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# Antibacterial activity of chitosan grafting nisin: Preparation and characterization

Xiaoming Zhu [a,c], Huan Wu [b], Jing Yang [b], Jun Tong [b], Jiayan Yi [b], Zhihai Hu [b], Jin Hu [b], Tan Wang [b], Lihong Fan [a,b,*]

[a] Hubei Collaboration Innovation Center of Non-power Nuclear Technology, Hubei University of Science and Technology, Xianning 437100, China
[b] College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, China
[c] School of Nuclear Technology & Chemistry and Biology, Hubei University of Science and Technology, Xianning 437100, China

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ABSTRACT

Nisin grafted chitosan was prepared by using microbial transglutaminase as biocatalyst. The transglutaminase-catalyzed reaction displayed high efficiency, high selectivity, mild reaction condition and environmental friendliness. The results revealed that the degree of substitution (DS) of nisin–chitosan could be controlled by adjusting the reaction time, the reaction temperature and the molar ratio of nisin to chitosan. And nisin–chitosan in different pH showed excellent solubility. In addition, *in vitro* antibacterial activity assessment, nisin–chitosan with the concentration of 0.008 mg/mL showed pronounced inhibitory effect against Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*) and Gram-negative bacteria (*Escherichia coli*). Furthermore, L929 mouse fibroblasts were cultured with nisin–chitosan, and the methylthiazol tetrazolium (MTT) assay showed that nisin–chitosan with the concentration from 0.005 to 0.01 mg/mL displayed low toxicity. The results may contribute to finding the application of nisin–chitosan in pharmaceutical and food industry fields.



## 1. Introduction

Over the last few years, considerable research has been conducted to develop wound dressings with an antibacterial property. Much research has been done concerning the application of natural plant polysaccharides because of their unique properties [1,2]. Chitosan is an antibacterial biopolymer derived from partial or total deacetylation of chitin [3]. It has been well known for its broad antibacterial activity, high biodegradability, and high compatibility with other incorporated compounds, due to the presence of high densities of amino and hydroxyl groups in the chitosan polymer structure [4]. And these two functional groups offer multiple possibilities for grafting desirable bioactive compounds [5]. In addition, chitosan can effectively fix certain enzymes such as lysozyme, nisin, lactoperoxidase system and organic or inorganic acids [6]. It has been evaluated for numerous applications in the field of medical, food, agricultural and chemical industries [7]. As the additive to wound dressings, chitosan attracts more and more attention. However, the application of chitosan is limited because of its poor solubility over pH 6.5. Hence, it is necessary to modify chitosan to improve its water solubility and antibacterial property.

The traditional wound dressings reduce the infections of the bacteria by adding the antibacterial agents, such as iodine, sliver ion and antibiotics. However, the frequent use may cause the argyria and bacterial resistance. Antibacterial peptides, which are induced by insects, are the kind of alkaline polypeptide. Most of them show a broad spectrum of antibacterial activity and rarely promote the rise of bacterial resistance. So the antibacterial peptides are considered to have broad application prospects in the pharmaceutical industry [5]. Hence, some researchers have put some effort into developing wound dressings with antibacterial peptides.

Nisin is one of the most studied bacteriocin produced from certain strains of *Lactococcus lactis* which is generally recognized as safe and is permitted for use in over 50 countries [8,9]. Structurally, it is a 34 amino acid polypeptide with a molecular weight of 3500 Da. Its molecular structure includes unusual amino acids and thioether ring which are responsible for the important functional properties, i.e. acid tolerance, thermo stability at low pH and a specific bactericidal activity [9–11]. It exhibits antibacterial activity against a broad spectrum of Gram-positive bacteria, including some pathogenic bacteria, such as *Clostridium botulinum*

* Corresponding author at: College of Chemical Engineering, Wuhan University of Technology, Wuhan 430070, China. Tel./fax: +86 27 87859019.
*E-mail address:* lihongfan2000@hotmail.com (L. Fan).

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and *Bacillus cereus*. However, it is ineffective against Gram-negative bacteria, yeasts, or molds [12,13]. Some studies suggest that nisin interferes successfully with cell-wall functions and the synthesis of cell-wall components in Gram-positive organisms, but it is ineffective against outer cell membranes of Gram-negative organisms. Nisin reportedly may lose activity above pH 4 and below 20 °C. And the efficiency of nisin depends on several parameters: pH, temperature and salt. These parameters play a major role on the solubility, bioactivity and stability of nisin. So it is necessary to modify nisin [14]. Cai et al. [10] prepared a complex of chitosan and nisin which was formed mainly by electrostatic interaction. The complexes with different concentrations and ratios showed high antibacterial activity against all tested bacteria and fungi. The introduction of nisin to chitosan may not only improve the antibacterial activity, but also enhance the stability and solubility of nisin [15,16].

However, the traditional physically blending materials are easily settled and stratified. The instability makes the materials difficult to show their characteristics and limits their application as well. And also the chemical crosslinking agents frequently induce toxic side effects or secondary reaction with unwanted products [17]. Hence, the enzyme-catalyzed reactions which are efficient, highly selective and environmentally friendly may attract more and more researchers [18,19]. Microbial transglutaminase (MTGase) as biocatalyst can catalyze an acyl-transfer reaction between the γ-carboxamide group of peptide-bound glutamine residues which are as acyl donors and a variety of primary amines which are as acyl acceptors [20,21]. It is calcium-independent and has a relatively low molecular weight [22,23].

In this study, MTGase was thought to catalyze the reaction between chitosan and nisin. In the process, chitosan was as the acyl acceptor and nisin was as the acyl donor. This study had the objective to develop and evaluate the antibacterial effect of chitosan grafted with nisin. It was also done to improve the solubility of chitosan and enhance the stability of nisin.

## 2. Materials and methods

### 2.1. Materials

Chitosan (Mw 520,000) with a 92% degree of deacetylation was supplied by the Golden-Bell (Cochin, India). Nisin (Mw 3500) was purchased from Huashun biological technology Co. Ltd. (Wuhan, China), without further purification. Microbial transglutaminase (MTGase) from *Streptoverticillium mobaraensis* was purchased from Huashun Biological Technology Co. Ltd. (Wuhan, China). Dubecco's Modified Eagle Medium (DMEM) was purchased from Thermo Co. Ltd. (Beijing, China). Fetal bovine serum (FBS) was purchased from Sijiqing Co. Ltd. (Zhejiang, China). Methylthiazol tetrazolium (MTT) was purchased from Sigma (Deisenhofen, Germany). Acetic acid, sodium hydroxide and other reagents used in this investigation were of analytical grade and without further purification. They were purchased from Sinopharm Group Chemical Reagent Corp.

### 2.2. Purification of MTGase

MTGase was first poured into 0.2 mol/L $Na_2HPO_4/NaH_2PO_4$ buffer solution (PBS, pH 5.0). The MTGase suspensions had to be centrifuged at the speed of 3000 RPM for 10 min. Then the supernatant was purified by dialysis through the 8000–10,000 molecular weight cut-off dialysis tubing in distilled water for 3 days. The dialyzed MTGase was lyophilized for 24 h to obtain the MTGase lyophilized power and stored at 4 °C.

### 2.3. Purification of nisin

Nisin was first poured into 0.1 mol/L acetic acid. The turbid liquid was centrifuged at the speed of 3000 RPM for 10 min. The supernatant was further purified by suction filtration for three times. The solution was purified by dialysis in distilled water through the 3000 molecular weight cut-off dialysis tubing for three days. Then the nisin was lyophilized for 24 h to obtain the nisin lyophilized power and stored at 4 °C.

### 2.4. Preparation of nisin–chitosan

In a typical reaction procedure, chitosan (1.0 g) was dissolved in 50 mL acetic acid and the solution was adjusted to pH 4.0. Nisin (0.1 g) was also dissolved in 50 mL acetic acid and the solution was adjusted to pH 4.0. And they were mixed together to form homogeneous solution. Thereafter MTGase power (0.15 g) was directly added into the solution to catalyze the reaction between chitosan and nisin. Magnetic stirring was continuous for 1.5 h at 30 °C. Then pH of the reaction solution was adjusted to 10.0 by sodium hydroxide to lead the enzyme to lose activity and purified by suction filtration. Subsequently, the transparent solution was further purified by dialysis in distilled water through the 8000–10,000 molecular weight cut-off dialysis tubing until the pH of the solution was neutral. The dialyzed product was finally freeze-dried to obtain the purified nisin–chitosan. The dried products were stored in vacuum desiccators over $P_2O_5$. Scheme 1 illustrates chitosan modified with nisin by using MTGase as catalyst.

### 2.5. One-factor-at-a-time experiment

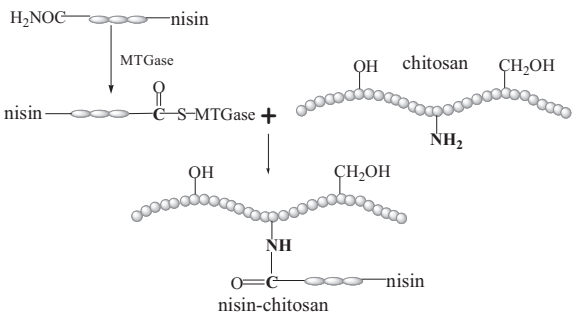
Four independent variables were investigated, including the reaction time (0.5, 1.0, 1.5, 2.0, 2.5 h), the reaction temperature (20, 30, 40, 50, 60 °C), the molar ratio of nisin to chitosan (0.4/200, 0.6/200, 0.8/200, 1.0/200, 1.2/200). Their variables were fixed at a certain value, while changing only one variable.

### 2.6. Fourier transforming infrared spectroscopy (FT-IR) analysis

FT-IR spectra of chitosan and nisin–chitosan samples were performed with a Nicolet 5700 Fourier transform infrared spectrometer (USA) in the wavenumber ranging from 400 to 4000 $cm^{-1}$. The samples were prepared by the KBr-disk method.

### 2.7. Measurement of the degree of substitution

The degree of substitution (DS) is defined as the number of amine groups substituted per repeating structural unit of the chitosan. In this work, the DS was measured according to the method of Fan et al. [24]. The concentration of nisin between 0.001 g/L and 0.05 g/L was linear relation with absorbance at 200 nm by UV




**Scheme 1.** Synthesis of chitosan modified with nisin by using MTGase as catalyst.