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Synthesis of peppermint oil-loaded chitosan/alginate polyelectrolyte complexes and study of their antibacterial activity

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ABSTRACT

Peppermint oil contains menthol, menthone and menthofuran as major components. It finds extensive applications in food, flavoring and pharmaceutical industries. However, it gets readily oxidised when exposed to air or ultraviolet light and is highly volatile in nature. So, to mitigate these shortcomings microencapsulation of peppermint oil is often used. In the present case a chitosan/alginate polyelectrolyte complex prepared by complex coacervation method was chosen as the encapsulating material for peppermint oil. The formation of polyelectrolyte complex was found to be dependent on pH, ratio between chitosan and alginate and amount of crosslinker, glutaraldehyde. The prepared complexes were characterized by fourier transform infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA) and scanning electron microscopy (SEM). The swelling and release studies were carried out in buffer solutions of pH 4, 7, 9 and phosphate buffered saline system (PBS, pH = 7.4). The complexes were tested for antimicrobial activity against *Proteus mirabilis*, *Enterobacter aerogenes*, *Bacillus subtilis* and *Staphylococcus aureus*. The highest zone of inhibition (25 ± 0.5 mm) was observed against *Bacillus subtilis*.

1. Introduction

Peppermint (*Mentha piperita* L.) is a herbaceous rhizomatous perennial plant and has long been known for its medicinal value. Peppermint oil (*Menthae piperitae aetheroleum*) is obtained from the fresh leaves of peppermint. The primary constituent L-menthol is the cause for its aroma. It finds extensive applications in pharmaceutical formulations, food products and cosmetics. It is applied to soothe headache, treat muscle pain, nerve pain, toothache, mouth inflammation, joint inflammation, itchiness, allergic rash and also acts as a mosquito repellent. It is also known to be effective against abdominal pain, irritable bowel syndrome (IBS), nausea, heartburn, cold and cough. Peppermint oil is a volatile liquid and as such it is susceptible to evaporation. Thus for prolonged shelf life microencapsulation technique has been used by several researchers [1–4].

Microencapsulation technique provides a means for protection from environmental agents like heat, air and cold and helps in storage and controlled release of fertilizers, vitamins, enzymes, food and flavor, pesticides etc. [5–8]. The encapsulated particles can be prepared by different ways viz. spray drying, phase separation and simple or complex coacervation [4,9]. Microcapsules produced by complex coacervation method are generally insoluble in water. They are also resistant to heat. Complex coacervation is a method of mixing oppositely charged polyelectrolytes in aqueous media and resulting in phase separated entities which are variously termed as complex coacervates, coacervates or polyelectrolyte complex. This phenomenon depends on various factors viz., pH of the medium, concentration of the polyelectrolytes, temperature etc. [10].

Natural polymers [11,12] are the materials of choice in the preparation of polymeric encapsulating systems for their natural abundance, low cost, eco-friendly and biodegradable nature.

In the present study, chitosan/alginate complex is used as the encapsulating agent for peppermint oil. Chitosan is a linear, natural, cationic polysaccharide composed mainly of β-(1–4)-linked D-glucosamine (the deacetylated unit) and *N*-acetyl-D-glucosamine (the acetylated unit). These units are randomly distributed in the

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molecule. It is non-toxic, biodegradable and biocompatible and as such finds applications in many delivery systems for bioactive molecules [5,11,12]. Sodium alginate is an anionic polysaccharide. It is a linear co-polymer composed of homopolymeric blocks of (1–4)-linked β-D-mannuronate and its C-5 epimer α-L-guluronate residues respectively. The complex coacervate was synthesized by reaction between chitosan and sodium alginate. The polyelectrolyte complex was formed due to electrostatic interaction between the negatively charged carboxylic acid groups of mannuronic and guluronic acid units in alginate and the positively charged amino groups of chitosan [13]. On formation of complex with alginate, chitosan still retains its characteristic property of biodegradability and biocompatibility but becomes mechanically stronger at lower pH which makes it an excellent wrapping material for the peppermint oil. The reaction parameters like concentration of polymer, pH of the medium and amount of cross-linking agent were optimized to get the maximum yield of the coacervate. The prepared coacervates were characterized by Fourier transform infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA) and scanning electron microscopy (SEM). The swelling and release studies were carried out in pH 4, 7, 7.4 (phosphate buffered saline system, PBS) and 9. The results confirmed the successful encapsulation of the peppermint oil in chitosan/alginate coacervate complex. The antimicrobial activity of the oil loaded coacervate was determined against four different bacteria: two gram negative viz., *P. mirabilis* and *E. aerogenes* and two gram positive viz. *B. subtilis* and *S. aureus*.

2. Experimental

2.1. Materials

Low molecular weight chitosan (degree of deacetylation, DD>75%, Sigma-Aldrich Inc., USA), sodium alginate (Himedia Laboratories, India), glutaraldehyde (50% solution in water, E. Merck India) and peppermint oil (Sigma-Aldrich Inc., USA) were purchased and used as received. *Proteus mirabilis* (MTCC No. 743), *Enterobacter aerogenes* (MTCC No. 111), *Bacillus subtilis* (MTCC No. 736) and *Staphylococcus aureus* (MTCC No. 96) were collected from the Defence Research Laboratory (DRL), Tezpur, India. Double-distilled water was used in all the experiments.

2.2. Methods

2.2.1. Optimization of reaction conditions for the formation of complex coacervates

2.2.1.1. Variation in the pH of the reaction medium. The formation of coacervates was studied as a function of pH. Nine different buffer solutions of pH (pH = 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0 and 5.2) were used to determine the pH at which polyelectrolyte complex formation became maximum. The buffer solutions were prepared by mixing 0.1 N acetic acid and 0.1 N sodium acetate in definite proportions. A known amount of chitosan solution of specific pH (250 mL) of concentration 0.3% (w/v) was stirred (600 rpm) at 30 °C until complete dissolution and then sodium alginate solution of same pH (250 mL) of concentration 0.3% (w/v) was added dropwise to the chitosan solution. The phase separation at different pH resulted in different weights of the coacervates which were used to determine the suitable pH at which maximum coacervate formation took place. The reactions were carried at room temperature.

2.2.1.2. Variation in the ratio of chitosan to alginate. Maintaining the optimum pH (3.6) determined from the previous experiment, the ratio of chitosan and alginate was varied. For this a series of experiments were carried out. 0.3% (w/v) of alginate and 0.3% (w/v) chitosan solutions were separately prepared in 250 mL acetate buffer (pH = 3.6) and mixed at 30 °C in the following volume ratios of alginate: chitosan (5:40, 10:35, 15:30, 20: 25, 25:20, 30:15, 35:10, 40:5). A total volume of 45 mL of the reaction mixture was maintained. The optimum ratio of chitosan to alginate for formation of maximum coacervate was determined by measuring the viscosity, turbidity, conductivity and UV absorbance of the supernatant liquid. Viscosity of the supernatant was measured by using Ostwald's viscometer at room temperature whereas the turbidity measurements were carried out in Nephelo Turbidity Meter 131(Systronics) in which the reference solution is a mixture of hexamethylenetetramine and hydrazinium sulphate. Conductivity measurements were carried out by microprocessor based conductivity/TDS meter (Model-1601) and UV absorbance was measured using UV-1800 (SHIMADZU) in the range 200–400 nm.

2.2.1.3. Preparation of peppermint oil loaded chitosan/alginate complex coacervates. A known amount of sodium alginate solution (250 mL) of concentration 0.3% (w/v) was stirred (600 rpm) at room temperature until complete dissolution. To this solution, peppermint oil (0.5–1.5 mL) was added and the reaction mixture was stirred for 15 min. A known amount of chitosan solution (200 mL) of concentration 0.3% (w/v) was stirred (600 rpm) at room temperature until complete dissolution. The peppermint oil containing sodium alginate solution was then added dropwise to the chitosan solution, when formation of coacervate complex took place. The reaction was carried out at pH = 3.6 while the volume ratio of alginate to chitosan was maintained at 5:4. The temperature of the reaction mixture was then increased to 45 °C and stirred (600 rpm) at this temperature for 1.5 h. At the end of 1.5 h, the temperature was lowered to 10–15 °C and glutaraldehyde (1.0–3.0 mL) was added dropwise. The temperature was then increased again to 45 °C and stirring continued for another 1.5 h. The crosslinked particles obtained were filtered, washed and then freeze dried. The flow chart for the preparation of peppermint oil loaded microparticles is shown in Fig. 1.

2.2.1.4. Water uptake study. The swelling behavior of sodium alginate/chitosan coacervates was studied at pH = 4, 7, and 9 and 7.4 (phosphate buffered saline system). The pre-weighed coacervates were immersed in solution of pH = 4, 7, 7.4 and 9 for 72 h. The swollen coacervates were weighed after the removal of excess buffer solution by drying with blotting paper. The swelling behavior was determined by measuring the change in the weight of the coacervates. The equilibrium swelling index was calculated from the following formula [14].

$$Swelling\ Index = \left[\frac{W_g - W_o}{W_o}\right] \times 100\%$$

where, W_g = weight of the coacervate after swelling and W_o = weight of the coacervate before swelling.

2.2.1.5. Preparation of calibration curve for peppermint oil. To determine the quantity of peppermint oil present in the coacervates, a standard calibration curve for peppermint oil was prepared. For that, known quantities of peppermint oil were dissolved in ethanol to prepare a series of solution and were scanned in the range of 200–450 nm by using [Shimadzu (Japan) 1800] UV–Vis spectrophotometer. The absorption