysis, data visualization, and the writing of the original draft. Dr. Michael Chae contributed to manuscript of all chapters' edits. Dr. Birendra B. Adhikari contributed to the design of texture analysis applied in section 4.1 and section 4.2, as well as section 4.1 edits. Dr. Vinay Khatri contributed to section 4.2 and section 4.3 edits. A version of section 4.1 has been published as Shui T., Adhikari B.B., Chae M., Bressler D.C., "Evaluation of thermally hydrolyzed specified risk materials cross-linked with glutaraldehyde for tackifier applications," *Progress in Organic coatings*, 2020, 140: 105535. A version of section 4.2 has been published as Shui T., Chae M., Bressler D.C., "Cross-linking of Thermally Hydrolyzed Specified Risk Materials with Epoxidized Poly (Vinyl Alcohol) for Tackifier Applications," *Coatings*, 2020, 10, 630. A version of section 4.3 has been submitted to *Renewable Energy* in 2020. All of the experiments were conducted by Tao Shui in Prof. David C. Bressler's lab at the University of Alberta except the following experiments: the nitrogen content in section 4.1 was analyzed with the help of Dr. Kelvin Lien in the analytical laboratory in the Department of Agricultural, Food and Nutritional Science; the texture analysis in section 4.1 and section 4.2 was performed in the Lipid Chemistry Lab (South Academic Building 5-52) in the Department of Agricultural, Food and Nutritional Science with the help of Dr. Ereddad Kharraz; the CHNS analysis and chlorine content analysis in

section 4.3 were performed by the Analytical and Instrumentation Laboratory of the Chemistry Department and the Natural Resources Analytical Laboratory, respectively. All the laboratories are located at the University of Alberta.

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List of Reactions

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ASTM	American Society for Testing and Materials
BSE	Bovine spongiform encephalopathy
CDI	N, N′-carbonyl diimidazole
CFIA	Canadian Food Inspection Agency
CHNS	Carbon, hydrogen, nitrogen, sulfur
CJD	Creutzfeldt-Jakob disease
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DSC	N, N′-disuccinimidyl carbonate
DSS	Disuccinimidyl suberate
DST	Disuccinimidyl tartrate
EGS	Ehylene glycol bis
FDA	Food and Drug Administration
FT-IR	Fourier transform infrared spectroscopy
HPLC	High performance liquid chromatography
HSD	Honestly significant difference
ISO	International Organization for Standardization
IMO	International Maritime Organization
LSD	Least significant difference
MS	Mass spectrometry
MUF	Methyl urea-formaldehyde
Mw	Weight average molecular weight
NHS	N-hydroxysuccinimide
OPA	O-phthalaldehyde
PA	Poly acrylate

PAM	Poly acrylamide
PEA	Poly (ether amine)
PEG	Poly (ethylene glycol)
PF	Phenol-formaldehyde
PrP	Prion Protein
PrP^{c}	The normal form of prion protein
PrP^{sc}	The infectious form of prion protein
PVA	Poly (vinyl alcohol)
PVA-EPC-Peptides	The products generated from the cross-linking reaction between SRM-derived peptides and epoxidized PVA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC-HPLC	Size exclusion high performance liquid chromatography
SRM	Specified Risk Materials
Sulfo-EGS	Sulfoethylene glycol bis
Sulfo-DST	Disulfosuccinimidyl tartrate
Sulfo-NHS	N-hydroxysulfosuccinimide
TGA	Thermogravimetric analysis
TNBSA	2,4,6-Trinitrobenzene sulfonic acid
TSEs	Transmissible spongiform encephalopathies
UF	Urea formaldehyde

Chapter 1: Introduction and objectives

1.1. Introduction

Bovine spongiform encephalopathy (BSE), also known as “mad cow disease”, is recognized as a transmittable neurodegenerative disease that affects cattle. BSE is believed to be caused by a high concentration of misfolded proteins, which are also termed as prions [1]. Since the BSE emergency initiated in the 1980s in England and continued with recent outbreaks in North America, certain cattle tissues, known as specified risk materials (SRMs), have been limited and even banned from utilization as animal feed, pet food, and fertilizers due to the risk of potential prion aggregation and infection [2]. The prohibition of the use of SRM tissues has resulted in economic losses for the rendering and processing industries since they were now forced to dispose of SRM by incineration or landfilling, which often required separation, storage and transportation.

Owing to the extreme resistance of prion proteins towards conventional decontamination techniques [3], a thermal hydrolytic method was approved by the Canadian Food Inspection Agency (CFIA), the Food and Drug Administration of the US (FDA) and the European Union [4-6]. This method was investigated as a potential pathway for the bioconversion of the infectious materials collected from the rendering process (SRM) into novel applications or value-added products. SRM-derived peptides, the protein

fragment/peptides obtained from SRM hydrolysates, were characterized with regards to their molecule weight, aqueous viscosity, and functional groups content [7] and were found to display outstanding properties including considerable binding strength, favourable biodegradability, potential carbon/nitrogen release, and water-solubility, all of which made peptides potentially useful as peptide-based composites. Furthermore, the abundant functional groups contained within SRM-derived peptides make it possible to modify their properties. In previous studies on SRM-derived peptides, cross-linking strategies were demonstrated to generate products with improved characteristics that made them more amenable in the applications of bio-based thermosetting plastics and bio-composites [8, 9]. Such approaches indicated the technical feasibility of recovering peptides from SRM as a potential industrial resource, addressing the environmental concerns and economic losses associated with several million tonnes of SRM disposal in North America each year.

Due to the biodegradability, sustainability and potential non-toxicity of the protein/peptide, the development of protein-based adhesives has re-emerged as a hot topic in recent years [10]. Moreover, the successful development of a specific binder for wood panels exhibited the potential of SRM-derived peptides as a feedstock resource [11]. However, the application of SRM-derived peptides as a binder for wood fibres (tackifiers) and wood particles has not been described previously. Therefore, the general objective of this project was to expand the boundaries of SRM bio-conversion platforms for wood binders,

including tackifiers and torrefied wood binders, through polymerization and bio-refining approaches. The cross-linking strategies employed for the conversion of SRM-derived peptides and the resulting enhancements are reported in the subsequent chapters of this thesis.

In chapter 2, the potential strategies for bioconversion of proteinaceous waste and rendering by-products into sustainable value-added industrial products, as well as the proteins/peptides bioconjugation strategies, are critically reviewed. The characterization of the peptides/protein fragments recovered from SRM hydrolysates is also mentioned in this chapter, based on the previous research achievements of our lab [7-11]. The review mainly focuses on the potential of the waste peptides and the available bioconjugation reagents used in protein modification to achieve the desired properties. The development of tackifiers and torrefied wood binders from SRM-derived peptides will be reported in subsequent chapters.

Chapter 3 lists all of materials and methods applied in this thesis. The development of bio-degradable tackifiers with promising water-resistance for hydro-mulch applications from SRM-derived peptides is first discussed in section 4.1 (chapter 4) [12]. Tackifiers function as an important adhesive in a hydro-mulch mixture, binding wood fibres and seeds of plants to the soil to restore vegetation to barren lands. Tackifiers demonstrate water-binding qualities that enable them to retain moisture for germination and growth, as well as moderate hydrophobicity to maintain binding strength during prolonged exposure to large amounts of water. To meet these demands, finding a delicate balance between

hydrophobicity and hydrophilicity is essential. Since SRM-derived peptides have been shown to display great hydrophilicity [3], glutaraldehyde cross-linking of peptides to increase hydrophobicity was studied as the main objective of this chapter. The temperature and the type of solvent used for the cross-linking reaction were investigated to reach the desired improvement in the hydrophobicity of the products. The binding strength, moisture-holding capacity and aqueous viscosity of the resulted products were also evaluated and shown to be comparable to the commercial tackifier guar gam, demonstrating the potential for using SRM as feedstock for sustainable tackifiers. However, there is probably some room for the improvement of binding strength exhibited in the products, when compared with other commercial tackifiers, starch and psyllium.

In section 4.2 (chapter 4), epoxidized poly (vinyl alcohol) (PVA) was synthesized and introduced into cross-linking of SRM-derived peptides for the development of novel tackifiers with enhanced binding properties [13]. The reaction conditions and the molar ratio of the reagents applied for the cross-linking reaction were reported to have significant effects on cross-linking behaviour. Epoxidized PVA, a newly synthesized cross-linker, successfully cross-linked with SRM-derived peptides, resulting in a remarkable increase in binding strength and moisture maintaining capacity of the product. Hence, as a supplement to the previous study in Chapter 3, this chapter indicates a promising avenue for the bioconversion

of SRM-derived peptides into a tackifier, potentially substituting for guar gum, psyllium and/or starch.

Section 4.3 (chapter 4) investigates the potential application of epoxidized PVA cross-linking peptides as a binder in the torrefied wood densification process [14]. Based on the optimized performance of pellets in terms of density and compression strength, the positive effect of cross-linked peptides was confirmed in the study. The elemental content and heating value of the resulting pellets were also characterized and confirmed to meet the standards of torrefied wood pellets for chlorine content, nitrogen content, sulphur content, and heating value, offering another value-added pathway for SRM-derived peptides that could be applied in the fuel industry.

Chapter 5, the last chapter of this thesis, provides a discussion on the outstanding feasibility of SRM-derived peptides as feedstock for the development of binders. An outlook on the usage of other hazardous proteinaceous wastes for the development of bio-based binders was also mentioned, via the same cross-linking strategies. The innovation of this Ph.D. project was discussed in this chapter, which focused on the construction of a technology platform for the conversion of proteinaceous waste materials. Meanwhile, this work contributes to the development of biodegradable binders, offering new cross-linking strategies to various protein studies.

1.2. Objectives

1.2.1. Short term objectives

- Evaluate the cross-linking between SRM-derived peptides and glutaraldehyde under the temperature range of room temperature to 80°C in water and/or methanol;
- Develop a PVA epoxidation process through epicholorohydrin modification that will facilitate cross-linking between PVA-epoxides and SRM-derived peptides in optimized conditions.

1.2.2. Long term objectives

- Develop a technology platform for chemical conversion of proteinaceous waste into industrial tackifiers and wood binders that have applications in hydro-mulching and torrefied wood pellets production, respectively;
- Investigate novel avenues to peptide-based products, such as adhesives, surfactants, flocculants, etc., through SRM-derived peptides cross-linking.

Chapter 2: Literature review

2.1. Prion diseases and prion transmission properties

The term “prion” is used to describe the infectious agent that is responsible for several neurodegenerative diseases found in mammals, including Creutzfeldt-Jakob disease (CJD) in humans and bovine spongiform encephalopathies (BSE) in cattle [15]. Prion diseases tend to cause irreversible damage to the brain or other neural tissues of the organism. Based on the studies of all known types of prions, it is hypothesized that prion proteins (PrP), which comprise prions and are typically found throughout the organism, become misfolded, making them infective and able to cause transmissible spongiform encephalopathies (TSEs) [16]. However, the process involved in formation of TSEs is currently unknown, leading to debate as to whether prions and/or other possible agents are the original source of infection. This uncertainty in prion study has been a problem in the search for a cure for prion disease. Meanwhile, the resiliency of prions against chemical and physical agents raises issues around prion disposal as prions cannot be completely destroyed by boiling, standard autoclaving methods, radiation, or exposure to alcohols or acids [17]. Therefore, the strategy for dealing with prions has become a pressing problem for both research and industry.

The study of prions began with Field’s investigations of scrapie and kuru, which indicated that the infectious ability of pathologically polysaccharides was only activated in

the host [18]. The mysterious infectious agent found in scrapie was also resistant to ionizing radiation [19], resulting in the hypothesis about TSEs infection that was raised by John Stanley Griffith in the 1960s [20]. To explain the formation and spread of prions, Griffith proposed the protein-only hypothesis for the replication of protein: an abnormal form of a cellular protein can convert normal proteins of the same type into its abnormal form, thus leading to replication [21, 22]. Moreover, the hypothetical infectious protein was first discovered and purified by Stanley in 1982, with the term "prion" resulting from an abbreviation of '**pr**oteinaceous **in**fectious particle'; this was followed by the discovery of prion protein (PrP), which accounted for the composition of prions [1]. Prusiner's work in this field earned him the Nobel Prize in Physiology or Medicine in 1997 [23], opening the gate for modern prion theory.

PrP can be found naturally throughout the bodies of humans and animals. According to the PrP theory, PrP displays non-infectious properties in its normal form (PrP^{c}), which can be eliminated by the proteases in the body [24]. However, when the infectious form of PrP (PrP^{Sc}) appears, PrP^{c} can be converted into its isoform, altering the interconnection of proteins [25]. PrP^{Sc} has a structure with a higher proportion of β-sheet compared to PrP^{c}, showing resistance to proteases. Furthermore, PrP^{Sc} was associated in an infection study with TSEs agents [25]. Nevertheless, the process of PrP^{Sc} formation has remained unknown.

Prion diseases may be acquired, familial, or sporadic [26]. Based on the current model of prion diseases, the prion infection was assumed to be transmissible, and complemented the manure theory [27, 28], which expounded on the possible spread of infectious agents into manure, accounting for the spread of prion diseases. Through the ingestion of infected animals, plants and even soil, prions could enter the biological cycle [27, 29]. The theory of prion transmission was supported by a 2011 observation of airborne transmission in laboratory mice, via aerosol particles [30]. Further evidence has also demonstrated the transmission of prions based on a 2011 study of urine-derived human menopausal gonadotropin [31]. The transmission property of prions has thus led to the development of precautions for the disposal of prions or potentially infectious material.

2.2. BSE emergency and potential peptides conversions

According to an overview of the rendering industry of the United States and Canada in 2015, around 30%-50% of the weight of livestock is not directly consumed by humans [32]. Parts of animals that are not consumed are instead used by the rendering industry to recover hide and skin, fats and oils, and protein-rich meals. The rendering industry used to recover these animals for processing into the products for oleo-chemical industry and the feed industry. These products can be of high-value, including cosmetics, commodity chemicals, pharmaceuticals and biofuels, while downstream feedstocks are used in low-

value products such as fertilizers [33]. Even though the sterilization process is invoked for environmental safety, including the elimination of *Clostridium perfringens*, *Listeria monocytogenes*, *Campylobacter jejuni* and *Salmonella* species [34], there is no evidence that all dangerous micro-organisms remain inactive after the rendering procedure, and thus downstream products still face high safety concerns [35]. Transmissible BSE, one of the most serious diseases of cattle, may be potentially stored after the procedure. Since the BSE emergency that began in the 1980s in England and spread to Europe, Japan, North America and other countries [36], certain animal parts, such as neck bones, tissue, brains, or eyes, have been limited and even banned from all related industries. These parts are listed as hazardous wastes and classified as specified risk materials (SRM), due to the risk that they may contain prion.

2.2.1. The history of BSE banning politics in Canada and the US

To defend against a potential BSE outbreak, the US and Canadian governments and food agencies initiated strict risk prohibition policies in 1997. Canada took the first steps of drafting the policy by banning proteins that had been identified and prohibited by the World Health Organization, derived from most mammals, in animal feed. However, BSE was not prevented from entering Canada; in 2003, a BSE-positive cow was discovered, forcing the government to ban the potential risky proteins in the food industry. The policy was then

extended to prohibit use in animal fertilizers in 2007 to eliminate potential carriers of prions [5, 37]. The Canadian Food Inspection Agency (CFIA), characterized certain animal parts - the skull, brain, trigeminal ganglia, eyes, spinal cord, and dorsal root ganglia from cattle over 30 months of age and the distal ileum and tonsils from cattle of all ages (Figure 2.1) - as specified risk materials (SRM), which are statistically related to the infection of BSE [38].

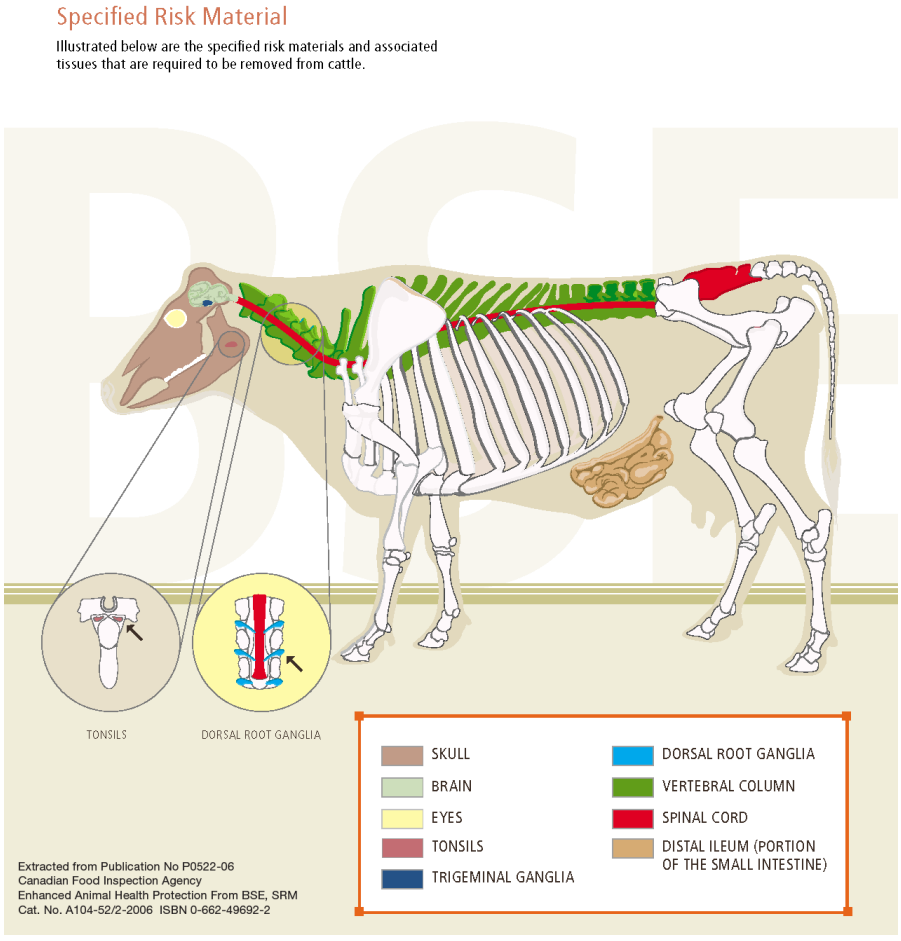


Figure 2.1. Specified risk materials (SRM): parts of cattle in Canada [38]. Used with permission of CFIA.

Additional procedures for the rendering industry, including the separation and disposal of SRM tissues, were legislated by the Canadian government in 2003, though these procedures completely changed the beef industry [4]. To segregate and dispose of more than 300,000 tonnes of SRM produced annually, companies spend an estimated CAD$75-200 per tonne of SRM [7]. As a downstream product of the rendering industry, fertilizer was also affected by the strict limitations on product composition. To meet Canadian requirements for fertilizer [4], only non-SRM bone meal and blood meal were used in the process, imposing serious restrictions on the source of the fertilizer feedstock. The import and export of cattle between Canada and other countries have also been modified and re-organized due to the policy change.

The US also established a feeding ban in 1997 and imposed several regulations regarding BSE defence and food safety. However, a case of BSE still appeared in Washington, resulting in a violent earthquake in US beef exports. A later discovery of BSE in 2005 forced the US government to establish the same classification of SRM as Canada, and add further restrictions on the usage of blood meal, fish meal and known-SRM bones [5]. Moreover, the disposal of SRM was strictly regulated in response to the appearance of BSE [5].

Compared to the previous rendering chain, a significant amount of proteinaceous material has been prohibited under these policies, reducing total product yield and feedstock conversion efficacy. Furthermore, rendering companies were required to separate, store,

transport, and dispose SRM water, which resulted in further expenses in the production process.

2.2.2. Thermal hydrolysis of SRM and peptide characterization methods

Due to the economic emergency that BSE brought to North America, it is necessary, especially for the rendering industry, to develop a cost-effective method of SRM bio-conversion into value-added products. With the nature of SRM, specifically the protein content, it is possible to develop strategies for SRM bio-conversion based on reported cases of protein modification/cross-linking for the production of renewable materials such as bio-plastics [38], adhesives [39], flocculants [40], and microbial growth media [41]. The only significant issue prior to the conversion is the removal of potential prions in SRM. There are currently 5 types of SRM disposal methods approved by the Canadian Food Inspection Agency (CFIA) [42]: landfilling, incineration, composting, burial, and hydrolysis.

Landfilling and burial take decades, require substantial amounts of land, and do not result in the generation of any value-added materials [43]. Furthermore, the increasing annual production of SRM will likely make this method infeasible in the future. Composting requires costly infrastructure for large-scale disposal and a substantial amount of time [44]. Incineration, while effective, requires much higher energy demands than general disposal methods such as landfilling and burial. Hydrolysis of SRM has emerged as an attractive

method of SRM disposal as it requires lower costs compared with incineration [38] and enables recovery of peptides or protein fragments that can potentially be valorized through various bioconversion processes [7]. To maximize the properties of SRM-derived peptides, such as the number of functional groups (i.e., amino groups, carboxyl groups and hydroxyl groups, etc.), molecular weight, nitrogen content, and water solubility, the parameters applied to SRM thermal hydrolysis become extremely important.

2.2.2.1. SRM thermal hydrolysis

Based on the hydrolysis method approved by the CFIA [43], both alkaline hydrolysis and thermal hydrolysis methods can be used for SRM disposal in prion removal. According to the reported protocols [4], alkaline hydrolysis is performed at a temperature of 150°C and a minimum 400 kPa pressure for 180 min/cycle with 15% w/v NaOH solution. On the other hand, the thermal hydrolysis of SRM is conducted at 180°C and 1200 kPa for 40 min/cycle with different amounts of pure water [4]. It is also useful to mention that in the thermal hydrolysis process, all instruments must be operated in a biosafety cabinet, followed by a 5% Environ LpH and 70% ethanol cleaning procedure to eliminate interactions of potential prions [45].

Although both of these protocols are effective in SRM hydrolysis, the peptides recovered under these protocols exhibit different properties. Mekonnen *et al.* compared

products derived from alkaline hydrolysis and thermal hydrolysis with different amounts of solution loadings based on the properties of the products in terms of molecule weight, free amino acid amount, protein concentration and water solubility [7]. According to their report, the alkaline hydrolysate of SRM displayed a lower and narrower molecular distribution than the thermal hydrolysate. Larger amounts of free amino acids were observed through alkaline hydrolysis (29.03 - 135.97 mg/g) indicating a higher degree of protein destruction as compared to thermal hydrolysis (5.69-9.74 mg/g) [7]. Moreover, the higher solubility and viscosity of the collected peptides in thermal hydrolysis [7], indicate thermal hydrolysis as a promising method for further adhesive, bioplastic, surface material and flocculent development.

2.2.2.2. SRM-derived peptides recovery and characterization methods

Before introducing the specific protein modification strategy for SRM conversion, recovery method characterizations are also necessary for the peptides produced from thermal treatment. For the general recovery process defined in our lab [46], the thermal hydrolysate of SRM was first diluted and filtered to remove any insoluble material. The collected filtrate was then washed with hexanes to facilitate the removal of any residual lipids. SRM-derived peptides can then be obtained through the lyophilisation of the collected aqueous fraction of the filtrate.

With regard to characterizations, several methods that have previously been used for protein studies and general polymer tests can be applied to SRM-derived peptides [38]. These methods can be classified into three types: functional groups characterization, molecular weight characterization and physical properties characterization.

The characterization of functional groups pays attention to two of the significant functional groups in SRM-derived peptides, which are also widely used in the study of amino acids/peptides/proteins: amino groups and carboxylic groups. Based on the method developed by Claudia *et al.* [47], free amino group concentration can be defined through the o-phthalaldehyde (OPA) reagent incubation of peptides, which forms coloured compounds upon reaction with the primary amines, followed by absorbency readings at 340 nm. This method was first applied for the characterization of SRM-derived peptides and their cross-linking products. However, the absorbency of the compound solution resulting from the OPA method keeps fluctuating with the incubation time and measuring time: less time will lead to the incompleted derivatization reaction, while longer time will cause the decomposition of reagents. Moreover, there is no preferred time mentioned in the method. This leads to the variation in the absorbency readings. Under the same mechanism, primary amino groups can also be treated with 2,4,6-trinitrobenzene sulfonic acid 5% (w/v) in methanol, forming orange-coloured compounds, followed by an absorbency reading at 335 nm, a process that is referred to as the TNBSA method [48, 49]. Since the reaction was stopped and stabilized

via adding HCl (1 N) and 10% sodium dodecyl sulfate solution after a suggested incubation (2 h), the TNBSA method gives a constant absorbency reading for the incubated peptides [48]. TNBSA method is thus used for SRM-derived peptides characterization in this study.

An adaptation of the titration method developed by Tropini *et al.* can be applied for the characterization of carboxylic groups [50]. From the conversion of $^{+}H_3N$-RCH-COO^{-} into $^{+}H_3N$-RCH-COOH indicated by the pH change from 6.0 to 3.0, the concentration of carboxylic groups can be characterized by the amount of 0.1 N hydrochloride (HCl) added for titration [51]. Other general characterization methods, direct or indirect, for functional groups, such as CHNS analysis, Fourier Transform Infrared Spectroscopy (FT-IR) and mass spectrometry (MS), can also be used in SRM-derived peptides study.

Another significant property of proteins/peptides mixtures is the molecular weight distribution. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) and size-exclusion high-performance liquid chromatography (SEC-HPLC) are effective ways to assess the molecular weight distribution of proteins/peptides in the SRM hydrolysates [7, 51]. However, as a requirement for SEC-HPLC, the samples should be completely soluble in the mobile phase, limiting the effectiveness of SEC-HPLC in the characterization of some hydrophobic or highly cross-linked samples.

Apart from chemical properties, general physical properties are more relevant to the application demands. With the development of materials science, a number of methods have

been used to examine these properties, such as thermal stability, aqueous viscosity and rheological property, which are essential in protein bio-conversion for the characterization of physical properties. Thermogravimetric analysis (TGA) and differential scanning calorimetry, for instance, are recognized as common methods for determining a product's thermal stability. Since cross-linking bonds help to improve the thermal stability of the material, while the number of cross-linking bonds is related to the cross-linking degree of the material, the difference in cross-linking degree can be estimated by comparing decomposition temperature and the mass of heating residuals of the cross-linking products in TGA. Cross-linking degree can also be estimated by comparing the glass transition point and the melting point of products achieved in differential scanning calorimetry analysis.

For the measurement of aqueous viscosity and rheological property, a viscometer and rheometer are used, respectively, to observe and predict the behaviour of the product solution. On the other hand, some specific physical properties must be investigated to meet the demands of the applications. These properties include solubility, binding property, moisture up-taking, product strength/hardness, and heating value, and are usually assessed by following the standard protocols or industrial regulations used in the target applications.

2.2.3. The potential conversion of SRM-derived peptides

According to Mekonnen *et al.*'s study, SRM-derived peptides were composed of 91.0 ± 1.7% proteins, 3.89 ± 0.2% ash, and 5.10 ± 1.5% residual lipids [7]. The molecular weight of SRM-derived peptides was reported as 2-14 kDa in their study [7]. Furthermore, the protein content and molecular weight of peptides derived from a different batch of SRM under same conditions was characterized as 90.0 ± 0.62% and 1.36-12.4 kDa, respectively, in the later study by Birendra *et al.* [46]. Also, the primary amines and carboxylic groups of SRM-derived peptides were characterized as 0.59 ± 0.05 mmol/g and 1.69 ± 0.06 mmol/g, in the study by Shui *et al*. [12], which has no significant difference to the values of 0.50 ± 0.02 mmol/g (primary amines) and 1.60 ± 0.05 mmol/g (carboxylic groups), reported by Birendra *et al.* [46]. These functional groups are targeted for SRM-derived peptides cross-linking in this study. All of these characterizations demonstrated a great reproducibility in the preparation of SRM-derived peptides. Given their solubility in water, their outstanding binding properties, and abundant functional groups, SRM-derived peptides can potentially be modified into protein-based products that can be applied in different fields, such as flocculants and adhesives (Figure 2.2). Moreover, compared to the artificial polymers used in these areas, SRM-derived peptides display promising biodegradability and possess considerable carbon/nitrogen content that can be used as carbon/nitrogen sources for plants,

animals and microorganisms. Therefore, determining directions and modification strategies for SRM-derived peptides bio-conversion have become essential.

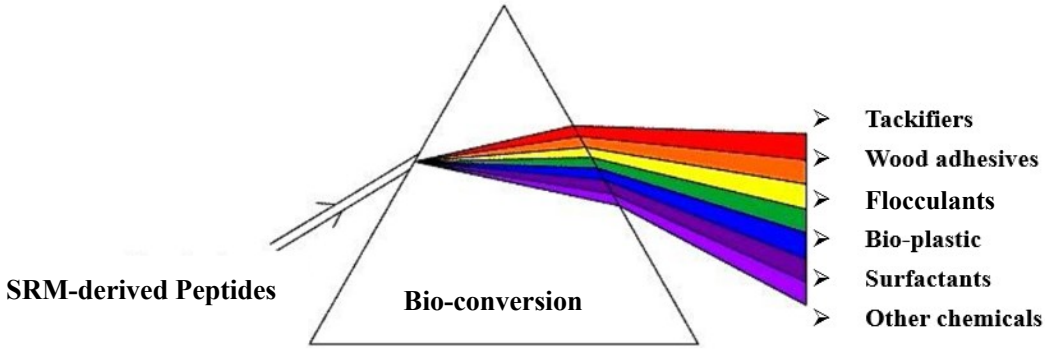


Figure 2.2. Potential bio-conversion model for SRM-derived peptides [38]. Adapted with permission of the publisher.

2.2.3.1. Peptide-based tackifier

To cope with the disturbance of native landscapes by agriculture, urbanization and natural evolution caused by normal rain events [52], one potential value-added opportunity for the use of SRM is to convert the peptides resulting from the thermal hydrolysis of SRM into tackifiers. Tackifiers function as a special type of adhesive in hydro-seeding or hydro-mulching applications, and help to protect soil from erosion in areas characterized by challenging soil conditions, high surface winds, and/or extreme weather conditions [53]. Tackifiers are typically mixed with water, fibre mulch, seed, fertilizer, and some soil

stabilizers (for severely burned and highly erosive areas), and facilitate plant re-growth and the maintenance of surface water levels [54]. Tackifiers must display longevity, erosion control, vegetative establishment and wet bond strength in order to be considered as important binding additives [55].

For erosion control and wet bond strength, the industry currently uses synthetic polymers as tackifiers, consisting of polyacrylamide (PAM) or polyacrylate (PA) or a copolymer of acrylate and acrylamide. However, the non-biodegradability of PAM complicates the process for wide application [56]. Similarly, guar gum, obtained from guar seeds, is currently the principal gum used as a tackifier (binder) in hydraulically applied mulches (hydro-mulches) for erosion control, or as a clumping agent in biodegradable cat litters [57, 58]. Due to price increases, to as much as $18.00/kg [59] for guar gum and other guar replacements such as psyllium or xanthan, and its dubious erosion control in rainfall, guar is usually combined with other, more cost-effective polymers for industrial applications. Natural or artificial polysaccharides, such as corn starch, are often used as alternative tackifiers due to the abundance of sugar sources, their considerable binding strength, and their relatively low price ($0.66-1.44/kg). In spite of these advantages, polysaccharides are limited in applicability due to doubts about their longevity and to their limited resistance to water [60].

Tackifiers usually show conflicting demands due to the complexity of the application environment, such as water-solubility vs water resistance, biodegradability vs longevity, etc. It is difficult for a single material to achieve all the balance points of these demands. Therefore, tackifiers have been developed as cost-effective and high cross-linked/polymerized products, using the by-products of agriculture, food processing and other industries as the feedstock [55]. Moreover, due to the cross-linking strategy involved in the conversion of these by-products to tackifiers, the potential toxicity of either the feedstock or related reagents is a concern for the hydro-mulch industry. To deal with the demand for tackifier investment, industries have paid more attention to protein fragments/peptides recovered from the rendering process, especially for SRM-derived peptides. SRM-derived peptides display desirable properties with regards to binding, degradability, carbon/nitrogen content and hydrophilicity [46], which allow them to be treated as potential feedstock for tackifiers in order to build wet bond strength and vegetative establishment. Also, the multiple functional groups allow peptides to be modified with desirable co-polymerization reagents to build erosion resistance and strengthen binding. SRM-derived peptides have also been recognized as a non-toxic feedstock with a relatively lower price than PAM and guar seeds [38, 58, 61]. Although there is currently no peptide-based formula available for hydro-mulches, it is still possible to develop SRM-derived peptides into a competitive tackifier by using the multiple chemical strategies for peptide-based adhesives [38].

2.2.3.2. Peptide-based wood adhesive

2.2.3.2.1 Wood board binders

Wood board binders were designed as components for engineered wood products, including plywood board, oriented strand board, parallel and laminated strand lumber and medium-density fiberboard [38]. Wood binders enhance dimensional stability, improve moisture resistance, and most importantly, facilitate the combination of materials. Combinations of different types of materials that fit market diversification are especially significant for industries such as construction [61], with wood binders as key ingredients in their production. Petrochemicals such as urea-formaldehyde (UF), methyl urea-formaldehyde (MUF) and phenol-formaldehyde (PF) are mostly used [38] to provide binding force and produce the desired enhancements of strength/hardness, density, water resistance, or thermal stability [62], but the use of formaldehyde and phenol risks the health of producers and consumers. The International Agency Research on Cancer has reported, for instance, that the risk of cancer increases with direct or indirect contact with these substances [63].

Due to the increasing environmental and health concerns of PF resins, renewable and non-toxic materials such as lignin and protein were examined as the feedstock for binder production. Lignin, mostly recovered from the pulp industry, is used as a replacement for phenol in the traditional PF formula, reacting with formaldehyde to increase product

viscosity [64]. The study of lignin demonstrates the feasibility of decreasing the use of PF systems, but is hard to apply practically due to the limited reactivity of lignin [65].

Moreover, proteins and peptides have been made into adhesives since the A.D. 50, using whale intestines as the feedstock [66]. The modern protein-based wood adhesive industry focuses on two major types of adhesive systems: protein-phenol-formaldehyde and formaldehyde-free [11]. In protein-phenol-formaldehyde adhesive systems, protein is combined with formaldehyde-based resins to create an irreversible incorporation into the phenol-formaldehyde network, replacing the phenol in the resins [67, 68]. According to Jiang *et al.*, protein-based adhesives can be created under this system by combining alkaline hydrolyzed feather protein with a mixture of phenol and formaldehyde, and then heating the ternary mixture at 90°C for 2.5 h [69]. In formaldehyde-free proteins adhesive systems, in contrast, the denatured protein is modified or cross-linked with the desired reagents to form the adhesive directly. Through cross-linking with different reagents, various proteinaceous by-products such as bones, blood, meat and SRM-derived peptides have been successfully converted into glue or adhesives and displayed greater binding strength than unmodified peptides [40, 69-73]. To generate rigid three-dimensional networks of polymers, and enhance the binder's water resistance and binding strength, reagents such as aldehydes, epoxy resins, polyols and maleic anhydrides can be used to modify or cross-link with proteins/peptides. This process will be discussed later.

2.2.3.2.2 Wood pellet binders

Wood chips, a source of alternative renewable energy, have been studied as feedstocks for fuels in order to cope with the challenge of greenhouse gas emissions [74-78]. To improve the energy density of wood chips (6.5 GJ/m^3) up to the level of coal (23-26 GJ/m^3), torrefaction and densification technologies are required, which could convert wood chips into torrefied wood pellets with an energy density of 16-22 GJ/m^3 [77, 79, 80]. Torrefaction is a thermal treatment under a low-oxygen environment at 200-300°C. As the first step for the production of torrefied pellets, torrefaction has been studied with regards to its kinetics and optimized conditions, due to its great function in increasing the energy density, softness, and hydrophobicity of the torrefied biomass to make high-grade pellets [74, 77, 80, 81]. However, compared to the research interest in the torrefaction process itself, few studies have been reported on the densification of torrefied particles, such as torrefied wood, into pellets [74].

Densification, the step that follows the torrefaction treatment, is a process that compacts biomass into solid particles of uniform shape and size (i.e., pellets, briquettes) by applying a mechanical force [74]. This process leads to a significant enhancement in bulk density. Torrefied pellets are not yet fully commercialized due to difficulties in producing durable pellets from torrefied biomass. However, the opportunity to expand the trade in torrefied pellets is still available because torrefied pellets can replace thermal coal without

substantial modification to industrial boilers. The current trade in white wood pellets (non-torrefied) accounts for more than 28.5 million tonnes of the annual world production of pellets [78].

Rumpf and Knepper's study of the mechanism of particle densification identified five major mechanism categories: attraction forces between solid particles, interfacial forces and capillary pressure to move liquid such as water onto surfaces, adhesion and cohesion forces between particles, formation of solid bridges, and mechanical interlocking to form closed bonds [82].

It is worth mentioning that the conditions of the densification process, including the temperature of the die, holding pressure, holding time, and load speed [76, 83-85], are essential in determining the properties of the pellets, including density, strength, moisture up-taking, and heating value [74]. To maintain the high density of the pellets, which reduces transportation and handling costs, the studies of Phanphanich in 2013 and Verhoeff *et al.* in 2011 on torrefied sawdust for pellets suggested high die temperature and holding pressure during the densification process for torrefied biomass [76-83]. Moreover, the mechanical strength of the pellets determines how easily the pellets will break during handling, transportation, and storage. Unlike a durability test used in large-scale production, which determines the percentage of mass retained after mechanical mixing [86], the strength of the pellets made by a single pellet pelletizer has been commonly estimated from the level of

breaking force established through compression testing [74]. Peng *et al.* reported a higher strength of the sawdust pellets at a temperature of 220℃ or above than that of the pellets made in the range of 70 to 220℃, indicating the effect of die temperature on pellet production [74]. Another essential parameter that affects pellet quality, moisture up-taking , is associated with the storage life of the pellets, which is measured by the mass change of pellets placed in a humidified chamber [83]. By treating the torrefied sawdust for pellet-making, Verhoeff *et al.* found that a higher die temperature (260℃) was preferable during the densification process, in order to yield a relatively low moisture up-taking value [76].

However, torrefaction and resulting reactions eliminate many of the functional groups in unreacted biomass to give strength and density increase [80]. Densification has less impact on improving the properties of pellets via forming new bonds. This leads to the demand on external binders to provide additional functional groups for the densification process. Binders provide the natural adhesion force that brings interlocked particles into close contact, assisting the development of solid bridges in particles by heating and compression during the densification process [81, 87-90]. Therefore, binders can theoretically improve the pellet strength without compromising other qualities. The significance of binders was first confirmed by Ghiasi *et al.* in their study of bio-coal production [77]. Also, potential binders such as lignin, starch, sawdust, calcium hydroxide, and sodium hydroxide were found to be effective in the torrefied biomass densification

process when used at a relatively high level (up to 30%) [81, 91-93]. However, the strict standards of the International Organization for Standardization (ISO) only allow a binder level of less than 4 wt.% for premium quality pellets made from thermally treated wood [94]; thus, wood binders are scarce in the current market [91]. This indicates a great opportunity for SRM to be converted into bio-degradable and cost-effective pellet binders.

2.2.3.3. Peptide-based flocculants

Flocculation is a process that results in particle aggregation in the colloidal suspension of a separated solid, and is mostly used in wastewater treatment [95]. In this process, reagents with positive charges, called flocculants, capture solids with negative charges from the colloidal suspension using electrostatic repulsion, forming stable precipitates or large matters. The quality of flocculants is thus judged by the length of precipitate formation time. To meet demands for flocculants, synthetic polymers including PAM, polyacrylic acid and polydiallyl dimethyl ammonium chloride are widely applied in wastewater treatment, and some extended fields such as oil recovery, paper manufacture, and floatation, due to their outstanding performance [96-99]. However, synthetic polymers raise concerns of biodegradability, shearing stability, and toxicity [38], thus leading to the use of renewable feedstocks, which are proteins/peptides, as surfactants.

Protein/peptides, especially SRM-derived peptides, display various advantages, such as low feedstock cost, outstanding solubility, potential release of carbon/nitrogen source as a fertilizer for plants and micro-organism, significant biodegradability, and non-toxicity, which are highly appreciated in flocculation application. By contributing to the formation of polyampholite chains and positively charged amines, protein/peptides can be as effective as synthetic polymers in the flocculation process [100]. Moreover, as Liu *et al.* have demonstrated in their study of soy protein, the function of protein/peptide in flocculation can be enhanced by methylation [101]. Protein methylation can be accomplished by esterification with alcohols in the desired pH range, capping the anionic functional groups, such as carboxylic groups, to increase the positive charge of the molecule. The resulting product can thus increase the speed of aggregation and precipitation, and can even approach the quality of PAM [101]. However, grafting is currently a more common strategy than methylation for the development of flocculants. This strategy was originally used for PAM grafting, by applying biodegradable materials, such as starch, to adjust the general cost, toxicity and solubility [102]. Even though grafting is scarcely mentioned in protein modification [38], this process can be theoretically applied to the development of protein/peptide-based flocculants by introducing bioconjugation into amino groups or carboxylic groups.

2.3. Cross-linking strategies for peptides

Cross-linking is fundamentally defined as the linking process that connects different types of molecules, usually reached via the formation of covalent bonds among the molecules [103]. Through the connection/linkage conducted with suitable reagents and reaction conditions, the chemical properties of the reagents can be modified, combined or even developed into new agents. For instance, catalyst/reactant immobilization can be achieved by cross-linked with gels or columns (solid phase), known as 'immobilized reactors', which was used for the separation of bio-ethanol and bio-diesel from the reactants [104=106]. Cross-linking strategies have also been used in biological studies for affinity-mediated enrichment, purification, or scavenging of biomolecules [107, 108]. In medical applications, modern medical technologies such as target antibodies and medical imaging have also been developed based on the cross-linking strategies [109-111].

As important components in cross-linking, the agents that act as the 'glue' to establish connections among other molecules are called cross-linkers. Appropriate cross-linkers are selected based on an accessible coupling reaction with one of the functional groups in the target molecules. This coupling reaction can be initiated under certain conditions directly, or via an additional activation process. About 1-2 dozen coupling

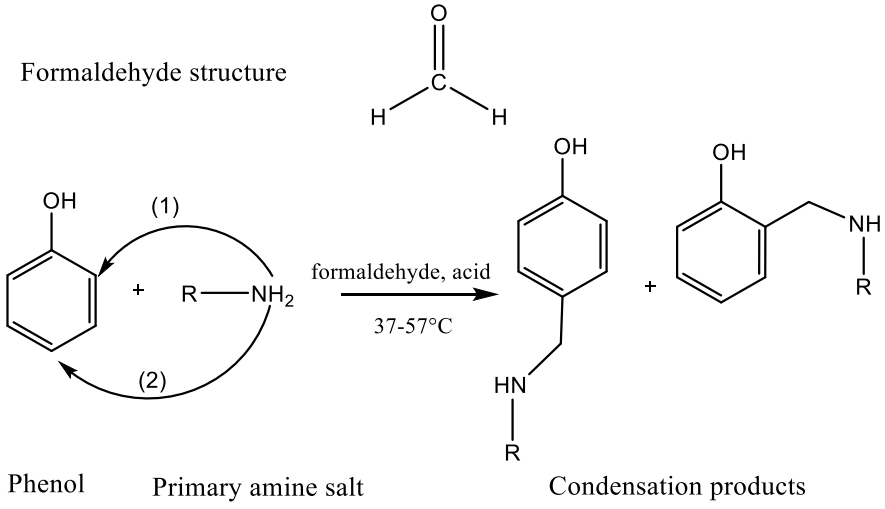
reactions between cross-linkers have been identified, demonstrating the outstanding diversity of cross-linking pathways [103].

2.3.1. Aldehydes

Aldehydes are notable among specific cross-linking reagents due to their wide applications in protein/peptide cross-linking. The use of aldehyde agents in protein studies has ranged from a single-carbon aldehyde (formaldehyde, forming aldehyde oligomers) to a five-carbon aldehyde (glutaraldehyde) [112, 113]. In addition, several aldehyde derivatives, such as pyridoxal polyphosphate, have been used in protein conjugation [114]. Two typical aldehyde agents, formaldehyde and glutaraldehyde, are discussed in this section as entry points and reference cases for aldehyde cross-linking.

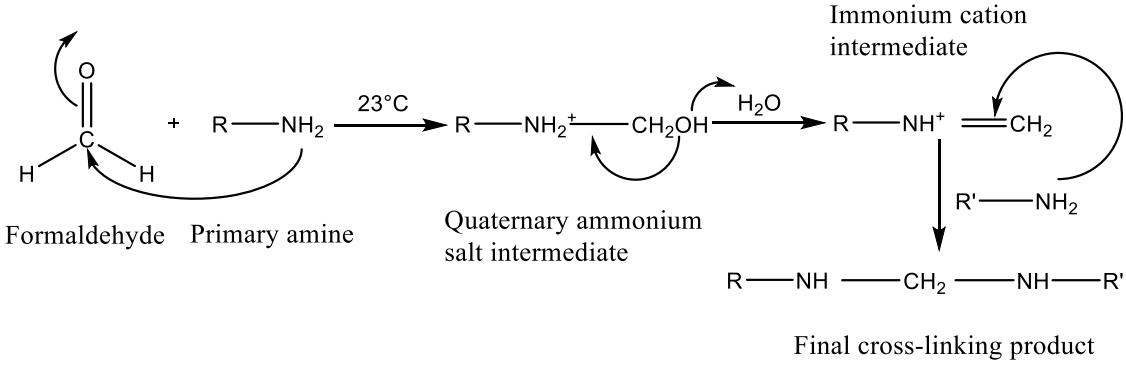
In most cross-linking cases, the cross-linkers used for peptide studies contain at least two reactive groups in one molecule. However, as the smallest aldehyde cross-linker with a mirror-symmetric structure (Reaction 2.1, formaldehyde structure), formaldehyde can lead to the conjugation of peptides or other compounds with amino groups by possessing two functional groups. It is notable that aqueous solutions of concentrated formaldehyde tend to form a low molecular weight oligo-formaldehyde, which functions as a homo-functional cross-linker. Since the formation of oligos can be greatly limited by diluting the concentration of formaldehyde, commercial formaldehyde has a concentration of 37%, and

methanol may be added as a stabilized surfactant. The pathway of formaldehyde cross-linking is mainly carried out by the Mannich reaction, which consists of formaldehyde condensation with ammonia and another compound with an active hydrogen [115]. In a typical reaction of formaldehyde with peptides/proteins, the hydrogen on the aromatic rings or ketones can be selected as active, making primary and secondary amines available for the formaldehyde condensation (Reaction 2.1). To optimize the yield of cross-linked products, 37% formaldehyde aqueous solution is usually preferred in 10-fold molar excess over the peptides/proteins under the conditions of 37-57℃ and 2–24 h to reach the acceptable conjugation [103]. Based on the mechanism of the Mannich reaction, formaldehyde has been commonly used in the immobilization of drugs and dyes, and the protein carrier hapten [116, 117].



Reaction 2.1. The structure of formaldehyde and the Mannich reaction occurs between an active hydrogen-containing compound (phenol) and an amine-containing molecule in the presence of formaldehyde.

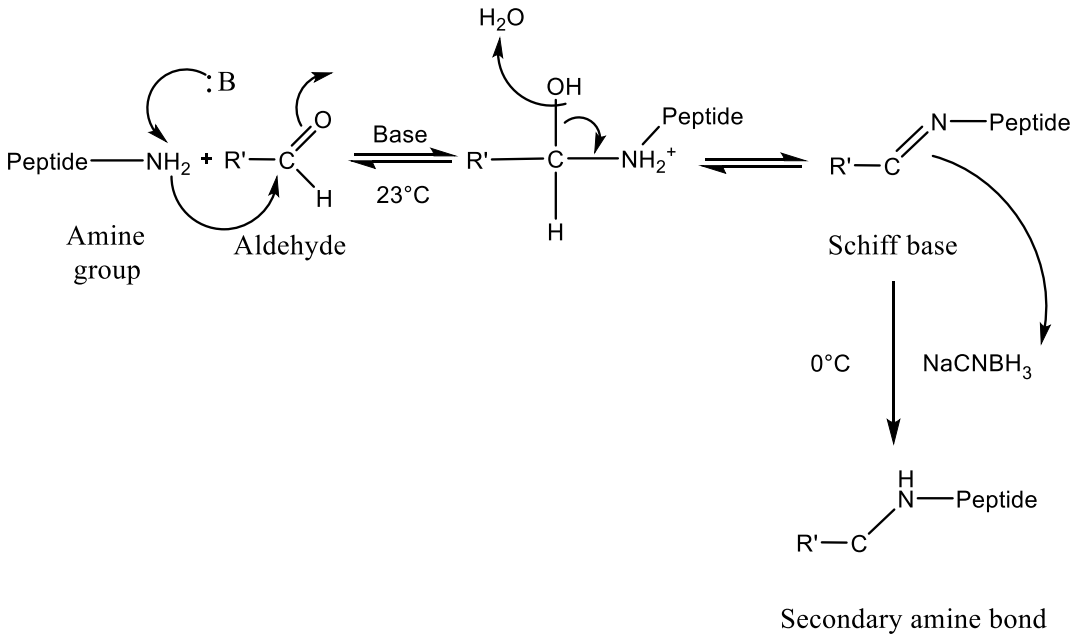
In addition to the Mannich reaction, another reaction pathway, the immonium ion mechanism, may be involved in the conjugation of formaldehyde with peptides [103]. In this pathway, formaldehyde first reacts with a primary amine to form an intermediate, a quaternary ammonium salt. In the second stage, this intermediate is spontaneously converted into a highly reactive immonium cation by losing one molecule of water [118]. Finally, the immonium cation attacks the nucleophiles of the peptides, leading to a methylene bond between two macro-molecules (Reaction 2.2). This specific pathway can occur simultaneously with the Mannich reaction, but only works for the cross-linking between an aldehyde and a nucleophile. Therefore, the pathways of formaldehyde cross-linking can be differentiated by the functional groups contained in the peptides.



Reaction 2.2. Immonium ion mechanism.

Glutaraldehyde, a linear five-carbon di-aldehyde, is used as an aldehyde cross-linker in various applications such as histochemistry, microscopy, cytochemistry, leather tanning, enzyme technology, chemical sterilization, and pharmaceutical sciences [118]. Glutaraldehyde is a clear, transparent, water-soluble, oily liquid that can effectively initiate cross-linking between proteins/peptides. However, the complex coupling mechanisms of glutaraldehyde in an aqueous system create uncertainty about the properties of the resulting products. Five types of reactions could probably take place in glutaraldehyde cross-linking study: the Schiff base (imine) formation, the Mannich reaction, the Michael addition, the Stork enamine alkylation, and aldol condensation [119]. As the mechanisms of the Mannich reaction have been mentioned previously, only the remaining four reactions are discussed below.

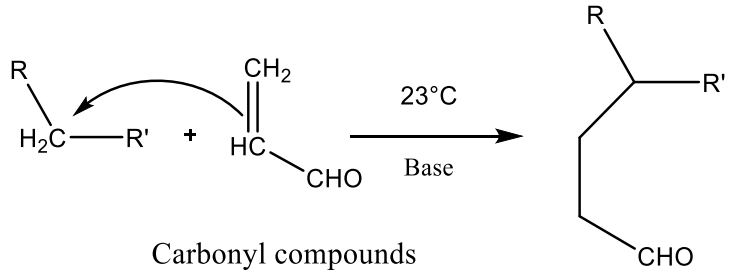
Schiff base formation, a common amino reaction, can be initiated by carbonyl groups such as aldehydes and ketones. However, the product of the Schiff base is recognized as an unstable intermediate with three free forms that are generated only under basic conditions. To achieve a stable product rather than the intermediate, sodium cyanoborohydride ($NaCNBH_3$) can be added as a follow-up treatment for the Schiff base product, leading to the reduction of the Schiff base bonds into constant secondary amine linkages (Reaction 2.3).



Reaction 2.3. Schiff base formation.

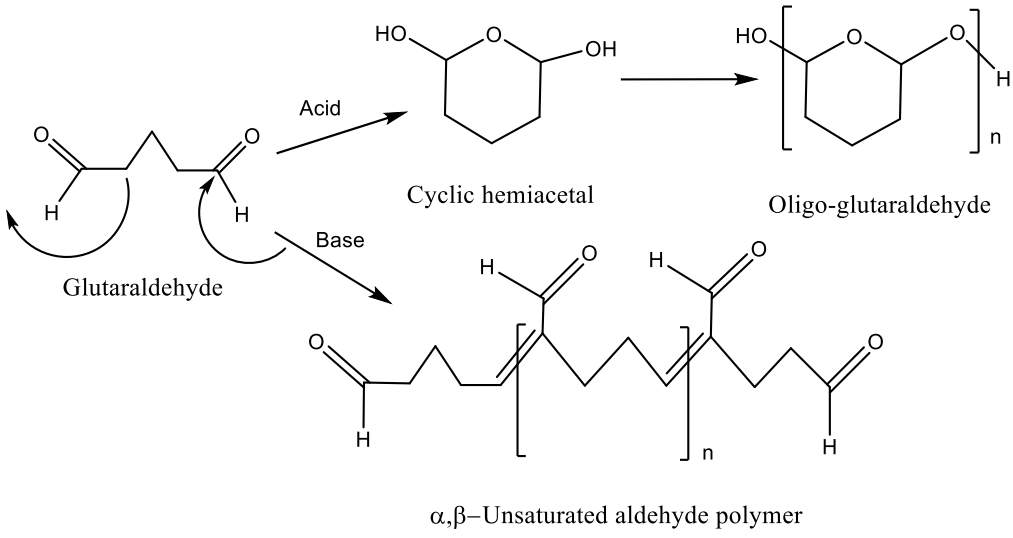
The Michael addition is a type of nucleophilic addition between a carbanion and an α, β-unsaturated carbonyl compound that forms a C-C bond (Reaction 2.4) [112]. Like the mechanism for the immonium ion, the initial step of Michael addition is a nucleophilic addition of a carbanion to an unsaturated carbonyl compounds with an electron-withdrawing group, forming the intermediate [119]. In the following step, the intermediate abstracts a proton from the base or other solvents, transforming into the final carbonyl product or an imine with great stability, which is different from the formation of a cation in the immonium ion pathway. As a special case of the Michael addition, the Stork enamine alkylation occurs between a ketone/aldehyde and a secondary amine, which is carbanion presenting in the proline and histidine residues contained within proteins/peptides. Meanwhile, the enamine

product formed during the Stork enamine alkylation does not produce a stable linkage and can be reversed or consumed by other reactions [119].



Reaction 2.4. Michael addition. R and R' are electron-withdrawing groups, making the methylenehydrogen acidic forming the carbanion on reaction under a base condition.

As one of the most considerable sources of uncertainty in glutaraldehyde cross-linking, aldol condensation is worthy of note here. Aldol condensation is responsible for both the self-polymerization of glutaraldehyde and condensation among aldehydes/ketones, which can be potentially catalyzed by peptides or proteins. On the one hand, the self-polymerization of glutaraldehyde may lead to cross-linked products that consist of linear glutaraldehyde oligomers, causing the unwarranted consumption of the glutaraldehyde **(**Reaction 2.5**)** [118]. On the other hand, the unstable ketene formed by the condensation of aldehydes/ketones may incorporate other functional groups in the final product. Therefore, the properties of the resulting product become unpredictable, especially with regards to polymer size, molecular structure, and formation of conjugating bonds, producing difficulties in glutaraldehyde usage and scale-up for the desired cross-linking.



Reaction 2.5. The self-polymerization of glutaraldehyde.

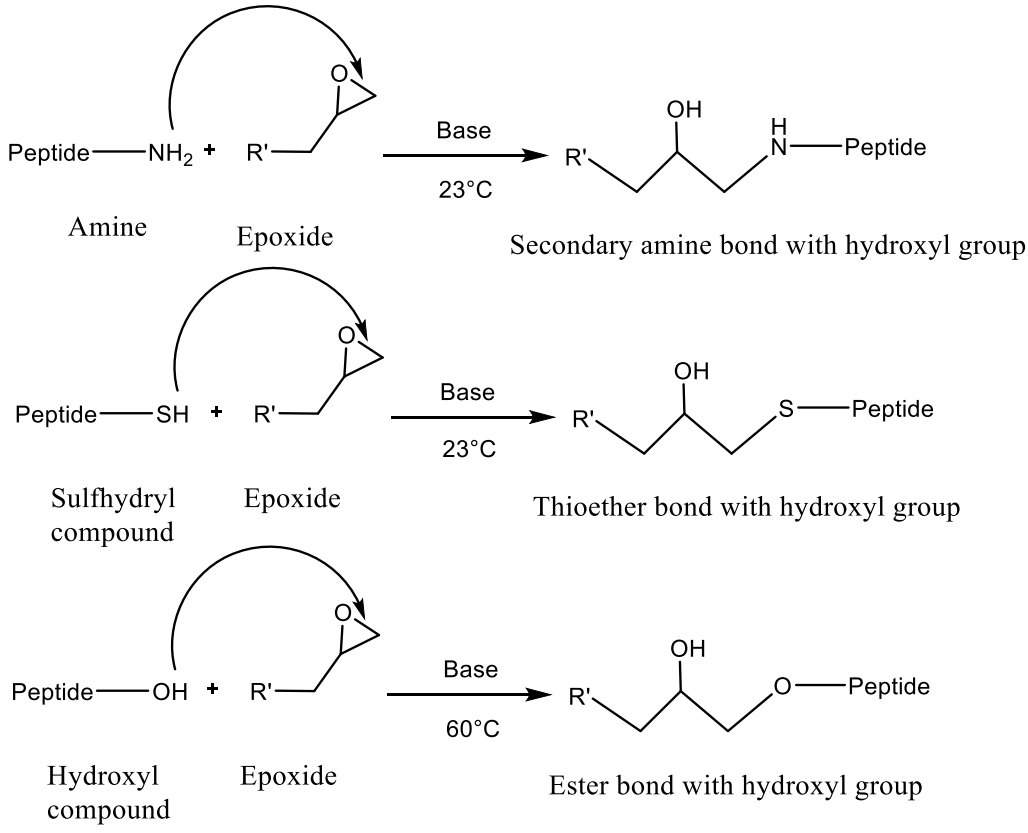
Despite the complexity of its cross-linking pathways, glutaraldehyde is commercially available at low cost, is highly reactive with primary amine ($-NH_2$) functionalities, and is generally thermally and chemically stable. All of these factors make glutaraldehyde a promising aldehyde reagent in the cross-linking of peptides/proteins [120, 121]. In various biological studies from the past 40 years, glutaraldehyde has played an important part in the fixation of enzymes and tissues [118]. Additionally, antibody-enzyme cross-linking techniques tend to use glutaraldehyde as the cross-linker in the production of vaccine immunogens [122].

For industrial applications, glutaraldehyde was identified as a feasible cross-linking reagent for the enhancement of properties in numerous protein/peptide studies, producing adhesives, hydrogels, or bioplastics. For example, Cheung *et al.* studied the mechanisms of

monomeric and polymeric collagen cross-linking by observing the cross-linking between glutaraldehyde and rat skin collagen at room temperature [123]. Their study confirmed an increased molecular length in the cross-linked products. Damink *et al.* conducted a similar reaction between sheepskin collagen and glutaraldehyde at 40°C and confirmed that cross-linking can affect the tensile strength of the collagens [124]. Ying *et al.* observed that cross-linking soy protein with different concentrations of glutaraldehyde at room temperature in aqueous solutions ranging from 4–80 μM significantly enhanced adhesion performance [125]. Wang *et al.* also reported swelling enhancement in cross-linking between soy protein and glutaraldehyde to produce hydro-gel [126]. Additionally, in the field of protein characterization, Payne used glutaraldehyde to cross-link with albumin at room temperature to generate a larger molecule, which was then applied as a marker in SDS-PAGE [127]. Finally, Nayef *et al.* examined the cross-linking between SRM-derived peptides and glutaraldehyde at 110-150℃ [112], reporting a high degree of insolubility in their cross-linked product as compared to the original peptides. The solubility changes observed in this report indicated that the water solubility of SRM-derived peptides can be adjusted by glutaraldehyde cross-linking, which can be beneficial in the development of hydrophobic peptide-based binders.

2.3.2. Epoxides

Epoxides with at least two epoxy groups in one molecule can be used as cross-linkers in peptide/protein conjugation. Epoxides can not only lead to reactions with amines, but also tend to capture other nucleophilic groups such as hydroxyl groups, sulfhydryls and carboxylic groups (Reaction 2.6). In alkaline conditions, the amino-epoxy coupling displays the highest priority among epoxide reactions due to the highest nucleophilicity of the amino groups, forming secondary amines and β-hydroxyl groups on the open epoxy rings. Moreover, a reaction between secondary amines and epoxides can take place, possibly reaching the maximum use of amino groups. Leon *et al.* conducted a mechanism study on the reactions between glycidyl ether and amines, demonstrating that the amino-epoxy coupling occurred rapidly, with lower temperature demands than epoxide reactions for carboxylic groups and hydroxyl groups [128]. The priority of primary amines, secondary amines and tertiary amines by glycidyl ether was higher than other groups (i.e. hydroxyl groups and carboxylic groups) demonstrated this study [128], demonstrating the dominant role of amino-epoxy coupling. On the other hand, by limiting the nucleophilicity of the amino groups, acidic conditions can promote the carboxyl-epoxy coupling during the peptide/protein conjugation.



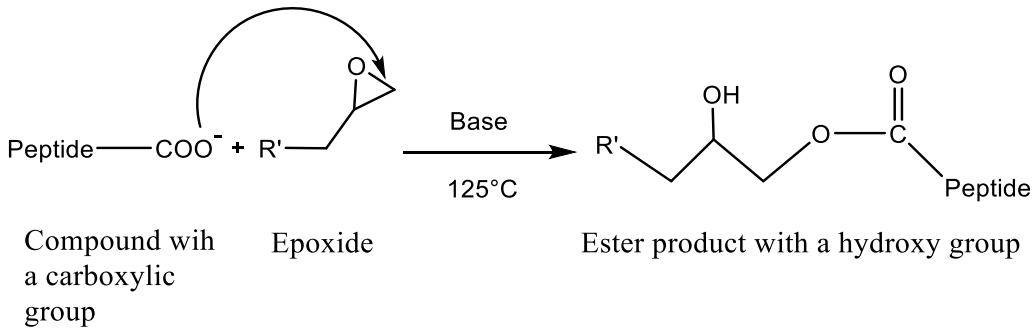
Reaction 2.6. Epoxides involving ring-open reactions.

As cross-linkers, epoxides can vary in the length of the molecular chain for different applications, ranging from 4-carbon, such as 1,2,3,4-diepoxybutane, to 12-atom, such as 1,4-(butanediol) diglycidyl ether, and even poly-epoxides [129-132]. For the conjugation of peptides/proteins, 1,4-butanediol diglycidyl ether is recognized as a common cross-linker in amino-epoxy coupling under moderate alkaline conditions. Based on Zeeman *et al.*'s modification study of dermal sheep collagen, a pH of about 8.5 was preferred for cross-linking with 1,4-butanediol [133]. Moreover, this study showed that at temperatures above 60°C with a 4 wt.% 1,4-butanediol diglycidyl ether concentration, relatively high reaction

efficiency and conversion rates were observed [133]. 1,4-butanediol diglycidyl ether can be used in additional applications of protein/enzyme immobilization for chromatography [134], demonstrating the significance of epoxides in cross-linking techniques.

By grafting epoxy groups to the polymer chain, poly-epoxides can be used in the cross-linking with amino groups, as a step toward the conjugation of polymers. This epoxy groups drafting process is called epoxidation. According to Motawie *et al.*, the epoxidation of poly (ethylene glycol) (PEG) can be accomplished by adding epichlorohydrin at different molar ratios, producing a water-soluble resin that contains significant amounts of epoxy groups [135]. Furthermore, Mingzhong *et al.*'s cross-linking study between epoxidized PEG and the amino groups of *Antheraea pernyi* silk fibroin demonstrated an increase in the water resistance, compliance, adhesion and tenacity of the cross-linked film product [136]. These studies provide alternative pathways of epoxide use and diversify conjugation strategies for the synthesis and modification of macromolecules.

Epoxides can also initiate reactions with carboxylic groups effectively through a ring-open process, forming esters in base conditions (Reaction 2.7). As a type of nucleophilic attack reaction, carboxyl-epoxy coupling requires a temperature of more than 60℃, a reaction time of 24 h, and KOH as a catalyst [128], demonstrating a pathway for the specific usage of carboxylic groups. The effect of epoxides on the conjugation of carboxylic groups is discussed further in the following section.



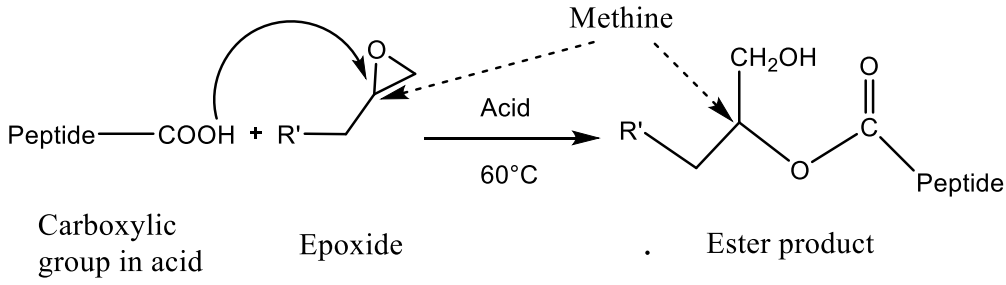
Reaction 2.7. Epoxide ring-open reaction with the carboxylic group in base conditions.

Epoxides, such as epichlorohydrin, 1,4-butanediol diglycidyl ether and some diglycidyl ether made from PEG and poly (vinyl alcohol) (PVA), can be used as potential cross-linkers in reactions with carboxylic groups [136, 137]. The coupling of carboxylic groups initiated by epoxide cross-linkers may consist of multiple pathways depending on the reaction conditions. In base conditions, hydroxyl ions first attack the epoxy rings, resulting in a ring-open process from the bond between the oxygen atom and the methylene group. The carboxylic group then intervenes in the non-oxygen position of the broken chain, forming an ester linkage and one hydroxyl group on the oxygen position of the broken chain (Reaction 2.7). Moreover, due to the generation of the hydroxyl group in the molecule, hydroxyl-epoxy coupling and hydroxyl-carboxyl coupling may be introduced into subsequent reactions, resulting in complex bonding of the products. It is worth noting that an activation agent, such as carbodiimide, is essential for the occurrence of hydroxyl-carboxyl coupling (esterification) in aqueous conditions because of the reversible reaction mechanism and by-production of water. Since hydroxyl groups have a higher nucleophilicity

than carboxylic groups in base conditions, the higher priority of hydroxyl-epoxy coupling might be an issue. Nevertheless, according to Leon *et al.*, at a temperature of 125℃ with 0.2% potassium hydroxide as a catalyst, a higher priority of carboxyl-epoxy coupling was observed compared to hydroxyl groups [128]. Leon *et al*. also demonstrated that when the carboxylic groups were consumed under the conditions of the study, an inherently faster coupling between the hydroxyl group and the epoxide was observed [128]. However, amino groups still display a higher priority in the coupling of epoxy groups in base conditions than carboxylic groups, complicating the use of carboxylic groups. Therefore, the conjugation bonds between epoxides and carboxylic groups containing molecules such as peptides can be achieved and adjusted for different applications.

On the other hand, acidic conditions open the epoxy ring from the bond between the oxygen atom and the methine, leading to the carboxylic groups attacking the methine (Reaction 2.8). Due to its inhibition effect on the nucleophilicity of the amines and hydroxyl groups in acidic conditions, epoxy-carboxyl coupling plays a dominant role among multiple coupling pathways, achieving the goal of carboxylic group use. Zhang *et al.* demonstrated this result in their study of the conjugation of polyglutamic acid and glycol glycidyl ether in the development of a high water-absorbing resin [138]. In their study, glycol glycidyl ether was applied to poly-glutamic acid at 60℃ at pH 5.0 for 44-48 h, leading to the relatively high consumption of carboxylic groups [137]. Additionally, Wang *et al.* further tested the pH

conditions of the conjugation between poly-glutamic acid and glycol glycidyl ether in the production of an advanced hydrogel at a range of 3.0-5.0, demonstrating pH 3.0 as the preferred pH condition [139].

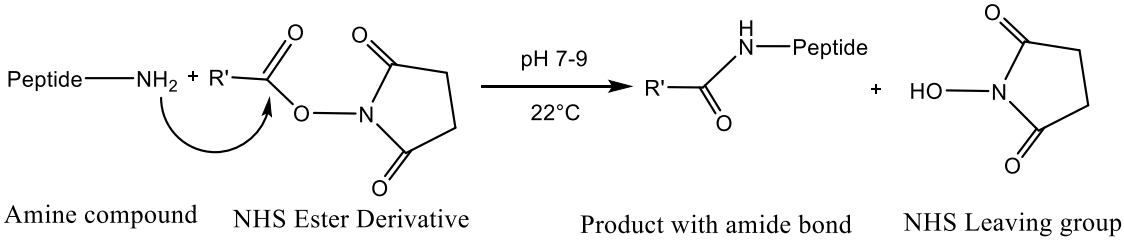


Reaction 2.8. Epoxide ring-open reaction with the carboxylic group in acidic conditions.

2.3.3. N-hydroxysuccinimide (NHS) esters

Even though carboxyl groups can theoretically react with amino groups to form peptide bonds among molecules, the reactivity of the carboxylic groups is relatively low without the presence of catalysts/enzymes. Therefore, this process cannot be considered as a novel conjugation strategy. However, with the introduction of N-hydroxysuccinimide (NHS) agents, carboxylate groups can be activated and converted into NHS esters, leading to direct coupling with amino groups under moderate conditions (Reaction 2.9). NHS ester-containing cross-linkers have been thus synthesized and applied as cross-linking agents since 1970 [140]. Moreover, to modify the solubility issue of common NHS esters in aqueous conditions, N-hydroxysulfosuccinimide (sulfo-NHS) esters are synthesized as water-soluble

cross-linkers, mostly used for the surface modification of membranes and cells [141]. In addition, both NHS and sulfo-NHS containing cross-linkers follow the same mechanism for amino coupling, producing acylated products with the release of NHS/sulfo-NHS groups. This reaction can occur with primary and secondary amines, respectively, creating irreversible amide and imide linkages with great stability. In the specific case of protein conjugation, NHS ester cross-linking reagents primarily react with the α-amines at the N-terminus and the abundant ε-amines of lysine side chains [103]. Nevertheless, the hydrolysis of NHS in aqueous conditions, which potentially occurs simultaneously with the coupling reaction, is noteworthy. As a reverse reaction of esterification, NHS ester hydrolysis shows high sensitivity to reaction conditions, including temperature, molar ratio and pH conditions. On the other hand, using an organic solvent instead of water, such as acetone, dimethylformamide (DMF) and dimethylsulfoxide (DMSO), might be a useful means of eliminating the hydrolysis of NHS esters.



Reaction 2.9. NHS Ester derivative-based amide formation.

Disuccinimidyl suberate (DSS), a typical NHS ester cross-linker, leads to the formation of irreversible eight-atom amide linkages between the amines of the molecules. DSS has been reported to produce cross-linking between vasoactive intestinal peptides and receptors, cross-linking of aorta membranes, and cross-linking between p75 peptides and receptors [142-144]. It should be noted that DSS is insoluble under aqueous conditions, while most amines are soluble in aqueous conditions. Therefore, to achieve homogeneous dispersion for cross-linking in aqueous conditions, DSS is usually pre-dissolved in an organic solvent, such as dioxane, DMF, or DMSO, followed by mixing with a phosphate buffer (pH 7-9) [144].

A similar dispersion procedure can also be applied to disuccinimidyl tartrate (DST), another NHS ester-containing cross-linker. DST targets cross-linking among molecules that contain α-amines and ε-amines, and is mostly used to characterize human properdin polymers and cell surface receptors [145, 146]. As a derivative of DST, disulfosuccinimidyl tartrate (sulfo-DST), modified from DST for its significant solubility under aqueous conditions, is attractive for protein-lipid conjugation and protein corticotropin-releasing factor binding [147, 148]. Additionally, ethylene glycol bis (EGS) and sulfoethylene glycol bis (sulfo-EGS), are similar to DST and sulfo-DST, respectively, and are used in amine reactions. The studies of EGS and sulfo-EGS specifically focused on their conjugation of proteins [149].

On the other hand, N, N′-disuccinimidyl carbonate (DSC), the smallest NHS ester cross-linker, displays high reactivity towards nucleophiles. Unlike other types of NHS ester cross-linking agents, DSC can be either used in aqueous conditions as a cross-linker, forming two molecules of NHS for the conjugation of amines, or applied in an organic solvent as an activation agent for hydroxyl groups, promoting reactions, such as esterification, with carboxylic groups and condensation with aldehyde groups [103]. The applications of DSC range from ligand coupling to amine-containing dendrimer conjugation [150, 151].

However, NHS ester cross-linkers are costly feedstock reagents and are mostly used for biology studies of cell membrane and micro-organism characterization. Hence, NHS esters may not be suitable as cross-linkers in the development of cost-effective peptide conversion.

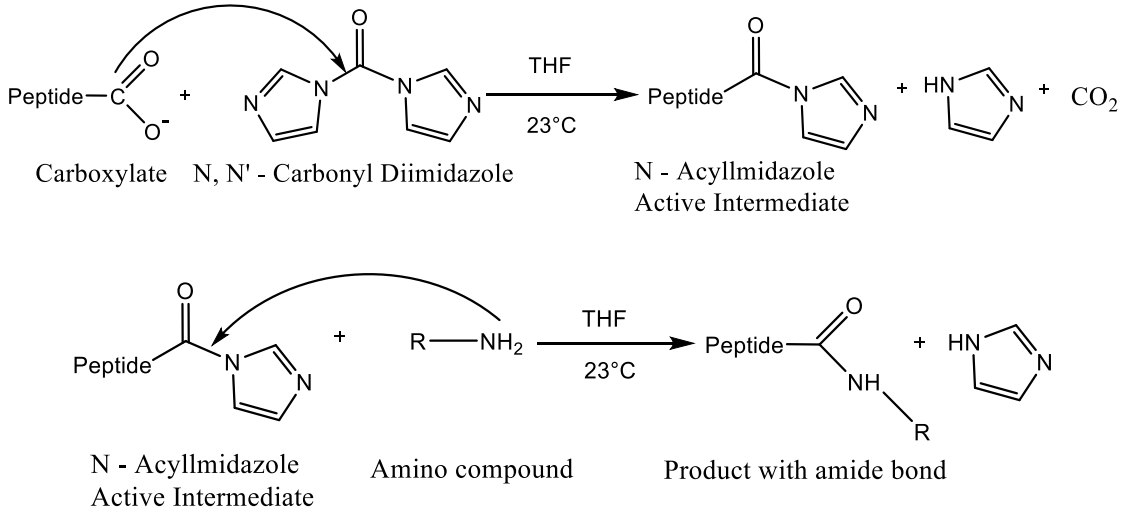
2.3.4. Amines

Amines that contain at least two amino groups in one molecule, including diamine (diethylamine), triamine (ternary alkylamine) and poly-amine (poly-ether amine, PEA), can be used as homo-functional cross-linkers in the conjugation of carboxyl compounds, forming peptide bonds with water as a by-product. However, due to the reversibility of the coupling reaction and the low nucleophilicity of carboxylic groups, the products of amino-carboxyl

coupling display relatively low conversion rates. Hence, amine cross-linkers are used mostly in the conjugation of monomers or small molecules, such as amino acids or diamines [103].

To overcome the issue of low conversion rates, a small amount of carbodiimide or its derivative is usually added as an activation agent and dehydrant for carboxylic acid conversion. The type of carbodiimide derivative used in the reaction is selected according to solubility in aqueous or organic conditions: water-soluble derivatives are used for the modification/conjugation of proteins or other macromolecules in aqueous conditions, which indicates a low requirement for the reaction degree, while water-insoluble derivatives are used for the synthesis of proteins or other polymers, showing a need for a relatively higher degree of reaction [152, 153]. It is worth mentioning that all carbodiimides and their derivatives initiate catalysis under the same mechanism: the agent first reacts with the carboxylic group to form a highly reactive and short-lived intermediate. An amine/alcohol reagent can then be attached to this specific intermediate, forming amide/ester bonds as linkages between the molecules. Taking N, N′-carbonyl diimidazole (CDI), a water-insoluble derivative, as a general example for amine-target carboxylate reactions, CDI first acts on the carboxylic group to form the intermediate of N-acyl imidazole, along with a leaving group of acyl imidazole (Reaction 2.10). The N-acyl imidazole is subsequently attacked by an amine, forming an amide bond to complete the amino-carboxyl coupling (Reaction 2.10). The catalysis of CDI was first utilized by Paul *et al.* and Anderson *et al.* to synthesize oligo-

peptides [154, 155] and was later extended to the immobilization of the enzyme lysozyme [156]. Other activation reagents, such as sulfo-NHS and DSC, also have wide applications in peptide/protein studies. However, the use of activation agents is complicated by the purification of products and the recovery of agents, making the process more expensive. Meanwhile, hydrolysis may lead to unnecessary consumption of the activation agents, making the selection of reaction conditions particularly important.



Reaction 2.10. CDI activation mechanism for amino-carboxyl coupling.

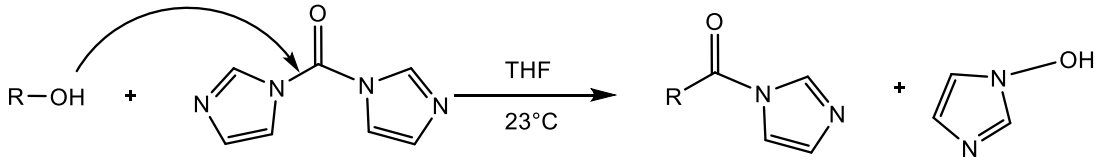
With the catalysis of carbodiimide under aqueous conditions, long chain peptides/proteins, such as γ-polyglutamic acid, can also cross-link with alkane-diamines, such as 1,3-propane diamine, 1,4-butane diamine, and 1,6-hexane diamine [157]. However, these cross-linking reactions take about a week to complete, as reported by Kunioka *et al.* [157]. Moreover, the maximum yield of the cross-linked products in this study was only

39.9%, demonstrating the limitation of alkane-diamine usage [157]. Another hydrogel study conducted by Lusiana *et al*. succeeded in a ternary conjugation between soluble wheat protein and poly (ethylene oxide) diglycidyl ether, via a 72 h coupling with ethyl diamines, indicating enhanced flexibility, insolubility and physical performance in the products [158]. Even so, the high toxicity of ethyl diamines still impedes the application of amine cross-linkers in protein/peptide conjugation.

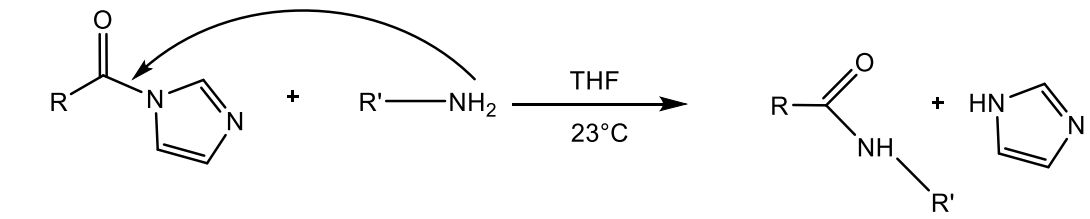
2.3.5. Alcohols

A reagent that contains at least two hydroxyl groups in one molecule can be used as a cross-linker in conjugation with peptides/proteins, ranging from glycol (diol) to poly (ethylene glycol) (polyol). Through the esterification of carboxylic groups, which mostly occurs under low pH and non-aqueous conditions, a hydrophobic linkage between peptides/proteins can be generated. However, without activation agents or catalysts, the conversion rate of the carboxylic groups is relatively low. Moreover, the reversible ester bonds require strict reaction conditions, such that the alcohol-peptide conjugation cannot easily be achieved directly. Therefore, to improve the performance of conjugation between hydroxyl groups and carboxylic groups, either functional group activation or hydroxyl group modification is commonly used.

NHS esters, can also be applied as an activation agent to approach the conjugation. For example, CDI, mentioned in the previous section, is a NHS ester that contains two acyl imidazole leaving groups. It can activate hydroxyl groups to form intermediates with great reactivity, resulting in cross-linking reactions with amines (Reaction 2.11). However, due to the simultaneous CDI activation of hydroxyl groups and carboxylic groups, the following amine-target couplings become complex in the system. Therefore, CDI is mostly added to activate a single functional group, and is widely used for peptide/protein immobilization [159]. Additionally, DSC, another NHS ester, is used for the hydroxyl group activation of poly (ethylene glycol) in anhydrous organic solvents, merely consisting of a carbonyl group containing two NHS esters. Succinimidyl carbonate derivatives, the intermediate of the activation process, demonstrate high reactivity towards nucleophiles, including amines and carboxylic groups, in organic or aqueous solutions (Reaction 2.12). It is worth mentioning that the low temperature and base conditions are preferred for the activation and for the following conjugation reaction in order to limit the hydrolysis of both NHS esters and the resulting products [103].

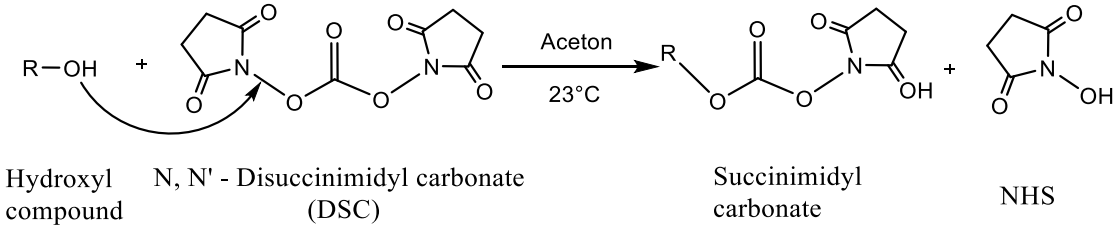


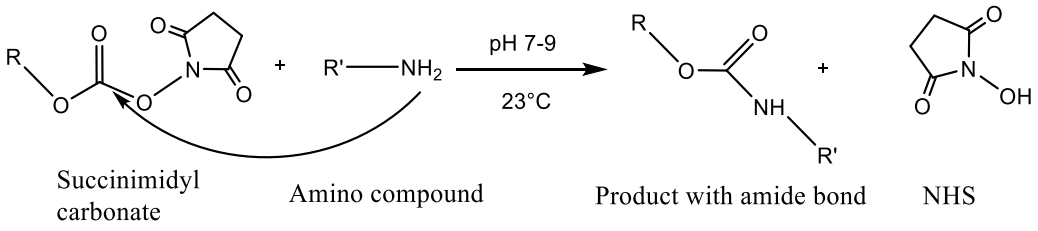
Hydroxyl compound | N, N' - Carbonyl Diimidazole | Active Intermediate



Reaction 2.11. CDI activation mechanism for amino-hydroxyl coupling.

By changing the pathway of cross-linking, the chemical modification of hydroxyl groups of alcohols is also commonly used to produce the peptide/protein conjugation. One simple modification strategy is the oxidation of hydroxyl groups into aldehydes. For example, diol alcohol can be oxidized into aldehydes by adding sodium periodate. This modification treatment is usually used to generate new cross-linking sites in glycols to produce conjugation of proteins or peptides [103]. Moreover, alcohol cross-linkers with oxidation agents show an advantage over aldehyde cross-linkers in the limited self-polymerization tendency in an aqueous system. However, the subsequent recovery of oxidization agents and the purification of products is complex and costly. In addition, due to the higher priority of aldehyde-amine coupling reactions, the carboxylic groups of the peptides are not frequently used, and the oxidation process is limited in its applications.





Reaction 2.12. DSC activation mechanism for amino-hydroxyl coupling.

In addition to the oxidation process for hydroxyl group conversion, alternative modification strategies can be applied by introducing highly reactive functional groups into the alcohol molecule, targeting peptide conjugation. Polyols, such as PVA and PEG [160], are usually used to accomplish coupling between carboxylic groups and hydroxyl groups, forming macromolecules with adjusted properties, and overcoming the low reactivity issue among polymers.

To adjust the hydrophilicity of the cross-linked product, PVA, one of the simplest polyols, is commonly modified into a cross-linker for the conjugation process by introducing epoxy groups into the molecule chain. As mentioned above, epoxy groups demonstrate significant reactivity to amines, hydroxyl groups and carboxylic groups, where the reaction priority can be controlled by changing the pH conditions. Therefore, PVA epoxidation demonstrates potential in coupling with carboxylic groups. In the preliminary study of epoxy group addition, Iulian *et al.* modified PVA into a poly-epoxides in the presence of epichlorohydrin and sodium hydroxide, showing the tendency of self-polymerization to

create a superabsorbent hydrogel [139]. In the study of the synthesis of resins, Milad *et al.* produced a conjugation between epoxidized PVA and 4-(4-aminobenzyl) benzylamine to enhance hydrophilic properties and swelling behaviour [161]. According to their study, base conditions at around 80℃ for 12 hr were preferred for the epoxidation of PVA, resulting in a relatively high conjugation rate among the reagents [161]. However, due to the self-polymerization property of the PVA epoxides, relatively few studies of peptide/protein conjugation have been conducted.

PEG can also be used as a polyol resource for the modification pathway, possessing fewer hydroxyl groups in one molecule compared with PVA. Due to the relatively high chemical stability of PEG derivatives, PEG is more commonly used as a cross-linker to couple the amines, hydroxyl groups, hydrosulfide groups, or carboxylic groups [103]. The process that adds reactive functional groups to the PEG chain or substitutes hydroxyl groups for other functional groups, known as PEGylation, has been attracting interest in cross-linking studies, especially in peptide/protein conjugation. According to Francesco's review of PEGylation technologies, PEG used to be modified into alkylate derivatives, such as PEG aldehyde and tosylated MPEG, in order to couple peptide amines [162]. Additionally, acyl derivatives can be synthesized from PEG, such as PEG-oxycarbonylimidazole, activated PEG alkyl acids, and PEG-p-nitrophenylcarbonate, leading to conjugation with molecules that contain halogen atoms, alkoxy groups and amines [162]. To accomplish binding in

carboxylic groups, PEG epoxides and PEG amines can be used as cross-linkers, with N-(-3-dimethylaminopropyl)-ethylcarbodiimide hydrochloride as an activation agent for carboxylic groups in some cases [162]. These PEG derivatives also display relatively high reactivity to amines that may compete with carboxylic groups. Moreover, PEG hydrazides, a newly released PEGylation product studied by Zalipki *et al.*, result in specific reactions with carboxylic groups in an acid medium with the catalysis N-(-3-dimethylaminopropyl)-ethylcarbodiimide hydrochloride [163], demonstrating the potential of using PEG for carboxylic groups conjugation.

2.3.6. Hetero-functional cross-linkers

As another major class of cross-linkers, hetero-bifunctional cross-linkers are reagent that contain at least two different reactive groups, coupling various functional groups for peptide conjugation, including amines, carbonyl groups, hydroxyl groups and sulfhydryl groups [103]. However, to reach the desired conditions for different reactions, hetero-bifunctional cross-linkers are often applied in multiple steps. Classified by their coupling targets, amine-sulfhydryl-reactive agents and carbonyl-sulfhydryl-reactive agents are discussed here as general hetero-functional cross-linkers in peptide studies.

Amine-sulfhydryl-reactive agents consist of both amine-reactive and sulfhydryl-reactive ends of the agent molecule. These agents are usually synthesized by combining an

NHS ester and an alkylating agent. The NHS ester targets the amine-reactive functional group, forming amide bonds as the linkage. Meanwhile, the alkylating agent reacts with the sulfhydryl group, resulting in thioether/disulphide bonds during cross-linking. Cross-linkers such as N-succinimidyl 3-(2-pyridyldithio) propionate, succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)-toluene, and succinimidyl-4-(N-maleimidomethyl)-cyclo-hexane-1-carboxylate are examples of this type of agent [164-166].

Carbonyl-sulfhydryl-reactive agents are characterized as newly raised cross-linkers, mostly used to attach carbonyl peptides to compounds that contain sulfhydryl groups. Carbonyl-sulfhydryl-reactive agents are designed to initiate the coupling reaction at the positions that are relatively far from critical active centers or binding sites of the peptides, thus preserving the molecular structure of the peptides for further treatment [103]. For instance, 3-(2-pyridyldithio) propionyl hydrazide and 4-(4-N-maleimidophenyl) butyric acid hydrazide are two of the commercial agents classified as carbonyl-sulfhydryl-reactive agents for macromolecule cross-linking [167, 168].

Even though the relatively high reaction activity and multiple functions of hetero-bifunctional cross-linkers are of interest to scientists, the high synthetic cost, complex application protocol, and purification methods involved in hetero-bifunctional cross-linker use are of particular concern. Thus, hetero-bifunctional cross-linkers are only used for research purposes in pharmaceutical development and microbiology studies.

2.4. Summary and outlook

The emergence of bovine spongiform encephalopathies has significantly changed the rendering process. Growing environmental concerns and additional cost of SRM disposal resulting from the SRM ban have posed considerable economic challenges to the rendering industry. With the concepts of sustainability and biodegradability introduced into modern materials, proteinaceous wastes produced during the rendering process are now being considered as potential feedstocks for the development of biodegradable polymers.

This chapter offers an overview of the impact of SRM policy on the rendering industry with a specific focus on SRM recovery and uses via possible bioconversion pathways. The chapter presents an extensive discussion of current bio-refinery achievements in the valorization of waste protein into bio-composites, including bio-based plastics, adhesives, flocculants and surfactants, demonstrating the potential of transforming SRM into safe, sustainable, cost-effective, and value-added products. The literature review outlines abundant cross-linking strategies in protein modification, depending on the multiple functions of the SRM to develop products with various desired properties, suggesting the feasibility of using SRM-derived peptides as a feedstock for the development of bio-based materials. Considering the limited reactivity of carboxylic groups in SRM-derived peptides [13] and the difficulty in product recovery (NHS ester and amine), amine-target reagents,

i.e., aldehyde, alcohol and epoxides, are selected to be used in this study. An aldehyde reagent, i.e. glutaraldehyde, is firstly used since it can consume the hydrophilic groups (amino groups) in the peptides, while forming hydrophobic bonds as a result, which probably can help to enhance the water resistance required by tackifier applications. Poly-alcohols and epoxides are then used in peptides cross-linking to increase the binding property by adding hydroxyl groups to peptides' chains. Further studies of SRM utilization as tackifiers and wood binders, as described in the following chapters, would benefit both the rendering industry and manufacturers of sustainable materials.

Chapter 3: Materials and methods

3.1. Materials

A batch of SRM was supplied by a large rendering facility in Western Canada, received in 2014. The torrefied wood was generously provided by Airex Energie (Laval, QC, Canada). According to the information provided by Airex Energie, the feed material of torrefied wood was spruce/fir sawdust. The torrefied material had undergone 30% weight loss on a dry mass basis, which is consistent with the weight loss value suggested by the references [74, 81].

The following chemicals/reagents were procured from Sigma-Aldrich (St. Louis, USA): L-leucine (reagent grade, ≥98%), (±)-epichlorohydrin (purum, ≥99%), hydrochloric acid (certified ACS, 37%) and poly (vinyl alcohol) (MW 13000-23000, 87-89% hydrolyzed).

The following chemicals/reagents were procured from Fisher Scientific (New Jersey, USA): glutaraldehyde (50 wt.%, certified), 0.1 M standard sodium hydroxide solution (0.0995 to 0.1005 mol/L at 20°C), 0.1 M standard hydrochloric acid solution (0.0999 to 0.1001 mol/L at 20°C), methanol (HPLC grade), hydroxylamine hydrochloride (97%), sodium hydroxide (certified ACS, 98.8%), hexane (certified ACS), acetone (certified ACS), cresol red (pure, indicator grade), and thymol blue (pure, indicator grade).

3.2. Methods

3.2.1. Thermal hydrolysis of SRM and peptides recovery

A dedicated 5.5 L stainless steel pressure vessel (Parr 4582, Parr Instrument Company, Moline, USA) equipped with a Parr reactor controller (Parr 4848, Parr Instrument Company, Moline, USA) was used for the thermal hydrolysis of SRM. According to the approved CFIA protocol [4] as well as the modified techniques developed by Mekonnen *et al*. [38], a standard laboratory protocol was used for the hydrolysis of SRM and recovery of peptides [3, 7, 38, 169]. In a typical run, 1.0 kg of SRM was mixed with 1.0 L of Milli-Q water in the Parr reactor vessel. The mixture was then subjected to hydrolysis at 180°C and

≥174 psi pressure for 40 min. For the recovery process, the hydrolysate was first diluted with 9.0 L Milli-Q water (hydrolysate:water ratio = 1:4.5, w/v), followed by agitation at 200 rpm in a shaker (Innova lab shaker, New Brunswick^{TM}, Mississauga, Canada) for 10 min. The slurry was then subjected to centrifugation (Avanti J-26 XP high-performance centrifuge, Beckman Coulter, Mississauga, Canada) at 7000 × g for 40 min. The supernatant was filtered (Whatman no. 4 filter paper, 20–25 μM pore size) to remove any insoluble material. The collected filtrate was then washed with hexane (1:1 v/v ratio) three times to facilitate the removal of any residual lipids. Lyophilization was applied as the final step on the aqueous fraction, affording a tan-coloured proteinaceous cake (total 34 ± 2% of the feed material), referred to hereafter as SRM-derived peptides [46]. Three batches of peptides derived from the same batch of SRM were used as triplicates for all of the experiments in this study. Following the methods for primary amine and carboxylic group determination described by Tropini *et al.* and Claudia *et al.* [47, 50], primary amine and carboxylic groups of SRM-derived peptides were characterized as 0.59 ± 0.05 mmol/g and 1.69 ± 0.06 mmol/g, respectively.

3.2.2. Cross-linking of SRM-derived peptides with glutaraldehyde

In a typical experiment, 2.0 g of SRM-derived peptides in 20 ml Milli-Q water (SRM-derived peptides:water ratio = 1:10, w/v) was reacted with glutaraldehyde at different

molar ratios (glutaraldehyde:primary amine groups in hydrolyzed SRM) at pH 7.0 as shown in Table 3.1. The reactions were held for 2 h at four different temperatures: 23°C, 55°C, 70°C, or 100°C. The resulting slurries were then characterized or processed into the final products.

Table 3.1. Design for cross-linking SRM-derived peptides with glutaraldehyde.

g Peptides	g Glutaraldehyde solution	mmol Glutaraldehyde	mmol Primary amines in peptides	Molar ratio of glutaraldehyde:primary amines
2.0	0.12	0.59	1.18	0.5:1
2.0	0.24	1.18	1.18	1:1
2.0	0.47	2.36	1.18	2:1
2.0	0.95	4.72	1.18	4:1
2.0	1.42	7.08	1.18	6:1
2.0	1.89	9.44	1.18	8:1

In reactions carried out in non-aqueous environments, methanol was used as the solvent instead of water, using the same molar ratios of glutaraldehyde and amine groups described in Table 3.1. The reactions were held for 2 h at 23°C, 55°C, or 64°C. The slurries

were studied or were processed into the products after the reaction. The control experiments consisted of subjecting SRM-derived peptides to the identical reaction conditions, but without any glutaraldehyde present.

3.2.3. Precipitate collection and nitrogen content determination

The various slurries resulting from the cross-linking reactions were characterized via nitrogen content analysis. After the reactor reached 23°C (room temperature), the water-insoluble materials formed in the slurry (i.e., precipitates) were collected by filtration (Whatman no. 4 filter paper, 20–25 μM pore size). The precipitate was then rinsed with water until the filtrate became clear. The precipitates were dried at 60°C overnight and were weighed before storage at 4°C in the fridge (Thermal Scientific™ General-Purpose Series Lab Refrigerators, Fisher Scientific, Ottawa, Canada). The collected dried precipitates were then transferred to a Leco C/N analyzer (Saint Joseph, USA) to determine nitrogen content. Briefly, samples were combusted at 950°C in pure O_2, followed by quantification of the N_2 produced. The amount of N_2 generated was then used to calculate the nitrogen content of the original sample.

3.2.4. Collection of products from glutaraldehyde cross-linked SRM-derived peptides

For the aqueous cross-linking system, the cross-linked products were collected as a powder after lyophilization of the reaction slurry. In the non-aqueous system, the reactions performed using methanol in lieu of water were processed through rotary evaporation of methanol affording a thick, resinous raw product, which was then diluted with 20 ml water and subjected to lyophilization. The final products were collected as powders for tackifier applications. The diagrams of the whole process are shown in Figure 3.1 and 3.2.

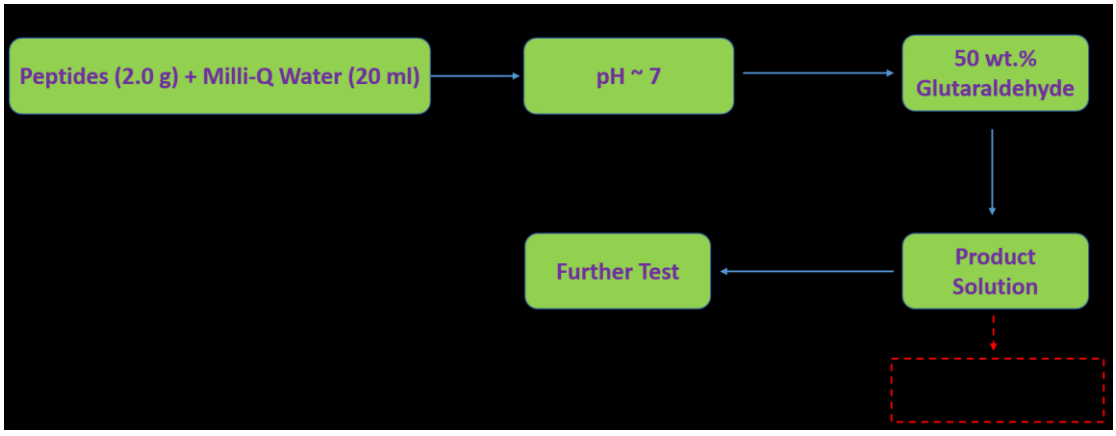


Figure 3.1. SRM-derived peptides cross-link with glutaraldehyde in the aqueous system.

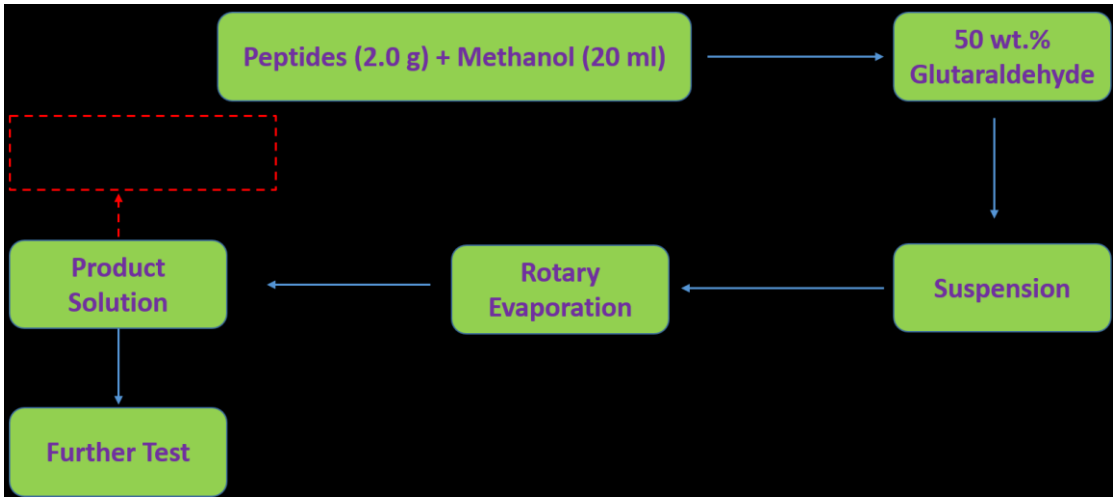


Figure 3.2. SRM-derived peptides cross-link with glutaraldehyde in the non-aqueous system (methanol).

3.2.5 Epoxidation of PVA

In a typical experiment, 1.14 g of PVA was dissolved in 5.0 ml Milli-Q water at 60℃ for about 10 min. Following this, 6.0 ml of epichlorohydrin was added into the pre-cooled PVA reactant at a molar ratio of 1:3 (epoxy groups in epoxidized PVA:primary amine groups in peptides) (Table 3.2). Other molar ratios (1:0.5, 1:1, 1:2, and 1:4) were also tested (Table 3.2). The reaction was carried out in an oil bath at a given temperature for 3 - 20 h. After the reaction, 1.20 ml of aqueous NaOH solution (0.75 - 7.00 mol/L) was added to the reaction at a rate of 200 µl/20 min to facilitate dechlorination. After cooling of the reaction to room temperature, the product solution was diluted with 15.0 ml Milli-Q water followed by a

hexane wash (30 ml) to remove excess epichlorohydrin. The hexane washed solution was later neutralized with 0.10 mol/L HCl, to produce epoxidized PVA.

Table 3.2. The molar ratio design for epoxidized PVA synthesis.

PVA (g)	No. of hydroxyl groups (mmol)	Epichlorohydrin (ml)	No. of epoxy groups (mmol)	Molar ratio of hydroxyl groups:epoxy groups
1.14	25.8	1.0	12.9	1:0.5
1.14	25.8	2.0	25.8	1:1
1.14	25.8	4.0	51.6	1:2
1.14	25.8	6.0	77.4	1:3
1.14	25.8	8.0	103.1	1:4

3.2.6. Epoxy content determination

An adapted method was used for measuring the epoxy content of the epoxidized PVA, which was based on an ultrasonication-assisted determination method developed by Zhipeng *et al.* [170]. In brief, the reaction indicator was prepared by mixing 0.1% cresol red

solution with 0.1% thymol solution at a volume ratio of 1:3, followed by pH adjustment to 7.0 with 0.01 mol/L sodium hydroxide aqueous solution. To measure epoxide content, epoxidized PVA (synthesized using 1.14 g PVA) was mixed and reacted with 10.0 mL of a hydrochloric acid-acetone solution (1:40 volumetric ratio), followed by ultrasonication (Crest P500 ultrasonic cleaner, Crest Ultrasonics Corp., New Jersey, USA) at 23℃ for 4 min. After this, 4 drops of reaction indicator were added to the solution. Subsequently, the solution was titrated with 0.10 mol/L NaOH standard solution. The blank (control) experiment was also conducted in the same manner but using 25.0 ml Milli-Q water (pH 7.0) instead of epoxidized PVA. The epoxy content was calculated using Equation 1 as follows [170]:

$$\textit{Epoxy content}\ (\text{mmol}) = [V_0\ \ (\text{ml}) - V\ \ (\text{ml})] \times C_{NaOH}\ \ \left(\frac{\text{mol}}{\text{L}}\right)$$

Where V_0 is the volume of NaOH standard aqueous solution consumed in the blank (control) experiment with Milli-Q water, V is the volume of NaOH standard aqueous solution consumed by epoxidized PVA reaction and C_{NaOH} is the concentration of NaOH used for the titration.

3.2.7. Cross-linking of SRM-derived peptides with epoxidized PVA

For the cross-linking reactions, SRM-derived peptides in different amounts (i.e., 8.00 g, 4.00 g, 2.00 g, and 1.50 g) were added to the entire epoxidized PVA reactant (15 ml) obtained from the epoxidation of 1.14 g PVA. After mixing, the pH of the reaction mixture

was adjusted to 9.0 using 3.00 mol/L NaOH/HCl before the reaction was carried out at a particular temperature (23-80℃) for 3-20 h in an oil bath. Subsequently, the reaction was neutralized with 0.10 mol/L NaOH, followed by sample collection through lyophilization. The products were termed as PVA-EPC-Peptides. The various reaction conditions examined for cross-linking of SRM-derived peptides with epoxidized PVA are shown in Table 3.3.

Table 3.3. Cross-linking reactions performed using epoxidized PVA and SRM-derived peptides.

Epoxidized PVA (mL)	No. of epoxy groups (mmol)	Mass of the SRM-derived peptides (g)	Molar ratio of Epoxy groups: primary amino groups
15	2.33	8.00	0.5:1
15	2.33	4.00	1:1
15	2.33	2.00	2:1
15	2.33	1.30	3:1

3.2.8. Primary amino groups characterization

The TNBSA method, a derivative method developed by Uptima (San Jose, USA) [48], was applied for the characterization of primary amino group content in the reacted product. L-leucine was used as the standard to establish a calibration line.

3.2.9. Carboxylic group characterization

The carboxylic acid groups remaining in the product powders were characterized by an adapted pH titration method [47]. The number of carboxyl groups was calculated from the relationship of volume and strength of acid consumed in the pH titration. The *Residual -COOH* (%) can be calculated as follows:

$$\textit{Residual -COOH}\ (\%) = \frac{N_{-COOH}\ (\text{mol})}{N'_{-COOH}\ (\text{mol})} \times\ 100\% = \frac{N_{-COOH}\ (\text{mol})}{m_{SRM}\ (\text{g}) \times\ 1.69\ \times 10^{-3}\ \left(\frac{\text{mol}}{\text{g}}\right)}\ \times\ 100\%$$

$$= \frac{C_{NaOH}\ \left(\frac{\text{mol}}{\text{L}}\right)\ \times\ V_{NaOH}\ (\text{ml})\ \times\ 10^{-3}}{m_{SRM}\ (\text{g})\ \times\ 1.69\ \times\ 10^{-3}\ (\frac{\text{mol}}{\text{g}})} \times\ 100\%$$

Where $N_{\text{-}COOH}$ is the number of carboxylic groups quantified in all of the products obtained from each of the cross-linking reactions shown in Table 3.1 and $N'_{\text{-}COOH}$ is the original amount of the carboxylic groups in the SRM-derived peptides that added for cross-linking, which can be calculated by multiplying the mass of peptides used (m_{SRM}) by the concentration of carboxylic groups in the SRM-derived peptides (1.69 x 10^{-3} mol/g). C_{NaOH}

is the concentration of the standard NaOH aqueous solution used for the titration, which is 0.10 mol/L. V_{NaOH} is the volume of the NaOH aqueous solution consumed during the titration.

3.2.10. Thermogravimetric analysis (TGA)

The thermal stability of the crosslinked products was studied with a Thermal Analysis Instruments Q500 (New Castle, USA) under a nitrogen atmosphere. Platinum pans were used as sample holders. Samples with a mass of 15-20 mg were inserted into the platinum pans and the temperature was ramped from 24°C to 100°C (held for 10-15 min to facilitate moisture removal), then heated to 350°C at a heating rate of 5°C/ min.

3.2.11. Compression analysis

The binding strength of tackifiers was assessed using a novel protocol developed in the lab that was based on measuring the compressive strength of "pucks". The pucks were made using protocols developed by our collaborators at Synermulch Erosion Control Products Inc. (Calgary, Canada). Briefly, 20 ml of a 2% (w/v) aqueous suspension of our SRM-based tackifiers were uniformly mixed with 4.0 g of MulchMax 101 wood fibres (Synermulch Erosion Control Products Inc., Calgary, Canada). The mixture was then transferred to an aluminum weighing dish (44 mm diameter inner base, Fisher Scientific, Ottawa, Canada) and cured at 40°C in an drying oven (Isotemp 625G Gravity Oven, Fisher

Scientific, Ottawa, Canada) for 48 h, followed by drying for 24 h at room temperature. Using a protocol developed in-house, the strength of the tackifier was assessed by determining the compressive strength of the pucks using a *TA.XTPlus* texture analyzer (Texture Technologies Corp. and Stable Micro Systems Ltd., Hamilton, Canada) with a TA-30 compression probe (76.2 mm diameter). The maximum force (N) recorded by the compression probe operated at a drop rate of 5 mm/s divided by the base area of the alumina dish (1520.5 mm^2) was calculated as the breaking stress (kPa) of the pucks.

3.2.12. Moisture holding capacity

To assess moisture holding capacity, samples (1.0 g) were first mixed with 20 ml Milli-Q water to produce a 5% (m/v) suspension. The suspensions were stirred on a magnetic plate for 2 h at 400 rpm and then transferred to a plastic Petri dish (100 × 15 mm, Fisherbrand, Ottawa, Canada). The dish was placed in a New Brunswick Innova 4900 Incubator Shaker (New Brunswick Scientific Co. Inc., Enfield, USA) at 22°C, where the relative humidity was maintained at 0.0% for 60 h. The residues in the dishes after drying were collected and subjected to moisture analysis (HE53 halogen, Mettler Toledo, Columbus, USA) to determine the residual moisture content.

3.2.13. Aldehyde titration

The aldehyde content of the cross-linked products was determined using the hydroxylamine hydrochloride titration method [171]. In a typical run, the cross-linked product obtained from 5.0 g unmodified peptides was collected and suspended in 50 ml Milli-Q water. The pH of the solution or suspension was then adjusted to 3.0 using 0.1 M HCl, which was then followed by the addition of 15 ml aqueous hydroxylamine hydrochloride (10%, w/v). The sample solution or suspension was titrated back to pH 3.0 using a 0.1 M NaOH solution. SRM-derived peptides in 5.0 g were also titrated as a control. The total aldehyde groups in the samples were calculated as follows:

$$N_{\text{-}CHO}\ (\text{mmol}) = 0.1 \times V_{NaOH}\ (\text{ml})$$

Where $N_{\text{-}CHO}$ is the total number of the aldehyde groups in the sample, V_{NaOH} (ml) is the volume of 0.1 M NaOH solution used during the titration.

3.2.14. Viscosity analysis

The aqueous suspensions of SRM-based tackifiers were analyzed by viscosity analysis using an OFI Model 900 Viscometer (OFI Testing Equipment Inc., Houston, USA) at a shear rate of 300 rpm and at ambient temperature. The suspension was made by adding

0.84 g of sample into 175 ml water followed by vigorous shaking for 5 min. The resulting suspension was then subjected to viscosity testing.

3.2.15. Preparation of densification powders

Prior to pelletization trials, the torrefied wood feedstock was pre-processed, which involved grinding using a blender (7500 series, Vitamix, Cleveland, USA) and sieve-screening to ≤300 μm. The torrefied wood powders were collected and stored in a container (945 mL in capacity, Plastic Containers with Lids, Fisherbrand™, Ottawa, Canada) at 23°C. The moisture content of the collected powders was measured at around 2.0-3.0 wt.% using a moisture analyzer (HE53 Halogen, Mettler Toledo, Columbus, USA).

To prepare densification powders with a specific binder, the ground torrefied wood powders were first mixed with the binder and/or Milli-Q water using a stainless steel coffee grinder (CBG 100S Bean Coffee Grinder, Black + Decker, Baltimore, USA). The moisture level of the mixture was then checked using the moisture analyzer. The steps of adding Milli-Q water, mixing, and checking moisture content were repeated until the desired moisture levels (3.0-10.0 wt.%) were reached.

3.2.16. Densification procedure

Densification experiments were conducted using an MTI-10K press single pelletizer (Measurement Technology Inc., Marietta, USA) to make torrefied wood pellets (Figure 3.3). A metal cylinder with a hollow channel of 6.35 mm inside diameter and 70 mm length, called the die unit, was placed on a metal pedestal. A piston of 6.30 mm in diameter and 90 mm in length was then inserted into the cylinder to facilitate densification. During the densification, the piston was pressed by a 25.0-mm-diameter mobile probe, which could be controlled to deliver the desired mechanical force for the formation of pellets. The die unit was wrapped with heating tape and preheated to a given temperature (23-175℃). The inside temperature of the die unit was monitored with a thermocouple, which was connected to the controller. In a typical run of sample densification, 0.50 g of the sample mixture was first loaded into the die to make a single pellet. The pistol was then inserted and pressed with a gradually increasing force at a velocity of 10 mm displacement/min until the pressing stress reached the desired level (50-350 MPa). This pressing stress was considered the holding pressure. After that, the pistol was held at the holding pressure for 1-5 min before increasing the pressure to the maximum value of 70-370 MPa (about 20 MPa higher than the holding pressure). The die was then placed on a metric hex nut (22 mm in diameter, 11 mm in height) to allow the probe to expel the pellet with the maximum-value pressure from the underneath

of the die. Finally, the pellet was taken out of the nut and stored in a Petri dish (100 × 15 mm, Fisherbrand™, Ottawa, Canada) at 23°C.

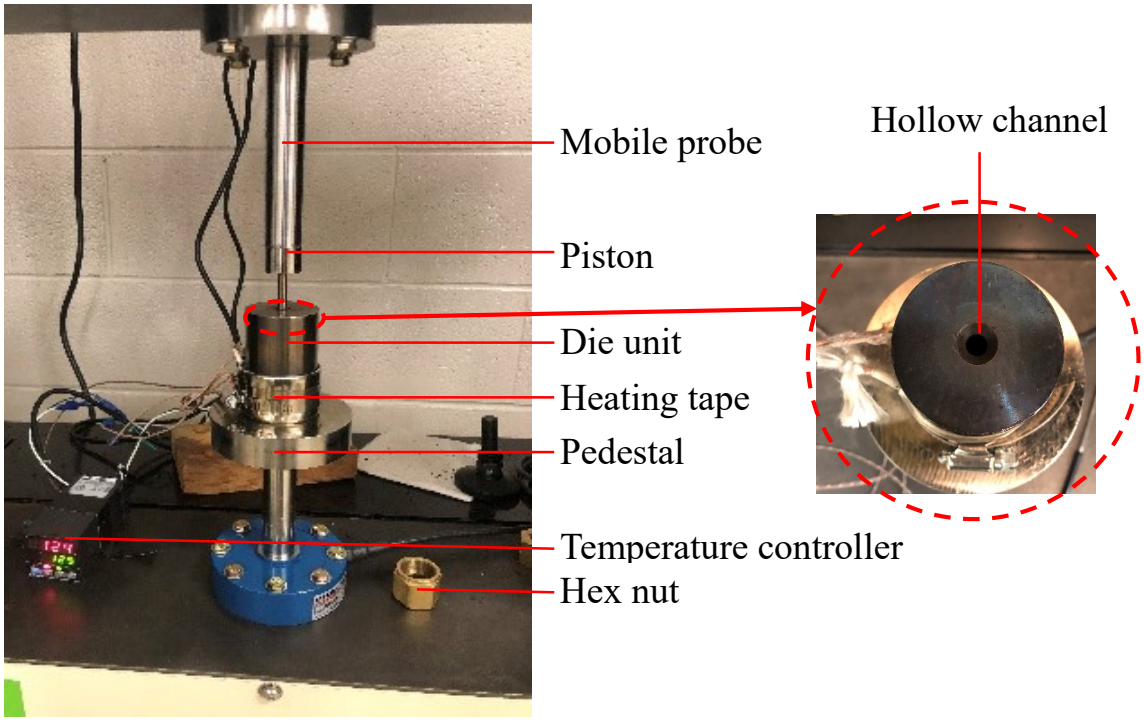


Figure 3.3. MTI-10K press single pelletizer.

3.2.17. Density determination for torrefied wood pellets

The mass of the pellet was determined using a balance (Balance XS105, Mettler Toledo, Mississauga, Canada). The diameter and the length of the pellet were measured with a digital calliper (Fisherbrand™, Ottawa, Canada). Since the pellet made by the single pelletizer was cylindrical in shape, the volume of the pellet could be calculated using the cubature formula for a cylinder. The density of the pellet was calculated as follows:

$$\rho_{pellet}\ (\mathrm{kg/m^3}) = \frac{W_{pellet}\ (\mathrm{g}) \times 10^6}{V_{pellet}\ (\mathrm{mm^3})} = \frac{W_{pellet}\ (\mathrm{g}) \times 10^6}{L_{pellet}\ (\mathrm{mm}) \times \pi \times (\frac{D_{pellet}\ (\mathrm{mm})}{2})^2}$$

Where ρ_{pellet} is the density of the pellet, W_{pellet} is the mass of the pellet weighed, V_{pellet} is the volume of the pellet, L_{pellet} is the length of the pellet, and D_{pellet} is the diameter of the pellet.

3.2.18. Compression stress determination for torrefied wood pellets

The compression stress at the breaking point of pellets was measured in both the radial and axial directions to estimate the overall strength of the pellet [172]. To measure the radial or axial compression stress, pellets made from the same mixtures were placed on a specific ion disc (31.5 mm diameter) under the MTI mobile probe before compression at a velocity of 10 mm displacement/min. The bottom surface of the pellet was set parallel to the surface of the disk for the radial measurement, and these two surfaces were placed perpendicularly to determine the axial measurement of Meyer hardness [74], shown in Figure 3.4. The maximum compression force (F/N) exerted to break a pellet in these measurements was recorded with the probe displacement, and the compression stresses of the pellets were calculated as follows:

Indentation depth (h (mm)) in radial compression test:

$$h\ (\mathrm{mm}) = D_{max}\ (\mathrm{mm}) - D_0\ (\mathrm{mm})$$

Where D_{max} is the displacement of the compressing probe at the point of pellet breaking, and D_0 is the displacement of the probe when the compressing probe first touches the pellet.

Radial compression stress (P_R (MPa)):

$$P_R\ (\text{MPa}) = \frac{F_R\ (\text{N})}{S\ (\text{mm}^2)} = \frac{F_R\ (\text{N})}{b\ (\text{mm})\ \times\ L_{pellet}\ (\text{mm})}$$

$$= \frac{F_R\ (\text{N})}{2 \times L_{pellet}\ (\text{mm}) \times \sqrt{\left(\frac{D_{pellet}\ (\text{mm})}{2}\right)^2 - \left[\frac{D_{pellet}\ (\text{mm})}{2} - h\ (\text{mm})\right]^2}}$$

$$= \frac{F_R\ (\text{N})}{2 \times L_{pellet}\ (\text{mm}) \times \sqrt{h\ (\text{mm}) \times \left[D_{pellet}\ (\text{mm}) - h\ (\text{mm})\right]}}$$

Where F_R is the breaking force in the radial direction measurement, S is the indentation area made by the compressed probe, b is the width of the indentation area, L_{pellet} is the length of the pellet, D_{pellet} is the diameter of the pellet, and h is the indentation depth.

Axial compression stress P_A (MPa):

$$P_A\ (\text{MPa}) = \frac{F_A\ (N)}{A\ (\text{mm}^2)} = \frac{F_A\ (\text{N})}{\pi \times (\frac{D_{pellet}\ (\text{mm})}{2})^2}$$

Where F_A is the breaking force in the axial direction measurement, A is the base area of a pellet cylinder, and D_{pellet} is the diameter of the pellet.

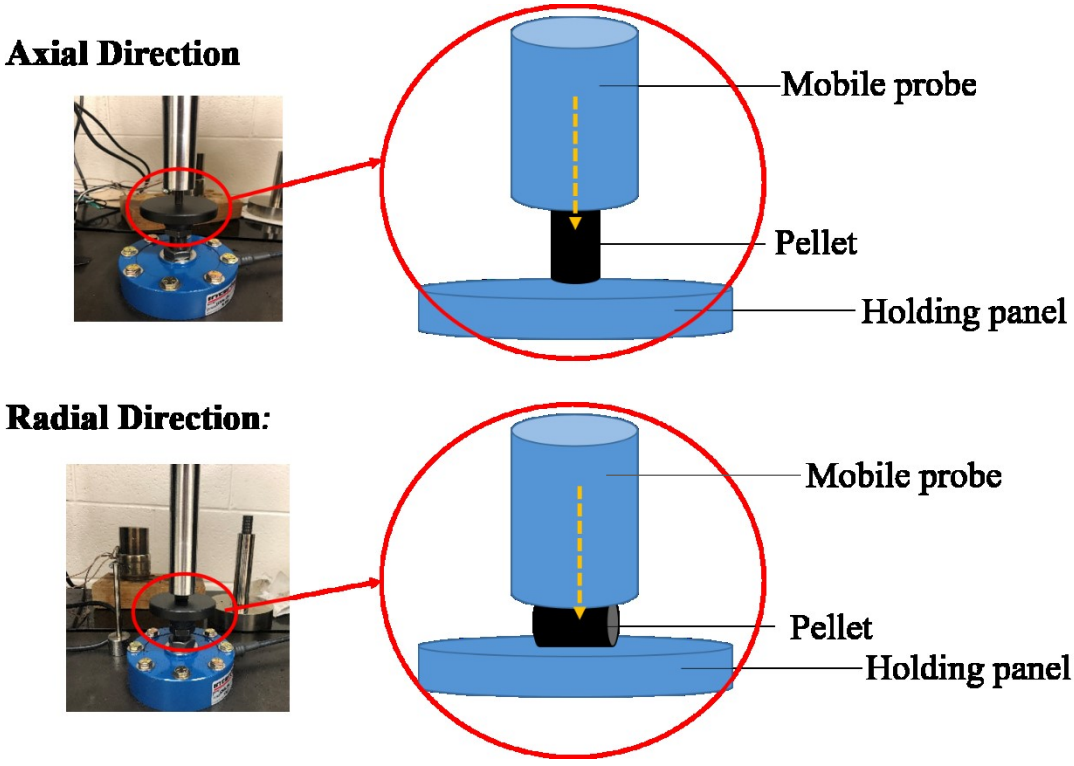


Figure 3.4. Compression stress determination of a pellet in both the axial and radial directions.

3.2.19. Moisture up-take of torrefied wood pellets

The method developed by Peng *et al.* was followed in this study [74]. A growth chamber (PGR 15, Controlled Environments Ltd, Winnipeg, Canada) was used to determine the moisture up-taking behaviour of pellets, including the maximum moisture up-taking value and the moisture up-taking with time. Before the test, pellets were first dried in a drying oven (Isotemp 625G Gravity Oven, Fisher Scientific, Ottawa, Canada) at 105°C for 3 h, and then stored in a Petri dish (100 × 15 mm, Fisherbrand, Ottawa, Canada) before being placed

into the chamber. The growth chamber was pre-set at 30°C and 90% relative humidity without any light. It is worth mentioning that the Petri dish was kept open when the pellets were placed into the chamber, but was covered during the measurement of pellet mass to reduce moisture loss. The weight of the pellets was measured every 30 min for the first 2 h, followed by measurements at 4 h, 8 h and 24 h. After 24 h, the weight was measured every 24 h until the weight became constant.

The moisture up-taking (wt.%) of the pellets can be calculated as follow:

$$Moisture\ \ up-taking\ \ (\text{wt.}\%) = \frac{W_{moisture}\ \ (\text{g})}{W_{pellet}\ \ (\text{g})} \times 100\% = \frac{W_{measurement}\ \ (\text{g}) - (W_0\ \ (\text{g}) - \text{W}_{\text{dish}}\ \ (\text{g}))}{W_0\ \ (\text{g}) - W_{dish}\ \ (\text{g})} \times 100\%$$

Where W_{pellet} is the weight of the pellet, $W_{moisture}$ is the weight of the moisture up-taken by the pellet, $W_{measurement}$ is the mass of the Petri dish with a pellet weighed at different measuring time, W_{dish} is the mass of the Petri dish without a pellet (weighed before the test). W_0 is the mass of the Petri dish with an oven-dried pellet weighed at the beginning of the test (0 h).

It is worth mentioning that the moisture attached to the dish may cause the moisture up-taking value overestimated. But since all the pellets were test simultaneously in the same chamber with the same lid model, the amount of moisture attached by the dish for each pellet

should be the same. Therefore, the results of the pellets can still be comparable to show the necessary difference in water absorption behavior.

3.2.20. Elemental analysis of torrefied wood pellets

The pellets (dry basis) made with and without a binder were ground with a blender (7500 series, Vitamix, Cleveland, USA) and measured with a Thermo Flash 2000 Elemental Analyzer (Thermo Fisher Scientific, Waltham, USA) to determine the content of carbon, hydrogen, nitrogen, and sulfur through sample combustion.

The chlorine (Cl) content of the binders (SRM-derived peptides and PVA-EPC-Peptides) was determined by the Cl^- content in the binder solution. The binder solution was made by dissolving SRM-derived peptides/PVA-EPC-Peptides (dry basis) in Milli-Q water at a concentration of 500 ppm. The resulting solution was measured with a Dionex Ion chromatography (IC) DX 600 system, with a 4 mm AS9-HC analytical column and electrolytic suppressor (Dionex, Sunnyvale, USA) to determine Cl^- content.

3.2.21. Heating value determination for torrefied wood pellets

An IKA C5000 Bomb Calorimeter (IKA-Werke, Staufen, Germany) was used to determine the calorific value of torrefied wood pellets obtained from 0.50 g powders.

Measurement in a calorimeter is accomplished via combustion processes in an oxygen atmosphere.

3.2.22. Statistical analysis

Tukey's HSD (honestly significant difference) [173] method was applied for all of the statistical analyses in section 4.1 and 4.2. Considering the large instrumental error given by the single pelletizer, Fisher LSD (Fishers Least Significant Difference) method was applied for the statistical analyses in section 4.3 to reduce the probability of Type II error (reject the difference) [174]. In a given figure, values or bars annotated with the same letter are statistically similar at a 95% confidence level.

Chapter 4: Results and discussion

4.1. Cross-linking of SRM-derived peptides with glutaraldehyde for tackifier applications [1]

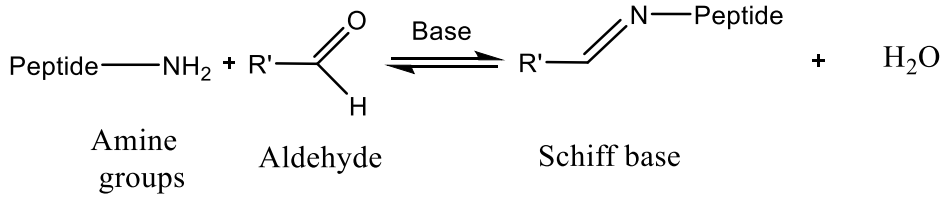
In this section, I provide proof-of-concept that peptides derived from the thermal hydrolysis of SRM can be used as a renewable tackifier. Furthermore, I show that cross-linking of the peptides with glutaraldehyde can be used to modulate characteristics of the tackifier, allowing it to possess lower solubility while maintaining identical moisture retention. In addition, a non-aqueous solvent (methanol) was introduced for the cross-linking of peptides to shit the Schiff base reaction into the product's side. Specifically, the use of methanol prevented aldol condensation by limiting the concentration of glutaraldehyde and increased the conversion rate by diluting the concentration of water during the reaction. Herein, I studied the effects of temperature and solvent type on cross-linking of SRM-derived peptides, and then evaluated the performance of the developed products as potential tackifiers for hydro-mulch applications.

4.1.1. SRM-derived peptides cross-linking characterization

To develop an effective tackifier for hydro-mulching applications, I subjected peptides derived from hydrothermally-treated SRM to glutaraldehyde. This cross-linking

[1] A version of this section has been published as a paper:
Tao Shui, Adhikari B.B, Michael Chae, David C. Bressler. *Progress in Organic Coatings*. 140 (2020) 105535.

strategy was employed to promote the generation of large polymers that have lower water solubility and could, therefore, persist longer in areas of high moisture. Since glutaraldehyde cross-linking of SRM-derived peptides has not been examined in great detail, initial studies were focused on achieving a better understanding of the cross-linking reaction in both aqueous and methanol systems. As a di-aldehyde cross-linker, glutaraldehyde can generate cross-linked structures through the reaction of its aldehyde groups with the primary amine groups of SRM-derived peptides through Schiff base formation (Reaction 4.1). By converting the hydrophilic amino groups into imine groups, glutaraldehyde cross-linking enhances the hydrophobicity of the SRM-derived peptides.



Reaction 4.1. Schiff base formation between aldehyde groups and SRM-derived peptides.

In order to exclude the potential interference from aldol condensation and self-polymerization, two types of control experiments were conducted at the same temperature, pH, and time as the cross-linking experiments: a) SRM peptides with water only; and b) glutaraldehyde solution (0.12-1.89 g, Table 3.1) with water only. While the SRM-derived peptides are fairly soluble in water, as evident from the results presented in Figure 4.1, a considerable amount of insoluble product (precipitate) was formed as a result of cross-

linking with glutaraldehyde. Conversely, no precipitates were formed in either of the control experiments. Taken together, this strongly suggests that the insoluble material formed is the cross-linked product (i.e., peptides cross-linked with glutaraldehyde), since the capping of the amine groups of the peptides will generate more hydrophobic products. Thus, the amount of precipitate formed was used to assess the degree of cross-linking.

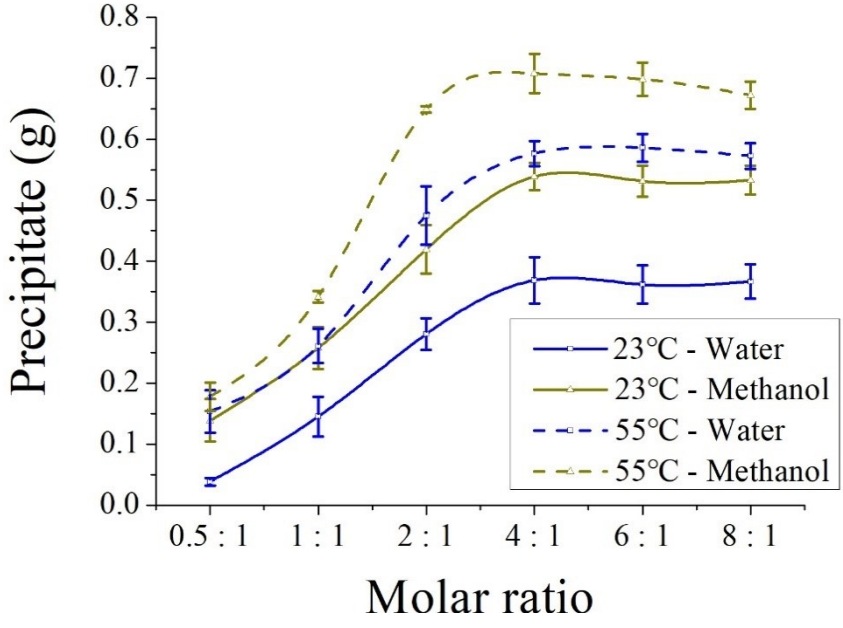


Figure 4.1. The impact of solvent type, temperature and molar ratio (glutaraldehyde:primary amine groups in SRM-derived peptides) on the mass of precipitate in the cross-linked products using 2.0 SRM-derived peptides. Plotted values represent the average mean ± standard deviation of triplicate experiments.

The effects of the molar ratio between glutaraldehyde and amine groups, reaction temperature, and the solvent type were initially evaluated by analyzing the mass of the

precipitate formed in the reaction of 2.0 g SRM-derived peptides with glutaraldehyde (Figure 3.4). It was found that a higher reaction temperature (55℃, dash lines) resulted in a higher mass of precipitate in both types of solvent at each molar ratio, except the 0.5:1 ratio in methanol. This suggests a higher degree of cross-linking is generally achieved in reactions performed at higher temperatures. Also, at a given temperature, the use of methanol as a solvent (brown lines) resulted in higher cross-linking compared to when water was used (blue lines). Regarding the effect of the molar ratio, the greatest amount of precipitate was observed at a molar ratio of 4:1 (glutaraldehyde:primary amine groups in SRM-derived peptides), indicating that this ratio achieved the highest amount of cross-linking. For any given system, higher molar ratios (6:1 and 8:1) achieved similar amounts of precipitate but used higher amounts of glutaraldehyde compared with the 4:1 ratio. Thus, the 4:1 ratio was determined to generate the highest amount of cross-linking with minimal glutaraldehyde input.

To provide further insights on the effects of temperature and solvent type, more detailed experiments were designed using a molar ratio of 4:1 and a wider range of temperatures (23–100℃) (Figure 4.2). Since some of the cross-linked product with less cross-linking degree or less molecular weight can still dissolve in the solvent, it is difficult to achieve the total yield of the cross-linked products. Also, the amount of water generated during the reaction can't be estimated, making it difficult to calculate the total yield of cross-

linked products via the mass equivalent of reactants. The mass of precipitates (g) was thus used in this study instead of precipitate percentage (%). To make the result comparable, 2.0 g SRM-derived peptides and 0.95 g glutaraldehyde were used for the following tests shown in Figure 4.2 and Figure 4.3. The aqueous system experiments showed that increasing temperature promoted higher levels of cross-linking until the temperature reached 64℃ (Figure 4.2). However, the mass of precipitate in methanol system was the same at 64℃ and 55℃ (Figure 4.2).

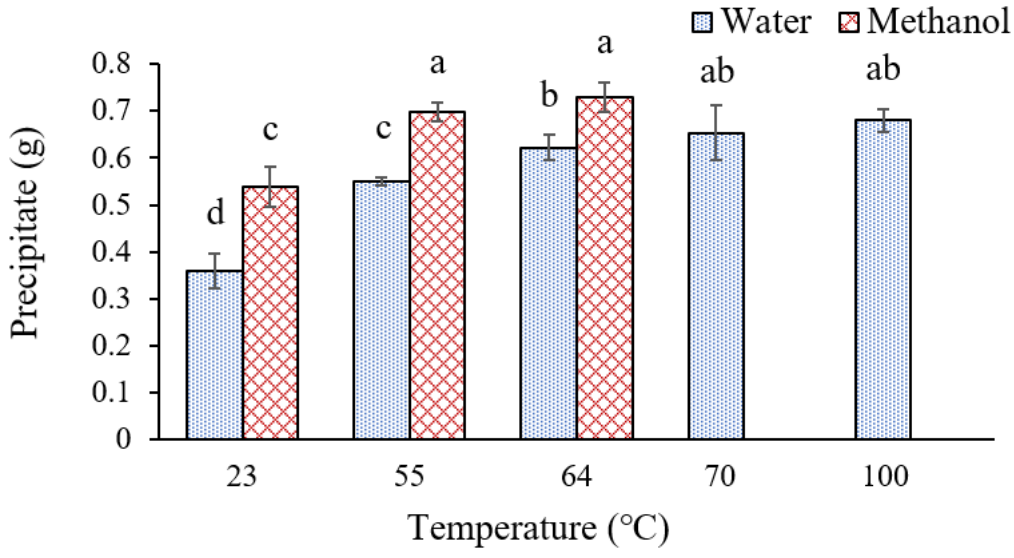


Figure 4.2. The effect of temperature on the amount of precipitate collected from the cross-linked products generated at a 4:1 ratio (glutaraldehyde:primary amine groups in SRM-derived peptides) in aqueous (water) and methanol systems using 2.0 g SRM-derived peptides. 70℃ and 100℃ were not approached for methanol system, since all the experiments were conducted in an open system with condensate water under the room

pressure. Values represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

However, the mass of the precipitates alone was not sufficient to characterize the cross-linking reaction between glutaraldehyde and SRM-derived peptides as the composition of the cross-linked products should also be taken into account. Due to the existence of side reactions (aldol condensation) [112], the precipitate obtained through cross-linking reactions may contain other by-products that may be undesirable. Thus, nitrogen content analysis was applied to help assess the precipitate composition. Since the nitrogen in the cross-linking system originates exclusively from the SRM-derived peptides, the amount of nitrogen in the precipitate should be directly proportional to the amount of SRM-derived peptides that participated in the cross-linking reactions and were thus retained in the precipitate.

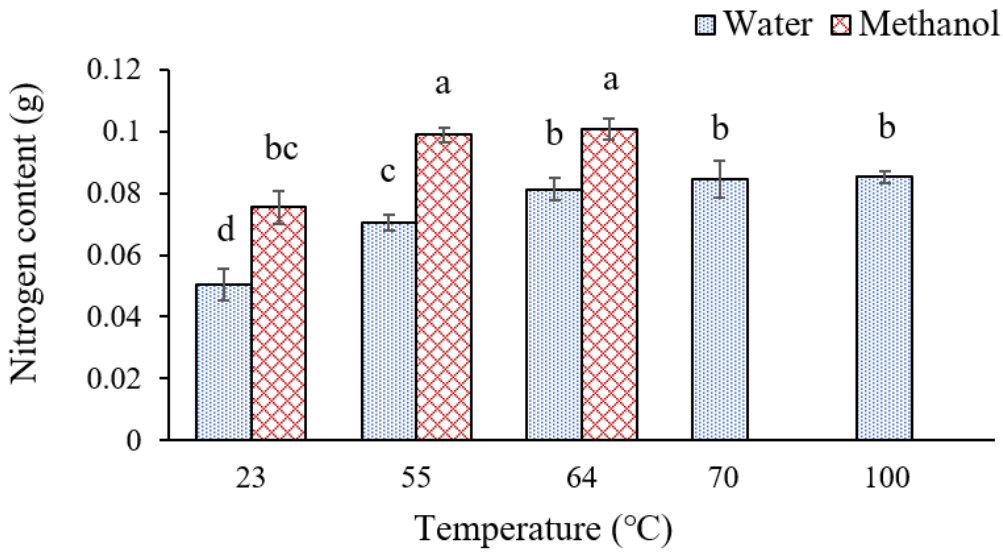


Figure 4.3. The effect of temperature on the amount of nitrogen present in the cross-linked products generated at a 4:1 ratio (glutaraldehyde:primary amine groups in SRM-derived peptides) in aqueous (water) and methanol systems using 2.0 g SRM-derived peptides.. Total nitrogen content in the precipitate was determined by multiplying the nitrogen concentration obtained using a Leco C/N Analyzer by the mass of the precipitate. Values represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

Based on nitrogen content analyses performed on the precipitate collected from the various cross-linking reactions (Figure 4.3), additional information regarding the effect of temperature and solvent type was obtained. The precipitates obtained at 23°C using the methanol system demonstrated similar nitrogen content to the aqueous system at 64°C, 70°C and 100°C. Furthermore, the precipitate formed in the water systems contained lower

amounts of nitrogen under all the temperatures compared to the nitrogen content of cross-linked products obtained at 55°C and 64°C using the methanol. This demonstrated that in the water system, a lower percentage of SRM-derived peptides were cross-linked and sequestered in the precipitate. In the methanol system, an increase in temperature from 23°C to 64°C seemed to promote cross-linking with a relatively high level of peptide involvement. This can be explained by the water removal process, which promotes Schiff base formation. Based on the reaction mechanism (Reaction 4.1), water is formed as a by-product in the reversible reaction that facilitates cross-linking of glutaraldehyde and the SRM-derived peptides. In the methanol system, even though water is a by-product of the cross-linking reaction, it is present at very low levels (methanol dilution), which favours the forward reaction and results in a higher cross-linking rate. Also, the water added with 50 wt.% glutaraldehyde solution was < 0.5 mL (1:4 molar ratios), which was negligible when compared to 20 mL methanol used in this study. It is worth mentioning that since water was introduced during the collection of the precipitate generated using the methanol system, the reverse reaction may have been favoured during the processing of the cross-linked product. However, this would likely have resulted in the removal of peptides during washing, leading to an underestimation of the amount of protein participating in the cross-linking reaction.

Even though the methanol system led to the promotion of precipitates during glutaraldehyde cross-linking, the carboxylic groups in the SRM-derived peptides could

potentially have reacted with the solvent through ester formation, adding uncertainty to the cross-linking reaction. Thus, I also monitored the level of carboxylic groups during the course of the reaction (Figure 4.4). It was clearly demonstrated that in the methanol system, there was no significant change in the concentration of carboxylic groups during the glutaraldehyde cross-linking or in the control reaction, which consisted of only peptides and methanol. To summarize the data obtained through the cross-linking studies, a temperature of 55℃ with a molar ratio of 4:1 (glutaraldehyde:primary amine groups in SRM-derived peptides) using the methanol system promoted the greatest cross-linking between SRM-derived peptides and glutaraldehyde.

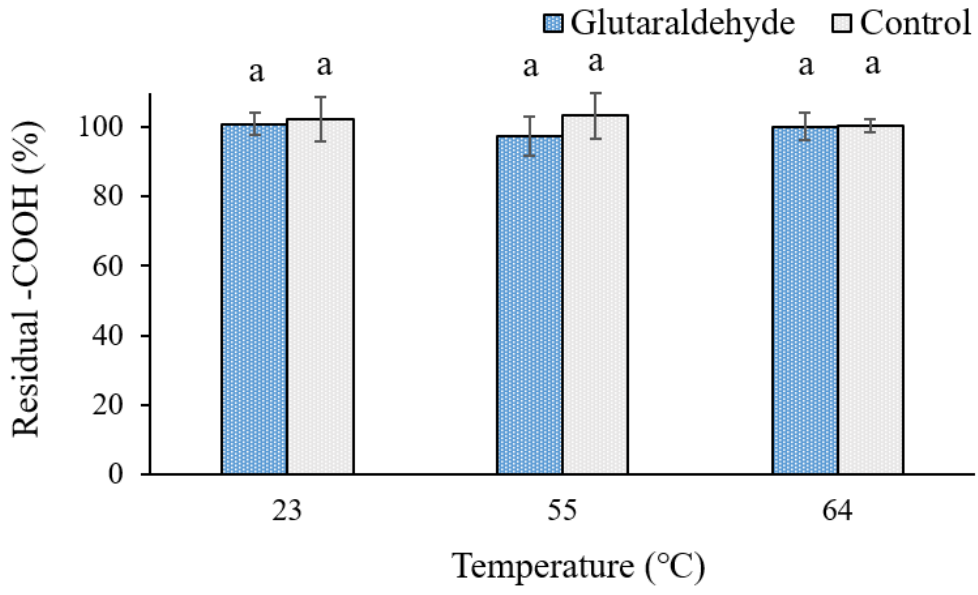


Figure 4.4. The effect of temperature on the carboxylic groups present in the cross-linked products generated in the methanol system. Values represent the average mean ± standard

deviation of triplicate experiments. The values that don't share the same letter are significantly different ($p<0.05$).

4.1.2. SRM-derived peptides based tackifiers characterization

Based on the cross-linking experiments described above, the methanol system at 55℃ was chosen for cross-linking reactions for all subsequent studies in section 4.1.2 focused on tackifiers characterization. However, although a molar ratio of 4:1 (glutaraldehyde:primary amine groups in SRM-derived peptides) was found to generate the greatest amount of the cross-linked product, I also explored other molar ratios as it was possible this variable impacted important tackifier properties, other than solubility. Therefore, in this section, cross-linked products obtained using various molar ratios were studied with regards to compression strength, moisture maintaining capacity, residual aldehyde groups, and viscosity. The objective of these studies was to determine the cross-linking ratio that achieves an optimal balance between hydrophobicity and hydrophilicity for tackifiers function.

4.1.2.1. Compression strength of SRM-derived peptides based tackifiers

In industry, one method for evaluating tackifiers is to generate "pucks" with tackifier and mulch (Figure 4.5A), followed by the evaluation of puck hardness using a

durometer (Figure 4.5B). However, the dramatic variance observed among replicates, as well as the dimensionless values given by a durometer, made it difficult to evaluate the different cross-linked products and commercially available tackifiers. Therefore, a novel testing method employing a texture analyzer was developed as an alternative measurement of puck strength (Figure 4.5B). The texture analyzer consists of a control panel, a TA-30 probe (76.2 mm diameter), and a load cell with a constant speed of compression or tension that can be controlled by the accompanying software. During testing of the sample, the force applied on the pucks, the distance travelled by the load cell, the size of alumina dishes and the time can be recorded and analyzed. The breaking stress determined during the compression testing of pucks with the texture analyzer displayed relatively low variability, which translates to greater reproducibility.

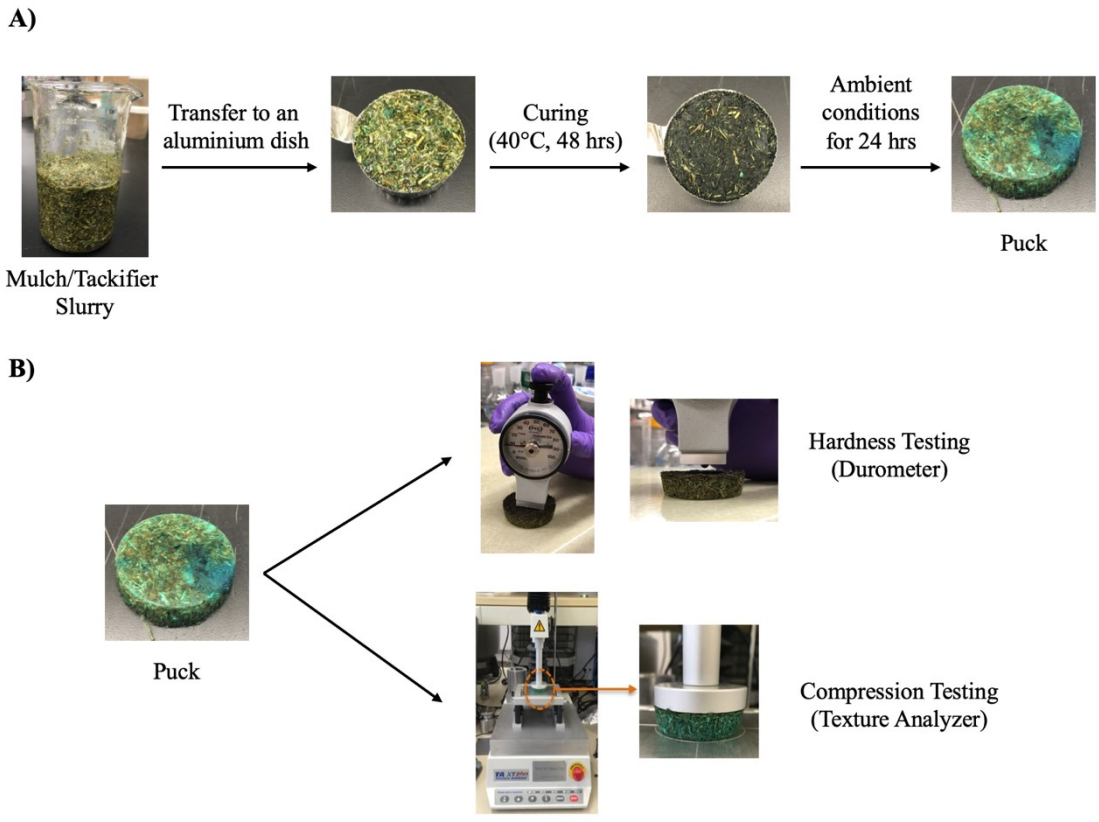


Figure 4.5. Puck creation and analysis methods. (A) Pucks were generated by mixing an aqueous suspension of tackifier (2% (w/v); 20 mL) with Mulchmax 101 wood fibres (4 g), then transferring to an aluminum dish (44 mm inner diameter). Pucks were cured, then left at ambient conditions prior to testing. (B) Tackifier quality was indirectly assessed through hardness testing using a durometer (industry method) or using a novel compression testing protocol employing a *TA.XTPlus* texture analyzer.

Of the three commercial tackifiers tested, psyllium displayed the greatest compression strength, followed by starch and finally guar gum (Figure 4.6). While the pucks made using SRM-derived peptides or glutaraldehyde cross-linked peptides displayed lower

compression strength than psyllium, they all performed comparably to guar gum. This demonstrates the potential of using SRM-derived peptides (both unmodified as well as cross-linked) as tackifiers in the erosion control industry. Furthermore, the peptides that were cross-linked with varying amounts of glutaraldehyde all demonstrated similar compression strength to one another, as well as to the unmodified SRM-derived peptides (Figure 4.6). This indicates that cross-linking peptides with glutaraldehyde does not negatively impact their adhesion qualities. In fact, pucks made using the cross-linked product generated at a 1:1 molar ratio of glutaraldehyde:peptides displayed similar compression strength as pucks made with starch. Combined with the solubility data acquired from section 4.1.1, these data demonstrate that glutaraldehyde cross-linking can lower the solubility of peptides, which enables the tackifier to endure in areas of high moisture, without negatively impacting adhesion strength.

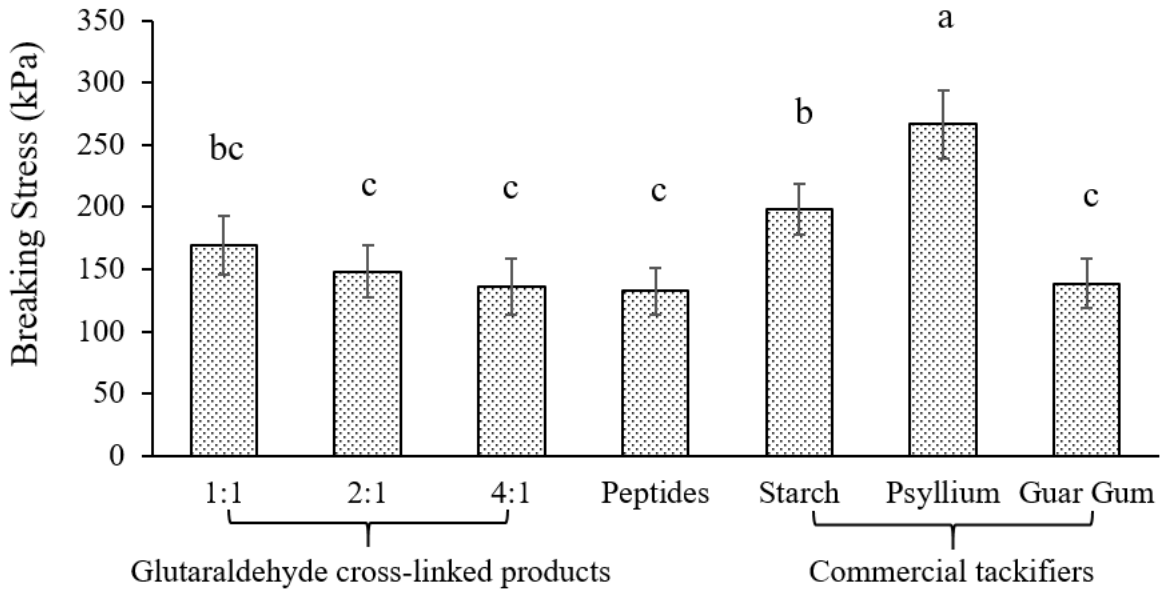


Figure 4.6. The compression strength of peptides, glutaraldehyde-peptides cross-linked products, and commercial tackifiers. Pucks generated using Mulchmax 100 and solutions of the tackifiers indicated above were subjected to compression testing using a *TA.XTPlus* texture analyzer to determine the breaking stress (kPa). 1:1, 2:1 and 4:1 represent for the glutaraldehyde-peptides cross-linked products formed using a 1:1, 2:1 and 4:1 ratio (glutaraldehyde:primary amino groups), respectively. Peptides represent for the unmodified SRM-derived peptides. Same size of alumina dishes and the compression probe were suggested to reproduce results. The values shown represent the mean ± standard deviation of triplicate experiments. Values annotated with different letters are significantly different ($p<0.05$).

4.1.2.2. Moisture holding capacity

The moisture holding capacity of the cross-linked products was also examined to help assess their ability to function as tackifiers. Although the generation of cross-linked products that are more hydrophobic (and thus not easily washed away during rainfalls) is desirable, the ability for tackifiers to sequester moisture is also beneficial as this water can potentially be used by seeds and plants to promote germination and growth. To evaluate the effect of cross-linking on the moisture holding capacity, a tackifier solution or suspension was placed in a humidity chamber set at 22.0℃ and 0.0% humidity. Under these conditions, the water-only control took 60 h for complete evaporation to occur. Thus, the sample slurries were left for 60 h in the incubator prior to the completion of moisture content measurements. Based on the results of this analysis, it can be observed that the cross-linked products and the unmodified peptides displayed the same water retention as starch and psyllium (Figure 4.7). This demonstrates that the SRM-derived peptides and the cross-linked products display similar water holding capacities as commercial tackifiers. Furthermore, consistent with the results of compression strength analysis, the hydrophilicity of SRM-derived peptides was not significantly affected by cross-linking with glutaraldehyde despite the fact that the cross-linked product had reduced solubility.

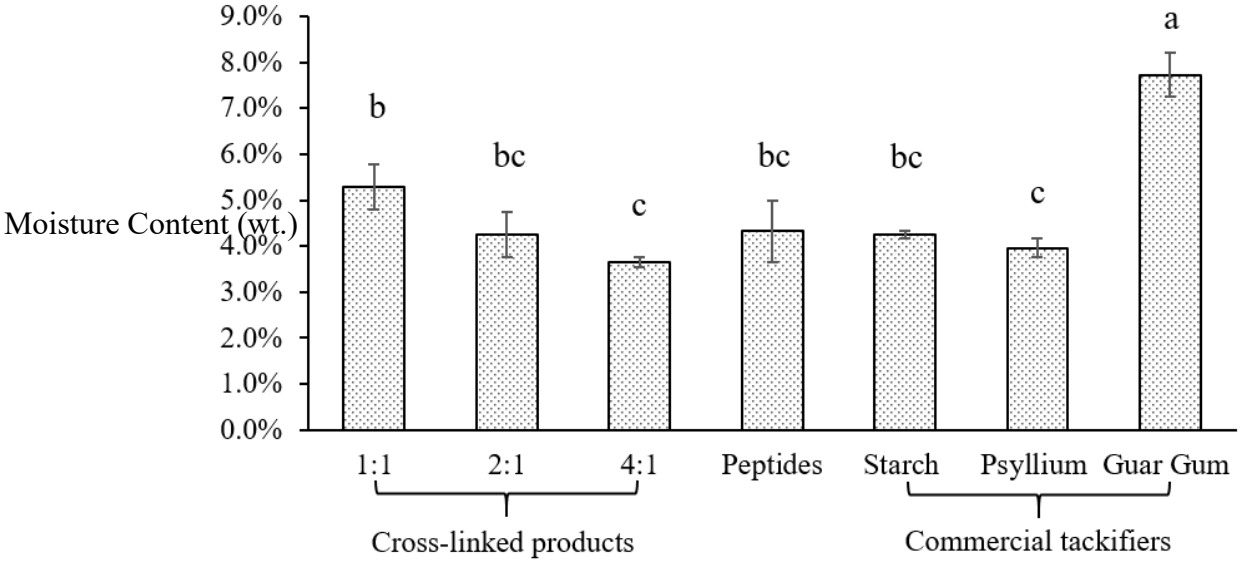


Figure 4.7. The water holding capacity of peptides, glutaraldehyde-peptides cross-linked products, and commercial tackifiers. 5% (w/v) solutions of the various tackifiers were placed in a humidity chamber set at 22.0℃ and 0.0% humidity. The moisture remaining after 60 h was determined and used to calculate the moisture content of the residual material. 1:1, 2:1 and 4:1 in the figure represent for the glutaraldehyde-peptides cross-linked products formed using a 1:1, 2:1 and 4:1 ratio (glutaraldehyde:primary amino groups), respectively. Peptides represent for the unmodified SRM-derived peptides. The values shown represent the mean ± standard deviation of triplicate experiments. Values annotated with a different letter are significantly different ($p<0.05$).

Moreover, the water holding capacity of the glutaraldehyde-peptides products generated at a 1:1 molar ratio was found to be higher than that of the cross-linked products produced at a 4:1 molar ratio (Figure 4.7), even though the compression strength of the pucks

generated using both tackifiers were statistically similar (Figure 4.6). Thus, subsequent characterization experiments focused on using products generated at a 1:1 molar ratio, though products generated using a 2:1 molar ratio were also tested to provide greater insights. The 2:1 molar ratio was used instead of the 4:1 ratio since less glutaraldehyde is needed, and the products generated from both of these reactions displayed similar qualities (Figure 4.6 and Figure 4.7).

4.1.2.3. Aldehyde groups remaining in the peptides-based tackifiers

To assess the degree of glutaraldehyde cross-linking (Schiff base formation, Reaction 4.1), the aldehyde and carbonyl groups remaining in the cross-linked product were characterized through titration. Each glutaraldehyde molecule contains two aldehyde groups, one at each end of the molecule, that could react with amine groups of SRM-derived peptides to facilitate cross-linking. Even though the primary amino groups in the peptides are expected to dominate the reaction, there are some other potential functional groups in the peptides that available for glutaraldehyde, such as carbonyl groups and phenolic hydroxyl groups. However, in this case, the cross-linking of peptides through glutaraldehyde would still be achieved. Although glutaraldehyde cross-linking is proposed to link two different peptide molecules, it is possible that only one of the two aldehyde groups in glutaraldehyde reacted with the peptides. In this case, the process would be classified as

a single modification of peptides, which would minimize formation of larger molecular weight products. Furthermore, there would be a significant amount of unutilized aldehyde groups, which is not ideal from a cost perspective.

The titration experiments demonstrated that the total number of aldehyde groups remaining in the products were statistically similar to the number of groups in the SRM-derived peptides. There were 0.65 ± 0.05 mmol of aldehyde/carbonyl groups in the 5.0 g of the SRM-derived peptides, and 0.53 ± 0.10 and 0.64 ± 0.05 mmol in the yield of 5.3 g and 5.4 g cross-linked products achieved from 5.0 g of the unmodified peptides, generated using a 1:1 and 2:1 ratio (glutaraldehyde:primary amino groups), respectively. If only one of the aldehyde groups in each of the glutaraldehyde molecules participated in the reaction, then the number of aldehyde/carbonyl groups in the product would be expected to increase. Thus, this titration experiment demonstrated that the reaction of both aldehyde groups with peptides was highly favored, resulting in the cross-linking of peptides. Furthermore, since there was no significant change in the amount of the aldehyde and carbonyl groups remaining in the cross-linked product when compared to the unmodified peptides, the risk of unreacted aldehyde groups is low. Additionally, since the toxicity of aldehyde groups stems from their high reactivity, it is likely that any remaining aldehyde groups will be consumed by other components of the hydromulch (i.e., the abundant hydroxyl groups present in the mulch fibres). Future work will be needed to confirm the low toxicity risk of the tackifiers.

4.1.2.4. Aqueous viscosity of tackifiers derived from peptides

Viscosity is an important parameter that is influenced by the concentration, shear rate, and the molecular structure of the material being examined [175]. For tackifier applications, products with low aqueous viscosity are desired to minimize clogging during the application of hydro-mulch, specifically during mixing and spraying. Since the hydrophobicity and molecular size were enhanced in the product generated through cross-linking of peptides with glutaraldehyde, it was important to assess the impact of these changes on the viscosity of the tackifiers.

In this study, the viscosities of the cross-linked products at 20°C were shown to be higher than that of the SRM-derived peptides, further confirming structural changes in the products as a result of reaction with glutaraldehyde. While the SRM-derived peptides in the water had a viscosity of 0.93 ± 0.11 cP, the viscosities of the aqueous solutions or suspension of cross-linked product were 1.37 ± 0.42 and 1.87 ± 0.32 cP for the material generated at a 1:1 and 2:1 ratio (glutaraldehyde:primary amino groups), respectively. However, even though the viscosity of the cross-linked products increased, it was still lower than that of the guar gum (26.0 ± 0.5 cP), a commercial tackifier that is currently used in hydro-mulching applications. Therefore, the cross-linked products generated at both 1:1 and 2:1 molar ratios

(glutaraldehyde:primary amino groups) would not likely lead to clogging issues during the hydro-mulching process.

4.1.2.5. Estimated cost of tackifiers derived from peptides

Since the process of converting SRM to tackifiers has not been optimized nor scaled-up to pilot scale, a technoeconomic assessment of the technology is not yet possible. Nevertheless, the potential for SRM to serve as a feedstock for the development of cost-effective tackifers is evident from the low cost of the necessary reagents. According to Alibaba, glutaraldehyde (50 wt.%) can be purchased for only US$ 2.6/kg. In addition, given that renderers are currently paying US$ 0.2/kg to dispose of SRM [7], it is highly likely that SRM could be procured for a low price. In fact, renderers may be willing to providing tipping fees to dispose of SRM. Given the low cost of the reagents, and the fact that the solvent (methanol) will be recycled, the feedstock prices by mass for glutaraldehyde-peptide tackifiers can be calculated as 0.49/kg and 0.71/kg for 1:1 and 2:1 products, respectively. Since both 1:1 and 2:1 cross-linked products showed the similar to guar gam while the water holding capacity of products is the same as starch, it is likely that the application rate of these cross-linked products is similar to that of starch, which is 75 lb/acre (guar gam: 40 lb/acre) [176]. Based on this, the feedstock prices by area for 1:1 and 2:1 cross-linked products can be inferred as ~US$ 16.7/acre and ~US$ 24.2/acre, respectively. These prices will be

competitive with the sale price of psyllium (US$ 56.8/acre) and starch (~US$ 34.0/acre), and will likely be substantially lower than that of guar gum (US$ 326.5/acre) for mulch fibre applications [59, 176, 177].

4.1.3. Summary

Through studies using a non-aqueous system (methanol) to facilitate the cross-linking between glutaraldehyde and SRM-derived peptides, a promising tackifier was successfully developed. Benefits associated with this SRM-based tackifier include a relatively low feedstock price, potential to biodegrade and serve as a nutrient source, the promising hydrophobicity (resistance to being washed away), and non-sacrificed hydrophilicity (moisture maintaining capability). The SRM-based tackifiers also showed comparable properties, such as compression strength and viscosity, to other renewable commercial tackifiers. Thus, it is possible that the tackifier described in this research could substitute for guar gum, psyllium, and/or starch in hydro-mulching applications.

4.2. Cross-linking of SRM-derived peptides with epoxidized poly (vinyl alcohol) for tackifier applications [2]

Section 4.1 section has shown that SRM-derived peptides could be potentially applied in the development of an industrial tackifier. However, compared with commercial tackifiers such as starch and psyllium, both SRM-derived peptides and glutaraldehyde cross-linked peptides (glutaraldehyde-peptides), exhibited lower binding strength and water holding capacity. Therefore, I studied other cross-linkers, such as epoxide cross-linkers, to develop a novel SRM-based tackifier with enhanced binding strength that is more comparable to commercial tackifiers. In this section, epoxidized PVA, an epoxide product of the reaction between PVA and epichlorohydrin, was synthesized and applied as a cross-linker for SRM-derived peptides under various conditions (i.e., temperature, molar ratio and reaction time) for tackifiers. This new cross-linking strategy strengthens the potential of using by-product waste from the livestock industry as a feedstock for the development of tackifiers.

4.2.1. Cross-linking of SRM-derived peptides with unmodified PVA

To develop a tackifier that is more comparable to starch and psyllium, PVA, a water-soluble synthetic polymer, was first introduced as the cross-linker. The abundant hydroxyl

[2] A version of this section has been published as a paper:
Tao Shui, Michael Chae, David C. Bressler. *Coatings*, 2020, 10, 630.

groups in the PVA molecule not only impart promising hydrophilicity, but also make the cross-linking with the amino groups and carboxylic groups of SRM-derived peptides possible for tackifier development. However, the amount of residual carboxylic groups in SRM-derived peptides after cross-linking with PVA was found to be 80.5 - 89.9% after 12 h at a 1:1 molar ratio (hydroxyl groups in PVA:carboxylic groups in peptides) using different solvents (i.e., water and dimethylformamide) and temperatures (i.e., 23 - 100℃ for water, 23 - 150℃ for dimethylformamide) (Figure 4.8). These results demonstrated a relatively low reactivity between hydroxyl groups of PVA and carboxylic groups of SRM-derived peptides.

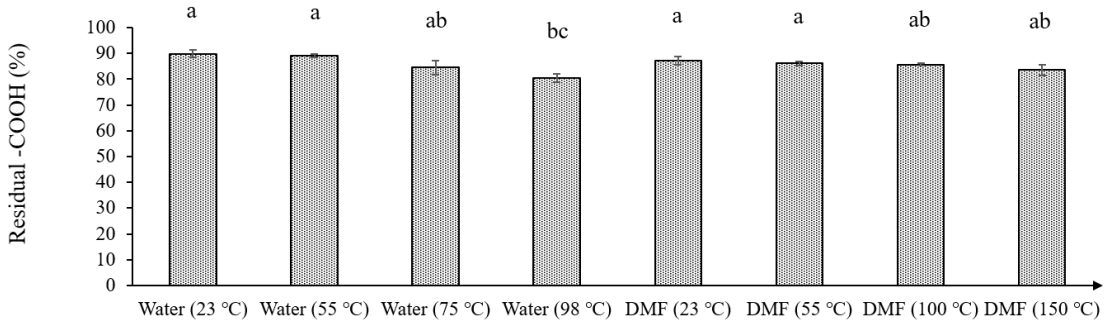
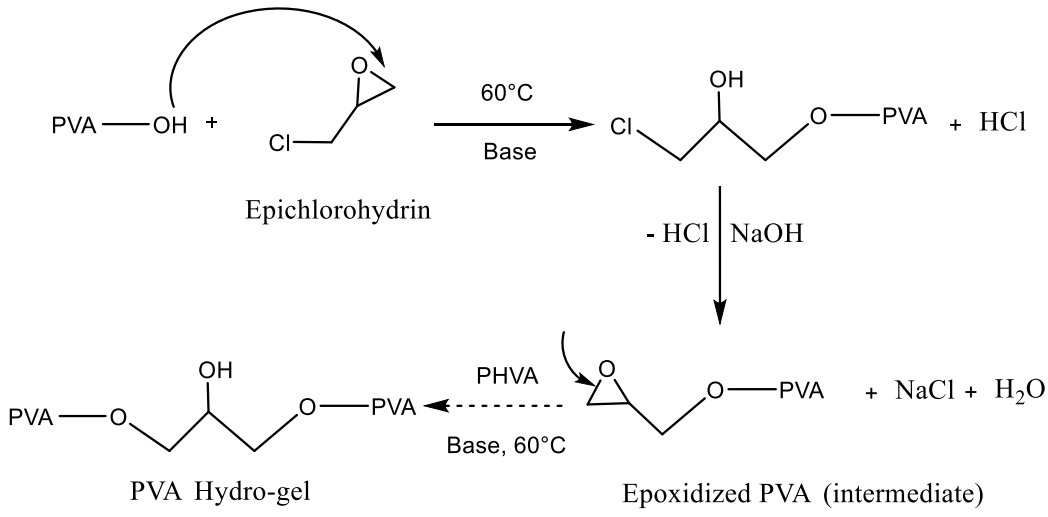


Figure 4.8. The residual carboxyl groups after reactions of SRM-derived peptides with PVA. Reactions were performed in aqueous (water) or non-aqueous (DMF; dimethylformamide) environments at a variety of temperatures. Values represent the average mean ± standard deviation of triplicate experiments. Values that do not share the same letter indicate that they are significantly different ($p<0.05$).

4.2.2. Synthesis and characterization of epoxidized PVA

In an attempt to improve the cross-linking reaction, I introduced an epoxide group into PVA, as epoxidized PVA is known to be highly reactive [128]. Epoxidized PVA is a substitutional product of PVA that can be generated via grafting of epoxy groups to its hydroxyl groups. Due to the promising reactivity of epoxy groups with both amino and carboxylic groups of SRM-derived peptides, epoxidized PVA was expected to be more effective for cross-linking of SRM-derived peptides.

According to the mechanism of polymer epoxidation, both poly (ethylene glycol) (PEG) diglycidyl ether and epoxidized PVA can be produced via reaction with epichlorohydrin [162, 178]. Moreover, compared with PEG diglycidyl ether, PVA possesses a higher amount of hydroxyl groups per molecule that might be useful as cross-linking sites, but can also facilitate self-polymerization (i.e., formation of a PVA hydro-gel; Reaction 4.2) during the epoxidation. The self-polymerization of PVA could result in wasted epichlorohydrin and reduce the epoxy content of the epoxidized products. Thus, the synthesis of epoxidized PVA with a high epoxy content is necessary for subsequent cross-linking of SRM-derived peptides. To this end, an extensive study on the reaction conditions (i.e., concentration of NaOH aqueous solution, temperature, and reaction time) was conducted to maximize production of the desired epoxidized product.

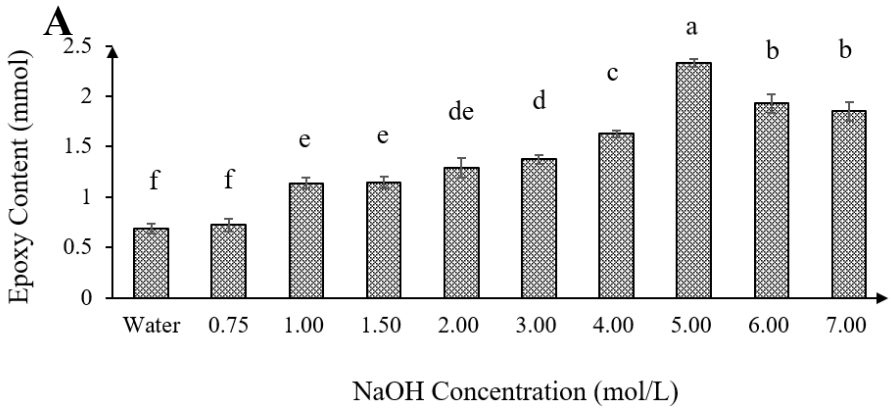


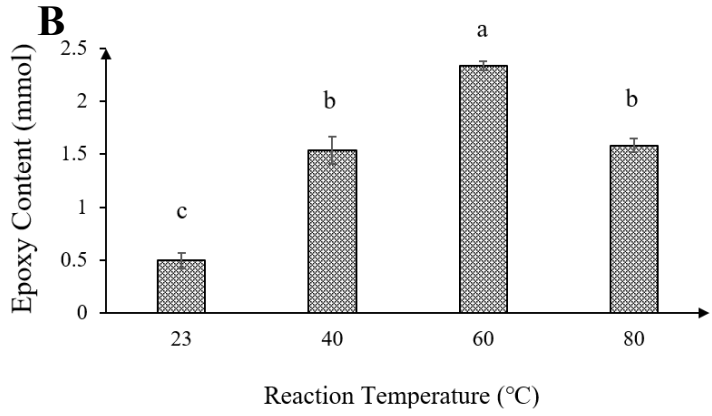
Reaction 4.2. The reaction pathway of PVA epoxidation using epichlorohydrin.

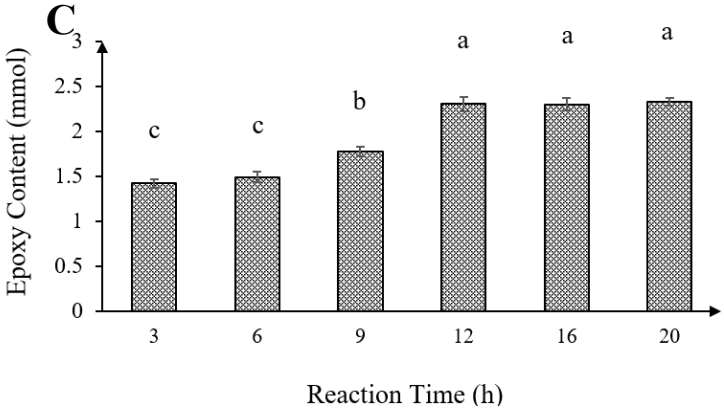
4.2.2.1. The impact of NaOH concentration

During the PVA epoxidation process, NaOH plays a key role in dechlorination by forcing the replacement of chlorine with an epoxy group (Reaction 4.2). In addition, NaOH functions to maintain an alkaline pH that is necessary for the substitution of hydroxyl groups. Based on conditions used for PEG diglycidyl ether synthesis [135], which has a similar reaction mechanism to PVA epoxidation, the impact of NaOH concentration on the epoxy content of the epoxidized PVA was studied (Figure 4.9A). Since the mass of epoxidized product yield was hard to approach due to the generation of salt, the amount of epoxy content (nmol) was used instead of the concentration of epoxy groups (nmol/g) in this study. Also, the epoxy contents of PVA and SRM-derived peptides were both characterized as 0 nmol.

To study the impact of NaOH concentration on the PVA epoxidation, the reactions were carried out at different NaOH concentrations using a 1:3 (hydroxyl groups in PVA:epoxy groups in epichlorohydrin) molar ratio at 60℃ for 12 h. Compared with the water control, NaOH started to improve the overall epoxy content of the reaction product at a concentration of 1.00 mol/L (Figure 4.9A). In addition, the epoxy content of epoxidized PVA increased with increasing NaOH concentration up to 5.00 mol/L. Concentrations higher than 5.00 mol/L resulted in lower epoxy content in the product (Figure 4.9A), which may have been caused by self-polymerization of epoxidized PVA. Furthermore, it is worth noting that the colour of PVA solution was changed from colourless to yellow at high NaOH concentrations (7.00 mol/L) during the epoxidation. This was consistent with the colour change observed by Cecelia *et al.*, which was reported to be caused by the hydrolysis of PVA [179]. Therefore, for subsequent reactions, NaOH was used at a molar concentration of 5.00 mol/L.


A
Epoxy Content (mmol)
2.5
2
1.5
1
0.5
0
f
f
e
e
de
d
c
a
b
b
Water
0.75
1.00
1.50
2.00
3.00
4.00
5.00
6.00
7.00
NaOH Concentration (mol/L)


B
Epoxy Content (mmol)
2.5
2
1.5
1
0.5
0
c
b
a
b
23
40
60
80
Reaction Temperature (°C)


C
Epoxy Content (mmol)
3
2.5
2
1.5
1
0.5
0
c
c
b
a
a
a
3
6
9
12
16
20
Reaction Time (h)

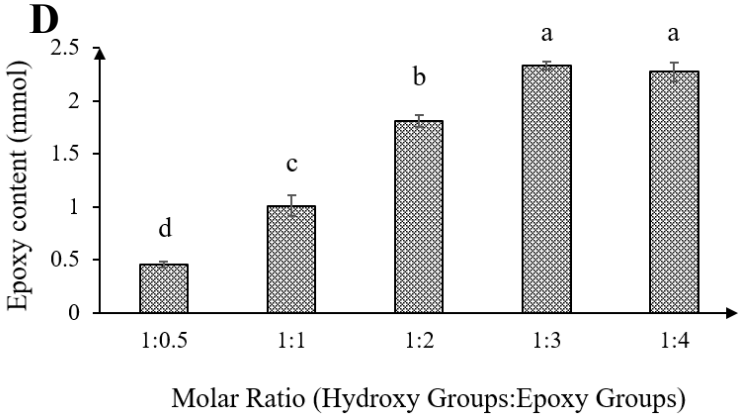

D
Epoxy content (mmol)
2.5
2
1.5
1
0.5
0
d
c
b
a
a
1:0.5
1:1
1:2
1:3
1:4
Molar Ratio (Hydroxy Groups:Epoxy Groups)

Figure 4.9. The impact of reaction parameters on the epoxy content of epoxidized PVA obtained from 1.14 g PVA epoxidation: **A.** The impact of NaOH concentration; reactions were performed at 60℃ for 12 h, using a 1:3 (hydroxyl groups:epoxy groups) molar ratio. **B.** The impact of reaction temperature; reactions were performed for 12 h, using a 1:3 (hydroxyl groups:epoxy groups) molar ratio and 5.00 mol/L NaOH. **C.** The impact of reaction time; reactions were performed at 60℃, using a 1:3 (hydroxyl groups:epoxy groups) molar ratio and 5.00 mol/L NaOH. **D.** The impact of molar ratio (hydroxyl groups in PVA:epoxy groups in epichlorohydrin); reactions were performed at 60℃ for 12 h, using 5.00 mol/L NaOH. Milli-Q water (Water) was used as a negative control for this study. Values in the figure represent the average mean ± standard deviation of triplicate experiments. Values that do not share the same letter indicate that they are significantly different ($p<0.05$).

4.2.2.2. The impact of reaction temperature

Temperature is another important parameter that affects the final equilibrium and reaction rate of the epoxidation process. To study the impact of temperature, the reactions were carried out at different temperatures at a 1:3 molar ratio (hydroxyl groups in PVA:epoxy groups in epichlorohydrin) for 12 h using 5.00 mol/L of NaOH. The results in Figure 4.9B indicated the increasing trend of epoxy content as the temperature increases from 23℃ to 60℃. Furthermore, a decreased epoxy content was observed at 80℃ in Figure 4.9B. Since

the newly generated epoxy groups in PVA molecule can further react with unreacted hydroxyl groups of PVA under basic conditions, resulting in the self-polymerization of PVA and the formation of hydro-gel as the final product [180-182], PVA epoxidation demands a great balance between the hydroxyl-epoxy groups reaction (grafting) and dechlorination (forming new epoxy groups). However, higher temperatures (≥ 80℃) might intensify the hydroxyl-epoxy groups reaction and decrease the nucleophilicity of amines by changing the pH of the system. This will break the balance and shit the system to the side of PVA self-polymerization, thus resulting in a decrease in epoxy groups in Figure 9B. Hence, 60℃ was the temperature used in subsequent PVA epoxidation.

4.2.2.3. The impact of reaction time

After examining the effect of epoxidation temperature and NaOH concentration, I then assessed the impact of reaction time. For these reactions, the reaction temperature was 60℃, the NaOH concentration was 5.00 mol/L, and the molar ratio was 1:3 (hydroxyl groups in PVA:epoxy groups in epichlorohydrin). As shown in Figure 4.9C, an increasing epoxy content in the epoxidized products was observed with increasing reaction time up to 12 h. At this point, the reaction reached equilibrium and no further increases in epoxy content were observed as the reaction time was increased to 20 h indicating the tendency of the epoxidized PVA to avoid self-polymerization. This can be explained by two possible reasons.

On one hand, due to the consumed NaOH during epoxidation, the pH of the solution was decreased to 6.0 after 12 h, which is relatively lower than the pH 11 - 12 required for hydroxyl-epoxy coupling as reported by Hermanson [103]. On the other hand, it is also possible that most of hydroxyl groups have already reacted and been substituted by the epoxy groups at 12 h, thus limiting self-polymerization. Taken together, a 12 h reaction time was employed for all future epoxidation reactions.

4.2.2.4. The impact of molar ratio

Finally, the effect of changing the molar ratio between the hydroxyl groups of PVA and epoxy groups of epichlorohydrin were studied. As shown by the results in Figure 4.9D, there was a strong correlation between the amount of epichlorohydrin used in the reaction and epoxy content in the reaction product to reach the balance between hydroxyl-epoxy reactions and dechlorination. The maximum epoxy content was observed in the reaction where molar ratios of 1:3 and 1:4 were employed. However, there was no significant difference in epoxy content using these two ratios. Therefore, in subsequent reactions, a 1:3 molar ratio was used as this had a lower requirement for epichlorohydrin.

4.2.2.5. Summary of the PVA epoxidation

By using the most promising reaction conditions identified in this section (i.e., 5.00 mol/L NaOH, 60℃ for 12 h, and a molar ratio of 1:3), a great reaction balance and a stable poly-epoxide with an epoxy content of 2.33 ± 0.03 mmol were achieved in aqueous conditions from the epoxidation of 1.14 g PVA. This is the first study to describe a synthesis method for epoxidized PVA, offering an alternative strategy for PVA cross-linking with amines or other proteinaceous materials. Additionally, the characterized epoxy content makes epoxidized PVA possible to react with primary amine groups (0.59 ± 0.05 mmol/g) or carboxylic acid groups (1.69 ± 0.06 mmol/g) of SRM-derived peptides at a certain molar ratio of functional groups. However, to optimize the epoxidation of PVA reaction, a future study on the reaction kinetics is needed.

4.2.3. Synthesis of PVA-EPC-Peptides composites

In this section, epoxidized PVA was used as a cross-linker to potentially react with amines and carboxylic acid groups in SRM-derived peptides. To this end, several reactions were setup in which the amount of peptides used was varied to change the molar ratio of the reactions (Table 3.3).

4.2.3.1. Assessing the degree of cross-linking through primary amine group quantification

Compared with other functional groups commonly found in peptides that can react with epoxides (i.e., -COOH, -OH, -SH; Reaction 2.6, Reaction 2.7, Reaction 2.8), primary amino groups possess the highest nucleophilicity and thus are most likely to react with epoxy groups [103]. Therefore, primary amino groups of SRM-derived peptides are likely the predominant target for the epoxide/PVA cross-linking reaction performed at alkaline pH (pH = 9.0, suggested by Leon *et al.* [128]). Based on this assertion, the rate of cross-linking between SRM-derived peptides and epoxidized PVA was assessed through quantification of residual primary amine groups in the reaction products.

4.2.3.1.1. Role of cross-linking temperature

Initial cross-linking studies were performed at various temperature to determine the most promising reaction temperature. As shown in Figure 4.10A, it was observed that relatively low levels of primary amino groups were observed in all the cross-linked products compared to the reaction with no epoxidized PVA, indicating the occurrence of the amine-epoxide cross-linking reaction. The lowest levels of residual amines (0.03 ± 0.01 mmol/g) were observed in reactions performed at 23°C (Figure 4.10A). However, as the reaction temperature was increased to 80°C, there was a small increase in the amount of residual

primary amino groups in the product, suggesting that higher temperatures have a slight inhibitory effect on the amine-epoxide reaction (Figure 4.10A). This may result as higher temperatures may favor competing reactions. For instance, a hydroxyl-epoxide reaction was reported to occur at 60-100℃ at a base condition in studies from Iulian *et al.* and Leon *et al.* [183, 184]. Therefore, 23℃ was used as the reaction temperature for subsequent cross-linking experiments.

4.2.3.1.2. Role of cross-linking time

The next studies examined the role of cross-linking time on the reaction between SRM-derived peptides and epoxidized PVA. As shown in Figure 4.10B, an increase in reaction time generally correlated with lower levels of residual primary amino groups. This suggests a direct relationship between the reaction time and the degree of cross-linking. Increasing the reaction time from 20 to 24 hours did not result in lower levels of residual primary amino groups, and thus subsequent cross-linking reactions were performed using a 20 h reaction time. However, since only the impact of a single factor was studied, a future study on the reaction kinetics is needed for this study to optimize the cross-linking reaction.

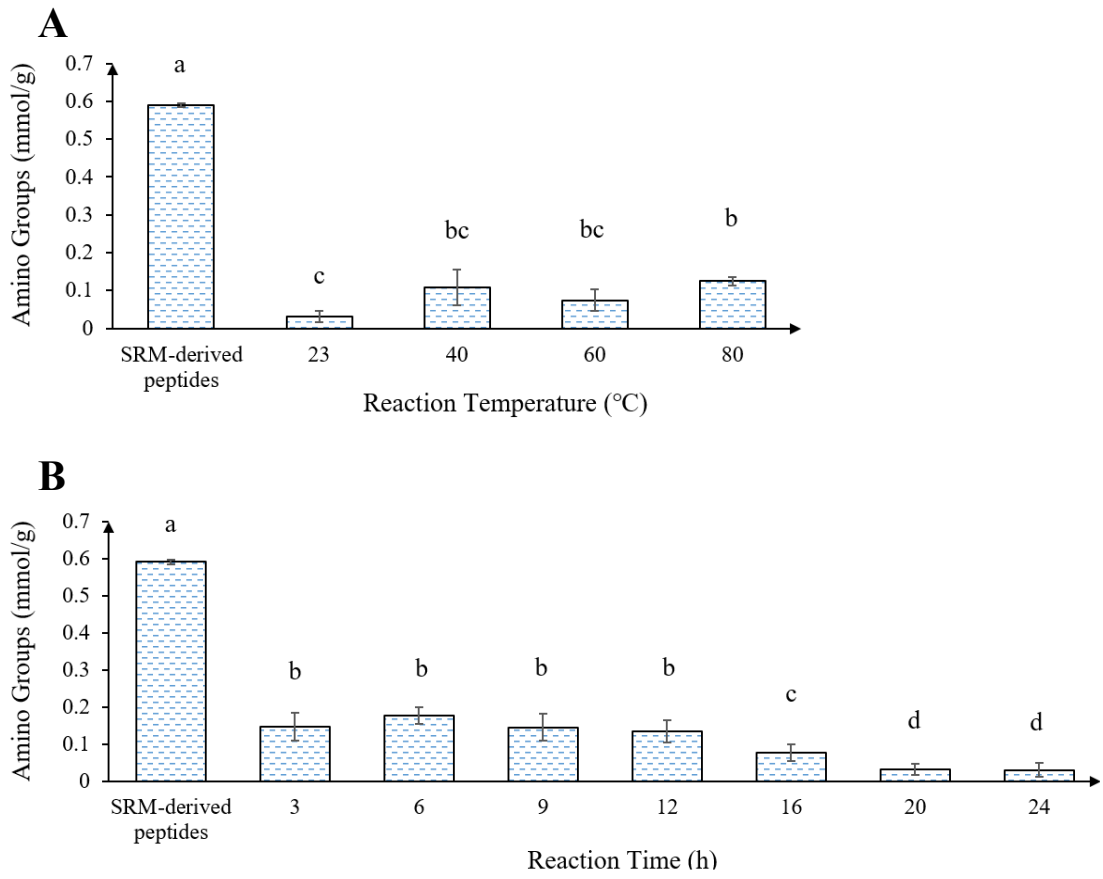


Figure 4.10. The role of reaction parameters on the primary amino groups remaining in the PVA-EPC-Peptides cross-linked at a molar ratio of 1:1 (epoxy groups in epoxidized PVA:primary amino groups in SRM-derived peptides): **A.** The role of cross-linking temperature; a reaction time of 20 h and a pH of 9.0 were employed for the reaction. **B.** The role of cross-linking time; a temperature of 60°C and a pH of 9.0 were employed for the reaction. A control reaction was performed in which SRM-derived peptides were added to 15.0 ml of Milli-Q water, in the absence of epoxidized PVA. Values represent the average

mean ± standard deviation of triplicate experiments. Values that do not share the same letter are significantly different ($p<0.05$).

4.2.3.2. Assessing the degree of cross-linking through carboxylic acid group quantification

The residual carboxylic groups in the products were also evaluated to assess the role of the carboxylic groups of the peptides in the cross-linking reaction. For this study, the reaction was performed at 23℃ for 20 h at a pH of either 5.0 or 9.0. An acidic pH was employed since it can promote the reaction of carboxylic acid groups with epoxides [128]. In contrast to previous experiments, 1.30 g of SRM-derived peptides was added to 20 ml epoxidized PVA to maintain a molar ratio of 1:1 (carboxylic groups in peptides:epoxy groups in epoxidized PVA). The control experiment was performed by adding 1.30 g of SRM-derived peptides to 20 ml water. Although carboxylic groups could theoretically react with epoxy groups and hydroxyl groups, the level of carboxylic groups in cross-linked products obtained at pH 5.0 and pH 9.0 (98.5 ± 1.0% and 98.2 ± 1.5%, respectively) remained unchanged compared to the control (97.4 ± 2.3% for pH 5.0 and 98.0 ± 1.4% for pH 9.0). This indicated a limited reactivity of carboxylic groups during the cross-linking. This is consistent with the ‘low carboxylic acid-epoxide reactivity’ observed in the study of Leon *et al.* [128].

4.2.4. Application of the PVA-EPC-Peptides product as a tackifier

The products obtained from amine-epoxide cross-linking were then subjected to a variety of testing to facilitate measurement of their tackifier qualities. For these studies, PVA-EPC-Peptides products generated from different molar ratios (epoxy groups in epoxidized PVA:primary amine groups in peptides) were studied to determine whether this had an impact on their binding strength, thermal resistance, moisture holding capacity and aqueous viscosity.

4.2.4.1. Compression strength of pucks produced using PVA-EPC-Peptides

To assess the binding strength of the various PVA-EPC-Peptides products, I employed compression analysis on 'pucks'. which gave us the breaking stress (kPa) as shown in Figure 4.11.

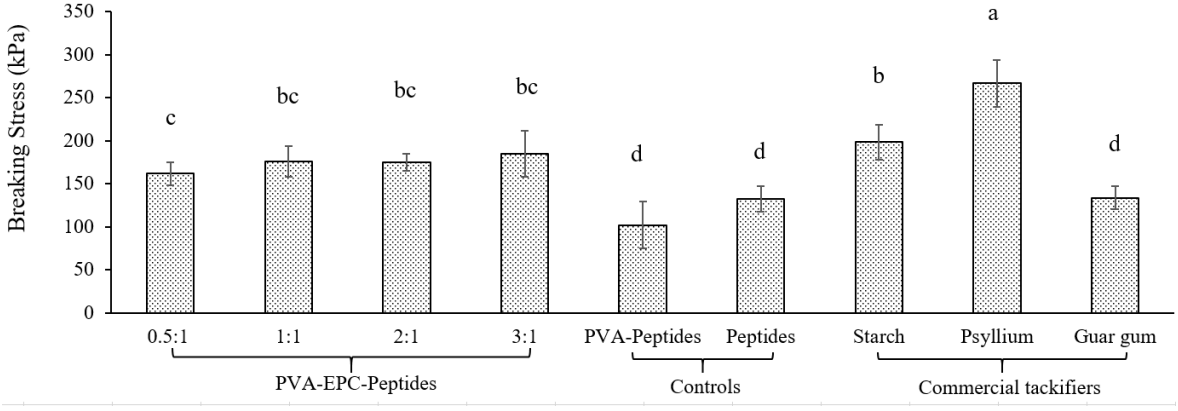


Figure 4.11. The compression strength of pucks. Pucks were prepared using MulchMax 100 wood fibres and a tackifier. The prepared pucks were cured at 40℃ for 48 h followed by a cooling process at room temperature for 24 h. The tackifier used was one of the Cross-linked products (PVA-EPC-Peptides generated at various molar ratios of epoxy groups:amino groups), Controls (Product obtained in reactions containing PVA-peptides product or SRM-derived peptides), or Commercial tackifiers (Starch, Psyllium, Guar Gum). PVA-peptides (Control) was obtained from the reaction between 1.14 g unmodified PVA and 4.00 SRM-derived peptides in 20 ml Milli-Q water at 23℃ and pH 9.0 for 20 h. The compression strength (i.e., breaking stress (kPa)) of pucks were assessed using a *TA.XTPlus* texture analyzer. Same size of alumina dishes and the compression probe were suggested to reproduce results. The values shown represent the mean ± standard deviation of triplicate experiments. Values annotated with different letters are significantly different ($p<0.05$).

The results in Figure 4.11 showed that all of the pucks generated using the PVA-EPC-Peptides products displayed an enhancement in compression strength, when compared to those made with unmodified SRM-derived peptides or PVA-peptides products. Moreover, the compression strength of pucks made from cross-linked products generated at different molar ratios of epoxy groups:amino groups were statistically similar to each other, with all having higher compression strength that those generated using guar gum, a commercial tackifier. Furthermore, pucks generated at cross-linking molar ratios of 1:1, 2:1, and 3:1

displayed similar compression strength to pucks generated using starch. Taken together, these data confirm the potential for the PVA-EPC-Peptides product to replace guar gum and starch in commercial tackifier applications.

PVA and epoxidized PVA were also used as tackifiers for compression strength measurement. However, the pucks generated using these reagents fell apart easily and had no integrity to be taken out from dishes for the following tests. Thus, it is clear that these reagents are not capable of functioning as tackifiers themselves.

4.2.4.2. Thermal resistance of PVA-EPC-Peptides

TGA analysis was then used to study the thermal resistance of PVA-EPC-Peptides. This testing method can provide insight into the degree of cross-linking in the sample and can be used to infer whether changes to the molecular structure have occurred. During the TGA process, the mass of all sample remained constant in the early stages, followed by a dramatic decrease (decomposition stage) with increasing temperature (Figure 4.12). It can be observed that the PVA-EPC-Peptides, obtained from the cross-linking at a 1:1 molar ratio (epoxy groups:amino groups), showed a slight decrease in the temperature range of 100-120°C. This can be demonstrated as the evaporation of moisture content. The PVA-EPC-Peptides and epoxidized PVA samples used for TGA were previously stored in the fridge at 4°C for 24 h after lyophilisation. However, these samples became sticky after the storage,

which probably due to the absorption of moisture. On the other hand, unmodified peptides, and PVA samples remained as dry powders during the storage. Thus, PVA-EPC-Peptides and epoxidized PVA samples contained some moisture content which would cause a slight decrease in mass in the temperature range of 100-120℃. Moreover, PVA-EPC-Peptides (1:1) displayed a similar decomposition temperature as the SRM-derived peptides (around 250℃), demonstrating that the PVA-EPC-Peptides likely possessed a molecular structure that remained similar to the unmodified peptides (Figure 4.12). However, both PVA-EPC-Peptides (1:1) and unmodified peptides showed lower decomposition temperature than PVA (around 275℃). Moreover, the PVA-EPC-Peptides cross-linked at molar ratios of 2:1 and 3:1 (epoxy groups:amino groups) displayed a relatively constant and consistent decomposition stage, in the temperature of 100-350℃, which was right in between the thermal behavior of epoxidized PVA and unmodified peptides (Figure 4.12). It can thus be inferred that the molecule of PVA-EPC-Peptides cross-linked at molar ratios of 2:1 and 3:1 appeared to have more volatile components or structures imparted by the epoxidized PVA. It is interesting to note that compared to the unmodified peptides and PVA, the products obtained from the cross-linking reaction at molar ratios of 2:1 and 3:1, had reduced thermal stability, which might limit the application of the PVA-EPC-Peptides in other bio-industrial strategies.

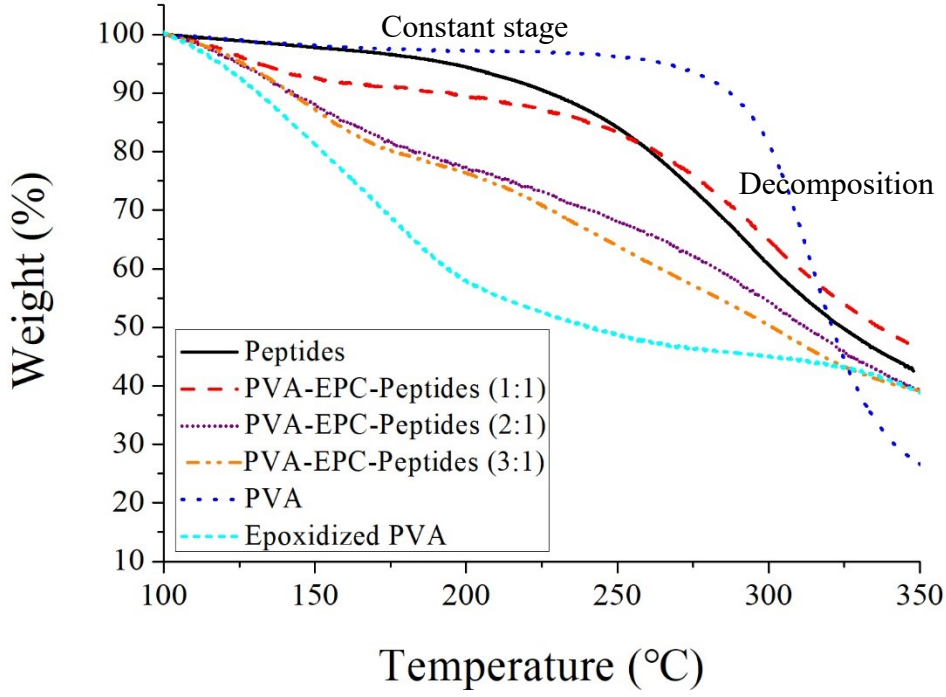


Figure 4.12. TGA on PVA-EPC-Peptides, SRM-derived peptides PVA and epoxidized PVA. TGA was performed on SRM-derived peptides, PVA-EPC-Peptides synthesized in cross-linking reaction at various molar ratios (1:1, 2:1, 3:1; epoxy groups:amino groups), PVA, and epoxidized PVA from 100℃ to 350℃ at a heating rate of 5°C/ min. The powders of epoxidized PVA used in TGA analysis were generated through lyophilization of the epoxidized PVA solution. Triplicates were applied for TGA analysis and all results were reproduced to 5% error or better. Since the replicates for each material were similar, only one run is shown above.

4.2.4.3. Moisture holding capacity of PVA-EPC-Peptides tackifiers

Moisture holding capacity was also studied as this is an important property for tackifiers. This is defined as the ability to retain the water obtained from surface water, rains and/or the hydro-mulching process. The moisture retained provides seeds and plant with water to promote plant growth. As shown in Figure 4.13, epoxidized PVA powders demonstrated the highest moisture holding capacity. This is likely due to the large number of hydroxyl groups present, which contributes to the relatively strong bonding with water. The PVA-EPC-Peptides obtained from the cross-linking reaction at 2:1 and 3:1 molar ratios (epoxy groups:amino groups) displayed increased water holding ability compared to the unmodified peptides and the product obtained from the cross-linking reaction preformed at a 1:1 molar ratio. This can be explained by the relatively high proportion of epoxidized PVA molecules that have been integrated into the product cross-linked at 2:1 and 3:1 ratios. Furthermore, the values measured for the products obtained from cross-linking reactions at 2:1 and 3:1 ratios were higher than that of starch and psyllium, and similar to the levels achieved by guar gum. This shows the competitiveness of the PVA-EPC-Peptides with commercial tackifiers with regards to water retention capacity. It is worth noting that even though PVA-EPC-Peptides cross-linked at a 1:1 ratio exhibited a reduced moisture retention (Figure 4.13), the values achieved were statistically similar to starch and psyllium. The different water holding capacities achieved for the various cross-linked products (PVA-EPC-

Peptides) also highlight the versatility of using peptide-derived tackifiers, enabling manufacturers a mechanism to modulate cross-linking ratios to produce tackifiers suited for a variety of geographical areas with different moisture retaining requirements. For instance, in areas where a significant amount of rain is common, PVA-EPC-Peptides cross-linked at a 1:1 ratio may be more applicable as water-holding capacity would not be as important. On the other hand, the outstanding water holding capability of PVA-EPC-Peptides products cross-linked at 2:1 and 3:1 ratios can probably help retain the necessary moisture for germination and growth of vegetation in dryer geographic locations (i.e., the desert).

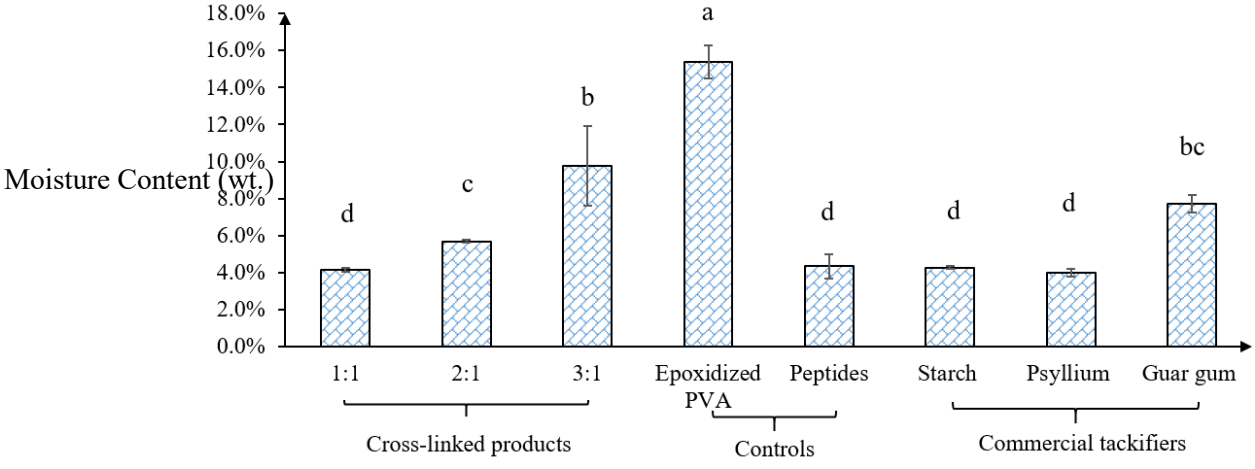


Figure 4.13. The water holding capacity of peptides, PVA, PVA-EPC-Peptides cross-linked products, and commercial tackifiers. 5% (w/v) solutions of the sample powders were added to Petri dishes, which were then placed in an incubator at 22.0°C and 0.0% relative humidity for 60 h. The moisture remaining after 60 h was determined and used to calculate the moisture holding capacity of the residual material. For the cross-linked products, the molar

ratio (epoxy groups in epoxidized PVA:primary amino groups in peptides) employed during the cross-linking reaction is indicated (i.e., 1:1, 2:1, or 3:1). Epoxidized PVA powders used for the test were generated via lyophilization of the epoxidized PVA solution. Peptides represent the unmodified SRM-derived peptides. The values shown represent the mean ± standard deviation of triplicate experiments. Values annotated with a different letter are significantly different ($p<0.05$).

4.2.4.4. Aqueous viscosity of PVA-EPC-Peptides

Tackifiers with low aqueous viscosity are generally preferred as this reduces fluid drag force during mixing and spraying and also minimizes the clogging of the hydro-mulching equipment. As indicated by the results of viscosity measurement, there was no significant difference between the viscosity of the aqueous suspensions of any of the cross-linked products, which were 1.00 ± 0.17 cP, 0.97 ± 0.15 cP and 1.00 ± 0.10 cP for the material generated from the cross-linking at 1:1, 2:1 and 3:1 ratios (epoxy groups:amino groups), respectively. Moreover, when compared with psyllium (1.50 ± 0.26 cP) and guar gum (~ 26.0 ± 2.43 cP), the SRM peptides and the various PVA-EPC-Peptides products displayed a lower aqueous viscosity in suspension, which provides further evidence that the peptides-based products are promising commercial tackifiers for hydro-mulching.

4.2.4.5. Estimated cost of tackifiers derived from peptides

A complete techno-economic assessment for production of PVA-EPC-Peptides cannot currently be completed as the process has not been optimized nor scaled-up to pilot scale. However, the SRM used to generate PVA-EPC-Peptides is of negative value, which greatly strengthens the process economics. Rendering companies pay ~US$ 0.2/kg for SRM disposal, and thus it may be possible for manufactures to collect tipping fees to dispose of the primary feedstock for PVA-EPC-Peptides [12]. According to Alibaba, epichlorohydrin (99.0%) and PVA (industrial grade) can be purchased for only US$ 1.0/kg and US$ 1.1/kg, respectively [185, 186]. Meanwhile, the hexane used during the process will likely be recycled via evaporation. Taken together, the feedstock prices by mass for PVA-EPC-Peptides tackifiers can be calculated as US$ 1.7/kg, US$ 2.5/kg, and US$ 3.0/kg for 1:1, 2:1 and 3:1 products, respectively. Since 1:1, 2:1, and 3:1 cross-linked products showed better binding property and similar water holding capacity when compared to guar gam, it is likely that the application rate of these cross-linked products is similar to that of guar gam, which is 40 lb/acre [176]. Based on this, the feedstock prices by area for 1:1, 2:1, and 3:1 cross-linked products can be inferred as ~US$ 30.8/acre, ~US$ 45.2/acre, and ~US$ 54.3/acre respectively. These prices will be competitive with the sale price of psyllium (US$ 56.8/acre) and starch (~US$ 34.0/acre), and will likely be substantially lower than that of guar gum (US$ 326.5/acre) for mulch fibre applications [59, 176, 177].

4.2.5. Summary

By facilitating the cross-linking between SRM-derived peptides and epoxidized PVA, a peptides-based tackifier was successfully developed and characterized. As a peptides-based product with great potential, all the PVA-EPC-Peptides products displayed comparable binding strength with commercial tackifiers, as well as a relatively low aqueous viscosity and feedstock price. In addition, by adjusting the molar ratio of the cross-linking reagents, the water holding capacity for PVA-EPC-Peptides can be varied to cater to different applications. Through this cross-linking strategy, a novel pathway is demonstrated for the bio-conversion of SRM-derived peptides into novel tackifiers, which may be applied to other cross-linking studies involving PVA and amines.

4.3. Development of a torrefied wood pellet binder from the cross-linking between SRM-derived peptides and epoxidized PVA [3]

Wood binders are mostly used to enhance the properties of pellets, especially pellet density and pellet strength. Since torrefied wood powders are generated from cellulose sources, they are expected to interact with the binders/adhesives developed for cellulosic materials. Cross-linking products produced from reactions between epoxidized PVA and SRM-derived peptides (PVA-EPC-Peptides) have demonstrated superior binding with wood fibres in Section 4.2. Therefore, in order to expand the application of this cross-linked product for other cellulosic materials, I conducted a study on the potential of PVA-EPC-Peptides to be used as a renewable and biodegradable torrefied wood binder. The properties of the pellets made with torrefied wood to which PVA-EPC-Peptides were added as a binder were characterized and evaluated in this section to verify the effects of this binder on pellet density, compression strength, moisture up-taking, elemental content, and heating value.

I also looked into other factors that influence pellet properties (i.e., die temperature, holding pressure, holding time, and moisture content) to obtain promising pellets that satisfy the current industry standards. Considering that the cross-linked products synthesized at molar ratios of 2:1 and 3:1 (based on primary amine groups in peptides:epoxy groups in

[3] A version of this section has been submitted to the journal in 2020:

Tao Shui, Vinay Khatri, Michael Chae, Shahabaddine Sokhansanj, Phillip Choi, David C. Bressler. *Applied Energy*.

epoxidized PVA) started to decompose at a temperature of 100°C, which is not suitable for a densification process (Figure 4.12). In further support of this selection, the cross-linked products generated using 2:1 and 3:1 ratios displayed excellent water absorption qualities (Figure 4.13), which may reduce the water-resistance of the resulting pellets and is thus not desirable. Herein, PVA-EPC-Peptides generated at a 1:1 molar ratio (epoxy groups:primary amino groups) were examined as binders in the torrefied wood densification process.

4.3.1. The density and compression strength of torrefied wood pellets

4.3.1.1. The impact of binder level

Initial studies were conducted to assess pellet density and compression stresses when different binder levels (1.0-5.0 wt.%, Table 4.1) of PVA-EPC-Peptides. Based on the conditions used by Peng *et al.*, a die temperature of 150°C, a powder moisture content of 3.0 wt.%, a holding pressure of 250 MPa and 1 min holding time were initially used for pellet densification [74]. During the tests, pellets made only with torrefied wood powders were applied as a control. In addition, unmodified SRM-derived peptides were mixed with torrefied wood to make the sample powders and were pelletized as another control to help assess the impact of cross-linking on SRM-derived peptides.

Table 4.1. The composition of the sample powders tested for densification.

Binder Level (wt.%)	Torrefied wood powders (g)	EPC-PVA-Peptides/ SRM-derived peptides (g)	Total moisture content	Total powders (g)
1.0	4.95	0.05	3.0 ± 0.2 wt.%	5.0 g
2.0	4.90	0.10	3.0 ± 0.2 wt.%	5.0 g
3.0	4.85	0.15	3.0 ± 0.2 wt.%	5.0 g
5.0	4.75	0.25	3.0 ± 0.2 wt.%	5.0 g

Pellets with high density take up less space at the same mass, which is favored to decrease the costs for transportation and storage. Moreover, pellets with high density can improve combustion efficiency, which is essential to the current industry [187]. The densities of the pellets generated with different levels of binder are shown in Figure 4.14. In comparison to the density of the pellets made without a binder, the density of the pellets made with PVA-EPC-Peptides or unmodified SRM-derived peptides was increased (Figure 4.14). Moreover, PVA-EPC-Peptides showed a greater improvement in pellet density when compared with the SRM-derived peptides, peaking at the 3.0 and 5.0 wt.% binder levels (Figure 4.14). One possible explanation for this density improvement would be that the

particles of PVA-EPC-Peptides and SRM-derived peptides were softer than torrefied wood particles, and could thus fill in the gaps between particles that would otherwise be filled with air. Another possible reason is that the introduction of additional binding forces by the binder promotes better binding between torrefied wood particles, decreasing the number and size of voids formed in the pellets.

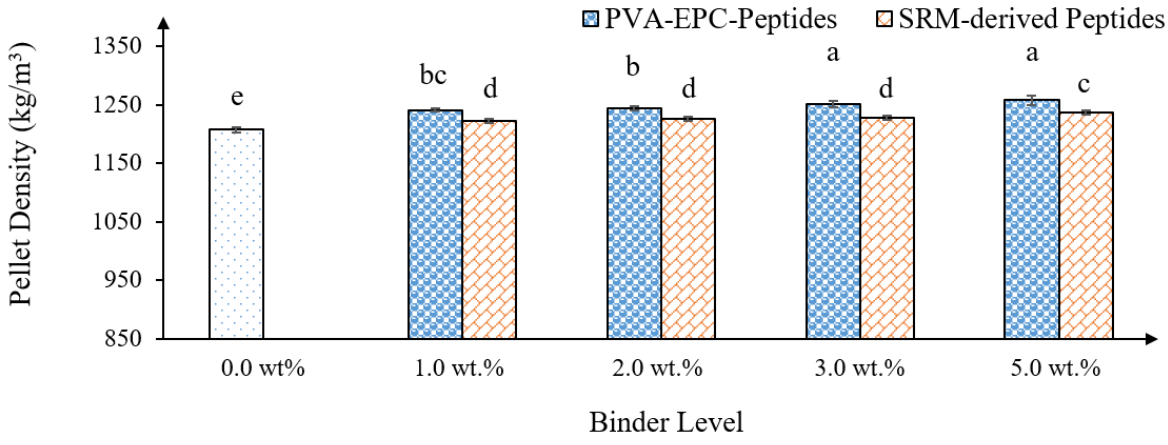


Figure 4.14. The density of the pellets produced at different binder levels. Conditions were as follows: 150°C die temperature, 250 MPa holding pressure, 1 min holding time and 3.0 wt.% moisture content of densification powders. PVA-EPC-Peptides refers to torrefied wood pellets made by adding PVA-EPC-Peptides. SRM-derived peptides refer to torrefied wood pellets made by adding SRM-derived peptides. Values in the figure represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

The radial and axial compression strength measurements were then studied to estimate the strength of the pellets. The torrefied wood pellets without a binder (0.0 wt.% binder level) exhibited the same compression strength as pellets made with unmodified peptides in both radial (Figure 4.15A) and axial tests (Figure 4.15B), indicating that the addition of unmodified peptides had no influence on the strength of the pellets. However, when observing the impact of PVA-EPC-Peptides, a strength enhancement in the pellets made using PVA-EPC-Peptides was noticed at all the binder levels, as demonstrated by higher breaking stresses of the pellets in both radial (Figure 4.15A) and axial (Figure 4.15B) tests. Furthermore, comparing the strength of the PVA-EPC-Peptides pellets at different binder levels demonstrated that the breaking stresses of pellets gradually increased with increasing the binder level of PVA-EPC-Peptides from 1.0 wt.% to 5.0 wt.%.

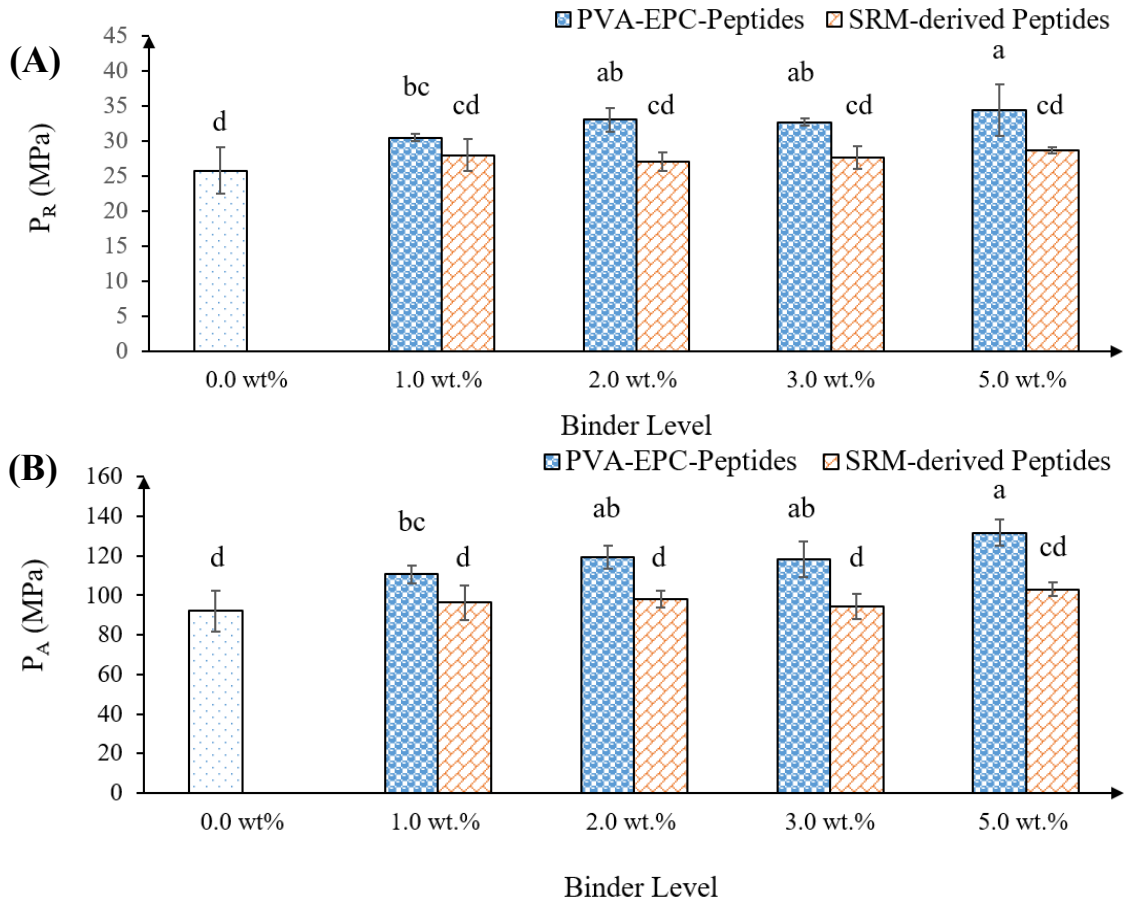


Figure 4.15. The radial (A) and axial (B) compression stresses of pellets produced at different binder levels. Conditions are as follows: 150°C die temperature, 250 MPa holding pressure, 1 min holding time and 3.0 wt.% moisture content of densification powders. PVA-EPC-Peptides refers to torrefied wood pellets made with an addition of PVA-EPC-Peptides. SRM-derived peptides refer to torrefied wood pellets with the addition of SRM-derived peptides. Values in the figure represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

Nevertheless, international standards and requirements can impact the binder level employed for pelletization. The International Standard Organization (ISO) specifies the binders/additives level to be ≤ 4.0 wt.% on a dry mass basis for the highest quality classes of thermally-treated wood pellets (i.e., TW1a and TW2b), shown in ISO/TS 17225-8:2016 [94]. In addition, the International Maritime Organization (IMO) specifies binders/additives level to be ≤ 3.0 wt.% for torrefied wood pellets that need to be transported on marine vessels [46]. Combining these requirements with the observation that there was no significant difference between 5.0 wt.% and 3.0 wt.% PVA-EPC-Peptides addition in either density or breaking stress tests, 3.0 wt.% of PVA-EPC-Peptides addition was applied for the following tests.

4.3.1.2. The impact of die temperature

As one of the essential factors in pellet production, die temperature affects the mechanical linkages formed within the powders during the densification process and may lead to further cross-linking of binders and improved adhesion. In their respective studies of sawdust densification, Peng *et al.* and Verhoeff *et al.* found that the strength of the torrefied pellets increased with increasing die temperature [74, 76]. Therefore, to study the impact of die temperature during the densification process, pellets were made using die temperatures

ranging from 22-175°C. The density and breaking stresses of both groups of pellets were then compared, as shown in Figure 4.16 and Figure 4.17, respectively.

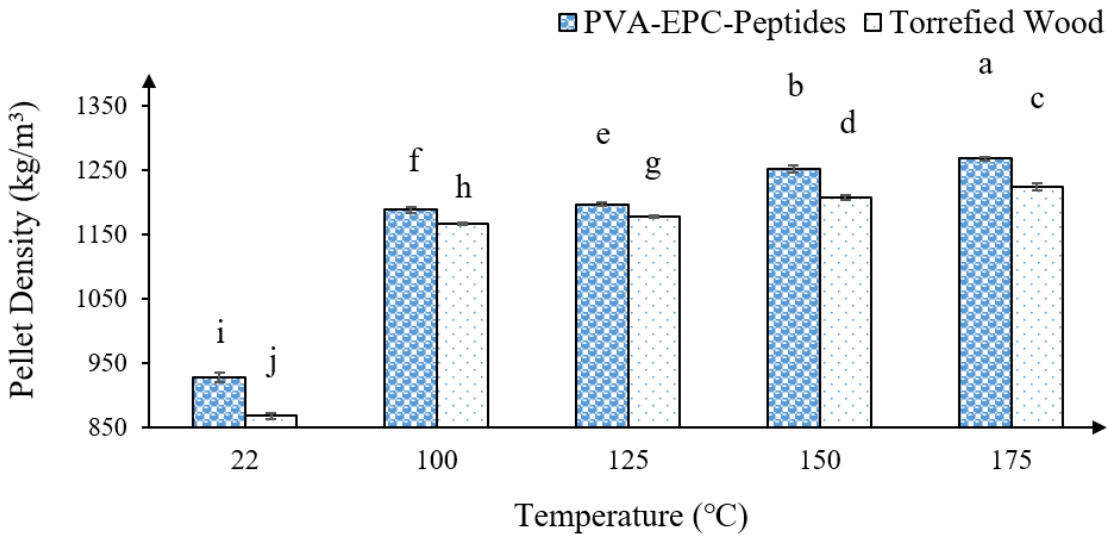


Figure 4.16. The density of the pellets produced at different die temperatures. Conditions were as follows: 250 MPa holding pressure, 1 min holding time and 3.0 wt.% moisture content of densification powders. PVA-EPC-Peptides refers to torrefied wood pellets made by adding 3.0 wt.% PVA-EPC-Peptides as a binder. Torrefied wood refers to pellets made with torrefied wood powders without a binder. Values in the figure represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

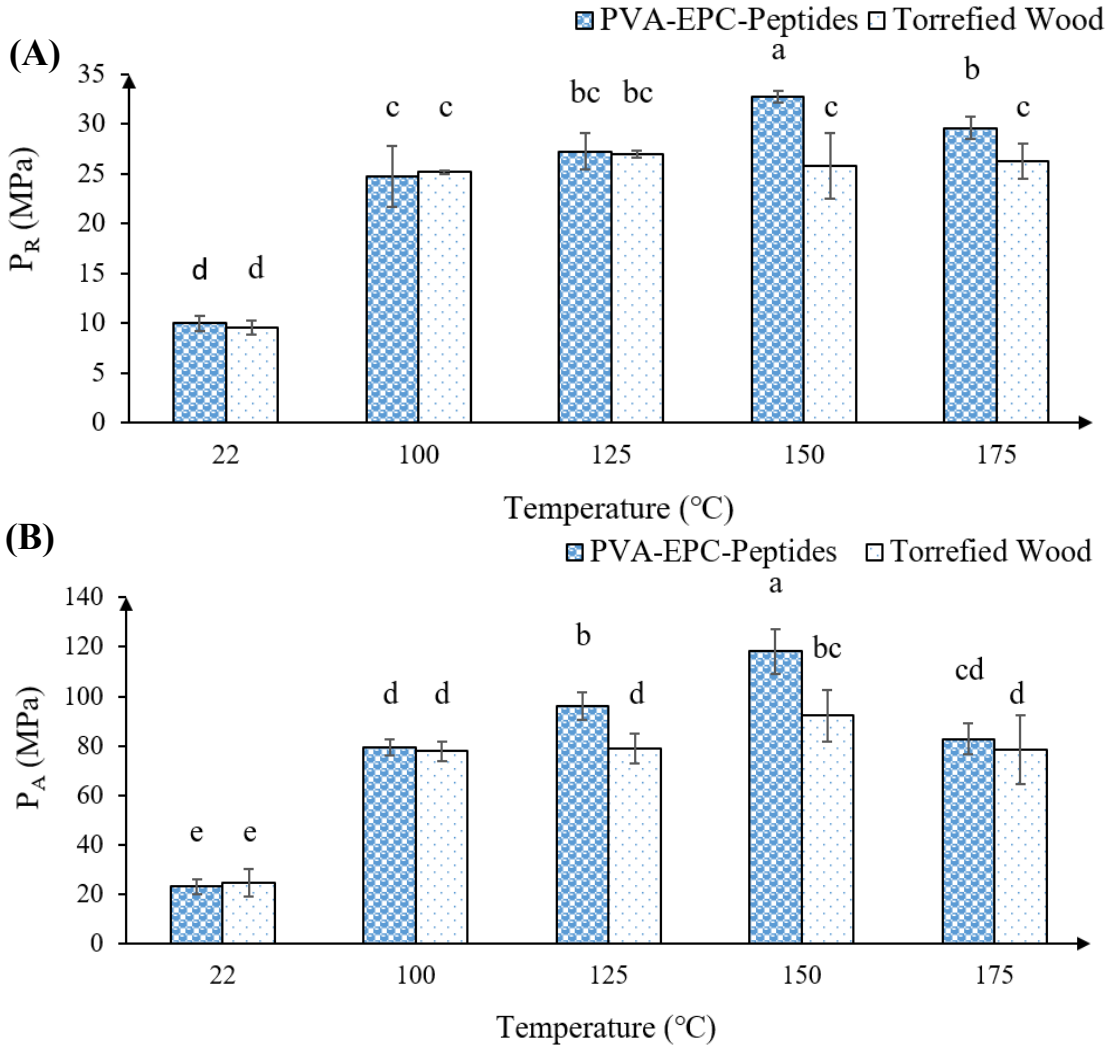


Figure 4.17. The radial (A) and axial (B) compression stresses of pellets produced at different die temperatures. Conditions are as follows: 250 MPa holding pressure, 1 min holding time and 3.0 wt.% moisture content of densification powders. PVA-EPC-Peptides refers to torrefied wood pellets made with the addition of 3.0 wt.% PVA-EPC-Peptides as a binder. Torrefied wood refers to pellets made from torrefied wood powders without a binder. Values in the figure represent the average mean ± standard deviation of triplicate experiments.

The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

Consistent with the conclusion of Peng *et al.* and Verhoeff *et al.* [74, 76], Figure 4.16 indicated an improvement in the density of both torrefied wood pellets without a binder (control) and PVA-EPC-Peptides pellets with respect to the increment in the die temperature. Moreover, due to the addition of PVA-EPC-Peptides, the density of the resulting pellets remained at a higher level than that of control pellets in Figure 4.16, demonstrating the function of PVA-EPC-Peptides as an effective adhesive for density improvement. This improvement was mainly because of two mechanisms: physical property of PVA-EPC-Peptides and chemical binding. The physical property of PVA-EPC-Peptides, i.e. softness and mechanical binding, can help to increase pellet's density at a low temperature (22℃), which is 60 kg/m^3 larger than the density of a control pellet. Due to the impact of temperature on torrefied wood pellets demonstrated by Peng *et al.* [74], the density difference between the control pellet and the PVA-EPC-Peptides pellet became smaller at 100℃ and 125℃, which is around 20 kg/m^3 shown in Figure 4.16. Moreover, the density difference was increased to 50 kg/m^3 when temperature reached 150℃, which can be demonstrated by the formation of additional chemical binding during the densification process.

However, in contrast to the positive effect of increasing die temperature on pellet density, the impact of temperature on pellet strength improvement peaked at a lower

temperature (Figure 4.17). In the radial compression test, the breaking stress of the control pellets stayed at the same level under the temperature range of 100-175℃, while the breaking stress of PVA-EPC-Peptides pellets peaked at 150℃ and decreased at 175℃ (Figure 4.17A). For the axial compression test, pellet strength for both control pellets and PVA-EPC-Peptides pellets peaked at 150℃ (Figure 4.17B). On the other hand, at a die temperature of 150℃, the radial and axial breaking stresses of the PVA-EPC-Peptides pellets were higher than those of the control pellets (Figures 4.17A and 4.17B). This indicated statistically a significant effect of the PVA-EPC-Peptides on pellet strength enhancement via introducing additional chemical binding among the torrefied wood particles. The formation of binding possibly happened at 125℃ to improve the axial pellet strength and peaked at 150℃ to increase the radial pellet strength. However, this speculation still needs to be proved in future experiments. Also, pellets started to break apart and became stuck in the die during densification at 200℃, indicating that a high die temperature (⩾175℃) may not be suitable for this study. The bulk density of industrial standard of ISO 17828 is higher than 600 kg/m^3 [188]. Assuming a 50% bulk porosity, the pellet density is estimated at about 1200 kg/m^3. The 150℃ densification thus meets the standard requirement.

4.3.1.3. The impact of holding pressure

As another essential factor for densification, the holding pressure represents the level of mechanical pressing force applied to form the shape of pellets in a single pelletizer. Thus, the holding pressure is expected to directly affect the pellets' properties, especially their density and strength. By varying the holding pressure (50-350 MPa), a study for the impact of holding pressure on pellet density and strength was performed.

It was observed in Figure 4.18 that the density of the pellets increased as the holding pressure increased. This can be explained by the decrease in pellet length due to the increasing compression force applied by the pelletizer. Moreover, the density required by ISO 17828 [188] has been achieved by all the pellets when the holding pressure was ⩾100 MPa. In addition, PVA-EPC-Peptides displayed an improvement in pellet density as compared to the pellets without a binder at the same holding pressure (Figure 4.18).

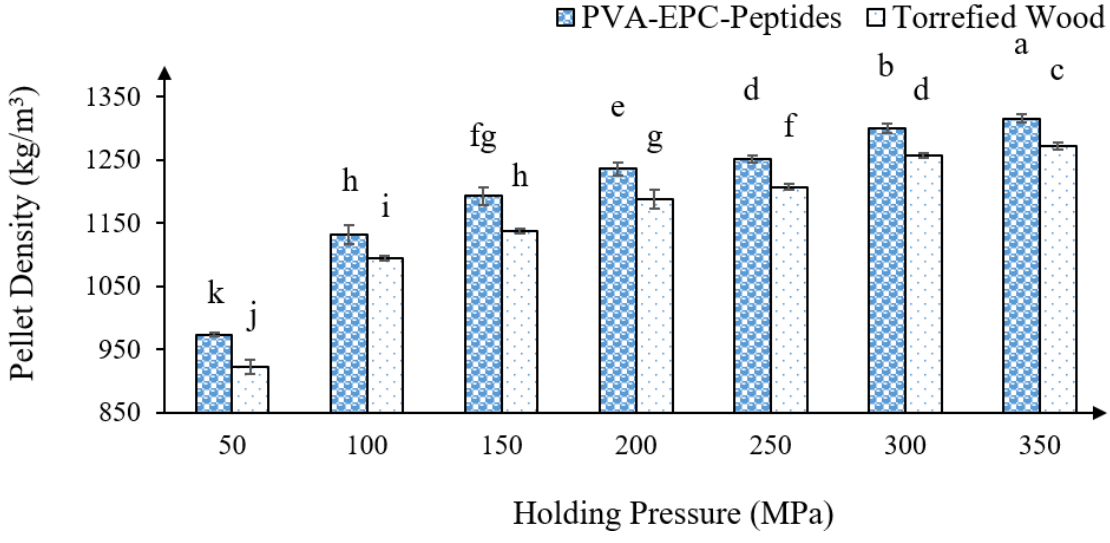


Figure 4.18. The density of the pellets produced at different holding pressure. Conditions were as follows: 150℃ die temperature, 1 min holding time and 3.0 wt.% moisture content of densification powders. PVA-EPC-Peptides refers to torrefied wood pellets made with the addition of 3.0 wt.% PVA-EPC-Peptides as a binder. Torrefied wood refers to pellets made from torrefied wood powders without a binder. Values in the figure represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

The breaking stresses of the pellets were also assessed, and showed that the radial (Figure 4.19A) and axial (Figure 4.19B) breaking stresses of the both pellets (i.e., PVA-EPC-Peptides pellets and torrefied wood pellets without a binder) increased with increasing holding pressure until the pressure reached 250 MPa, which was consistent with the results achieved by Verhoeff *et al.* [76]. In addition, the breaking stresses of the pellets were maintained at a constant level at a pressure range of 250-350 MPa. Therefore, 250 MPa was chosen as the holding pressure for subsequent studies. Moreover, regarding the function of PVA-EPC-Peptides, both the radial and axial breaking stresses of the pellets made with this binder were higher than that of the pellets made only with torrefied wood at the same holding pressure (Figure 4.19). These results further confirmed the strength enhancement of pellets generated using PVA-EPC-Peptides as a binder.

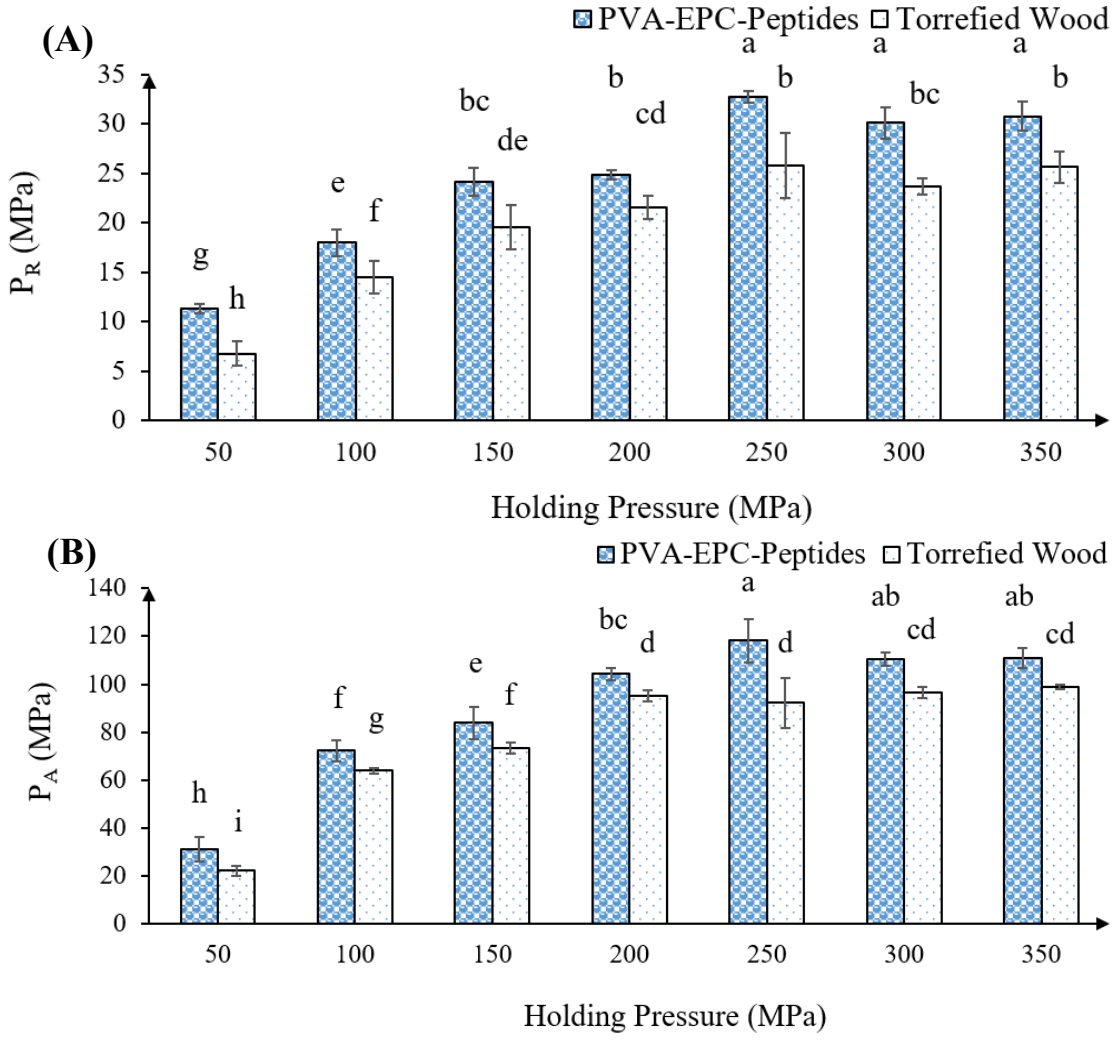


Figure 4.19. The radial (A) and axial (B) compression stresses of pellets produced at different holding pressure. Conditions are as follows: 150℃ die temperature, 1 min holding time and 3.0 wt.% moisture content of densification powders. PVA-EPC-Peptides refers to torrefied wood pellets made with the addition of 3.0 wt.% PVA-EPC-Peptides as a binder. Torrefied wood refers to pellets made from torrefied wood powders without a binder. Values

in the figure represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

4.3.1.4. The impact of moisture content and holding time

In a typical densification process, the small amount of water present in the densification powders will quickly evaporate due to the relatively high temperature of the die (150°C) applied for the process, particularly at higher holding times. Since the amount of the moisture retained in the pellets can directly affect the density and strength of pellets, tests to determine the impact of moisture content and holding time on pellet density (Figure 9) and pellet strength (Figure 10) were performed. As shown in Figure 4.20, moisture content and holding time exhibited no significant influence on pellet density. Regarding pellet strength, Figure 4.21 exhibited that holding time had no significant effects on the radial and axial breaking stresses of the resulting pellets. This suggests that densification powders formed a stable pellet within 1 min, regardless of whether binder was added. However, the moisture content of the powders exhibited a negative effect on the pellet strength, demonstrated by the decreasing breaking stress with increasing moisture content of the densification powders in Figure 4.21, which was also observed by Peng *et al.* in their pellet study [84]. Even though a high moisture content was related to the poor physical performance of the pellets, water still played an important role in feedstock softening and

lubrication during the densification process [189]. Moreover, the pellets made from powders with 2.0 wt.% moisture content displayed cracks during the study, indicating a decrease in pellet quality. Based on this, a 3.0 wt.% moisture content was chosen for the following studies. It is worth noting that an enhancement was observed in the density and breaking stress of PVA-EPC-Peptides pellets, when compared with that of the pellets without a binder under the same holding time and moisture content (Figure 4.21), consistent with the adhesive function of PVA-EPC-Peptides on the pellets properties as shown in previous sections.

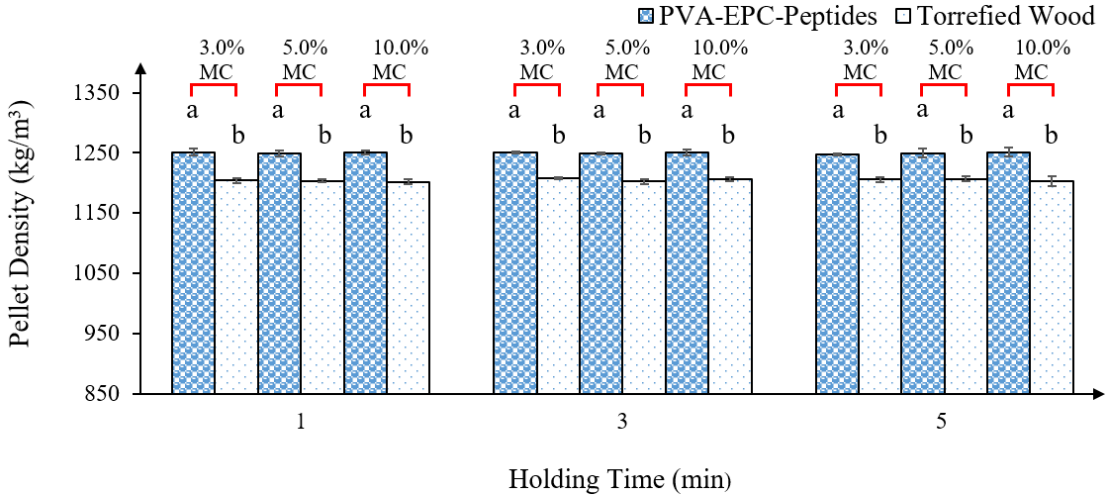


Figure 4.20. The density of the pellets produced under different moisture content and holding time. Conditions were as follows: 150°C die temperature and 250 MPa holding pressure. MC refers to the moisture content of the powders before densification. PVA-EPC-Peptides refers to torrefied wood pellets made with the addition of 3.0 wt.% PVA-EPC-Peptides as a binder. Torrefied wood refers to pellets made with torrefied wood powders without a binder. Values in the figure represent the average mean ± standard deviation of

triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

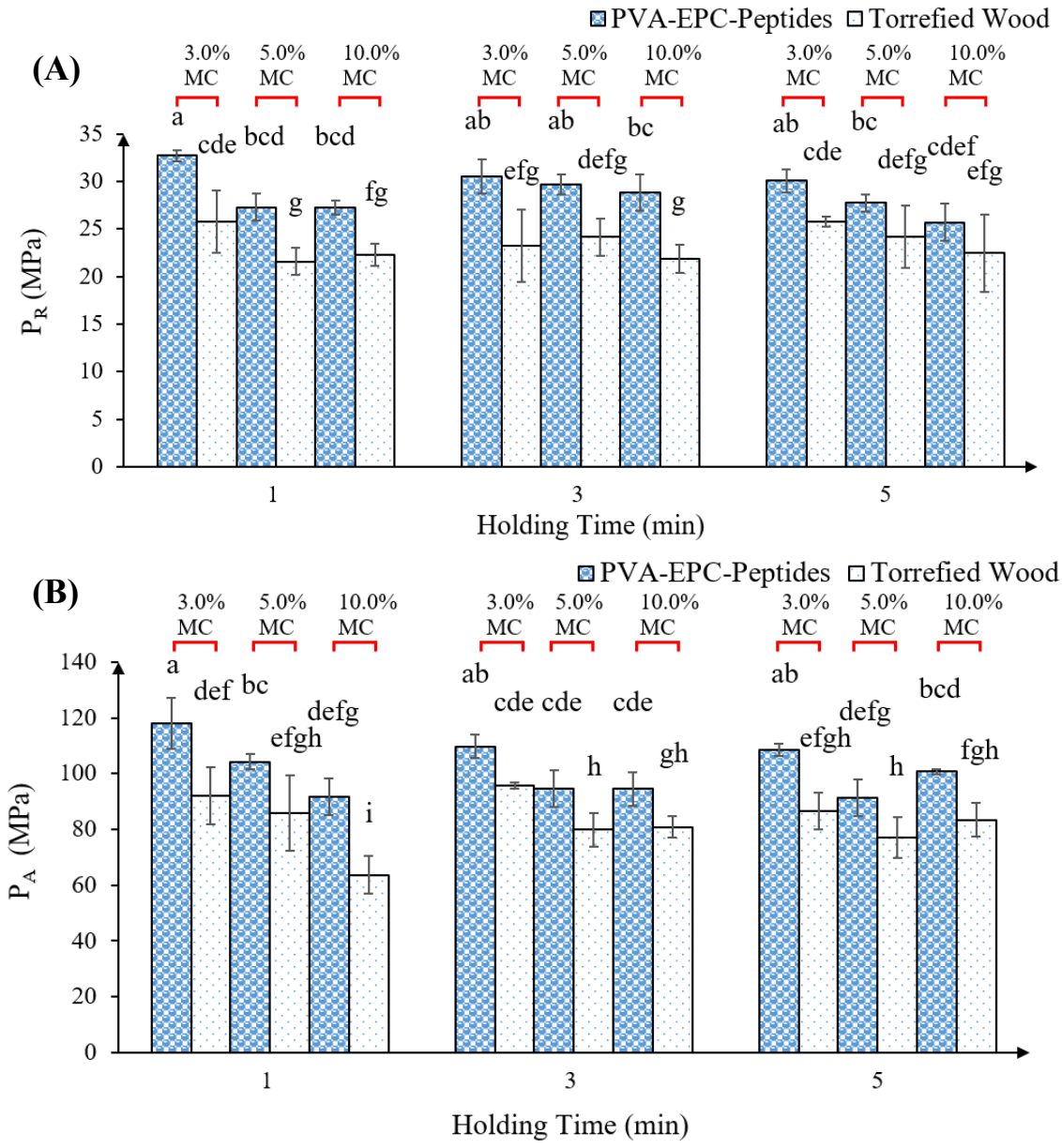


Figure 4.21. The radial (A) and axial (B) compression stresses of pellets produced under different moisture content and holding time. Conditions are as follows: 150°C die temperature and 250 MPa holding pressure. MC refers to the moisture content of the powders

before densification. PVA-EPC-Peptides refers to torrefied wood pellets made with the addition of 3.0 wt.% PVA-EPC-Peptides as a binder. Torrefied wood refers to pellets made from torrefied wood powders without a binder. Values in the figure represent the average mean ± standard deviation of triplicate experiments. The values that do not share the same letter indicate that they are significantly different ($p<0.05$).

4.3.2. Moisture up-take of torrefied wood pellets

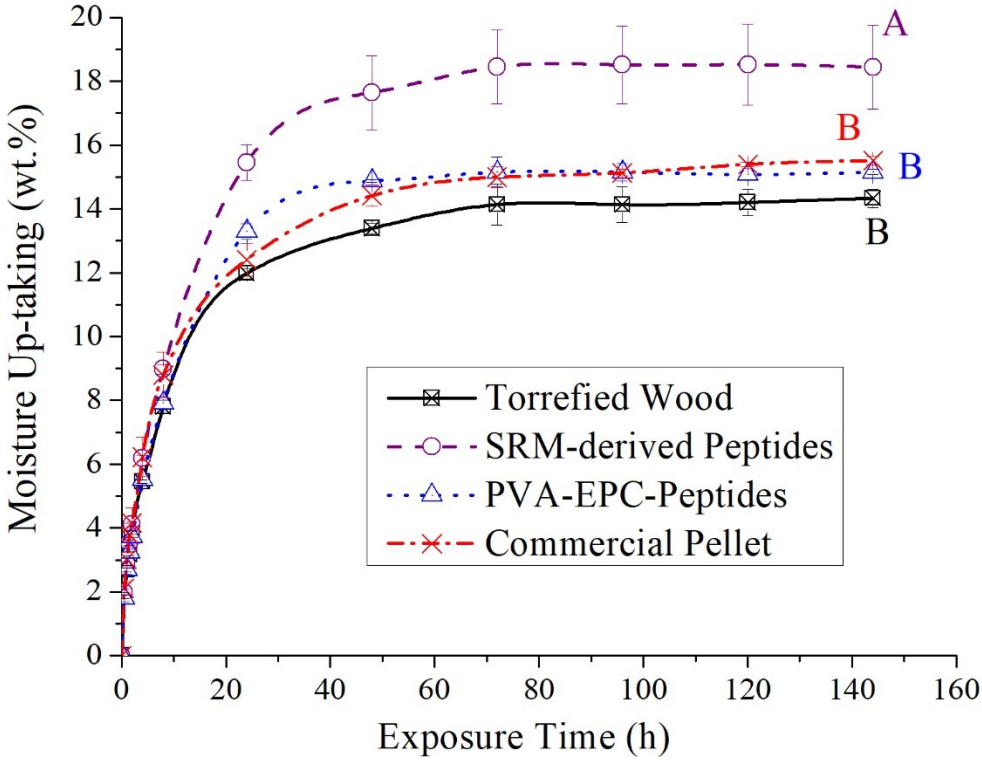


Figure 4.22. The moisture up-taking of pellets. A humidity chamber (90% relative humidity) at 30°C was used for this analysis. Torrefied wood refers to pellets produced without a binder. SRM-derived peptides and PVA-EPC-Peptides refer to pellets produced with 3.0 wt.%

unmodified peptides and PVA-EPC-Peptides, respectively. Commercial pellet refers to the pellets provided by our collaborators. Plotted values represent the average mean ± standard deviation of triplicate experiments. Values that do not share the same letter have significantly different ($p<0.05$) moisture up-taking values after 144 h.

The moisture absorbed by the pellets during transportation and storage is directly related to the storage life of the pellets. A high moisture up-take level or hygroscopicity may loosen the inner structure of the pellets, resulting in a decrease in pellet density and strength. Also, the combustion process requires a limited level of moisture to maximize the energy output (≤ 10 wt.% on wet basis, [94]). Therefore, a relatively low level of moisture up-take is essential for production of torrefied wood pellets. For this study, powders were pelletized under the conditions identified in the previous section.

Measuring the moisture up-take rate of each pellet revealed that it took more than 72 h for all the pellets to reach saturation, as shown in Figure 4.22. In addition, moisture up-take at saturation of the PVA-EPC-Peptides pellets remained at the same level as the pellets without a binder and the commercial pellets, indicating that the hygroscopicity of the pellets was not modified by PVA-EPC-Peptides. On the other hand, the water holding capacity of PVA-EPC-Peptides (1:1) was similar to unmodified peptides shown in section 4.2.4.3., indicating the similar hygroscopicity of PVA-EPC-Peptides (1:1) to that of unmodified peptides. Nevertheless, compared to pellets made with unmodified SRM-derived peptides, the

moisture up-take of the PVA-EPC-Peptides pellets was lower. The possible reason for unchanged PVA-EPC-Peptides pellets' moisture up-take is that the relatively high temperature and pressure provided by the pelletizer may lead to cross-linking between the hydroxyl groups and the carboxylic groups during the densification process. This would consume most of the hydrophilic groups in PVA-EPC-Peptides and could even provide some hydrophobic characteristics due to the formation of ester bonds. This possible feature helps PVA-EPC-Peptides pellets to retain the same level of moisture up-take as the pellets without a binder, which is desirable for the pellets industry. However, this speculation still needs to be proved in future experiments.

The moisture attached to the dish may cause the moisture up-taking value overestimated. But since all the pellets were test simultaneously in the same chamber with the same lid model, the amount of moisture attached by the dish for each pellet should be the same. Therefore, the results of the pellets can still be comparable to show the necessary difference in water absorption behavior.

4.3.3. Composition of torrefied wood pellets

4.3.3.1. Elemental analysis of torrefied wood pellets

Torrefied wood pellets made with and without binders (EPC-PVA-Peptides or SRM-derived peptides) were characterized with regards to carbon, hydrogen, nitrogen, and

sulfur (Table 4.2) to assess the effect of binders on the elemental content. The carbon and hydrogen content in the pellets generated with a binder, regardless of which binder, were statistically similar to those made without a binder (Table 4.2). Sulfur was not observed in any of the pellets. Conversely, the nitrogen content was increased in the pellets made with a binder since peptides, a nitrogen source, were introduced (Table 4.2). According to International Industrial wood pellets specification there are 3 different types of pellets: I1, I2 and I3 [188], where I1 is the highest grade of a pellet. Fuel specification standard of ISO 16948 for industrial torrefied wood pellets requires a maximum nitrogen content of 0.3 wt.% for I1 and I2 classes, and 0.6 wt.% for the I3 class [188]. Therefore, use of 3.0 wt.% PVA-EPC-Peptides as a binder resulted in pellets that met the nitrogen requirement for the production of the I3 class of torrefied wood pellets, making PVA-EPC-Peptides feasible as a binder for pellets production. Nevertheless, a further study is needed to reduce the nitrogen content of PVA-EPC-Peptides for the production of I1 and I2 pellets.

Table 4.2. C, H, N, S analysis of torrefied wood pellets made with/without binders formed at a 150℃ die temperature, 250 MPa holding pressure, 1 min holding time and 3.0 wt.% moisture content. ND refers to “not detected”. Values in the table represent the average mean

± standard deviation of triplicate experiments. The values that do not share the same letter are significantly different ($p<0.05$).

Binder	% C	% H	% N	% S
No binder	51.7 ^{a} ± 0.3	5.78 ^{h} ± 0.27	< 0.2 ^{z}	ND
SRM-derived peptides (3.0 wt.%)	51.9 ^{a} ± 0.2	5.91 ^{h} ± 0.39	0.71 ^{x} ± 0.10	ND
PVA-EPC-Peptides (3.0 wt.%)	51.6 ^{a} ± 0.2	5.77 ^{h} ± 0.22	0.48 ^{y} ± 0.08	ND

4.3.3.2. Chlorine analysis of PVA-EPC-Peptides and SRM-derived peptides

Chlorine content is strictly limited in wood pellet production. During the combustion of pellets, the relatively high chlorine content is associated with a risk of instrument corrosion due to HCl emission. Therefore, ISO 16994 specifies that the chlorine content limits for the I3 class of industrial torrefied wood pellets is ≤ 0.1 wt.% [188]. The chlorine content of the PVA-EPC-Peptides was determined through the ferrithiocyante method (EPA Method 325.2 [190]) and was quantified at 3.10 ± 0.1 wt.%. Since the chlorine content of the torrefied wood powders is negligible (~1.0×10^{-7} wt.%, [46]), addition of 3.0 wt.% PVA-EPC-Peptides led to a total of 0.09 ± 0.00 wt.% of chlorine in the pellets, which satisfied the chlorine standard of the I3 class of pellets. This also demonstrates the feasibility of PVA-EPC-Peptides to be used for pellets production. However, to produce I1

and I2 pellets, a further study is needed on reducing the chlorine content of PVA-EPC-Peptides via removing the salt in PVA-EPC-peptides.

4.3.4. Heating value of torrefied wood pellets

The heating value or calorific value is the most important property of pellet destined for fuel applications (ASTM D5865, [191]). It is directly related to the amount of energy released by the combustion of the pellets, which can be converted into other forms of energy, such as heating and electricity. The heating values of the pellets made with different binders were statistically the same, measured as 20.6 ± 0.1 MJ/kg for the pellets made with unmodified peptides (3.0 wt.%) and 20.5 ± 0.0 MJ/kg for the pellets made with PVA-EPC-Peptides (3.0 wt.%), respectively. Moreover, 20.6 ± 0.1 MJ/kg was measured as the heating value of the pellets without a binder. This indicated that neither PVA-EPC-Peptides nor unmodified SRM-derived peptides affected the heat generation of the resulting pellets. In addition, the heating values of all the pellets satisfy the ISO/TS 17225-8 fuel specification standards for the industrial pellets of all classes (I1-I3), which is $\geqslant$16.5 MJ/kg [188]. Moreover, the heating value of all the pellets was close to that of coal (20-32 MJ/kg, [80]), further confirming the potential of pellets as a substitute for coal.

According to the Airex Energy's report, the price of torrefied wood pellets is around US$ 9.8/GJ (US$ 0.20/kg) in Canada [192]. Also, based on the feedstock price provided in

section 4.2.4.5, the feedstock price of PVA-EPC-Peptides (1:1) would be US$ 1.7/kg. Since the heating value of the pellets made with 3.0 wt.% PVA-EPC-Peptides is 20.5 MJ/kg, the price of PVA-EPC-Peptides pellets can be estimated as US$ 11.9 kg/GJ, which is still closed to the US$ 9.8/GJ.

4.3.5. Summary

Section 4.3 examined the essential properties of pellets resulting from the addition of PVA-EPC-Peptides as a binder for the densification of torrefied wood powders, including density, compression strength, moisture content, elemental analysis and heating value measurement. Through a densification process performed under the preferred condition established in this study, pellets made with 3.0 wt.% PVA-EPC-Peptides statistically showed a significant improvement in density (around 4%) and strength (around 27% for radial strength and around 28% for axial strength) without compromising hydrophobicity as compared to pellets without a binder. Furthermore, these pellets met the requirements for the I3 class of industrial pellets with regards to density, nitrogen content, chlorine content and heating value. PVA-EPC-Peptides is a cross-linked product generated from SRM-derived peptides, which are associated with a relatively low feedstock price. This makes PVA-EPC-Peptides a promising torrefied wood pellet binder to improve the strength of pellets and provides further evidence that a binder can be obtained from proteinaceous waste. This study

not only expands the application of SRM-derived peptides, but may also help to make pellets a more attractive option than coal.

Chapter 5: Conclusions, outlook and further recommendations.

5.1. Conclusions and outlook

As a strategy for BSE containment, SRM has been separated and landfilled with great caution of Canada since 1997. This implementation strategy has succeeded in the defence of BSE, preventing new BSE infection cases from emerging recently. Yet meanwhile, this strategy has brought forward some problems to the government and industry. On one hand, landfilling is considered to be insufficient with the increasing SRM production. On the other hand, the rendering industry suffers from the additional costs brought by the SRM separation, transportation, storage and disposal. To address these problems, thermal hydrolysis was proposed by the government as a fast SRM disposal method and optimized in our lab, producing SRM-derived peptides, a promising biodegradable feedstock, for value-added applications. The potential of SRM-derived peptides was proved previously in our lab studies for the development of bio-plastic and wood panel adhesives, taking the first step to SRM bio-conversion. Herein, as follow-up steps, my Ph.D. project has extended the applications of SRM-derived peptides, creating values for the rendering industry. My project also contributes to growing the number of peptide modification strategies for the development of sustainable products.

Tackifiers, a wood fibre binder used in hydro-mulching processes, must be adhesive, water-soluble and biodegradable, mostly matching the properties of SRM-derived peptides. However, the binding strength and water-resistance of the SRM-derived peptides has not reached a necessary level to compete with the commercial tackifier. To address the issue of water-resistance, glutaraldehyde cross-linking is posted to add hydrophobicity via increasing the molecular weight of the products and consuming hydrophilic functional groups in peptides. Moreover, a methanol system is introduced as an innovation to improve the conversion rate, offering a new strategy for glutaraldehyde cross-linking. New characterization methods for tackifiers, texture analysis (4.1.2.1) and moisture holding analysis (4.1.2.2) have been developed in this thesis, making it possible to assess the binding property with less variance and evaluate the moisture holding capacity of tackifiers easily. These parameters can be compared and standardized against a reference for the development of tackifiers. Through these methods, the feasibility of this glutaraldehyde-peptides tackifier as a substitute for guar gum in hydro-mulching applications has thus been demonstrated.

During the development of a peptides based tackifier, a lower binding strength of the SRM-derived peptides was observed, when compared to psyllium and starch. Even though glutaraldehyde cross-linking successfully increases the hydrophobicity of the product, the binding strength of the product remained an issue due to the similar values obtained when compared to the unmodified peptides. By introducing cross-linking between

SRM-derived peptides and epoxidized PVA, a novel peptides-based tackifier was developed and characterized, successfully solving the binding capacity issue. Furthermore, as a newly synthesized polymer in this thesis, epoxidized PVA not only offers a way to address the low-reactivity of PVA, but also adds adjustable moisture holding capacity to the peptides via changing the molar ratio of the cross-linking reagents. The latter makes the customizing of tackifiers possible.

Following the outstanding binding strength observed through the cross-linking between epoxidized PVA and SRM-derived peptides for tackifier applications, the application of the cross-linking product was extended to other cellulosic materials. Torrefied wood, the main feedstock of torrefied wood pellets, gains the most attention of mine due to its great sustainability to be processed into the fuel. Moreover, torrefied wood shows an urgent need for a binder to increase the density and strength of the resulted pellets, while the properties of our peptides match the need well. To explore the feasibility of using SRM-derived peptides as a torrefied wood pellet binder, the product obtained by epoxidized PVA-peptides crosslinking was added during the densification (pelletization) of torrefied wood powders, as a part of this thesis. These studies demonstrated a statistically significant improvement in pellet density and compression strength without compromising the hydrophobicity, confirming the great potential of both SRM-derived peptides and epoxidized

PVA cross-linking strategy. It also offers a promising binder for the pellets production that can meet the needs of pellets industry.

In summary, the two different cross-linking strategies employed in this thesis have described a promising avenue to the commercialized bio-conversation of SRM-derived peptides into a novel and biodegradable binder for hydro-mulching or torrefied wood production. These competitive strategies can also be applied to technology platforms for the re-utilization of proteinaceous waste materials. Specifically, for the tackifier application, this thesis provides alternative answers to address the deficiencies of the SRM-derived peptides, producing promising products that potentially satisfy the hydro-mulching industry and rendering industry. For the torrefied wood application, this thesis contributes to the strength enhancement of torrefied wood pellets, that can combat against the breakage of pellets during storage and transportation. This definitely creates value for wood industry, especially in the competitive fuel market.

5.2. Future recommendations

As discussed in the previous section, the reaction kinetics of each glutaraldehyde cross-linking, PVA epoxidation, and epoxidized PVA cross-linking need to be studied in the future to achieve the optimized reaction factors. Also, a future characterization is needed to

verify the mechanism of the unchanged hygroscopicity of PVA-EPC-Peptides pellet shown in section 4.3.2.

Moreover, currently, the strategy mentioned in this thesis for SRM-derived peptides, either glutaraldehyde cross-linking or epoxidized PVA cross-linking, is studied for one aspect of improvement, i.e., glutaraldehyde cross-linking for water-resistance. In fact, both tackifiers and torrefied wood binders demand a great adhesion property for binding, along with considerable water-resistance for longevity usage/storage. A great balance is thus needed for these two competing properties via combining both of the cross-linking strategies. Due to the different solvent used in these strategies, it is difficult to approach the glutaraldehyde-epoxidized PVA-peptides product by mixing the reagents together for a one-step-reaction. Considering the relative low solubility of glutaraldehyde cross-linked peptides in aqueous system, it would be highly recommended to first reach a pre-product (PVA-EPC-Peptides) from epoxidized PVA cross-linking in aqueous conditions. The pre-product could be collected via spray drying after the first reaction and then cross-linked with glutaraldehyde in methanol. The final product could be separated from the methanol via rotary evaporation. In order to reach the desired balance in the product generated from this two-step-reaction, I would first set the epoxidized PVA-peptides cross-linking at the molar ratio of 0.5:1 (epoxy groups:primary amino groups in peptides). That is because a 0.5:1 molar ratio can still lead to an improvement of the binding property of the product, which

was demonstrated in section 4.2.4.1, while theoretically leaving half of the primary amino groups unreacted for the glutaraldehyde cross-linking. Also, the molar ratio of the following glutaraldehyde cross-linking would then be set at 1:1 and 2:1 (glutaraldehyde:theoretically remaining primary amino groups), to reach the same molar ratio used in secton 4.1.1. Briefly, the molar ratio of the whole process would set at 0.5:1:0.5 and 0.5:1:1 (epoxy groups:primary amino groups in peptides:glutaraldehyde). Other conditions (temperature, reaction time and pH) for each reaction would be set the same as the conditions recommended in section 4.1.1 and section 4.2.3. On the other hand, it may be worth mixing two cross-linked products directly at a 1:1 mass ratio, for tackifier applications and torrefied wood pellet binder applications. It would be of great benefit to approach the combination of two cross-linking strategies to meet the demands of related industries. This combination would also promote the development of a novel conversion platforms and demonstrate the feasibility of future commercialization for this peptides based product.

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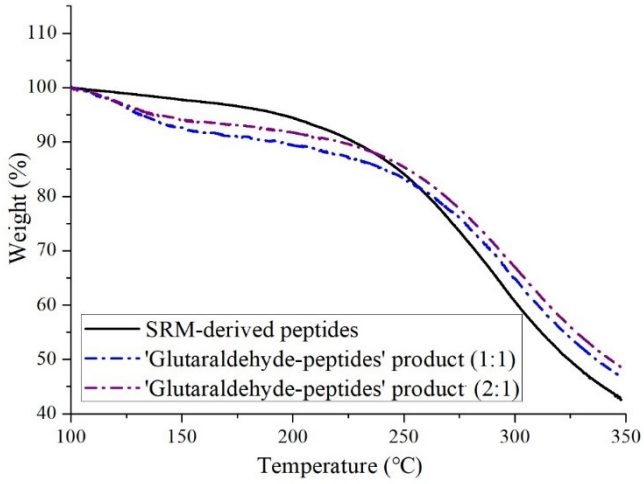
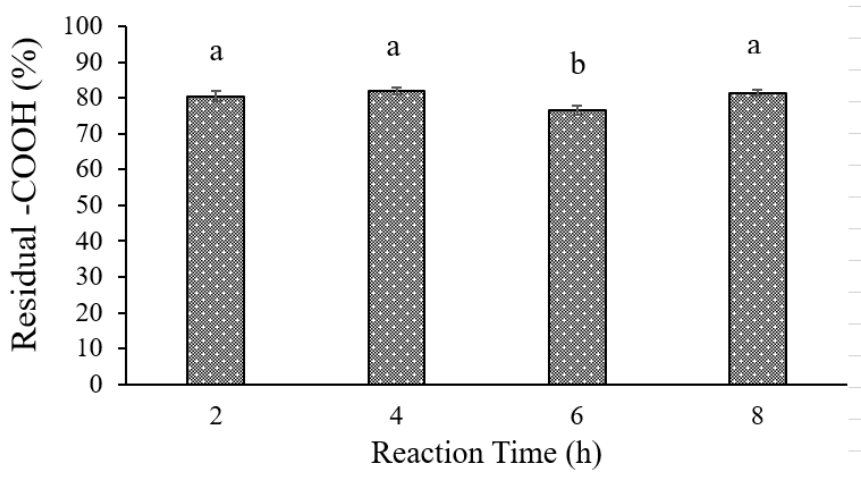
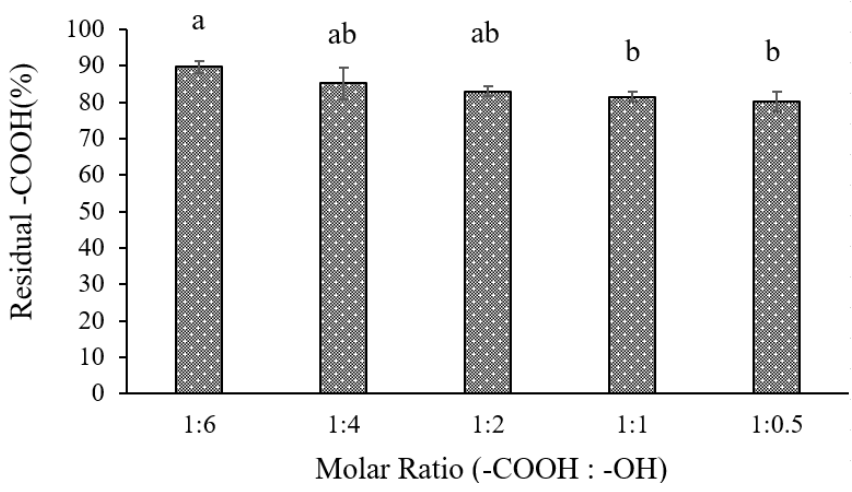
Appendix



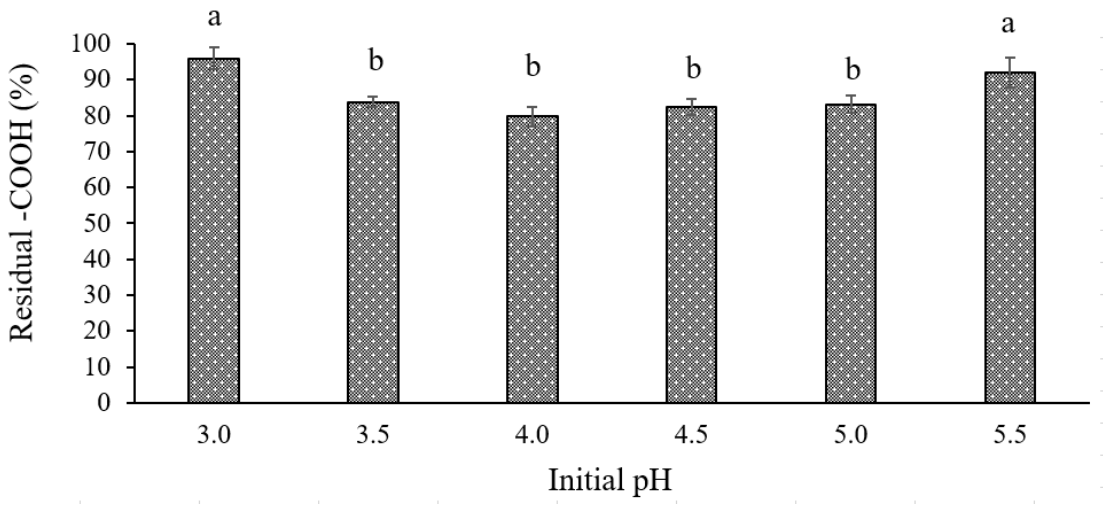
Appendix 1. TGA analysis of products obtained through cross-linking of SRM-derived peptides with glutaraldehyde from 100℃ to 350℃, at a heating rate of 5°C/ min.1:1 and 2:1 were the molar ratio of cross-linking reactants (glutaraldehyde: primary amines in SRM-derived peptides). Triplicate samples were applied for TGA analysis and all the results were reproduced to 5% error or better. Since the replicates were similar, only one run is shown in the figure above.



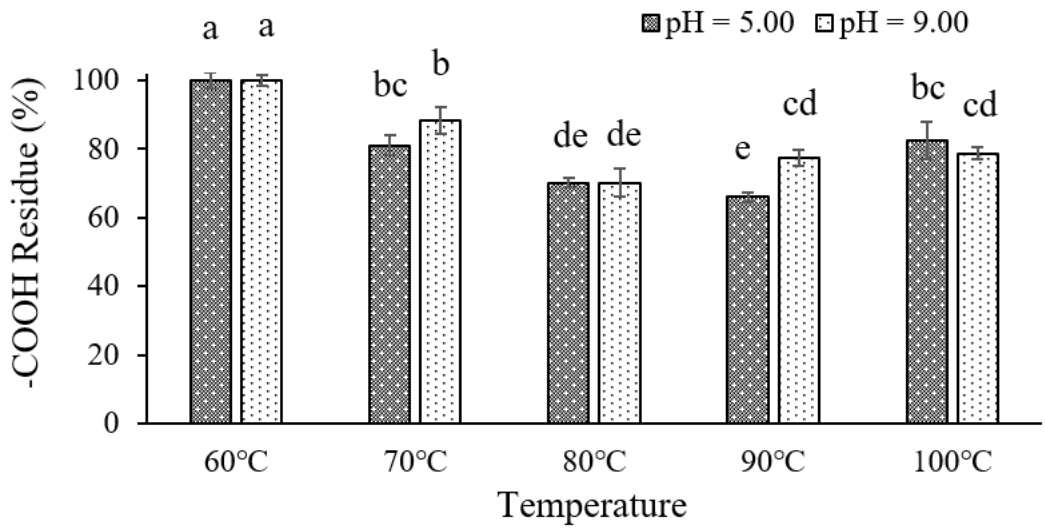
Appendix 2. The effect of reaction time on the carboxylic groups present in the PVA cross-linked products generated at 100°C at a 1:1 molar ratio (-COOH in SRM derived peptides: -OH in PVA) in water (initial pH 5.0). Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



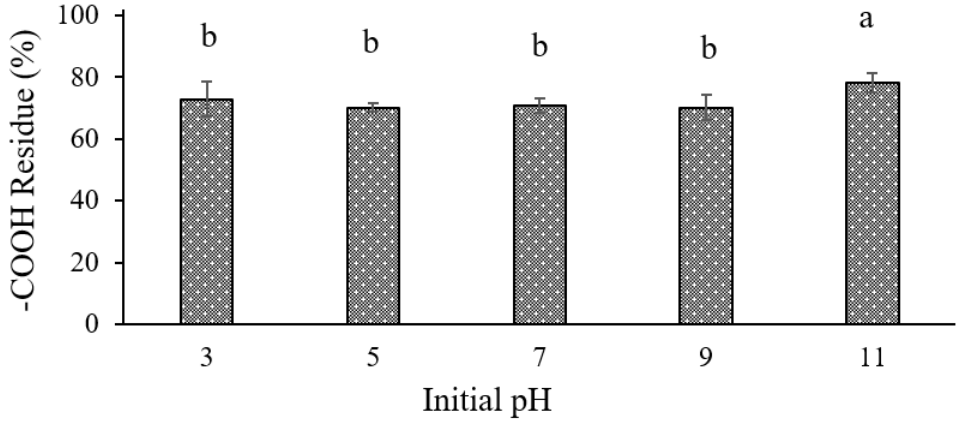
Appendix 3. The effect of molar ratio (-COOH in SRM derived peptides: -OH in PVA) on the carboxylic groups present in the PVA cross-linked products generated at 100°C for 2 h in water (initial pH 5.0). Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



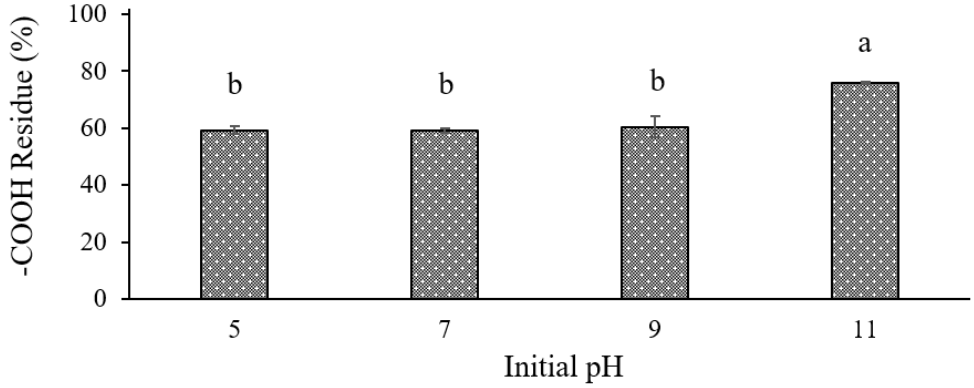
Appendix 4. The effect of initial pH on the carboxylic groups present in the PVA cross-linked products generated at 100°C at a 1:1 molar ratio (-COOH in SRM derived peptides: -OH in PVA) for 2 h in water. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



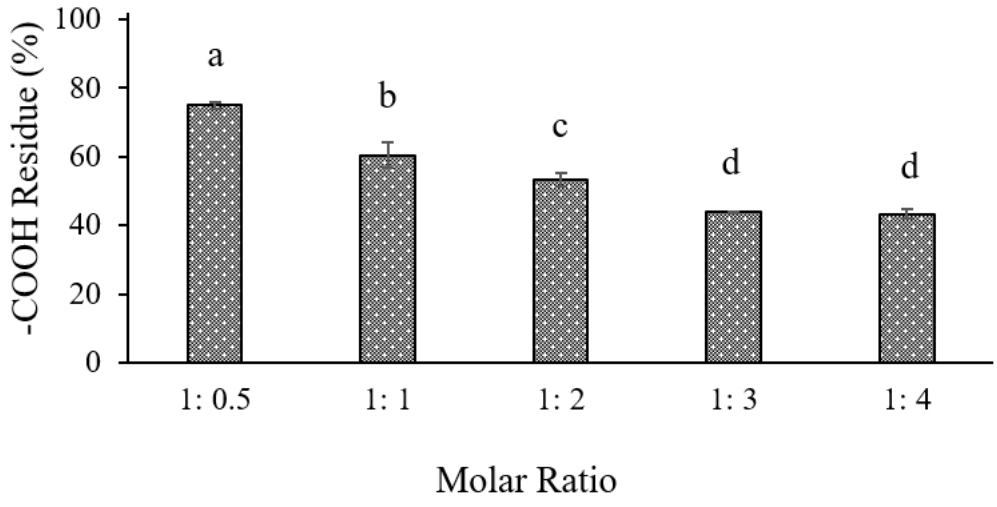
Appendix 5. The effect of temperature and initial pH on the carboxylic groups present in the epoxidized PVA cross-linked products generated at a 1:1 molar ratio (-COOH in SRM derived peptides: epoxy groups in epoxidized PVA) for 20 h in water. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



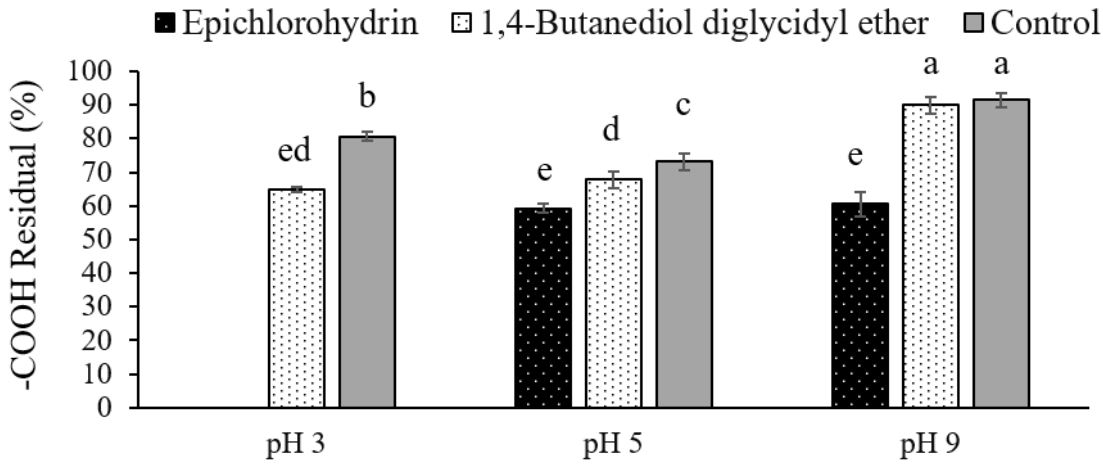
Appendix 6. The effect of initial pH on the carboxylic groups present in the epoxidized PVA cross-linked products generated at 80℃ at a 1:1 molar ratio (-COOH in SRM derived peptides: epoxy groups in epoxidized PVA) for 20 h in water. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



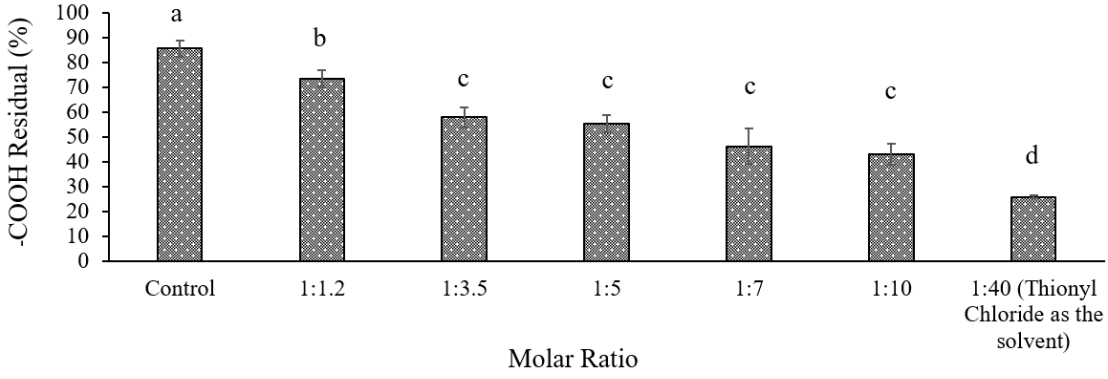
Appendix 7. The effect of initial pH on the carboxylic groups present in the epichlorohydrin cross-linked products generated at 80°C at a 1:1 molar ratio (-COOH in SRM derived peptides: epoxy groups in epichlorohydrin) for 20 h in water. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



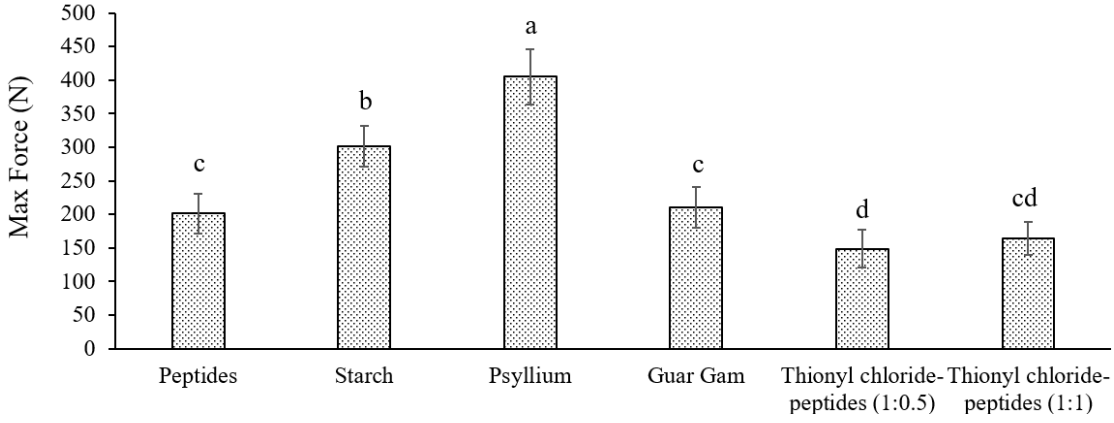
Appendix 8. The effect of molar ratio (-COOH in SRM derived peptides: epoxy groups in epichlorohydrin) on the carboxylic groups present in the epichlorohydrin cross-linked products generated at 80°C at pH 5.0 for 20 h in water. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



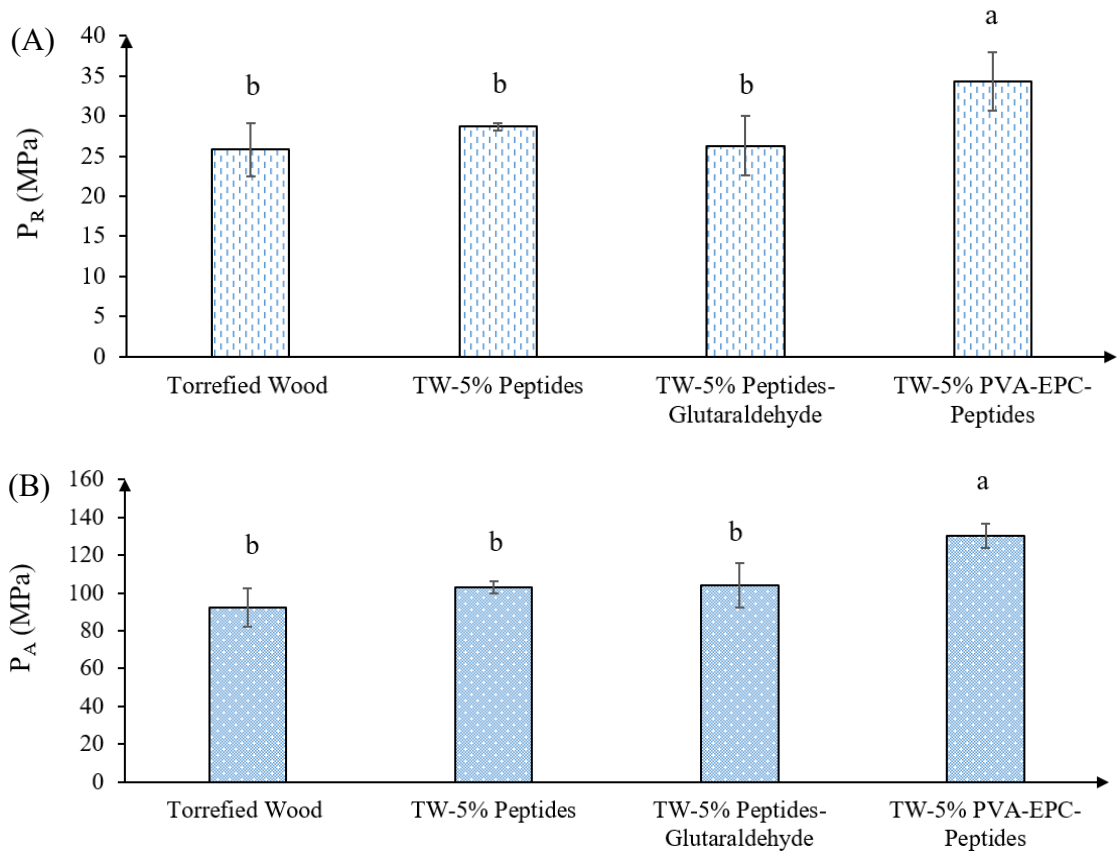
Appendix 9. The effect of initial pH on the carboxylic groups present in the products using different cross-linkers (epichlorohydrin and 1,4-butanediol diglycidyl ether) generated at 80°C at 1:1 molar ratio (-COOH in SRM derived peptides: epoxy groups in the reagents) for 20 h in water. Control refers to the reaction with no cross-linkers. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



Appendix 10. The effect of molar ratio (-COOH in SRM derived peptides: thionyl chloride) on the carboxylic groups present in the thionyl chloride modified products generated at 60°C for 3 h in hexane. Control refers to the reaction with no thionyl chloride. Values represent the average mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



Appendix 11. The compression strength of pucks made with peptides, thionyl chloride-peptides cross-linked products, and commercial tackifiers. Pucks generated using Mulchmax 100 and solutions of the tackifiers indicated above were subjected to compression testing using a TA.XT*Plus* texture analyzer to determine the breaking force (N). 0.5:1 and 1:1 represent cross-linked products generated using thionyl chloride at 60℃ for 3 h using a molar ratio of 1:0.5 and 1:1 (carboxylic groups in SRM-derived peptides: thionyl chloride), respectively. Peptides represent testing performed using unmodified SRM-derived peptides. The values shown represent the mean ± standard deviation of triplicate experiments. Tukey's HSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).



Appendix 12. The radial (A) and axial (B) compression stresses of torrefied wood pellets made with or without a binder. Pelletization was performed at a die temperature of 150℃, 250 MPa holding pressure, 1 min holding time and 3.0 wt.% moisture content of densification particles. TW was the abbreviation of torrefied wood. PVA-EPC-Peptides represented the product obtained from the cross-linking between SRM-derived peptides and epoxidized PVA. TW-5% Peptides refer to the torrefied wood pellets with the addition of 5.0 wt.% SRM-derived peptides. TW-5.0% Peptides-Glutaraldehyde refer to the torrefied wood pellets with the addition of 5.0 wt.% glutaraldehyde-peptides cross-linked products. TW-5% PVA-EPC-Peptides refers to the torrefied wood pellets made with an

addition of 5.0 wt.% PVA-EPC-Peptides. Values in the figure represent the average mean ± standard deviation of triplicate experiments. Fisher's LSD method is used for the statistical analysis. Values that do not share the same letter are significantly different ($p<0.05$).