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Research Article

Physicochemical Properties of Edible Chitosan/Hydroxypropyl Methylcellulose/Lysozyme Films Incorporated with Acidic Electrolyzed Water

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The treatment with acidic electrolyzed water (AEW) is a promising disinfection method due to its effectiveness in reducing microbial population. The aim of the study was to evaluate physicochemical properties of chitosan/HPMC films incorporated with lysozyme and acidic electrolyzed water. In the composite films, decreasing film solubility and increasing concentration of sodium chloride solution and prolongation of electrolysis time were observed. Electrolysis process with sodium chloride induces spongy network of film structure. The use of AEW has not changed chemical composition of films which was proved by ^{1}H NMR, MALDI-TOF, and FT-IR spectroscopy. The research confirmed that electrolysis significantly improved thermomechanical properties of the examined films. The contact angle values of the films were quite similar and ranged between 56° and 73°. The increase of salt concentration used in the electrolysis process had an impact on increasing flexibility of samples. Application of electrolyzed water in commonly used food processing systems is possible. Fusion of AEW and biopolymers may provide better integration with coated food product and multidirectional protecting effect.

1. Introduction

Packaging is used to maintain appropriate sensory characteristics and nutritional value of food products. It also has protective function against the adverse effect of environment during transportation and storage; additionally, it has an impact on appearance of the final product. In recent years, the application of edible films and coatings based on biopolymers like proteins, polysaccharides, and lipids has raised attention of many researchers [1, 2] mainly because of the fact that biodegradability of protective coatings containing natural polymers reduces the application of synthetic polymers difficult for utilisation [3]. Requirements for the packaging from natural materials depend on the characteristics of food products and their changes during production and storage [4]. Many authors focus on modification of hydrocolloids designed to produce packaging materials with the most preferable properties and multifunctional applications [5, 6].

Chitosan and cellulose derivatives as hydroxypropyl methylcellulose (HPMC) are promising materials for creating edible coatings or films. HPMC is a water soluble cellulose ether hydrocolloid with good film forming characteristics. It is used as a film former, tablet binder, and coating, stabilizing, suspending, and viscosity increasing agent [7, 8]. Cellulose derivative films are poor water vapour barriers, which are the result of the inherent hydrophilic nature of polysaccharides and cellulose derivative films' poor mechanical properties. Improving the moisture barrier would be by incorporation of hydrophobic compounds, such as fatty acids, into the cellulose ether matrix to develop a composite film [9].

Table 1: Experimental design.

Variants	Variability factors		Constant factors				
	NaCl (%)	Electrolysis time (min)	Chitosan (%)	HPMC (%)	Lysozyme (%)	Glycerol (%)	Lactic acid (%)
N0.001E0	0.001						
N0.01E0	0.01	0					
N0.1E0	0.1						
N0.001E5	0.001						
N0.01E5	0.01	5	1	1	0.2	25 (of dry weight of used polymers)	0.5
N0.1E5	0.1						
N0.001E10	0.001						
N0.01E10	0.01	10					
N0.1E10	0.1						

Chitosan is a derivative of chitin, a polysaccharide with linear chain of linked 2-acetamido-2-deoxy-D-glucopyranose units. This material is biodegradable, biocompatible, and nontoxic. Due to its film forming properties and multiple uses in applications of coatings production, drug delivery, nutrients, and controlled release of food ingredients and separation techniques, it is a valuable polymer [10]. It has a high modulus along with low elongation at break owing to the high glass transition temperature (Tg) and crystallinity. Therefore, mixing or copolymerizing chitosan with different polymers can influence its morphology and plasticity [11].

Edible films are not good barriers against water vapour; however, they could be used as a carrier of active compounds, antimicrobial agents, or preservatives, which protect food quality [12]. Such active ingredients of edible films can be lysozyme or acidic electrolyzed water.

Lysozyme is muramidase (N-acetyl-muramyl-hydrolase) which decomposes β-1,4-glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine in the cell wall of polysaccharides in bacteria. It possesses lytic properties and can be applied as an antiseptic substance [13].

Acidic electrolyzed water is an antimicrobial agent generated by membrane electrolysis of sodium chloride solution. It is characterized by low pH, high oxidation-reduction potential, and free chlorine which is a major germicidal component [14, 15]. The free chlorine in electrolyzed acidic water is associated with a combination of hypochlorous acid and chlorine gas [15]. AEW has the advantages of nonirritating response of mucous membranes and skin tissue [16]. Acidic electrolyzed water can be also used as a component in biomolecular system, such as hydrogels. Polymer hydrogel composites have been synthesised and characterised for their application in electrochemically controlled drug release devices. Polymer hydrogels give the possibility of obtaining various advanced functional polymers. Electrosensitive hydrogels are commonly made of polyelectrolytes and an insoluble, swellable, polymer network with ionic groups [11].

To the best of our knowledge, there is no published study regarding characterization of edible films incorporated with acidic electrolyzed water. Interactions between hydrocolloids and acidic electrolyzed water could affect physicochemical properties of edible films.

Therefore the aim of the present study was to evaluate the physicochemical properties of chitosan/HPMC films incorporated with lysozyme and acidic electrolyzed water. Further investigations will be correlated with antibacterial properties of examined films and their effectiveness in food products.

2. Materials and Methods

2.1. Apparatus. Electrolyzed salt solutions (acidic electrolyzed water, AEW) were generated using a water generator (own design batch type generator, equipped with two titanium electrodes coated with 0.6 μm layer of platinum) by membrane electrolysis of diluted salt solutions (0.001, 0.01, and 0.1%) in various time spans (0, 5, and 10 minutes). The diluted aqueous solutions of sodium chloride (analytical quality, POCH) were added to both the anode and the cathode chambers (2 L in each chamber) of the ionizer.

2.2. Material. A low molecular weight chitosan (CH) obtained from shrimp shells was supplied from Sigma-Aldrich with 20–200 cP viscosity and DD = 75–85%; hydroxypropyl methylcellulose (HPMC) in the METHOCEL SX product was obtained from Dow Chemical Company; lysozyme isolated from hen egg whites with the 2000 U/mg activity was purchased from Ovopol Company (Poland), in addition to lactic acid (PURAC FCC 80), glycerol (analytical quality, POCH), and 80% sodium chloride (analytical quality, POCH).

2.3. Preparation of Polymer Films. The film stock solutions were prepared by dissolving known amount of film material (chitosan, HPMC) in (non)electrolyzed sodium chloride solutions in the amount shown in Table 1 and 0.5% of lactic acid continuously stirred, using mechanic stirrer CAT R-250 (stirring rate 400 rpm), for 16 hours. The lysozyme solution was prepared by dissolving known amount of lysozyme in (non)electrolyzed sodium chloride solutions. Glycerol (as a plasticizer) was added to the homogenous solutions; then all the solutions were mixed together to obtain the final concentration of components presented in Table 1. The final solutions were degassed by centrifugation and poured into a

glass Teflon coated plate of 80 mm × 200 mm and dried for 72 hours. Dehydration was carried out in Binder KBF-LOC 240 chamber at 4°C and relative humidity of 60%. Dried films were taken off and cut out for further tests.

2.4. Film Characterization

2.4.1. Film Solubility in Water. The analysis was performed according to the method described by Pinotti et al. [17]. The percentage of total soluble matter (% solubility) was calculated by the following formula:

$$\% \text{ solubility} = \frac{\text{Initial dry weight} - \text{Final dry weight}}{\text{Initial dry weight}} \times 100. \quad (1)$$

The samples were analysed at least in triplicate.

2.4.2. Scanning Electron Microscopy (SEM). Cross section of the obtained films was performed using EVO LS 15 Zeiss scanning electron microscope. The samples were sputtered with gold for 150 s using Scancoat 6 type (Edwards, London, England) and examined using 20 kV voltage.

2.4.3. Fourier Transform Infrared Spectroscopy (FT-IR). The ATR FT-IR method was used for each sample testing. The spectra were recorded at a resolution of 2 cm^{-1} by 64 scans between 450 and 4000 cm^{-1} in Infinity AR60 spectrometer (ATI Mattson).

2.4.4. ^1H NMR Spectroscopy. The samples were then characterized by nuclear magnetic resonance (NMR) spectroscopy. The nuclear magnetic resonance measurements were performed on a Bruker Avance III. The solid-state CP MAS NMR experiments were performed using the technique of cross-polarization with magic-angle spinning. The spectra for ^{13}C and ^1H nuclei were obtained, respectively, at 100.61 MHz and 400.15 MHz frequency in a MAS BB DVT wide-band probe with 4 mm diameter of zirconium (ZrO_2) rotor. The isotopically labeled *L*-[1-*13*C]-*tyrosine* (Tyr) was applied in order to optimize the parameters for the CP MAS ^{13}C NMR and also to attain the first-order Hartmann-Hahn matching condition. Spectroscopic parameters are as follows: for *^{13}C CP MAS spectrum:* measurement temperature: 298 K, rotation speed: 8 kHz, relaxation time: 3 s, pulse: 90° for ^1H 4 μs, contact time: 2 ms, spectral width SWH: 40 kHz, TD = 3.5 k, and SPINAL decoupling; for *^1H MAS spectrum*: measurement temperature: 298 K, rotation speed: 8 kHz, relaxation time: 2 s, pulse: 90° for ^1H 4 μs, spectral width SWH: 40 kHz, and TD = 16 k.

2.4.5. MALDI-TOF Mass Spectrometry. The samples for the experiment were prepared by mixing 10 μL of examined solution with 10 μL of matrix solution (an aqueous solution of 2,5-dihydroxybenzoic acid (DHB) at a concentration of 10 mg/mL) in Eppendorf tube with a capacity of 0.5 mL. 2 μL of the sample was placed on a measuring plate and left to evaporate the solvent. The plate was then placed in an ion source of the mass spectrometer Voyager-Elite (PerSeptive Biosystems, Framingham, CT, USA). The ionization technique which was applied was matrix assisted laser desorption/ionization (MALDI). The wavelength of the laser radiation and accelerating voltage were at 337 nm and 20 kV, respectively. The positive ions were subjected to registration using ion time of flight (TOF) analyser with the reflection of ions (reflectron). The recorded mass range varied from 300 to 3500 (*m/z*). Mass spectrum is the sum of 200 spectra. The measurements were taken using an external mass calibration based on defined reference mixture spectrum of polyethylene glycols. Processing of spectra was carried out using Data Explorer v. 4 (Applied Biosystems, Foster City, CA, USA).

2.4.6. Dynamic Mechanical Thermal Analysis (DMTA). The tests were performed using Rheometric Scientific DMTA Mk III. The loss modulus, storage modulus, and loss tangent (tan δ) were measured at the temperature range from −80°C to +50°C, with heating rate 2°C/min and frequency of 1 Hz.

2.4.7. Contact Angle Measurements. The measurements were taken with Contact Angle Analyzer (Surface Electro Optics Company) using the sessile drop method. The drop of distilled water (6 μL) was placed on the surface (2 cm^2) of the coating sample.

2.5. Statistical Analysis. The experiments were made in triplicate. The effects of two independent categorical variables such as time of electrolysis and concentration of sodium chloride were evaluated. The obtained data were analyzed using a 2-way factor analysis of variance (ANOVA) using Statistica 10 (StatSoft, Poland). Differences between means were established by Duncan test with 5% significance.

3. Results and Discussion

3.1. Film Solubility in Water. It was found out that the use of the acidic electrolyzed water significantly influences solubility of edible protective films. The films containing 0.1% sodium chloride solution after 10 minutes of electrolysis (N0.1E10) present the lowest solubility of 6% (Figure 1). The highest solubility of 44% was observed in N0.01E0 film. The decreasing film solubility with increasing concentration of sodium chloride solution and prolongation of electrolysis time were observed in composite films (Figure 1). The solubility can be adjusted to enhance the possible applications by controlling the parameters of the solvent used in film formulation [17]. Water solubility is an indicator of the film's water affinity [18]. High solubility may be an advantage for some applications. In some cases, a water-insoluble film is preferred in order to provide water resistance and improve food integrity; in other cases, edible films with high water solubility may be required [1]. The required degree of solubility of the material may be changed depending on intended applications [19].

3.2. Scanning Electron Microscopy (SEM). The scanning electron micrographs of films are shown in Figure 2. The SEM (a), (b), and (c) micrographs showed smooth, compact, and

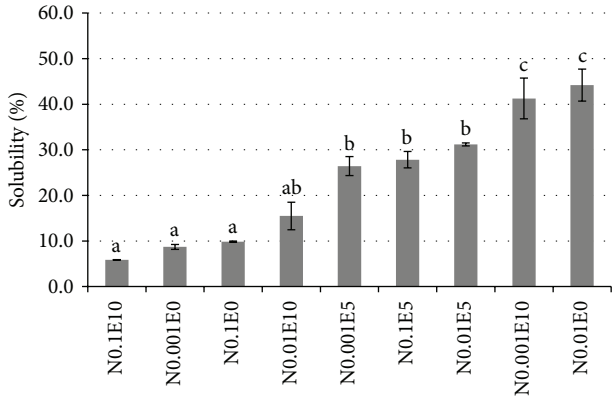

Figure 1: Film solubility in water. Different letters indicate significantly different groups determined by Duncan's test ($P < 0.05$).

homogeneous surface, and no pores or cracks were detected. These results are consistent with those described by Yin et al. [20], Rotta et al. [21], and de Moura et al. [22], where micrographs of cross section confirmed the partial miscibility between chitosan and HPMC. The N0.001E0 and the N0.01E5 films revealed multilayer structures, which could be associated with limited chain mobility [17]. It is related to specificity of used polymers (HPMC, chitosan, and chitooligomers) and causes differences in formation of their chains and functional groups. Electrolysis process with NaCl induces spongy network of film structure, which is shown in Figure 2. Process of electrolysis with NaCl as electrolyte causes lack of creation of crystal structure of components in LCAEW. By subjecting the electrodes to direct current voltage, two kinds of electrolyzed water with different characteristics were produced. In anode chamber of ionizer are present only chloride compounds, such as Cl_2, HOCl, HCl, and chloride ions [15]. Sodium ions are transferred to the cathode as a result of membrane electrolysis and obtained water is not used in this study.

3.3. Fourier Transform Infrared Spectroscopy (FT-IR). The FT-IR spectra of examined films and their ingredients are shown in Figure 3. The spectrum of N0.2E30 sample shows the absorption peak at about 3232 cm^{-1}, which is assigned to stretching vibrations from overlapping O–H and N–H bonds. These findings are in accordance with the results obtained by Leceta et al. [23]. The vibrations indicate inter- and intramolecular hydrogen bonds interaction. The signals observed at 3426 cm^{-1}, 3377 cm^{-1}, 3303 cm^{-1}, and 2878 cm^{-1} were associated with O–H stretching vibration, NH_2 asymmetric stretching, N–H stretching, and C–H stretching, respectively. Similar peak absorption was also reported in the work of Leceta et al. [23]. Characteristic absorption bands of NAG units were observed for C–O stretching of secondary amides at 1656 cm^{-1} and for N–H bending (primary amine) at 1597 cm^{-1}. The absorption bands near 1378 cm^{-1} are associated with the symmetrical skeletal deformation of CH_3 group and those at 1421 cm^{-1} with the O–H (primary alcohol). The absorption bands at 1032 cm^{-1} (stretching vibration of C–O–C ring) are characteristic for the glucopyranose ring. Praxedes et al. [24] indicated that the band peaks at 1409 cm^{-1} and 1316 cm^{-1} correspond to the vibration of –OH and –CH groups in the pyranose ring. Specific bands of (1,4)-glycosidic bridges were observed at 1153 and 897 cm^{-1} [25, 26]. Praxedes et al. [24] observed at the peak of antisymmetric stretching C–O–C glycosidic linkage at 1080 cm^{-1}. Many authors present different wave numbers at which there are adequate absorption bands [27]. The small band with low absorption at 1733 cm^{-1} refers to the acylated group –OH [28]. No significant differences in the positions and intensities of the bands of all the samples were observed. It means that polysaccharides were not degraded by lysozyme or acidity of electrolyzed water. Despite the fact that electrolyzed water has low pH, basically it is a weak acid [15].

3.4. ^1H NMR and ^{13}C NMR Spectroscopy. No significant differences of intensities and widths of ^{13}C individual resonance signals for experimental films were observed (Figure 4). The stability of the fundamental structure of the films was proved by the lack of significant changes in ^{13}C CP MAS NMR spectra. The interactions between large amounts of protons as well as high value of their dipole coupling resulted in broadening of the proton signals in ^1H MAS NMR spectra. Some significant differences of ^1H MAS NMR spectra were noted between N0.001E0, N0.01E0, N0.001E5, N0.01E5, and N0.001E10 and N0.01E10, N0.1E0, N0.1E5, and N0.1E10 samples. The intense signal at about 4.4 ppm was observed in spectra of N0.001E0, N0.01E0, N0.001E5, N0.01E5, and N0.01E10 films. The peak originates predominantly from water protons. Three strong signals at 5, 3.5, and 1.5 ppm were noted. They originate from water protons or glycerol, CH group of chitosan and HPMC (or CH_2 of glycerol), and CH_3 of HPMC. Changes in intensity and width of these signals are probably correlated with different content and cross-linking structures of the films.

3.5. MALDI-TOF Mass Spectrometry. The analysis of the compounds consisting of sugar units conducted by MALDI enables the observation of the oligosaccharides in the molecular weight range from a few to several thousand daltons [29]. Although lysozyme was added to potentially increase antimicrobial activity, it also could cause polysaccharides hydrolysis, which was investigated by others [30]. Park et al. [31] and Zimoch-Korzycka and Jarmoluk [13] noted that lysozyme may cause polysaccharides hydrolysis and their products enhanced inhibition efficacy against many bacteria species. Therefore the research was carried out to identify potential oligomeric products of chitosan and HPMC hydrolysis. The intense peaks of matrix dominate in the mass range from 300 to 500 *m/z*; therefore the mass range over 500 *m/z* was taken into account (Figure 5). No peaks of other *m/z* values, which would create a series of constant mass differences between peaks, indicating the presence of oligomeric products with different composition, were reported. The optimum temperature required for the maximum activity of lysozyme proved to be 37°C [32]. Uncontrolled enzymatic degradation was inhibited by sample storage under cooling condition.

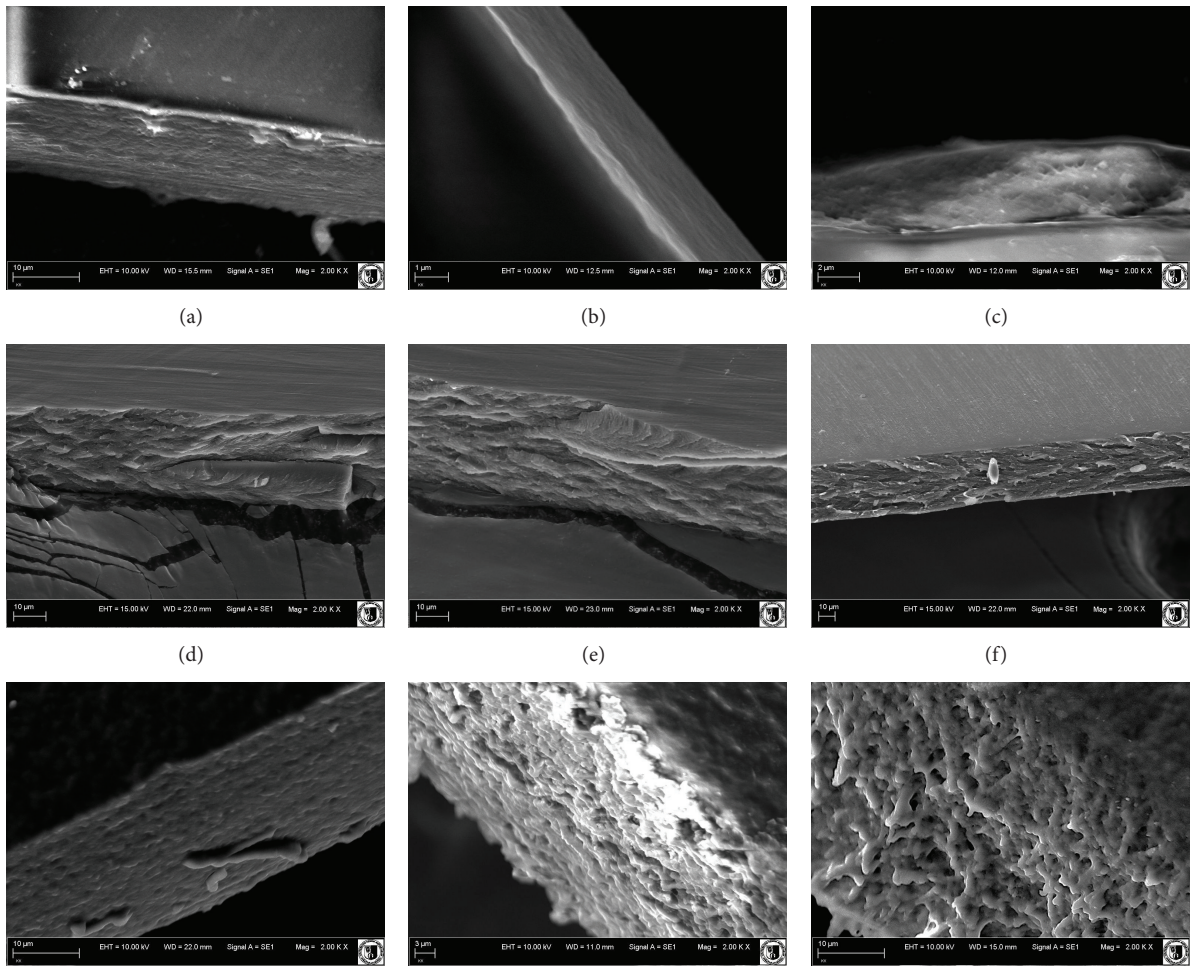

Figure 2: SEM micrographs of the cross section of (a) N0.001E0, (b) N0.001E5, (c) N0.001E10, (d) N0.01E0, (e) N0.01E5, (f) N0.01E10, (g) N0.1E0, (h) N0.1E5, and (i) N0.1E10 films.

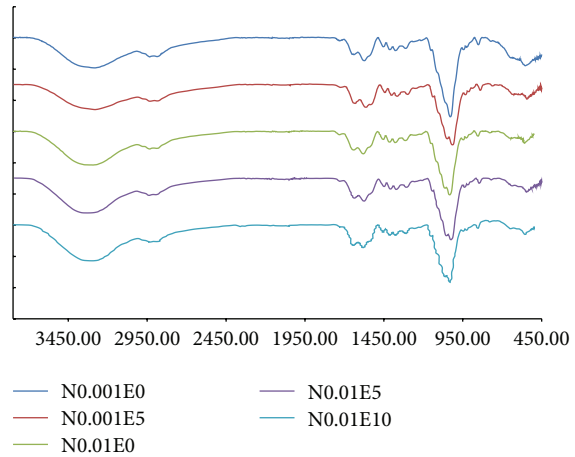

Figure 3: Spectra of Fourier Transform Infrared (FT-IR) for edible films. Reference films: N0.01E0 and N0.001E0; films incorporated with acidic electrolyzed water: N0.01E5 and N0.01E10.

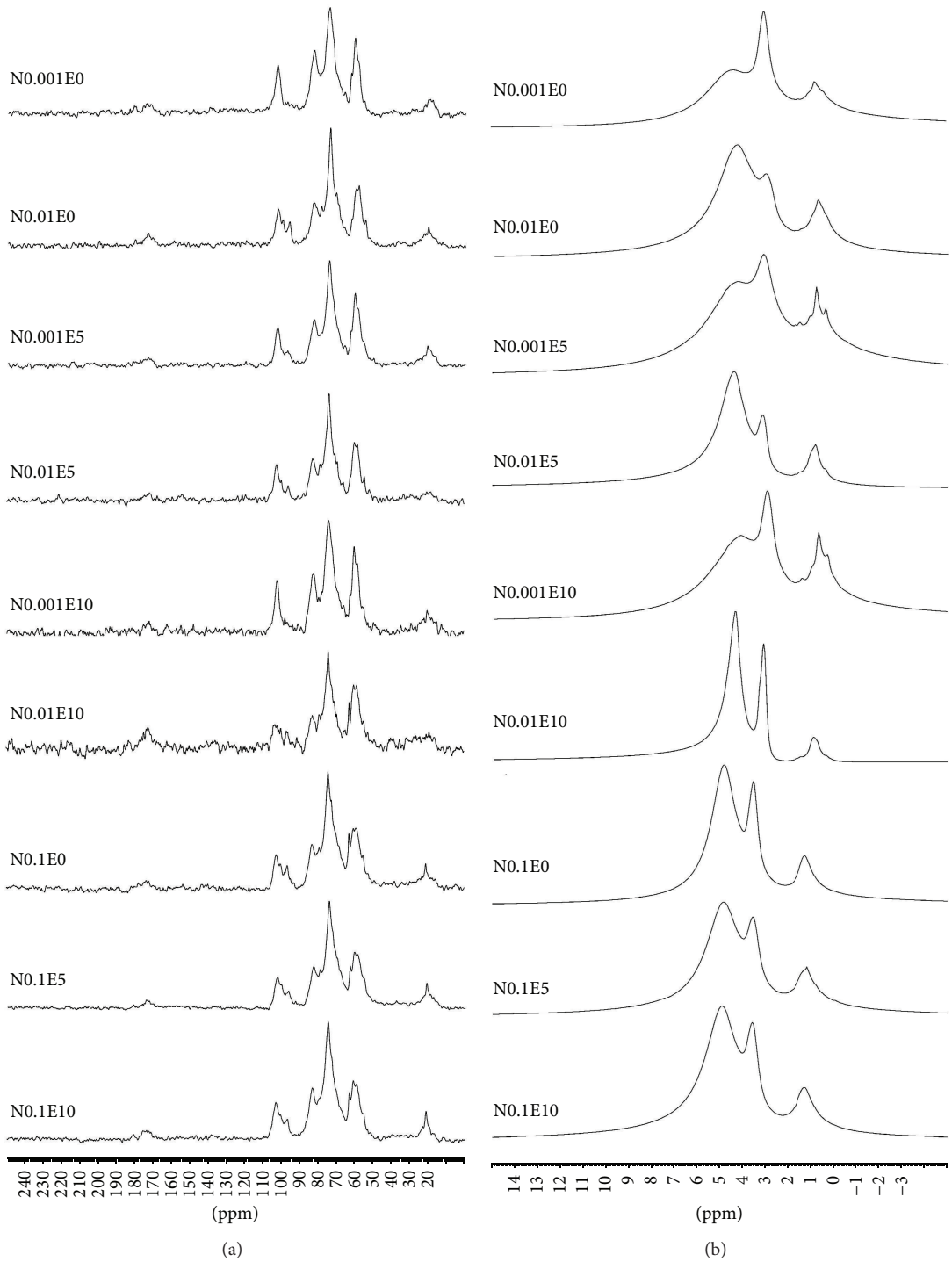

Figure 4: ^{13}C NMR (a) and ^{1}H NMR (b) spectra of examined edible films.

Oligomeric products of chitosan hydrolysis were not found, which was confirmed by MALDI-TOF analysis. Chitosan degradation could lead to reduction of film homogeneity which was not desirable in this study.

3.6. Dynamic Mechanical Thermal Analysis (DMTA). Figure 6 presents the dependence on dynamic mechanical behaviour for selected films (N0.01E0, N0.01E5, N0.01E10, N0.001E0, and N0.1E10) in order to show possible differences caused by AEW. A remarkable signal at 0°C for N0.001E0 and N0.01E0 was noted and probably is associated with the phase transition. The analysis of N0.001E0 and N0.01E5 films showed less intense and more significant peaks at 0°C and −30°C, respectively. Sample N0.01E10 (like N0.1E10 sample) comprises the second loss tangent peak at about 0°C. Rotta et al. [21] and Martínez-Camacho et al. [33] observed a

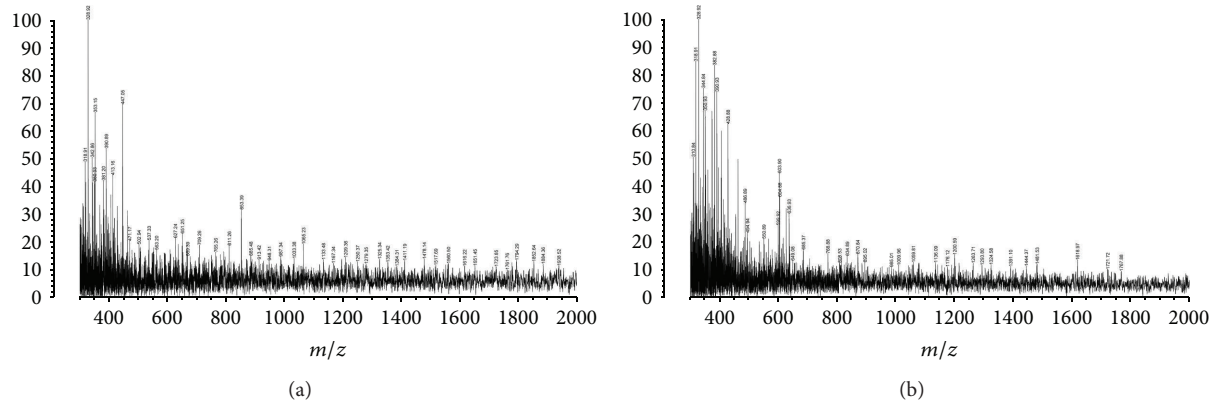

Figure 5: MALDI-TOF mass spectrometry analysis of edible films based on acidic electrolyzed water (N0.1E10) (a) and its reference (N0.1E0) (b).

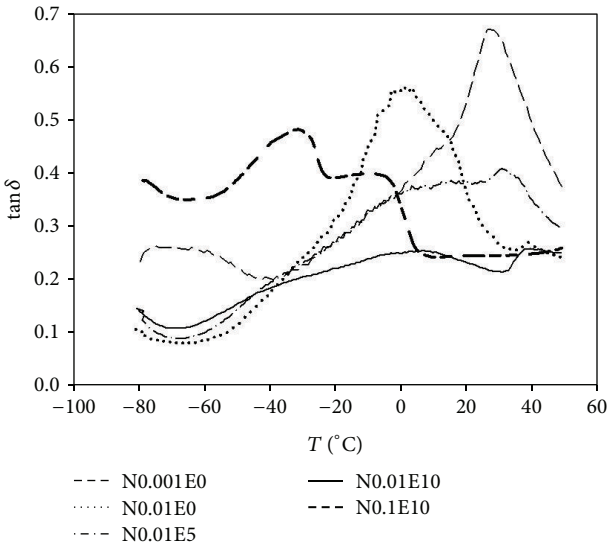

Figure 6: DMTA spectra of tan δ for N0.001E0, N0.01E0, N0.01E5, N0.01E10, and N0.1E10 films.

Tg value of chitosan and hydroxypropyl methylcellulose at 203°C and 164°C, respectively, whereas Kittur et al. [34] and Neto et al. [35] have not found any evidence of Tg. The maximum in the tan δ graph corresponds to transition temperature of the amorphous regions of the polymer (α relaxation) [36]. Tg is strongly dependent on both the film composition and the moisture content and can denote stability of a film. As water content of the amorphous material increases, Tg decreases. Double tangent peak was observed in N0.1E10 film, which suggests the presence of two phase transitions or weak homogeneity of the material. Two separate peaks on the graph of the loss tangent were probably associated with higher NaCl content. Aggregation of fillers would result in the heterogeneous collections of polymer matrix and filler. It was found that with increasing NaCl concentration in examined films α relaxation moves toward higher temperatures and its maximum is lower. The use of AEW caused a shift of the main relaxation to a higher temperature which probably indicates restricted molecular movement [37]. Close to 0° loss tangent values indicate high flexibility of the sample. Process of electrolysis significantly improved thermomechanical properties of polymeric coatings. It was observed that 5-minute electrolysis improved N0.01E5 elasticity by 27.28% and elongation of electrolysis time promotes reduction of tan δ by 54.55% compared to the untreated sample N0.01E0 (0.55°). Electrolysis process created spongy network of films, which was confirmed by SEM analysis. When the porosity of material increases, the elasticity increases also [38].

3.7. Contact Angle Measurements. The photographs of wetting films are shown in Figure 7. The study revealed that there was no significant deformation between films which contained AEW. The destruction of films is caused by dissolution of the sample by its contact with water. This process is accompanied by the formation of stress, which results in deformation of the sample. Among the tested samples N0.01E0 film was subject to insignificant deformation. There are two types of terminal groups in the chemical structure of examined films: hydrophobic methyl group ($-CH_3$) and hydrophilic hydroxyl group (CH_3-OH). During the first phase of the analysis methyl groups were exposed to the outside, which prevented water penetration into the interior of the surface film. The tested surface of the coating was wetted with water, which probably caused its dynamic reorganization. Hydrophilic hydroxyl groups were gradually appearing on the material surface. The water drop was extended, but the penetration was insignificant. Chemical stability of N0.1E0, N0.001E5, N0.1E5, and N0.1E10 films was observed during the time of measurement. It was noted that the contact angle values of the films were quite similar and were in the range 56°–73°. This evidenced insignificant differences in chemical structure surface of the test samples. Contact angles greater than 90° indicate surface hydrophobicity and below 90° correspond to hydrophilicity of the surface [39]. Contact angles below 90 degrees can indicate that examined films are characterized by high wettability. Park et al. [31] observed that the addition of lysozyme causes increased hydrophobic

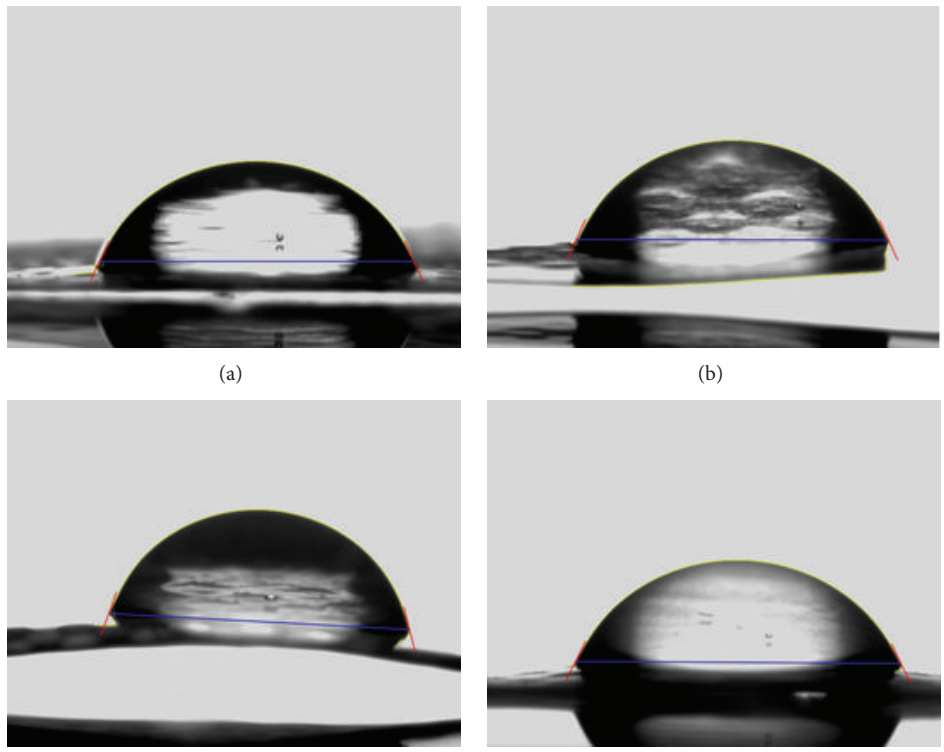

Figure 7: Images of contact angle for (a) N0.001E0, (b) N0.01E0, (c) N0.01E5, and (d) N0.01E10 films.

side chains in film structure and may be responsible for the decrease in hydrophilicity of lysozyme-chitosan films.

4. Conclusions

Control of AEW production parameters allows modifying properties of edible protective films, including their solubility and wettability, while electrolysis process improves elasticity of the polymeric films. The use of AEW has not caused undesirable changes in chemical composition of films which was proved by ^{1}H NMR, MALDI-TOF, and FT-IR analysis. The use of low salt concentration guarantees obtaining a homogeneous coating surface and desirable cohesion with all components. Application of acidic electrolyzed water generator is possible to be implemented into already existing food processing system. Fusion of AEW with biopolymers and lysozyme is easy to introduce and will provide better integration with coated food product.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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