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The Combined Effect of Additives and Processing on the Thermal Stability and Controlled Release of Essential Oils in Antimicrobial Films

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ABSTRACT: Essential oils (EOs) have a long history as food preservatives and many advantages compared to synthetic preservatives. The major limitation of EOs as antimicrobial agents incorporated into a polymeric matrix is their low thermal stability and their high volatility from polymers. This study suggests a new methodology approach to bypass the key limitations of adding EOs into active packaging, as food preservatives. Antimicrobial active films based on linear low density polyethylene (LLDPE) and Montmorillonite (MMT) were produced by incorporating the EO thymol in a stepwise procedure. The production of the film composites, containing thymol, has been conducted, under a protocol of three stages: (1) production of a highly porous nano composite master batch; (2) impregnation of thymol into the master batch; and (3) cast film extrusion. Adding MMT incorporated together with a foaming agent at stage-(1) increased significantly the heat stability of the EO during melt processing at stage-(3). Finally, the film obtained showed a high antimicrobial activity. The results obtained have proven the synergistic interaction between MMT, foaming agent and thymol. As the MMT content in the film increased, the loss of EO in the processing decreased, leading to a natural antimicrobial film with improved antibacterial properties. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40564.

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INTRODUCTION

In recent years, there is a growing demand for synthetic preservatives free food, and at the same time a demand for food products with an extended shelf life.[1–5] One proposed solution for these requirements is controlling the atmosphere conditions within the packed goods, to inhibit the development of bacteria growth. To date, various methods exist to incorporate active agents into a package. Antimicrobial activity can be achieved by adding active pads containing volatile antimicrobial agents into packages, incorporating antimicrobial agents directly into polymers,[6–10] spreading antimicrobials coatings onto polymer surfaces,[3,11,12] immobilizing antimicrobials by chemical grafting, using polymers that have inherent antimicrobial activity,[13] etc.[14] EOs are known to have antimicrobial and antioxidant properties. Most EOs are approved for contact with food and are classified as generally recognized as safe (GRAS) by the U.S. Food and Drug Administration.[6,11,15] Various studies have examined the implementation of various EOs for the development of films with antibacterial properties, including thymol,[1,8,12,16] carvacrol,[1,2,8,17] eugenol,[7] cinnamaldehyde,[3] and rosemary[10] in different polymers.[1,2,8,12,16–18] Thymol is a component found in EOs of oregano and thyme; it has a phenolic structure and is known as very active against proliferation of different bacteria and fungi. The antibacterial properties of thymol and similar compounds are associated with their lipophilic character enabling the accumulation in cell membranes of microorganisms,[19] leading to the disruption of cell structure.[20]

Nano clays have being used in different polymers for improving polymers mechanical and barrier properties.[10,21,22] In addition to these properties, it was found that Montmorillonite (MMT) nano clays (NC) have high adsorption capacity according to specific surface treatment, from hydrophilic to hydrophobic. Clay minerals have been usually modified with quaternary amine cations, replacing the exchangeable inorganic sodium, potassium or calcium ions on the clay surface.[23]

The main goal of this work is to increase the thermal stability of EOs during processing using NCs to control the migration rate from the final antimicrobial film. The concept of foaming NC composites, to create porosity to optimize the EO absorption and the contact between NCs and EOs, is a new approach that has not been studied yet. This innovative approach can be utilized to create antibacterial nano composite packaging, containing high concentration of volatile substances as active agents.

EXPERIMENTAL

Materials

The polymer used in this work was a linear low density polyethylene (LLDPE) LL-118-BRASKEM with a Melt flow index (MFI) of 1.0 g 10 min^{-1} (ASTM D1238 standard −190°C, 2.16 kg), and a density of 0.916 g cm^{-3}. Thymol (99.5%) was purchased from Sigma-Aldrich [Figure 1(a)]. PE-g-MAH Bondyram-4108 was purchased from Polyram-Israel. The foaming Agent-TRACEL PO 2201 was generously accepted from Tramaco. The organically modified clay Cloisite 15A, Cloisite 30B and Cloisite Na supplied by Southern Clay Products/Rockwood Additives, was dried in a vacuum oven at 110°C overnight before processing [one can see Cloisite 15A and Cloisite 30B formulas in Figure 1(b,c) respectively].

Sample Preparation

Different formulations were obtained using a three stage methodology for minimal loss of the active substance during melt processing: (i) master batch compounds were produced by melt mixing in a EUROLAB Digital 16 ('Prism') twin screw corotating extruder ($D = 16$ mm, $L/D = 24$), using a uniform temperature profile of 200, 220, 220, 220, 220, and 230°C at the die, and operated at a screw speed of 250 rpm. The materials were physically mixed in a plastic bag, and were added to the extruder at the same upstream feed port. The melt compound strands were continuously cooled down to solidify under water at 23°C, which then cut to granules with granulating machine. Different concentrations of NCs and foaming agent were introduced in the polymer using a maleic anhydride based compatibilizer; (ii) the impregnation of EO in the foamed pellets was done by mixing the pellets with EO in a closed tank at 70°C for 24 h. (iii) Antimicrobial films were produced by dry mixing the EO containing master batches with neat LLDPE in MICRO-TRUDER ('RandCastle') Cast-film single screw extruder ($D = 13$ mm, $L/D = 20$), using a temperature profile of 200, 210, 220, 220, 220, and 230°C at the die. The machine was operated at a screw speed of 70 rpm, to obtain film of 100 μm wall thickness and width of 150 mm. Samples without active ingredient were also prepared and used as control. The composition of the films is shown in Table I.

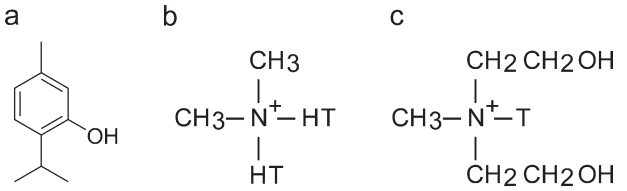

Figure 1. (a) Thymol EO, (b) Cloisite 15A where HT is hydrogenated tallow (∼65% C18; ∼30% C16; ∼5% C14), and (c) Cloisite 30B where *T* is tallow (∼65% C18; ∼30% C16; ∼5% C14).

Table I. List of Film Sample Formulations

	Sample name	Nano clay wt %	Foaming agent wt %	PE-g-MAH wt %	LLDPE wt %	Essential oil (thymol) wt %
1	NC1FAEO	1	1.3	5	89.7	3
2	NC3FAEO	3	1.3	5	87.7	3
3	NC5FAEO	5	1.3	5	85.7	3
4	NC7FAEO	7	1.3	5	83.7	3
5	NC10FAEO	10	1.3	5	80.7	3
6	NC1EO	1	0	5	91	3
7	NC3EO	3	0	5	89	3
8	NC5EO	5	0	5	87	3
9	NC7EO	7	0	5	85	3
10	NC10EO	10	0	5	82	3
11	LL0FAEO	0	1.3	5	90.7	3
12	LL0EO	0	0	5	92	3
13	NC1FA	1	1.3	5	92.7	0
14	NC3FA	3	1.3	5	90.7	0
15	NC5FA	5	1.3	5	88.7	0
16	NC7FA	7	1.3	5	86.7	0
17	NC10FA	10	1.3	5	83.7	0
18	NC1	1	0	5	94	0
19	NC3	3	0	5	92	0
20	NC5	5	0	5	90	0
21	NC7	7	0	5	88	0
22	NC10	10	0	5	85	0
23	LL0FA	0	1.3	5	93.7	0
24	LL0	0	0	5	95	0

Material Characterization

The active films were characterized using different techniques in order to investigate their ability to absorb and control the release of the EO. Rheological and antimicrobial properties were studied as well.

Quantitative Analysis of EO Concentration in the Film. The amount of thymol in the samples was determined by UV–visible spectroscopy. The EO containing films were cut into small pieces and immersed in 2-propanol (1 mL 2-propanol for every 20 mg of film) for extraction. Thymol was then extracted by refluxing for 60 min. To 100 mL volumetric flasks containing 10 mL of a Standard Buffer Solution of Boric Acid + Potassium Chloride, 0.2 *M*, 1 mL of the extraction solution and 4 mL of 2-propanol were added. The solution was mixed and then, 1 mL of chloroimide solution (Gibbs reagent) was added. After gentle mixing the solution turned blue as a result of the chemical reaction between thymol and Gibbs reagent. The reaction mixture was allowed to stand for 15 min and then distilled water was added to the volumetric flasks making up a 100 mL solution. The absorbance of the various solutions was measured at $\lambda = 590$ nm, using a UV–visible 1650PC spectrophotometer, Shimadzu. The thymol content was calculated from a calibration curve.

Analysis of the EO Migration. The migration characterization was done using two different techniques: (i) quantification of the extracted antimicrobial EO from the film by UV–visible spectroscopy (as detailed in Quantitative analysis of EO concentration in the film) and (ii) by headspace analysis, using an autosampler headspace GC–MS to evaluate the time-dependent migration of EO to the headspace of glass vials. The headspace was analyzed by gas chromatography using a Thermo GC-MS system (Finnigan Trace GC ultra, Finnigan Trace DSQ) equipped with HTA HT200H headspace autosampler; RESTEK column, RTX-5MS Phase. Column length was 30 mm, the inner diameter was 0.25 mm, and the film thickness was 0.25 μm. Each sample was held in the autosampler conditioning oven for incubation in 40°C for 2 min before 0.5 μL was injected. The GC temperature profile was: initial temperature was 100°C, ramped to 200°C at 15°/min for a total run time of 8.17 min. Helium was the mobile gas phase; the carrier constant flow was 0.7 mL/min, split flow was 10 mL/min, the split ratio was 14 : 1. MS transfer line was at 200°C. MS : ion source temp was 250°C, mass range was 30–650, scan mode was full scan, and scan per second was 2.5. The retention times of thymol were 4.96 min. 50 mm^2 film samples were incubated in 20 mL headspace vials for 2 min at 40°C before extraction. Quantitative analysis of EO was carried out using a calibration curve.

Parallel Plate Rheometer. Viscoelastic behavior of the different composites was characterized in the melt state by a dynamic oscillatory shear rheometer (TA Instruments ARES ex2000) using parallel plate geometry with a sample diameter of 25 mm and a plate gap of 2 mm. The samples were made by compression molding at 200°C. The measurements were carried out at 190°C, using a constant strain of 1% and with angular frequency range of 0.1–100 rad s^{-1}. These conditions were confirmed to be within the linear viscoelastic region of the materials. All measurements were carried out under nitrogen to minimize polymer degradation and moisture absorption.

Wide Angle X-ray Diffraction. The intercalation and exfoliation of clay in the polymer matrix were determined by X-ray diffraction using a Bragg–Brentano diffractometer (Phillips PW1710; Cu Ka radiation, 40 Kv 40 mA) at room temperature. A rectangular film sample with a length of 25 mm, width of 10 mm and thickness of 1 mm was made using compression molding at 200°C, and was scanned at 2Θ range from 1 to 10 at a scanning rate of 0.02°/s.

Scanning Electron Microscopy (SEM). The morphologies of different samples (un-foamed/foamed master batch with different concentration of NCs; with and without EO) were observed using ASPEX EXPLORER SEM. The samples were obtained by compression molding at 200°C in a hot press (Carver laboratory press, Model 2697-5) and were cryogenic fractured in liquid nitrogen.

Mechanical Properties. Tensile test of antimicrobial films were carried out on testing machine (Instron model 5543). The tests were performed using a load cell of 500 N at a crosshead speed of 100 mm/min and a gauge length of 50 mm. The tensile specimens were strips with width of 12.7 mm. Stress at yield (MPa), stress at break (MPa) and strain at break were obtained according to ASTM 882.

Thermal Properties. Melting temperature (Tm), heat of fusion (ΔHm), crystallization temperature (Tc), and heat of crystallization (ΔHc) were determined from DSC thermograms of the samples with two repetitions. DSC tests were conducted using a TA DSC TA Q-2000 instrument. 8 mg of films was introduced in aluminum pans (40 μL) and were submitted to the following thermal program: heating from 23 to 150°C at 10°C min^{-1} (2 min hold), cooling at 10°C min^{-1} to 23°C (2 min hold) and heating to 150°C at 10°C min^{-1}.

Optical Microscope. Analysis of thymol crystals formed by a result of the blooming effect was made using an optical microscope (Zeiss Axioplan Hamburg, Germany).

Antibacterial Activity. The antibacterial activity of the various films was evaluated and compared using *Escherichia coli* ATCC 8739 as the experimental model. *E. coli* bacteria were grown overnight in Nutrient Broth media (NB, Sigma) under shaking (250 rpm) at 37°C. In the following day, the overnight culture was diluted in a fresh NB medium to OD = 0.1, which approximately corresponds to 10^8 colony forming units (CFU) per mL, and grown for 1.5 h to allow the cells to enter a logarithmic state until OD = 0.6 was reached. Then, the bacteria were diluted into NB 1% (1 : 100) to obtain a stock solution with a working concentration of 10^5 CFU/mL. 3 mL from the stock solution were taken into each well in a 6-well plate (DE-GROOTH). Each of the various films was laid on top of the well in a way that there was no direct contact between the film and the bacterial solution, thus the antibacterial activity if achieved would be due to the migration of the oil from the film to the bacterial solution through the headspace of the well. In light of the EOs evaporation, there was a separation between the films, thus each plate received one film and the empty wells

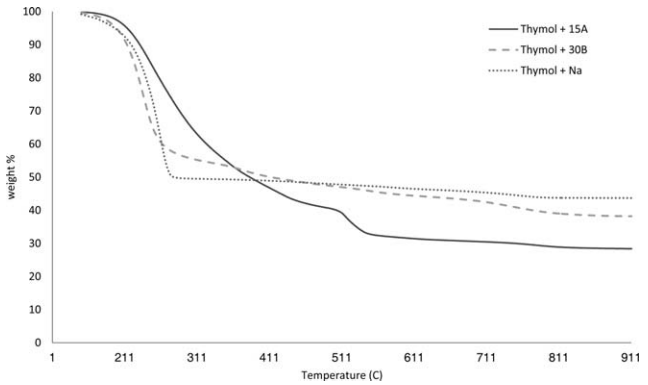

Figure 2. Thermal stability of thymol in different type of NCs (by TGA).

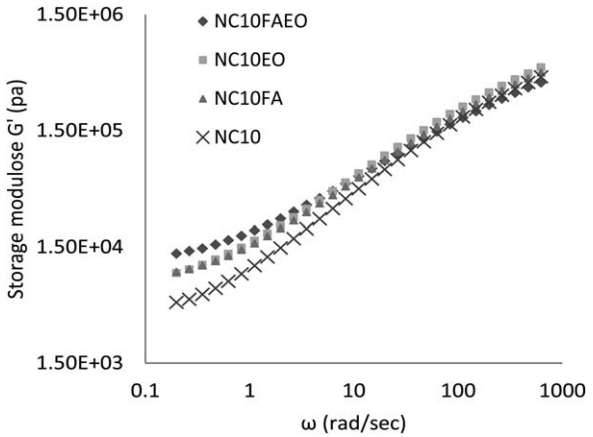

Figure 3. The influence of foaming agent, EO and the combination of both on the *G'* value for 10 wt % NC versus frequency.

were filled with an equal volume of water. The plates were then incubated on a shaker (100 rpm) at 37°C for 20–24 h. In the day after and in order to determine the CFU in each treatment, serial dilutions were carried out and the cells were spotted onto NB agar plates. The NB plates were incubated at 37°C for 20 h. Cell growth was monitored and determined by viable cell count.

Thermogravimetric Analysis. Thermo Gravimetric Analysis was done using TGA Q 50-TA Instruments. The samples (3–5 mg) were heated from ambient temperature to 700°C at a heating rate of 10°C/min and the data of weight loss vs. temperature were recorded. Each test was done twice.

RESULTS AND DISCUSSION

Thermal Gravimetric Analysis (TGA)

The selection of the optimal NC was done after TGA analysis. The most significant factor is the interaction between the nano particles with the EO. This factor is related to the surface treatment affecting the chemical affinity between the different materials. TGA results can be used as an indication for the chemical affinity. In order to study the interactions between thymol and different surface treated NCs, samples were prepared by mechanical mixing of thymol with NCs at a ratio of 50–50 at 70°C in a closed container for 24 h. The results seen in Figure 2 can be understood as an indication of the chemical affinity between the EO and the NC. While Cloisite Na (without surface treatment) does not affect thymols evaporation rate, Cloisite 15A, with a nonpolar surface treatment (organic modifier of dimethyl, dihydrogenated tallow, quaternary ammonium) interacts with thymol leading to a delay in the evaporation rate. Cloisite 30B (organic modifier of methyl, tallow, *bis*−2-hydroxyethyl, quaternary ammonium) also affects thymol evaporation rate but it was considered less appropriate due to the high polarity of its surface treatment enabling a poor dispersion in polyethylene matrices.

Parallel Plate Rheometery

The rheology of different polymer/NC systems was analyzed using parallel plate rheometer. Storage modulus (*G'*) vs. time or frequency was determined in order to estimate the degree of dispersion of NCs in the polymer matrix.[25–29] Storage modulus (*G'*) is a rheological function sensitive to nano particles dispersion in the polymer matrix.[26] It is assumed that better NCs dispersions could lead to enhanced diffusion of the EO in the polymer. Different combinations of foaming agent, NC and EO were analyzed as a function of angular frequency. Figure 3 shows the results of storage modulus for samples with a fixed NC concentration (10 wt %). High values of *G'* at lower frequencies could be noticed for samples containing EO and foaming agent, indicating a synergism between the two, leading probably, to better dispersion of NCs. Figure 4 shows *G'* values at a fixed frequency of 0.1987 rad/s, as a function of NC concentration. No significant differences could be noticed for low NC concentrations. For high concentrations, agglomerates are probably formed in the polymer matrix. According to Durmus et al.[26] high *G'* values at low frequencies indicate improved NC dispersions. Therefore, it can be concluded that the addition of a foaming agent together with the EO resulted in a better dispersion of the NC in the polymer matrix leading to a solid-like behavior confirmed by the increase of the *G'* values.

Scanning Electronic Microscopy (SEM)

The addition of NCs leads to a visible change in the morphology of the material. The porosity degree rises and the texture becomes coarser as can be seen in Figure 5. The films produced from a master batch containing a foaming agent present micro porosity. Films without EO have shown NC agglomerates.

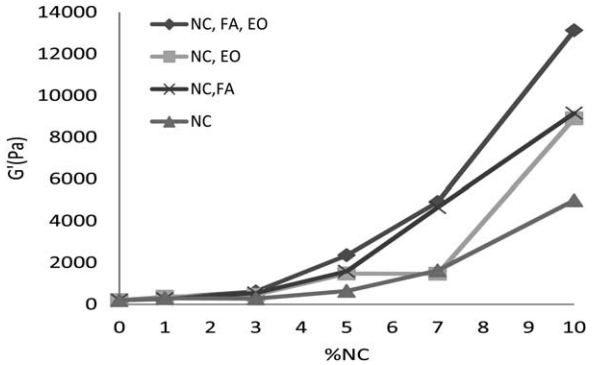

Figure 4. *G'* value for different NCs concentration, at the same frequency of 0.1987 rad/s.

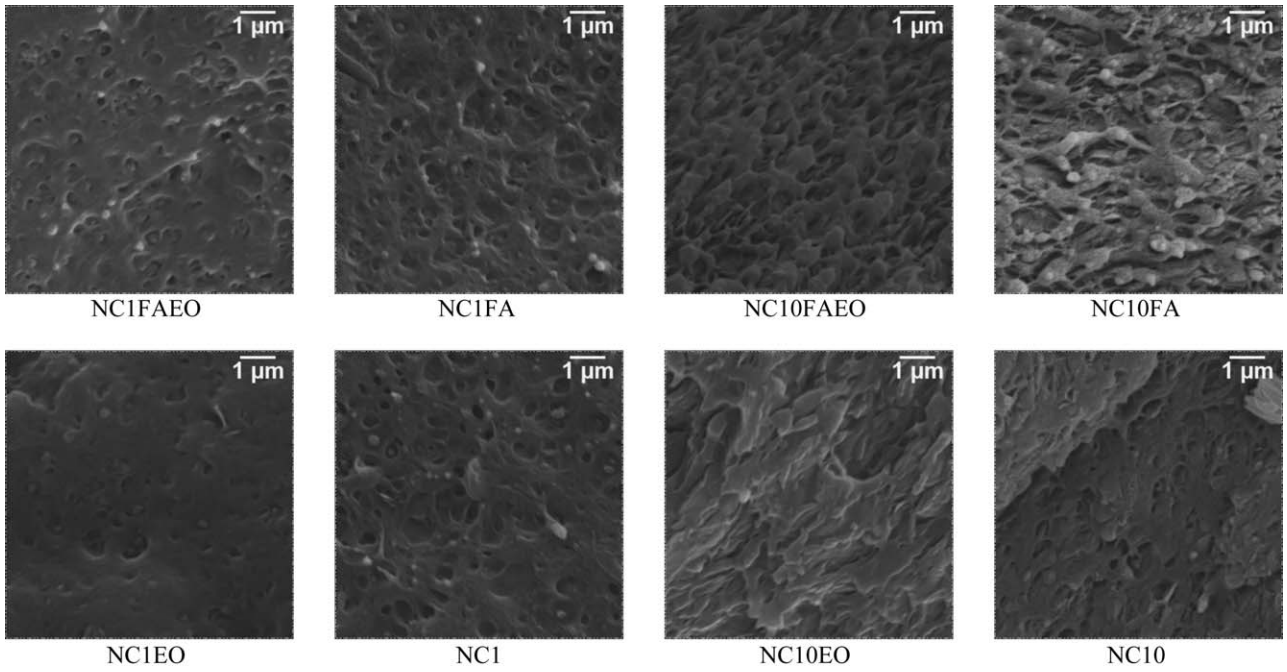

Figure 5. SEM images of 1% wt NC and 10% wt NC films with and without EO/foaming agent.

Wide Angle X-ray Diffraction Analysis

The ordered arrangement of silicate layers of the NCs can be discerned from X-ray diffraction patterns. When polymer penetrates the clay interlayers (intercalation) it results in an increase in interlayer spacing and a shift of XRD peaks toward lower angles. Partial or complete exfoliation of the ordered clay structure usually can be seen as a further shift to lower angles and a broadening or disappearance of the characteristic XRD peaks.[27] As can be seen in Figure 6, the addition of EO to the master batch causes a decrease in the intensity of the peak, indicating an increase in the interlayer spacing of the NCs. According to Persico et al.[2] EO positively interacts with NC, leading to a good dispersion of the EO into the clay galleries. As a result, the swelling of the NC stacks also promoted and enabled a larger polymer/clay interfacial area. The higher path tortuosity obtained improved the barrier properties of the film and delayed the release of EO.

According to the results exhibited in Figures 7 and 8, it can be concluded that the addition of a foaming agent to the master batch resulted in a decrease of the peak intensity and a broadening of the peak, indicating a possible expansion of the clay galleries. It probably occurred as a result of shear forces generated during the foaming. A synergistic effect between the foaming agent and the EO could be seen, leading to better NC dispersion in the polymer matrix, improving the intimacy between the different components in the film.

Quantitative Analysis of EO Concentration in the Film

The films were obtained using extrusion cast at 230°C. High temperatures processing may cause significant loss of EO. However, the presence of additives in the film can significantly affect the degree of loss. When comparing films with different clay concentrations, it was found that as the clay content increased the loss of EO during processing decreased significantly. In addition, when the clay concentration was above 5 wt %,

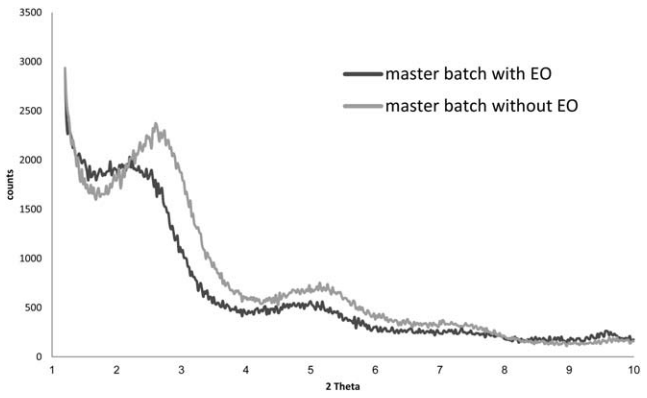

Figure 6. XRD results of foamed master batch with (dark gray) and without (light gray) EO for 9% wt NC.

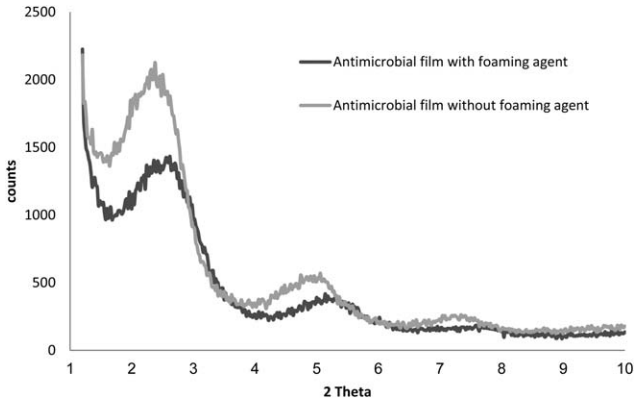

Figure 7. XRD results for 3% wt NC with (dark gray) and without (light gray) foaming agent.

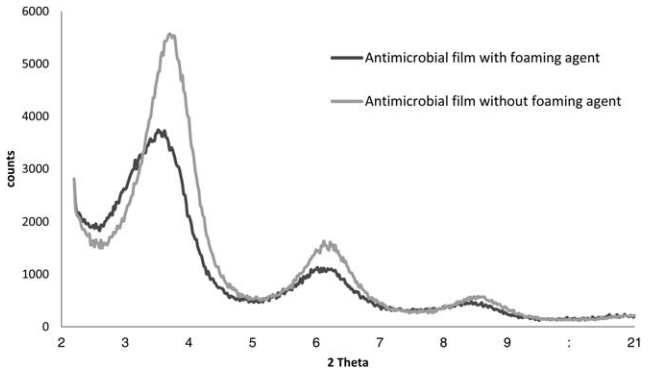

Figure 8. XRD results for 7% wt NC with (dark gray) and without (light gray) foaming agent.

foaming the master batch using a foaming agent increased the amount of EO absorbed and reduced its loss during processing. The initial concentration of the EO was 3 wt %. The final concentration depended on the interactions between the EO, the NCs and the polymer. An important factor to be considered is the solubility parameter of the blend components. The information about the solubility parameters were taken from HSPiP (Hansen Solubility Parameters in Practice) software v4 (http://www.hansen-solubility.com). The solubility parameters of the selected hydrophobic polymer, the EO and the NCs are as follows: $\delta = 8.16$, 10.22, 9.125 $(\text{cal/cm}^3)^{0.5}$ respectively. When the solubility parameters of the blends component are similar, or, the interactions between the components are strong enough to overcome the solubility parameters gap, the system will be miscible. The present system significantly delays the EO desorption from the antimicrobial film. This can lead to less antimicrobial activity since the EO may not diffuse out of the film. However, if the solubility parameters gap cannot be bridged, noncompatible system will be achieved, and, the EO will quickly evaporate from the film. In order to obtain a controlled migration system, the solubility parameter gap should be low, leading to a compatible system. The results of EO concentration in the film after processing are shown in Table II and Figure 9.

Migration Phenomenon

Migration depends on the conditions in which the test is being performed[30] and on the type of media it occurs in (different simulants, air, etc).[3,6,8,11,17] Volatile antimicrobial substances, such as EO, can act on foods even without direct contact between the food and the package, due to the presence of EO molecules in the headspace of the package. For this reason it is of great importance to determine the kinetics of the migration to the headspace. If a film is inserted in an open container, the large osmotic pressure gradient will result in fast migration. However, when the film is stored in a closed container the migration rate will slow down until the equilibrium. Two different migration tests were performed. In the first test the amount of EO in the films exposed to air for different periods of time, was measured, using extraction and colorimetric test (Figure 10). In the second test the amount of EO released to the headspace of a closed glass vial was evaluated by an auto sampler headspace GC-MS (Figure 11). In this test, the vials were kept at room temperature, and the samples were conditioned for 2 min at 40°C prior to injection.

A fast initial release could be noticed from the results presented in Figure 10. This phenomenon is typically referred to as 'burst release'.[31] This leads to a high initial active substance delivery reducing the controlled release effectiveness.[31]

The presence of NC affects the EO migration kinetics, as can be seen in Figures 10 and 11. The migration rate is reduced as the amount of the NC in the film increased. However, although the initial rate of migration depends on NC concentration, the equilibrium concentration reached was the same for all samples.

According to Helmroth,[32] the migration prediction of a specific combination of migrant, polymer and food simulant can be obtained by the model parameters values; the diffusion coefficient (D), that represents the migration rate, and the partition coefficient (K), that represents the ratio of the migrant concentration in the film to the migrant concentration in the food simulant at equilibrium. When the diffusion coefficient decreases, the time to reach the equilibrium increases. The diffusion coefficient of three different film compositions; NC1FAEO, NC5FAEO, and NC10FAEO were calculated using eqs. 1 and 2. Equation 1 shows that for large values of t, a plot of ln $(M_{\text{inf}} - M_o)$ versus t can give a straight line with a slop of θ. The value of θ can be useful for the calculation of the diffusion coefficient from eq. 2.[33]

$$\ln(M_{\text{inf}} - M_t) = \ln\left(\frac{8M_{inf}}{\pi^2}\right) - \frac{D\pi^2 t}{\mathrm{l}^2} \quad (1)$$

When M_{inf} is the total mass desorbed after infinite time, M_t is the total mass desorbed from the film at time t, D is the diffusion coefficient, and l is film thickness.

Table II. EO Concentration in the Film After Processing Compared to the Concentration Before Processing

	STDEV	Average EO concentration in the film		STDEV	Average EO concentration in the film
LL0EO	0.26%	1.35%	LL0FAEO	0.10%	1.08%
NC1EO	0.21%	1.39%	NC1EAEO	0.03%	1.35%
NC3EO	0.35%	1.37%	NC3FAEO	0.32%	1.38%
NC5EO	0.20%	1.51%	NC5FAEO	0.29%	1.60%
NC7EO	0.26%	1.83%	NC7FAEO	0.46%	1.73%
NC10EO	0.37%	1.94%	NC10FAEO	0.46%	2.05%

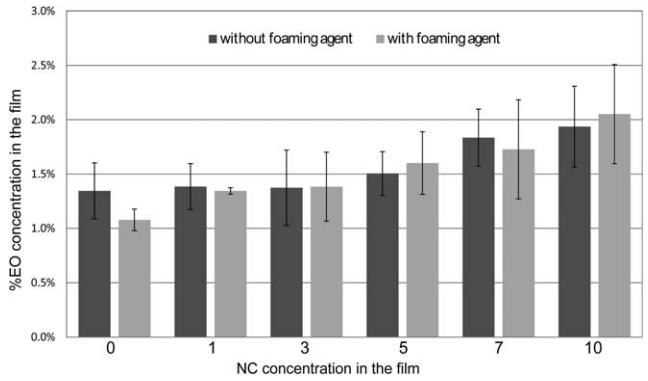

Figure 9. EO in the film after extrusion casting vs. NC concentration and foaming agent presence average.

$$\theta = -\frac{D\pi^2}{l^2} \quad (2)$$

The diffusion coefficient of NC1FAEO, NC5FAEO, and NC10FAEO were 8.11E−09, 5.07E−09, and 2.03E−09, respectively. The calculated diffusion coefficients in combination with the results presented in Figure 11 show that the change in the curve shape and the diffusion coefficient changes as a result of NC concentration in the different films. As the NC concentration in the film increased, the diffusion coefficient and the migration rate decreased.

Blooming Phenomenon

Blooming can occur when an additive is present in the polymer at a concentration above its saturation limit. Additives can diffuse through the polymer, precipitating on the surface. This phenomenon is most common when the melting point of the additive is below that of the polymer.[34] This effect can cause significant loss of the active substance reducing the antimicrobial activity of the final film. Blooming can damage film surface, affecting the ability to perform secondary processes such as printing, welding, etc. EOs can diffuse through the polymer leading to blooming when the films are scroll packed. The presence of NCs in the film can help to reduce this phenomenon, as can be seen in Figure 12. The images were taken six months after the film processing. As the concentration of the NC in the film increases, blooming decreases. The influence of the NC

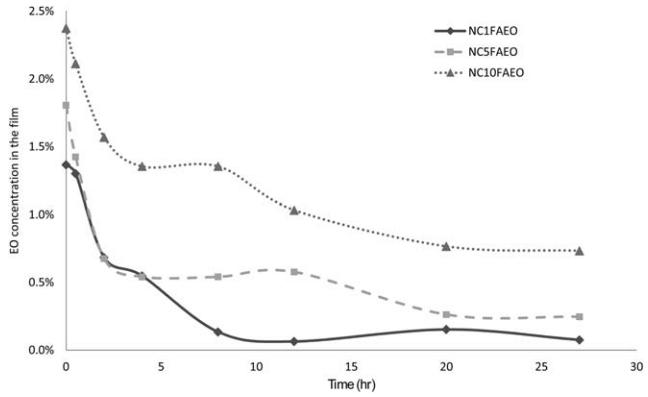

Figure 10. EO content in the film vs. time in exposed films at room temperature.

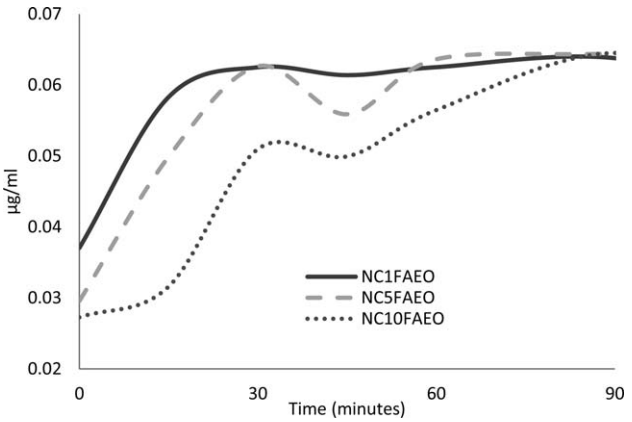

Figure 11. Release of EO into the vial headspace vs. time.

concentration on blooming phenomenon can be explained by the chemical affinity between the NC and the EO, and the prolonged tortuosity path generated by the dispersion of the NCs in the polymer matrix.

Antibacterial Activity of the Films

The antibacterial properties of the various films were tested against *E. coli*, a common food-born bacterial pathogen. As can be seen in Figure 13, all the films completely eradicated the bacteria except NC10FAEO, which only caused partial elimination. This result can be explained by a strong interaction between the EO, the NCs and the foaming agent. The presence of EO and a foaming agent did help to achieve a better dispersion of the NC within the polymeric matrix; however, it also led to a better interaction between the EO and the NCs which resulted in a lower antibacterial activity. Since a difference between films containing increasing concentrations of NCs and films containing both NCs and a foaming agent could not be detected (all showed complete bacterial killing) it was decided to challenge the system by loading 10^5 bacteria (the initial bacteria quantity) each day until the films would fail to prevent bacterial growth. As presented in Table III, the NC1EO film managed to eliminate all the bacteria only in the first day of the experiment and was not able to handle more bacterial loading cycles, while films containing increasing concentrations of NC (i.e., NC5EO and NC10EO) still retained their antibacterial activity. Moreover, films containing increasing concentrations of NCs with the addition of a foaming agent exerted a prolonged antibacterial activity in comparison to the ones containing NCs alone (Table III), suggesting that the foaming agent helped at dispersing and exfoliating the NCs in the polymer matrix, increasing the interaction between the EO and the NCs. As a result, a controlled release of the oil was obtained. Nevertheless, in agreement with the results obtained in Figure 13, NC10FAEO film was shown to be much less effective, further corroborating the notion that the EO might be "locked" within the film. To further establish that addition of a foaming agent and NCs slows down the release of the oil into the head space of the well to affect the bacteria growth, and that the results obtained in Table III are indeed due to a controlled release of the oil throughout the experiment period and not resultant of higher amounts of oil in the films, NC5EO and NC5FAEO films were treated with

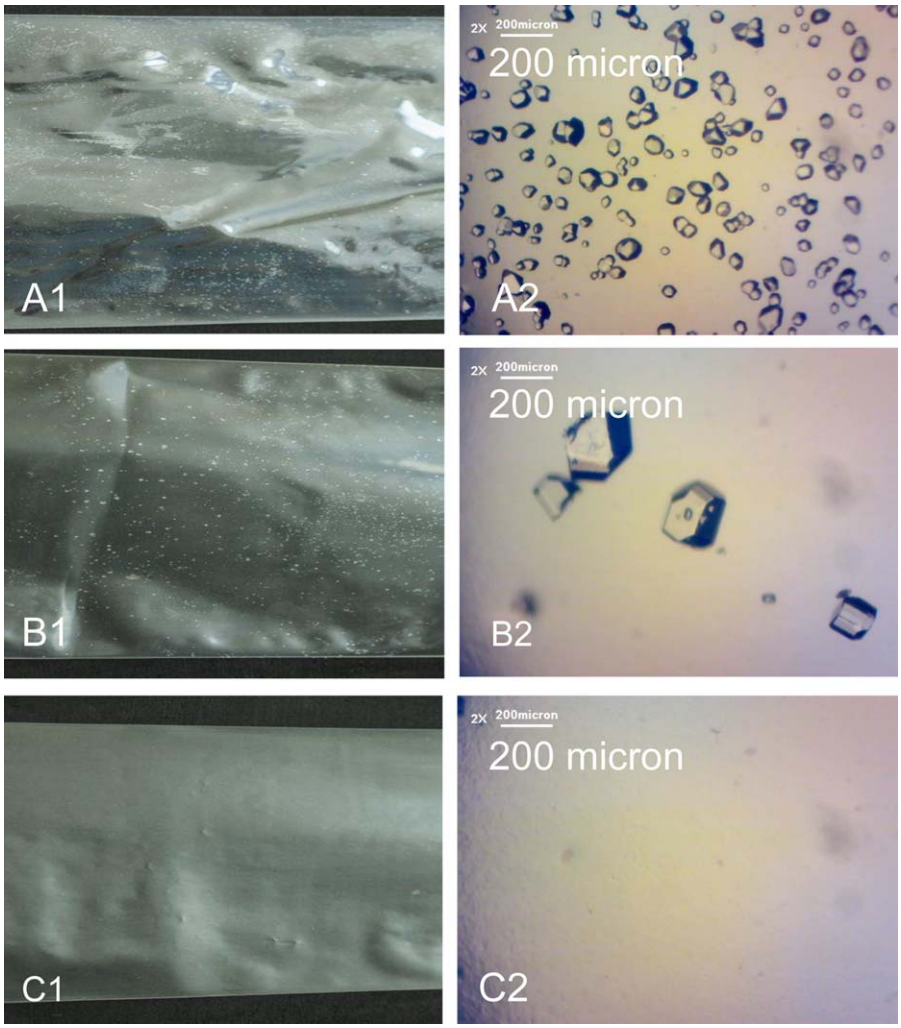

Figure 12. Film containing 1% wt NC-NC1FAEO (A1, A2), 5% wt NC-NC5FAEO (B1, B2), 10% wt NC-NC10FAEO (C1, C2). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

bacteria in the same manner as in Figure 13 and Table III. The only exception was that following the first incubation, one plate for each film was replaced with a new cover plate and the film was taken out (designated as 'new' in the table) while a second plate was left with the film (designated as 'same' in the table). Both NC5EO and NC5FAEO have managed to cope with two bacterial loading cycles no matter whether the film was taken out after the first day of the experiment or not (Table IV). After the third loading cycle, the 'new' NC5EO failed to kill the bacteria while the 'same' NC5EO provided only 2 log reduction compared to the control (Table IV). This suggests that the NC5EO film did not maintain major amounts of EO within the film to provide a slow release advantage and it is likely that most of the oil was released to the medium in the first 24 h. However, for the NC5FAEO film a clear difference between the 'new' NC5FAEO and the 'same' NC5FAEO was observed in the fourth day, where the 'new' film almost did not affect the tested bacteria ($\sim$ 1 log less than the control, i.e. untreated bacteria), while the 'same' film still presented excellent antibacterial activity, eradicating all the bacteria in the medium (Table IV). This highlights that in the presence of the foaming agent the oil was still maintained in the film and not completely released in the first loading cycles. Taken together, the results provide evidence that the oil release was delayed by the addition of a foaming agent, which enhanced the interaction between the oil and the clays, culminating in a gradual oil-releasing system.

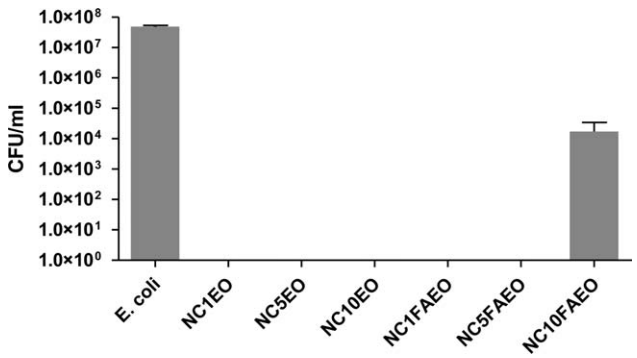

Figure 13. Antibacterial activity of thymol-containing films. *E. coli* bacteria were grown and treated with the various films as described in the experimental section.

Table III. Antibacterial Activity of Thymol-Containing Films

Time (days)	1	2	3	4	5	6
Film type						
Untreated E. coli	5.90E + 07	1.45E + 08	2.60E + 08	1.50E + 09	1.95E + 08	2.00E + 08
NC1EO	0	3.90E + 07	4.40E + 07	9.00E + 07	1.50E + 08	1.15E + 08
NC5EO	0	0	0	2.51E + 06	2.50E + 07	2.40E + 07
NC10EO	0	0	0	2.01E + 05	3.70E + 07	6.50E + 07
NC1FAEO	0	0	0	0	6.50E + 07	8.00E + 07
NC5FAEO	0	0	0	0	3.20E + 07	3.10E + 07
NC10FAEO	1.09E + 04	9.50E + 03	9.50E + 05	8.50E + 06	1.55E + 07	2.05E + 07

E. coli bacteria were treated as in Figure 13, only that each day 10^5 bacteria were added to the plates. The numbers represent CFU/mL.

Table IV. Antibacterial Activity of Thymol-Containing Films

Time (days)	1	2	3	4	5
Film type					
Untreated E. coli	4.40E + 07	1.80E + 08	5.95E + 07	9.00E + 08	1E + 08
NC5EO new	0	0	1.17E + 08	6.00E + 07	1.15E + 08
NC5EO same	0	0	1.07E + 05	1.10E + 07	3.40E + 07
NC5FAEO new	0	0	0	5.14E + 07	4.45E + 07
NC5FAEO same	0	0	0	0	3.35E + 07

E. coli bacteria were treated as in Table III, only that after the first day of incubation, the film from one plate was taken out and a new cover was added instead (designated as 'new') while the second plate remained with the film for the whole experiment (designated as 'same'). The numbers represent CFU/mL.

Mechanical Properties

Three different antimicrobial films were tested using an Instron 4481 tensile test machine for evaluation the influence of EO and/or NC on the mechanical properties compared to neat polymer (LLDPE). The results are shown in Table V. It can be seen that the EO slightly decreases elongation and stress at yield.

Thermal Properties

The thermal properties of three antimicrobial films were examined using DSC in order to evaluate the influence of EO and/or NC on the thermal properties of the films compared to neat polymer (LLDPE). The results are shown in Table VI. As a result of EO and/or NC presence, Tc increased by 2–4°C and Tm decreased by 4–5°C, on average. The influence of the nano particles on the crystallization temperature (Tc) referred to its effect on the nucleation of the polymer matrix crystals. The clay can provide heterogeneous surface increasing the crystals nucleation rate. The interfacial interactions between the surface treatment of the NCs and active groups of polymer chains can reduce molecule mobility, reducing the crystals growing rate.[35] The addition of NC to the polymer probably led to a crystallization mechanism characterized by a fast primary process during the initial stages and by slower secondary process[2] resulting in a higher number of smaller crystals. According to Persico et al.[2] the diffusional chain mobility promoted by the EO enhances the ability of the PE to be self nucleated resulting in an increase of the Tc. At the same time, plasticization effect of EO can be observed in decreasing ΔHTm values; meaning that less energy is needed for breaking the crystalline arrangement.

Table V. The Influence of NC and EO on Mechanical Properties of Antimicrobial Films

	Stress at yield (MPa)	Strain at break (%)
LLDPE	8.9	1020
LL0EO	8	971
NC10FA	9.3	764
NC10FAEO	8.4	906

Table VI. The Influence of NC and EO on Thermal Properties of Antimicrobial Films

	Tc (C)	Tm (C)	ΔHTc (J/g)	ΔHTm (J/g)
LLDPE	111.7	127.6	55.4	71.8
LL0EO	113.9	123.5	52.2	70.3
NC10FA	115.1	122.9	53.4	72.8

CONCLUSIONS

Antimicrobial layered silicate nano composite films were prepared by three stages melt compounding procedure of LLDPE in the presence of Cloisite 15A organo-modified MMT, Tracel 2201 foaming agent and thymol EO. According to parallel plate and XRD result, the presence of thymol facilitates the organoclay dispersion in the film; moreover, the incorporation of a foaming agent creates a synergistic effect leading to a better dispersion in the film. As a result of improved dispersion of clays in the polymer matrix, and improved chemical affinity between

the nano composite and the EO, desorption was delayed and a controlled release was obtained. Depending on the concentration of clays in the film, desorption delay caused a decrease in the antimicrobial activity. It can be concluded that the antimicrobial activity of the films is related not only to the EO concentration in the film, but also to the ability of the film to desorb the EO to the headspace.

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