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Effect of Emulsion Condition of Oil Phase on Microstructure and Anti-Fungal Properties of Emulsified Films Based on Carboxymethyl Cellulose

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ABSTRACT

Background: A comparison of the various microstructural and antimicrobial properties of emulsified films indicated significant differences. This study aimed to determine the effect of emulsion condition of oil phase on microstructure and anti-fungal properties of emulsified films based on carboxymethyl cellulose.

Methods: Emulsified films containing macro and nano emulsion of cinnamon essential oils were prepared at different concentrations (0.25%, 0.5% and 1 W/V). The anti-fungal test was performed in three replicate for each sample. The count of fungal spores was undertaken under the microscope by using the Neubarer cell until reaching the spore number of 10^6 CFU/ml. The films were cut using sterilized punch and placed on an inoculated culture medium. The plates were placed inside the incubator at 25°C for 24 hours. The output data were reported as the diameter of the non-growth halo and the anti-fungal index.

Results: The decrease in the size of emulsion droplets below 100 nm resulted in a better dissemination from the cell wall of microorganisms and the anti-film efficiency improved. The microscopic images were an indicator of smooth surfaces for nano-emulsion films similar to control films, and the nano-droplets had better stability in the film matrix.

Conclusion: This study introduced a new kind of nano-active packaging film with some of its improved functional properties.

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Introduction

Oils, vegetable and animal fats, essential oils, waxes and glyceride derivatives, do not produce continuous and independent films due to its lipid nature, and lack of polymer structure. But because of their high inhibitory properties in relation to water vapor permeation, they can be used in composite film production, along with other biopolymers such as proteins and polysaccharides. One of the methods used in the production of double layer composite films is molding a layer of lipid on the surface of the biopolymer, (protein

or polysaccharide). However, due to the multi-stage method, (two molding stages and two drying stages), film formation with a layered structure, creating fine cavities on the surface (superficial cracking), and lack of uniformity (surface cohesion) led to use the direct diffusion of lipid compounds into the film-forming solution (1).

In spite of the reduction in the number of production steps (one molding stage and one drying step), in order to avoid the phase separation, the use of an emulsifier in the emulsion film forming solution will be necessary. The characteristics of the films produced by this method are dependent on the type of manufacturing method, the type and amount of the components (the ratio of the amount of hydrocolloids and lipids), the lipid and hydrocolloid phase compatibility and the microstructure uniformity (2).

Macro emulsions are unstable thermodynamic emulsion systems that are known as conventional emulsions, and their droplet size range is usually between 0.1 and 100 µm. Often in scientific literature, emulsions with a droplet size of about nanometers (typically in the range of 20 to 200 nanometers) are referred as nano emulsion. Nano emulsions have a high degree of stability against the phenomena of deposition and creaming. This method is used as a suitable solution in food technology, as it can store essential oils more in the biopolymer structure compared to conventional emulsions (3).

The close contact between the constituents in the dried film can be improved by the use of the nano emulsification technique, which can improve the microstructure of the emulsified films. The antimicrobial activity of the intrinsic essential oils in surfactant micelles with nanometer sizes, is affected in two directions. First, the amount of the release of the over coated essential oils deposited inside the nano liposomes or nano emulsion vesicles decreases significantly, and secondly, the permeability of the cell membrane of microorganisms will be improved, due to increased bioavailability, non-polar bioactive compounds (3).

Nano liposomes of cinnamon essential oil have been used to inward overlap in the gelatin biopolymer bed. The average size of nano-liposome droplets after applying ultrasound was about 107 nanometers. The images obtained from the scanning electron microscope (SEM) indicated that films containing nano liposomes of essential oil had a denser cross-section and a smoother surface (4). Another study showed that the average droplet size of the pre-emulsion of essential oil obtained from a high shear homogenizer, using an ultrasound probe, decreased from the macro meter scale to a nano meter scale. They observed that the over coated of the nano emulsion droplets have improved the anti-fungal effect of these two essential oils in comparison to the macro emulsion in the biopolymer matrix (3).

The microscopic images of sodium caseinate films covered with nano emulsion of essential oils have been assessed indicating that reducing the size of the droplets results in greater oil content stability in the film matrix (5). Based on our knowledge, there has been no study on the comparison of macro and nano emulsion solutions of cinnamon essential oil on the micro structural and anti-fungal properties of carboxymethyl cellulose emulsified films. Therefore, our aim of this study was to produce carboxymethyl cellulose (CMC) emulsified films containing macro and nano emulsion of cinnamon essential oil by high shear homogenizer and high shear-ultrasound combined method, respectively; and to compare the effect of these two types of emulsion solution on micro structural properties and microbial deterrence.

Materials and Methods

CMC, with an average molecular weight, 41000 g/mol (Applied grade), was obtained from Caragam Parsian (Tehran, Iran). An analytical grade of glycerol was purchased from Dr. Mojallali Chemical Labs (Tehran, Iran). Polysorbate 80 (Tween 80) was purchased from Merck (Darmstadt, Germany). Cinnamon Essential Oil, (Cinnamomum zeylanicum) was prepared from Exire Gole Sorkh Pharmaceuticals (Mashhad, Iran). Fungal species of *Aspergillus nigers* strains (ATCC 64974) and *Mucor racemous* (IBRC-M number 30117), were prepared for microbial test from microorganism bank (Tehran, Iran).

For emulsification of cinnamon essential oil, the production of macro emulsion solution was undertaken by adding various amounts (0.125,0.25, 0.5 grams) of Tween 80 equivalent to 50% W/W of surfactant to oil ratio (SOR) to 20 ml of double distilled water and homogenization by a high shear homogenizer (JANKE & KUNKEL, Germany) at 20,000 rpm for 1 min. The cinnamon's essential oil was added to each emulsion solution at levels of 0.25, 0.50 and 1 g which was equivalent to 0.25, 0.50, and 1 W/V (essential oil to emulsion), and again homogenization was performed for 2 min. The prepared macro emulsion solution at 20 kHz, 400 W, and a range of 70 S for 10 min was subjected to sonication by ultrasound probe (FAPAN, Iran) to prepare nano emulsion solution.

The Particle Sizer (Malvern instruments, UK) was used to determine the average droplet size (D_z) and distribution index (PdI) of emulsion solutions. First, the emulsion solutions were diluted in distilled

water (3) to prevent multiple scattering, and the droplet interactions of the oil phase in the emulsion in proportion (1 to 10). The PdI index was used in emulsion to express the size distribution of droplets. The values close to zero indicated the homogeneous emulsion solution. The average droplet size index, D_Z, was expressed in terms of the intensity of diffusion. The mean droplet size index was calculated using the relation, while $D_{i=}$Size of each drop, $D_{Z=}$Average size of droplets, and Si=the intensity of Droplet size distribution

$$(S_i \approx D_i^6).\ D_z \approx \frac{\sum S_i}{\sum (\frac{S_i}{D_i})}$$

In order to make films, a previously used method was applied with some modifications. First, 80 ml of double distilled water was heated in the water bath, until it reached to 85°C, then 1.5 g of CMC was added to hot water, and a magnetic stirrer, at 800 rpm, in the temperature of 85°C was stirred for 60 minutes, and finally the glycerol, in 0.75% w/w biopolymer was added as a plasticizer. The stirring was continued in this situation for 10 min. The biopolymer solution temperature was reduced to 60°C to prevent the active compounds of the cinnamon's essential oil from degrading and mixed with 20 ml of macro and nano emulsions which was prepared in the previous step. In order to prepare a homogeneous solution, the emulsion solution was stirred at 500 rpm at 60 for 30 min. The film forming solution was inoculated under ambient conditions for 5 min using vacuum pump (DV-3E 250, USA), then 100 ml of each emulsion film forming solution, at a flat surface of the teflon plates (PTFE) Molded at 15×15 cm in size and dried at 40°C for 18 hours at the incubator (6).

Micro structural analysis of surface and cross section of emulsion films containing different concentrations of macro and nano emulsion solutions of cinnamon essential oil was investigated using SEM (Tescan, Czech Republic). Liquid Nitrogen was used to cut the films and then the cut films were placed on the copper base and coated with gold (DSR1 Nanostructural Coating Co., Iran). After coating with a thin layer of gold, the films were photographed at a voltage of 10 KV and a magnification of 5,000.

To determine the anti-fungal features of emulsion films, a disc diffusion method described before was used with some minor modifications (7). In order to activate the primary suspensions of *A. niger* strains (ATCC 64974) and *M. racemous* (IBRC-M number 30117), the potato dextrose agar (PDA) culture medium was used. The count of fungal spores was done under the microscope by using the Neubarer cell until reaching the spore number of 10^6 CFU/ml. The PDA solution was poured into plates of 80 mm diameter.

After hardening of the culture medium, the counted fungal strains in the serum physiology was spread over the surface of the culture medium. The films were cut using sterilized punch and placed on an inoculated culture medium. Then, the plates, were placed inside the incubator at 25°C for 24 hours. The diameter of the non-growth halo around the films was measured using a digital ruler. Anti-fungal index (AI%) was calculated using the equation: $A.I=D_i/D_p \times 100$, while D_i=Non-growth halo (mm), and D_p=The plate diameter (80 mm).

SPSS software (Version 21, Chicago, IL, USA) was used to analyze the data. One-way analysis of variance (ANOVA) and Duncan's test were used to compare the minimum significant difference between data. The tests were performed three times for each sample and the data were reported as mean±SD. A $P<0.05$ was considered statistically significant.

Results

The average droplet size based on the distribution intensity (D_z) and the dispersion of the droplet size distribution (PdI) for both macro and nano emulsion solutions are presented in Table 1. The average size and the distribution of the droplet size decreased by increasing the input energy to the emulsion solution. The high shear homogenizer was used as a relatively low mechanical energy method to produce

Table 1: Droplet diameters (Z-average), Polydispersity Index (PdI) and ζ-potential values of macro (ME)- and nano (NE-emulsions of cinnamon essential oil (CEO) at different concentration.

Emulsion type	Z-average (nm)	PdI
ME-CEO (0.25%)	242.1±1.01^c	0.39±0.04^c
ME-CEO (0.50%)	263±1^b	0.63±0.03^b
ME-CEO (1%)	362.2±1.07^a	0.72±0.02^a
NE-CEO (0.25%)	59.2±1.06^e	0.39±0.01^c
NE-CEO (0.50%)	80.08±1^d	0.24±0.02^d
NE-CEO (1%)	80.02±1^d	0.25±0.06^d

Values were given as mean±standard deviations. Different superscripts in the same column indicate significant differences ($P<0.05$).

a macromolecular solution. The droplet size range in the macromolecular solution was between 262 to 362 nm. The ultrasound method was used to reduce the mean droplet size to a range of 100 nm, and the droplet size ranges from 59 to 80 nm.

The surface micro structure images and the cross section of emulsified films were shown in Figure 1 and 2, respectively. CMC-based films and nano emulsion films containing cinnamon essential oil had smooth, uniform and interconnected surfaces. In contrast, films containing macro emulsion had a heterogeneous and irregular structure. These differences in the surface structure are due to the different dimensions of drops of cinnamon essential oil in the film matrix because of the difference in the intensity of unstable phenomena such as flocculation, coalescence and creaming. Droplets at nanometer level compared to macro droplets had better stability against unstable phenomena because of high zeta potential, smaller droplets and low droplet size distribution. Therefore, the interaction between the bio polymer matrix and the nano droplets of the cinnamon's essential oil can prevent the separation of the phase during drying.

Based on SEM images, the cross-section of films containing nano emulsion had a uniform, dense structure, with coherent layers (Figure 2). On the contrary, in macro emulsion films, a highly sponge-like structure was visible. These differences can be due to flocculation and coalescence because of the distribution and the size of larger droplets. Macro droplets can migrate to the surface of the film during the drying process and throughout the creaming phenomenon, which results in the formation of micro imperfections, cavities and fractures throughout the matrix. This discrete, high-pores with high cavities structure can have negative effects on the mechanical properties and barriers of films.

The anti-fungal test results were shown in Table 2. As expected, the oil-free control film did not have the ability to inhibit the growth of fungal. By increasing the concentration of oily essential oil, the anti-fungal index for both films containing macro and nano emulsion increased. Anti-fungal indexes of films containing macro emulsion improved from 14.16% and 20.82% to 18.81% and 25%, respectively, for *A. nigers* and *M. racemous* at 1% concentration for films containing nano emulsion. The decrease in the size of drops of oily essential oil from the macro droplet surface to the over coated nano droplets has an important effect on the improvement of anti-fungal properties against *A. nigers* and *M. racemous*, as a source of corruption in bakery products and sweet fruits respectively.

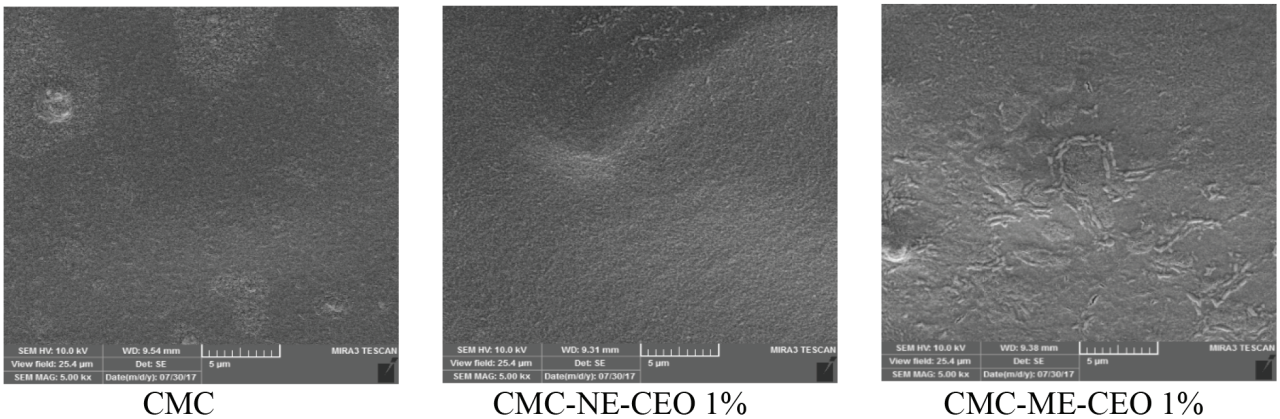

Figure 1: SEM images of the surface of the CMC control film and films loaded with ME (CMC-ME-CEO) and NE (CMC-NE-CEO) at selected concentration of 1% CEO.

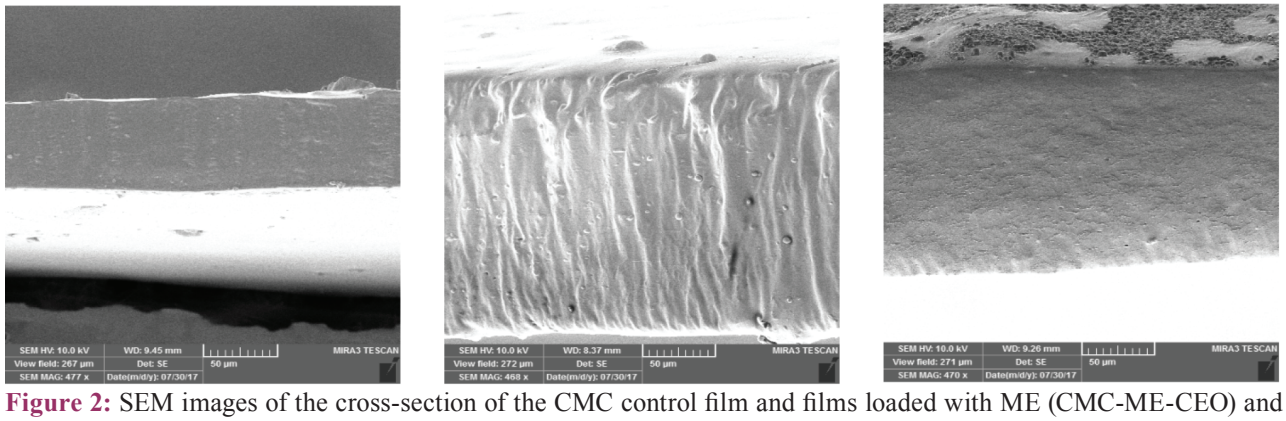

Figure 2: SEM images of the cross-section of the CMC control film and films loaded with ME (CMC-ME-CEO) and NE (CMC-NE-CEO) at selected concentration of 1% CEO.

Table 2: Inhibition diameter and Antifungal Index percentage (AI) of *A. nigers* and *R. mucurium* in control and films containing ME or NE of CEO in different concentration after 24 h at 25°C.

Film types	Inhibition diameter of *A.niger* (mm)	Inhibition diameter of *M.racemous* (mm)	A.I of *A.niger* (%)	A.I of *M. racemous* (%)
CMC	0^{d}	0^{c}	0^{d}	0^{c}
CMC-ME-CEO (0.25%)	9.5 ± 0.01^{c}	0^{c}	11.87^{c}	0^{c}
CMC-ME-CEO (0.50%)	10.38 ± 0.09^{c}	13.33 ± 0.77^{b}	12.97^{c}	16.66^{b}
CMC-ME-CEO (1%)	11.33 ± 0.01^{bc}	16.66 ± 2.88^{ab}	14.16^{bc}	20.82^{ab}
CMC-NE-CEO (0.25%)	11.5 ± 0.01^{bc}	15 ± 0.01^{ab}	14.37^{bc}	18.75^{ab}
CMC-NE-CEO (0.50%)	13.16 ± 1.89^{b}	16.66 ± 2.88^{ab}	16.45^{b}	20.82^{ab}
CMC-NE-CEO (1%)	15.05 ± 2.08^{a}	20 ± 0.01^{a}	18.81^{a}	25^{a}

Values were given as mean±standard deviations. Different superscripts in the same column indicate significant differences ($P<0.05$).

Discussion

The distribution of droplets of over coated essential oil in emulsion films has a significant effect on their various inhibition and micro structural properties (3, 8, 9). PdI as a dimensionless factor indicates the dispersion of the size of droplets spread in the emulsion (10). Sonication creates inter surface waves and forms micro bubbles, which, as a result of their destruction, reduce the size of oil droplets to a range of less than 100 nanometers (11). Sonication had a significant effect on reducing both droplet size (D_z) and size distribution (PdI) in emulsion of cinnamon essential oil ($P<0.05$). Hashemi-Gehroei et al. (2017) indicated that with an increase in the time of applying sonication to the emulsion solution, the distribution and size of droplets reduced in the essential oil of *Zataria multiflora* in the films matrix on the basil gum (12).

An increase in the concentration of cinnamon essential oil in both macro and nano emulsion resulted in an increase in droplet size ($P<0.05$). This phenomenon can be due to the accumulation of droplets due to the ineffectiveness of surfactants to completely cover the surface between droplets. Similar result was reported before on the effect of basil and oregano oily concentrations on the size of droplets in a chitosan biopolymer solution (13). Microstructure analysis is used to better understanding of the effects of different formulations on the micro structure and inhibition of emulsified films.

The anti-fungal activity, CMC-based films containing macro and nano emulsion of cinnamon essential oil, at different concentrations, against the *A. nigers* species and *M. racemous* species, using a disk diffusion method were studied. Different mechanisms have been proposed to improve the anti-fungal characteristics of nano-emulsion films compared to macro emulsion films, which can be the reduced selectivity of cell membranes against mass transfer through resonance in the mechanism of passive absorption in the membrane, and improvement in bioavailability and facilitating the release of active compounds in the form of nano droplets from the cell membrane, resulting in greater accumulation in cellular plasma (10, 11).

Conclusion

Comparison of various micro structural and antimicrobial properties of carboxymethyl cellulose containing macro and nano emulsion films of cinnamon essential oil indicated significant differences. The decrease in the distribution and size of the emulsion droplets resulted in the further absorption of the biopolymer to the nano droplets surface and, as a result, the stability of the nano emulsion in the emulsified film matrix improved. Microscopic images indicated a smooth and uniform surface and dense cross section for nano emulsion films. In contrast, macro emulsion films have high-pores with high cavities structure, due to flocculation droplets in film matrix in the form of creaming. Reduction in the size of the emulsion solution droplets through combined homogenizes high shear ultrasound resulted in a better diffusion of cinnamon essential oils from the cell membranes of microorganisms. Hence, the anti-fungal performance of nano emulsion films was improved.

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Conflict of Interest

None declared.

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