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Preparation and antimicrobial activity of oregano essential oil Pickering emulsion stabilized by cellulose nanocrystals

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ABSTRACT

An effective antimicrobial system was developed by using oregano essential oil (OEO) Pickering emulsion. The Pickering emulsion was stabilized by cellulose nanocrystals (CNCs), which were from microcrystalline cellulose (MCC) by selective oxidization ammonium persulfate. Factors affecting the stability of OEO Pickering emulsions, such as oil/water ratio, CNCs concentration, ionic concentration and pH, were studied and analysed by the measurement of the droplet sizes and Zeta potential. The results showed that the emulsions had good stability at higher CNCs concentration and pH values, or at lower oil/water ratio and salt concentration. In addition, the antimicrobial efficacy of OEO Pickering emulsions against four food-related microorganisms was evaluated by determining the minimal inhibitory concentration (MIC). The results showed that prepared OEO Pickering emulsions could efficiently inhibit the growth of four tested microorganisms by destroying the integrity of the cells' membrane. The research would have a certain leading meaning on the design and use of OEO Pickering emulsions stabilized by CNCs for the delivery of antimicrobial essential oils in the food and other industries.

1. Introduction

Essential oils (EOs) are aromatic and volatile oily liquids obtained from plants. Many kinds of plant essential oil are known for their antibacterial, antifungal, antiviral, antioxidant, and biological modulation properties [1–5]. Within the great variety of EOs, oregano essential oil (OEO) from *Origanum vulgare* L is well known for its antioxidative and antimicrobial activities [6]. These activities are probably due to the presence of carvacrol and thyme [6–8]. In recent years, OEO has been widely used in the food, pharmaceutical, cosmetic, and feed industries [6,9–11].

However, like other EOs, OEO could easily evaporate or decompose during food processing, drug formulation and preparation of antimicrobial films, owing to direct exposure to heat, pressure, light or oxygen [12]. In order to overcome the susceptibility and improve the stability of OEO during processing and storage, many methods have already been developed by different encapsulation methods [11–13].

Pickering emulsions are of any type, oil-in-water (o/w), water-in-oil (w/o), or even multiple stabilized by solid particles instead of surfactants [14–16]. The high resistance to coalescence is a major benefit of the stabilization by solid particles requiring an interfacial solid material which should be partially wet by both liquid phases and should be weakly flocculated [14,17,18]. In addition to their mechanical properties, Pickering emulsions present the double advantage of being extremely stable and requiring a very small quantity of particles [19,20]. For volatile oils, like some perfume oils, the research showed that the resistance to oil evaporation of water-insoluble oils from o/w emulsions is larger for NP-stabilized emulsions than for surfactant-stabilized systems [21,22]. Cellulose nanocrystals (CNCs) are regarded as ideal biomaterial due to their nice properties, such as low density, low carbon footprint, chemical tenability, environmental sustainability, and anticipated low cost. It is of particular interest for various applications such as cosmetics, pharmaceutics, or medical implants. The use of CNCs as stabilizer of Pickering emulsions is expected to be advantageous over some inorganic nanoparticles when the above advantages are considered [23–25]. The utility of CNCs to stabilize Pickering emulsion has been demonstrated by Kalashnikova and Zoppe respectively [20,25]. And it have been improved that CNCs were able to stabilize Pickering emulsions thus it can resist to creaming or centrifugation [22]. Furthermore, in our previous research, we had successfully prepared D-limonene Pickering emulsion stabilized by CNCs [26]. However, the influence of CNCs Pickering emulsion on the bioactivity of EOs was not clarified.

In this study, CNCs were prepared from microcrystalline cellulose (MCC) by ammonium persulfate (APS) hydrolysis, which was electrostatically stabilized by carboxylic groups installed on the surface which promotes uniform aqueous dispersions. To the best of our knowledge, OEO Pickering emulsions stabilized by CNCs have not been studied yet. In addition, carboxylic groups were installed on the surface of CNCs, which would change the surface charge of OEO Pickering emulsions. Moreover, Ziani and his coworkers have found that surfactant

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charge could affect the antimicrobial efficacy of surfactant-stabilized emulsions [27]. Therefore, it is much important to evaluate whether OEO Pickering emulsion is more or less effective than the OEO itself. Thus, the present research focused on the fabrication, characterization and antibacterial effects of OEO Pickering emulsions stabilized by CNCs, and factors that may influence the properties of Pickering emulsion, like CNCs concentration, ionic strength and pH. In addition, the cellulose structure, crystallinity index and thermal stability were also investigated using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and Thermogravimetric analysis (TGA) techniques. OEO Pickering emulsions were prepared by sonicating OEO and CNCs aqueous dispersion, and their characterization were carried out by static light scattering experiments, optical microscopy. At last, their antimicrobial efficacy was assessed against four food-related microorganisms by determining the MIC values and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

Ammonium persulfate (APS), potassium chloride, microcrystalline cellulose (MCC) and potassium hydroxide were all were purchased from Beijing Chemicals (Beijing, China). Oregano essential oil (OEO) was purchased from Guanxiang Chemicals Trading Co. Ltd. (Changsha, China) and stored at 4 °C. Glutaraldehyde, kanamycin sulfate was purchased from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China).

2.2. Preparation and characterization of CNCs

CNCs were prepared according to the method reported by Leung and his coworkers with a little modification [24]. In brief, MCC (1 g) was added to APS (100 mL, 1 M) and the mixture was heated to 60 °C for 16 h, then a suspension of CNCs was obtained. The suspension was centrifuged at 12000 rpm for 10 min. The supernatant was removed. Water was added to the CNCs pellet followed by mixing and repeated centrifugation/washing until the solution conductivity was $\approx 5\ \mu S\ cm^{-1}$ (pH 4). The product was lyophilized to yield a white powder. A white aqueous suspension of CNCs was obtained after ultrasonic treatment of CNCs powders in water.

MCC and CNCs were subjected to an X-ray diffractometer to characterize their structures [28]. A Shimadzu IR Prestige-21 Infrared spectrophotometer was also used for the analysis of MCC and CNCs. The thermal stabilities of MCC and CNCs were characterized using simultaneous thermal analyzer filled with a manual liquid nitrogen cooling system.

2.3. Preparation and of characterization OEO Pickering emulsion

Practically, a certain amount of OEO was added to CNCs aqueous suspension in a plastic vial. Then the mixture was sonicated at 0.4 kW power level in an ice bath to avoid overheating of the samples. The power level was applied by alternating 3 s sonication with a 2 s standby for 3 min and allowed to stand for a period of time before characterization. A series of emulsions were prepared with different CNCs concentrations, pH, and ionic strength.

Optical micrographs of the CNC-stabilized OEO Pickering emulsions were captured by an Olympus BX 51 optical microscope fitted with a digital camera (Olympus, DP 50). Emulsions were placed directly onto a microscope slide and viewed under the magnification from 10 to 40 times.

The mean particle radius and particle size distribution of OEO Pickering emulsions were measured using a Malvern 2000 granulometer apparatus equipped with a He-Ne laser (Malvern Instruments, U.K.) with Fraunhofer diffraction. The samples were diluted using deionized water at ambient temperature prior to analysis to avoid multiple scattering effects. In all the experiments, the error bars were calculated based on the standard deviation from three independent measurements.

The zeta potentials of the OEO Pickering emulsions at different pH values and salt concentrations were measured using a particle electrophoresis instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, and Worcestershire, UK) at 25 °C. All the OEO Pickering emulsions samples were freshly prepared, and the emulsions are homogeneous and stable. The emulsions were diluted using buffer solution at the pH and KCl concentration of the samples to avoid multiple scattering effects. The zeta potential was reported as the average and standard deviation of measurements made on three freshly prepared samples, with three readings taken per sample.

2.4. Antimicrobial activity of OEO Pickering emulsion

Four food-related microorganisms were used to assess the antimicrobial properties including the Gram-positive *S. aureus* ATCC 6538, *B. subtilis* ATCC 6633, the Gram-negative *E. coli* ATCC 8739, and the yeast *S. cerevisiae* ATCC 9763. All strains were provided by China General Microbiological Culture Collection Center, and maintained on slants of Nutrient Agar (NA, Abxing, Beijing, China) for bacteria and Yeast Peptone Dextrose Agar (YPDA, Abxing, Beijing, China) for the yeast at 4 °C.

Active cultures were prepared by transferring a loop of cells from the agar slant to a test tube containing 5 mL of Nutrient Broth for bacteria and YPD Broth for the yeast. The cultures were then incubated overnight to the logarithmic phase of growth at 37 °C for bacteria (8–10 h) and 30 °C for the yeast (12–16 h) under mild agitation (150 rpm in a rotary shaker). Culture purity was examined by streaking each culture on plates of Nutrient Agar for bacteria and YPD Agar for yeast [2]. The inoculums were prepared from overnight broth cultures and suspensions were adjusted to the required microbial concentration at 600 nm using the UV–visible spectrophotometer [29]. As a guideline, at an OD 600 nm of 1.0, cultures of strains contain approximately 10^5–10^6 CFU/mL. The cultures were then diluted 100-fold using fresh medium severally to determine the minimal inhibitory concentration (MIC).

MIC is cited by the most researchers as a measure of the antimicrobial performance of antimicrobial agents. MIC was determined by a broth dilution method with some modifications as described by Weerakkody, Caffin, Turner and Dyke. After adding 300 μL OEO Pickering emulsions to the first tube containing 6 mL of broth, 3 mL of the mixed medium from the first tube were then transferred to the second one, and this procedure was continued so as to make successive 2-fold dilutions up to the last. Therefore the final concentrations of each treatment solution from the first tube to the last were 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78 and 0.39 μL/mL, respectively. A 100 μL suspension of tested microorganisms were added to each tube. A negative control tube contained broth and microorganism. Meanwhile, a positive control tube contained 50 μg/mL of kanamycin sulfate in broth and microorganism. MIC was defined as the concentration in the lowest serial dilution of OEO Pickering emulsions which resulted in the lack of visible microorganism growth in tubes after 24 h (bacteria) and 48 h (yeast) [29].

In order to examine the mechanism of OEO Pickering emulsions against cell membranes, SEM studies were carried out as previously reported with some modifications [30]. Logarithmic growth phase cells of 4 tested microorganisms (each approximately 10^6 CFU/ml) were treated with each MIC value of OEO Pickering emulsions. Then, samples were incubated at room temperature for 3 h. After incubation, cells were harvested by centrifugation for 15 min at 5000 r/min, and washed three times with 0.1 M phosphate buffer solution (PBS, pH 7.0). Then they were resuspended in PBS containing 2.5% glutaraldehyde and kept for 4 h at 4 °C to fix the cells. After centrifuging, the cells were further dehydrated in water-ethanol solutions at various ethanol concentrations (30%, 50%, 70%, 90% and 100%) for 15 min each. After that the ethanol was replaced twice by Isoamyl acetate. Finally, the samples were lyophilized and fixed on SEM support, and then sputter-coated