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Microencapsulation of norfloxacin in chitosan/chitosan oligosaccharides and its application in shrimp culture

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ABSTRACT

Norfloxacin chitosan/chitosan oligosaccharide microcapsules (NCCM) were prepared by emulsion-chemical crosslinking method. The characteristics of obtained microcapsules were evaluated by scanning electron microscopy, Fourier transform infrared spectroscopy and release experiments. Cumulative release profile of norfloxacin from the chitosan microcapsules in natural seawater was measured and the controlled release of drugs was at a uniform rate in 48 h. The chitosan microcapsules were applied onto the antibacterial study of the shrimp culture in natural seawater. It is observed that the seawater in the NCCM added groups was relatively clear and the biomass of *Vibrio* increased slowly in contrast to the control and norfloxacin groups. The inhibition rate of *Vibrio* in norfloxacin groups obvioursly decreased after the 5th day, whereas, it remained high and stable during experiment period in NCCM groups. The results showed that the chitosan microcapsules as release materials have excellent antibacterial effects on *Vibrio* in the farming of *Penaeus vannamei* Boone. The controlled release could obviously reduce dosage of antibiotics and delivery times, and effectively improve the utilization rate of norfloxacin drugs for shrimps

1. Introduction

Chitosan is one of the most important partially deacetylated derivatives obtained by chitin from crab and shrimp shells, which is the second abundant polysaccharide next to cellulose in nature [1]. As a natural polyaminosaccharide with advantages of nontoxicity, biocompatibility and biodegradation, chitosan is widely used as a pharmaceutical excipient in oral drug formulations in order to improve the dissolution of poorly soluble drugs or for the sustained release of drugs [2,3]. Chitosan possesses primary amino groups in its structure and the number of these amino groups is related to the rate of antimicrobial activity. Many reports showed that chitosan as an attractive biomaterial in terms of biomedical applications, has a certain antibacterial strength and antibacterial spectrum [4,5].

Norfloxacin, (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolone-carboxylic acid, NFX), which has potent activity against Gram-positive and Gram-negative bacteria, and limits activity against anaerobes, is a synthetic, broad- spectrum fluoroquinolones antibacterial agent and extensively used in shirmp farming to treat bacterial infections [6]. Vibriosis is a common bacterial disease which can cause high morbidity and mortality in farmed shrimp [7]. In most of the commercial shrimp farms, shrimp farmers control vibriosis by water management and feeding the feedstuff which mix with antibiotics such as NFX during initial period [8]. However, there are some disadvantages to limit widespread usage of NFX, for example, low bioavailability and requests frequent dosage and long-term administration [9]. Frequent dosing is not only tedious but also poses many side effects [10,11]. The intensive and extensive use of antibiotics is widely criticized and has led into their accumulation and contamination in the water environment [12,13]. Therefore, a new administration system for dosage of antibiotics and controlled release is necessary for use of NFX in shrimp aquaculture.

Microencapsulation was early studied in 1929 to prepare gelatin sphere by coacervation. This technology is used for stabilization of particles and protection and/or isolation of active core material from surroundings which allows materials to be handled more easily for application[14,15]. Thus, the advantages of microencapsulation are described as the controlled release of encapsulated bioactive materials, protection of encapsulated materials from oxidation, and imparting stability to environmental stress [16]. Furthermore, microencapsulation provides an effective and long lasting method for the release of antifungal drugs, and has drawn a great amount of attention due to its potential applications in the

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fields of medicine, biomedicine and environmental engineering, *etc* [17–19]. In recent years, chitosan microcapsules have been developed for site specific drug release such as antibacterial, antitumor and anticancer [20–22]. Up to now, there is no report on the application of chitosan microcapsules as release materials in marine aquaculture.

In this study, chitosan was used as the shell material to encapsulate NFX and the norfloxacin chitosan/chitosan oligosaccharide microcapsules were prepared by emulsion-chemical crosslinking method using glutaraldehyde as crosslinker. The influences of feed radio and the amount of crosslinker were investigated and the microcapsules were characterized by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). The drug-loaded microcapsules would continuously release NFX in natural seawater and were applied onto the antibacterial study in the shrimp farming.

To the best of our knowledge, this work represents the first attempt of using chitosan microcapsules loaded NFX as release materials in the aquaculture system. In recent years, the use of antibiotics as feed additives to treat diseases has increased with the development of shrimp culture. Antibiotic residues have been found in sediment and wild organisms in aquaculture areas and may affect coastal or marine ecosystems and human health. However, the potential use of antibiotics in aquaculture industries was paid minimum consideration in regard to human health. As the shrimp is being consumed by large amount of consumers they may serve as carrier for antibiotics to the next consumers in the food chain. Hence measures to limit the use of antibiotics by decreasing the dose is of significant importance.

2. Materials and methods

2.1. Materials

Norfloxacin (NFX) and glutaraldehyde (50%, water solution) were purchased from Aladdin Reagent Co., Ltd. Chitosan (CS, average molecular weight 120 kDa, acetylation degree 97%) was purchased from Qindao Hecreat Bio-tech Co., Ltd. China. Chitosan oligosaccharide (CSO, average molecular weight 1500 Da, acetylation degree 90%) was obtained from Zhaoqing Changlong Biotechnology Co., Ltd. All chemicals and reagents were of analytical grade. Acetonitrile was all of HPLC grade and from Merck. All water used was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). A 200 mg L^{-1} NFX stock standard solution was prepared in 0.1 M hydrochloric acid solution and placed in the dark at 4 °C. Working solution was prepared immediately before experiment by appropriate dilution.

2.2. HPLC analysis

This was performed on a Hitachi L-2000 series HPLC system containing an L-2130 binary pump, an L-2200 autosampler, an L-2300 column compartment and an L-2455 diodearray detector. Chromatographic separation was carried out on an Agela C18 column (250 mm × 4.6-mm-*i.d.*, 5 μm) purchased from Hitachi (Japan). The column thermostat was set at room temperature. The mobile phase was 0.025 M phosphate solution (pH adjusted to 3.00 by triethylamine) and acetonitrile (87:13, v/v). The flow rate was 1.0 mL min^{-1} and the detector was set to 278 nm.

2.3. Preparation of NCCM

The NCCM were prepared by emulsion-chemical crosslinking method. Briefly, CS and CSO were mixed and dissolved in 8 mL of 1% (v/v) acetic acid solution. A certain amount of NFX was dissolved in 2 mL of 0.1 M hydrochloric acid solution and then the NFX solution was added to chitosan/chitosan oligosaccharide solution. Afterwards, the mixture was dispersed into 100 mL of liquid paraffin solution containing surfactant span-80 (2%, w/v) in the water bath heating at 50 °C and agitated by mechanical stirring at speed of 1000 rpm for 1 h. Subsequently, glutaraldehyde water solution was dropped into the mixture slowly, and the system was kept stirring at 800 rpm for 3 h. After centrifugal separation, the microcapsules were washed with petroleum ether and isopropyl alcohol successively. The NCCM product were harvested and dried under vacuum overnight until constant weight. The influences of feed radio and the amount of crosslinker were investigated in the preparation process.

Blank chitosan/chitosan oligosaccharide microcapsules (CCM) were prepared and washed using the same manner, but without the addition of the NFX.

2.4. Morphology observation and FTIR spectra

The external morphology of microcapsules was analyzed by a Hitachi S-4800 cold field emission scanning electron microscope (Tokyo, Japan). All samples were prepared by wetting the slide glass with a small drop of diluted particle dispersion. Before scanning electron microscopy experiments, the dried specimen was coated under vacuum with a thin layer of gold. FTIR spectra were recorded in powder form in KBr discs in the range of 4000–500 cm^{-1} on a Tensork-27 spectrophotometer from Bruker Ltd., Germany.

2.5. Determination of drug loading content and encapsulation efficiency

The NCCM were dispersed in 0.1 M hydrochloric acid solution and heated in water bath for 2 h until drug was fully released from microcapsules. Afterwards, the microcapsules were separated by centrifugation and NFX in the filtrate was detected by HPLC method at 278 nm. The calculation of drug loading content (DLC) and encapsulation efficiency (EE) were defined according to the following Eqs.

$$\text{DLC}\ (\%) = \frac{\textit{The mass of NFX encapsulated in the microcapsules}}{\textit{The mass of the total microcapsules}} \times 100 \quad (1)$$

$$\text{EE}\ (\%) = \frac{\textit{The mass of NFX encapsulated in the microcapsules}}{\textit{The mass of NFX used in the process}} \times 100 \quad (2)$$

2.6. Drug release studies

The drug release study was performed in natural seawater. A certain amounts of NCCM were added to 100 mL natural seawater. 5 mL of medium was collected at appropriate time intervals and the content of drug released from the NCCM in the medium was determined by HPLC. The fresh natural seawater with the same volume of 5 mL was then added into the medium to maintain the total volume.

2.7. Shrimp experiment and antibacterial studies

Penaeus vannamei Boone was purchased from hatchery of Tianheng, Qingdao. The shrimps were acclimatized to standard conditions at 28(±2) °C and constant aeration. After the initially biometric analysis (10.1 ± 1.1 cm), shrimps were divided into three groups and randomly stocked at a density of 10 shrimps in each