Improving material properties of canola protein films using modified nanocrystalline cellulose for food packaging applications

by

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Abstract

The demand for biopolymer-based food packaging increases day by day with the increasing concerns over environmental and economic sustainability. Agricultural by-products such as canola meals are becoming more popular among researchers due to their high availability at a low cost. Canola protein isolate derived from the canola meal has already proven its film-forming abilities desirable for food packaging applications. However, compared to petroleum-based plastic food packaging, intact canola protein-based films possess poor mechanical properties and high hydrophilicity that limit their use in food packaging. Nanocrystalline cellulose (NCC) is an excellent reinforcing material that enhances the properties of biopolymers. However, their hydrophilicity and agglomeration in the polymer matrix limit their excellent properties. The objective of the first study of the thesis was to modify the NCC using the TEMPO (2,2,6,6-Tetramethylpiperidine-1-oxyl) method to obtain TEMPO modified NCC (TM-NCC) and fabricate the films using the solvent casting method at different levels of unmodified NCC (U-NCC) or TM-NCC (0, 1, 3, 5% w/w of protein). The second study aimed at a different modification where oleic acid (OA) was used to modify NCC and obtain OA modified NCC (OA-NCC). Then the films were fabricated using U-NCC or OA-NCC (0, 1, 3, 5, 7, 9% w/w of protein). In both studies, the effect of modified NCC and U-NCC on the films' mechanical, barrier, and thermal properties were evaluated. TM-NCC significantly increased the films' tensile strength, resulting in the highest value (8.36 ± 0.85 MPa) for TM-NCC 5% films. Moreover, both U-NCC and TM-NCC enhanced the films' water barrier and thermal properties compared to control. In the second study, OA-NCC significantly enhanced mechanical, barrier, and thermal properties compared to control and U-NCC resulting maximum of 3.44 ± 0.32 MPa for OA-NCC 3% films and a minimum of 0.054 ± 0.004 g mm/m^2 KPa h water vapor permeability for OA-NCC 9% films. Overall, modification of NCC enhanced the properties of canola protein films due to the enhanced interactions and compatibility of the nanomaterials in the polymer network. This study provides a new platform of value addition to the canola industry's main byproduct.

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Dedication

This thesis is dedicated

To my beloved father and mother,

Thilakarathna Dissanayake and Shantha Dissanayake

for their endless love and unimaginable dedication to making me who I am today…

to my husband, Rivindu

for his love and patience with me…

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List of abbreviations

CNF- Cellulose nanofibers

CPI- Canola protein isolate

FTIR- Fourier transformed infrared spectroscopy

NC- Nanocellulose

NCC-Nanocrystalline cellulose

OA- Oleic acid

OA-NCC- Oleic acid-modified nanocrystalline cellulose

PDCAAS- Protein digestibility corrected amino acid score

SEM- Scanning electron microscopy

SPI- Soy protein isolate

TEMPO- 2,2,6,6- Tetramethylpiperidine-1-oxyl

TGA- Thermogravimetric analysis

TM-NCC- TEMPO modified nanocrystalline cellulose

XRD- X-ray diffraction

Chapter 1: Introduction

The role of food packaging in food safety and food quality is inimitable. Food packaging contributes to the prevention of food waste during storage and transport. On the other hand, food packaging protects food by acting as a chemical and physical barrier against microbial contaminations, light, temperature, moisture, odors, and physical damage (Han et al., 2018). In terms of production cost and performance, petroleum-based plastics have acquired great interest from the package processing industries. They can be introduced as versatile materials with excellent thermal, tensile, and moisture barrier properties (Asgher et al., 2020). However, the negative environmental impact of petroleum-based food packaging suppresses its superior qualities. Around 30% of plastic packaging is not available for recycling or reuse due to practical difficulties in sorting and contaminating organic materials or chemicals. Therefore, the fate of most of the plastic food packaging ends up in the oceans and open ecosystems creating global plastic pollution (Dilkes-Hoffman et al., 2018).

With that concern, there is an increasing demand for sustainable food packaging materials developed from bio-polymers (Qasim et al., 2020). As the world's second-largest produced oilseed crop, canola produces a considerable amount of by-product (meal) after the oil extraction process. The current use of canola meal is limited to the animal feed industry as its use in direct food applications is limited by the antinutritive factors present in the canola (Bandara et al., 2018; Fetzer et al., 2020). Canola protein's thermoplastic properties and film-forming ability were already proven by many studies indicating the possibility of its use in future packaging applications (Manamperi et al., 2010; Zhang et al., 2016). However, similar to most other proteins, canola also shows high hydrophilic nature and poor mechanical properties. Several approaches have been taken to overcome these issues in canola protein-based packaging developments, including the use of plasticizers, blending with other polymers, the use of nanomaterials, and the use of crosslinking agents (Chang & Nickerson, 2014, 2015; He et al., 2019; Li et al., 2017; Mao et al., 2013; Osorio-Ruiz et al., 2019; Shi & Dumont, 2014; Zhang et al., 2019). Even though these studies were able to improve some properties of films up to a satisfactory level, at the same time, another film property was compromised. Therefore, there is a need to improve film properties while avoiding the negative impact on existing properties.

Nanocrystalline cellulose (NCC) plays a vital role as an additive in food packaging. NCC has the potential to increase the tensile, barrier, thermal, antimicrobial, and antioxidant properties of food packaging materials (Zhang, Zhang, Cao, & Jiang, 2021). Despite these functionalities, NCC faces some drawbacks, such as high hydrophilicity, poor exfoliation in the polymer matrix, and agglomeration due to abundant surface hydroxyl groups. As a solution for these drawbacks, several strategies are used to modify the surface of NCC and thereby reducing the hydrophilicity and promoting proper exfoliation that ultimately helps to show desirable functions of NCC in film formulations (Ilyas et al., 2019). Esterification with fatty acids, substituting hydroxyl groups with carboxylic groups, covalent grafting, and ionic complexations are some recently used surface modifying techniques (Liang et al., 2020). TEMPO (2,2,6,6- Tetramethylpiperidine-1-oxyl) mediated modification of NCC is becoming popular due to its selective and fast oxidation. This modification converts the surface hydroxyl groups of NCC into carboxylic groups to increase the surface charge and thereby enhance the proper exfoliation of NCC in the polymer matrix (Da Silva Perez et al., 2003; Mahendra et al., 2020). On the other hand, introducing long-chain hydrophobic hydrocarbon structures onto the surface of NCC enhances the hydrophobic properties of the material that can be used to improve the hydrophobic properties of the prepared films (Wei et al., 2017). Unfortunately, the literature based on these types of modified NCC in protein-based food packaging is limited.

We hypothesized that the use of modified NCC will improve the properties of the films compared to the films with unmodified (U-NCC) and films without NCC (control). The objectives of the overall thesis were to modify the NCC using two methods (TEMPO modification and oleic acid (OA) modification) in two different studies, fabrication of canola protein films using modified NCC and U-NCC, and characterization of prepared films to evaluate mechanical, barrier, and thermal properties of the films. The thesis results would be a novel addition to the Canadian canola industry as it would provide a platform to add value to its by-product by successfully using it in food packaging developments.

Chapter 2: Literature Review

2.1. Canola protein

2.1.1. History and current status of canola industry

Canola (*Brassica napus*) was developed in Canada, and the story of canola began in 1942. In 1978, the trademark "canola" was registered in Canada (Manitoba Canola Growers, 2021). Compared to rapeseed, canola is different in reduced glucosinolate and erucic acid content. Canola oil contains <2% of erucic acid, and canola meal contains <30 µmol/g of glucosinolates (The Canola Council of Canada, 2021). Today, canola is the world's second-largest oilseed crop after soybean, where the rapeseed production was 72 million metric tons for 2020/2021. From that, 19 million metric tons have been produced in Canada, becoming the highest-produced country (World's soybean production: 366 million metric tons). Due to Canada's high canola production, 6 million metric tons of canola meal have been produced, exporting more than 80% of that (5 million metric tons) (USDA, 2021). However, the current use of canola meals has been limited to the animal feed industry, indicating a high meal availability for value-added products (Bandara et al., 2018).

2.1.2. Defatting and extraction of canola protein

Apart from the conventional defatting of canola seeds, several techniques have been reported, such as cold-pressing, alternate solvent defatting (supercritical CO_2), microwave-/ ultrasound-assisted defatting, and solvent-free defatting. Defatting methods significantly contribute to the functional properties of extracted canola protein from the defatted meal. Seed cleaning, pre-conditioning to obtain the required moisture content, and flaking using roller mills to physically break the seed coat and cell walls to expel the oil bodies are initial steps of the defatting process (Fetzer et al., 2020).

The conventional defatting method involves the inactivation of enzymes to avoid the formation of sulfur-containing compounds that are not desirable for the final product by heating up to 85-95 °C for 30-40 minutes. The best example of this is hydrolyzing glucosinolates into non-desired compounds by myrosinase. Moreover, the inactivation of enzymes such as lipases helps simplify the process by agglomerating the proteins and oils (Spielmeyer et al., 2009). Direct solvent extraction of oil is not optimum for canola due to the high oil content present in the seed (50%).

Therefore, full pressing or pre-pressing is followed by solvent extraction to extract the oil from seeds. After solvent extraction, the obtained meal is toasted at around 130 °C to evaporate residual hexane. Therefore, the protein quality is highly compromised due to the harsh conditions used in conventional defatting (Fetzer et al., 2020; Galves et al., 2019; Sun et al., 2008). On the other hand, the roasting of canola seeds up to 160 °C increases the canolol (4-Vinylsyringol) content of canola oil by 120 times compared to unroasted seeds by decarboxylation of sinapic acid. The strong antioxidant activity of canolol increases the value of the final food product by preventing lipid oxidation and thereby increasing shelf-life (Spielmeyer et al., 2009).

Cold-pressing canola seeds involve only mechanical treatments where the temperature is not elevated beyond 40 °C and produces a high-quality meal with strong colour, taste, and odour. However, the meal contains 15%-18% of higher oil content than traditional defatting that requires further oil removal through solvent extraction (Fetzer et al., 2020; The Canola Council of Canada, 2021). In addition, the Cold-press technique minimizes the protein denaturation and maintains the functional properties of proteins, such as solubility, which is destructed in traditional defatting (Fetzer et al., 2018).

Extraction of oil using supercritical fluids such as CO_2 at temperature and pressures above critical levels is tested to use in industrial oil extraction. The critical temperature and pressure of CO_2 are 31 °C and 74 bars which are not harsh for the final product. Moreover, supercritical fluids show better diffusivity compared to liquids. As a result of high diffusivity and low viscosity, supercritical fluids can easily penetrate solutes and different matrixes. All these properties of supercritical fluids can be adjusted by changing pressure and temperature in the system (Khaw et al., 2017). This method is becoming more popular due to the CO_2's non-toxic, non-flammable, non-corrosive, and inert behavior. Moreover, it is a cheap ingredient to use at the industrial level. CO_2 can be easily removed from the meal by decompressing without using any solvent removal process (Gaber et al., 2018). Several studies have reported this method is used in canola oil extraction (Cvjetko et al., 2012; Koubaa et al., 2016; Pederssetti et al., 2011).

Microwave- or ultrasound treatments are used in solvent extraction methods to obtain a high oil yield and reduce solvent and extraction time. Protein yield from the extracted meal is also increased due to the reduction in extraction time and temperature. Further, oxidative stability and the quality of oil and meal is enhanced (Fetzer et al., 2020). Solvent-free extraction methods are becoming popular due to the use of solvents' (i.e. hexane) adverse environmental effects, flammability, cost,

and negative health effects. However, ethanol is introduced as a green bio solvent where it also extracts compounds such as tocopherol, which increases the oil quality. On the other hand, extracting insoluble compounds such as pigments, sugar, and phosphatides into oil is an issue with ethanol-based defatting (Sánchez et al., 2018). In that case, aqueous enzymatic extraction provides a novel platform for extracting oil from oil seeds. Oil is separated from the other components using a combination of enzymes, including carbohydrates and proteases. However, a thin layer of an emulsion between oil and the aqueous phase and an upper layer of oil-bound protein hydrolysate in the sediment is retained after centrifugation. The residual oil and protein from emulsion and precipitate should be recovered by washing and demulsification steps (Zhang et al., 2007). However, the need for centrifugation procedures and demulsification processes (boiling, freezing and thawing, phase inversion) limits the economic feasibility of enzymatic extraction (Fetzer et al., 2020; Zhang et al., 2007).

Once the oil is extracted from the seed, the resultant meal is used for protein extraction. The most common method of extracting canola protein is alkaline extraction, followed by isoelectric precipitation. Canola's two major proteins, cruciferin and napin, show their isoelectric points at pH 7.25 and 10-11, respectively (Akbari & Wu, 2015; Chmielewska et al., 2020). Perera et al. (2016) reported low molecular weight proteins are soluble in acidic pH between 1.5 and 4.5, while large molecular weight proteins are soluble in basic pH between 5.5-10.5. However, due to the presence of other proteins in the canola, the range of isoelectric points runs from pH 4-11, where sequential precipitation is required to precipitate different proteins (Manamperi et al., 2010). Depending on the canola variety, the major isoelectric point of canola proteins is shown between pH 3.5-5.5 and precipitate at acidic or neutral pH conditions (Manamperi et al., 2011). Akbari and Wu (2015) isolated napin and cruciferin by an integrated method where acidic washing at pH 4 (separate napin), alkaline extraction/ washing of the precipitate at 12.5 pH, and acidic precipitation steps (remaining napin in the extract and cruciferin in the precipitate) involved. Zhang et al. (2020) evaluated the effect of pH on the components of protein isolate obtained through alkaline extraction and acid precipitation. The highest protein yield with an intact protein structure was obtained at alkaline extraction at pH 9 and acid precipitation at pH 4.5. The lowest content of anti-nutritional factors was reported at pH 4.5 where the decrease in protein charge and structure stabilization of protein results in weakening electrostatic interactions between protein and positively charged glucosinolates and phytic acid. Moreover, H bond, hydrophobic, ionic, and

covalent interactions were decreased between protein and sinapine. Several studies have been published based on the extraction of canola protein isolate by alkaline extraction and acidic prepitation (Akbari & Wu, 2015; Aluko & Mcintosh, 2001; Manamperi et al., 2010).

2.1.3. Protein and amino acid (AA) profile of canola and functional properties

After oil extraction, a canola meal contains 36-40% of protein (DWB, moisture 12%). The presence of high protein content, residual oil, and fiber make canola meal ideal for animal feed production, and currently, the meal is used in dairy cow rations and feeds of broilers, layers, and fish (Wanasundara et al., 2016). Different proteins are present in canola, such as storage proteins, oil body proteins, trypsin inhibitors, and lipid transfer proteins. Cruciferin (globulin type) and napin (albumin) are the two predominant proteins found in canola seed at the content of 60% and 20%, respectively that come under storage proteins. Oleosin, caleosin, and steroleosin are oil body proteins present in the canola (Perera et al., 2016). Cruciferin (11S or 12S, 300-350 KDa) contains six subunits/protomers where protomers exist as two trimer units. Non-covalent bonds such as H bonds, electrostatic interactions, hydrophobic interactions, H bonded salt bridges, and van der Walls interactions contribute to assembling two trimers to form a hexamer. Each protomer contains two polypeptides, α chain and β chain that are linked through disulfide bonds. Inter-chain disulfide bonds between α and β polypeptide chains also play an important role in hexamer assembly (Wanasundara et al., 2016). Napin (1.7-2S, 12.5-14.5 KDa) structure is formed by one large and one small polypeptide chain linked by two inter-chain disulfide bonds. Moreover, two intra-chain disulfide bonds are formed between the cysteine residues of the large polypeptide chain (Perera et al., 2016).

Canola protein possesses a well-balanced AA profile rich with sulfur-containing AAs due to the high amount of cysteine present in the napin (Campbell et al., 2016). Table 1 shows the AA composition of canola protein isolate (CPI) reported by a few studies, AA composition of soy protein isolate (SPI), and AA requirement recommended by FAO/WHO/UNU (2007) for adults. CPI and SPI show almost similar amino acid profiles while meeting the recommended levels of FAO/WHO/UNU (2007). Protein digestibility corrected amino acid score (PDCAAS) value of CPI was reported as 0.86, while it was 0.84 for SPI in a study conducted to evaluate the nutritional value of canola protein and soy protein (Fleddermann et al., 2013). According to the study's results, the true N digestibility of CPI and SPI were 93.3 ± 1.9% and 94.9 ± 0.4%, respectively

(Fleddermann et al., 2013). Moreover, several studies have reported that the PDCAAS value of canola is at least more than 0.75 (Chmielewska et al., 2020; Hertzler et al., 2020). Moreover, commercially available canola protein products such as >80% napin rich Supertein™, 80% cruciferin rich Puratein™, 60-65% of globulin and 30-35% albumin contained Isolexx™, and 35-60% napin and 40-65% cruciferin contained canolaPRO™ also contain the required amounts of essential AAs (Chmielewska et al., 2020).

Table 1. Amino acid percentage of CPI, SPI, and FAO/WHO/UNU recommendation

Protein source	**CPI** (Shi & Dumont, 2014)	**CPI** (Fleddermann et al., 2013)	**CPI** (Alashi et al., 2014)	**SPI** (Fleddermann et al., 2013)	**Amino acid requirement by FAO/WHO/UNU** (FAO/WHO/UNU, 2007)
Essential AA					
Histidine	2.45	2.53 ± 0.03	3.6	2.44 ± 0.06	1.5
Isoleucine	3.03	3.33 ± 0.05	4.3	4.04 ± 0.04	3.0
Leucine	7.52	6.96 ± 0.29	7.9	7.68 ± 0.39	5.9
Lysine	4.74	4.78 ± 0.12	5.4	6.43 ± 0.21	4.5
Methionine	1.91	1.57 ± 0.17	1.9	1.17 ± 0.03	1.6
Cysteine	0.63	1.78 ± 0.27	0.7	1.13 ± 0.02	0.6
Phenylalanine	4.36	3.82 ± 0.03	5.0	4.99 ± 0.14	3.0 (Phenylalanine
Tyrosine	3.02	2.68 ± 0.03	3.9	3.73 ± 0.09	+ tyrosine)
Threonine	4.13	4.37 ± 0.12	5.2	3.98 ± 0.05	2.3
Valine	3.77	4.12 ± 0.01	5.8	4.16 ± 0.09	0.6
Tryptophan	-	-	0.9	-	-
Non-essential AA					
Alanine	4.66	4.23 ± 0.01	4.8	4.23 ± 0.13	-
Arginine	6.32	6.79 ± 0.19	7.9	7.36 ± 0.28	-
ASX*	8.32	8.34 ± 0.44	9.7	11.6 ± 0.6	-
GLX**	17.28	19.1 ± 0.9	15.7	19.4 ± 1.1	-
Glycine	5.38	4.92 ± 0.01	5.7	4.04 ± 0.09	-

Proline	5	5.58 ± 0.21	5.7	5.21 ± 0.15	-
Serine	4.71	4.15 ± 0.22	5.3	5.36 ± 0.30	-

ASX* Aspartic acid or asparagine, GLX** Glutamic acid or glutamine.

Values are given as either mean or mean ± standard deviation (SD) for the percentage of amino acid present in the protein

2.1.4. Undesirable components present in canola

Applications of canola proteins in food products are limited by undesirable compounds such as glucosinolates, erucic acid, fibers, phytates, and phenolic compounds. The development of low glucosinolate and low erucic acid cultivars improves the composition of canola (Aider & Barbana, 2011). The digestive products of glucosinolates such as nitriles, isothiocyanates, epithionitriles, and thiocyanates cause anti-nutritive, toxic, and off-flavor effects (Zhang et al., 2020). Phytates reduce the bioavailability of divalent cations (Ca^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, and Fe^{+2}) by complex formation/chelating and negatively affect the mineral absorption. Moreover, phytates act as an inhibitor of starch (Aider & Barbana, 2011). Fibers contain insoluble dietary fiber (cellulose and lignin) and soluble dietary fiber (hemicellulose, pectin) fraction where the high level of these compounds negatively affect the digestibility of the canola products that are resulting in a decrease in nutritional value (Chmielewska et al., 2020).

Phenolic compounds are present in canola meal in three forms free, esterified, and bound phenolic acids. Protein-bound phenolic acids are stabilized by covalent H, hydrophobic, and/or ionic bonds (Aider & Barbana, 2011). Phenolic compounds are the principal reason behind canola protein's dark color and unpleasant flavors. During oxidation, canola protein produces dark colors. The best example of this phenomenon is the formation of quinones from phenolic compounds after enzymatic or non-enzymatic oxidation under alkaline conditions that can react with proteins resulting in dark brown or dark green color (Xu & Diosady, 2002). Sinapine is the most predominant phenolic compound, followed by sinapic acid choline ester present in canola. Sinapine causes reduced palatability due to bitterness and astringency and negatively affects nutrient uptake (Zhang et al., 2020). Apart from that, phenolic acids, including sinapic acid and soluble and insoluble tannins, are other phenolic compounds present in the canola (Chmielewska et al., 2020).

Regardless of the disadvantages of these antinutritional compounds, many potential health benefits have been reported. Phenolic compounds are rich in bioactivities such as anti microbial, cardio-protective, anti-inflammatory, anti-allergenic, vasodilatory, anti-therogenic, and anti-thrombotic activities (Chmielewska et al., 2020). In terms of phytates, they can prevent diabetes, kidney stones, atherosclerosis, carcinogenicity, and curiosity (Greiner et al., 2006). As such, glucosinolates act as cancer-preventing agents, crop protectors, and flavors precursors (Mailer et al., 2008). Dietary fibers in canola are beneficial in cancer prevention and cardiovascular diseases (Chmielewska et al., 2020).

2.1.5. Non-food applications of canola protein

Due to the limitations associated with food-related applications of canola protein, several non-food applications have been reported in the areas of packaging, adhesives, plastics, and delivery systems (Bandara et al., 2018). The ability to use canola protein in a wide range of applications results from its desirable functional properties, including solubility, emulsification, foaming, gelation, water binding, and adhesion and cohesion properties (Chmielewska et al., 2020). Due to the hydrophilic and amorphous nature and low mechanical properties of canola protein films, several strategies have been used to improve the mechanical, water barrier, and thermal properties of canola protein-based packaging films, including the use of plasticizing agents, nanomaterials, and crosslinkers (Yachuan Zhang et al., 2018). Bandara et al. (2017) developed a wood adhesive using canola protein isolate in which the graphite oxide was used to improve the adhesion strength. Wang et al. (2014) also developed a wood adhesive using canola protein isolate-poly(glycidyl methacrylate) conjugates that increased the adhesive strength and water resistance. The principle behind adhesive fabrication is breaking the structure of canola protein by disturbing the H, disulfide, and other interactions within the protein and thereby exposing a larger, flexible, and interwoven polypeptide chain to interact with the wood surface (Wang et al., 2014).

Delivering bioactive compounds using biopolymer-based delivery systems is becoming more popular due to their safety, biocompatibility, and biodegradability. Due to several functional groups, proteins can interact with active compounds and hold them in their 3-dimensional gel network (Akbari & Wu, 2016). Akbari & Wu (2016) fabricated cruciferin nanoparticles to encapsulate brilliant blue and β-carotene compounds. The results reported a ∼200 nm particle size and non-toxicity to the Caco-2 monolayer. Moreover, hydrophobic and electrostatic interactions

were the driving forces in the formation of particles. Few studies have been reported on the canola protein-based-delivery systems (Akbari et al., 2017; Xu & Dumont, 2015), but there is a huge potential for this area in the future due to the proven functional properties of canola protein to act as encapsulating agents and high availability of canola meal as a biocompatible resource.

2.2. Food Packaging

2.2.1. Current status of petroleum-based food packaging

The primary role of food packaging is to ensure food safety and preserve food quality to minimize food waste. Due to the relatively low production cost, excellent properties, and technological innovations, traditional packaging made from petroleum-based resources has become the leader in the food packaging industry (Nilsen-Nygaard et al., 2021). Polypropylene, polyester, polyethylene, polyethylene terephthalate, polyvinyl chloride, and polystyrene are common petroleum-based polymers used in packaging applications, and open eco systems and oceans have become their final destination creating a huge need for a sustainable solution (Mendes & Pedersen, 2021). Global plastic production in 2019 is 368 million tons which were only 2 million tons in 1950. Of the total produced plastics, 39.6% accounts for the packaging. Roughly 9.2 billion tons of plastics have been produced globally, and 5 billion tons of that have ended up in the environment (Plastic Soup Foundation, 2019). On the other hand, humans and animals can be exposed to microplastics through ingestion, inhalation, and skin contact resulting in adverse health effects such as neurotoxicity, oxidative stress, and male infertility (D'Angelo & Meccariello, 2021). With these concerns, the need for biodegradable and sustainable food packaging materials arises. Figure 1 illustrates a simple classification of materials used for food packaging based on biodegradability and source.

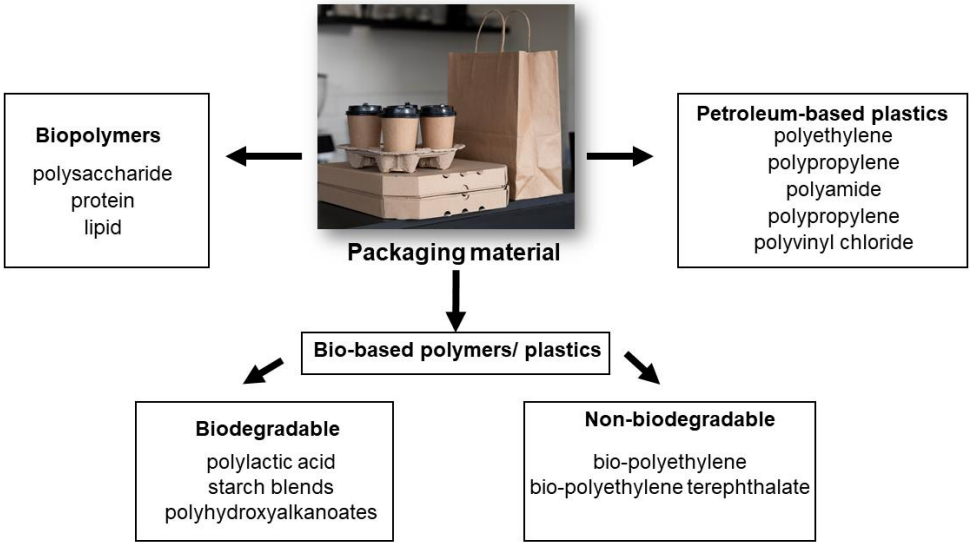

Figure 1. Classification of materials used to produce food packaging based on the source and biodegradability

2.2.2. Biodegradable food packaging

2.2.2.1. Protein-based food packaging materials

Relative abundance, film-formation capacity, biodegradability, and nutritional value of proteins are the major factors contributing to increasing the demand for proteins used in food-packaging applications in recent years (Calva-Estrada et al., 2019; Liu et al., 2017). Proteins have acquired varied functional characteristics due to 20 different types of AAs in their structure (Benichou et al., 2004). Globular proteins made from these amino acids can unfold and cross-link to create new structures that are desirable as food packaging films (Schmid & Müller, 2018). Compared to the films derived from lipids and polysaccharides, protein-based films have better mechanical properties due to their unique structure. Covalent (disulfide bonds or cross-linking), electrostatic, hydrophobic, or ionic interaction between protein chains facilitates film formation with better characteristics (Chiralt et al., 2018). As discussed by Calva-Estrada et al. (2019), the film formation ability of proteins depends on the molecular weight, conformation, charge, flexibility, and thermal stability of the protein. On the other hand, the mechanical properties of the protein films are affected by the nature of AA sequences, degree of elongation, and interactions among protein chains.

Raw materials used to develop protein-based films can be categorized as animal and plant origin proteins. Many proteins can be obtained at a low cost for film preparation due to the high availability of protein sources. Whey protein, sesame protein isolate, gluten, gelatin, lentil protein concentrate, and CPI are some of the by-products of the dairy industry (Moosavi et al., 2020), sesame oil industry (Sharma & Singh, 2016), wheat starch industry (Moosavi et al., 2020), poultry industry (Tew et al., 2017), lentil processing industry (Apodaca et al., 2020), and canola oil industry (Bandara et al., 2018) respectively, that could be potentially used to develop food packaging films. Table 2 summarizes the advantages and limitations of commonly used proteins in film formulations.

Table 2. Advantages and limitations of commonly used proteins as food packaging films

Protein	Advantages	Limitations	Reference
Gelatin	Excellent barrier properties against oxygen and aroma compounds, odorless and tasteless, excellent film-forming capacity	High sensitivity to humidity, limited thermal stability, and mechanical properties during processing	(Ramos et al., 2016)
Casein	Better barrier properties against oxygen	Poor water resistance, high brittleness, poor mechanical strength, and elasticity	(Picchio et al., 2018)
Whey protein	Good film-forming capacity, excellent barrier properties against oxygen	Low tensile strength, intrinsic structure, high	(Oymaci & Altinkaya, 2016)

		water vapor permeability	
Keratin	Good film-forming capacity	High brittleness, low breaking strength	(Strnad et al., 2019)
Albumin	High transparency and elasticity	High water vapor permeability	(Calva-Estrada et al., 2019)
Soy protein	Good film-forming capacity	High moisture sensitivity, weak mechanical properties	(Liu et al., 2017)
Wheat gluten	Good film-forming capacity, selective gas barrier properties	High water vapor permeability, poor mechanical properties	(Nataraj et al., 2018)
Zein	Good film-forming capacity, good barrier properties against moisture and oxygen	Structural brittleness	(Guiyun Chen et al., 2019)
CPI / concentrate	Good film-forming capacity	Poor mechanical properties, poor barrier properties	(Li et al., 2019)

Lower water vapor resistance and lower mechanical strength are major limitations of proteins compared to synthetic polymer-based packaging (Nogueira & Martins, 2018). Several techniques have been used to overcome the limitations of protein-based films through functionalization or modification. Cold plasma is an effective technique that causes surface cleaning, surface ablation

or etching, crosslinking, and modification of functional properties. Mechanical properties of whey protein films and gluten films were improved using low-pressure glow plasma created from air and argon at 50W for 10 minutes and 5 minutes, respectively. However, there was no significant effect of plasma on solubility and water vapor permeability of films (Moosavi et al., 2020). The development of bilayer films is another alternative to overcoming issues of protein-based films. The principle of this method is to combine the advantageous properties of two materials into one single structure and thereby improve the properties of the final film. The bilayer films prepared from the following materials combinations hake protein isolate and zein (PI/Z), hake protein isolate and wheat gluten (PI/WG), and zein and wheat gluten (Z/WG) were compared with individual hake protein isolate films, whet gluten films, and zein films to evaluate the properties of films. Greater tensile strength could be observed from the hake protein isolate films and by-layers of it. This may be due to facilitating molecular entanglement by unfolded proteins during fish protein isolation. Molecular entanglement facilitates the molecular sliding of the protein that promotes greater interactions and, thereby, good mechanical and resistant properties. From these combinations, PI/WG film was identified as the most appropriate film for food packaging as it resulted in better mechanical, optical, and structural properties than other films (Nogueira & Martins, 2018). Picchio et al. (2018) used the crosslinking technique to improve the physicochemical properties of casein-based food packaging films. The authors have used tannic acid, which is a plant-derived phenolic acid, to crosslink casein. The study results clearly showed that crosslinking enhanced the tensile strength, water resistance, thermal stability while decreasing the degree of swelling at the equilibrium. Also, plasticizers are added to the film formulations as a solution for most protein-based films' brittleness. In general, plasticizers are small molecules that reduce protein-protein interactions and induce the film's flexibility, extensibility, and dispensability. Glycerol, sorbitol, xylitol, glycols, sugars, and triethanolamine are commonly used plasticizers in protein-based film formulations (Calva-Estrada et al., 2019). Hybridization with other biopolymers, reinforcing nanostructural materials, and incorporating natural and synthetic preservatives are the other major techniques used to improve the material properties and functionality of natural polymer-based films.

Chicken feathers can be introduced as one of the major by-products in the poultry industry. Around 91% of the components of the chicken feathers are protein, and the protein fraction is mainly composed of β-keratin, which is called hard keratin. The β-keratin structure is hard to disrupt

because it consists of non-covalent and covalent interactions. Electrostatic forces, hydrogen bonds, and hydrophobic forces are the non-covalent fraction, and disulfide bonds are the covalent fraction. Disulfide bonds result from the presence of Sulphur containing cysteine that improves the keratin material's strength and thermal and chemical properties. However, due to the hydrophobic nature resulting from disulfide bonds and non-covalent interactions, keratin cannot be easily dissolved in many solvents, including water, dilute acids, alkaline solutions, and most organic solvents, which limit its applications (Garrido et al., 2019). Therefore, several methods have been introduced to solubilize keratin, such as physical treatments, including high temperature and high-pressure steam, and chemical treatments, including acid and alkaline chemicals that hydrolyze feathers by disruption of disulfide and peptide bonds. Moreover, biological treatments such as using keratinolytic microorganisms and their enzymes (keratinase) can be used for keratin hydrolysis. However, its industrial feasibility is limited as it is long-lasting with a very slow reaction rate (Garrido et al., 2019; Sinkiewicz et al., 2017). Due to the poor mechanical properties of keratin, keratin is blended with other polymers to obtain packaging films with enhanced properties (Ramirez et al., 2017).

Among the biopolymers extracted from plant-based by-products, soy protein, zein, and gluten are the most commonly used biopolymers in food packaging applications (Chiralt et al., 2020). Soybean is the largest oilseed crop grown worldwide (Delgado et al., 2018), and soybean meal can be introduced as one of the major by-products of the soy oil industry. Soybean meal is a cheap protein source as it contains approximately 50% (w/w) protein (Zhang, Yang, Zhang, Hu, & Zhao, 2017). SPI is a low-cost, biocompatible, and nutritious source with its superior film-forming capacity. The films prepared from SPI exhibit moderate mechanical properties and superior oxygen and oil barrier properties (Han, Yu, & Wang, 2018). However, low water resistance and insufficient mechanical strength restrict the application of SPIs in the production of films. Therefore, it is necessary to blend the soy protein with other polymers or chemicals to obtain good quality packaging films (Ye et al., 2019).

Canola meal is the major by-product of the canola oil industry, and oil-free canola meal contains 36-40% protein (w/w dry weight basis). However, the other non-protein components, such as fiber, polymeric phenolics, phytates, and sinapine which are present in seed coat and cellular components, limit the suitability of canola meal for food use (Wanasundara et al., 2016). These antinutritive factors lead to poor physiochemical properties, objectionable color, bad taste, and

poor digestibility (Tan et al., 2011). Napin also limits its applications in foods due to its allergenicity (Wanasundara et al., 2016). Nevertheless, due to higher availability, low cost, and issues related to anti-nutritional factors (which makes canola less desirable for food application), canola protein can be a promising starting material for food packaging application (Bandara et al., 2018; Zhang et al., 2016). Table 3 summarizes the material properties of canola protein-based packaging materials reported in the literature.

Table 3. Properties of canola protein-based packaging materials reported in the research studies

Canola protein-based study	**Tensile strength (MPa)**	**Elongation at break (%)**	**Water vapor permeability (g mm/m^2 h kPa)**	**Other properties/ remarks**	**Reference**
Effect of protein and glycerol concentration	~ 6.5 (7.5% protein, 30% glycerol)	10.18 ± 0.91 (5% protein, 50% glycerol)	~ 0.5 (5% protein, 30% glycerol)	The increase in protein and glycerol concentration decreased the tensile strength and water barrier properties	(Chang & Nickerson, 2015)
Effect of plasticizers and genipin	12.7 ± 0.61 (5% protein, 50% sorbitol, 1% genipin)	10.2 ± 0.91 (5% protein, 50% glycerol)	0.5 ± 0.04 (5% protein, 50% sorbitol, 1% genipin)	Films with genipin showed highest tensile properties.	(Chang & Nickerson, 2014)
Effect of crosslinking using 1,4-butanediol diglycidyl	~ 9.5 (32 g protein + 80 mg BDDE)	~ 160 (control films)	-	Thermal stability of the films increased by branching reaction	(Li et al., 2017)

ether (BDDE)				between the protein fractions	
Effect of sodium doecy sulfate (SDS, protein denaturant) and stearic acid (SA, co-plasticizer)	3.8 ± 0.2 (25% glycerol, 15% SDA, 0% SA)	551.4 ± 48.9 (40% glycerol, 5% SDS, 0% SA)	-	The addition of SDS decrease the thermal stability and increased the water absorption capacity due to the reduced hydrophobicity	(Shi & Dumont, 2014)
Effect of NCC	~ 3.9 (36% NCC)	~ 140% (0% NCC)	-	The incorporation of NCC enhanced the thermal stability of the films	(Osorio-Ruiz et al., 2019)
Effect of acylation	2.4 (with 100% relative degree of modification with lauroyl)	151% (non-modified protein)	-	With the increase in the relative degree of modification, oxygen permeability of the films increased	(Fetzer et al., 2021)

Effect of succinylation	5.02 ± 0.12 (5% succinylated protein)	123.6 ± 4.90 (5% succinylated protein)	0.32 ± 0.02 (5% succinylated protein)	The lower degree of succinylation improved the thermal stability of the films)	(He et al., 2019)
Effect of protein hydrolysate and blending with chitosan	~ 23.46 (12% degree of protein hydrolysis + chitosan blends)	~ 40 (12% degree of protein hydrolysis + chitosan blends)	~ 0.35 (3% degree of protein hydrolysis + chitosan blends)	12% degree of hydrolysis showed the better antimicrobial properties	(Zhang et al., 2019)

2.2.2.2. Polysaccharide-based food packaging

Polysaccharides have a high potential to use in food packaging materials due to their highest availability and inherent characteristics such as biodegradability, biocompatibility, and non-toxicity (Nešić et al., 2020). A variety of polysaccharides such as cellulose and its derivatives, chitosan, starch, alginate, pectin, carrageenan, and guar gum are used in film formulations with different properties (Cazón et al., 2017; Nešić et al., 2020; Zhao et al., 2019). Polysaccharides are a good source to form films with barrier properties against gases, mainly oxygen and carbon dioxide. Also, some of the polysaccharide-based films show excellent tensile strength values that are similar to synthetic polymers. However, polysaccharide-based films exhibit poor water vapor barrier properties and are far away from values percentage of elongation from the desirable values found in synthetic polymers (Cazón et al., 2017). As shown in Table 4, several methods have been introduced to overcome the limitations of polysaccharides and their derivatives to use them in developing food packaging materials.

Table 4. Solutions reported in the literature for some of the limitations of polysaccharides and polysaccharide derivatives used in food packaging

Polysaccharide or polysaccharide derivative	Focused limitations	Developed methods to overcome the limitations	Reference
Methylcellulose films	Higher susceptibility of physical properties to moisture and biodegradability	Crosslinking with glutaraldehyde	(López de Dicastillo et al., 2016)
Carboxymethyl cellulose	Absence of antioxidant and antimicrobial properties	Incorporation of antimicrobials and antioxidants into film formulation Ex: Chinese chives root extract	(Riaz et al., 2020)
Hydroxypropyl cellulose	Hydrophilicity	Addition of nanometric fillers	(Lopez-Polo et al., 2020)
Chitosan	Poor water barrier properties	Incorporation of lipid fraction into film formulation Ex: beeswax	(Hromiš et al., 2015)
	Inadequate antioxidant properties	Incorporation of active compounds Ex: caraway essential oil	(Hromiš et al., 2015)

Starch	Poor water vapor barrier properties	Introduction of hydrophobic compounds Ex: rapeseed oil	(Basiak et al., 2017)
	Poor mechanical strength	Addition of nanomaterials	(Mohammadpour Velni et al., 2018)
Pectin	Poor tensile strength, water vapor barrier properties, and thermal stability	Combination with polyvinylpyrrolidone	(Nešić et al., 2017)
	Poor tensile strength and water barrier properties	Preparation of nanocomposite using NC	(Chaichi et al., 2017)

Cellulose can be introduced as the most abundant biopolymer on the earth, and it is a linear homopolysaccharide of D-glucopyranosyl units linked by β-(1→4) glycosidic bonds. It can be obtained from various sources, including wood pulp, cotton fibers, and plant-based wastes such as husk and shell, peel, and sugar cane bagasse (Nešić et al., 2020). On the other hand, cellulose has attractive properties such as high mechanical strength, low density, low cost, high durability, good film-forming capacity, high chemical stability, and the ability to make chemical derivatives. However, as raw material, cellulose suffers from the drawback of its insolubility in water and most organic solvents due to the inherent inter and intra-molecular hydrogen bonds (Duan et al., 2016). Due to the high occurrence of hydroxyl groups in cellulose structure, extremely strong intermolecular interactions are result which in turn result in high stiffness, fibrous aspect, infusibility, and insolubility characteristics (Otoni et al., 2018). Several techniques have been developed to dissolve cellulose, an essential step in film preparation (Cazón et al., 2017).

Regarding availability, low cost, natural renewable sources, and environmental issues, cellulose obtained from agro-industrial waste and by-products have gained significant attention in food packaging applications. Microcrystalline cellulose, a widely used form of cellulose, is

commercially produced from highly costly hardwoods and purified cotton. Therefore, there is a need to produce cellulose and its derivatives from renewable sources in an economically viable manner (Sundarraj & Ranganathan, 2018). As summarized by Sundarraj and Ranganathan (2018), there are cheaper sources of cellulose from agricultural wastes such as cereal straws, soybean husk, flax fibers, flax straw, sugarcane bagasse, mulberry barks, pineapple leaf, corn cobs, brewer's spent grains, hazelnut cells, bagasse pulp, orange mesocarp wastes, wheat straw, groundnut husks, coconut husks, and corn residues. Pomaces from different fruits and vegetables such as apple, cucumber, carrot, and tomato were treated with hot water, acidic solution (1/0.5M HCl), alkali solution (1M NaOH), and oxidative reagent (1-2% NaClO) to obtain sugars, pectic polysaccharides, hemicellulose, lignin, and cellulose. Such a biorefinery process produces several by-products at low costs. Resulted cellulose can be used as reinforcing materials in food packaging while reducing the amount of waste related to fruit and vegetable processing industries (Szymańska-Chargot et al., 2017).

After cellulose, chitin is the most abundant biopolymer on earth found in structural materials of crustaceans, insects, and fungi. Chitosan is obtained from the deacetylation of chitin. As there is nitrogen in chitosan, it is different from other polysaccharides, and its major monomer is (1-4)-linked 2-amino-2-deoxy-β-D-glucose. The presence of amino groups affects chitosan's various chemical, physical, and biological properties, such as solubility, viscosity, film formation, ion binding, and antimicrobial properties. Moreover, nitrogen composition, N/C ratio, molecular size, degree of acetylation, and polydispersity index of the chitosan depend on the source of chitin (Priyadarshi & Rhim, 2020). Due to its inherent antimicrobial properties, pure chitosan films can be used to delay qualitative and nutraceutical traits changes, prevent microbial growth, protect antioxidant activity and increase the shelf life of foods (Wang et al., 2018). In addition, chitosan films exhibit low gas permeability and better resistance to fat and oil (Hromiš et al., 2015). However, many research studies are carried out to improve the properties of chitosan-based films further to use them in packaging applications. Hybridization with other biopolymers, reinforcing with nanomaterials, and incorporating natural and synthetic preservatives are highlighted.

Starch is another studied biopolymer for food packaging applications due to its high availability, low cost, and biodegradability (Mohammadpour Velni et al., 2018). The basic unit of starch is glucose, and it has two components, namely amylose and amylopectin. Around 20-30% of starch's structure is amylose and has a helical structure of α-D-glucose units linked through α(1 → 4)

glycosidic bond. Around 70% of the starch structure is amylopectin, consisting of linear glucose units added to the structure through α(1 → 4) glycosidic bond and branched units through α(1 → 6) glycosidic bond at each 24-30 glucose repeating units. This branched structure is soluble and easily degraded due to the availability of many endpoints for enzyme reactions (Niranjana Prabhu & Prashantha, 2018). The semi-crystalline structure of the starch depends on the amylose/amylopectin ratio. Short-branched chains in the amylopectin result in crystalline regions, while amorphous regions result from the amylose and the branching points of the amylopectin. The brittleness of starch films results from the amorphous regions formed by amylose. On the other hand, excellent oxygen barrier properties of starch result from the high ordered hydrogen-bonded network structure. Therefore, high content of amylopectin or increased crystallinity enhances the barrier properties of starch films (Cazón et al., 2017). However, despite the higher potential of starch to use in food packaging films, films prepared with pure starch show a very brittle structure, low mechanical properties, and high-water vapor permeability (Mohammadpour Velni et al., 2018). Several techniques have been used to overcome these issues and improve their properties, such as adding lipid and hydrocolloids, reinforcing agents, additives, and active compounds (Pelissari et al., 2018).

Pectin is a family of natural polysaccharides found in the cell walls of peels in several fruits, such as apple peel and citrus peel (Cazón et al., 2017). It consists of a chain of D-galacturonic acid linked through α-(1–4) bonds. Around 80% of carboxylic groups present in galacturonic acid are methyl esterified. Therefore, the behavior of pectin in solutions that influence the properties of pectin-based materials is affected by the ratio of esterified to non-esterified galacturonic acid. However, due to neat pectin films' brittle and hand-breakable characteristics, pectin needs to be further modified (Nešić et al., 2017). Also, poor barrier and thermomechanical properties and weak water resistance limit its applications in packaging films (Chaichi et al., 2017).

2.2.2.3. Lipid-based food packaging

In general, lipid-based substances are used in packaging films to limit moisture migration. Due to the non-polarity of these hydrophobic substances, they can effectively act as moisture barriers. Composite films using lipids can be developed either by incorporating a hydrocolloid film-forming solution (emulsion method) or depositing them onto the pre-prepared hydrocolloid film (Debeaufort & Voilley, 2009). The water barrier efficiency of the lipid-based films is affected by

the composition and structure of the edible films. Debeaufort and Voilley (2009) discussed that the water vapor permeability of emulsion-based films is higher than that of bilayer films. The permeability of the emulsion-based films is closer to that of protein- or polysaccharide-based films. Other than the structure and composition, hydrophobicity, interactions with other film components, and physical state (solid/liquid) of the lipid affect the barrier properties (Pérez-Gago & Rhim, 2013). According to Mohamed, El-Sakhawy, and El-Sakhawy (2020), lipids used in food packaging can be classified under several classes such as fats and oils (triglycerides), essential oils, waxes, resins, emulsifiers (phospholipids), and plasticizers. Even though lipids are excellent moisture barriers, the films made from lipids are relatively inflexible and opaque. On the other hand, casting solid lipids at room temperature is difficult as they need solvents or high temperatures to facilitate casting (Pérez-Gago & Rhim, 2013). In terms of oxygen barrier properties, lipids are not as efficient as proteins or polysaccharides due to their strong affinity for oxygen. Also, some lipid-based compounds, such as unsaturated fatty acids, are sensitive to oxygen, resulting in rancidity that causes off-flavors. Therefore, it is hard to produce pure lipid-based food packaging materials, but hybridization with hydrocolloids results in excellent packaging materials with better properties and mechanical properties (Debeaufort & Voilley, 2009).

Chain length is one of the major factors influencing the water barrier property of lipid-based substances such as fatty acids. With the increase in carbon atom number of fatty alcohols and fatty acids (from 14 to 18), the moisture barrier property increases. This is due to the increase in the non-polar portion of the molecule that minimizes water solubility and moisture transfer. Therefore, carboxylic acids such as stearic and palmitic show good barrier properties. However, higher moisture permeabilities result from fatty acids containing more than 18 carbon atoms, such as arachidonic and behenic acids. This can be explained by the polymer's heterogeneous structure induced by very long chains and thereby the decreased moisture barrier properties. On the other hand, functional groups of the lipid molecule affect the film's hydrophobicity or moisture barrier properties. Better barrier properties of stearyl alcohol compared to triglycerides and fatty acids are due to the presence of hydroxyl groups in the stearyl alcohol structure. Hydroxyl groups of stearyl alcohol have less affinity to water than carbonyl and carboxyl groups. Likewise, saturated or unsaturated states, liquid or solid-state, polymorphism, film preparation technique, and film thickness also affect the barrier properties. Due to the polarity of unsaturated fatty acids compared to saturated fatty acids and different crystallization tendencies, they are less efficient in acting as

moisture barriers. Therefore, stearic or palmitic acid films are better barriers than films made from oleic acid. (Debeaufort & Voilley, 2009).

Waxes are the superior water vapor barrier to other lipids due to their excellent hydrophobicity resulting from the high amount of long-chain fatty alcohols and alkanes. Candelilla wax, beeswax, carnauba wax, and palm wax are some of the high potential waxes that could be used in food packaging (Syahida et al., 2020). Syahida et al. (2020) used palm wax to improve the physical, mechanical, and water barrier properties of fish gelatin-based food packaging films. Palm wax consists of long-chain saturated fatty acids, mostly palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$). Due to the large portion of saturated fatty acids, palm wax is less susceptible to traditional autooxidation in which double bonds are mostly attacked. Based on the study done by Syahida et al. (2020), the incorporation of palm wax into gelatin films enhanced the water barrier properties and resistance to UV light. Also, palm wax resulted in more flexible and less stiff films than control gelatin films.

Essential oils are aromatic chemical substances that are extracted from the plant materials such as leaves, seeds, flowers, buds, stems, and bark. As summarized by Ribeiro-Santos, Andrade, Melo, and Sanches-Silva (2017), a variety of essential oils are used in active food packagings, such as cinnamon, wintery savory, oregano, clove, rosemary, bergamot, lemon thyme, ginger, turmeric, lemongrass, pimento, nutmeg, citral, garlic, peppermint, and mustard. Atarés and Chiralt (2016) have discussed the diverse results that have been obtained for the tensile properties of essential oil incorporated films. The effect on the tensile strength is affected by the lipid characteristics and its interactions with the polymer matrix. The tensile strength reduction of lipids or essential oil incorporated films is due to the partial replacement of stronger polymer-polymer interactions by weaker polymer-oil interactions. On the other hand, the authors have explained that the increase in tensile strength of films after essential oil addition is due to the induced rearrangement of the polymer network and also the cross-linking of the chain by a vast range of compounds present in the essential oil (Atarés & Chiralt, 2016). Major limitations of essential oil in packaging applications are the strong aroma and lack of reproducibility due to the great diversity of bioactive compounds' content (Ribeiro-Santos et al., 2017).

Emulsifiers or plasticizers are used in packaging formulations to improve certain properties of the films, such as water resistance, optical properties, surface morphology, and solubility in water. Emulsifiers increase wettability and the adhesion of the film by reducing surface tension. Also, the water vapor permeability of the films is decreased due to the hydrophobic characteristics of

emulsifiers (Nur Hanani & Aelma Husna, 2018). Lecithin is a widely used emulsifier, and due to the presence of amphiphilic phospholipids, it can quickly adsorb on the oil-water interface. Therefore, it plays an excellent role in stabilizing emulsion films (Aydogdu et al., 2020).

2.2.2.4. Polymers synthesized from biobased monomers

Even though biopolymer-based packaging is an excellent solution for environmental pollution, they are still unable to provide physicochemical properties like synthetic plastics. Poor water barrier properties and mechanical strength are highlighted among them. Renewable, biodegradable, synthetic polymers can be introduced to solve this issue better. However, blending with other polymers and adding compounds such as plasticizers, nucleating agents, and bioactive compounds is still needed to improve bio-based synthetic polymers' physicochemical properties. (Nilsuwan et al., 2020). On the other hand, all the bio-based polymers are not biodegradable.

Polylactic acid is a bio-based, biodegradable aliphatic polyester potentially used in food packaging applications. Also, polylactic acid is generally considered recognized as safe (GRAS) by the US Food and Drug Administration (FDA). Lactic acid (2-hydroxy propionic acid) is the basic monomer of polylactic acid, and it can be produced either by bacterial fermentation of carbohydrates or by chemical synthesis (Castro-Aguirre et al., 2016). However, bacterial fermentation is industrially preferred as chemical synthesis has many limitations, such as limited production capacity, high manufacturing cost, and the inability to produce only the desired L-lactic acid isomer (Castro-Aguirre et al., 2016). Many reasons are behind the wide use of polylactic acid in food packaging applications. Mainly, polylactic acid does not create environmental contamination as in the disposal of synthetic plastics, and its biocompatibility eliminates the health issues related to contact and consumption of food packaging. Furthermore, in prepared polylactic acid-based packaging, they show an acceptable hydrophobicity, transparency, resistance to UV, bright appearance, and ease of processability (Khosravi et al., 2020). However, polylactic acid still shows some limitations, such as limited mechanical and barrier properties that challenge the application of pure polylactic acid in commercial level food packaging production (Castro-Aguirre et al., 2016).

Polyhydroxyalkanoates are aliphatic, semi-crystalline, thermoplastic, and biodegradable polyester widely used in the packaging industry. Poly(3-hydroxybutyrate) is a commonly synthesized polyhydroxyalkanoate with comparable physical properties to isotactic polypropylene and

polystyrene (Cherpinski et al., 2018). The promising properties of polyhydroxyalkanoates, such as hydrophobicity, nontoxicity, nonlinear optical activity, piezoelectricity, gas/water impermeability, and thermoplasticity, make it an ideal candidate for food packaging (Masood, 2017). Microorganisms produce polyhydroxyalkanoates to store energy in granular forms as lipid inclusions. Polyhydroxyalkanoates can be produced in both aerobic and anaerobic conditions by gram-positive and gram-negative bacteria (Raza et al., 2018). However, due to the high cost of synthesis, narrow thermal processing properties, low strength, and high brittleness limit the application of Polyhydroxyalkanoates in food packaging (Cherpinski et al., 2018).

2.2.2.5. Polymers extracted from microbial biomass

Even though plants are considered the major contributor to cellulose, various bacteria also produce cellulose (Esa et al., 2014). Bacterial cellulose is secreted as an extracellular protective layer by the bacteria, and it is used in a vast range of applications, including edible food packaging material. Bacterial cellulose structure is composed only of glucose monomers. Therefore, it shows excellent properties such as a high degree of polymerization, high water holding capacity, high mechanical strength, and high crystallinity. Due to the fine network, biodegradability, and high-water resistance properties, bacterial cellulose has become a promising material for food packaging. However, as with most polysaccharides, it should be combined with other polymers to improve the physicochemical properties of packaging material (Esa et al., 2014). Compared to plant-based cellulose, the structure is similar in both; however, bacterial cellulose is free from lignin, hemicellulose, and pectin compared to plant cellulose. Therefore, the purification process of bacterial cellulose is easy and consumes less energy. However, there are many challenges to commercial-level bacterial cellulose production due to the lack of efficient fermentation systems and the high cost of production (Azeredo et al., 2019).

Xanthan gum, a high molecular weight extracellular polysaccharide produced by *Xanthomonas campestris* bacteria, is widely used in food applications as a thickening agent and stabilizer. The primary structure of xanthan gum is composed of β-1,4 linked D-glucose chain, and every second glucose unit is substituted by side chains containing glucuronic acid between two mannose units (Talens & Chiralt, 2019). Water solubility, non-toxicity, high viscosity at low concentrations in aqueous media, strong shear-thinning behavior, and stable rheological behavior in a wide range of

pH, temperature, and ionic strength make xanthan a potential source for food packaging applications (Ferreira et al., 2016).

Pullulan is another potential exopolysaccharide obtained from the fermentation medium of the *Aureobasidium pullulans*, a fungus-like yeast to prepare food packaging materials. Due to its impressive film-forming properties produce colorless, tasteless, odorless, transparent, heat-stable, and oxygen impermeable films (Silva et al., 2018). Apart from that, it exhibits considerable mechanical strength, adhesiveness, film and fiber formability, and enzymatic degradability (Farris et al., 2014). However, due to the hydrophilic nature, brittleness, and absence of active functions, the formation of pure-pullulans-based packaging is limited (Silva et al., 2018).

Gellan gum is an anionic, linear polysaccharide secreted by *Sphingomonas elodea* bacteria. The structure of the gellan gum is composed of linear tetrasaccharide repeating units. Tetrasaccharide repeating unit is made from two β-D-glucose, one β-D-glucuronic acid, and one α-L-rhamnose. Gellan gum can form stable gels in the presence of cations. The ability is affected by the gellan gum concentration, the chemical nature of the cations, and ionic strength. It can provide resistance to active compounds in acidic environments (Rukmanikrishnan et al., 2020). Pure gellan gum-based films exhibit limitations such as hydrophilic nature, brittleness, and poor mechanical properties. Blending with other natural polymers, adding active compounds, and using crosslinkers are some of the attempts taken to overcome these limitations in food film formulations (Du et al., 2019).

Some polysaccharides such as alginate and carrageenan are obtained from algae to use in food packaging applications (Ferreira et al., 2016). Alginate is a linear polysaccharide that is produced by brown seaweeds and some bacteria such as *Azotobacter vinelandii* or mucoid strains of *Pseudomonas aeruginosa* (Senturk Parreidt et al., 2018). The structure of the alginate is composed of 1-4-linked β-D-mannuronate (M blocks), α-L-guluronate (G blocks) (Wang, Shankar, & Rhim, 2017), and segments of alternating mannuronic and glucuronic acids (MG blocks). The relative proportion of these three blocks affects the physical properties of alginate. Due to the low cost and excellent functional properties such as thickening, stabilizing, gel-producing, and suspending, alginate is a better component of biodegradable food packaging (Ferreira et al., 2016). Alginate-based food packaging films show high mechanical strength and high transparency. However, poor water vapor permeability and low flexibility limit its use in packaging applications (Wang et al., 2017).

Carrageenan is a sulfated anionic linear polysaccharide explicitly obtained from the Rhodophyceae family of red seaweeds. The structure of the carrageenan is composed of α-D-1,3 and β-D-1,4 galactose residues in which up to 40% of the weight is sulfated (Ferreira et al., 2016). Based on the amount and position of the sulfate group on the repeating units, carrageenan is classified into three major groups called lambda (λ), kappa (k), and iota (ι). Among these three types, k-carrageenan is mostly used in industrial applications (Sedayu, Cran, & Bigger, 2018). Due to a considerable amount of sulphonic groups, k-carrageenan films are formed through the self-aggregation of its helical structure. However, poor mechanical, thermal, and barrier properties of the k-carrageenan films limit their applications in food packaging. Blending with other polymers or nano-reinforcing fillers improves the properties of carrageenan (Kassab et al., 2019; Yadav & Chiu, 2019). On the other hand, the inherent brittleness of the pure carrageenan films is overcome by adding plasticizers to the film formulation (Sedayu et al., 2018)

2.2.3. Active packaging with natural and synthetic antimicrobials

Active packaging is an innovative concept that has been introduced to maintain and prolong the shelf-life of foods while protecting the quality, safety, and integrity of food products. In general, active packaging systems are divided into active scavenging systems (absorbers) and active-releasing systems (emitters). Absorbers remove undesired compounds such as moisture, carbon dioxide, oxygen, ethylene, or odor from the food or food environment, while emitters add the compounds such as antimicrobial compounds, carbon dioxide, antioxidants, flavors, ethylene, or ethanol to the food or packaging headspace (Yildirim et al., 2018).

The presence of oxygen in food packaging accelerates food spoilage due to fat and lipid oxidation or promotes microbial growth due to spoilage and changes the organoleptic properties of the food. The most commonly used oxygen scavengers in food packaging are iron, platinum group metal, unsaturated hydrocarbons, α-tocopherol, ascorbic acid, enzymes, and microorganisms (Dey & Neogi, 2019). Oxygen scavenging film with a palladium-based catalytic system was developed for linseed oil to prevent lipid oxidation. The film removed the oxygen in the headspace (2% volume), and palladium efficiently converted hydrogen and oxygen into the water. Compared to the normal atmosphere and modified atmosphere without palladium, a modified atmosphere with palladium significantly reduced the lipid oxidation in linseed oil. This was measured by analyzing primary and secondary oxidation products such as conjugated dienes and trienes, hydroperoxides, and

volatile and non-volatile aldehydes over 168 days (Faas et al., 2020). Oxygen scavenging films prepared from gallic acid in the presence of sodium carbonate were used to evaluate the film surface color and oxygen absorption at different temperatures and relative humidity. Gallic acid absorbs a large amount of O_2 under alkaline conditions. The results revealed that the rate of oxygen absorbance in gallic acid incorporated polymer was lower than that of gallic powder due to the action of the polymer as water and oxygen barriers (Pant et al., 2017). Low-density polyethylene (LDPE) incorporated polyisoprene film was prepared by Gaikwad, Singh, Shin, & Lee (2020) to use the oxygen scavenging activity of polyisoprene. The allylic carbon and hydrogen bonds in polyisoprene undergo oxidative degradation due to their lower bond energy and remove the oxygen from the headspace of the packaging. Gaikwad et al. (2020) showed that incorporating polyisoprene enhanced the mechanical properties of films but affected the barrier properties. Further, packed beef jerky in LDPE/polyisoprene films showed a decreased microbial count, red color retention property, acceptable TBARS (Thiobarbituric acid reactive substance), and pH (Gaikwad et al., 2020).

Excess moisture in food packages leads to microbial growth and foggy films. In addition, the packaging materials with poor water permeability will lead to water accumulation inside the package due to the respiration of fresh products such as fruits and vegetables, drip of tissue fluid from cut meat and poultry products, and temperature fluctuations. This may result in shelf life reduction and quality loss of food products due to the bacterial and mold growth inside the packaging (Ozdemir & Floros, 2004). The most common ways of controlling this phenomenon are using moisture scavengers such as sodium chloride, silica gel, zeolites, cellulose fibers, and other moisture-absorbing substances (Wyrwa & Barska, 2017). In dry products with low moisture levels, sachet forms contain these substances. Blankets or desiccant pads absorb water in food products with high moisture content, such as meat, poultry, and fish. Superabsorbent polymeric films can be used as another approach to absorb moisture in which a moisture absorbent layer is present (Ozdemir & Floros, 2004). Fructose, sorbitol, cellulose, and cellulose derivatives are organic-based moisture absorbents. However, these organic moisture absorbers are relatively high cost and absorb a lower amount of moisture than inorganic absorbers such as silica gel, calcium oxide, calcium chloride, potassium chloride, potassium carbonate, activated alumina, and bentonite. Other than that, some polymer-based materials are also used as moisture absorbers, such as starch copolymers, polyvinyl alcohol, and absorbent resin (Gaikwad et al., 2019).

Some polymers themselves act as antimicrobial agents, and therefore, they can be used to develop food packaging materials. Chitosan is one such polymer, and it has a broad-spectrum antimicrobial activity against both gram-positive and gram-negative bacteria and fungi. Environmental conditions such as pH, type of microorganism, and neighboring components, and its structural conditions such as molecular weight, degree of deacetylation, derivative form, concentration, and source affect the antimicrobial property of chitosan (Hosseinnejad & Jafari, 2016). Also, chitosan has been blended with other antimicrobial substances to develop active food packaging in many research studies (Hafsa et al., 2016; Ma et al., 2015, 2016).

Antimicrobial peptides are another unique group used in active food packaging due to their antibacterial, antiviral, antifungal, and antitumor activities. These peptides are introduced as protein fragments produced by invertebrates, vertebrates, and microorganisms. Nisin is one of the most common antimicrobial agents produced by *Lactococcus lactis* strains. Due to its harmless and heat-stable properties and high availability, it is widely combined with different polymers to develop active food packaging (Santos et al., 2018). Saini, Sillard, Naceur Belgacem, and Bras, (2016) grafted nisin on carboxylated cellulose nanofibers to produce packaging materials and prepared films that exhibited excellent antimicrobial properties. Many studies have been done using nisin to produce active food packaging (Correa et al., 2017; Cui et al., 2017; Zehetmeyer et al., 2016).

Plant secondary metabolites are promising antimicrobial substances due to their inherent bioactivity and can be used in active food packaging as they are environmentally friendly, versatile, and low-cost materials. The antibacterial mechanisms of plant secondary metabolites are not yet fully understood due to their complexity. However, it may involve several mechanisms such as disturbance of bacterial ion transport, interruption of bacterial signals, inhibition of ATP production, and coagulation of the cytoplasm. Further, exposure to plant secondary metabolites can lead to a reduction in enzymatic activity, loss of turgor pressure, changes in DNA synthesis, and inhibition of different metabolic functions of bacteria (Al-Jumaili et al., 2018). Bioactive compounds in plants are categorized based on their chemical classes and biochemical pathways. The main chemical groups are phenolic compounds (phenolic acid, hydroxycinnamic acid, stilbenes, flavonoids, and anthocyanins), tannins, terpenoids, phenylpropanoids, lignans, resins, alkaloids, furocoumarins, naphthodianthrones, and glycosides (cardiac glycosides, glucosides,

saponins, glucosinolates, and anthraquinone) (Cvjetko Bubalo et al., 2018). Table 5 illustrates some of the recent development in packaging materials using several plant extracts.

Essential oils are naturally occurring combinations of secondary plant metabolites and aromatic substances produced by various plant parts such as seeds, flowers, buds, leaves, stems, and bark (Al-Jumaili et al., 2018; Ribeiro-Santos et al., 2017). As essential oils are volatile compounds, the antimicrobial activity of films prepared with essential oils exhibits even without direct contact with the food (Becerril et al., 2019). Various essential oils are used in food packaging development, such as oregano, rosemary, clove, ginger, thyme, cinnamon, citrus, peppermint, and tea tree oil (Akram et al., 2019). Essential oils show antibacterial, antifungal, allelopathic, and antioxidative properties (Aridoğan et al., 2002). Due to the great diversity of compounds present in essential oils, the lack of reproducibility has become a significant obstacle in essential oil-based packaging materials. Moreover, their strong aroma restricts their usage in certain food applications. On the other hand, the migration of essential oils from food packaging to food should be monitored to ensure the safety of packed food and the quality of food packaging (Ribeiro-Santos et al., 2017). Nanocarriers are used to encapsulate essential oils before incorporation into films to overcome the issues related to essential oils, such as poor bioavailability, low solubility, poor sensory properties such as strong aroma, and quick release (Rehman et al., 2020).

Table 5. Applications of several plant extracts in active food packaging

Type of packaging film	Plant extract	Key findings	References
Zein edible film impregnated with *Zataria multiflore* Boiss. essential oil and monolaurin	*Zataria multiflore* Boiss.	-Films exhibited antibacterial activity against *Listeria monocytogenes* and *Escherichia coli*. Except for 1% monolaurin films, all other films with antimicrobial compounds significantly reduced the growth of both *E. coli* and *L. monocytogenes* (log reduction	(Moradi et al., 2016)

		of 0.7-1 CFU/g) on the minced beef meat -Gram-negative *E. coli* was more resistant than gram-positive *L. monocytogenes* - Due to the oxygenation of monoterpenes in *Z. multiflora* Boiss, high total phenolics content was shown by the films containing 3% *Z. multiflora* Boiss compared to control zein films and films impregnated with monolaurin	
Mango kernel extract incorporated SPI and fish gelatin films	Mango kernel extract	-Incorporation of mango kernel extract enhanced the tensile strength of both films -Incorporation of mango kernel extract decreased the water solubility -Incorporation of mango kernel extract improved the antioxidant activity of both films. However, SPI films showed higher antioxidant activity compared to fish gelatin films (Effect on	(Maryam Adilah et al., 2018)

		antimicrobial properties of the films has not been reported)	
Mango peel extract incorporated fish gelatin films	Mango peel extract	-Incorporation of mango peel extract decreased the water vapor permeability and solubility of films -Colour ed tint films and reduction in transparency were resulted due to the hydrogen bond linkages between fish gelatin molecules and phenolic content -Higher free radical scavenging activities resulted from higher concentrations of mango peel extracts (Effect on antimicrobial properties of the films has not been reported)	(Adilah et al., 2018)
Cinnamaldehyde incorporated polylactic acid films	Cinnamaldehyde	-Incorporation of cinnamaldehyde showed an effective antibacterial effect against *Staphylococcus aureus* and *Escherichia coli*. Control films reported $5.33 \times 10^7 \pm 1.15 \times 10^7$ and $1.67 \times 10^7 \pm 5.77 \times 10^6$ CFU/ml for *S. aureus* and *E. coli* respectively where no	(Villegas et al., 2017)

		CFUs were reported for the films with cinnamaldehyde -Cinnamaldehyde improved the thermo-mechanical properties of films	
Amaranthus leaf extract incorporated polyvinyl alcohol and gelatin films	Amaranthus leaf extract	-Antioxidants, antibacterial, and intelligent properties were given to the films by *Amaranthus* leaf extract -Presence of amaranthus leaf extract increased the zone of inhibition from 0 to 30 ± 1, 33 ± 2, and 26 ± 1 mm against *S. aureus*, *Bacillus cereus*, and *E. coli*, respectively. -Films containing *Amaranthus* leaf extract showed increased protection against UV light -Incorporation of *Amaranthus* leaf extract reduced the water solubility and water vapor permeability of films -Incorporation of *Amaranthus* leaf extract improved the mechanical properties of films - *Amaranthus* leaf extract changed the color at basic pH	(Kanatt, 2020)

		due to the presence of betalains (1-6: pink, 7-8: light pink, 9: yellow)	
Clove oil-coated low-density polyethylene (LLDPE) films	Clove oil	-Clove oil coating improved the UV-barrier property of LLDPE films -Incorporation of clove oil exhibited a strong antimicrobial effect in films against *Salmonella typhimurium* and *L. monocytogenes*. Films with clove oil reduced the initial microbial load of both bacteria in minced chicken samples by 2-3 log cycles after one day compared to neat films. In minced chicken samples, the growth of these bacteria was completely restricted on the 5^{th} day of storage, and there was no further growth until the 21^{st} day of the storage period -Chromic acid treatment of LLDPE films created a porous surface on films which allowed the successfully coating with clove oil	(Mulla et al., 2017)

		-There was no change in mechanical properties due to the incorporation of clove oil. However, melting and crystallization temperatures of films were decreased due to the plasticization effect of clove oil	
Oregano oil incorporated whey protein-based films	Oregano oil	-Films with 1% and 1.5 % oregano oil showed the highest antimicrobial effect against *Penicillium commune* compared to control and films with 0.5% oregano oil based on the diameter of the zone of inhibition. Control and films with 0.5%, 1%, and 1.5% oregano oil showed 0.00 ± 0, 0.07 ± 0.10, 1.3 ± 0.8, and 1.7 ± 0.5 cm inhibition zones respectively. CFU/ml was reported as 5.13×10^5, 3.17×10^5, 3.14×10^5, and 1.98×10^5 for the control and films with 0.5%, 1%, and 1.5% oregano oil. -Highest tensile strength has resulted in the films with 1% oregano oil	(Oliveira et al., 2017)

		-Compared to control, 1.5% oregano oil film showed a lower water solubility. However, water vapor permeabilities of all the films containing oregano oil were higher than control	
Chitosan films with cinnamaldehyde	Cinnamaldehyde	-Incorporation of cinnamaldehyde enhanced the antimicrobial activity against *S. aureus*, *E. coli*, and fungus *Candida albicans*. Further, *C. albicans* showed a higher inhibition effect compared to bacteria by resulting in larger inhibition areas at 1.5 and 2.0 ratio of cinnamaldehyde carbonyl groups to chitosan amino groups compared to control -Incorporation of cinnamaldehyde improved the UV barrier properties of films -Cinnamaldehyde release from the film was affected by the polarity of the medium and the amount of cinnamaldehyde in the emulsion	(Chen et al., 2016)

	-The mechanical properties and water barrier properties depended on the amount of cinnamaldehyde

2.3. Role of nanotechnology in food packaging

2.3.1. Different nanomaterials used in food packaging

The introduction of nanofillers into bio-composite packaging films exhibits an increasing demand in the current packaging industry. This is mainly to overcome the drawbacks such as poor mechanical, thermal, and barrier properties of biopolymers (Zubair & Ullah, 2020). Moreover, nanofillers can give additional functionalities to packaging films, such as antimicrobial, biosensor, and oxygen scavenging functions (Vilarinho et al., 2018). In composite films, nanofiller dispersion and interfacial interaction between the polymer matrix and nanofiller can be introduced as the most crucial factors that decide the overall properties of the nanocomposite. Therefore, uniformly dispersed nanofillers in the polymer matrix should be obtained to form a percolation network with a better reinforcing effect (Kwak et al., 2020). Nanofillers used in food packaging applications can be classified under several groups, and figure 2 illustrates the classification of nanofillers under several categories

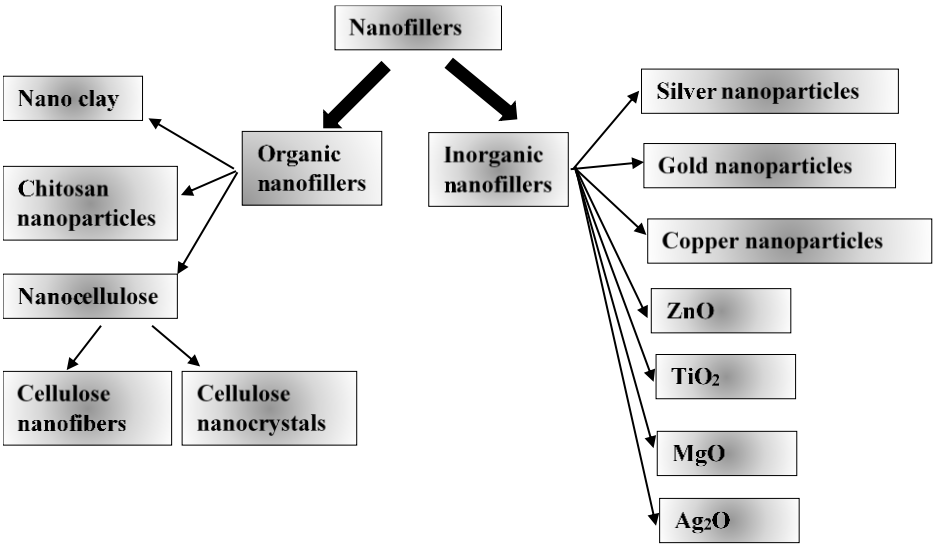

Figure 2. Classification of nanofillers used in several food packaging materials

2.3.2. Nanocrystalline cellulose

Cellulose nanoparticles (CNs) or nanocellulose (NC) are the most common biodegradable reinforcing material used in food packaging applications. NC exhibits remarkable mechanical properties and contains several surface functional groups supporting modifications and cross-linking with other materials. Moreover, high aspect ratio, biodegradability, and biocompatibility increase its applications in various valuable products (Liang et al., 2020). Production of cellulose nanoparticles involves two main steps: pre-treatment of raw materials and fibrillation. Purified-individual cellulose fibers will be obtained from the pre-treatment step to obtain cellulosic fibers. Different pre-treatments are applied based on the source of raw material and desired size and morphology of the final CNs. The material structure is opened to facilitate access to the cellulose microstructure during the fibrillation step. Maintaining a desired degree of polymerization should also be considered when pre-treating the raw materials. In the fibrillation step, cellulosic fibers are transformed into cellulose nanofibrils (CNFs) or cellulose nanocrystals (CNCs) using separate or combined mechanical treatment, acid hydrolysis, or enzymatic hydrolysis. Wood fiber or other cellulosic materials are acid-hydrolyzed to obtain CNCs 3-20 nm wide and 50-500 nm in length (Vilarinho et al., 2018). CNCs are also called NCC or cellulose whiskers, and they are rod-like, highly crystalline solids in which amorphous regions are solubilized by acid hydrolysis (Liang et al., 2020). CNF can be produced from the same raw materials using mechanical processes without chemical and biological treatments. CNFs are 4-50 nm wide and longer than 500 nm of linear or branched chains (Vilarinho et al., 2018).

Zhao et al. (2020) evaluated the mechanical and barrier properties of chitosan (cationic polymer) and alginate (anionic polymer) films prepared by blending and layer by layer methods. The authors reported that the concentration of CNF (0%, 1%, 3%, 5%, 7%, and 9%) affected the films' tensile strength, water barrier properties, and opacity. A gradual linear increase of turbidity values of alginate films ($p<0.05$) could be observed with the increase in CNF concentration due to the high aspect ratio of CNFs. In chitosan films, a dramatic increase in turbidity ($p<0.005$) values could be seen initially with the increase of CNFs concentration. However, further increase of the CNF's concentration decreased the turbidity values due to the aggregation at higher concentration. In terms of water vapor permeability, all the films showed a decreased tendency towards water vapor permeability with increased CNFs concentration (except chitosan films prepared by blending method). This can be due to the increased tortuosity and passage length of water molecules. The

lowest water vapor permeability was observed in layer-by-layer prepared chitosan films with 7% CNF. This result was attributed to the electrostatic interaction between the adjacent chitosan and CNFs. The loosely packed poriferous due to the staked accumulation of CNF and CNF aggregation leads to the increase of water vapor permeability of chitosan films made by the blending method with the increase in CNF concentration. Due to the high stiffness caused by CNF, alginate films showed an increase in tensile strength with increased CNF concentration. Moreover, the tensile strength of blending films was elevated than layer-by-layer films due to the better compatibility between CNF and alginate. Chitosan films prepared using the layer-by-layer method showed increased tensile strength with the increase of CNF concentration (except 1% CNF). This was explained by the strong electrostatic interaction between chitosan and CNF that facilitates a compact structure. On the other hand, chitosan films prepared from the blending method showed a negative effect on tensile strength with CNF concentration. The authors suggested that CNF at 1% has started to produce insufficient interactions between CNF and chitosan, leading to inhomogeneous and fragile films (Zhao et al., 2020).

In another study (de Souza Coelho et al., 2020), starch-based nanocomposite films were developed by adding CNCs obtained from grape pomace. Acid hydrolysis of grape pomace resulted in CNCs used in 1, 2, 5, 10, and 15g/100g of starch concentration in film formulation. The lowest water vapor permeability was observed in 1% and 2% CNCs concentrations, where the control treatment showed the significantly highest water vapor permeability compared to other treatments regardless of CNC concentration. It was assumed that CNC had been well dispersed in the starch matrix at 1% and 2% CNC concentration leading to the tortuosity of water molecules in the starch matrix. As discussed by the authors, the further increase of CNC resulted in the aggregation of nanostructures, leading to enhanced water vapor permeability. There was a significant increase in tensile strength of 5, 10, and 15% CNC films compared to 1 and 2%. However, there was no significant difference between control and 1 and 2% CNC films. In terms of elongation at break, there was a significant difference between control, films with small concentrations of CNC (1%, 2%), and films with a high concentration of CNC (5%, 10%, 15%). The highest elongation at break value resulted from the control and smaller concentrations than high concentrations. The decrease in elongation at break with the increase in CNC concentration is due to the restriction of movement of the starch matrix because of the promotion of strong interactions between cellulose and starch due to their similarities in chemical structures. On the other hand, young modulus significantly

increased with 5, 10, and 15% CNC films, compared to 1 and 2% CNC films and control. This could be due to the reinforcement effect caused by high CNC concentrations resulting from strong H bond interactions between CNC and starch molecules. By considering these results, authors have reported that mechanical reinforcement of films was caused by a 5% CNC concentration (de Souza Coelho et al., 2020).

2.3.3. Limitations of nanocrystalline cellulose

In the elementary cellulose unit, H bonds are formed between hydroxy groups of glucopyranosyl unit and oxygen of adjacent units. These junctions between the NC chains are crucial in transferring mechanical loads among chains to result in mechanically robust materials. H-bonds and van der Waals forces are the dominant bonds that cause strong interactions between CNC particles. The hydrophilicity of NC has become a significant issue in NC-related applications due to the penetration of water molecules through the junctions between the NC units in the elevated ambient humidity or direct contact with water. As a result of this process, water molecules compete for interfibrillar hydrogen bonding and cause higher motion of chains, thereby drastically reducing mechanical strength. Several methods have been introduced to decrease the hydrophilicity of NC, such as cross-linking and surface modifications, including acetylation, carboxymethylation, ionic complexation, and covalent grafting (Liang et al., 2020). The cross-linking process results in a functional modification to NC without disintegration to improve stability and hydrophobicity. Even though chemicals such as toluenesulfonic acid, hexanoic acid, butyryl, benzoyl, naphtoyl, diphenyl acetyl, and stearoyl are popular as chemical cross-linkers, attention toward the environmentally friendly, non-toxic cross-linkers such as citric acid and succinic acid has been increased (Herrera et al., 2017).

2.3.4. Different methods of modification of nanocrystalline cellulose

Several surface modification techniques have been reported in the literature to overcome the high hydrophilicity and accumulation of nanomaterials. The most prominent methods are esterification, silylation, etherification, oxidation, and polymer grafting. Moreover, non-covalent surface modifications such as adsorption of surfactants and polymer coating have also been reported. However, one of the significant limitations associated with surface modification is limiting the

modification only to the surface without damaging the integrity of the crystal (Tajul Islam et al., 2007).

NC aggregation is known as hornification due to the H bonds between abundant surface OH- groups. Esterification with carboxylic acids is a hydrophobic modification method where it increases the hydrophobicity of the final product due to the introduced fatty acids. Also, esterification limits the agglomeration by reducing available surface OH- groups (Rusmirović et al., 2017). Yoo and Youngblood (2016) used a green one-pot method to esterify NCC using fatty acids, biodiesel, and plant oils. An aqueous lactic acid syrup was used as the reactive solvent, which was re-usable. In another study, a pyridine/ toluenesulfonyl chloride system was used to esterify CNF with OA at 50 °C for 4 hours. Esterified CNF showed improved tensile and Young's modulus values, thermal stability, and water barrier properties (Almasi, Ghanbarzadeh, Dehghannya, et al., 2015). Likewise, this method has been reported in a few other studies where the properties of NC have been improved after modification (Chen et al., 2020; Rusmirović et al., 2017; Uschanov et al., 2011).

Silylation is a chemical reaction where the silyl groups are reacted with NC to enhance the compatibility with non-polar compounds (Almasi, Ghanbarzadeh, Dehghannya, et al., 2015). Widely used silylation agents are

3-aminopropyltriethoxysilane (APS), methacryloxypropyl-trimethoxysilane (MPS) and diethylenetriaminopropyltrimethoxysilane (TAS) (Indarti et al., 2019). Due to the high affinity between OH- groups and silane coupling agents, there is a potential for loss of original morphology of NC due to the high degree of silation that ultimately results in crystal disintegration. Therefore, attention should be given to maintaining the reaction in an optimum degree of silylation where cellulose morphology is preserved (Ghasemlou et al., 2021). Peresin et al. (2017) used APTES ((3-Aminopropyl)triethoxysilane)-chemistry strategy to modify the surface of CNF films. A UV Ozonator activated both sides of the CNF films before surface modification. This surface modification resulted in a significantly thinner layer on a nanometer scale compared to layers obtained from conventional coating or laminating techniques. The films showed significantly lower oxygen permeability and hydrophilicity than reference CNF film without surface modifications (Peresin et al., 2017).

Hydroxypropyl methylcellulose is also water-soluble, odorless, tasteless, generally recognized as a safe cellulose derivative, and produced by etherification of alkaline cellulose with methyl

chloride and propylene oxide. These reactions substitute hydroxyl groups with methoxyl and hydroxypropyl groups, respectively. The substitution degree is the average number of replaced hydroxyl groups by methoxyl groups in an anhydrous glucose unit. Studies have proved a correlation between the chemical structure of the hydroxypropyl methylcellulose and the physical and mechanical properties of films made from modified NC (Otoni et al., 2018). Therefore, methylcellulose is a promising cellulose derivative raw material made from the etherification of NC. As summarized by Cazón et al. (2017), films made from hydroxypropyl cellulose and methylcellulose are efficient oxygen, carbon dioxide, and lipid barriers but with high water vapor permeability. On the other hand, methylcellulose and hydroxypropyl methylcellulose form films with better thermal resistance properties. Crosslinking is a better solution for this issue as it results in rigid structures by strong covalent attachment onto the surface of the polymer. Thereby, crosslinking enhances the films' water resistance and mechanical strength (López de Dicastillo et al., 2016).

TEMPO (2,2,6,6- Tetramethylpiperidine-1-oxyl) mediated oxidation of NC results in conversion of surface hydroxyl groups of NC to carboxylic groups that results in increased surface charge. As a result, individual NC repels each other and properly exfoliates. This conversion can improve the compatibility of NC in the polymer matrix to act as a reinforcing agent (Mahendra et al., 2020). Even though several approaches have been reported in the literature based on TEMPO mediated oxidation (Buffa, Grela, Aranguren, & Mucci, 2016; da Silva Perez, Montanari, & Vignon, 2003; Fraschini, Chauve, & Bouchard, 2017; Indarti, Marwan, Rohaizu, & Wanrosli, 2019; Lin, Bruzzese, & Dufresne, 2012; Mahendra et al., 2020), a limited number of research studies have been reported on their use in food packaging (de Castro et al., 2018). This method is becoming popular due to its selective oxidation of surface hydroxyl groups, fast oxidation, and better-controlled nature (da Silva Perez et al., 2003). Other than oxidizing the surface OH- group by methods such as TEMPO-mediated oxidation, carboxylation of NC can also be achieved by chemical grafting or physical adsorption of carboxyl group onto the NC surface and oxidation of OH- groups by breaking down the bond between C2 and C3 (Chu et al., 2020).

In a study by Kwak et al., 2020, gelatin films' tensile properties and barrier properties were enhanced using di-aldehyde NC. Periodate oxidation reaction, which shows a strong selectivity and absence of significant side reactions, was used to modify the surface of CNCs to obtain di-aldehyde cellulose nanocrystals (D-CNC). Periodate oxidation selectively converts two secondary

hydroxyl groups in the C2 and C3 positions of the anhydrous glucose units into aldehyde moieties to produce D-CNC. Interestingly, all the D-CNC films showed significantly higher tensile strength and young's modulus values than chemically un-modified CNC films with 20% CNC, while the other way around for the elongation at break. This was attributed to the ability of covalent cross-linking and the physical reinforcement effect of aldehyde-functionalized NC. Furthermore, the water insolubility and morphological stability of the D-CNC films also resulted from the covalent-based cross-linking reaction between the aldehyde group of D-CNCs and the amine group of gelatins, which resulted in the formation of Schiff base (Kwak et al., 2020).

Polymer grafting is another way of reducing the hydrophilicity of NC by grafting hydrophobic groups. In a way, esterification, silylation, and etherification reactions can be introduced as polymer grafting techniques where covalent bonding involves. Apart from that, surface physical adsorption is another green and cost-effective way to reduce H bonds between intermolecular OH-groups of NC by adsorption of hydrophobic compounds using H bonds, electrostatic interactions, and Van der Waals interaction (Chu et al., 2020). However, surface adsorbed NC can flocculate due to the hydrophobic interactions between hydrophobic groups of the surfactants used for the adsorption. In addition, reduced electrostatic repulsion between NC due to the neutralization by cationic surfactants also could be a reason for the flocculation. Anionic surfactants are used to overcome this issue as they increase NC's surface charge, resulting in repulsion forces. On the other hand, hydrophobicity is also enhanced due to the absence of hydrophobic interactions, which hinders the hydrophobic groups (Xie & Liu, 2021).

Negatively charged groups such as sulfates and carboxylates are introduced to NC's surface to support the easy dissociation from raw materials and the formation of a stable dispersion due to the electrical repulsion. When the negatively charged NC is added to the negatively charged matrix, electrostatic repulsion facilitates the homogeneous suspension, even morphology, and further contribution to film strength. However, the agglomeration of NC itself is a major issue in reinforcing films with high concentrations of NC. On the other hand, when NC is added to the oppositely charged matrix, electrostatic attractions facilitate the compact structure between NC fillers and polymer. However, the excess amount of fillers causes heterogeneous suspension and thereby unsatisfied film properties and compact films due to serious agglomeration between matrix and NC. Therefore, the amount of nanofillers added to film formulation is an important factor in obtaining the film's desired properties (Zhao et al., 2020).

Chapter 3: Reinforcing canola protein matrix with chemically tailored nanocrystalline cellulose improves the functionality of canola protein-based packaging materials

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Dissanayake, T., Peng Chang, B., Mekonnen, T. H., Senaka Ranadheera, C., Narvaez-Bravo, C., & Bandara, N. (2022). Reinforcing canola protein matrix with chemically tailored nanocrystalline cellulose improves the functionality of canola protein-based packaging materials. Food Chemistry, 383, 132618. https://doi.org/10.1016/J.FOODCHEM.2022.132618

3.1. Abstract

Canola protein derived from the canola industry byproduct is a potent biopolymer source to develop sustainable food packaging materials, but it has limitations due to its poor mechanical and barrier properties. Nanomaterials such as NCC have shown promising potential in improving material properties. The current study aimed to enhance the functionality of canola protein-based films using TEMPO ((2,2,6,6-Tetramethylpiperidin-1-yl)oxyl) modified nanocrystalline cellulose (TM-NCC). TEMPO modification was performed using TEMPO/NaClO/NaBr based oxidation. Modified and unmodified nanocrystalline cellulose (U-NCC) were used at different weight ratios to prepare the films. TEMPO-mediated oxidation converted 19.61 ± 3.53 % of primary –OH groups into –COOH groups. The addition of U-NCC and TM-NCC significantly increased the tensile strength reporting the highest value of 8.36 ± 0.85 MPa for 5% TM-NCC, which was only 3.43 ± 0.66 MPa for control films. Interestingly, both U-NCC and TM-NCC enhanced the films' water barrier and thermal properties compared to control.

Keywords: Canola protein, food packaging, chemical modification, TEMPO, nanocrystalline cellulose, tensile strength

3.2. Introduction

Among the potentially available biopolymer sources for packaging applications, canola protein holds a unique position due to its higher availability at a low cost (Bandara et al., 2018; Yachuan Zhang et al., 2018). Canola (*Brassica* spp.) is the world's second-largest oilseed crop grown after soybean. Even though the canola seed contains 17-26% (dry matter basis) of protein content, its use in human food products is limited due to antinutritional factors (Bandara et al., 2018). Canola meal (around 60% w/w of seed after oil extraction) is the primary byproduct of the oil recovery process and is mainly used in animal feed formulations (Bandara et al., 2018) and contains 37% of protein content (Yachuan Zhang et al., 2018). Few studies have been reported on developing canola protein-based packaging films using extracted canola protein isolate from the canola meal (Chang & Nickerson, 2014, 2015; Li et al., 2017; Osorio-Ruiz et al., 2019; Shi & Dumont, 2014). Therefore, canola protein can be introduced as a sustainable source with a proven film-forming ability for packaging applications due to its high availability at a low cost compared to other proteins (Bandara et al., 2018; Yachuan Zhang et al., 2018). However, similar to other proteins and many other biopolymers, canola proteins tend to produce packaging films with brittle, hydrophilic, and poor tensile properties (Yachuan Zhang et al., 2018). The studies mentioned above have used several techniques to address these issues, including the use of plasticizers (Chang & Nickerson, 2014, 2015; Shi & Dumont, 2014), crosslinking agents (Chang & Nickerson, 2014; Li et al., 2017), denaturant agents (Shi & Dumont, 2014), and nanomaterials with various degrees of success (Osorio-Ruiz et al., 2019).

Even though published studies reported the potential of canola protein to fabricate films, the reported values for mechanical strength were still not comparable to petroleum-based packaging, indicating further improvements are required. On the other hand, the use of nanomaterials plays a significant role in food packaging, but to the best of our knowledge, the use of nanomaterials for improving the properties of canola protein-based films has been not reported in the literature except one study with limited success (Osorio-Ruiz et al., 2019). Moreover, modification of NCC has proved the improvement in NCC's properties, but the use of modified NCC in protein-based food packaging is also limited. Therefore, further studies are needed targeting novel techniques and modified ingredients to further the development of the film properties.

Regardless of NCC's reinforcing properties, the abundance of –OH groups on the surface of the NCC accelerate the agglomeration of NCC in the polymer matrix resulting in poor exfoliation (W.

Jiang et al., 2020). Therefore, surface modifications of NCC can be used to improve interactions between NCC and polymer matrices, thereby improving exfoliation. Researchers have used a wide range of techniques including, but not limited to, esterification/acetylation (Le et al., 2020), silylation (Indarti et al., 2019), oxidation (Buffa et al., 2016; Habibi et al., 2006), and polymer grafting (Soeta et al., 2020) for surface modification of NCC. TEMPO ((2,2,6,6-Tetramethylpiperidin-1-yl)oxyl) mediated oxidation of NCC converts the primary –OH groups present on the surface of NCC into –COOH groups (Buffa et al., 2016; Fraschini et al., 2017). Compared to other modification methods, TEMPO-mediated oxidation can be performed relatively quickly and efficiently using minimal chemicals. Most importantly, TEMPO selectively oxidizes primary hydroxyl groups of NCC in the presence of secondary hydroxyl groups. Therefore, based on the application, the degree of oxidation can be controlled by changing experimental conditions (Buffa et al., 2016; Da Silva Perez et al., 2003; Fraschini et al., 2017). Until today, there are no published scientific studies based on the use of TEMPO modified NCC (TM-NCC) in canola protein-based films to the best of our knowledge. In this study, we hypothesize that TM-NCC will increase the functional properties of canola protein-based packaging films due to an improvement in the exfoliation of NCC and the subsequent nano-reinforcement. Therefore, this study's objectives aimed to modify the NCC using TEMPO mediated oxidation, exfoliate both TM-NCC and unmodified NCC (U-NCC) at different addition levels to prepare the canola protein-based films and characterize their mechanical, thermal, and surface morphology properties.

3.3. Materials and methods

3.3.1. Chemicals and materials

NCC was donated for research purposes by InnoTech Alberta, Edmonton, AB, Canada, and Richardson International (Lethbridge, AB, Canada) donated the canola meal. For the antimicrobial study, two bacterial strains, including *Listeria innocua* (NRRL 33314 - as a representative of the Gram-positive group) and *Escherichia coli* (ATCC 11775 – as a representative of the Gram-negative) were sourced from the Department of Food and Human Nutritional Sciences, University of Manitoba bacterial culture collection. NaOH (≥ 97.0% purity) and HCl were purchased from ACS chemicals. TEMPO (98.0% purity) was purchased from TCI America, and NaBr (99.5% purity) was purchased from Acros Organics. NaClO (14.5% available chlorine) and trans-

cinnamaldehyde (≥ 98.0% purity) were purchased from Alfa Aesar. Anhydrous ethanol and glycerol (≥ 99.5% purity) were purchased from Ricca Chemical and Fisher chemicals. MacConkey agar, Listeria selective agar, and brain heart infusion (BHI) broth were purchased from Thermo Fisher Scientific (Oxford brand), and 3.5 kDa dialysis tubes (snakeskin) were purchased from Thermo Scientific.

3.3.2. Canola protein extraction

Protein was extracted from the canola meal according to the method described by Manamperi et al., (2010) with slight modifications. First, the canola meal was ground and passed through a 0.5mm screen using Hosokawa milling and classifying system (Hosokawa Micron Powder Systems, Summit, NJ, USA). Then the ground meal was mixed with mili-Q water in a 1:10 (w/v) ratio. The pH of the slurry was adjusted to 12.0 using 3 M NaOH a stirred for 30 min at 300 rpm at room temperature to solubilize the proteins. Non-protein components were precipitated and separated by centrifugation (Beckman Coulter floor-stand centrifuge, 10000 g, 15 min, 4 ^{o}C), and the solubilized protein in the supernatant was collected. The pH of the supernatant was readjusted to 4.0 to precipitate the protein again, and the resulting precipitate was collected by centrifugation at 10000 g for 15 min in 4 ^{o}C. Precipitated protein was freeze-dried using a semi-pilot plant freeze dryer (SP VirTis, Oakville, ON.) and stored at -20 ^{o}C until further use. The proximate analysis of extracted protein reported 81.07 ± 0.85 % (DWB, dry weight basis) of protein content.

3.3.3. TEMPO mediated oxidation of NCC

Surface oxidation of NCC was performed using TEMPO/ NaBr/ NaClO system according to the methods described by Buffa et al. (2016) and Fraschini et al. (2017) with slight modifications. An aqueous suspension of NCC (61.68 mmol of anhydrous glucose units) was prepared by mixing 10 g of NCC with 200 ml of de-ionized water and sonicated for 30 min (at 15s intervals for every 30s in an ice bath) at 60% amplitude, 20 kHz frequency and 500 W power output (Fisher Brand Model 505 probe sonicator, Fisher Scientific, Newton, Connecticut, USA). TEMPO (107.06 mg, 0.69 mmol) and NaBr (1.27 g, 12.35 mmol) were separately dissolved in 200 ml of de-ionized water under constant magnetic stirring at 500 rpm. Then, previously prepared NCC, TEMPO, and NaBr solutions were mixed while stirring at 900 rpm. In a separate beaker, NaClO solution (NCC: NaClO molar ratio = 1: 2.5)) was prepared, and pH was adjusted to 10 using 1 M HCl. Then NaClO

solution was added to the NCC/TEMPO/NaBr suspension slowly while maintaining the pH at 10-10.5 using 0.5 M NaOH solution. The reaction was considered finished when the pH was constant (for 1 minute) without further consumption of NaOH (~1.5 hr), and 20 ml of absolute ethanol was added to quench the reaction. Following the termination of the reaction, the pH of the solution was adjusted to 7 using 0.5 M HCl and kept for another 15 min under stirring. The resulting suspension was dialyzed for five days using a 3.5 kDa dialysis tube against excess de-ionized water with frequent water changes. After dialysis, the final product was sonicated for 3 min (15s intervals for every 30s), freeze-dried, and stored at -20 ºC until further use.

3.3.4. Degree of oxidation (DO) of NCC

The degree of oxidation (DO) or percentage of converted primary –OH groups into –COOH groups were calculated based on the conductometric titration method previously described by Da Silva Perez et al. (2003). A TM-NCC suspension was prepared by dispersing around 30 mg of oven-dried TM-NCC in 30 ml of 0.1 M HCl followed by sonication for 3 min to improve dispersion. Prepared dispersion was titrated against 0.1 M NaOH, and conductivity (mS/cm) was measured using a conductivity meter (Model 06-662, Fisher Scientific, Pittsburgh, PA, USA). The titration curve was used to estimate NaOH volume spent to react with –COOH groups (V_2-V_1). Equation (1) was used to calculate the DO/–COOH group content.

$$DO = \frac{162 \times C \times (V_2 - V_1)}{w - [36 \times C \times (V_2 - V_1)]} \qquad (1)$$

Where C = NaOH concentration (mol/dm^{-3})

V_1 and V_2 = NaOH (l) amount corresponding to carboxyl groups

w = weight of the oven-dried sample (g)

3.3.5. Fabrication of canola protein films reinforced with U-NCC and TM-NCC

Films were fabricated using the solvent casting method based on the method described by Agarwal et al. (2020) with slight modifications. For the film-forming solution, 3.5g of canola protein isolate (5% w/v protein of final distilled water volume) was dispersed in 50 ml of distilled water, and pH was adjusted to 10 using 0.5 M NaOH. The resulting dispersion was stirred for 30 min (650 rpm at room temperature), heated in a water bath (Model 1227, VWR International, Cornelius, OR, USA) at 70 °C, 100 rpm for 20 min, cooled down to room temperature, and glycerol was added to the solution as a plasticizer at a ratio of 1:0.5 (w/w of protein: glycerol). Then, the canola

protein/glycerol dispersion was homogenized (Fisher brand 850, Fisher Scientific, ON, Canada) at 14000 rpm for 5 min. Next, the nanomaterial samples at different addition levels (TM-NCC or U-NCC at 0, 1, 3, 5 % w/w of protein) were separately dispersed in 20 ml of de-ionized water, sonicated for 2 min at 60% amplitude, 20 kHz frequency, and 500 W power output, and slowly added to the previously prepared canola protein/glycerol dispersion while stirring. Then, the nanomaterials and cinnamaldehyde (0, 1% w/w of protein) were added into canola protein/glycerol dispersion in two steps where the dispersion was homogenized at 14000 rpm for 5 min after the addition of each compound. In the end, sonication was carried out again for 5 min at 60% amplitude, 20 kHz frequency, and 500 W power output. The resulting dispersion was named as "film-forming solution" and used in fabricating films. The film-forming solution was cast on hexagonal polystyrene dishes (0.43 ml/cm^2), air-dried for 2 days, and films were peeled off. After that, the films were conditioned at 23 °C and 50% relative humidity (RH) for 48 hrs prior to their mechanical and chemical characterizations.

3.3.6. Characterization of NCC (U-NCC and TM-NCC) and packaging films

3.3.6.1. Effect of TEMPO-mediated oxidation on surface functional groups of NCC and impact of NCC addition on protein secondary structure

Fourier transformed infrared spectroscopy (FTIR) was performed for the initial NCC powder (U-NCC, TM-NCC) samples to characterize the impact of TEMPO mediated oxidation on their surface functional groups and for film samples to understand the impact of U-NCC and TM-NCC on protein secondary structure. Dried nanomaterials/film samples were scanned using an ATR-FTIR (Nicolett 6700, Thermo Electron Inc, Madison, WI, USA), and IR spectra in the range of 400-4000 cm^{-1} were collected using 120 scans at a resolution of 4 cm^{-1}. The collected IR spectra were analyzed using Origin Pro 2021 software (OriginLab Corporation, Northampton, MA, USA). The effect of NCC on protein secondary structures was analyzed by 2nd derivative method followed by peak fitting.

3.3.6.2. Impact of TEMPO-mediated oxidation on the crystallinity and exfoliation in the protein matrix

X-ray diffraction (XRD) was used to study the crystallinity of initial NCC samples (U-NCC and TM-NCC) and their exfoliation properties. A Siemens/Bruker X-ray diffractometer (D5000, BrukerNano Inc, Madison, WI, USA) equipped with a Cu X-ray tube (configured to Bragg-Brentano reflection geometry) was used to collect diffraction angle at a 2θ range of 5-50 degrees using 0.02° step size and 1s step time. Background subtraction and data processing were conducted using Jade software (Materials Data and the International Centre for Diffraction Data, Newtown, USA). Data visualization and graphing were done using Origin 2021 software (OriginLab Corporation, Northampton, MA, USA).

3.3.6.3. Impact of U-NCC and TM-NCC on the surface morphology

Field emission scanning electron microscopy (FESEM) was used to study the impact of NCC addition and TEMPO-mediated oxidation of NCC on surface morphological properties of canola protein-based films. A 1 cm × 1cm film sample was cut from each film, and 60% gold (Au) and 40% lead (Pd) was coated using a Denton vacuum. SEM images were collected using a FESEM (Quanta 650 FEG, FEI Company, Hillsboro, OR, USA) at ×2000 magnification.

3.3.6.4. Mechanical properties of the nano-reinforced canola protein-based films

Canola protein films were conditioned at 50% RH, 23 °C, for 48 hrs before the experiment according to the requirement of ASTM D882 (ASTM D882-18, 2018) standards. Tensile strength, elongation at break, and elastic modulus were studied using Shimadzu AGS-X Universal Tester (Shimadzu America INC, Columbia, MD, USA) coupled with a 500 N load cell. The test was performed at a crosshead speed of 5 mm/min using samples with a dimension of 50 (l) x 9.5 (w) x 2.5 (t) mm according to ASTM D882 (ASTM D882-18, 2018). At least five specimens per film were tested, and the mean ± standard deviation (SD) of the tensile strength, elastic modulus, and elongation at break were recorded to analyze the mechanical properties.

3.3.6.5. Water vapor permeability (WVP)

WVP of the prepared films was studied gravimetrically according to the method described by ASTM E96/E96M-16 with slight modifications (ASTM E96/E96M-16, 2016). Prepared

packaging films were conditioned at 50% RH and 23 °C at least for 48 hrs before the experiment, as per the ASTM standards. Disposable cups with a 4.24 cm mouth diameter and 4.02 cm height were filled with distilled water up to ¾ of the cup. Canola protein films were placed on the top of the cup after applying vaseline on the circle edge of the cup. The weight of the films with the cup and water was measured and kept in a controlled environmental chamber (50% RH, 23 °C). After 24 hrs, the weight of the films with the cup and the remaining water was measured. WVP of the films was then calculated using Equation (2).

$$WVP = \frac{Weightchange(g) \times thicknessofthefilm(mm)}{Areaofthecupmouth(m^2) \times vaporpressuredifference(kPa) \times time(hours)} \quad (2)$$

3.3.6.6. Surface hydrophobicity using contact angle analysis

The water contact angle between canola protein films and distilled water was measured to understand the U-NCC and TM-NCC induced surface hydrophobicity changes using a contact angle goniometer (Model No: 200-00-115, Rame-hart Inc, Mountain Lake, NJ, USA) along with DROPimage Standard software. For every sample, 10 measurements were taken at a time interval of 0.001 s, and the average of the acceptable angles was taken for the analysis.

3.3.6.7. Water solubility

The effect of nanomaterials on the water solubility of the canola protein-based films was studied according to a test method described by González et al. (2019) with slight modifications. First, prepared films were cut into 2 cm × 2 cm and placed in a drying oven at 105 °C for 24 hrs to obtain the dry weight ($W_{\text{initial dry weight}}$). Then the film samples were immersed in a falcon tube filled with 50 ml of distilled water and placed in a water bath while stirring at 100 rpm at 25 °C for 24 hrs. The soaked samples were again kept in a drying oven at 105 °C for 24 hrs, and the final dry weight ($W_{\text{final dry weight}}$) was measured. Finally, the water solubility percentage of the films was measured using Equation(3) shown below.

$$Water\ solubility\ \% = \left(\frac{W_{initial\ dry\ weight} - W_{final\ dry\ weight}}{W_{initial\ dry\ weight}}\right) \times 100 \quad (3)$$

3.3.6.8. Thermal properties

Thermogravimetric analysis (TGA) was performed for powder (U-NCC, TM-NCC) and film samples to evaluate the thermal degradation behaviors using TGA-2 system (Mettler Toledo,

Mississauga, ON, Canada). Approximately 5-10 mg of samples were heated up to 600 °C at a 10 °C/min rate under a constant flow nitrogen environment (30 mL/min). The thermal gravimetric curves and the first-order derivative gravimetric curves were obtained and analyzed using Origin Pro 2021 software (OriginLab Corporation, Northampton, MA, USA).

3.3.6.9. Impact of U-NCC and TM-NCC on antimicrobial properties of cinnamaldehyde

The effect of TEMPO modification on the antimicrobial activity of cinnamaldehyde in the films was evaluated using the disk diffusion assay method as previously described by Agarwal et al. (2020) with slight modifications as described below. *Listeria* selective agar (Oxford formulation) and MacConkey agar (Oxford formulation) Petri dishes were prepared according to the manufacturer's instructions. *E. coli* and *L. innocua* stock cultures were inoculated into BHI broth and incubated at 37 °C for 24 hrs. After incubation, 100 µl of inoculated broth was spread on polystyrene agar plates (Listeria selective agar and McConkey agar) using cotton swaps. Films were cut into disk shapes (1 cm in diameter), and disk-shaped film samples were placed on the middle of the petri dish with inoculated culture medium. Then, the Petri dishes with film samples were incubated for 48 hrs at 37 °C, and the zone of inhibition diameter was measured. The antimicrobial activity of the films was calculated in terms of the diameter of the zone of inhibition as it is relatively proportional to the antimicrobial activity.

3.3.7. Statistical analysis

Data collected was analyzed using a two-way ANOVA ($p < 0.05$) followed by a Tukey test for mean separation using Minitab 2021 software (Minitab LLC, State College, Pennsylvania, USA). The figures and illustrations were created using Origin 2021 software (OriginLab Corporation, Northampton, MA, USA). NCC and cinnamaldehyde percentages were considered as the two factors with 7 (0%, U-NCC 1%, U-NCC 3%, U-NCC 5%, TM-NCC 1%, TM-NCC 3%, TM-NCC 5%) and 2 (0%, 1%) levels respectively. All the treatments (films) were triplicated, and data were reported as mean ± SD.

3.4. Results and Discussion

3.4.1. Characterization of unmodified and TEMPO modified NCC

TEMPO-mediated oxidation selectively converts the primary –OH groups on the NCC surface into –COOH groups through an aldehyde intermediate in a reaction medium where NaClO acts as the primary oxidant and NaBr acts as the co-oxidant/co-catalyst during the reaction (Calderón-Vergara et al., 2020; Li et al., 2015). At the end of the conversion, the surface charge of the NCC increases as a result of the introduction of new –COOH groups to the surface that is present in the form of sodium salts of carboxylate ions (Calderón-Vergara et al., 2020). Figure 3(a) illustrates the oxidation mechanism of NCC through TEMPO/NaClO/NaBr reaction system. In the present study, the NCC: NaClO molar ratio of 1:2.5 was selected for the modification as reported with a previously optimized study by Buffa et al. (2016). They observed high crystallinity and high DO for modified NCC after TEMPO-mediated oxidation. The conductometric titration was used to estimate the DO, and the titration curve is shown in Figure 3 (b), and the calculations are shown in supplementary materials. In the present study, the DO was calculated to be 19.61 ± 3.53 % after the oxidation process, indicating that 19.61 ± 3.53 % of primary –OH groups were converted into –COOH groups. These DO values achieved in this study are comparable with the results reported in previous studies. Buffa et al. (2016) reported DO of 26.60% and 19.83% for oxidized samples at the NCC: NaClO ratio of 1: 1.25 and 1:1, respectively. Habibi et al. (2006) observed an increase in DO% from 2.9% to 9.7% when NCC: NaClO molar ratio was increased from 1: 0.06 to 1: 0.5. The DO observed in this study (19.61 ± 3.53 %) is lower than the value (26.60%) reported by Buffa et al. (2016) for the same NCC: NaClO molar ratio. The variation in the DO at the same NCC: NaClO molar ratio could be attributed to the variations in the source of NCC, and its initial –OH group concentration.

Figure 3 (c) shows the FTIR spectra of U-NCC and TM-NCC samples. The broad peak around 3300 cm^{-1} represents NCC's O–H stretching vibrations, while the low-intensity peak around 2900 cm^{-1} represents C-H stretching vibrations of $–CH_2$ groups (Calderón-Vergara et al., 2020; Li et al., 2015; Rafieian et al., 2014). In NCC, the band between 1203 cm^{-1} – 1111 cm^{-1} occurred primarily due to the C–O–C stretching vibrations of the glycosidic bond, and a peak around 1032 cm^{-1} is arising due to the C–O vibration at C_2, C_3, and C_6 carbon molecules (Calderón-Vergara et al., 2020). Similar to previously published work, all the peaks showed a slight shift with the TEMPO-mediated oxidation where shifting took place from 2893 cm^{-1} to 2897 cm^{-1}, 1203 cm^{-1} to 1201 cm^{-}

[1], and 1032 cm^{-1} to 1030 cm^{-1}. Slight shifts in peaks could be a result of changes in ionic interactions and molecular compatibility in the NCC due to the modification (Shen et al., 2017). The peak in U-NCC around 1635 cm^{-1} has resulted from the O–H bending of absorbed water (Li et al., 2015), and a cluster of peaks around 1300 cm^{-1} could be attributed to the several C-H vibration modes (Rafieian et al., 2014). A marked difference between the spectra of U-NCC and TM-NCC appears at the peak around 1597 cm^{-1} in TM-NCC, which is not visible in the U-NCC sample. The peak at 1597 cm^{-1} is mainly a result of asymmetric C=O (carbonyl) stretching of the sodium salt of the –COOH (Rafieian et al., 2014). The appearance of this new peak confirms the successful conversion of –OH groups of NCC into –COOH groups during the TEMPO oxidation. Moreover, in previous studies, C=O stretching vibrations of sodium salts of carboxylic acids were reported at 1608 cm^{-1} (Fraschini et al., 2017), 1610 cm^{-1} (Li et al., 2015), 1602 cm^{-1} (Calderón-Vergara et al., 2020), 1599 cm^{-1} (Shen et al., 2017), and 1598 cm^{-1} (Rafieian et al., 2014), which are very closer to the peak (1597 cm^{-1}) observed in the current study.

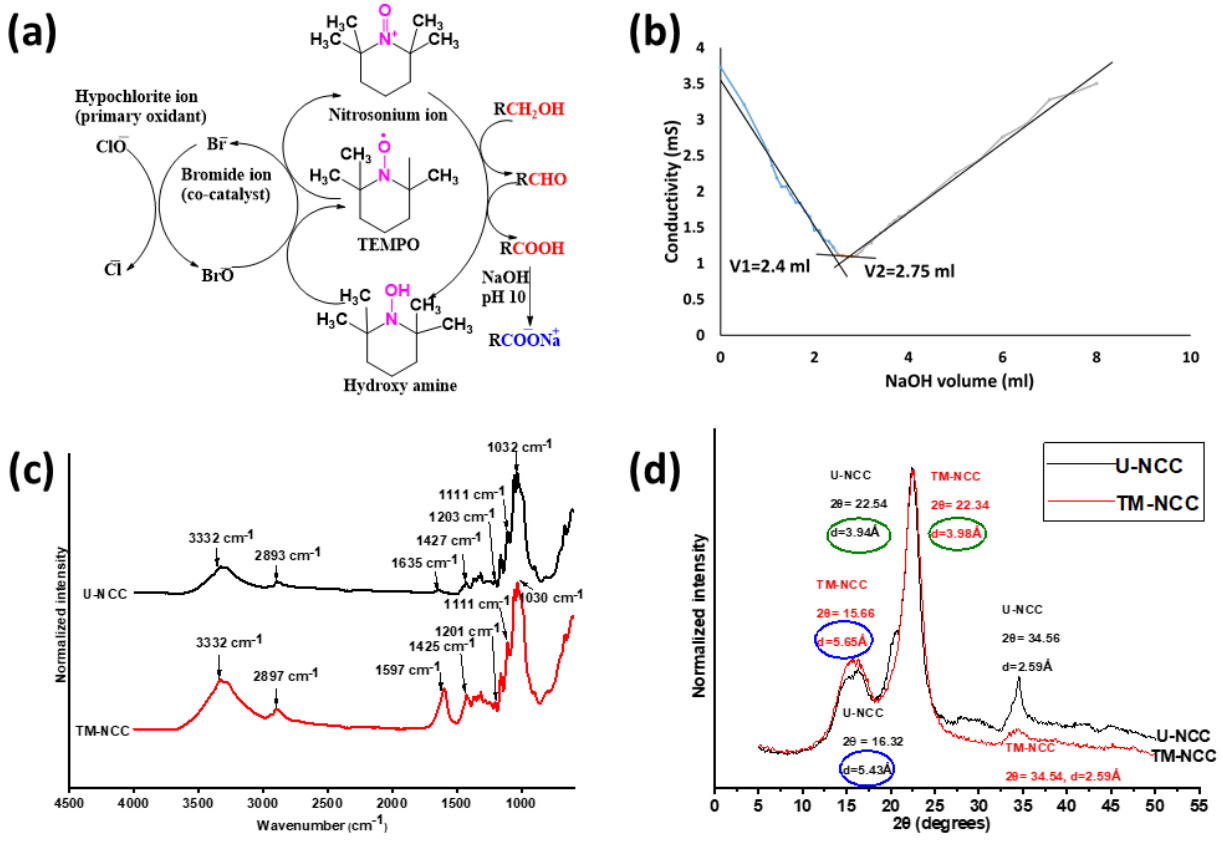

Figure 3. Comparison of unmodified and TEMPO modified NCC samples (a) Illustration of the oxidation process of NCC using TEMPO/ NaClO/ NaBr system (modified based on the figure

reported by Fraschini et al. (2017)) (b) Conductometric titration curve for TM-NCC to calculate the degree of oxidation (c) FTIR spectra of U-NCC and TM-NCC powder samples (d) XRD patterns of U-NCC and TM-NCC powder samples

Figure 3 (d) shows the XRD patterns of the U-NCC and TM-NCC samples and interlayer spacing (d) values of modified and unmodified samples according to Bragg's law (Sin $\theta = n\lambda/ 2d$). Both U-NCC and TM-NCC shows three major crystalline peaks at a diffraction angle (2θ) of 15°, 22°, and 35° that correspond to the characteristic crystalline peaks of cellulose (Trilokesh & Uppuluri, 2019). TEMPO-mediated oxidation did not change the crystallinity significantly; however, there is a decrease in the intensity of the peak for TM-NCC at a diffraction angle of 22°, probably due to the marginal destruction of crystallinity of NCC due to the introduction of –COOH groups onto the surface of the NCC. Also, a slight shift of the peaks for diffraction angle from 16.32° to 15.66° and 22.54° to 22.34° indicates a slight increase in the distance between d spacing (interlayer spacing) of the NCC from 5.43 nm to 5.65 nm and 3.94 nm to 3.98 nm. The change in interlayer spacing may also be due to the introduction of –COOH groups between the NCC planes, which increases the distance between NCC planes.

3.4.2. Characterization of canola protein films

3.4.2.1. Exfoliation of U-NCC and TM-NCC in the canola protein films

Proper exfoliation of nanomaterials in the biopolymer matrix is one of the key factors affecting film properties and functionality. The TEMPO modification aims to increase the surface charge of the NCC and thereby prevent the aggregation of NCC due to the repulsion between the charged surfaces of the NCC. Figure 4 shows XRD patterns of the canola protein films at different U-NCC and TM-NCC addition levels. Figure 4(a) shows the crystallinity of U-NCC and TM-NCC with the characteristic peaks shown around diffraction angles of 15°, 22°, and 35°. In the XRD pattern of the controlled sample (canola protein films without NCC addition), a major peak can be observed around a diffraction angle of 20° that is characteristic of canola proteins (Bandara & Wu, 2018). Up to 3% (w/w protein: U/TM-NCC) addition levels, both U-NCC and TM-NCC films did not show any distinct peaks corresponding to the NCC in the XRD pattern indicating proper exfoliation of U-NCC and TM-NCC in the canola protein matrix. However, when the U-NCC amount is increased up to 5% (w/w protein: U-NCC), a clear peak appears around a diffraction

angle of 22°, corresponding to the characteristic crystalline peaks of NCC as shown in Figure 4 (a). This is an indication of aggregation of U-NCC in the canola protein matrix at the level of 5% (w/w protein: U-NCC). For the films prepared with 5% (w/w) TM-NCC, a slight peak appeared around 22°, but not as prominent as in 5% (w/w) U-NCC indicating TM-NCC aggregation in the polymer matrix is lower than U-NCC aggregation. Similar aggregation behaviors at higher nanomaterial concentrations were reported with several previous studies with NCC, graphite oxide, bentonite, and surface-modified montmorillonite (Bandara et al., 2017a, 2017b; Bandara & Wu, 2018). Repulsive interactions generated due to increased –COOH groups and slightly increased interlayer spacing (d spacing) of TM-NCC could be the potential reasons behind improved exfoliation of TM-NCC in the canola protein matrix.

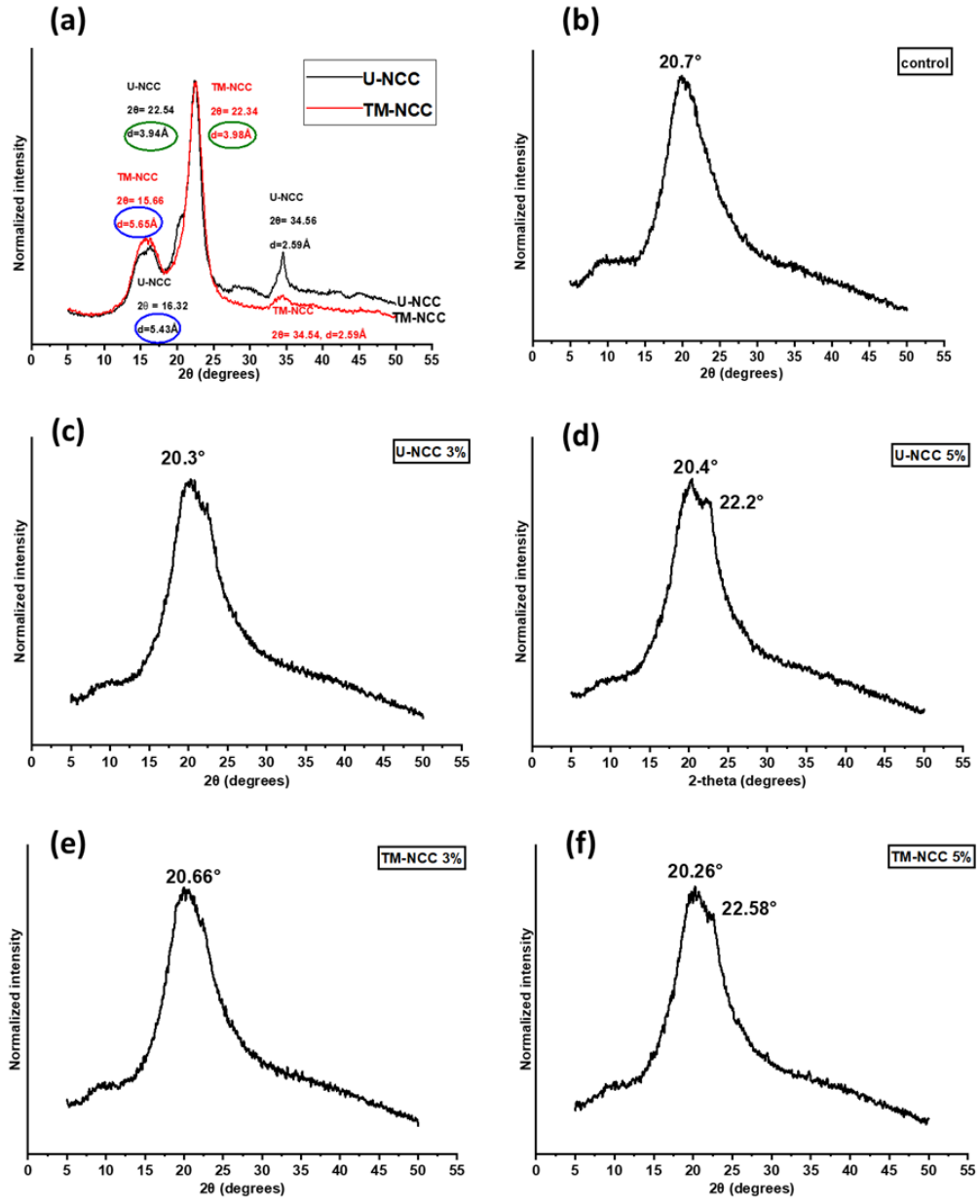

Figure 4. X-ray diffraction patterns of canola protein films at different concentrations of U-NCC and TM-NCC (a) U-NCC and TM-NCC powder samples (b) control films (no nanomaterials) (c) films with 3% U-NCC (d) films with 5% U-NCC (e) films with 3% TM-NCC (f) films with 5% TM-NCC

3.4.2.2. Secondary structural changes of the canola protein film

Changes in the canola protein secondary structure as affected by U-NCC and TM-NCC addition and film processing conditions were evaluated by differentiating amide-I peak using the 2nd

derivative method followed by peak fitting. Previously reported data on protein secondary structural changes were used to determine the corresponding secondary structure for each fitted peak (Bandara & Wu, 2018; Kong & Yu, 2007). As shown in the Figure 5, protein films show four main secondary structures; β-sheets (1621, 1631-1634, 1671, 1676, 1677 cm-1), α-helix (1652-1654 cm-1), turns (1692-1694, 1696 cm-1), and coils (1684 cm-1). Secondary structural change was not visible at the 1% (w/w) U-NCC addition levels based on the fitted spectra compared to control samples. However, at 1% (w/w) TM-NCC, there is an increase in the relative proportion of the turns and a decrease in the proportion of β-sheets. This change was present in the 3% (w/w) U-NCC and TM-NCC levels, with additional coil structures in 3% (w/w) U-NCC. This may probably be due to the increased interactions between nanomaterial and protein that open the protein structure and increase the buried structures and functional groups. On the other hand, increased protein-protein and protein-nanomaterial interactions can enhance the tensile properties of the films, as discussed in the next section (Bandara et al., 2017a). At the 5% (w/w) U-NCC addition levels, the relative proportion of the β-sheets has decreased while the proportion of the turns has increased. However, this change was not dominant at the 5% (w/w) addition level, mostly because of slight aggregation of NCC in the polymer matrix that limits the number of interactions with the protein. It is also compatible with the XRD patterns that indicate slight aggregation of U-NCC at the 5% (w/w) level.

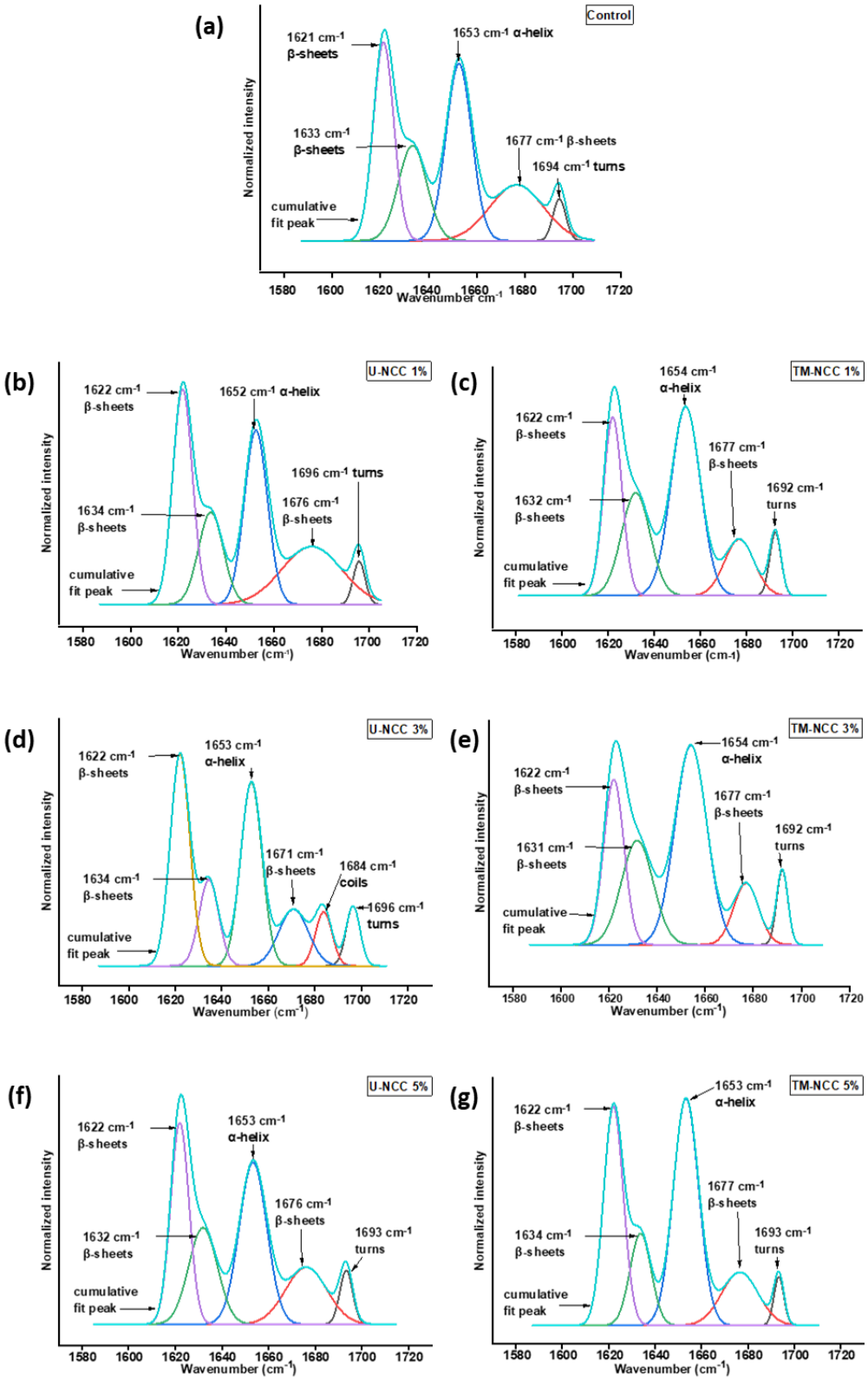

Figure 5. Illustration of protein secondary structural changes using peak fitting of amide-I peak

3.4.2.3. Mechanical properties of the nanoreinforced canola protein-based films

The effect of TEMPO-mediated oxidation and different addition levels on the mechanical properties of the canola protein films was evaluated by measuring tensile strength, elastic modulus, and elongation at break. Since cinnamaldehyde was used as a separate factor to investigate its impact on the antimicrobial properties of the packaging material, the effect of cinnamaldehyde on the mechanical properties of the films was also discussed in this section, even though it's not the objective of this study. Both NCC and cinnamaldehyde significantly affected the film functionality, but there was no interaction effect between the two factors. Figure 6 (a) shows the changes in tensile strength of the films due to TEMPO modification, nanomaterial addition level, and incorporation of cinnamaldehyde. Films with U-NCC at all addition levels were significantly different ($p<0.05$) from each other, and tensile strength has increased with the increasing U-NCC addition levels (w/w). Films with TM-NCC also showed the same trend but significantly improved values indicating that both U-NCC and TM-NCC can enhance the films' tensile strength. Osorio-Ruiz et al., (2019) also observed a similar increase in tensile strength of canola protein-based films when the unmodified NCC content was increased. Compared to control films, all the films with U-NCC and TM-NCC showed significantly higher tensile strength. The most important finding of the study was tensile strength of the films was significantly enhanced due to the TEMPO modification from 5.09 MPa to 5.91 MPa at 1% level and from 6.70 MPa to 8.36 MPa at a 5% level. At the 3% level, it has increased from 5.99 MPa to 6.50 MPa, but the increase was not statistically significant.

Previous studies have reported that enhanced tensile strength could result from improved nanomaterial exfoliation, resulting in enhanced tensile properties of the canola protein-based adhesives (Bandara et al., 2017a). Results of the current study supported this as TM-NCC was expected to be exfoliated well in the protein matrix due to increased surface charge, which was also confirmed by the XRD patterns. Protein-protein and protein-NCC interactions play a major role in determining tensile properties. When the NCC are introduced to the protein-polymer matrix, they interact through various molecular interactions that support the stress transfer through the polymer, which ultimately enhances the tensile strength (Osorio-Ruiz et al., 2019). It is explained by replacing weak protein-protein interactions with strong protein-NCC interactions. The improvement in tensile strength observed in this study could result from the increased number of protein-nanomaterial interactions owing to the mutual polarity of NCCs and proteins that could

result in polar-polar interaction and hydrogen bonding among others. Moreover, it is suggested that proper exfoliation further increases the number of interactions (Bandara et al., 2017a; Osorio-Ruiz et al., 2019). Therefore, films with TM-NCC showed higher tensile properties than films with U-NCC at the same nanomaterial addition levels. The tensile strength value (8.36 MPa) observed for the films with 5% (w/w) TM-NCC in this study was significantly higher than the tensile strength values reported in literature where NCC was used in protein-based films (González et al., 2019; Osorio-Ruiz et al., 2019; Rojas-Lema et al., 2021).

On the other hand, the addition of cinnamaldehyde also significantly increased the tensile strength of the canola protein-based films. According to reported literature, the addition of essential oil can increase or decrease the tensile properties depending on the interactions between the polymer and essential oil, the nature of the essential oil and biopolymer, and the dispersibility of the essential oil in the polymer matrix (Agarwal, et al., 2020). Balaguer et al. (2011) reported the ability of cinnamaldehyde to acts as a crosslinking agent in protein matrices. Therefore, the enhanced tensile strength observed in this study with the addition of cinnamaldehyde could be attributed to light crosslinking of the protein via the cinnamaldehyde, leading to the observed enhancement in the tensile strength. Furthermore, rearrangement of protein molecules within the biopolymer matrix due to new molecular interactions between protein and cinnamaldehyde and increasing the continuity of protein matrix could also help improve tensile properties (Hasheminya et al., 2019).

Figure 6(b) shows the elastic modulus of the prepared films. The addition of both U-NCC and TM-NCC significantly increased the elastic modulus of the canola protein-based films. Furthermore, the addition of cinnamaldehyde also significantly increased the elastic modulus. in agreement with the possibility of crosslinking. Rafieian et al. (2014) reported a similar trend for tensile strength and elastic modulus for the protein-based films, which agrees with the results of this study.

Elongation at break (Figure 6(c)) of the films with U-NCC reduced from 86% to 76% when the concentration of U-NCC increased from 1% to 5% but did not show a statistical significance. However, films with TM-NCC showed a significant reduction in elongation at the break where values have dropped from 116% to 73% when the TM-NCC concentration increased from 1% to 5%. This could be due to the limited flexibility of protein molecules due to increased interactions between protein molecules and protein-nanomaterial interactions (Chang & Nickerson, 2015; Osorio-Ruiz et al., 2019). Most interestingly, all films with nanomaterials showed significantly higher elongation at break percentages than control films, even though control films have the

lowest tensile strength. The suspected reason for this observation is an overall improvement in the integration of the protein films with the incorporation of the NCCs leading to enhancement in toughness and thus improved elasticity.

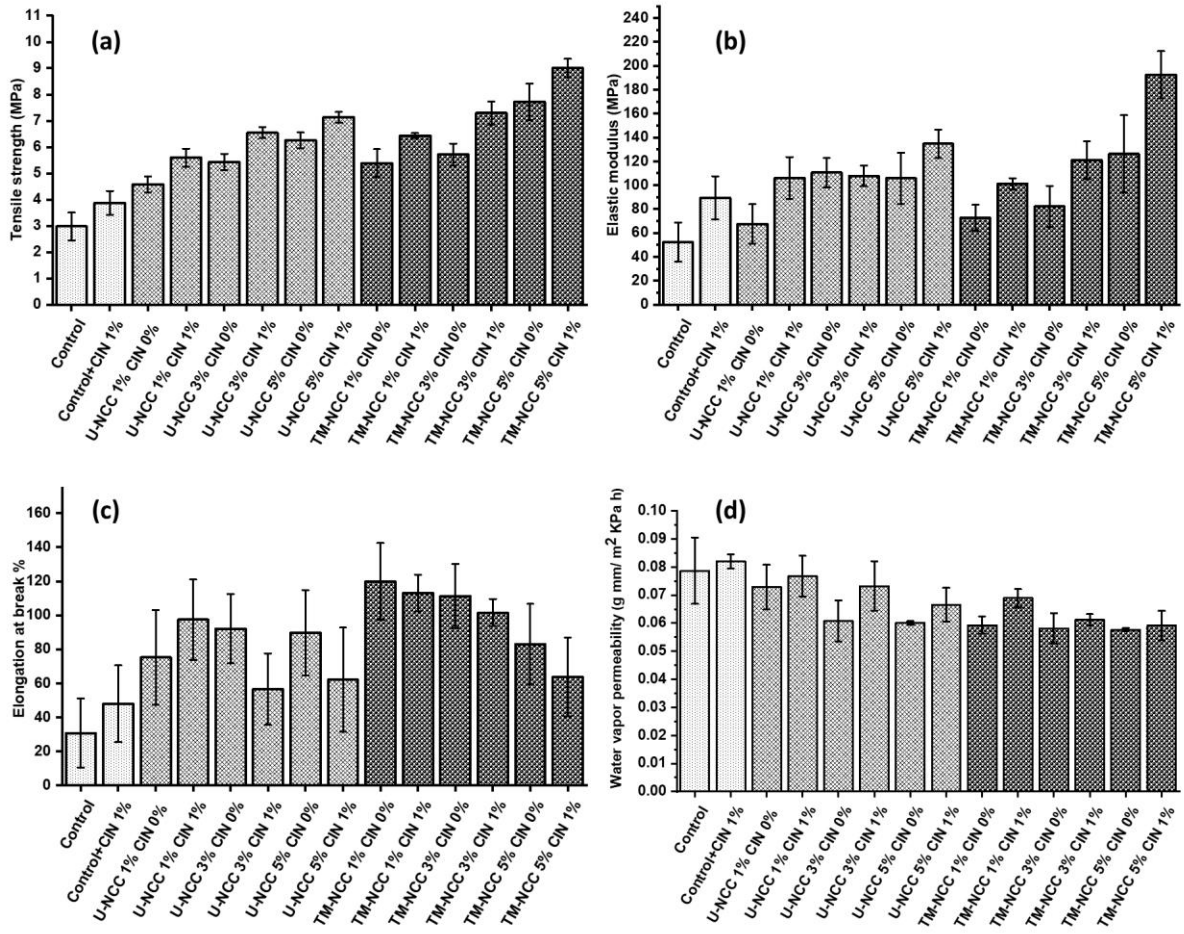

Figure 6. Mechanical and water barrier properties of the canola protein films affected by U-NCC and TM-NCC amount (a) tensile strength (b) elastic modulus (c) elongation at break percentage (d) WVP (CIN = cinnamaldehyde). All the values are given as average ± SD

3.4.2.4. Moisture/water barrier properties of the canola protein films

The moisture barrier properties of the film and their interaction with moisture were evaluated using WVP, solubility, and contact angle measurements, respectively. WVP is an important property of food packaging materials that evaluates the moisture transmission rate through packaging (Çakmak et al., 2020). As shown in Figure 6(d), there is a slight change in the WVP of the films, with the increasing amount of nanomaterial, especially with TEMPO-modified NCC. However,

this cannot be concluded as the difference is not statistically significant. It was expected to observe a reduction in WVP with the presence of nanomaterials and with the increase in their amounts due to the increased tortuous path (Hasheminya et al., 2019). However, the lack of reduction in WVP, could be due to the canceling out the effect of the tortuous path by the hydrophilicity of the NCC as these two phenomena act in opposite ways. In another study, it is suggested that reduction in WVP due to the tortuous path is almost similar to an increase in WVP due to the hydrophilicity of nanomaterials where the net effect is zero and results in no significant difference among films (Hasheminya et al., 2019). In films with TM-NCC, tortuosity may be higher than those with U-NCC due to the improved exfoliation; however, hydrophilicity is also higher in TM-NCC than in U-NCC due to the surface –COOH groups.

The contact angle of the films was measured to evaluate the surface hydrophobicity of the films (Table S1 – Supplementary information). As illustrated in figure 7(c), when the contact angle value exceeds 65°, the surface is considered hydrophobic (Rocca-Smith et al., 2016). Except for the control film, all other films with nanomaterials showed contact angle values more than 65° indicating an increase in surface hydrophobicity in canola protein-based films. However, there was no significant impact on hydrophobicity with different nanomaterial addition levels, except the control film and films with 3% and 5% TM-NCC. One of the reasons for the increase in surface hydrophilicity of the films is the migration of glycerol (plasticizer used in all the films) to the surface of the film during the drying process. Also, evaporation rate, unidirectional flow of film-forming solution, and contact with the hydrophobic surface affect the surface hydrophilicity of the films (Rocca-Smith et al., 2016). The reason for the higher surface hydrophobicity of films with 3% and 5% TM-NCC compared to control film may be due to the holding and sustained release of glycerol by TM-NCC due to the enhanced exfoliation in the film matrix.

Films' susceptibility to water was also evaluated using the solubility percentage of the films (Table S1 – Supplementary information). The water solubility of the films was expected to reduce when there is good compatibility between protein and NCC that limits the interactions between protein and water. Moreover, more interactions should occur when the amount of the nanomaterial is increased, thereby reducing solubility (González et al., 2019). However, all the films, including control, did not show a significant difference in solubility. It may be due to the highly hydrophilic nature of U-NCC and TM-NCC that interact with water regardless of their strong interactions with the protein.

3.4.2.5. Surface morphology of the canola protein-based films

The microstructure of the film's surface was analyzed using SEM images. As shown in Figure 7(a), the control film showed a rough and heterogeneous surface, which is confirmed by the images shown in Figure 7(b). This is explained by the disruption of protein-protein interactions due to partial denaturation of proteins during the formulation of film-forming solution and rearrangement of protein molecules during the drying process (Tongnuanchan et al., 2012). However, with the addition of U-NCC and TM-NCC up to 3% (w/w), the film's surface became smoother and homogeneous. It indicates that both U-NCC and TM-NCC stabilize the protein network due to the strong interactions in the protein matrix. At the 5% (w/w) level, it showed a rougher and heterogeneous surface than 3% (w/w) addition but still showed a smoother surface than control samples. This result is confirmed by the films' XRD patterns where nanomaterials' aggregation started to appear at the 5% (w/w) addition level. Also, SEM images of the films with TM-NCC are smoother and more homogeneous compared to the films with U-NCC, which could be attributed to the improved exfoliation of TM-NCC in the protein matrix. Previous studies obtained similar results where small NCC amounts (1-3%) resulted in more homogeneous and smoother surfaces due to improved compatibility between protein and nanomaterials in the film-forming solution (Rojas-Lema et al., 2021). These results also reflect the enhanced tensile strength of the films with the increased amount of nanomaterials and modifications.

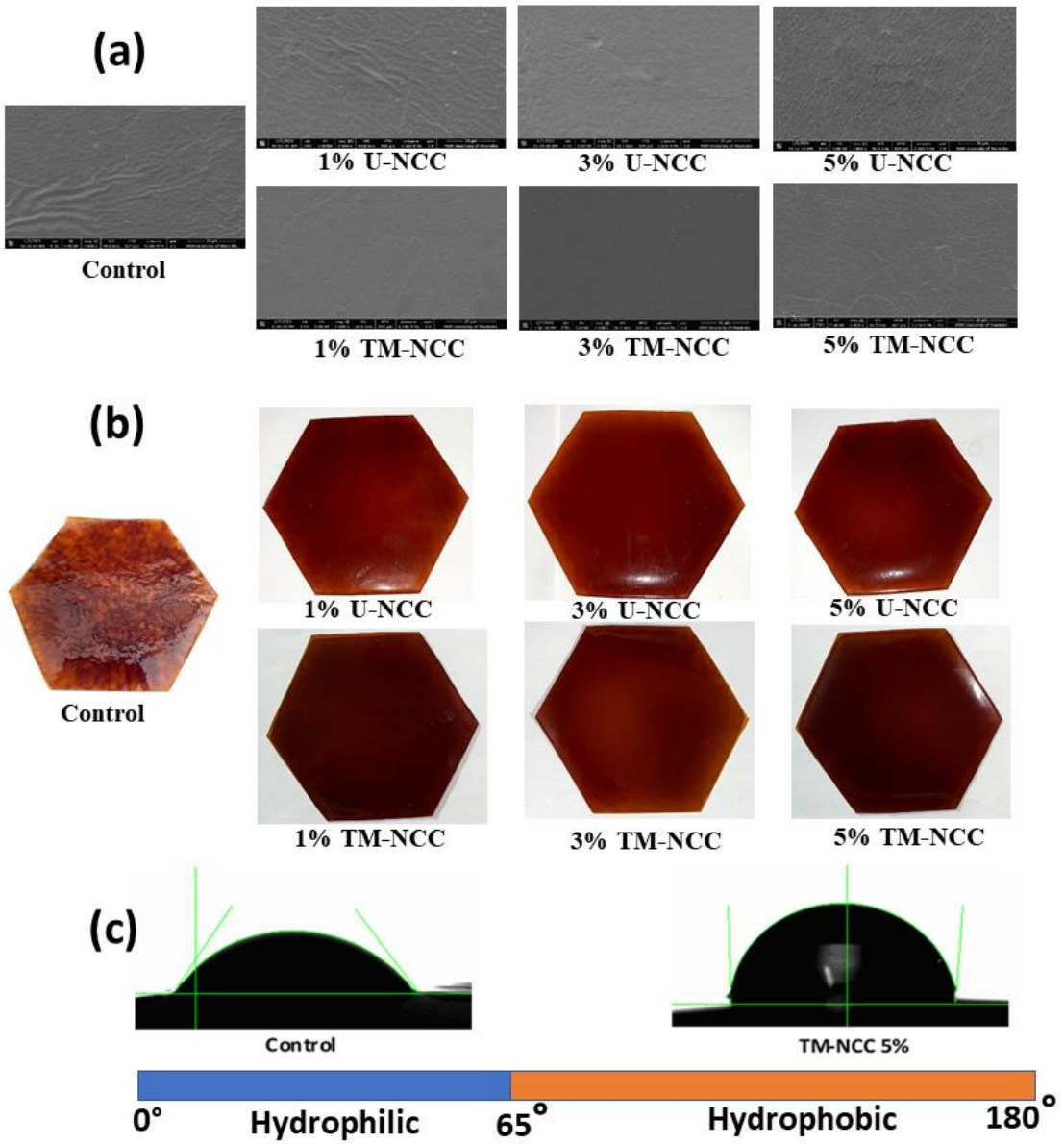

Figure 7. (a) SEM images of the surface of canola protein films at X2000 magnification (b) macroscopic images of canola protein films (c) graphical illustration of the relationship between contact angle and surface hydrophilicity

3.4.2.6. Thermal properties

Weight loss of the canola protein-based films as a function of temperature is represented by the TGA curves shown in Figure 8(a). Three main stages of weight loss were observed. Degradations at 30-125 °C, 125-280 °C, and 280-500 °C correspond to the moisture loss of the films, glycerol evaporation, and protein and NCC degradation, respectively. These three stages have been reported by Osorio-Ruiz et al. (2019) and Rojas-Lema et al. (2021) for NCC-incorporated canola protein and fava bean protein films. Among all the TGA curves, the highest weight loss was noted for the control films. For 1% (w/w) and 3% (w/w) films, the highest weight loss was observed for the films prepared by exfoliating U-NCC than that of TM-NCC. This thermal stability is resulting from strong interactions present in the polymer matrix due to the presence of nanomaterials and its further increase due to the TEMPO modification. However, at 5% (w/w) addition level, films with both U-NCC and TM-NCC showed less thermal stability than lower nanomaterial addition levels, as explained by XRD results where aggregation of nanomaterials started. The remaining weight percentage of the films at 600 °C is given in Table S2 in the supplementary material. DTGA curve shown in Figure 8(b) also confirms this result where the highest maximum decomposition temperature increased in the films with nanomaterials compared to control. Overall, all the films with nanomaterials exhibited higher thermal stability than control, while 1% (w/w) and 3% (w/w) TM-NCC showed the highest thermal stability.

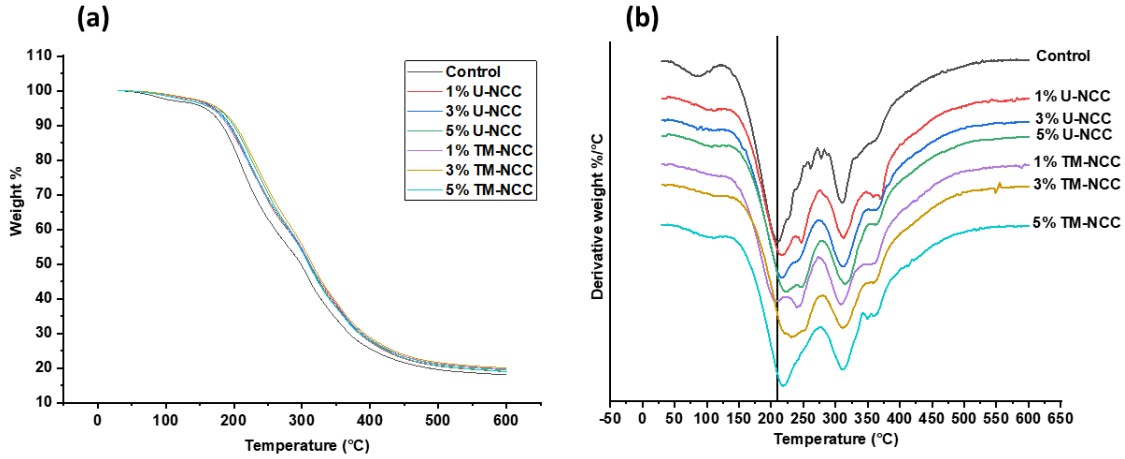

Figure 8. Analysis of thermal properties of the films using TGA (a) TGA curve (temperature vs weight percentage change) (b) DTGA curve (temperature vs the first derivative of weight percentage)

3.4.2.7. Antimicrobial properties

Incorporating essential oils (EO) in food packaging material is an innovative method that is currently under intensive investigation in academia and industry to protect food from microbial spoilage and shelf life extension (Agarwal et al., 2020). Even though the primary function of EO is providing antimicrobial activities, they affect the packaging material properties, such as tensile strength, water vapor permeability, and flexibility (Agarwal et al., 2020; C. Chen et al., 2021). Cinnamaldehyde is a widely used EO in food packaging applications due to its proven antimicrobial properties (Villegas et al., 2017). However, one of the key objectives of this study was to understand the impact of U-NCC and TM-NCC addition and their addition levels on the antimicrobial efficacy of cinnamaldehyde as measured by zone inhibition assay (Figure S1 – Supplementary information). As shown in Table S3 (supplementary information), there was no significant effect from the presence or absence of nanomaterial, TEMPO modification, or the nanomaterial addition level on the antimicrobial activity of cinnamaldehyde. Only the presence or absence of cinnamaldehyde significantly affected the antimicrobial properties of the films. Only at 5% (w/w) addition levels of U-NCC and TM-NCC zone inhibition were observed with or without cinnamaldehyde against *E. coli*. However, in the published literature, nano and micro cellulose have affected the antimicrobial activity of the essential oils in several ways. Chen et al. (2021) reported that micro-fibrillated cellulose controlled the release of cinnamaldehyde from starch/polyvinyl alcohol films. The current study results confirmed the cinnamaldehyde's excellent antimicrobial properties regardless of nanomaterials' effect on packaging films and EOs, which may be due to the cinnamaldehyde’s excellent antimicrobial efficacy, which other factors cannot mask.

3.5. Conclusion

The current study evaluated the effect of TEMPO-mediated modification of NCC on several film properties. FTIR and XRD analysis confirmed the conversion of surface –OH groups of the NCC into –COOH groups via TEMPO modification. TM-NCC significantly enhanced the film’s tensile strength values compared to control and films with U-NCC due to proper exfoliation of nanomaterials in the film matrix, reporting an average of 8.36 ± 0.85 MPa for TM-NCC 5% films. However, in terms of water barrier properties, there was not a significant effect from TEMPO modification, which was assumed due to the zero net effect resulting from robust polymer network

that consequently improves the barrier properties and increasing hydrophilicity of the polymer network due to the hydrophilic nature of TM-NCC which decrease the barrier properties. TM-NCC enhanced the thermal properties of the films due to the strong interactions in the polymer network, as evidenced in the TGA analysis. Considering the significant improvement observed in the canola protein-based films and their functionality, nanoreinforcing protein matrix with chemically tailored NCC can be introduced as an efficient method to enhance the tensile properties without compromising water barrier, thermal, and antimicrobial properties. This would be a novel addition to reinforcing materials for protein-based packaging applications. Based on our knowledge, this is the first time using NCC modified by TEMPO in protein-based packaging applications. Also, the outcome of this work will provide a scientific base and technology to create novel value-added applications for the major byproduct of the canola oil processing industry.

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Chapter 4: Improving properties of canola protein-based nanocomposite films by hydrophobically modified nanocrystalline cellulose

4.1. Abstract

Canola protein isolate derived from the canola meal is a sustainable source for developing food packaging materials due to its proven film-forming properties, but poor mechanical properties and high hydrophilicity limit its applications. The current study aimed to improve the properties of canola protein nanocomposites using hydrophobically modified nanocrystalline cellulose (OA-NCC). Canola protein nanocomposites were fabricated using unmodified NCC (U-NCC) and OA-NCC (0, 1, 3, 5, 7, and 9% w/w of protein) nanofillers via a solvent casting method, and the prepared nanocomposites were characterized to evaluate the film properties. The tensile strength of the canola protein nanocomposites was significantly enhanced by OA-NCC, resulting in a maximum of 3.44 ± 0.32 MPa for OA-NCC 3% nanocomposites. In addition, the elongation at break percentage was increased up to 130% by OA-NCC 7% nanocomposites. Moreover, the thermal stability and water barrier properties (from 0.096 ± 0.004 (control) to 0.054 ± 0.004 (9% OA-NCC) g mm/m^2 KPa h) were also improved because of the improved cohesiveness due to the OA-NCC and proteins interactions.

Keywords: canola protein, oleic acid, nanocrystalline cellulose, hydrophobicity, tensile properties

4.2. Introduction

Biopolymer-based packaging materials are becoming more popular among consumers, industry, and scientists due to the increasing environmental pollution of petroleum-derived traditional plastics. A vast range of food packaging materials, including pure biopolymers and biocomposites of biopolymers, have been introduced in several scientific studies to achieve environmental and economic sustainability while ensuring food safety. Proteins, polysaccharides, and lipids are the three main biopolymers directly extracted from biomass as raw materials for biodegradable packaging production (Grujić et al., 2017). Relative abundance, availability, and cost are essential factors for fabricating economically sustainable packaging materials. In this context, gelatin, soy protein, whey protein, and fish protein derived from agri-food industry byproducts and wastes are gaining considerable attraction in the packaging industry (Grujić et al., 2017; Kaewprachu et al., 2016). In Canada, the byproduct of the canola oil industry has excellent potential to be used in packaging applications due to its abundance and low cost (Bandara et al., 2018). On the other hand, a few studies have shown the film-forming ability of canola protein, with limited success, providing the opportunity for further enhancement of functional properties (Chang & Nickerson, 2014, 2015; S. Li et al., 2017; Osorio-Ruiz et al., 2019; Shi & Dumont, 2014).

Proteins are appealing raw materials for food packaging due to their film-forming ability and uniqueness of the films prepared from different proteins. The presence of 20 different amino acids in protein structure results in a vast range of functional groups that ultimately support various interactions between proteins and other materials (Kaewprachu et al., 2016). Therefore, the functionality of the protein-based films is highly dependent on the sources of the proteins and experimental conditions (Kaewprachu et al., 2016). Proteins can produce strong, viscoelastic, and cohesive films; however, despite these excellent material properties, the major disadvantages of protein-based films are their brittleness and higher susceptibility to moisture due to their hydrophilic nature (Calva-Estrada et al., 2019). In addition, even though proteins show good mechanical properties due to various types of interactions, they are still in the inferior range compared to synthetic polymers (Calva-Estrada et al., 2019). The addition of plasticizers is an effective way of enhancing film's flexibility and reducing brittleness. The compatibility between protein and plasticizer is affected by the level of denaturation, protein type, and plasticizer variety. However, the hydrophilic nature of common protein plasticizers, such as glycerol, tends to attract more water and increase the water vapor permeability (WVP) (Chang & Nickerson, 2014).

Lipid-based materials are used to enhance the water barrier properties of the films due to their low polarity that reduces the polar-polar interactions with water. Galus (2018) developed soy protein films with different concentrations of rapeseed oil and reported a significant reduction in WVP in films containing 3% oil compared to control from 5.12×10^{-10} $gm^{-1}Pa^{-1}s^{-1}$ to 3.62×10^{-10} $gm^{-1}Pa^{-1}s^{-1}$, though tensile strength was reduced from 1.93 MPa to 0.91 MPa. Similarly, the addition of walnut oil and almond oil reduced the WVP of whey protein films; however, 1% oil addition showed a reduction in tensile strength compared to the control, while 0.5% oil addition improved tensile strength values (Galus & Kadzińska, 2016). These results demonstrate that most of the time, the addition of oils improves the water barrier properties while compromising the tensile properties. Therefore, there is a need to improve the film's barrier properties without compromising the tensile strength. The use of reinforcing materials such as NCC is a common method in material science to improve tensile properties. The addition of NCC can improve the strength and stiffness of the material due to the percolation effect that describes the behavior of NCC as a filler that increases the H bonds within the polymer. On the other hand, the tortuosity effect resulting from NCC due to the increased path length for H_2O diffusion through the film enhances the barrier properties. Despite these properties, the agglomeration of NCC in the polymer matrix due to the abundance of hydrophilic –OH groups hinder its effective use in biopolymer-based packaging (Azeredo et al., 2017).

The introduction of lipid-based molecular structures to NCC is explored as a surface modification technique to improve the hydrophobicity of NCC (Chen et al., 2020; Fotie et al., 2020). Chen et al. (2020) observed an increase in tensile strength and hydrophobicity of the films after adding stearic acid esterified NCC, mainly due to the increased filling effect and the Van der Waals forces between nanomaterial and polymer matrix and the introduction of hydrophobic long-chain fatty acids respectively. Though esterification of NCC has been reported in several studies (Agustin et al., 2018; Almasi, Ghanbarzadeh, Dehghannya, et al., 2015; Chen et al., 2020; Rusmirović et al., 2017), to the best of our knowledge, incorporating them within protein-based composites is extremely limited. Especially, the method reported by Chen et al. (2020) to modify NCC without using harmful chemicals except ethanol is ideal for food applications. We hypothesize that the interaction of OA with NCC would improve the mechanical, barrier, and thermal properties of the nanocomposites. Thus the objectives of the study were to modify the NCC using OA, develop canola protein nanocomposites using modified NCC (OA-NCC) and unmodified NCC (U-NCC),

and characterize the nanocomposites in terms of mechanical, barrier, and thermal properties. This would be a novel addition to the food industry as no reported studies were found in the literature that aimed at both hydrophobic and mechanical properties of canola protein-based nanocomposites using modified NCC.

4.3. Materials and Methods

4.3.1. Materials

Canola meal and NCC were provided by Richardson International (Lethbridge, AB, Canada) and InnoTech Alberta (Edmonton, AB, Canada), respectively, for research purposes. Food grade OA was purchased from Spectrum Chemical Corporation. NaOH (≥ 97.0% purity) and HCl (36.5-38% w/w) were purchased from ACS chemicals. Anhydrous ethanol (89.5 to 91.5% v/v ethanol) was purchased from Ricca Chemicals. Snakeskin dialysis tubes of 3.5 kDa and glycerol were purchased from Thermo Fisher Scientific (Ottawa, ON, Canada). Unless otherwise specified, all other chemicals were purchased from Fisher Scientific, Ottawa, ON, Canada.

4.3.2. Canola protein extraction

Canola protein was extracted according to the previously reported method by Manamperi et al. (2010) with slight modifications. Ground canola meal was passed through a 0.5 mm mesh screen using Hosokawa milling and classifying system (Hosokawa Micron Powder Systems, Summit, NJ, USA). Residual oil in the canola flour was removed by Soxhlet extraction for 24 hrs with hexane and dried in a fume hood for 2 days. For the protein extraction, the ground canola meal was mixed with mili-Q water at a ratio of 1:10 (w/v), and the pH of the solution was adjusted to 12 using 3 M NaOH. Then the slurry was stirred for 30 min, centrifuged (Beckman Coulter floor-stand centrifuge, 10,000 × g for 30 min at 4 °C) and the supernatant was collected. Then the pH of the supernatant was adjusted to 4 using 3M HCl to precipitate the protein, and centrifuged (10,000 × g for 15 min at 4 °C) to collect the precipitated protein. The protein precipitate was freeze-dried (SP VirTis, Oakville, ON) and stored at -20 °C until further use. Kjeldahl analysis of protein isolates reported 81.07 ± 0.85 % (DWB) of protein content with a 2.52 ± 0.23 % moisture content.

4.3.3. Modification of NCC with OA

NCC modification was carried out based on the method described by Chen et al. (2020) with slight modifications. NCC (5g) was dispersed in 170 ml distilled water and sonicated for 5 min at 60% amplitude, 20 kHz frequency, and power output of 500 W using a probe sonicator (Model 550, Fisher Scientific, Newton, Connecticut, USA). Then 255 ml of anhydrous ethanol (water: ethanol = 2:3) was added to the dispersion, sonicated for another 30 min. Following sonication, 20 g of OA was added to the solution, sonicated under the same conditions for another 30 min, and allowed to react for 8 hrs at 78 °C under magnetic stirring. After the reaction, the product was centrifuged (12,000 rpm, 20 min) to separate the unreacted OA and repeat the same process with sonication for 2 min to further separate and remove any unreacted OA. The resulting samples (OA-NCC) were dialyzed against excessed de-ionized water for 48 hrs using 3.5 kDa dialysis tubes, freeze-dried, and stored at -20 °C until further use.

4.3.4. Weight gain percentage (WGP %)

The weight gain percentage of the NCC due to the introduction of oleic acid was calculated based on the weight difference between U-NCC and OA-NCC. The following equation (1) was used to calculate the WGP % (Wei et al., 2017).

$$WGP\,\% = (W_{OA-NCC} - W_{U-NCC})/W_{U-NCC} \times 100 \quad \text{——————} \quad (1)$$

Where $W_{OA\text{-}NCC}$ = Weight of the NCC after modification and $W_{U\text{-}NCC}$ = Weight of the NCC before modification.

The molar ratio ($MR_{NCC:OA}$) between one anhydrous glucose unit and OA was calculated using the below formula (2).

$$MR_{NCC:OA} = \frac{(W_{OA-NCC} - W_{U-NCC}) \times MW_{OA}}{(W_{U-NCC} - MW_{AGU})} \quad \text{——————} \quad (2)$$

Where MW_{AGU} = molecular weight of one anhydrous glucose unit and MW_{ACID} = molecular weight of oleic acid

4.3.5. Fabrication of canola protein-based nanocomposites with U-NCC and OA-NCC

Based on our previous work, Canola protein films were fabricated using a modified solvent casting method (Agarwal et al., 2020). Canola protein (3.5 g) was dispersed in 50 ml of distilled water

(final concentration of 5% (w/v) protein in film-forming solution after all components added), pH was adjusted to 10 using 0.5 M NaOH, stirred for 30 min at 650 rpm in room temperature, and then in a water bath (Model 1227, VWR International, Cornelius, OR, USA) at 100 rpm and 70 °C for 20 min. After cooling to room temperature, 50% (w/w of protein) glycerol was added and homogenized for 5 min at 14000 rpm (Fisher brand 850, Fisher Scientific, ON, Canada). Then U-NCC or OA-NCC was dispersed at 0, 1, 3, 5, 7, or 9% (w/w of protein) concentration in 20 ml of distilled water by sonicating for 2 min (amplitude 60%, frequency 20 kHz, power output 500 W) and homogenizing for 5 min at 14000 rpm. The final film-forming solution was prepared by mixing the initial protein/glycerol mixture and U-NCC/OA-NCC dispersion, followed by sonication (amplitude 60%, frequency 20 kHz, power output 500 W) for 5 min. Polystyrene dishes were used to cast the nanocomposites (0.43 ml/cm^2) and air-dried for 5 days before peeling off the film. Nanocomposites were conditioned in a controlled environment of 50% relative humidity (RH) and 23 °C before characterization (ASTM D882-18, 2018; ASTM E96/E96M-16, 2016).

4.3.6. Characterization of U-NCC, OA-NCC, and canola protein-based nanocomposites

4.3.6.1. Changes in NCC surface functional groups and secondary structural changes in protein

NCC (U-NCC, OA-NCC) and canola protein nanocomposites were characterized for their chemical interaction and protein secondary structural changes using Attenuated Total Reflectance - Fourier transformed infrared spectroscopy (ATR-FTIR), where IR spectra in the range of 400-4000 cm^{-1} at a 4 cm^{-1} resolution and 120 scans (Nicolett 6700, Thermo Electron Inc, Madison, WI, USA). The impact on surface functional groups of NCC due to the OA esterification was analyzed using the IR spectra. The secondary structural changes of protein as a result of adding U-NCC and OA-NCC were analyzed by generating the 2^{nd} derivative of the IR spectra followed by peak fitting of the amide I peak using Origin 2021 software (OriginLab Corporation, Northampton, MA, USA).

4.3.6.2. Crystallinity of NCC and exfoliation of NCC in nanocomposites by X-ray diffraction (XRD)

The initial NCC (U-NCCOA-NCC) and canola protein nanocomposites were analyzed using a Siemens/Bruker X-ray diffractometer (D5000, BrukerNano 219 Inc, Madison, WI, USA) to

characterize their crystallinity and exfoliation, respectively. The Cu X-ray tube was configured to Bragg-Brentano reflection geometry, and the diffraction angles were collected in the range of 5-50° using 0.02° step size and 1s dwell time. Jade software (Materials Data and the International Centre for Diffraction Data, Newtown, USA) was used for background subtraction and data processing, while Origin 2021 software (OriginLab Corporation, Northampton, MA, USA) was used for data visualization.

4.3.6.3. Surface morphology analysis by Field emission scanning electron microscopy (FESEM)

The impact of U-NCC and OA-NCC addition on the surface morphology of canola protein-based nanocomposites was studied using FESEM images at ×2000 magnification (Quanta 650 FEG, FEI Company, Hillsboro, OR USA). The nanocomposites were cut into 1 cm × 1 cm squares and coated with 60% gold (Au) and 40% lead (Pd) using a Denton vacuum before imaging.

4.3.6.4. Impact of U-NCC and OA-NCC addition on tensile properties

The tensile strength, elastic modulus, and elongation percentage at break of the canola protein-based nanocomposites were characterized according to the ASTM D882 (ASTM D882-18, 2018) standards using a Shimadzu AGS-X Universal Tester (Shimadzu America INC, Columbia, MD, USA). The samples were conditioned for 48 hrs at 23°C and 50% RH before characterization. Samples of 50 (*l*) x 9.5 (*w*) x 2.5 (*t*) mm were tested at 5 mm/min crosshead speed using a 500 N load cell. At least four specimens per one sample were tested, and mean ± SD (standard deviation) was reported for tensile strength, elastic modulus, and elongation at break percentage.

4.3.6.5. Water vapor permeability (WVP) using gravimetric cup method

The canola protein-based nanocomposites were conditioned for 48 hrs at 23°C and 50% RH according to the ASTM E96/E96M-16 method (ASTM E96/E96M-16, 2016), prior to the WVP test. WVP of the nanocomposites was calculated after 3 hrs using the gravimetric cup method (ASTM E96/E96M-16, 2016). The test was conducted in a controlled environmental chamber using disposable cups with 4.24 cm diameter and 4.02 cm height. Distilled water was filled up to three-quarters of the disposable cup, and canola protein nanocomposites with the same diameter of the cup mouth were placed on the cups using water-resistant adhesives. The weights of the cup,

film, and water were measured, kept in the controlled environmental chamber for 3 hrs at 23°C and 50% RH, and the weight of the cup, film, and the remaining water was measured again. The below equation (3) was used to calculate the WVP of the nanocomposites.

$$WVP = \frac{Weight\ change(g) \times thickness\ of\ the\ film(mm)}{Area\ of\ the\ cup\ mouth(m^2) \times vapor\ pressure\ difference(kPa) \times time(hours)} \quad \text{——————} \ (3)$$

4.3.6.6. Wettability of the nanocomposites using contact angle

The impact of U-NCC/OA-NCC on the wettability and surface hydrophilicity of the prepared nanocomposites was characterized by measuring the contact angle between the film and distilled water. A contact angle goniometer (Model No: 200-00-115, Rame-hart Inc.NC, Mountain Lake, NJ, USA) equipped with DROPimage software was used to characterize contact angle. The contact angle value for the left or right side of one replicate given by the instrument represented ten measurements taken at 0.001 s time intervals. The average of the acceptable angles from replicates was considered for the data analysis.

4.3.6.7. Water solubility

The impact of U-NCC and OA-NCC addition on the water solubility of the canola protein nanocomposites was analyzed according to the method described by González et al. (2019) with slight modifications. The initial dry weight of the 2 cm × 2 cm film samples ($W_{initial\ dry\ weight}$) was measured after keeping them in a drying oven for 24 hrs at 105 °C. Next, the oven-dried nanocomposites were immersed in conical tubes containing 50 ml distilled water, and the tubes were stirred at 100 rpm in a water bath at 25 °C for 24 hrs. The immersed samples were again oven-dried at 105 °C for 24 hrs, and the final dry weight ($W_{final\ dry\ weight}$) was recorded. The below equation (4) was used to calculate the water solubility percentage of the canola protein nanocomposites.

$$Water\ solubility\ \% = \left(\frac{W_{initial\ dry\ weight} - W_{final\ dry\ weight}}{W_{initial\ dry\ weight}} \right) \times 100 \quad \text{——————} \ (4)$$

4.3.6.8. Thermogravimetric analysis (TGA)

Thermal stability of modified and unmodified NCC samples (U-NCC, OA-NCC) and canola protein nanocomposites were analyzed using a Thermogravimetric analyzer (TGA-2, Mettler Toledo, Mississauga, ON, Canada). The thermogravimetric curves of the samples were obtained by heating approximately 5-10 mg of sample up to 600 °C and at a rate of 10 °C/minute in a constant nitrogen flow environment (30 ml/minute). Data visualization and analysis of thermogravimetric curves and first-order derivatives of thermogravimetric curves were performed using Origin 2021 software (OriginLab Corporation, Northampton, MA, USA).

4.3.7. Statistical analysis

A one-way ANOVA ($p < 0.05$) followed by a mean comparison using the Tukey test was used to statistically analyze the collected data using Minitab 2021 software (Minitab LLC, State College, Pennsylvania, USA). NCC addition levels were considered as the input variables (with 11 levels based on the amount of NCC presented in the film w/w of protein - Control-0%, U-NCC 1%, U-NCC 3%, U-NCC 5%, U-NCC 7%, U-NCC 9%, OA-NCC 1%, OA-NCC 3%, OA-NCC 5%, OA-NCC 7%, OA-NCC 9%). All the treatments (nanocomposites) were prepared in triplicate, and the results were reported as mean ± SD. The figures and graphs were generated using Origin 2021 software (OriginLab Corporation, Northampton, MA, USA).

4.4. Results and Discussion

4.4.1. Modification of NCC using oleic acid

The hydrophilicity of NCC due to the presence of abundant -OH groups is one of the major barriers that limits its applications in packaging. On the other hand, H bonds resulting between –OH groups lead to aggregation and reduce NCC dispersibility in the polymer matrix. Therefore, hydrophobic modification of NCC before using them in packaging applications is a critical technique to enhance the barrier properties of the packaging materials. Surface adsorption and chemical grafting are the major techniques used for hydrophobic modification of NCC. However, an excessive introduction of hydrophobic groups can also lead to the aggregation or flocculation of NCC in the polymer matrix mainly due to the hydrophobic interactions among the added hydrophobic groups and reduced electrostatic repulsion between NCC due to neutralization (Xie & Liu, 2021). The method developed by Chen et al. (2020) was selected for the NCC modification in this study mainly due

to the optimal modification level as well as its minimal use of chemicals (ethanol only), which aligns with green chemistry principles.

After modification, the NCC's weight gain percentage (WGP%) was calculated to be 8.08 ± 1.52%, where the molar ratio between NCC and OA ($MR_{NCC:OA)}$ was calculated as 0.047 (Equations 1 & 2), indicating 1 mol of anhydrous glucose reacted with 0.047 mol of OA which confirms that not all the –OH groups in NCC react with OA. Figure 9(a) shows the interactions between -OH groups of NCC and OA. The successful modification of NCC using OA was confirmed by the FTIR spectra of U-NCC and OA-NCC, as shown in Figure 9(b). A new peak at 1709 cm^{-1} in OA-NCC indicated the carbonyl group originating from OA in OA-NCC, which was absent in the U-NCC. However, based on the FTIR data, there was no evidence to support the theory of esterification between NCC and OA, as claimed in the method developed by Chen et al. (2020). The carbonyl group of an ester bond typically shows an FTIR peak in the range of 1730 cm^{-1} to 1750 $cm^{-1,}$ as evident in the previous studies. For example, Rusmirović et al. (2017) observed an absorption peak at 1736 cm^{-1} for esterified NCC with oleic, sunflower, and linseed fatty acids. Moreover, Freire et al. (2006), Wei et al. (2017), Peng et al. (2016), and Yoo & Youngblood (2016) confirmed the esterification of NCC using absorption peaks at 1735-1750 cm^{-1}, 1730 cm^{-1}, 1740 $cm^{-1,}$ and 1750 cm^{-1} respectively. Moreover, Almasi, Ghanbarzadeh, Dehghannia, et al. (2015) claimed that the moving shift in carbonyl peak from 1710 to 1745 was a clear-cut indication of the esterification.

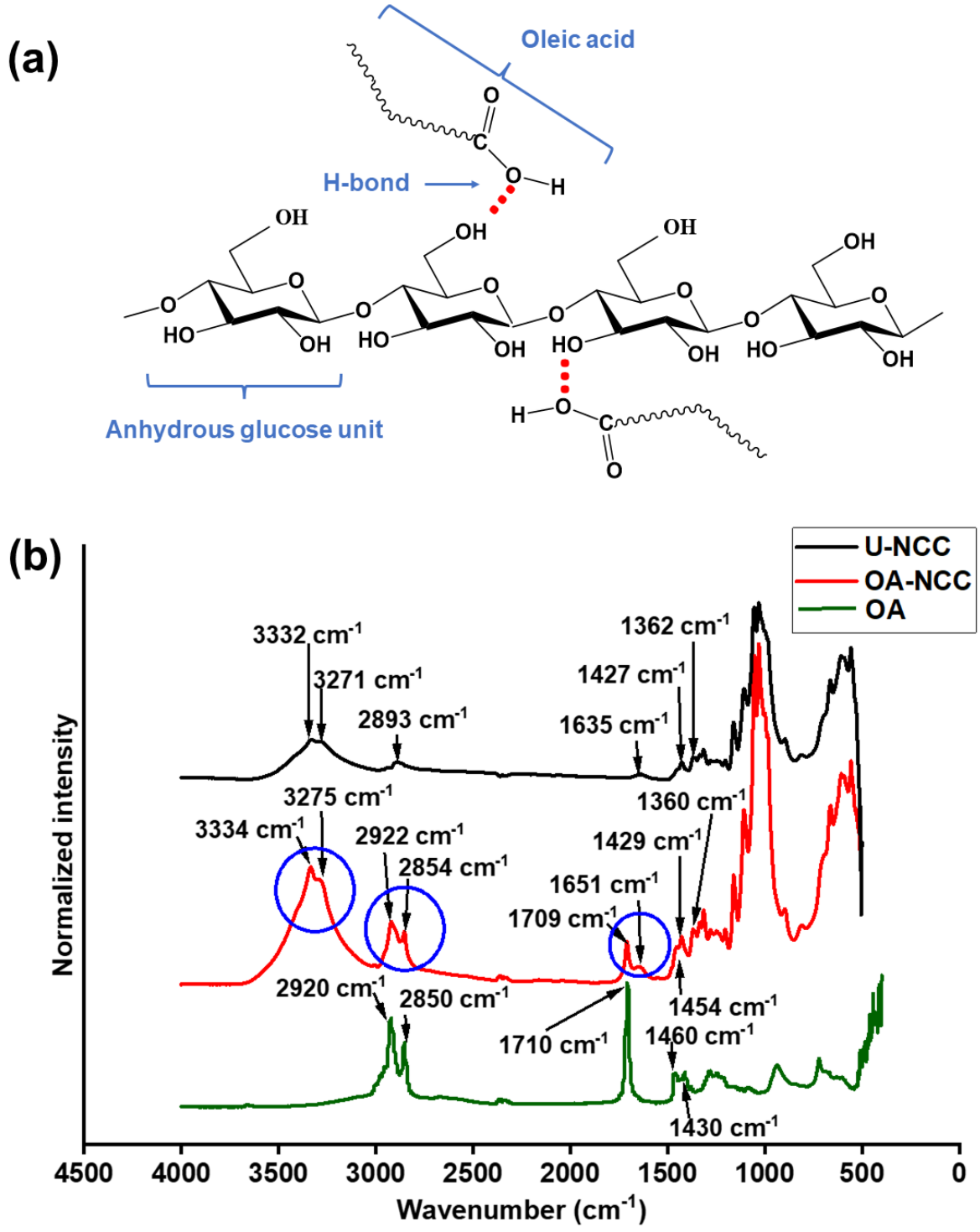

Figure 9. (a) The reaction between -OH groups of anhydrous glucose and -OH groups of OA (b) FTIR spectra of U-NCC and OA-NCC indicate the interactions between OA and NCC

Nevertheless, the current study showed no significant shift in the oleic acid's carbonyl peak at 1710 cm^{-1} following modification. Therefore, it can be concluded that the method used in this NCC modification help create physical interaction between NCC and OA, but not esterification. Figure 10 shows a schematic illustration of the potential physical interactions between NCC and OA after modification. Physical interaction between NCC and OA could result from several mechanisms such as mechanical interlocking, adsorption, wetting, diffusion, and weak boundary layers (Quddus et al., 2014). H bond formation can be increased when the NCC has more accessible surface –OH groups and shallow and narrow grooves on the surface to open additional –OH groups. On the other hand, the diffusivity of OA towards the NCC planes also determines the number of potential H bonds between NCC and OA. The presence of a higher number of H bonds per unit area of NCC (higher molecular surface area) and higher surface roughness of the NCC plane could lead to a higher degree of adsorption of OA onto NCC NCC (Quddus et al., 2014). Chen et al. (2015) reported H bonds between OA and cellulose sulfate in composite films, where stable oil-in-water emulsions were formed and stabilized via H bond formation between –COOH groups of OA and –OH groups of cellulose sulfate.

The absorption band between 2850 cm^{-1} - 2950 cm^{-1} in Figure 9(b) results from the C–H stretching vibrations of the carbon chain of OA and NCC's skeleton (Rusmirović et al., 2017). The single peak appeared at 2893 cm^{-1} in U-NCC shifted to a double peak at wavelengths of 2922 and 2854 cm^{-1} in OA-NCC. This could result from introducing additional methylene groups from the long hydrocarbon chain of OA. Previous studies reported the same observation for the NCC, where additional methylene groups were introduced through esterification (Almasi, Ghanbarzadeh, Dehghannya, et al., 2015; Q. Chen et al., 2020; Rusmirović et al., 2017). Moreover, an increase in the band intensity at 3200-3400 cm^{-1} due to –OH vibrations was another evidence for introducing additional –OH groups. Rusmirović et al. (2018) reported an increase in the peak's intensity corresponding to –OH stretching vibrations were due to the H bonds between NCC and fatty acids. The peak given in 1635 cm^{-1} for U-NCC could be due to the absorbed water present in U-NCC (Li et al., 2015). Rusmirović et al. (2017) and Wei et al. (2017) also observed the peak at 1637 cm^{-1} and 1640 cm^{-1} from absorbed water molecules of NCC.

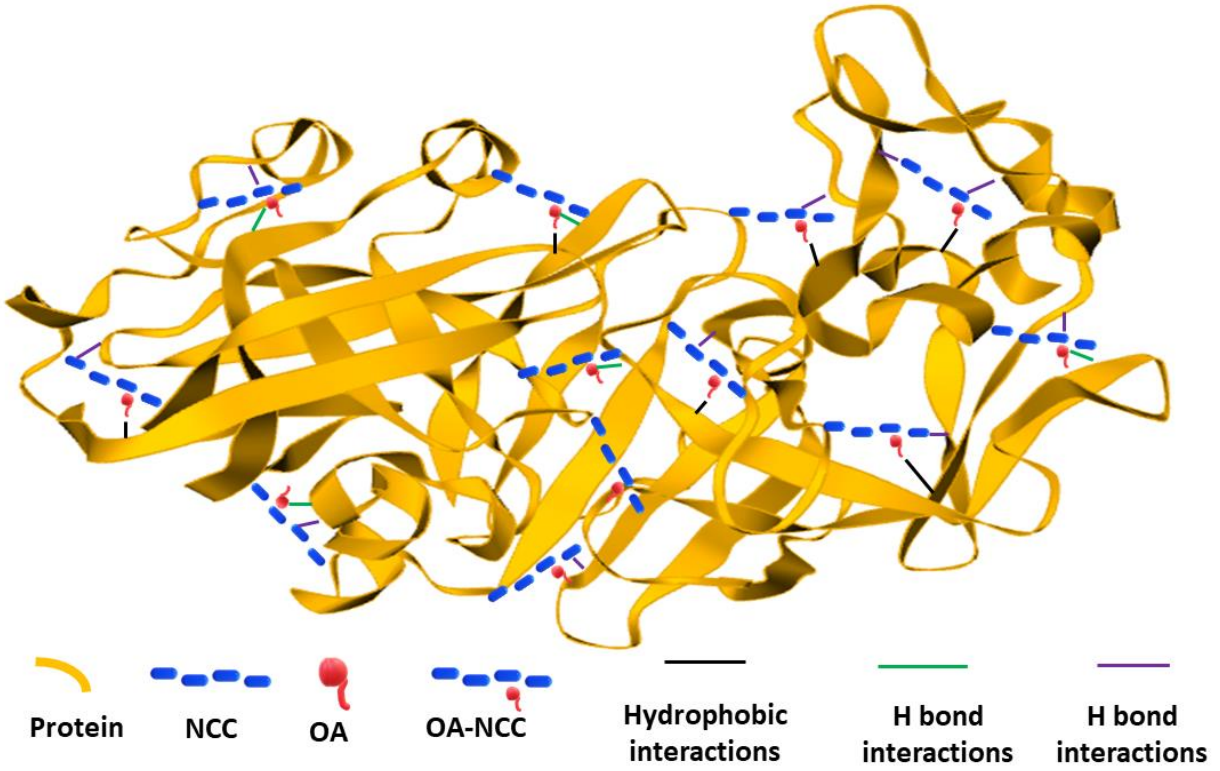

Figure 10. Graphical illustration of structure polymer network consists of protein and OA-NCC stabilized through several interactions, mainly hydrophobic interactions between hydrophobic moieties of the canola protein and hydrophobic tail of the OA, H bond between –COOH groups of canola protein and –OH groups of OA and H bonds between –COOH groups of canola protein and –OH groups of NCC

Figure 11 shows the XRD spectra of U-NCC and OA-NCC samples, indicating modification-induced crystallinity changes. Both U-NCC and OA-NCC showed characteristic diffraction peaks of NCC around 15°, 16°, 22°, and 34° diffraction (2θ) angles (Bandara & Wu, 2018; Freire et al., 2006; Trilokesh & Uppuluri, 2019). Comparing U-NCC and OA-NCC, no significant difference between the two spectra was observed, confirming that the crystalline structure of the NCC was not affected during the OA modification. Therefore, it is expected that the mechanical strength of U-NCC is retained in OA-NCC as well (Wei et al., 2017). Interlayer spacing (d) values for crystal planes were calculated using Bragg's equation ($\sin\theta = n\lambda / 2d$) for U-NCC and OA-NCC. A slight increase in the d values of OA-NCC in crystalline peaks around 15° and 16° compared to U-NCC could be attributed to the introduction of OA to the cellulose crystal planes that increase interlayer

spacing between crystal planes. However, there was no considerable change in d spacing at 22° and 34° diffraction angles.

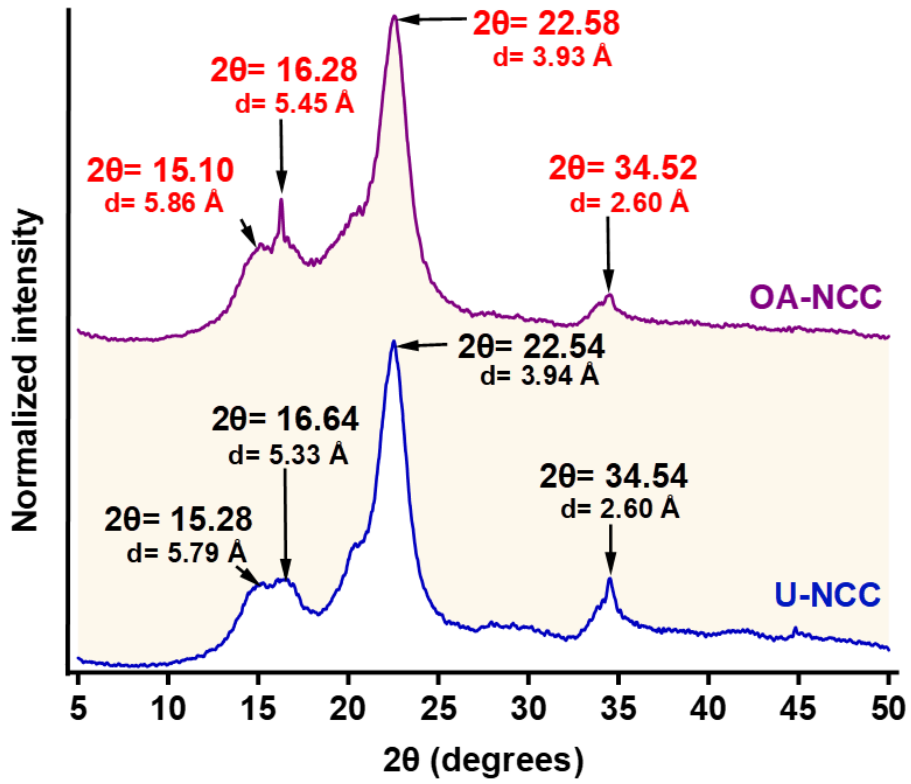

Figure 11. XRD spectra of U-NCC and OA-NCC showing crystallinity of the NCC before and after modifications

4.4.2. Characterization of canola protein-based nanocomposite films

4.4.2.1. Protein structural changes of canola protein-based nanocomposite films

Cruciferin (60% of total proteins), the major protein present in canola protein isolate, is made with six subunits/protomers that are slightly different from each other. An acidic α-chain polypeptide and basic β-chain polypeptide make the protomer of the cruciferin (Perera et al., 2016; Wanasundara et al., 2016). Napin (20% of total proteins), the other major protein in canola, consists of one small (4 KDa) and one large (9 KDa) polypeptide chains linked by disulfide bonds. Cruciferin's β-chain is buried within the protomers where the α-chain is exposed to the outside (Perera et al., 2016). The monomer units assemble as trimers via inter-chain disulfide bonds, and two trimers assemble into a hexamer via non-covalent H bonds, electrostatic interactions,

hydrophobic interactions, and van der Wall interactions (Perera et al., 2016). Therefore, cruciferin hexamer is sensitive to environmental conditions such as pH, temperature, and ionic strength. Temperature (70-80 °C) and pH changes can cause partial denaturation of tertiary structure, expose the buried binding sites of trimers, and expose hydrophobic functional groups (Perera et al., 2016). During the film preparation process in this study, canola protein was heated to 70 °C for 20 min, exposing the buried hydrophobic groups inside the cruciferin trimers while providing more functional groups to interact with other ingredients in the polymer matrix.

Cruciferin's secondary structure is composed of β-sheets (dominant), α-helix, β-turn, and random structures, while napin's secondary structure is predominated by α-helices. As shown in Figure 12, considerable differences between the secondary structure of the proteins in different nanocomposites cannot be observed except in the nanocomposites with 3% U-NCC. All other nanocomposites shows four fitted peaks corresponding to the secondary structures at 1623 cm^{-1} (β-sheets), 1650-1651 cm^{-1} (α-helix), 1677-1678 cm^{-1}(β-sheets), and 1694-1695 cm^{-1} (turns) (Bandara & Wu, 2018). The use of similar film-forming conditions such as protein amount, pH, temperature, and sonication time could be the reason for similarities in protein secondary structures between different nanocomposites. Therefore, proteins in different film formulations had similar exposed functional groups to interact with other molecules in the film-forming solution except the 3% U-NCC, which showed a difference in secondary structure compared to the others. At the 3% NCC addition level, the relative proportion of the β-sheets decreased while coil structures appeared at 1681 cm^{-1}. This could be NCC-induced protein secondary structural changes. Jiang et al. (2019) also confirmed that the NCC induced secondary structural changes in whey protein concentrate, where an increase in NCC amount resulted in an increase in α-helices and a decrease in β-sheets and random coils. Based on that, it is assumed that NCC can induce protein secondary structural changes, but in the current study, only the nanocomposites with 3% U-NCC could express that at a significant level. A lower amount of NCC (<1%) might not be enough to interact with the protein, and levels higher than 3% U-NCC may result in slight aggregation and thereby reduction in available U-NCC for interaction with protein. Due to the reduced interactions, higher levels may not able to induce secondary structural changes. In terms of OA-NCC, it is assumed that direct protein-NCC interactions were compromised by protein-OA-NCC interactions, which reduced the secondary structural changes. In summary, 3% U-NCC could be suggested as the optimum level to open the protein structure through protein-nanomaterial interactions.

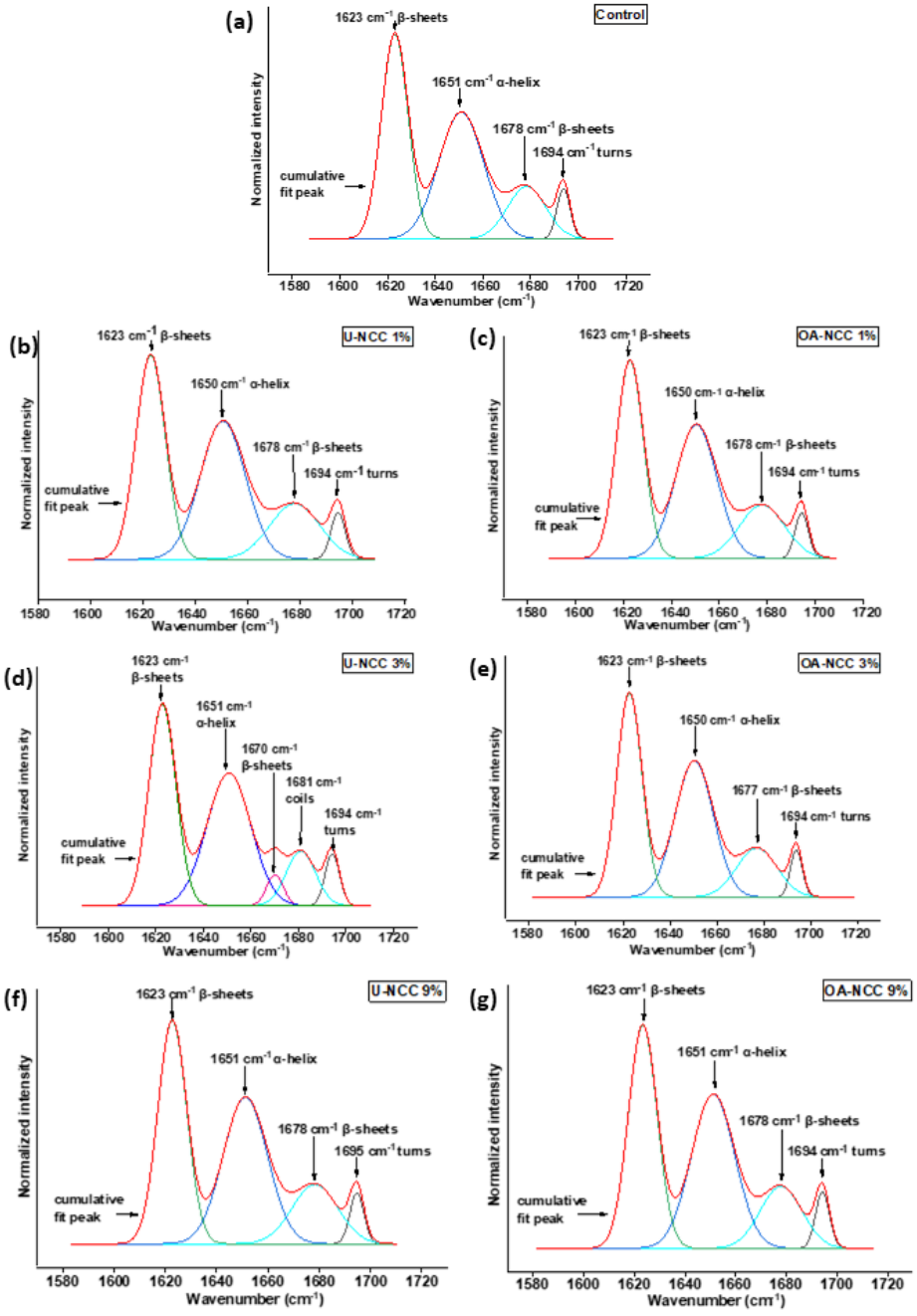

Figure 12. Protein secondary structure analysis by peak fitting of Amide I peak of FTIR spectra

4.4.2.2. Effect of U-NCC and OA-NCC on NCC exfoliation of the polymer matrix

Proper exfoliation of NCC in the polymer matrix is essential in polymer applications as it can ultimately impact the mechanical properties of the nanocomposites. Aggregation of nanomaterials can negatively affect the mechanical and thermal properties of the nanocomposites due to the lack

of molecular interactions in the polymer matrix (Xie & Liu, 2021). Complete and random exfoliation of nanomaterials in the protein matrix is an essential aspect of determining the material's strength. Exfoliation or aggregation of nanomaterials in a polymer matrix can be characterized by analyzing the diffraction peaks of the initial nanomaterial in the XRD spectra of the final nanocomposite/film (Bandara et al., 2017a). Figure 13 shows the diffraction peaks of canola nanocomposites affected by different amounts of U-NCC and OA-NCC. Canola protein has a broad diffraction peak of around 20°, which has already been reported in several previous studies (Bandara et al., 2017a; Bandara & Wu, 2018). Control and nanocomposites with U-NCC 1% and OA-NCC 1% addition only showed the characteristic canola protein diffraction peak, indicating proper exfoliation at 1% addition levels. However, considering 3% addition, nanocomposites with 3% OA-NCC did not show characteristic NCC diffraction peaks, while 3% U-NCC showed a slight diffraction peak around 22°, similar to NCC. However, this peak was not prominent. Therefore, level 3% can be identified as the maximum level where NCC is properly exfoliated in the canola protein network. However, from 5% addition levels, nanocomposites prepared with both U-NCC and OA-NCC showed the characteristic diffraction peak of NCC at 22°, and the intensity of the peak increased with the increased NCC addition levels. However, OA-NCC showed lower diffraction peak intensities at 22° compared to U-NCC in every nanomaterial addition level, indicating a better exfoliation of OA-NCC in the protein matrix than U-NCC. This difference between U-NCC and OA-NCC exfoliation could be due to the improved compatibility of the OA-NCC with the protein matrix compared to U-NCC, mainly due to the potential of hydrophobic interactions (Bandara et al., 2017). During film preparation, proteins were heated to 70 °C, which exposed the hydrophobic groups. Therefore, OA-NCC creates hydrophobic interactions with proteins due to the presence of a hydrophobic long carbon chain in OA's structure, thereby supporting exfoliation in the protein matrix where there is no hydrophobic moiety in the U-NCC's structure. Also, NCC and OA interactions can reduce the possible interactions between NCC molecules, preventing NCC aggregation.

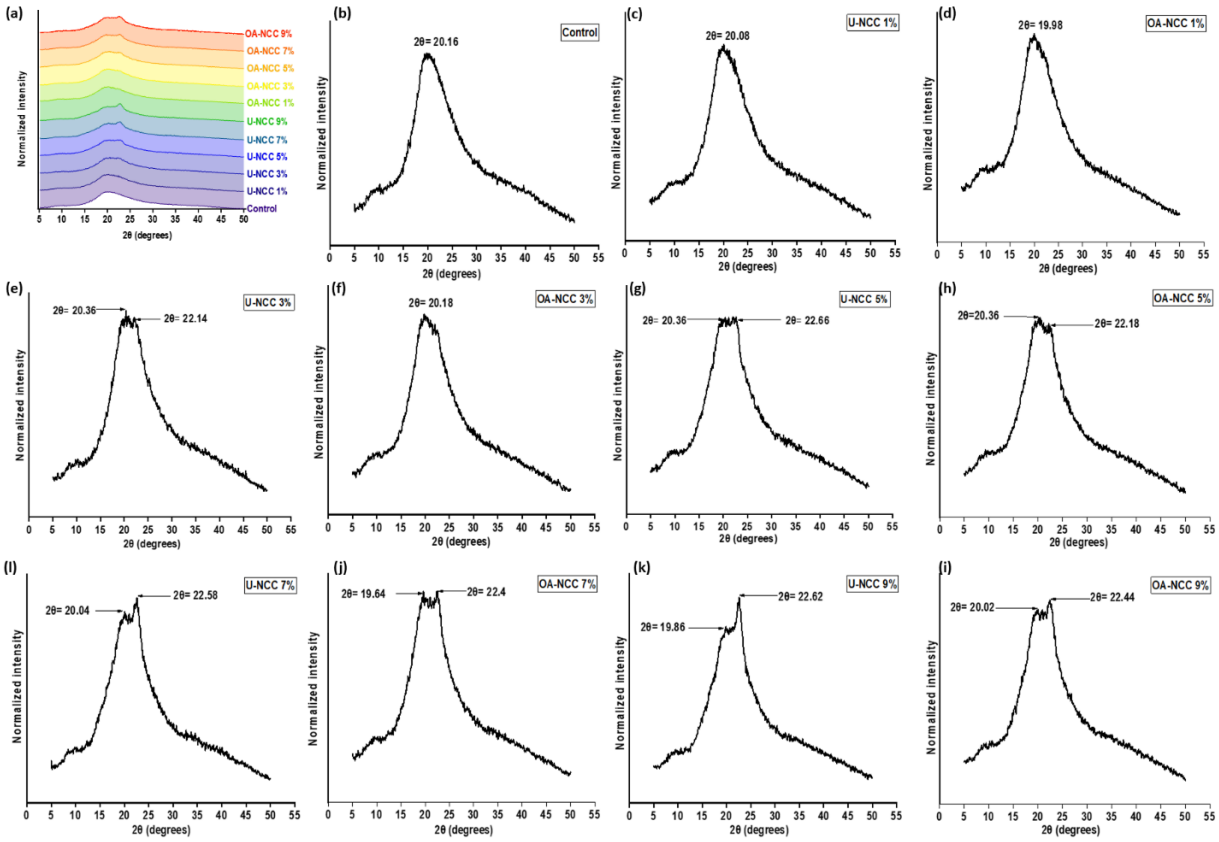

Figure 13. XRD spectra of canola protein nanocomposites that are affected by different amounts of modified and unmodified NCC (U-NCC and OA-NCC)

4.4.2.3. Surface morphology of the canola protein-based nanocomposites

Figure 14(a) shows the macroscopic images of canola protein nanocomposites with different U-NCC and OA-NCC addition levels. The control film showed a rough and aggregated surface compared to nanocomposites made with NCC (U-NCC and OA-NCC) addition. The aggregation of proteins in the control film could result from hydrophobic interactions between exposed hydrophobic sites of the proteins during the rearrangement of the polymer network in the drying process. However, when NCC is present, it interacts with protein molecules and reduces protein aggregation by creating the protein-NCC network. However, in the nanocomposites with U-NCC 1% and OA-NCC 1%, protein aggregation can still be observed, probably because a 1% addition of NCC was insufficient to create strong protein-NCC interactions that could prevent protein aggregation. In the macroscopic images, all the 3%, 5%, 7%, and 9% nanocomposites showed smooth and homogeneous surfaces without any aggregation, as shown in figure 14(a). This could

directly result from increased protein-NCC interactions, which prevents protein aggregation. Figure 14(b) shows the surface morphology of the nanocomposites using SEM images at ×2000 magnification. Similar to macroscopic images, control and films with U-NCC 1% and OA-NCC 1% show aggregated and rough surfaces. However, when comparing the OA-NCC films and U-NCC films, U-NCC films showed improved surface smoothness while OA-NCC films showed rough surfaces, which can not be observed in macroscopic images. This difference between OA-NCC composites and U-NCC composites in SEM images is explained by the heterogeneity imparted by OA as a result of NCC modification. The presence of OA-NCC may affect the interactions in the polymer matrix in ways such as hydrophobic interactions between exposed hydrophobic moieties of protein and the hydrophobic moiety of OA-NCC, H bonds between –COOH and –OH of proteins, and –OH of OA-NCC. As a result, OA-NCC can produce a more heterogeneous and rougher surface than U-NCC. Similar results were obtained by Galus (2018) for the films made from soy protein isolate when rapeseed oil was incorporated. The addition of rapeseed oil into a protein film-forming solution resulted in a heterogeneous film structure with a discontinuous, rough, and irregular surface (Galus, 2018). SEM images observed clear lipid droplets for those films due to the poor miscibility of oil and protein. Moreover, the author of that study reported that gravitational phase separation that occurred during film drying could also impact the surface roughness (Galus, 2018). However, in the current study, miscibility was enhanced as OA was attached to NCC before mixing with protein which is not the same as directly adding OA into protein. Yu et al. (2018) also prepared soy protein composite films with NCC and pine needle extract. The authors reported that pores presented in soy protein films disappeared in the SEM images with the addition of NCC due to the filling effect of NCC. Moreover, a heterogeneous structure was obtained due to the addition of pine needle extract, indicating that when the film matrix contained more heterogeneous ingredients, it resulted in a heterogeneous film structure (Yu et al., 2018).

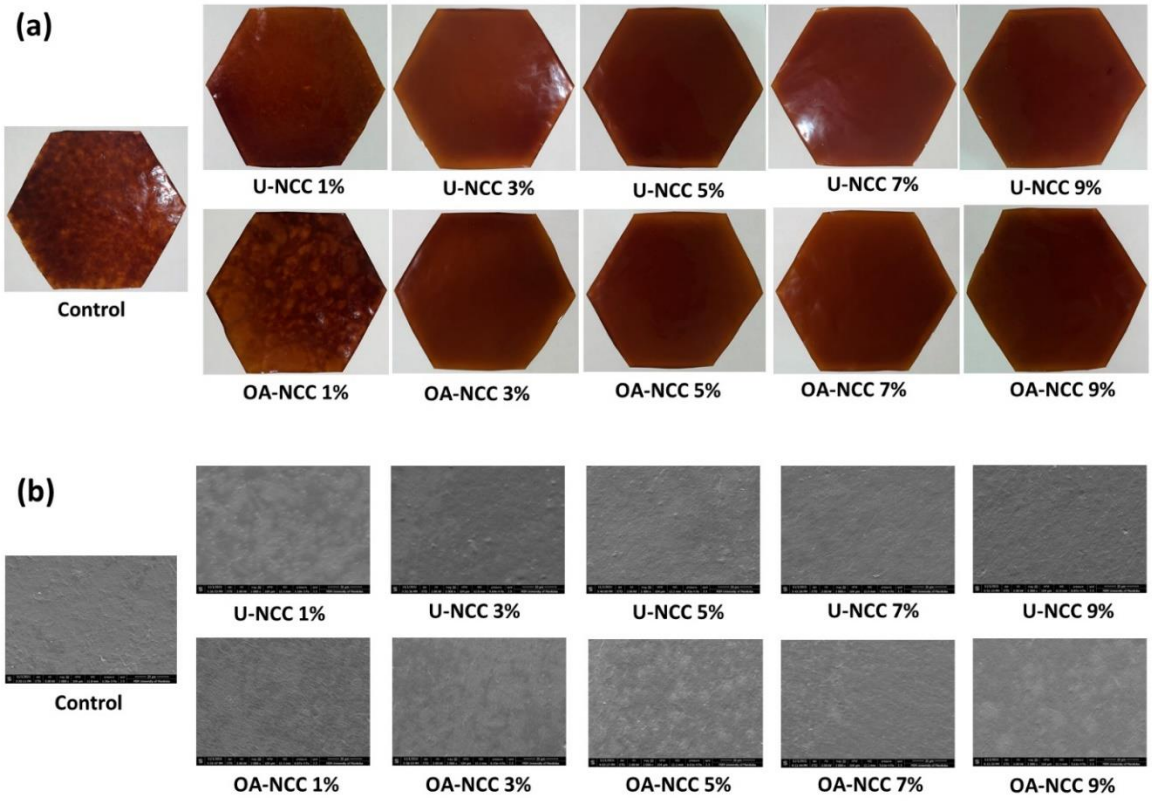

Figure 14. The surface morphology of the canola protein nanocomposites is shown by (a) macroscopic images and (b) SEM images at ×2000 magnification that illustrates the changes in surface morphology of the canola protein films affected by modification of nanomaterial and amount of nanomaterial

4.4.2.4. Impact of U-NCC and OA-NCC on tensile properties of the canola protein-based nanocomposites

Tensile properties of the canola protein-based nanocomposites were evaluated by measuring tensile strength, elastic modulus, and elongation at break percentage. Tensile strength measures the maximum stress that a material can withstand under a given force before breaking (ASTM D882-18, 2018). Tensile properties of the food packaging materials play an essential role as they should be flexible, resistant to environmental influences, and non-breakable during deformation to protect the food during handling, storage, and transportation (Aji et al., 2018). The addition of U-NCC and OA-NCC did not affect the thickness of the films, where films reported an average of 0.25 mm ± 0.01 thickness regardless of the NCC addition levels. As shown in Figure 15(a), except

for U-NCC 1% films, all others exhibited significantly higher tensile strength values than the control (1.85 MPa ± 0.15). Even though there was a trend of increasing tensile strength with the increased amount of U-NCC, only 7% (2.82 MPa ± 0.17) and 9% (2.87 MPa ± 0.28) w/w U-NCC addition levels showed a statistically significant increase in tensile strength compared to 1% (2.14 MPa ± 0.10) w/w U-NCC. Similar results were reported by Zhao et al. (2021), where tensile strength has increased from 4.25 MPa to 6.02 MPa for soy protein isolate films when the NCC amount was increased from 0 to 7 %. However, a further increase of NCC addition by up to 9% reduced the tensile strength of the films. Authors attributed these changes to the strong H bonds and ionic interactions between NCC and soy protein isolate due to the high surface area of the NCC (Zhao et al., 2021). Moreover, the authors reported 9% NCC might result in non-uniform nanomaterial distribution due to the aggregation of NCC at higher addition levels.

In another study by Fitriani et al. (2021), 7% NCC addition resulted in the highest tensile strength of 5.1 MPa for whey protein isolate films, whereas it was only 0.281 MPa for control films. The authors have attributed this improvement to the inter-and intra-molecular H bonds between protein and NCC, high compatibility of NCC in the protein matrix, and high strength and chain stiffness of the NCC (Fitriani et al., 2021). However, at the 10% NCC addition level, the tensile strength was significantly reduced due to the NCC aggregation and non-uniform distribution in the protein matrix (Fitriani et al., 2021). NCC aggregation at a higher addition level was confirmed by the current study's XRD data, as shown in Figure 13. Even with slight aggregation of NCC, which started at the 5% U-NCC addition level, a significant reduction in tensile strength could not be observed. Even though the aggregation could be seen, it may not be enough to reduce the tensile strength significantly. However, looking at Figure 15(a), it can be expected that a further increase of U-NCC beyond 9% will not increase the tensile strength.

Comparing the impact of U-NCC and OA-NCC on the tensile strength of the films, 1% OA-NCC (3.27 MPa ± 0.32), 3% OA-NCC (3.44 MPa ± 0.32), and 5% OA-NCC (3.33 MPa ± 0.34), showed significantly higher tensile strength than 1% U-NCC (2.14 MPa ± 0.10), 3% U-NCC (2.47 MPa ± 0.17), and 5% U-NCC (2.57 MPa ± 0.30), respectively. The enhancement in tensile strength with NCC modification could result from facilitating interactions between NCC and proteins in the film-forming solution. Due to the introduction of a hydrophobic long carbon chain to the NCC structure, more hydrophobic interactions could occur between protein and OA-NCC, which was not possible between U-NCC and proteins. On the other hand, OA-NCC and proteins can interact

through the H-bonds between –OH and –COOH groups present in both structures. After 3% OA-NCC level, tensile strength started to decrease, reporting a significant reduction between 3% OA-NCC and 9% OA-NCC. This may result from the replacement of protein-NCC interactions by protein-OA interaction at higher OA-NCC addition levels. However, even though the OA was introduced to the film-forming solution in this study, tensile strength was not compromised. One of the significant limitations in food packaging development is reducing tensile strength when hydrophobic substances are introduced to the film. This tendency was reported by several studies where the addition of hydrophobic substances enhanced the barrier properties compromising the tensile strength of the films (Galus, 2018; Monedero et al., 2009; Soazo et al., 2011; Ye et al., 2019). This limitation was overcome in the current study by modifying NCC with OA without directly adding the OA into the film-forming solution. Due to the presence of NCC, it may enhance the interactions within the polymer matrix, acting as a crosslinker with the attached OA moiety in its structure.

Figure 15(b) shows the elastic modulus of the films affected by different amounts of U-NCC and OA-NCC. Elastic modulus is calculated by dividing stress by strain in the elastic region of the stress-strain curve, giving an idea about the material's resistance to deformation (ASTM D882-18, 2018). Similar to tensile strength, all the films with U-NCC and OA-NCC showed significantly higher elastic modulus values than control except those with 1% U-NCC. It could be due to the presence of NCC in the protein matrix that stabilized the molecules through interactions and thereby reduced the deformation of material at the given force. However, a significant difference could not be observed between the same amount of U-NCC and OA-NCC. An increase in the elastic modulus of the films with the addition of nanomaterials was also reported by previous studies (Martelli-Tosi et al., 2018; Qazanfarzadeh & Kadivar, 2016; Rojas-Lema et al., 2021). The increased amount of H bonds between NCC and protein may lead to the increased cohesion of the films, ultimately increasing the films' tensile strength and elastic modulus (Rojas-Lema et al., 2021).

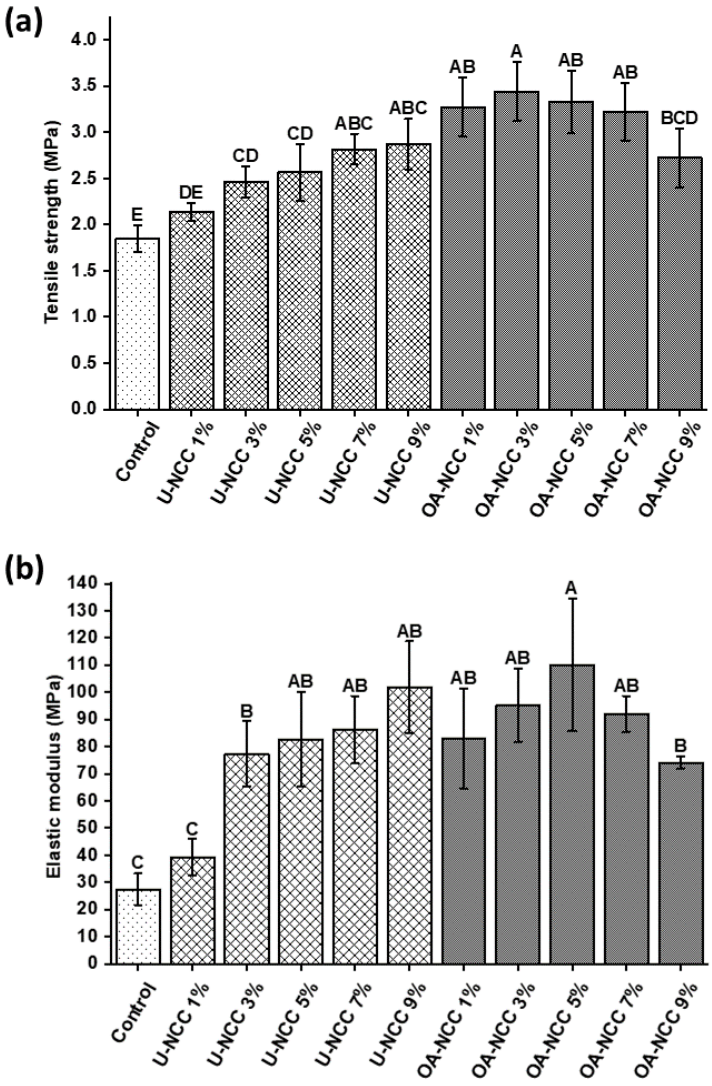

Figure 15. (a) Tensile strength and (b) elastic modulus of the canola protein-based nanocomposites as affected by different amounts of U-NCC and OA-NCC addition. Films were prepared in triplicates (n=3), and at least four specimens were tested for each treatment/film. One-way ANOVA followed by the Tukey test was used for the statistical analysis. All the values shown are average values of the films, and SD is marked using the bars on the top of the columns. Different letters on the top of the bars indicate significant differences between values ($p < 0.05$)

Elongation at break percentage of the film shows the maximum elongation of the film as a percentage value compared to the initial length (Cazón et al., 2017). In general, the addition of

nanomaterials reduces the elongation at break percentage of the material due to the reduced flexibility that was resulted from the strong interactions between nanomaterial and protein network (Qazanfarzadeh & Kadivar, 2016). Due to this reason, in most of the studies, the trend of elongation at break percentage values was reported as the opposite trend of the tensile strength values (Osorio-Ruiz et al., 2019; Qazanfarzadeh & Kadivar, 2016). However, in the present study, the modification of NCC with OA significantly enhanced tensile strength and elongation at break percentage values, unique from previous studies. As shown in Figure 16, all the films with OA-NCC showed significantly higher elongation at break percentage values than the control and those with U-NCC addition. As previously explained, when the interactions are strong, the flexibility of the molecules to move becomes limited, and elongation at break percentage decreases. However, in the current study, elongation at break percentage values was not compromised, most probably due to the enhanced flexibility given by OA in the polymer network. Moreover, all the OA-NCC films showed elongation at break percentage values higher than 100%, which was comparatively higher than the other values shown by other protein-based films reported in the literature (Zhang et al., 2016).

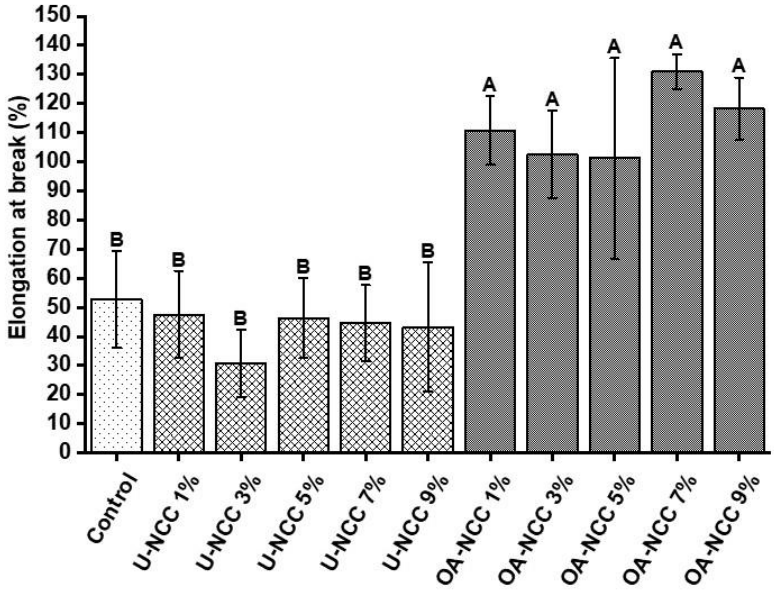

Figure 16. Elongation at break percentage of the canola protein films affected by different amounts of U-NCC and OA-NCC. Films were prepared in triplicates (n=3), and at least four specimens were tested for each treatment. One-way ANOVA followed by the Tukey test was used for the statistical analysis. All the values shown are average values, and SD is marked using the bars on

the top of the columns. Different letters on the top of the bars indicate significant differences between values ($p < 0.05$)

4.4.2.5. Impact of U-NCC and OA-NCC on moisture barrier properties of the canola protein-based nanocomposites

Figure 17 Shows the WVP values of the canola protein-based nanocomposites under different U-NCC and OA-NCC addition levels. Measuring the WVP of the nanocomposites is important as it measures the ability of the material to control the transportation of the water vapor between food components and the surrounding environment. Water transportation through the nanocomposite occurs through the absorption of water molecules to the nanocomposite surface and diffusion through it. Due to the hydrophilic nature of the proteins, in general, they show high WVP values (Zhang et al., 2016). Based on the current study's results, regardless of the modification, all the films with NCC showed significantly lower WVP values compared to the control (0.096 ± 0.004 g mm/m^2 KPa h). The lowest WVP value was reported in the films with a 9% w/w OA-NCC addition level (0.054 ± 0.004 g mm/m^2 KPa h). There was no significant difference among the nanocomposites with different amounts of U-NCC. The presence of NCC enhances the diffusion path of the water molecules due to the tortuosity (Hubbe et al., 2017). On the other hand, due to the hydrophilic nature of NCC (Xie & Liu, 2021), it can hinder the effect of tortuosity on WVP. Hydrophobic modification of NCC has effectively overcome that issue due to the reduction of hydrophilicity of NCC by introducing hydrophobic OA onto the NCC surface. In the nanocomposites with OA-NCC, there was a clear trend of decreasing WVP with the increasing amount of OA-NCC. Moreover, after the level of 3%, nanocomposites with OA-NCC showed significantly lower WVP compared to the nanocomposites with the same amount of U-NCC due to the enhanced hydrophobicity of the OA-NCC. Previous studies are also align with these result where hydrophobic modification of NCC increased the barrier properties of the final product (Balasubramaniam et al., 2020; Chen et al., 2019; Li et al., 2019).

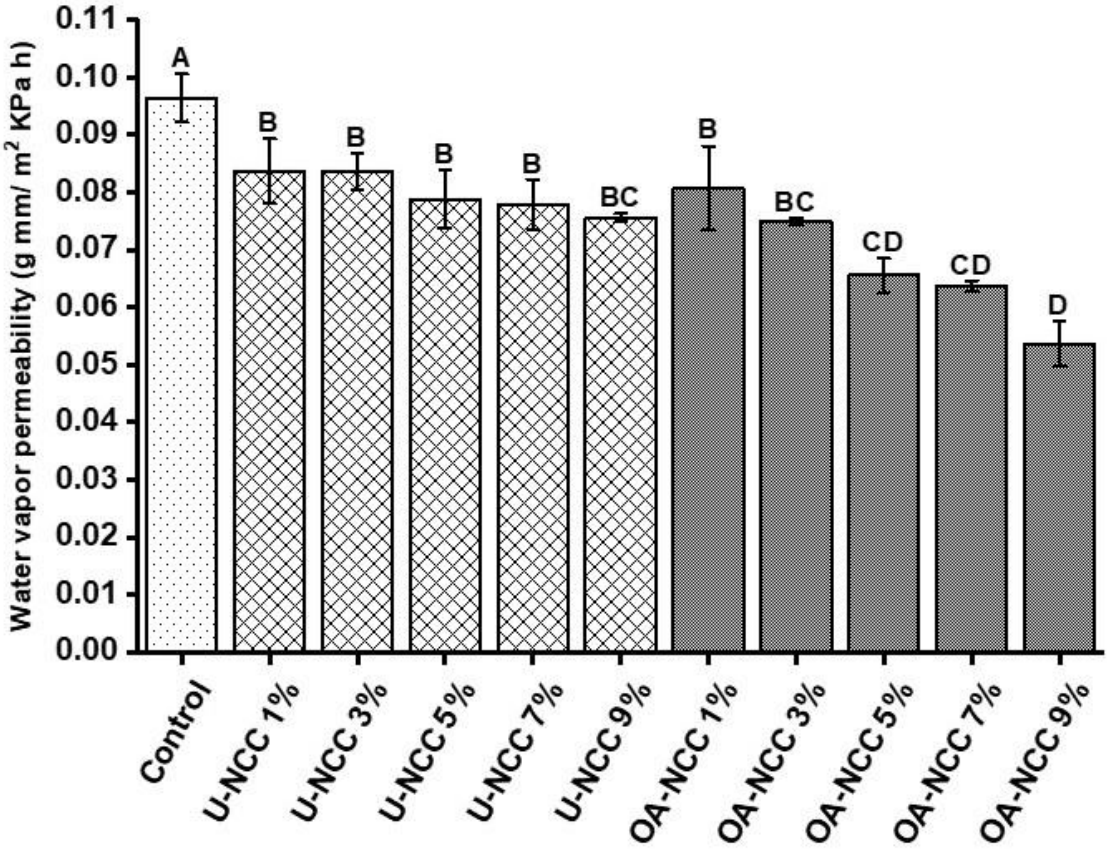

Figure 17. WVP of the canola protein films as affected by different amounts of U-NCC and OA-NCC addition levels. Films were prepared in triplicates (n=3), and all three replicates were tested. One-way ANOVA followed by the Tukey test was used for the statistical analysis. All the values shown are average values of the films, and SD is marked using the bars on the top of the columns. Different letters on the top of the bars indicate significant differences between values ($p < 0.05$)

The contact angle is a measurement of hydrophobicity of the nanocomposites, where the high contact angles show the ability to limit the hygroscopic property of the nanocomposites (Tang & Jiang, 2007). Different reference values have been given in the literature to demarcate the hydrophilicity and hydrophobicity of the materials. According to Fernandes et al. (2020), the contact angle values of more than 90° are considered hydrophobic surfaces, and according to Rocca-Smith et al. (2016), the reference value for hydrophobicity is at 65°. As shown in Figure 18, there was no significant difference between control nanocomposites and other nanocomposites with OA-NCC and U-NCC. Even though it was expected to increase the hydrophobicity of the

surface of the nanocomposite due to the introduction of OA-NCC, the results did not support that theory. One possible explanation for that could be the presence of hydrophobic moieties of OA-NCC inside the polymer network, where the surface is almost composed of protein and glycerol which was used as the plasticizer for the films. During the drying process, glycerol can migrate to the surface of the nanocomposites, which is affected by many factors such as drying time, the surface area of the films, environmental conditions, and compatibility with other components present in the film-forming solution (Zhang et al., 2021). Therefore, regardless of the internal composition of the nanocomposites, all nanocomposites may contain glycerol on the surface. This could be the reason for showing very few contact angle values for the films reporting the highest value, around 32°, indicating all the films had hydrophilic surfaces. However, there was a trend of decreasing contact angle among the OA-NCC films when the amount of OA-NCC increased. Again, this is explained by increased glycerol migration in higher OA-NCC contents due to the incompatibility of OA-NCC and glycerol in the polymer matrix.

The water solubility of the canola protein-based nanocomposites prepared using different amounts of U-NCC and OA-NCC did not show a significant difference and reported values in the range of 26%- 30%. Also, during the solubility test, all the film samples retained their original shape without degradation. It was expected to have the partial denaturation of proteins during film preparation and exposure of more hydrophobic groups of the proteins, reducing protein solubility. Therefore, regardless of nanomaterial, protein plays a role in nanocomposites' solubility. Another possible explanation for that could be the actions of NCC in opposite directions. In the first case, NCC interacts with hydrophilic groups present in the protein structure through H bonds to enhance the cohesiveness of the polymer matrix. This reduces protein interactions with water and thereby reduces solubility (González et al., 2019; Zhang et al., 2021). Secondly, NCC can interact with water due to its hydrophilic nature and thereby increasing solubility (Sukyai et al., 2018). Condés et al. (2015) also reported that maize starch nanocrystals did not affect the solubility of amaranth protein films except for 12% nanocrystals that further increased the solubility.

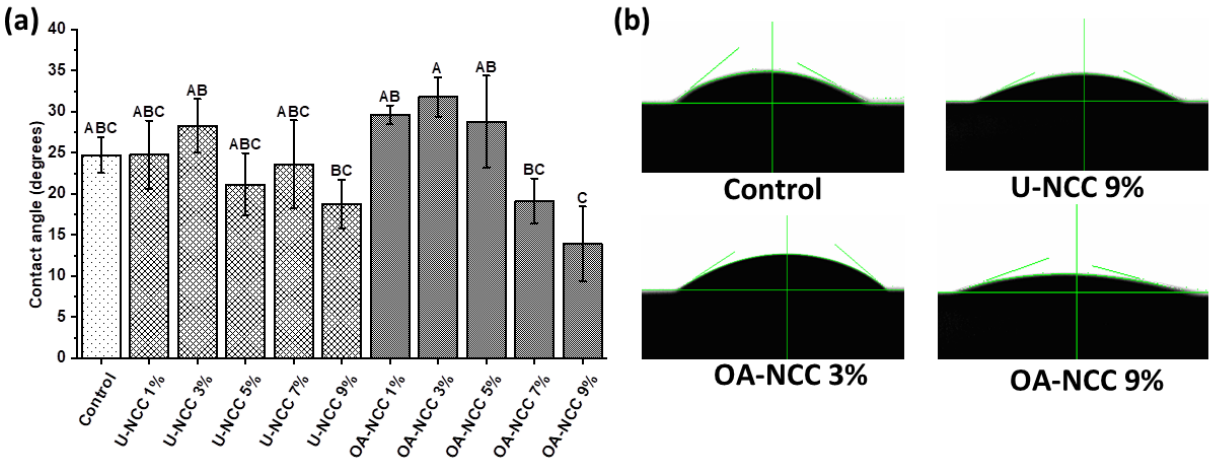

Figure 18. The contact angle of the canola protein films that were affected by different amounts of U-NCC and OA-NCC. Films were prepared in triplicates (n=3), and all 3 replicates were tested. One-way ANOVA followed by the Tukey test was used for the statistical analysis. All the values shown are average values of the films and SD is marked using the bars on the top of the columns. Different letters on the top of the bars indicate significant differences between values ($p < 0.05$). (b) Photographs of the water droplets on control, U-NCC 9%, OA-NCC 3%, and OA-NCC 9% films showing contact angles

4.4.2.6. Impact of U-NCC /OA-NCC on thermal properties of nanocomposites

TGA analysis was performed to evaluate the thermal stability of the nanocomposites by measuring retained weight % of the films at different temperatures from 0- 600 °C. Figure 19(a) shows the weight % vs temperature of the nanocomposites, and Figure 19(b) shows the first derivative of weight % vs temperature of the nanocomposites. All nanocomposites showed three stages of weight loss, mainly due to moisture evaporation, glycerol evaporation, and protein and NCC degradation at 30-125 °C, 125-280 °C, and 280-500 °C, respectively. Previous studies have also reported similar three stages of weight loss in nanomaterial-loaded protein-based films (Osorio-Ruiz et al., 2019; Rojas-Lema et al., 2021). Osorio-Ruiz et al. (2019) observed the addition of NCC increased the maximum decomposition temperatures in DTGA curves and lower residual mass % for control films due to the enhanced thermal properties of canola protein films with added NCC. Based on the TGA curve, except for the films with U-NCC 1%, all other films showed lower weight loss % at different temperatures compared to the control in this study. This could be due to the stronger interactions between protein and NCC than protein-protein interactions. Also, in U-

NCC films, weight loss % reduced with the increased amount of U-NCC. This is also explained by the increased number of protein-NCC interactions when the U-NCC amount is higher in the polymer matrix.

Contrary to that, weight loss % increased with the increasing amount of OA-NCC due to the lower thermal stability of OA (Almasi, Ghanbarzadeh, Dehghannia, et al., 2015). Moreover, Jandura et al. (2000) reported a reduction of crystalline order in cellulose fibers due to the introduction of organic acids, which can reduce thermal stability. In DTGA curves, the addition of U-NCC and OA-NCC showed a shift of maximum decomposition temperatures towards high temperatures indicating enhanced thermal properties. Comparing all the films, U-NCC 9% films and OA-NCC 1% films showed higher thermal stabilities compared to other films. Regardless of the thermal instability of OA, the higher thermal stability of OA-NCC could be a result of OA association with NCC without separately adding to the film-forming solution.

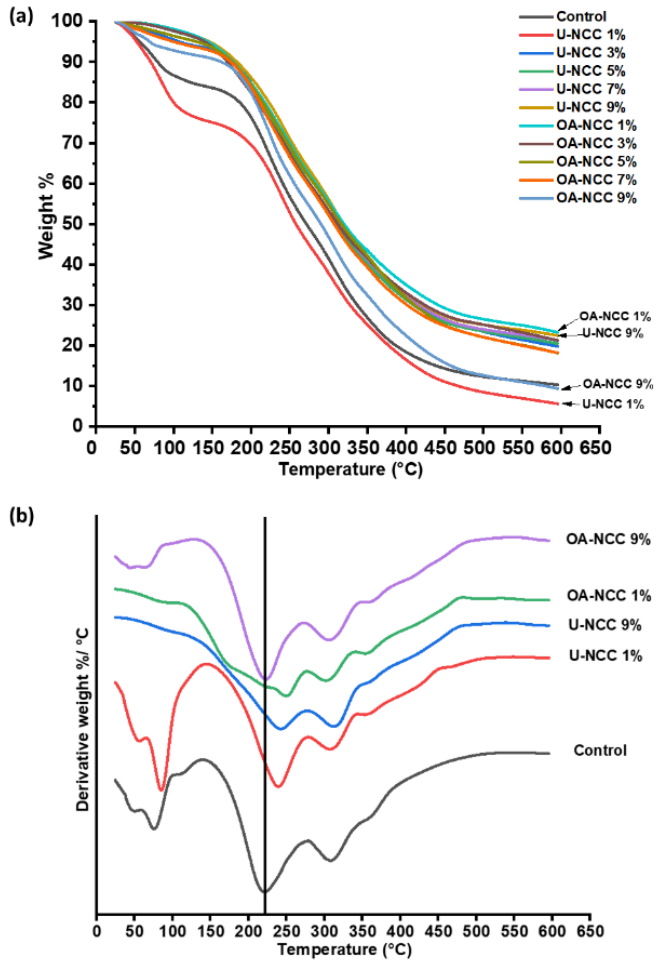

Figure 19. TGA curves (temperature vs weight percentage) for canola protein films affected by different amounts of U-NCC and OA-NCC (b) DTGA curves showing temperature vs the first derivative of weight percentage for control, U-NCC 1%, U-NCC 9%, OA-NCC 1%, and OA-NCC 9% films

4.5. Conclusion

The current study was carried out to evaluate the effect of OA-NCC on the properties of the canola protein-based nanocomposites. NCC was modified using OA, and the introduction of OA onto the surface of NCC was confirmed using FTIR analysis of OA-NCC and U-NCC. As a result of OA introduction, hydrophobicity of NCC was enhanced, and the ability of NCC to interact with hydrophobic moieties was also improved. The addition of OA-NCC enhanced the tensile, water barrier, and thermal properties of the canola protein films compared to U-NCC addition. These changes can be attributed to the OA moieties of OA-NCC, which can provide the platform for hydrophobic interactions between protein and OA-NCC and OA-protein interactions through H bonds. As a result of enhanced interactions, the cohesiveness of the polymer matrix was improved and ultimately resulted in enhanced tensile strength and thermal stability. Most interestingly, due to the presence of OA in OA-NCC form, the flexibility of the films was significantly increased. In terms of water barrier properties, due to the enhanced hydrophobicity of NCC by introducing a long hydrophobic carbon chain of OA, the water vapor permeability of the nanocomposites was decreased. However, the effect of OA-NCC on solubility and surface hydrophilicity was not significant, potentially due to the prominent role of protein and glycerol migration during the film preparation and drying process. As a result, OA-NCC could not reduce the surface wettability of the films, and future studies should focus on the prevention of glycerol migration. Overall, OA-NCC is an efficient way to fabricate canola protein-based nanocomposites with enhanced mechanical, barrier, and thermal properties where it was not easy to achieve all these properties simultaneously. Therefore, the outcome of this study will provide a platform to use the byproducts of the canola oil industry sustainably in material applications.

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Chapter 5: Conclusions and future research directions

The development of canola protein-based food packaging films is a better way of utilizing the canola oil industry's by-product sustainably. Film formation was possible even for control films where only canola protein isolate and glycerol (plasticizer) were used in the film-forming solution. However, protein aggregation could be observed in control films that were absent in films with nanomaterials, giving the first indication of the compatibility of nanomaterials with the protein network. Moreover, regardless of modification, NCC improved the canola protein film's mechanical, barrier, and thermal properties. The thesis is composed of two different studies where two different NCC modifications were used to improve the film properties.

Summary of the findings of 1st study- TEMPO mediated modification

- TEMPO-mediated modification converted 19.61 ± 3.53 % of primary –OH groups into –COOH groups
- XRD analysis of films indicated the proper exfoliation of TM-NCC compared to U-NCC in films
- TM-NCC significantly increased the tensile strength of the films compared to the control and films with the same amount of U-NCC (at 1% and 5% levels) and reported the highest value of 8.36 ± 0.85 MPa for TM-NCC 5% films
- The addition of cinnamaldehyde significantly increased the tensile properties of the films
- All the films showed significantly higher elongation at break percentage values compared to control, but with the increased amount of TM-NCC (from 1% to 5%), elongation at break percentage significantly decreased from 116% to 73%
- WVP of the films significantly decreased in all the films with nanomaterials, except U-NCC 1%. However, there was not any significant difference among the different levels of nanomaterials. WVP was in the range of 0.05-0.08 g mm/m^2 KPa h
- Only 3% and 5% TM-NCC films showed significantly increase surface hydrophilicity compared to control
- There was no significant difference between the films in terms of solubility. All the films reported solubility in the range of 26-32%
- Thermal stability of the films was enhanced by U-NCC and TM-NCC, and at 3% and 5% levels, TM-NCC further enhanced the thermal properties of the films compared to U-NCC

- Antimicrobial properties of the cinnamaldehyde present in the films against *E. coli* and *L. innocua* were not affected by the modification of NCC

Overall, TM-NCC significantly enhanced the tensile strength and thermal stability of the films compared to U-NCC but did not negatively affect any other property.

Summary of the findings of 2nd study- OA modification

- The interacted molar ratio between NCC and OA was 0.047 (1 mol of anhydrous glucose units reacted with 0.047 mol of OA)
- XRD spectra indicated proper exfoliation of OA-NCC in the polymer matrix compared to U-NCC
- Except for U-NCC 1% films, all other films showed significantly higher tensile strength values compared to control reporting a maximum of 3.44 ± 0.32 MPa for OA-NCC 3% films. In 1%, 3%, and 5% levels, OA-NCC showed significantly higher tensile strength values compared to U-NCC, but in 7% and 9% levels, there was no significant difference
- All the films with OA-NCC showed significantly higher elongation at break values (>101%) compared to control and films with U-NCC that showed (<53%) and there was no significant difference between the levels of nanomaterials
- All the films with NCC showed significantly decreased WVP values compared to control reporting the lowest value of 0.054 ± 0.004 g mm/m^2 KPa h for OA-NCC 9% films. OA-NCC showed significantly lower WVP values compared to the films with the same amount of U-NCC at the levels of 5%, 7%, and 9%. There was no significant difference at the levels of 1% and 3%
- There was no significant difference between the films in terms of solubility. All the films reported solubility in the range of 26-30%
- The addition of nanomaterial showed improved thermal properties in the films. With the increased amount of U-NCC, thermal stability increased while with the increased amount of OA-NCC, thermal stability decreased

Overall, OA-NCC significantly enhanced tensile strength, water barrier properties, and thermal stability (only at 1% and 3%) of the films compared to control, but did not negatively affect any other property.

Based on the overall results, TM-NCC and OA-NCC could be used to increase the properties of canola protein-based films without compromising any other existing desirable property.

Future research directions:

The current study only focused on improvement in mechanical and water barrier properties of the films. Even though the results of the study showed improvement in those properties, future research is needed on focusing on other material properties such as solubility percentage and surface hydrophilicity/wettability of the films. On the other hand, one of the important properties of food packaging is its antimicrobial properties. In the first study, the effect of modification on the antimicrobial properties of cinnamaldehyde was evaluated. However, to produce antimicrobial packaging, concerns should be taken from the beginning of the study. Attention is required starting from selecting un-contaminated raw materials and followed by maintaining sterile conditions during film fabrications to avoid contaminations. Also, antimicrobial properties should be evaluated using different antimicrobial agents at different levels, where whole separate research should be planned on that. For that, the best conditions for material properties can be selected from current studies' results.

Another thorough investigation should be conducted on the environmental factors that affect the properties of films. Drying conditions such as temperature and RH, the velocity of air around, and also the type of the molds can affect the film properties. On the other hand, the effect of other parameters such as level of protein denaturation, homogenization, sonication, and also other ingredients such as plasticizers and cross-linkers should be evaluated.

Considering all these factors, more studies on different aspects should be conducted to develop a food packaging material with optimum film properties. Also, it is very hard to compare the different studies' results by looking only at numbers, as they have been conducted under different conditions. Therefore, it is suggested to compare several strategies reported in the literature under one experimental design at the end to evaluate the most impactful strategy. The current study can be introduced as the initiation required for the beginning of that long journey towards the industrial level production of canola protein-based food packaging materials.

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Appendix

The calculation for the DO

Calculation of Sample 1:

$$DO = \frac{162 \times \mathrm{C} \times (V_2 - V_1)}{w - [36 \times \mathrm{C} \times (V_2 - V_1)]}$$

C = NaOH concentration (mol/dm^{-3})

V_1 and V_2 = NaOH (l) amount corresponding to carboxyl groups

w = weight of the oven-dried sample (g)

C= 0.1

$V_2 - V_1$ = (2.75-2.4) 10^{-3}

W = 36.3 × 10^{-3}

DO = 0.1618

DO % of sample 1 = 16.18%

Likewise, calculations were done for sample 2 and sample 3.

DO % of Sample 2= 23.23%

DO % of Sample 3= 19.41%

Average DO %= 19.61%

Standard deviation = 3.53

Table S1. Contact angle and solubility of the films

Film	Contact angle (degrees)	Solubility %
Control	60.7± 13.0^{b}	30.6± 2.5^{a}
1% U-NCC	68.5± 14.2^{ab}	29.5± 2.9^{a}
3% U-NCC	71.8± 18.4^{ab}	30.0± 2.9^{a}

5% U-NCC	72.8± 10.6^{ab}	26.8± 7.1^{a}
1% TM-NCC	75.2± 17.8^{ab}	29.5± 2.1^{a}
3% TM-NCC	82.8± 13.5^{a}	29.1± 1.7^{a}
5% TM-NCC	81.3± 6.5^{a}	31.6± 6.8^{a}

All the data are given as mean ± SD. Different letters indicate significant difference among the means based on the Tukey test ($p < 0.05$)

Table S2. Remaining weight percentage of the films at 600°C resulted from TGA

Film	Weight % at 600°C
Control	17.99
1% U-NCC	18.92
3% U-NCC	19.56
5% U-NCC	19.43
1% TM-NCC	19.63
3% TM-NCC	19.96
5% TM-NCC	18.96

Weight % at 600°C was obtained from the TGA graphs and reported in the table

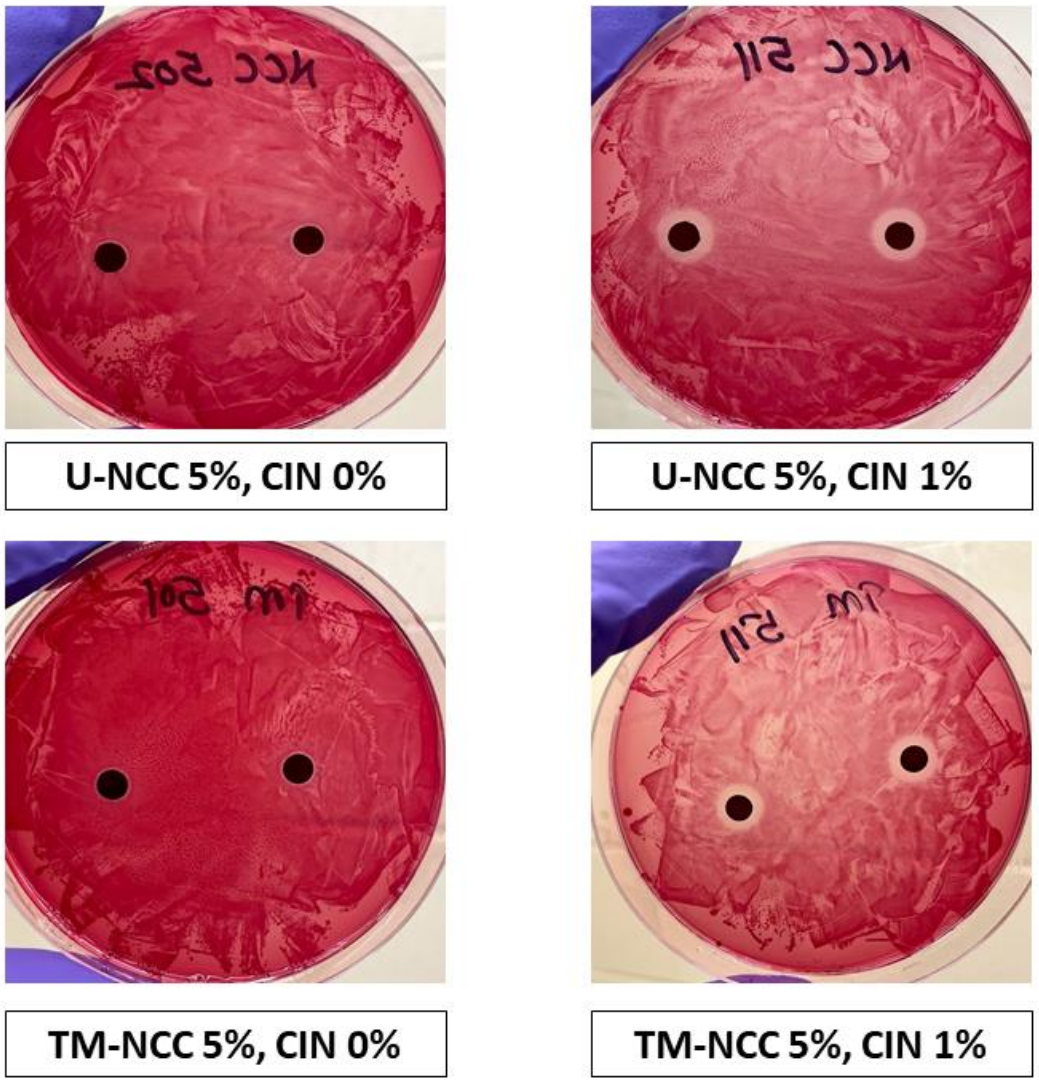

Figure S1. Zone of inhibition resulted by 5% U-NCC and TM-NCC films with and without cinnamaldehyde (CIN) against E. coli in McConkey agar plates

Table S3. Zone of inhibition measured against *E. coli* and *L. inocua*

Film	Zone of inhibition against *E. coli* (cm)	Zone of inhibition in against *L. innocua* (cm)
Control + 0% CIN	0 ± 0	0 ± 0
Control + 1% CIN	1.07 ± 0.15	1.87 ± 0.06
1% U-NCC + 0% CIN	0 ± 0	0 ± 0
1% U-NCC + 1% CIN	1.00 ± 0.17	1.70 ± 0.46
3% U-NCC + 0% CIN	0 ± 0	0 ± 0
3% U-NCC + 1% CIN	0.87 ± 0.21	1.63 ± 0.23
5% U-NCC + 0% CIN	0 ± 0	0 ± 0
5% U-NCC + 1% CIN	0.97 ± 0.06	1.70 ± 0.17
1% TM-NCC + 0% CIN	0 ± 0	0 ± 0
1% TM-NCC + 1% CIN	0.93 ± 0.06	1.93 ± 0.06
3% TM-NCC + 0% CIN	0 ± 0	0 ± 0
3% TM-NCC + 1% CIN	0.83 ± 0.15	1.93 ± 0.12
5% TM-NCC + 0% CIN	0 ± 0	0 ± 0
5% TM-NCC + 1% CIN	0.93 ± 0.06	1.87 ± 0.15

All the data are given as mean ± SD