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Nanocomposites of cellulose nanofibers incorporated with carvacrol via stabilizing octenyl succinic anhydride-modified ϵ -polylysine

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ABSTRACT

Food packaging plays an extremely important role in the global food chain, allowing for products to be shipped across long distances without spoiling. However, there is an increased need to both reduce plastic waste caused by traditional single-use plastic packaging and improve the overall functionality of packaging materials to extend shelf-life even further. Herein, we investigate composite mixtures based on cellulose nanofibers and carvacrol via stabilizing octenyl-succinic anhydride-modified epsilon polylysine (MePL-CNF) for active food packaging applications. The effects of epsilon polylysine (ϵ PL) concentration and modification with octenyl-succinic anhydride (OSA) and carvacrol are evaluated with respect to composites morphology, mechanical, optical, antioxidant, and antimicrobial properties. We find that both increased ϵ PL concentration and modification with OSA and carvacrol lead to films with increased antioxidant and antimicrobial properties, albeit at the expense of reduced mechanical performance. Importantly, when sprayed onto the surface of sliced apples, MePL-CNF-mixtures are able to successfully delay/hinder enzymatic browning, suggesting the potential of such materials for a range of active food packaging applications.

1. Introduction

As the competition for shelf space has never been more intense, future packaging for the food industry must deliver a more compelling value proposition to the consumer [1]. Active packaging can be considered a natural progression in packaging innovation by performing some desired role in food preservation in addition to providing an inert barrier to external conditions [2]. Among available active packaging technologies, the incorporation of preservatives into packaging materials rather than adding them directly to the food is generally preferred, as the food remains chemically unchanged, while still serving a preservative function as part of its protective packaging [3,4].

The current interest in active packaging has focused on synthetic biodegradable polymers and natural polymers such as poly(lactic acid) (PLA), poly(hydroxy-alkanoates) (PHAs), thermoplastic starch, and cellulose nanofibers (CNF) [5]. Cellulose nanofibers are a promising bio-based nanomaterial that is a few nanometers in diameter and a few

hundred to several thousand nanometers in length [6]. Owing to their biodegradability [7], good film-formability [8], remarkable mechanical and barrier properties [9–13], and their highly functionalized surface [14], CNF have become widely exploited as a polymeric matrix for the incorporation of active molecules in recent years [3,15]. Extensive research on the incorporation of active compounds into nanocellulose-based films and coatings has indeed highlighted their potential as an effective carrier for a wide range of antimicrobial and antioxidant agents [6,16–21].

Among various natural compounds, essential oils and their components have recently attracted widespread attention for active packaging applications [22]. Particularly, compounds with phenolic groups such as carvacrol (5-isopropyl-2-methylphenol) are highly effective as antimicrobial and antioxidant agents, thus they have been intensely studied for food preservation [23–26]. Carvacrol is well known for its antimicrobial activity against a broad spectrum of typical foodborne microorganisms, including *Botrytis cinerea*, *Escherichia coli* [27–30], *Staphylococcus aureus*

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[30,31], *Candida albicans* [32], *Listeria innocua*, *Saccharomyces cerevisiae*, and *Aspergillus niger* [33]. Additionally, the antioxidant properties of carvacrol have been recognized for many years [34–36]. For these reasons, carvacrol has been incorporated into various substrates, including chitosan [37], starch [38], PLA [39], among others [40]. Although the highly hydrophobic nature of carvacrol may hinder its association with cellulose nanofibers, different methods can overcome this drawback, such as encapsulation and emulsification [41]. In a previous study, Lavoine et al. [42] developed a controlled release system based on paper coated with TEMPO-oxidized CNF and β -cyclodextrine (β -CD) in which the hydrophobic cavities were able to entrap the carvacrol by simple impregnation and drying treatment. Subsequently, the combined action of TEMPO-oxidized cellulose nanomaterials and β -CD to deliver carvacrol has been exploited by others [43,44]. To the best of our knowledge, no other strategies able to include carvacrol into nanocellulose matrices have been described in the literature. Despite this, recent studies are focusing on the incorporation of essential oils or their components into CNF or CNF-containing biopolymers [45–48].

Epsilon-Poly-L-Lysine (ϵ -polylysine or ϵ PL) is a food-grade cationic homopolyamide of L-lysine produced by *Streptomyces albulus* [49]. Because of its excellent antimicrobial activity and heat stability, ϵ PL has been used as a natural food preservative against a wide spectrum of Gram-positive and Gram-negative bacteria, yeasts, and molds [49–51]. Besides, hydrophobically modified ϵ -polylysine graft copolymers could be synthesized by reacting ϵ PL with octenyl succinic anhydride (OSA) [52–54] that is widely used to yield amphiphilic hydrocolloids approved by the Food and Drug Administration (FDA) for food application purposes [55,56].

In the present study, a new method based on octenyl succinic anhydride-modified ϵ -polylysine (OSA- ϵ PL) and surface-carboxylated (TEMPO-oxidized) CNF was developed as a potential strategy to entrap carvacrol for active packaging applications. Herein, the use of carvacrol and ϵ -polylysine was investigated in relation to their incorporation into CNF-based composites, placing particular emphasis on the resulting morphological, optical, mechanical, antioxidant, and antibacterial properties. A potential synergistic effect of combining carvacrol and ϵ -PL into TEMPO-oxidized CNF was assumed to be an advantage over previous methods as improved antimicrobial and antioxidant properties could be provided to the CNF matrix. As a proof of concept, the carvacrol-loaded ϵ PL-CNF composites were also tested as spray coatings to hinder the enzymatic browning of sliced apples, demonstrating the potential of such materials for active food packaging.

Although the literature presents several studies using nanocellulose embedded in essential oils, no studies were found that reported the joint use of carvacrol and ϵ -PL to develop CNF-based active packaging.

2. Experimental

2.1. Materials

Never-dried elemental chlorine free cellulose fibers from bleached softwood pulp (*Picea abies* and *Pinus* spp., chemical composition 81.3 % cellulose, 12.6 % hemicellulose, 0 % lignin and 0.3 % ash [57]) were obtained from Stendal GmbH (Berlin, Germany). 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO), 2,2-Diphenyl-1-picrylhydrazyl free radical (DPPH, ≥ 97.0 % purity), Deuterated chloroform (CDCl_3), Dimethyl Sulfoxide (DMSO), ethanol, and sodium hypochlorite solution (NaClO , 12–14 % chlorine) were obtained from VWR. Sodium hydroxide (NaOH), and 3-Aminopropyl triethoxysilane (APTES, 99 %) were obtained from Sigma-Aldrich. Sodium bromide (NaBr , ≥ 99 %) and sodium bicarbonate (NaHCO_3 , ≥ 99.7 %) were supplied by Carl Roth GmbH & Co. 2-octenylsuccinic anhydride (OSA, cis- and trans- mixture, > 95.0 %) and carvacrol (> 98.0 %) were purchased from Tokyo Chemical Industry Co. Ltd. Poly epsilon L-lysine (ϵ PL, average MW 3500–4500 Da) was purchased from Carbosynth Limited. All chemicals were used as received without any further purification.

2.2. Synthesis of CNF via TEMPO-mediated oxidation

CNF were prepared via TEMPO-mediated oxidation as described in our previous work [58], whereby never dried cellulose pulp (33.3 wt% total solids) was dispersed in water to a final concentration of 2 wt%. TEMPO and NaBr were dissolved in water, and subsequently mixed with the cellulose pulp suspension at 0.1 and 1.0 mmol/g cellulose, respectively. The suspension pH was then adjusted to 10 by dropwise addition of 2 wt% NaOH. Finally, NaClO was added at 10 mmol/g cellulose, and the reaction proceeded under mild agitation at room temperature for 4–5 h. Following, the TEMPO-oxidized cellulose fibers were washed several times with distilled water, and subsequently ground using a Supermass Colloider (MKZA10-20 J CE Masuko Sangyo, Japan) at an applied energy of 9 kWh/kg. The CNF suspension was stored in a refrigerator at 4 °C until further use. TEMPO catalyzed oxidation produces individualized fibrils without significant aggregation and introduces functional carboxylate groups at the cellulose surface [59]. Morphological and chemical characteristics of the obtained CNF are available in the Supporting Information, Table S1.

2.3. Synthesis of OSA- ϵ PL graft copolymer & loading of carvacrol (MePL)

A 1:1 mixture of ϵ -polylysine and 2-octenyl succinic anhydride was reacted for 18 h in DMSO at 40 ± 5 °C to facilitate grafting, following the procedure of Yu et al. [53]. The reaction mixture was then dialyzed in 3500 Da molecular-weight-cut-off (MWCO) dialysis membrane against 95 % EtOH, 0.01 % NaHCO_3 , and deionized water (twice sequentially) to remove any unreacted moieties or possible hydrolysates. The dialyzed mixture was then lyophilized to obtain the final OSA- ϵ PL graft copolymers. Subsequently, carvacrol was loaded into the hydrophobic domain of self-assembled OSA- ϵ PL graft copolymers, adapting a previously published method [54]. Briefly, 50 mg of OSA- ϵ PL and 10 mg of carvacrol were dissolved in 10 mL DMSO and dialyzed for 6 cycles against distilled water (3500 Da MWCO). The mixture was then filtered through a 0.45 μm filter and lyophilized to obtain carvacrol-loaded OSA- ϵ PL, herein referred to as MePL.

2.4. Preparation of MePL-CNF composite films

Based on our preliminary investigations, MePL-CNF film forming mixtures were prepared by blending 0.5 wt% CNF aqueous suspension with MePL to a final concentration of 0.10 wt% or 0.25 wt% (1:5 or 1:2 ratio with respect to CNF solid weight). Mixtures and subsequent films are denoted as MePL010-CNF and MePL025-CNF, respectively. In each case, MePL was slowly added to the CNF aqueous suspension and vortex mixed until fully suspended. MePL-CNF film forming mixtures were subsequently stirred for at least 1 h before further use to ensure homogeneity. For comparison, ϵ PL-CNF mixtures were also prepared by simply dissolving pure ϵ PL into CNF aqueous suspensions at the same concentrations as for MePL (denoted as ϵ PL010-CNF and ϵ PL025-CNF). As above, the ϵ PL-CNF mixtures were vortexed and subsequently stirred prior to further characterization/use. ϵ PL-CNF and MePL-CNF wet films were formed by vacuum filtration of the above-described formulations using a Buchner funnel and 0.65 μm filter (Durapore DVPP membrane filter, Merck Millipore Ltd.). Note that all films were formed from the same volume of ~ 0.5 wt% film-forming mixtures, yielding a basis weight of ~ 20 g/m² for each film. The wet films were subsequently hot pressed at 90 °C and 20 bar for 30 min. Dried films were stored at room temperature until characterization; as required, they were cut to size using a laser cutter (Nova24 60 W, Thunderlasers). CNF-only films were also fabricated using the same procedure for comparison.

2.5. Analyses of suspensions

The degree of substitution of α -amino groups of ϵ PL by nucleophilic

reaction with OSA was calculated via ^1H NMR (Bruker 300 spectrometer operating at 300.13 MHz) in CDCl_3 following the description of Yu et al. [53]. FT-IR spectra of lyophilized OSA- ϵ PL, M ϵ PL, and their starting materials were recorded using a Bruker Tensor 27 FT-IR spectrometer in attenuated total reflectance (ATR) mode. Additionally, lyophilized film forming mixtures of ϵ PL-CNF composites, with (M ϵ PL010-CNF, M ϵ PL025-CNF) and without carvacrol (ϵ PL010-CNF, ϵ PL025-CNF), were chemically analyzed by using FT-IR technique and compared with CNF-only material. All spectra were recorded between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans per sample.

The aggregation behavior of composite and CNF-only mixtures was characterized via dynamic light scattering (DLS, 0.025 wt% in water), and zeta potential (0.25 wt% in 10 mM NaCl) using a ZetaSizer Nano ZS (Malvern), whereby all measurements were repeated in triplicate with results presented as the average \pm standard deviation. The mean values of measured parameters were compared using Fisher's least significance difference (LSD) test, where the F-test was significant and with a significance level of $p \leq 0.05$. To this aim, a one-factor comparative experiment was performed, whose levels were represented by the film forming mixtures (CNF, ϵ PL010-CNF, ϵ PL025-CNF, M ϵ PL010-CNF, M ϵ PL025-CNF).

Particle morphology of the various composites was evaluated by high-resolution transmission electron microscopy (TEM, JEOL, JEM 2200fS) and atomic force microscopy (AFM, Bruker ICON3). Note that for AFM, samples were deposited on a freshly cleaved mica substrate modified with 0.05 % APTES at a sample concentration of ~ 2 g/L, and measured in tapping mode. TEM images were obtained after the samples (0.001 wt%) were deposited onto carbon-coated copper grids, stained by 1 wt% uranyl acetate.

2.6. Analyses of films

Scanning electron microscopy (SEM, FEI NanoSEM 230) was used to characterize the surface and cross-section microstructure of composite and CNF-only films.

Tensile testing was performed using a micromechanical testing device equipped with a 5 N load cell. Samples were strained at 1 $\mu\text{m/s}$ until breakage to generate stress-strain curves. The Young's modulus was calculated from the initial linear portion of the curves, tensile strength was determined as the maximum stress achieved during testing, and toughness was calculated by numerically approximating the area under the stress-strain curve. Samples were tested in at least quintuplicate, with results presented as the average \pm standard deviation. To differentiate CNF composites of each series from those of pristine CNF, all pairwise comparisons of means of measured mechanical properties were performed using Fisher's least significance difference (LSD) test, where the F-test was significant ($p \leq 0.05$).

Optical properties were evaluated via UV/Vis (UV3600 UV-vis NIR Spectrophotometer, Shimadzu). Film transparency was measured using an integrating sphere and BaSO_4 backplate, while film haze was calculated as the ratio of diffuse transmittance (no backplate) to film transparency.

Contact angle (CA) measurements were performed using the video-based optical contact angle measuring system OCA 20 (DataPhysics Instruments GmbH, Filderstadt, Germany). The contact angle of water in air at 20 °C and 65 % RH was determined by the sessile drop method by gently placed a 5 μL droplet on the film surface (5 \times 20 mm strips). The drop shape was processed by an image analysis system and calculated with an accuracy of $\pm 0.1^\circ$. The contact angle was taken as an average of at least twenty measurements, each being obtained as an average of the left and right contact angles of the water drop.

2.7. Assessment of active properties

DPPH free radical scavenging activity of all film forming mixtures was determined according to the procedure reported by Feng et al. [60]

[60] with slight modifications. In a test tube, 1.0 mL of film forming mixture was added to 4.0 mL of freshly prepared 0.1 mM DPPH ethanolic solution. The mixture was well shaken in a Vortex (3000 rpm), then stored in the dark for 30 min. The mixture was then centrifuged at 5000 rpm for 15 min and the supernatant was collected. The absorbance of the latter was read at 517 nm, using a Cary 1E UV-vis spectrophotometer (Varian). A blank comprising distilled water and ethanol, and a control containing only the DPPH solution were also run. DPPH radical scavenging activity was calculated as percent of inhibition (%) using the following formula:

$$\text{Radical scavenging activity\%} = \left(1 - \frac{A_i - \bar{A}_j}{A_c}\right) \times 100 \quad (1)$$

where A_i is the absorbance of the sample, \bar{A}_j is the absorbance of the control, and A_c is the absorbance of the blank. All samples were assayed in at least triplicate for each condition.

The film's antimicrobial activity was tested against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. Films were cut into 10 mm diameter discs using a laser cutter as described above and were sterilized via exposure to UV light for 30 min under ambient conditions. Microbial cultures (*E. coli* DSMZ 1103, *S. aureus* ATCC 6538, and *C. albicans* ATCC 90028) were precultured in 5 mL of 30 % tryptic soy broth (TSB) plus 0.25 % glucose and incubated overnight at 37 °C at 40 rpm. Precultures were diluted to OD₆₀₀ nm 0.001. Pre-cut films were then added to 1 mL of the diluted microbial culture and incubated for 2 h at 37 °C and 40 rpm. The films were finally placed on a PC-agar plate (Sigma-Aldrich 70152) which was incubated overnight at 37 °C prior to imaging. All samples were tested in at least triplicate for each condition.

2.8. Spraying test on apples

A suspension containing ϵ PL-CNF or M ϵ PL-CNF with a 1:5 or 1:2 ratio of active molecules with respect to CNF was prepared in order to form a 0.5 wt% solids mixture (as detailed above). This suspension, named as film forming mixture was sprayed onto the surface of locally purchased apples using an atomizing sprayer (Ultra-airbrush, Harder & Steenbeck GmbH & Co. KG). Spraying only took place on the exposed surface of a half of apple (fruits were cut in half through the core immediately before spraying), while the remaining half was used as a control. The bare and coated half-apples were imaged with a digital camera for 1 h to evaluate the effects of the spray coating on fruit browning.

3. Results and discussion

In this study, the development of functional cellulose nanofibers incorporated with carvacrol via stabilizing OSA-modified ϵ -polylysine (M ϵ PL-CNF) was investigated (Fig. 1). The interaction between the components was verified by ^1H NMR and FTIR-ATR spectroscopies. Firstly, ^1H NMR demonstrated the occurrence of the hydrophobic modification of α -amino groups of ϵ PL by nucleophilic reaction with OSA, which served as preparation for the subsequent steps. The ^1H NMR spectra of OSA, ϵ PL and OSA- ϵ PL samples are shown in Fig. 2a. Compared to pure ϵ PL, OSA- ϵ PL graft copolymer showed additional peaks between 0.7 and 2.7 ppm attributed to the octenyl succinate group substitution [61]. Furthermore, the shift of the methine group of ϵ PL from 3.8 to 4.0 ppm is indicative that some of the amine groups were acylated. The degree of substitution was found to be 23.9 %, which agrees well with previous findings using a similar OSA: ϵ PL feed ratio [53].

OSA- ϵ PL conjugation as well as the subsequent interaction with carvacrol to get M ϵ PL was further investigated via FTIR (Fig. 2b). The absorption of all ϵ PL-containing samples included contributions from the neutral amide NH and side chain amine NH_2 groups at 3400–3100 cm^{-1} , from charged (protonated) side chain NH_3^+ groups in the range

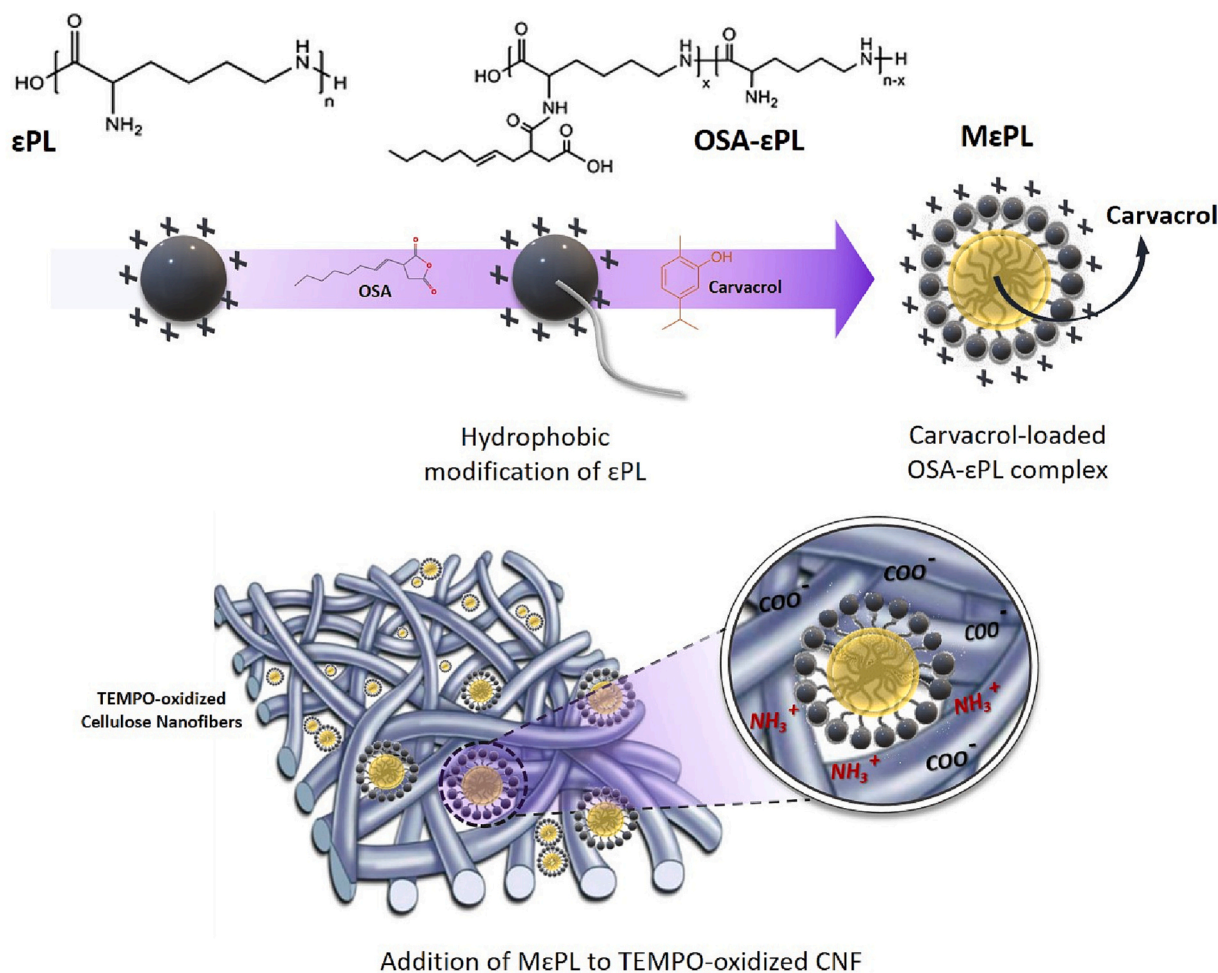


Fig. 1. Schematic representation of MεPL-CNF composite synthetic pathway.

3100–2500 cm^{-1} [62] along with from amide I, II and III groups between 1700 and 1200 cm^{-1} [63,64]. The pure ϵ PL samples showed a broad absorbance peak centered around 3218 cm^{-1} identifying the stretching band of N–H groups. The position of this band shifted to around 3240 cm^{-1} and 3250 cm^{-1} in the OSA- ϵ PL and M ϵ PL spectra, respectively, suggesting the existence of hydrogen bonding between the N–H groups of ϵ PL and the carbonyl (C=O) groups from OSA [53]. The weak band at approximately 3070–3050 cm^{-1} may be assigned to the overtone of the N–H stretching band (amide-II band) at 1560–1530 cm^{-1} [53,65]. The stretching modes of protonated -NH_3^+ side chain groups also fall in the range at ca. 3030–3050 cm^{-1} [62,66,67]. The intensity of this band was stronger upon hydrophobic substitution of ϵ PL with OSA. Also, the two absorption bands of C–H stretching at 2925 and 2860 cm^{-1} were naturally more prominent than for pure ϵ PL [53]. After the substitution, a redshift from 1662 to 1645 cm^{-1} occurred in the amide-I band, and a significant change was observed in the adsorption intensity at 1535 cm^{-1} in the amide-II band. The band at 1390 cm^{-1} , designated as methyl C–H bending, was significantly intensified in the OSA- ϵ PL and M ϵ PL spectra versus pure ϵ PL. Although the absorption of M ϵ PL was very similar to that of OSA- ϵ PL, the addition of carvacrol led to the appearance of a new peak at 1014 cm^{-1} and a small shoulder at 950 cm^{-1} that might be traced back to the aromatic functionalities of the incorporated carvacrol [68–70].

Afterward, composite films were prepared from a mixture of negatively charged TEMPO-oxidized cellulose nanofibers (CNF) and the positively charged ϵ PL or M ϵ PL polypeptides. Successful composite formation was investigated via FTIR (Fig. 2c). Compared with pure CNF, broadening and shifting of the band at 1600 cm^{-1} combined with the

appearance of a new shoulder at 1662 cm^{-1} occurred in all composite spectra, which may be attributed to the electrostatic interaction between COO^- of CNF and NH_3^+ of ϵ PL and the C=O and –NH vibration bands in amide groups of the peptide ([64,71,72]; C. [73]). As expected, these distinctive bands were more prominent with an increase in ϵ PL concentration in the composite films and upon the addition of carvacrol.

The addition of ϵ PL or M ϵ PL to the CNF matrix led to clear aggregation, as evidenced by the increase in size via DLS, and decrease in surface charge via zeta potential. As shown in Fig. 3a, the impact of this addition on z-average diameter and zeta potential was greater upon increasing the ϵ PL (or M ϵ PL) content. Note that, although some of the aggregates are too large to be reliably measured by DLS, the relative differences between samples are still assumed to be meaningful.

At 0.25 wt%, the partitioning of ϵ PL at the surface of nanoparticles was such as to cause a rapid change in zeta potential from -42.7 mV to a positive value of 26.5 mV for ϵ PL-CNF sample. This suggested a complete neutralization of the negatively charged colloidal surface of CNF. Nevertheless, M ϵ PL caused less aggregation than ϵ PL, and resulted in minor change in zeta potential. As the carvacrol molecules were physically entrapped into the hydrophobic domain of the self-assembled OSA- ϵ PL block copolymers via the dialysis method, this might be due to the compact packing of the hydrophobic core of the polymer complex that acted reducing the overall surface charge density of M ϵ PL as compared to the pure ϵ PL. Consequently, the neutralization of negatively-charged nanofibers by ϵ PL is attenuated to some extent, reducing aggregation and thereby suppressing DLS size increase. According to previous reports [53,54], the amphiphilic OSA- ϵ PL copolymer can self-assemble to form a complex layer at the oil-water

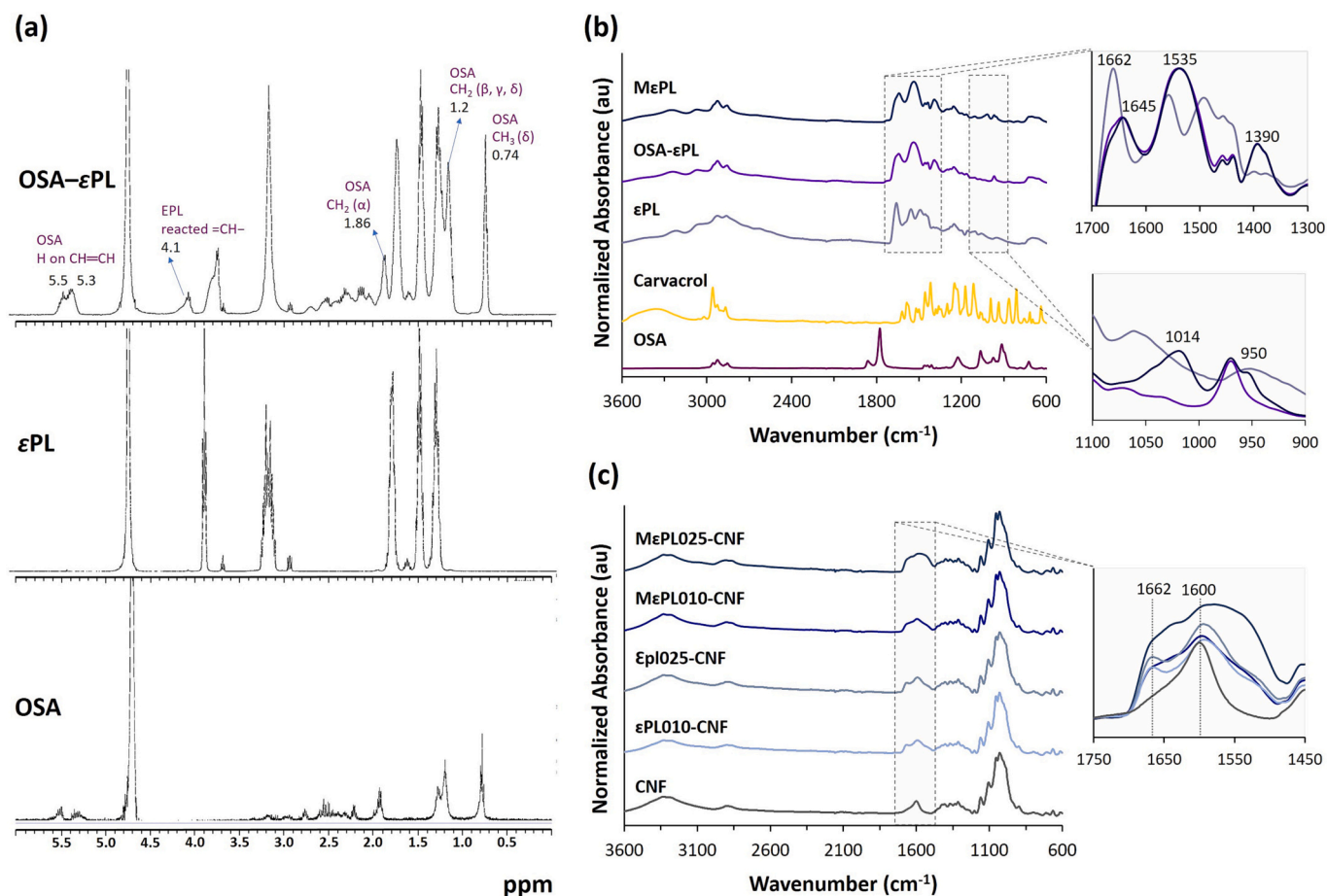


Fig. 2. Hydrophobic modification of ϵ PL and loading of carvacrol via stabilizing OSA-modified ϵ PL: (a) ^1H NMR spectra of OSA, ϵ PL and OSA- ϵ PL. (b) Normalized FTIR-ATR spectra of OSA- ϵ PL, MePL, and their pure components ϵ PL, OSA and carvacrol; areas of interest are shown in the inset spectra. (c) Normalized FTIR-ATR spectra for CNF-only films, and ϵ PL/MePL-CNF composite films at varying ϵ PL loading concentrations; the 1750–1450 cm^{-1} portion of the spectra is shown in the inset.

interface, which may serve as a microenvironment to water-insoluble bioactive compounds and thus increase their water solubility. It could be hypothesized that, as the organic solvent DMSO inside the dialysis bag was gradually replaced by water (non-solvent for the hydrophobic block), carvacrol was progressively loaded into self-associated polymeric complexes, having a hydrophobic core of OSA and a cationic shell of ϵ PL (Fig. 1). Afterwards, the cationic shell of the complexes is expected to promote their electrostatic attraction to the negatively charged CNF, while the hydrophobic core still retained the poorly water-soluble carvacrol molecules. The presence of CNF nanoparticles in the vicinity of MePL interfacial layer may additionally contribute to stabilizing the oil-water interface.

MePL-CNF was morphologically analyzed and compared to ϵ PL-CNF and CNF-only by TEM and AFM imaging. TEM evidenced a more uniform distribution of MePL within the CNF matrix in comparison to pure ϵ PL having the same loading concentration. In the TEM images (Fig. 3b), the CNF sample showed a network of randomly assembled nanofibers, while the composite samples (both containing 0.10 wt% of ϵ PL or MePL with respect to CNF weight) were also made up of nearly spherical nanoparticles (diameter \sim 30–40 nm), which adhered to the needle-like nanofibers. In MePL010-CNF, these spherical nanoparticles appeared much well-dispersed throughout the surrounding nanofibers as compared with ϵ PL010-CNF. As the loading concentration of ϵ PL increased to 0.25 % by weight, more spheres of ϵ PL (or MePL) were adhered along the cellulose nanofibers, resulting in a partial concealment of the underlying nanofibers (Supporting Information, Fig. S1). Some globular particles clearly appeared also in the AFM image of

MePL010-CNF (Fig. 3c), while they were not visible in the composite without carvacrol. The large globular particles (50–100 nm) visible in Fig. 3c are attributable to carvacrol loaded OSA- ϵ PL complexes that are agglomerated, while the smaller particles might be the same isolated complexes. At higher loadings (0.25 %), the entanglement and aggregation increased drastically, thereby making ϵ PL025-CNF and MePL025-CNF composites unable to be imaged via AFM.

The microstructure of the composite films was highly dependent on the loading concentration of ϵ PL or MePL as evidenced by SEM imaging. Cross-section micrographs of CNF-only films versus composite films at low and high ϵ PL or MePL loadings are shown in Fig. 4a (SEM images of the film surface are available in the Supporting Information, Fig. S2). At 0.10 wt% ϵ PL or MePL, a homogeneous, compact, and dense lamellar-like structure is observed, demonstrating a minor impact on film morphology compared with CNF only films. Conversely, the incorporation of 0.25 wt% ϵ PL or MePL led to an irregular, less compact morphology, with some large pores evidenced throughout the film cross-section (these could be formed from trapped air bubbles during the drying process, as mixtures with this high ϵ PL/MePL loading were qualitatively much more viscous [74]). Furthermore, it is likely that the increase in ϵ PL content reduces the cross-links between CNFs and the alignment of the nanofibers in the network, thereby increasing the spatial distance within the film matrix [73]. As a result, depending on the ϵ PL or MePL loading concentration the films showed different thicknesses of 9 ± 1 , 11 ± 1 , 24 ± 3 , 14 ± 2 , 30 ± 5 μm for CNF, ϵ PL010-CNF, ϵ PL025-CNF, MePL010-CNF, MePL025-CNF, respectively.

Film transparency was assessed by UV-vis spectroscopy (Fig. 5a),

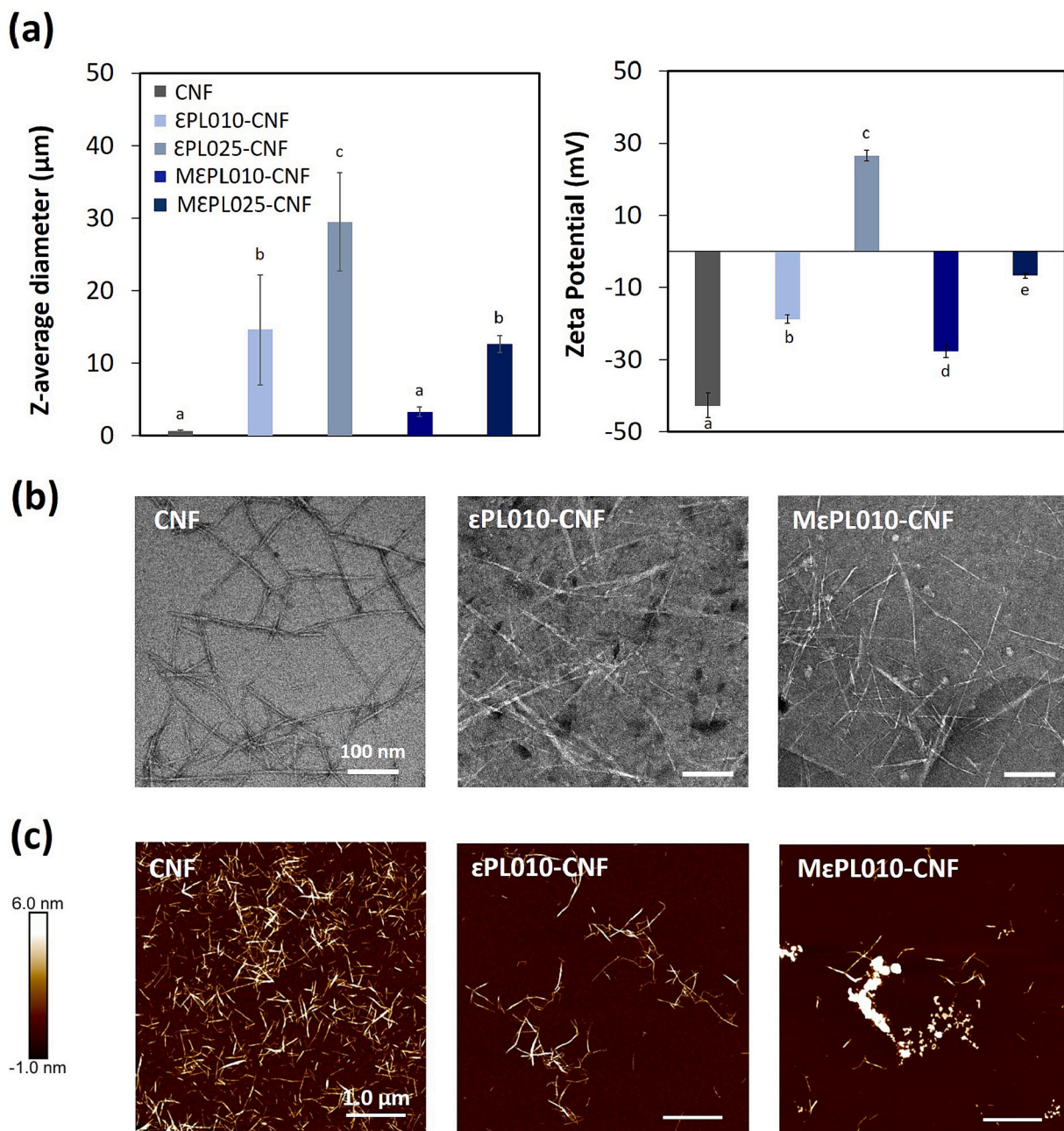


Fig. 3. (a) Comparison of the z-average diameter (as a qualitative measure of aggregate size) and the zeta potential (mean values with standard deviations) of ϵ PL-CNF and M ϵ PL-CNF composites. Same letter indicates statistical differences smaller than least significant difference (LSD) (not significant for $p \leq 0.05$). Microstructural characterization of composite mixtures: (b) TEM and (c) AFM micrographs for CNF-only, ϵ PL010-CNF, and M ϵ PL010-CNF suspensions.

whereby all films exhibited excellent transparency ($> 80\%$) in the visible range, regardless of ϵ PL/M ϵ PL loading. However, there were notable differences in film transparency in the UV range ($< 400\text{ nm}$), whereby the addition of M ϵ PL led to a concentration-dependent absorbance of UV light. This is likely due to the high concentration of phenolic residues in carvacrol, which have a strong absorption in the UV region (maximum absorption at $\sim 275\text{ nm}$) [75,76]. These results are consistent with previous studies in which UV barrier properties have been observed via the incorporation of essential oils (or their components) into a film matrix [77–79]. This UV-blocking property could be extremely beneficial for food packaging applications, providing protection against oxidative deterioration (i.e., discoloration, loss of nutrients, off-flavors production) caused by UV radiation [77]. Notably, the incorporation of ϵ PL and M ϵ PL into CNF films did result in an increase in film haze (scattered light, Supporting Information, Table S2), which is likely due

to increased film heterogeneity/porosity as observed via SEM.

Composite film mechanical properties were assessed via tensile testing (Fig. 5b, additional information in Table S2). For all samples, the stress-strain curves showed a relatively linear profile, indicative of purely elastic deformation and good interfacial adhesion/stress transfer during loading, typical for CNF-based films [80]. Compared to CNF only films, ϵ PL010-CNF and M ϵ PL010-CNF films showed an increase in tensile strength (from 59 MPa to 156 and 75 MPa, respectively, Fig. 5d), elongation at break (from 0.7% to 1.3 and 1.4%, respectively Fig. 5c), and toughness (from 0.22 MJ/m³ to 1.17 and 0.62 MJ/m³, respectively, Fig. 5f). This is likely due to the intercalation of ϵ PL/M ϵ PL between individual CNF, increasing the interfacial adhesion/inter-molecular interactions within the film [77]. This increase in the mechanical properties of CNF films following the addition of ϵ PL is consistent with data from a previous work, in which amino-cellulose nanofiber films (ϵ -PL-

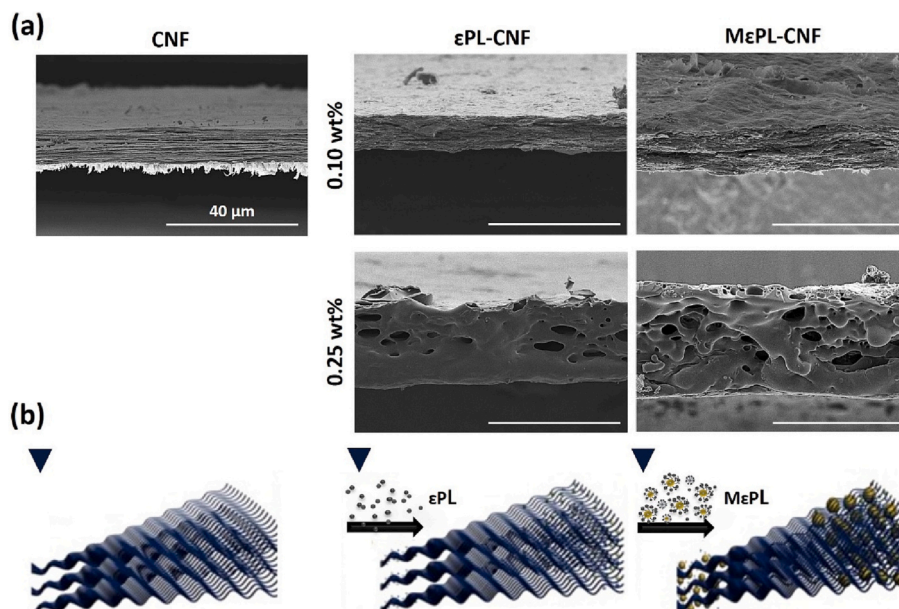


Fig. 4. (a) Scanning electron micrographs (magnification 5000 \times) of the cross-section of films made by CNF-only, ϵ PL010-CNF, ϵ PL025-CNF, M ϵ PL010-CNF, and M ϵ PL025-CNF. (b) Schematic illustration of films 3D network.

TOCNs) prepared by grafting ϵ -PL onto TEMPO-oxidized cellulose (TOCN) exhibited higher mechanical properties than TOCN films, attributing this behavior to the increase in hydrogen bonding due to the introduction of amine and amide bonds on polypeptide structures [81]. However, upon increased ϵ PL/M ϵ PL loading, mechanical properties decreased to values below even the CNF control. This systematic decline in mechanical performance is likely due to the increased heterogeneity/presence of void spaces in the film microstructure, as observed via SEM. This was also consistent with previous findings, where the tensile strength of composite nanocellulose-based films containing ϵ -PL was slightly increased from 36.2 MPa to 38.7 MPa than that of the control film and then decreased by further increasing the content of ϵ -PL [72].

The surface wettability of composite films was investigated by water contact angle (θ_w) as an indicator of the hydrophilic or hydrophobic character of a film surface [60]. As can be seen from contact angle measurements (Table S2 in Supporting Information), using the OSA-modification pathway rendered the M ϵ PL-CNF composite films more hydrophobic compared to those produced with pure ϵ PL at the same loading concentration. The θ_w values between 63° and 64° for CNF-only film, and both ϵ PL-CNF composite films were indicative of rather hydrophilic surfaces. A significant increase ($\theta_w \sim 78$) in contact angle was observed for both M ϵ PL films, indicating that the hydrophobicity of those composite films was greatly improved by the incorporation of hydrophobic OSA- ϵ PL loaded with carvacrol. This is important for food packaging applications, as the increased hydrophobicity would lead to less diffusion of water into the film.

Active properties of the composite films were assessed in order to demonstrate the advantage of incorporating both preservatives, ϵ PL and carvacrol, into CNF films. First, the antioxidant capacity of all film forming mixtures was determined as via DPPH radical scavenging activity, and the results are summarized in Fig. 6a-c. The effects of an antioxidant on DPPH are dependent to its ability to act as an electron donor (i.e., hydrogen-donor), being able to pair the odd electron of DPPH radical, thereby turning its color from purple to yellow [82]. From Fig. 6a, it is clearly seen that the strong absorption at 517 nm of DPPH from its odd electron was visibly reduced when the electron paired with a free radical scavenger in the film forming mixtures and, as expected, the degree of discoloration of DPPH was directly proportional to the percentage of electrons scavenged by the reaction system (see Fig. 6c). The antioxidant activity of neat CNF for DPPH, was equal to 59.2 ± 8.8

% (Fig. 6b). Low levels (0.10 wt%) of ϵ PL and M ϵ PL led to percentages of radical removal very close to that of the CNF control, whereas a 19.4 % increase was recorded when 0.25 wt% ϵ PL was loaded to CNF (albeit largely statistically insignificant). Nevertheless, among all cases, best radical inhibition, ~ 80 %, was achieved through the high-level incorporation of carvacrol via OSA-modified ϵ PL (M ϵ PL025), largely imputing this result to carvacrol molecules, which act as effective antioxidants. The antioxidant capacity of carvacrol has been proven to depend on the steric and electronic effect of its ring, besides the presence of the hydroxyl group which is capable of donating hydrogen atoms [77,83,84]. These findings are consistent with results of other authors, who incorporated carvacrol in chitosan [37], poly(lactic acid)/poly(ϵ -caprolactone) [69], starch [38], and flaxseed gum [85] films.

The antibacterial efficacy of ϵ PL-CNF and M ϵ PL-CNF composite films was qualitatively tested against Gram-positive *S. aureus*, Gram-negative *E. coli*, and fungal *C. albicans* (Fig. 6d and Fig. S3 of the Supporting Information). After exposure to ϵ PL and M ϵ PL, all three microbial strains showed clearly reduced growth on agar plates compared to those exposed to CNF-only films, demonstrating the antimicrobial activity of both ϵ PL and M ϵ PL. Furthermore, the concentration-dependent activity was observed against all three strains. Higher concentration of M ϵ PL exhibited higher activity, whereas ϵ PL displayed an opposite behavior, with the higher activity from the lower concentration. The latter might be due to the non-homogeneous dispersion of 0.25 wt% ϵ PL within the CNF matrix. ϵ PL has well-documented broad-spectrum antibacterial activity arising from its primary amine groups and positive charge [86,87]. This activity is typically more pronounced against Gram-positive bacteria, ([88]; [73]) as was observed here. Carvacrol is well known for its antimicrobial activity, whereby the presence of its phenolic residues can interact with the cell membrane of microorganisms causing loss of membrane structural integrity [38,77]. Furthermore, the hydroxyl groups on benzene rings of carvacrol play a crucial role in the antimicrobial activity of this phytochemical as they are capable of disrupting the equilibrium of inorganic ions and pH homeostasis in the cytoplasm of bacteria [38]. The poorer sensitivity of Gram-negative bacteria to carvacrol is due to differences in the structure and permeability of the bacterial cell membrane, such as the outer membrane that surrounds gram-negative microorganisms restricting the diffusion of hydrophobic compounds [33]. This behavior agrees with the results obtained herein. Against *Candida*, instead, carvacrol seems to

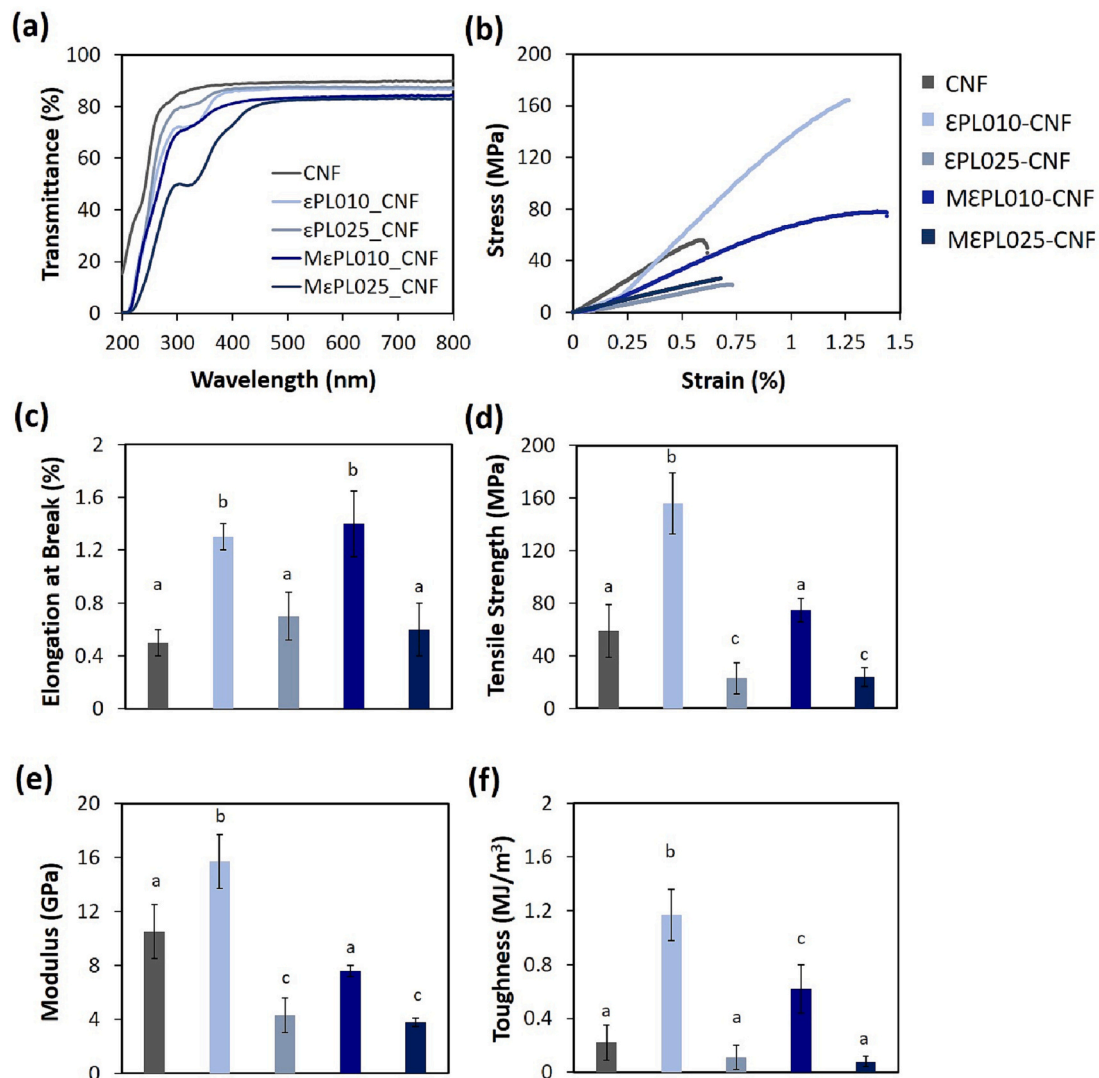


Fig. 5. Optical and mechanical characterization for CNF-only, ϵ PL010-CNF, ϵ PL025-CNF, M ϵ PL010-CNF, and M ϵ PL025-CNF films: (a) Representative UV-vis transmittance spectra, (b) representative stress-strain curves, (c) elongation at break, (d) tensile strength, (e) Young's modulus, and (f) Toughness. Error bars indicate the standard deviation of the mean of at least five replicates. Same letter indicates statistical differences smaller than least significant difference (LSD) (not significant for $p \leq 0.05$).

exert its antifungal activity by disrupting membrane integrity, and blocking ergosterol biosynthesis [89].

Finally, as a proof of concept for active food packaging, the effectiveness of ϵ PL-CNF and M ϵ PL-CNF spray coatings in preserving oxidation-sensitive foods was verified in locally purchased apples, which served as a model system. Importantly, in all cases, the spray coating was almost completely undetectable, as evidenced by the images taken directly after spraying (0 min) in comparison with the uncoated apples. This is crucial for practical application as active food packaging, as food products should retain their visual appeal. By comparing the images of control samples (uncoated sliced apples) with those of sprayed sliced apples (Fig. 7), it clearly appears that all film forming mixtures were effective in preventing enzymatic browning for up to 60 min post-spraying (Supporting Information, Video S1). Notably, at 60 min post-spraying, the control bare apples as well as those coated with CNF-only showed considerable enzymatic browning of the apple flesh. It can therefore be reasoned that the loading of active compounds, ϵ PL and carvacrol, within CNF films are effective in delaying/inhibiting the oxidative reactions that lead to browning of the fruit surface.

4. Conclusion

In summary, composite mixtures of CNF with carvacrol loaded modified- ϵ PL (M ϵ PL) were prepared via simple electrostatic complexation, whereby the addition of increasing amounts of M ϵ PL led to noticeable aggregation, as demonstrated via DLS, AFM, and TEM measurements, which hindered the optical transparency and mechanical properties of the films formed thereby. However, this high loading of M ϵ PL also led to dual-functional CNF-based composites with significantly improved antioxidant activity (as measured via DPPH assay) and increased broad-spectrum antimicrobial activity against *S. aureus*, *E. coli*, and *C. albicans*, compared with either CNF or ϵ PL alone. Importantly, M ϵ PL-CNF composites were verified as sprayable active food packaging, whereby the enzymatic browning of sliced apples was drastically reduced after coating. Taken together, these results open the possibility to use such CNF-based composites for food packaging applications based solely on sustainable, natural materials. Nevertheless, additional studies may be required to further investigate key aspect of food packaging materials, including barrier properties, sensory evaluation, as well as to demonstrate compliance with the general safety requirements.

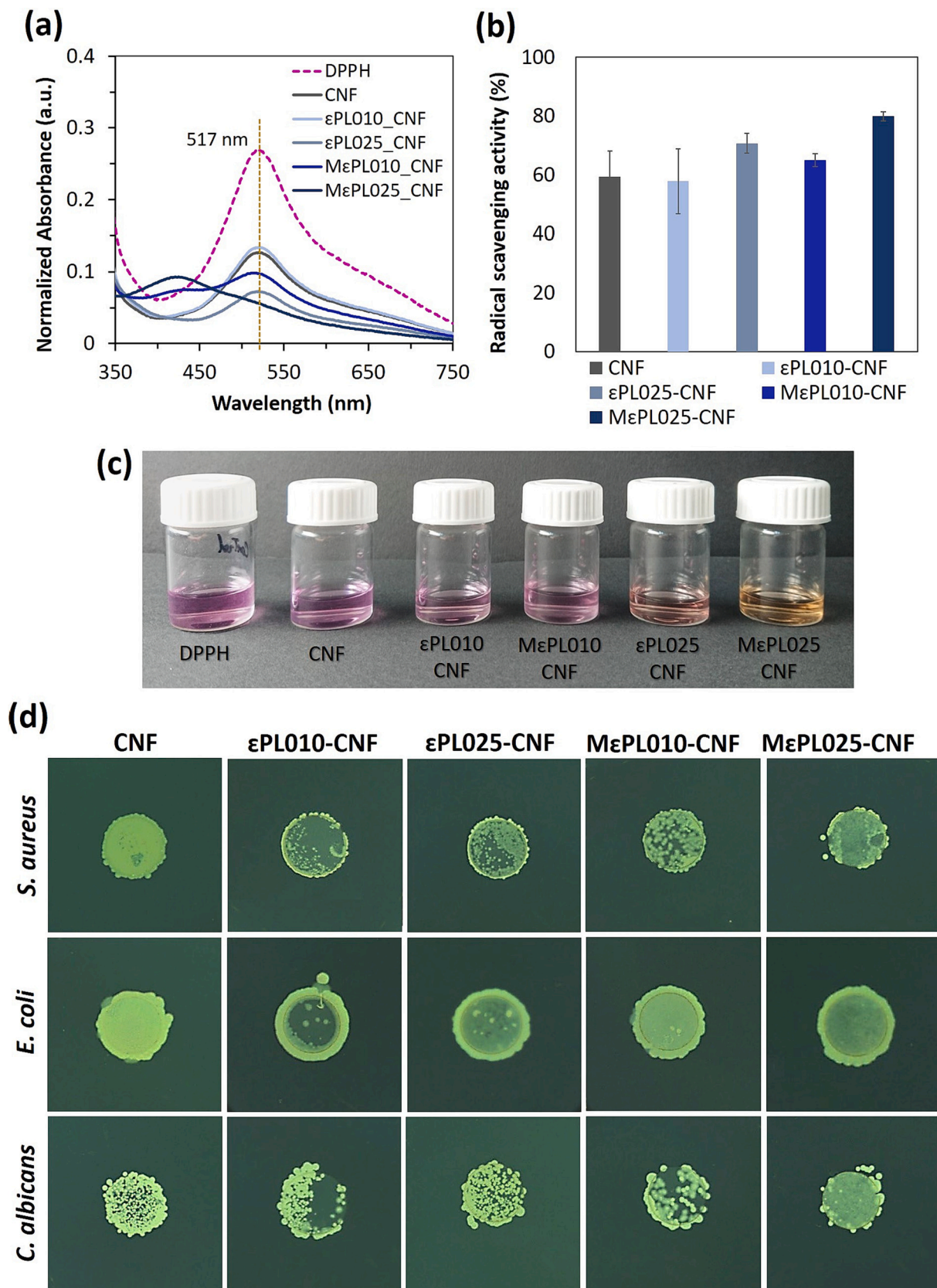


Fig. 6. Assessment of active properties of composite films: (a-b) evaluation of the radical scavenging activity of the film forming mixtures with varying ϵ PL and M ϵ PL content via DPPH assay. (c) Photographs of DPPH assay taken immediately after reading absorbance. (d) Antimicrobial activity of CNF-only film versus ϵ PL-/M ϵ PL-CNF composite films with varying loading concentration against *S. aureus*, *E. coli*, and *C. albicans*.

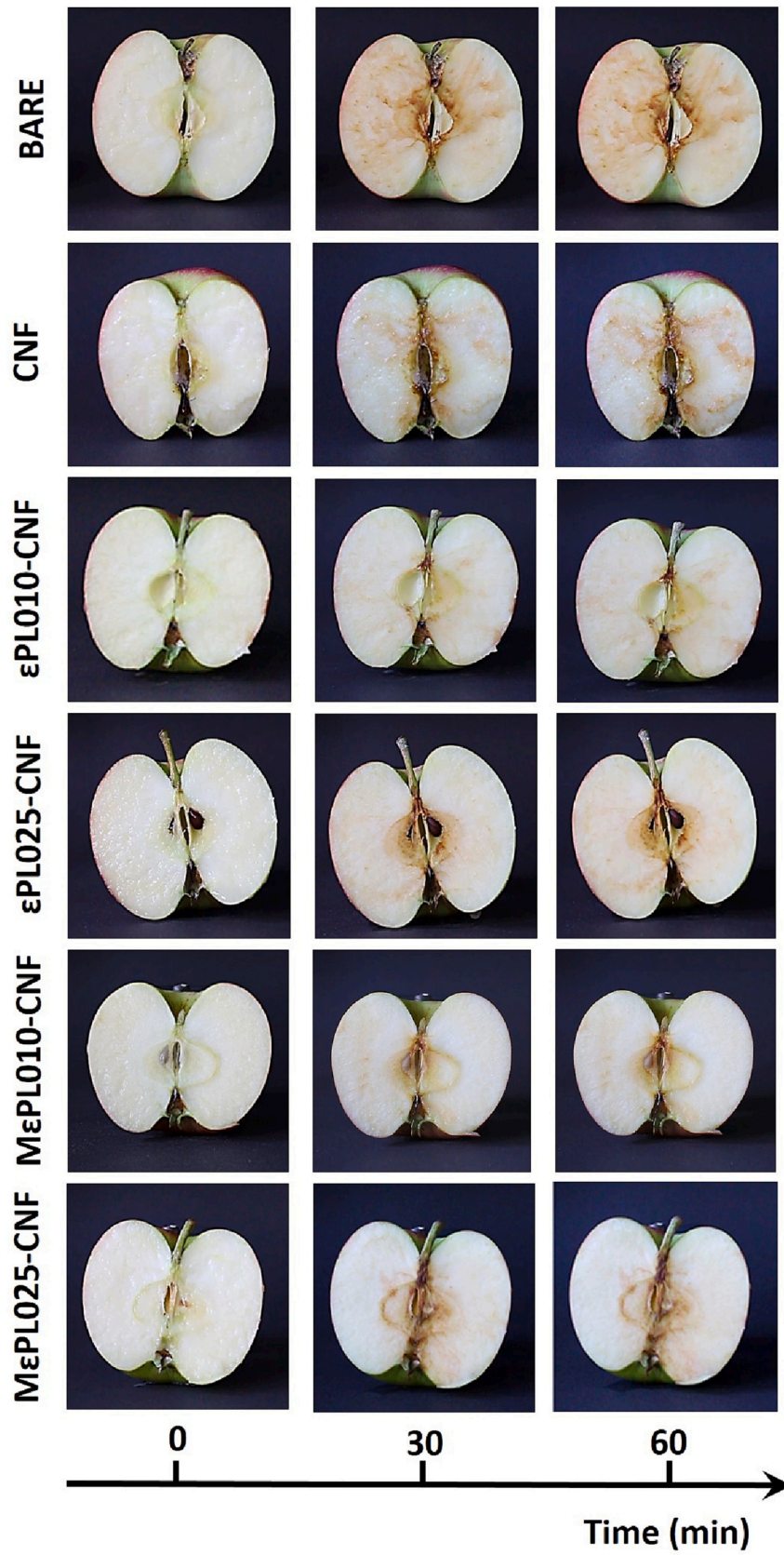


Fig. 7. Visual appearance of bare and sprayed sliced apples over 60 min post-spraying.

Additional TEM images of cellulose nanofibers containing ϵ PL and $M\epsilon$ PL at 0.25 wt% along with SEM images of films made thereof at high and low loading concentrations are provided. Images of the antimicrobial assay on agar plates are reported for three repeats of CNF-only films versus all ϵ PL-/ $M\epsilon$ PL-CNF composite films. Data on mechanical properties, haze, and water contact angle of the composite CNF films are also provided. A video recording of bare and sprayed sliced apples over 60 min post-spraying is available. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2023.124869>.

CRedit authorship contribution statement

Experiments were designed by L.A., K.D., G.S., and G.N., and were conducted by L.A. and K.D. NK performed the AFM measurements. QR was responsible for the antimicrobial assay. L.A. wrote the manuscript with input from all coauthors. All authors reviewed and commented on the manuscript.

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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