FORMATION AND CHARACTERIZATION OF SUCCINOYL CHITOSAN PARTICLES LOADED WITH WARNERIN

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Abstract

This study aims to obtain nanoparticles based on succinoyl chitosan loaded with Warnerin (War-SCNPs), low molecular weight cationic peptide. The nanoparticles of succinoyl chitosan (SCNPs) were prepared by salt coacervation method, and Warnerin loading efficiency on SCNPs was reached 75% in the optimum conditions, particularly, ration (SCNPs : peptide) was equal to (1.75 : 1, μg / ml). Formed War-SCNPs were stable, had a weak electric charge from (-4.4) to (-14,6) mV. Determining of SCNPs size showed that a main fraction of SCNPs had the size of 160 nm, and after the peptide sorption War-SCNPs size increased up to 330 nm. The experimental data of this study will likely impact War-SCNPs use as therapeutic delivery systems of the peptide to be administered parenteral.

Key words: *succinoyl chitosan, chitosan nanoparticles, warnerin, coacervation method.*

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1. Introduction

For the past years the delivery of biologically active compounds using biodegradable carriers is attracting a great deal of interest [1-3]. In particular, protein molecules such as biologically active peptides, therapeutic proteins, are generally show excellent results against several diseases. Encapsulation or conjugation of peptides and proteins with biopolymeric nanocarriers is frequently used to save and prolong their therapeutic activity, and these are also the very promising drug delivery systems *in vivo* [4].

A wide range of biopolymers are currently used as a matrix for the active compounds, and chitosan occupies a special place among all materials. Chitosan is the unique biopolymer thanks to wide variety of its properties, particularly, biodegradability, biocompatibility and low toxicity [5,6]. It is known that a delivery system based on chitosan should be able to enhance adsorption, to improve penetration of proteins / peptides across the cell membrane and to provide a sustained release of biologically active substances [7].

The main disadvantage of chitosan is its low solubility in water at neutral physiological pH in the range of 6.5-7.5. Therefore, to improve chitosan solubility, this biopolymer is subjected to depolymerization or modification by introducing additional ionic groups. For example, chitosan is acylated by succinic anhydride to provide N-succinoyl derivatives of chitosan with different degree of substitution depending on the ratio of reactants. Solubility of the obtained derivatives increases by several times at pH values more than 6.5.

Designing of particles on the basis of succinoyl derivatives can be performed in several ways. First, N-succinoyl chitosan particles could be formed as a result of self-assembly, beginning from the substitution degree of 30-40%, and due to the presence of sufficient amount of the protonated amino groups in the structure. Second, the additional crosslinking by a polyanion ─ tripolyphosphate (TPP) is possible for the aim of obtaining of a more stable system [8]. Finally, for the formation of particles based on succinoyl chitosan with a high substitution degree (of 60-80%) it can be used a coacervation method, in which a solution of strong electrolyte shall be used as the precipitating agent.

Succinyl residue in the molecule of succinoyl chitosan could be considered as a low molecular weight linker. The use of a linker in drug delivery systems based on biopolymers is governed by two reasons. First one is associated with the lack of necessary functional groups in a polymer chain or in a drug for their combination with each other. The second reason is related to the need of specific binding (amide, ether, etc.) between the carrier and the drug, which will be gradually broken down in the body.

The aim of this study is to design a Warnerin delivery system using N-succinoyl chitosan (SC).

2. Materials and methods

2.1. Materials

2.1.1. N-succinoyl chitosan

In this study to prepare the particles, the N-succinoyl chitosan (ZAO «Bioprogress», Moscow region, Russian Federation) from the crab chitosan was used. There were the following characteristics of chitosan: a molecular weight (MW) of 300 kDa and a deacetylation degree (DD) of 85%. The degree of substitution (SD) in N-succinoyl chitosan was determined by spectral method - proton magnetic resonance (PMR) and comprised 70% (Fig. 1).

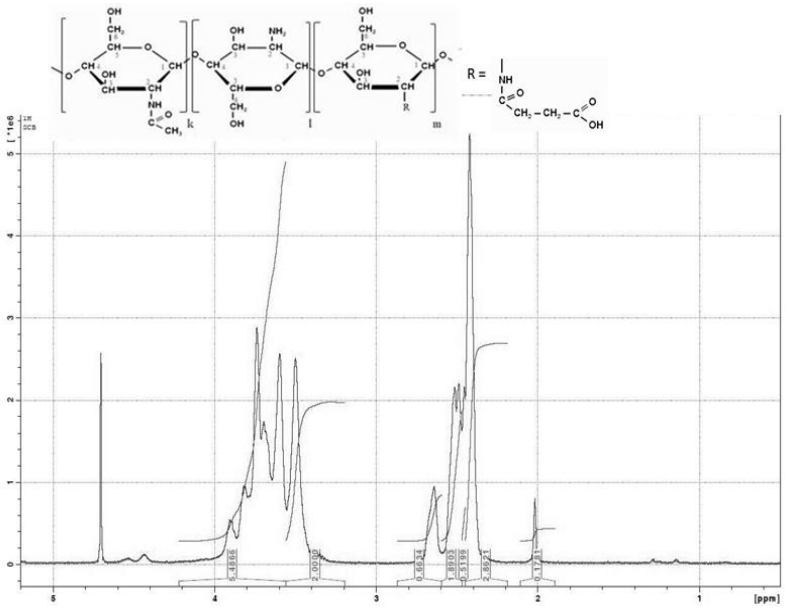

Figure 1. ^{1}H-NMR spectrum of N-succinoyl chitosan.

2.1.2 Warnerin

A low molecular weight cationic peptide Warnerin (MW 2999 Da) has been used in this study as peptide loaded on the particles obtained. It was isolated from the cultivation medium of *Staphylococcus warneri* IEGM KL-1. The peptide consists of a significant amount of cationic and hydrophobic amino acid residues and 3 residues of unique amino acid lanthionine. Lanthionine residues provide the peptide molecule significant rigidity. An important characteristic of Warnerin is pronounced bactericidal activity against Gram-positive bacteria at a wide pH range and the preservation of biological properties after sterilization procedures [9].

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2.2. Methods

2.2.1. Preparation of N-succinoyl chitosan nanoparticles (SCNPs) by coacervation methods

Nanoparticles based on succinoyl chitosan were prepared by the method as reported elsewhere [10]. To obtain nanoparticles from N-succinoyl chitosan by the salt coacervation method, 0.12% solution of the derivative in distilled water was prepared under vigorous stirring at 22^0C. Then, 1% solution of $CaCl_2$ was gradually added to the derivative solution until the appearance of opalescence, which was measured at a wavelength of 600 nm. The resulting concentration of SC in the solution was equal to 0.1%, calcium ions - 0.14%, the ratio of the reagents' volumes ─ 1:0.2, correspondingly. Stirring was left for 30 min. SCNPs were separated by centrifugation (20 min 14000g), and then nanoparticles were resuspended in distilled water. To maintain the stability of the nanoparticles PEG-2000 (20 mg / ml) was added in the suspension. The yield of freeze-dried SCNPs was 10%.

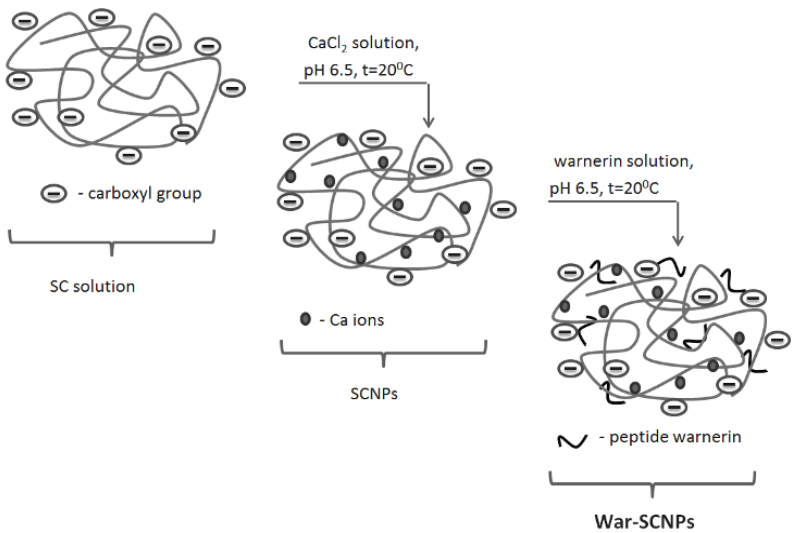

Figure 2. Scheme of the formation of N-succinoyl chitosan nanoparticles loaded with Warnerin.

2.2.2. Additional purification of warnerin

When using methods of ultrafiltration, ion exchange and reverse phase chromatography Warnerin was obtained in a homogeneous state [9]. To achieve a positive effect during the process of sorption peptide on SCNPs, Warnerin was subjected to additional purification using dialysis in regenerated cellulose membranes with a pore size of 1000 Da, and with repeated change of dialysis water. Dialysis was performed for 24 hours at 6^0C. After freeze-drying Warnerin yield amounted 3%.

2.2.3. Sorption of warnerin on SCNPs

Sorption of Warnerin on SCNPs (350 micrograms / ml) was carried out by adding the Warnerin solution (initial concentration - 1 mg / ml), 50, 100, 200 μg / ml to nanoparticles resuspended in deionized water with the addition of PEG (20 mg / ml). The reaction was performed for 30 min at 22^0C. Then, nanoparticles loaded by Warnerin were centrifuged (20 min, 14,000 rev / min), and the amount of unbound peptide was measured in the supernatant at 214 nm to determine the loading efficiency (the percent of peptide binding).

2.2.4. Characterization of prepared SCNPs

2.2.4.1. Dynamic Light Scatterning (DLS)

The size and zeta potential of SCNPs were determined using the method of Dynamic Light Scattering (DLS) with the device 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation, Vernon Hills, IL, USA). Measurements were carried out in bi-distilled water at a scattering angle of 90^0 and a laser light wavelength at 661 nm. Zeta (ζ) potential was determined in 10 mM KCl (Sigma) using the identical equipment with additional unit Zeta-PALS. Measurements were carried out at 25.0 ± 0.1° C.

2.2.4.2. Atomic Force Microscopy (AFM)

Morphology and sizes of prepared nanoparticles were observed using the method of Atomic Force Microscopy (AFM), (NT-MDT INTEGRA Prima. A single drop of nanoparticles solution was applied as a thin layer on a substrate of mica, and allowed to dry at room temperature. Images of nanoparticles were obtained with semicontact mode using cantilevers NSG01.

3. Results and discussion

3.1. Optimization of warnerin loading on SCNPs

Currently there are various methods for preparing the nanoparticles based on biopolymers [11]. In this study the method of salt precipitation (coacervation and precipitation) was used. For the process of nanoparticles formation 1% calcium chloride solution was selected as a precipitating agent. Succinoyl chitosan nanoparticles with negative charge (-18) mV were selected as a matrix for peptide Warnerin which was positively charged and had a zeta potential of about (+6) mV. The formation of complex of succinoyl chitosan nanoparticles and Warnerin occurred at a room temperature. Warnerin was loaded on the pre-formed nanoparticles; the peptide concentration varied from 50 to 200 μg/ml. As a result of the experiment it became clear that the maximum amount of peptide loaded on SCNPs at the Warnerin : nanoparticles ratio being equal to 100 μg / ml: 175 μg/ ml. Loading efficiency of Warnerin was 75% (Table 1)

3.2. Characterization of SCNPs and War-SCNPs

War-SCNPs were fully analyzed after preparing. The zeta potential of the particles, which value increased from (-18.9) mV (for control particles unloaded with peptide) to (-4.4) mV for different nanoparticles: peptide ratios indicated that the interaction between Warnerin and SCNPs was the ionic one.

Table 1. Warnerin loading on SCNPs.

Concentration of peptide added for loading, μg / ml	**Zeta potential, mV**	**Peptide adsorption, %**
200	- 14.6 ± 2.5	74
100	- 4.4 ± 2.9	75
50	- 13.0 ± 2.8	30
0 (control)	- 18.9 ± 3.2	0

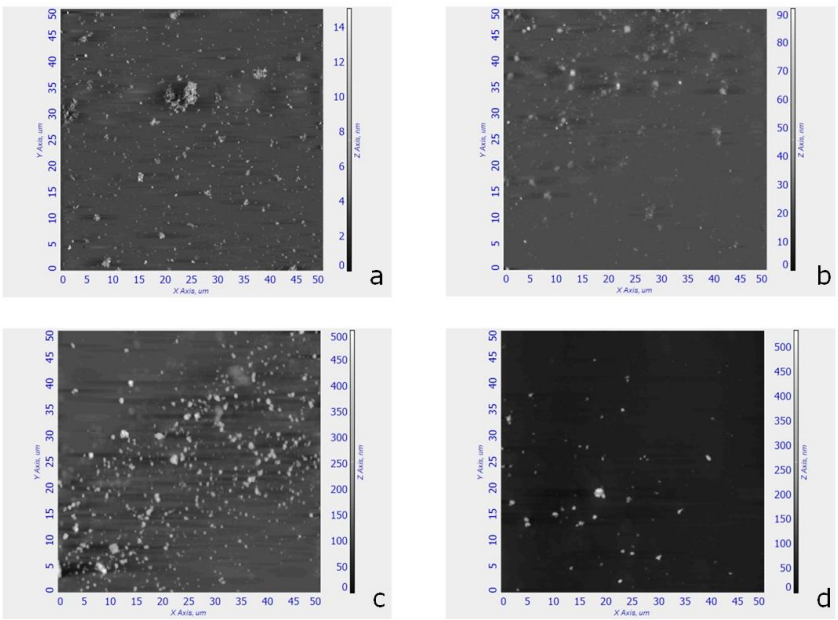

Figure 3. AFM images of the control SCNPs (a) and War-SCNPs with different amount of peptide b) 50 μg, c) 100 μg, d) 200 μg.

Warnerin loading efficiency on SCNPs was 75% under the optimum conditions: ration (SCNPs, μg/ml): (peptide μg/ml) was equal to (1.75 : 1). These results correlate with the neutralization (73%) of the negative charge on the particles by the positive charge on the peptide molecules. That is Warnerin sorption (under experimental conditions) occurs mainly due to ionic interactions.

To determine the SCNPs sizes we used two alternative methods - (DLS) and (AFM). DLS results indicated that the main fraction of SCNPs had the size of 160 nm, and after the peptide sorption War-SCNPs size increased up to 330 nm. The analysis of particles by AFM method indicated that their size increases

is in the correlation with the different polymer amounts used. The particle size increased from 50 nm (Fig. 3a) to 300 nm (Fig. 3d). It is reasonable to assume that the difference in the nanoparticles' size obtained by these two methods could be explained by the distinctions in a sample preparation [10]. We have determined the particle's morphology using the AFM method. The obtained results indicated the homogeneity of nanoparticles and that SCNPs and War-SCNPs have correct spherical shape.

4. Conclusions

As a result of experimental study, there were prepared and characterized succinoyl chitosan nanoparticles loaded by low molecular weight cationic peptide Warnerin, adsorption efficiency which comprised 75%. Formed nanoparticles were stable, has a weak electric charge that can serve as a prerequisite for their use as delivery systems for the therapeutic peptide which will be injected parenterally.

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6. References

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