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Ciprofloxacin chitosan conjugate: combined antibacterial effect and low toxicity

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In this study, we used crab shell chitosan (Bioprogress, Russia) with a viscosity-average molecular weight (MW) of 3.5×10^4 and a degree of acetylation of 26%, viscosity-average molecular weight (MW) of 7.1×10^4 and a degree of acetylation of 28% and viscosity-average molecular weight (MW) of 17.2×10^4 and a degree of acetylation of 24%. Ciprofloxacin, DCC (*N,N′*-dicyclohexylcarbodiimide), NHS (*N*-hydroxysuccinimide) were purchased from Sigma Aldrich, USA. Other chemicals and solvents were obtained from commercial sources and used as received without further purification.

The ^{1}H NMR spectra were recorded on a Bruker spectrometer (Germany) operating at a frequency of 400 MHz using D_2O/CF_3COOH 50/1 (v/v) as a solvent.

Ultrasonic treatments were carried out in an ultrasonic bath (USB300X, ITA) equipped by temperature control device. The ultrasonic bath can work at frequencies of 22 kHz, 30 kHz, 45 kHz, 70 kHz, 80 kHz, 100 kHz, 250 kHz, or 300 kHz with a variable power output from 120 to 300 W. The ultrasonic energy was delivered from the bottom of the bath to water by six coupled transducers. For each experimental run, the reaction mixture was loaded into a tube, and then placed in the water bath and fixed at the same position during the ultrasound treatment.

Synthesis of ciprofloxacin-chitosan conjugates

Under ultrasound-free conditions, chitosan (0.5 g) was dissolved in 1% acetic acid (20 ml), the pH of the solution was adjusted to 3; then ciprofloxacin, DCC and NHS (1.5, 3.5 or 6

equiv. each) were added, and the reaction mixtures were stirred at 50 °C for 5 hours. The formed polymers were precipitated by addition of acetone (25 ml). The precipitated polymers were dissolved in water and dialyzed against distilled water and freeze-dried.

The synthesis of the mentioned above chitosan derivatives under ultrasonic conditions differs from that under ultrasound-free conditions in that 0.5, 2.0 or 3.5 equiv. of ciprofloxacin, EDC and NHS were added, and the reaction mixtures were treated with ultrasonic irradiation at 100 kHz, 280 W for 20 min at 50 °C.

Antibacterial activity

The *in vitro* antibacterial activity of the chitosan-based antibacterial systems was evaluated by the agar well diffusion method [S1-S3]. The antibacterial effect was studied against *Staphylococcus aureus* (RCMB 010027) and *Escherichia coli* (RCMB 010051). The activity was determined by measuring the diameter of the inhibition zone (in mm). Each inhibition zone was measured three times by a caliper to get an average value. Ampicillin and gentamicin were used as reference antibacterial drugs [S4].

Toxicity study

The *in vitro* toxicity was estimated by the MTT test. Solutions or nanosuspensions of the tested samples were prepared by serial dilution in alpha-MEM culture medium. A 0.1 mL volume of each of nanosuspension was added to a confluent monolayer of cells cultured in a 96-well plate. Cells were incubated for 24 h at 37 °C in an atmosphere containing 5% CO_2. The cells were washed twice with PBS and then 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, 0.1 ml, 0.5 μg ml^{-1}) in PBS was added, and the mixture was incubated for 4 h. The supernatant was then replaced with 96% ethanol (0.1 ml), and the absorbance was measured at 535 nm.

Statistical analysis

The statistical significance of differences between the samples was determined by a one-way analysis of variance (ANOVA) using JMP 5.0.1 software (SAS Campus Drive, Cary, NC). Mean values, where appropriate, were compared by applying the Student's *t*-test at a significance level $p < 0.05$.

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