Original article

Physical properties of biopolymers containing natamycin and rosemary extract

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Summary Antifungal biopolymers were prepared by incorporating natamycin (NA) and NA + rosemary extract (RE) into wheat gluten (WG) and methyl cellulose (MC) films. Interaction between antimicrobial agents and biopolymers was determined with mid-infrared spectroscopy and scanning electron microscopy (SEM). Water vapour permeability and mechanical properties of these films were also measured. Mid-infrared spectroscopy did not indicate any interaction. SEM observations showed that NA crystallises at high concentrations in biopolymers. There were no significant changes in water vapour permeabilities of biopolymers containing active agents at $P < 0.05$. While NA incorporation did not result in any changes in mechanical properties of WG films a reduction in tensile strength was observed for MC films containing high concentration of NA. In general, active agent incorporation into WG and MC films did not result in any considerable changes in their physical properties that could affect their application.

Keywords Methyl cellulose film, natamycin, rosemary extract, wheat gluten film.

Introduction

Recent studies on food packaging especially focus on development of biopolymers that have additional functions such as antimicrobial and antioxidant properties besides traditional role of a packaging film. These types of packaging films could be effective in delaying or inhibiting microbial growth originating from post-processing contamination. Biopolymers could be prepared from renewable sources such as whey protein, corn protein zein, alginates and starch, and provide protection of food against moisture, gases and vapour. Various antimicrobial agents such as bacteriocins and plant extracts were incorporated alone or in combination into different types of biopolymers (Quattara *et al*., 2000; Cutter *et al*., 2001; Ozdemir & Floros, 2003; Min *et al*., 2005). Combinations of antimicrobial agents might have enhanced antimicrobial effect compared with individual agents owing to their synergistic action. In our previous study, it was shown that wheat gluten (WG) and methyl cellulose (MC) films with natamycin (NA) have antifungal activities against *Aspergillus niger* and *Penicillium roquefortii*. Although rosemary extract (RE) did not have any inhibitory effect against these two fungi it enhanced the inhibitory activity of NA in WG and MC films (Ture *et al*., 2007). Only NA containing casein coatings and cellulose-based films were used to prevent mould spoilage in cheese (Yildirim *et al*., 2006; de Oliveira *et al*., 2007).

Incorporation of antimicrobial and/or antioxidant agents into films adds new functionality to packaging. However, addition of these agents into the film may cause changes in the structure and the properties of the films. Modification in the structure could arise from interactions between added agent and the film. Mid-infrared spectroscopy and scanning electron microscopy (SEM) could be useful in determining these interactions (Pranoto *et al*., 2005; Maizura *et al*., 2007). While some agents did not have any effect on the barrier and mechanical properties of the biopolymers, modifications were also reported in several studies depending on the nature of the antimicrobial agent and the biopolymer (Ko *et al*., 2001; Pranoto *et al*., 2005; Bifani *et al*., 2007).

The aims of this study are to determine the effect of NA, RE and combination of NA and RE incorporation on water vapour barrier and mechanical properties of WG and MC films, and also to evaluate the interaction between active agents and films by infrared spectroscopic and SEM analysis.

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Materials and methods

Materials

Pimalac® used as NA source was provided by Mayasan (Istanbul, Turkey). All reagents were of analytical grade and were purchased from Sigma (St. Louis, MO, USA).

Rosemary extraction

Rosemary leaves were collected from Urla region of Turkey and extraction was performed immediately. RE was produced using a modified procedure of Madsen *et al*. (1998). Rosemary leaves (12 g) were homogenised in 70 mL of absolute ethanol at 26 000 r.p.m. (Heidolph Silent Crusher M Homogenizer, Germany) for 5 min. The solution obtained was stirred for 30 min in dark and cenrifugated at 5000 r.p.m. (Nüve NF 615, Turkey) for 5 min. The supernatant was collected and 30 mL of ethanol was added to precipitate and the same procedure was repeated twice. In the last extraction step, 20 mL of ethanol was used. The collected supernatant was evaporated under vacuum at 40 °C for approximately 1 h with a rotary evaporator (Heidolph Laborata4000, Germany). The obtained extract was vacuum-filtered through 5 μm cellulose nitrate filter. Approximately 25 mL of extract was obtained.

Preparation of WG and MC films and incorporation of active agents

WG films were prepared according to a method by Pochat-Bohatier *et al*. (2006) with some modifications. Fifteen grams of WG (Sigma-Aldrich, Germany) was dissolved in 31.5 mL of absolute ethanol with mixing. Then, 0.03 g of sodium sulphite, 3 g of glycerol and 63 mL of distilled water were added to the solution and mixed with a magnetic stirrer. The pH of the film solution was set to 4 with acetic acid and the solution was mixed and heated to 70 °C in a magnetic stirrer. Ten gram of film solution (fs) was spread onto 8.5-cm diameter polystyrene petri dishes and dried at 30 °C overnight.

A procedure by Turhan & Sahbaz (2004) with some modifications was used for the preparation of MC films. Three gram of MC (Sigma-Aldrich) was mixed with 50 mL of ethanol. Fifty millilitre of distilled water was added and homogenised. After addition of 1 mL glycerol, the solution was heated to 80 °C. Ten gram of fs was spread onto polystyrene petri dishes and dried at 30 °C overnight.

NA-containing films were prepared exactly like control films, but NA in powder form was added to films just before spreading fs onto petri dishes at a temperature of 50–55 °C. After addition of NA, the fs was mixed for about 5 min with a magnetic stirrer. Concentration of NA was in the range of 0.2–40 mg per 10 g fs.

RE-containing films were produced by replacing a certain volume of ethanol and water in film formulation with RE (2.3 mL alcohol and 0.7 mL water for 3 mL RE in 10 g fs). NA + RE-containing films were made according to procedures explained before and adding RE and NA before spreading fs onto plate. The average thickness of films (mm) was measured randomly at ten points with a hand micrometer (SHAN Electronic, China).

Measurement of mechanical properties

The films were conditioned at 50% relative humidity and at about 23 °C in desiccators for 48 h before the measurement of mechanical properties. Relative humidity was adjusted by placing saturated solution of magnesium nitrate inside desiccators. Tensile strength (TS), elastic modulus (EM) and elongation at break (EB) of films were tested according to ASTM Method D882 (1996) by using Mechanical Testing Machine (AG-I 250 kN, Schimadzu, Japan). Films were cut into 25 mm × 100 mm strips. Two hundred and fifty Newton load cell was used for both films. Initial grip separation was 50 mm and head speed was set to 50 mm min^{-1}. At least six replicates were performed in each case.

Measurement of water vapour barrier properties

The films were conditioned at 50% relative humidity and at about 23 °C in desiccators for at least 48 h before the measurement of water vapour permeability (WVP). WVP of films was measured according to WVP Correction Method (McHugh *et al*., 1993). Glass jars containing water were sealed with films and placed in desiccators containing saturated solutions of $MgNO_3$ (50% relative humidity). The desiccators were kept in a room at 25 °C. The area of jar mouth was 17.34 cm^2, and the jar depth was 7 cm. The gap between the film and water in the jar was 1.4 cm. Fans operating at speeds of 2 m s^{-1} were also placed in the desiccators and the fan speed was measured with an anemometer (Turbo Meter, Hayward, CA, USA). Three replicates of each film were tested. The weight changes of glass jars were measured and plotted against time. WVP Correction Method (McHugh *et al*., 1993) was used in calculation of relative humidities of the films' undersides and WVP values.

Fourier transform infrared spectroscopy measurement

Infrared spectra of the films were obtained in 4000–650 cm^{-1} range with a Perkin Elmer Spectrum 100 Fourier transform infrared spectrometer (Perkin Elmer Inc., Wellesley, MA, USA) equipped with a deuterated tri-glycine sulphate (DTGS) detector. A horizontal

attenuated total reflectance (HATR) sampling accessory (ZnSe crystal) was used to collect the spectral data of the films. The resolution was set at 4 cm^{-1} and the number of scans collected for each spectrum was 64.

SEM analysis

The films were conditioned at 50% relative humidity in a desiccator for at least 48 h before SEM analysis. The films were coated with 100–200 Å thickness of gold. Cross-section of films were scanned with XL-30S FEG electron microscope (Phillips, The Netherlands).

Statistical analysis

WVP and mechanical properties data were analysed by analysis of variance (ANOVA) using the MINITAB (version 14.10; State College, PA, USA). The means were compared using Fisher least significant difference (LSD) method at $P = 0.05$.

Results and discussion

While MC film used in this study is carbohydrate-based and WG is prepared from one of the major protein fraction of wheat. NA and RE were added to these films to provide antimicrobial properties (Ture *et al.*, 2007). NA is an amphoteric compound produced by *Streptomyces natalensis*. RE contains phenolic diterpenes such as carnosic acid, carnosol, rosmanol, epirosmanol, isorosmanol, methyl carnosate and other phenolic acids, such as rosmarinic acid (Schwarz & Ternes, 1992; Cuvelier *et al.*, 1996). The physical properties of films which were proved to have antifungal activities were investigated in this study.

Both WG and MC films were homogenous, thin and flexible. While films prepared from WG were opaque MC films were clear. Adding active agents to films did not have any effect on their visual appearance. The thicknesses of prepared films are presented in Table 1. There were no significant differences in thicknesses between control and NA or NA + RE-added films except MC containing 20 mg NA per 10 g fs.

Mechanical properties of films

Mechanical properties of control and NA, NA + RE-containing WG and MC films are listed in Table 1. Incorporation of NA into WG film did not cause major changes in its mechanical properties. As antimicrobial synergy between NA and RE was observed at low NA concentration (Ture *et al.*, 2007), mechanical properties of the films containing 0.5–2 mg NA per 10 g fs in combination with RE were tested. A decrease in TS (15.5%) and EM (50%) of WG films was observed at 2 mg NA per 10 g fs + 1.5 mL RE per 10 g fs. In addition, the elongation increased with increasing NA concentration for WG films containing RE.

For MC films, TS decreased at 10 and 20 mg NA per 10 g fs, and reduction in TS at the highest NA concentration corresponds to a value of 38.3% relative to the control. As it was observed in SEM pictures, high

Table 1 Mechanical and water vapour properties of wheat gluten (WG) and methyl cellulose (MC) films

				Mechanical properties			
Film	Natamycin (mg per 10 g fs)	Rosemary extract (mL per 10 g fs)	Thickness (mm)	Tensile strength (MPa)	Elastic modulus (N mm^{-2})	% elongation at break	Water vapour permeability (g mm m^{-2} kPa h)
WG	0	0	0.25 ± 0.028[a]	2.06 ± 0.27[a]	28.79 ± 11.62[a]	224.80 ± 53.01[a]	6.85 ± 0.56[a]
	2	0	0.22 ± 0.036[a]	2.01 ± 0.28[a]	28.21 ± 9.26[a]	225.32 ± 49.18[a]	5.99 ± 2.23[a]
	5	0	0.23 ± 0.022[a]	2.00 ± 0.17[a]	23.29 ± 3.93[a]	254.76 ± 56.80[a]	6.36 ± 0.67[a]
	10	0	0.24 ± 0.026[a]	2.10 ± 0.19[a]	27.25 ± 3.29[a]	227.87 ± 41.00[a]	6.47 ± 0.95[a]
	20	0	0.24 ± 0.023[a]	2.23 ± 0.11[a]	29.62 ± 5.18[a]	225.10 ± 20.97[a]	6.42 ± 0.65[a]
	1	1.5	0.23 ± 0.034[a]	2.02 ± 0.21[a]	20.62 ± 3.44[a]	260.92 ± 11.00[a]	5.99 ± 0.71[a]
	1.5	1.5	0.23 ± 0.037[a]	2.20 ± 0.09[a]	21.19 ± 3.43[a]	278.12 ± 16.72[a]	6.04 ± 0.74[a]
	2	1.5	0.23 ± 0.037[a]	1.74 ± 0.13[b]	14.68 ± 1.08[b]	295.95 ± 15.42[b]	6.26 ± 0.25[a]
MC	0	0	0.063 ± 0.009[a]	36.63 ± 7.53[a]	313.23 ± 56.89[bc]	73.98 ± 13.22[b]	3.50 ± 0.25[a]
	2	0	0.069 ± 0.01[a]	37.17 ± 11.95[a]	380.73 ± 58.36[a]	60.45 ± 17.67[a]	3.43 ± 0.57[a]
	5	0	0.066 ± 0.01[a]	33.80 ± 6.11[a]	346.03 ± 51.79[ac]	66.47 ± 6.58[ab]	3.20 ± 0.25[a]
	10	0	0.065 ± 0.01[a]	27.28 ± 3.80[b]	263.87 ± 31.97[b]	66.78 ± 2.51[ab]	3.79 ± 0.41[a]
	20	0	0.075 ± 0.005[b]	22.59 ± 4.98[b]	299.90 ± 27.21[bc]	56.76 ± 9.18[a]	4.11 ± 0.74[a]
	1	1.5	0.064 ± 0.009[a]	36.09 ± 5.36[a]	426.75 ± 35.47[a]	62.20 ± 8.03[a]	3.88 ± 0.23[a]
	1.5	1.5	0.062 ± 0.01[a]	32.34 ± 12.48[a]	357.80 ± 39.38[b]	62.10 ± 16.61[a]	3.63 ± 0.59[a]
	2	1.5	0.066 ± 0.01[a]	35.45 ± 13.92[a]	341.89 ± 69.43[b]	70.46 ± 21.73[a]	3.65 ± 0.46[a]

[a–d]Similar letters show that there is no statistical difference between different levels in the same column within each group at $P > 0.05$.
fs, film-forming solution.

concentrations of NA caused a disruption in film matrix resulting in changes in strength properties of the film. A slight increase in EM of NA-containing MC films was observed at low NA concentration. % EB values of MC films were also affected by NA incorporation, and a slight decrease was observed. Addition of low concentration of NA in combination with RE did not result in any significant changes in TS of MC films. These changes might be because of the weakening of some of the chemical bonds in the polymer structure.

Water vapour barrier properties of films

WVP of the control and active agent containing WG and MC films are provided in Table 1. WVP of WG and MC control films were 6.85 and 3.50 g mm m^{-2} kPa^{-1} h^{-1}, respectively. Although Gontard *et al.* (1993) reported lower WVP values for WG films, results of our study were similar to Pommet *et al.*'s (2003) study. The differences could be attributed to film formulations as parameters such as protein concentration and plasticiser amounts could alter WVP values. Differences in WVP measuring conditions also cause differences in WVP values. WVP of MC film was in the range (3.02–4.36 g mm m^{-2} kPa h) of what was reported by Park *et al.* (1993). There was no significant difference between WVP of control films and films containing active agents. Therefore, NA and NA + RE incorporation into WG and MC biopolymers did not have any effect on water vapour barrier properties of these films. Active agents did not probably cause any significant structural changes or plasticising effect in the films that would result in an increase in free volume. As will be explained in the section on infrared spectroscopy there were also no significant interactions between active agents and films. Therefore, WVP values also did not change with addition of active agents.

Mid-infrared spectroscopy

Infrared spectra of control films and films containing antifungal agents at various concentrations were

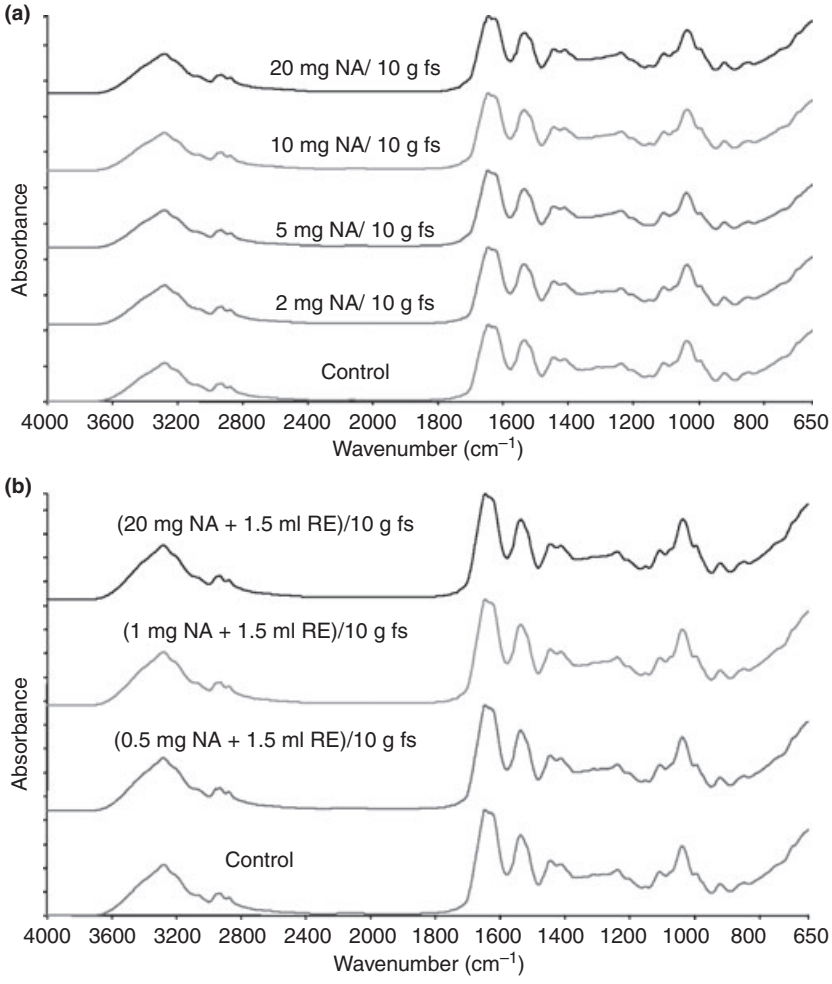

Figure 1 Fourier transform infra red spectra of wheat gluten films containing: (a) natamycin and (b) natamycin + rosemary extract.

recorded to study the interaction between added agents and the biopolymers (Figs 1 and 2). Absorption bands corresponding to N–H stretch at 3280 cm^{-1}, –CH, $–CH_2$ and $–CH_3$ stretching at 2935–2877 cm^{-1}, amide carbonyl group at 1650 cm^{-1}, $–CH_2$ and $–CH_3$ groups at 1445–1415 cm^{-1} and C–N stretch at 1039 cm^{-1} were observed for WG films. MC films had absorbance bands at 3400 cm^{-1} (O–H stretching), 2835–2940 cm^{-1} (C–H stretching), 1645 cm^{-1} (C–O), 1450–1315 cm^{-1} ($–CH_2$ and $–CH_3$ groups) and 1100–1000 cm^{-1} (C–O–C) which are similar to that were reported in literature (Zaccaron *et al*., 2005). All spectra belonging to control films and films containing NA and NA + RE have similar infra red absorbance patterns and formation of any new peaks or shifts in the peaks were not observed. Therefore, it could be concluded that there was no interaction between added agents and the WG and MC films. Pranoto *et al*. (2005) also did not observe any interaction between incorporated antimicrobial agents (garlic oil, potassium sorbate and nisin) and biopolymer, chitosan.

Scanning electron microscopy

SEM pictures of cross-sections of control films and films containing antifungal agents were also obtained to visually examine the structure of the films. Structure of WG control film was homogeneous as reported by Pochat-Bohatier *et al*. (2006; Fig. 3a). There was no visual change in the structure of 2 mg NA per 10 g fs containing WG film (not shown). However, continuity of the film was disturbed at high NA concentrations and

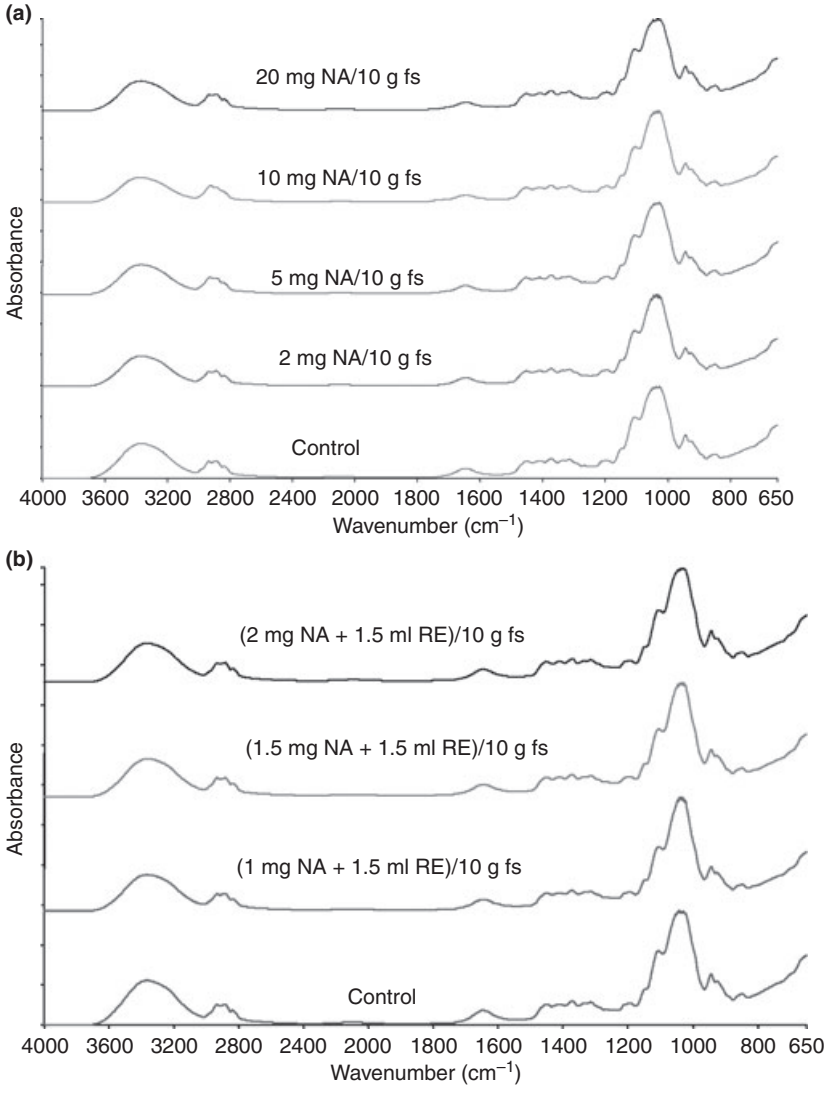

Figure 2 Fourier transform infra red spectra of methyl cellulose films containing: (a) natamycin and (b) natamycin + rosemary extract.

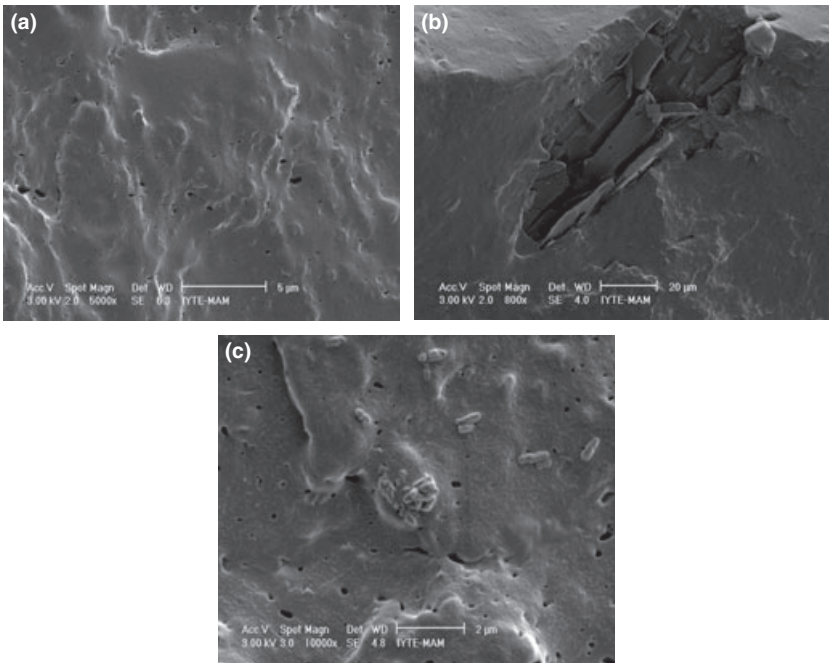

Figure 3 Standard electron microscopic pictures of wheat gluten films: (a) control; (b) 20 mg natamycin/10 g fs and (c) (2 mg natamycin + 1.5 mL rosemary extract)/10 g fs (fs, film-forming solution).

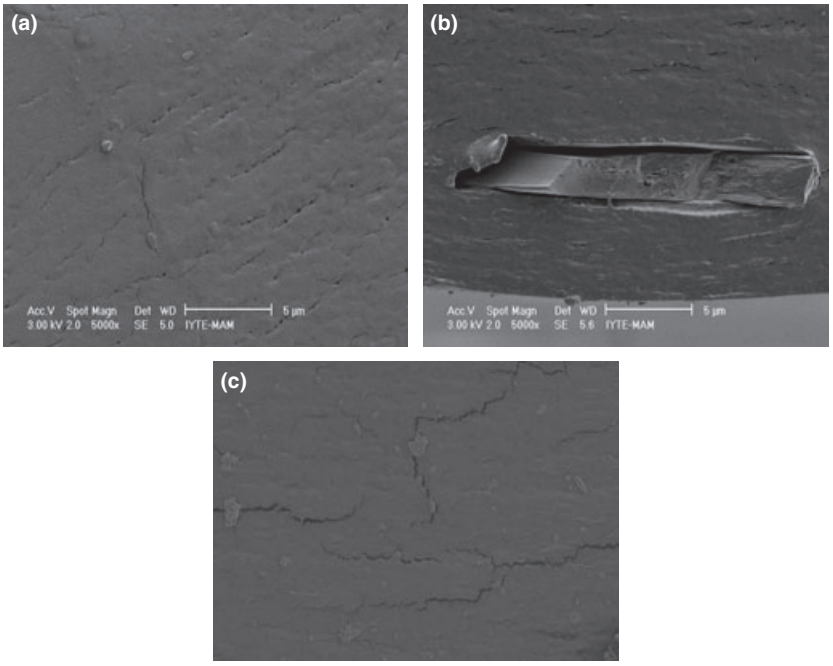

Figure 4 Standard electron microscopic pictures of methyl cellulose films: (a) control; (b) 20 mg natamycin/10 g fs and (c) (2 mg natamycin +1.5 mL rosemary extract)/10 g fs (fs, film-forming solution).

holes were formed (Fig. 3b). In addition, NA crystalline structures were observed in the films. It was reported that NA suspension at pH 6.5 has a stable crystalline form (Stark, 2003). With the addition of RE (1.5 mL per 10 g fs), besides NA, into WG films, small rod-shaped particles were observed in the film (Fig. 3c). These rod-shaped particles are probably RE as films containing only RE had these particles.

MC control films also had a homogeneous structure (Fig. 4a). 2 mg NA per 10 g fs did not cause any change in the structure of the MC film. However, at high NA concentrations irregularities in the film structure were

seen. As it was observed in WG films, there were crystalline structures in the film at 20 mg NA per 10 g fs (Fig. 4b). Small particles as well as cracks occurred when RE (1.5 mL per 10 g fs) was incorporated into MC films in combination with NA (Fig. 4c).

From SEM observations, it could be concluded that NA homogeneously distributed in WG and MC films at low concentrations while it formed crystals at higher levels.

Conclusions

As a conclusion, high NA concentration in biopolymers resulted in discontinuity of the film structure. However, barrier properties were not affected from antimicrobial agent addition. Some changes were observed in mechanical properties depending on the antimicrobial concentration and film type.

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