Development of chitosan nanoparticles for anti-cancer drug delivery systems

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Abstract. Chitosan is a natural polymer that is often used in nanotechnology because it has such useful properties as biodegradability and biocompatibility. Chitosan has many benefits, but they are outweighed by drawbacks such insolubility, aggregation at physiological pH, and insufficient cargo release in the cytosol. In order to obtain chitosan nanoparticles that can be used as drug delivery systems, we optimized the synthesis technology by selecting MES buffer pH 6.3 and using a BSA coating step to reduce nanoparticle aggregation. Deposition of the chemotherapeutic agent etoposide in chitosan nanoparticles has been proven and confirmed by IR spectra, although future experiments are needed to tune conditions for stronger interactions. The chitosan nanoparticles that we have obtained can become suitable nanomaterials for biomedical applications as promising carriers for drug delivery.

1 Chitosan nanoparticles as potential drug delivery systems

Chemotherapy is the mainstay of cancer treatment, but it is not always effective enough and can cause significant harm to the body. Side effects occur due to the non-selective action of the drugs on normal cells. The properties of tumor tissue are significantly different from normal tissue and combining a conventional chemotherapy drug with a system directed against tumor cells will reduce the side effects [1]. Nanoparticles are one possibility for targeted drug delivery. Nanoparticles are a group of substances ranging in size from 1 to 1000 nm and exhibiting a variety of shapes and properties. They offer suitable means of time-controlled or site-specific drug and bioactive agent delivery [2].

Polysaccharides are a sizable class of hydrophilic polymers of natural origin with high biocompatibility that are frequently used in nanotechnology. This is mainly because they have beneficial characteristics in biological systems, such as biodegradability, biocompatibility, and low toxicity [3]. Chitosan is widely used polysaccharide that a derivative of the natural polymer chitin. The major drawbacks affecting efficiency of chitosan include its insolubility at physiological pH and the deficient release of the cargo in the cytosol [4]. The goal of our study was to optimize the technology for obtaining chitosan nanoparticles under physiological conditions. The nanoparticles were produced by ionotropic gel formation

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using the sol-gel transition of chitosan polymers in the presence of a polyanionic crosslinking agent.
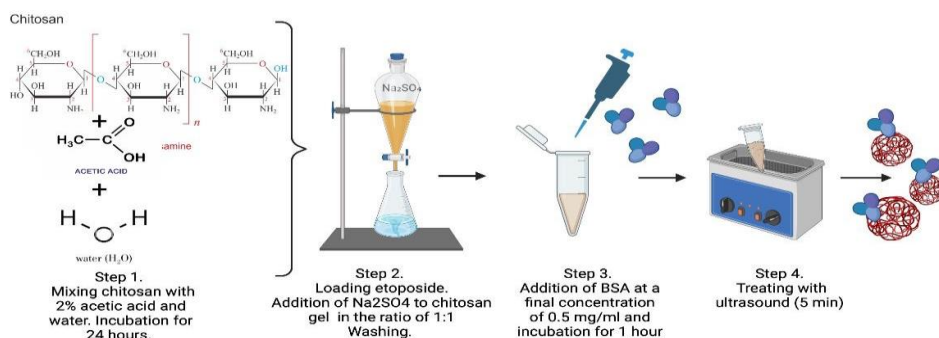
1.1 Preparation of chitosan nanoparticles (ChiNPs) loaded with etoposide

1.1.1 Synthesis of chitosan gel loaded with etoposide

Chitosan powder was mixed with acetic acid (2 %) and dissolved for approximately 24 hours to prepare 1,5% stock solution. The chitosan solution was then purified of the undissolved residue by the centrifugation (15 min, 5000g). To the clarified chitosan solution, 1000 μM etoposide was added to reach final concentration of 20μM and incubated for 30 minutes. A gelling agent solution (Na_2SO_4) was added drop by drop using separating funnel (5-8 drops/sec) with vigorous stirring (about 600 rpm) for 10 min after adding the gelling agent. Then the particles were precipitated in a centrifuge (15 min, 5000g) and the supernatant was drained. Precipitate washing was performed 3 times with deionized water by cycles of dispersions and sedimentations in a centrifuge for 15 min, 5000g. Samples were stored at + 4 °C

1.1.2 Synthesis of ChiNPs-BSA loaded with etoposide

The chitosan gel was suspended by taking 1 gram per 5 ml of deionized water. To prepare samples, 1000 μM each of a suspension of chitosan particles was taken and sedimented by centrifugation. Bovine serum albumin (BSA) was dissolved in a 25 mM MES buffer (pH 6.3) at a concentration of 1 mg/ml. Then BSA solution was added to the particles and the final concentration of BSA in solution was 0.5 mg/ml. The incubation was done for 1 hour. Rapid mixing of the samples was performed using a vortex with a speed of 700 rpm. BSA were added to the mixture to take up otherwise unoccupied sites on the particle surface to prevent particle aggregation. Remove excess BSA by centrifugation. The particles were washed at least twice with a MES buffer to be sure that the unbound protein is completely removed. Nanoparticles were resuspended in 50 μl of buffer and store at +4 °C. The overall scheme of obtaining chitosan -BSA nanoparticles loaded with etoposide is shown in Figure 1.

Fig.1. The overall scheme for obtaining of chitosan -BSA nanoparticles loaded with etoposide.

1.2 Characterization of chitosan nanoparticles (ChiNPs) loaded with etoposide

1.2.1 Characteristics of chitosan

When using chitosan, it is critical to know its characteristics. The chitosan we used was produced from crab shell, had a degree of deacetylation ≥80% and a molecular weight 200 kDa.

1.2.2 Study of the aggregation of chitosan particles in different buffers
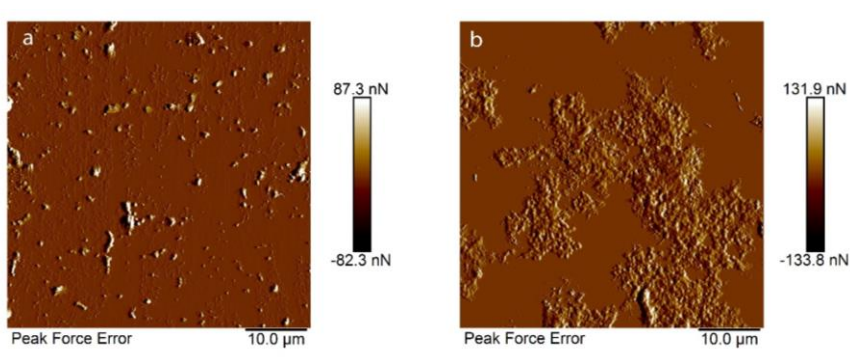
We studied the aggregation of chitosan particles in different buffers at different pH: borate (pH 8.01), phosphate (pH 7.4) and MES [2-(N-morpholino) ethane sulfonic acid] (pH 6.3) buffers. The samples were examined using the atomic force microscope (AFM Bioscope Resolve, Bruker, USA).

2 Results

## 2.1 Study of ChiNPs aggregation by atomic force microscopy

We studied the aggregation of chitosan particles in different buffers at different pH. Chitosan nanoparticles suspension of 50 μl was applied to a slide glass and incubated for 20 min at room temperature. The liquid was then removed from the slide with a filter paper and the sample was left to dry for 20 min. The samples were examined using an atomic force microscope (AFM Bioscope Resolve, Bruker, USA).

In Figure 2, we see that the aggregation of chitosan strongly increases depending on pH. Particles dissolved in PBS form small aggregates, and we did not see particles smaller than one micron. And in the borate buffer we see the formation of stable large clusters of aggregates, which do not decrease even after treatment with ultrasound.

Fig. 2. Study of the aggregation of chitosan particles in different buffers by atomic force microscopy. a- phosphate-buffered saline (pH-7.4). b- borate buffered saline (pH-8.01).

The most optimal buffer for our purposes was 25 mM MES [2-(N-morpholino) ethane sulfonic acid], pH 6.3 buffer, in which we recorded minimal aggregation of chitosan and the formation of stable nanoparticles about 100-200 nm in size (Figure 3). The obtained nanoparticles were also modified by sorption with BSA and treatment with ultrasound, which significantly reduces the aggregation of nanoparticles.
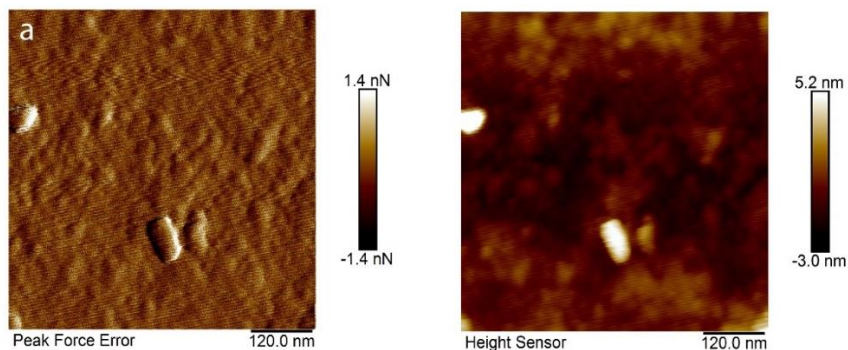
Fig. 3. A size study of chitosan nanoparticles by AFM
in 2-(N-morpholino) ethane sulfonic acid (25 mM MES buffer pH-6.3).

2.2 Study of ChiNPs size by scanning electron microscopy

The nanoparticle size was investigated using a ZEISS Sigma scanning electron microscope (ZEISS, Germany). The samples were prepared using the critical point drying technique. In Figureы 4 and 5, we observe the formation of stable nanoparticles with sizes in the range of 100-200 nm under optimized conditions, which corresponds to the AFM results.
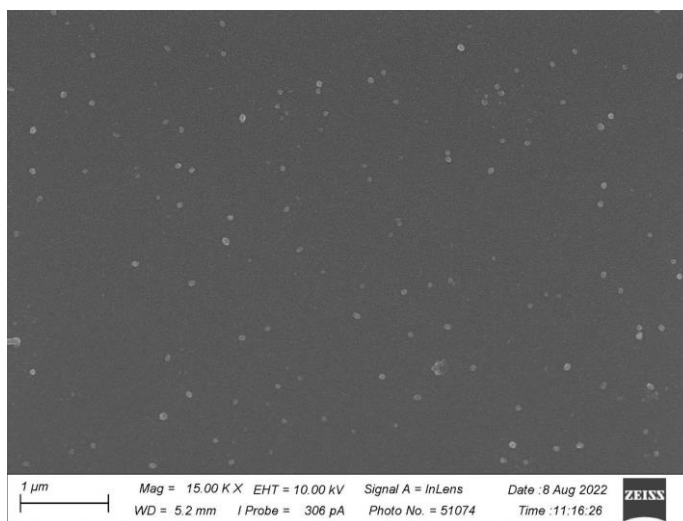
**Fig. 4.** SEM image of the nanoparticle at 15,000x magnification.
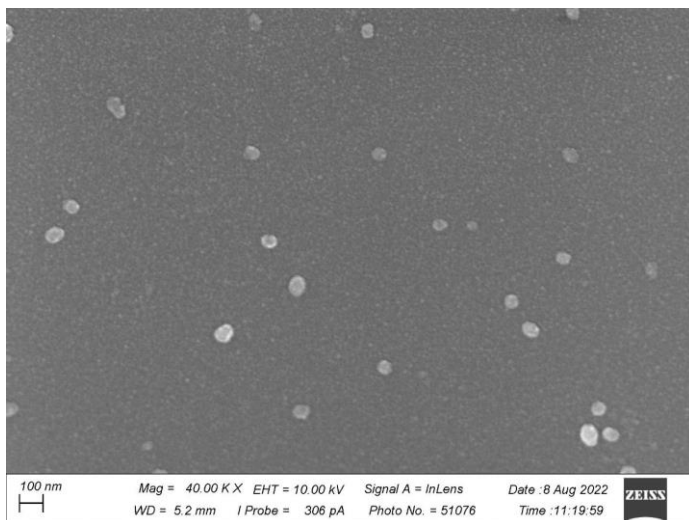
Fig. 5. SEM image of the nanoparticle at 40 000x magnification

2.3 Analysis of etoposide deposition in chitosan nanoparticles

The loading of drugs into chitosan NPs systems was performed during particle preparation. We chose etoposide as the drug, it inhibits DNA topoisomerase II, thereby inhibiting DNA re-ligation. This causes critical errors in DNA synthesis at the premitotic stage of cell division and can lead to apoptosis of the cancer cell [5]. The main absorption bands in the spectrum of chitosan (Figure 6, a) are at 3406, 1662, and 1081 cm-1. They are due to the valence vibrations ν(O-H), ν(C=O) in acetylated monosaccharide residues, and ν(C-O-C), respectively. The peak at 1588 cm-1 refers to the strain vibrations δ(N-H) of the NH2 amino group.

The formation of chitosan nanoparticles is apparently carried out by molecular cross-linking via deacetylated amino groups, which are transferred to the charged (-NH$_3^+$) state depending on pH conditions. This corresponds to a shift of the δ(N-H) vibration band to the low-frequency region up to 1534 cm-1 (Figure 6, b).

Most peaks after etoposide introduction do not change their position (Figure 1, c) in comparison with the spectrum of nanoparticles without additives (Figure 6, b), including the band at 1534 cm-1. It is worth noting the appearance of three bands of medium intensity at 1733, 1691 and 1645 cm-1. They may refer to asymmetric, symmetric, and intermolecular valence vibrations of the ν(C=O) carboxyl group of etoposide.
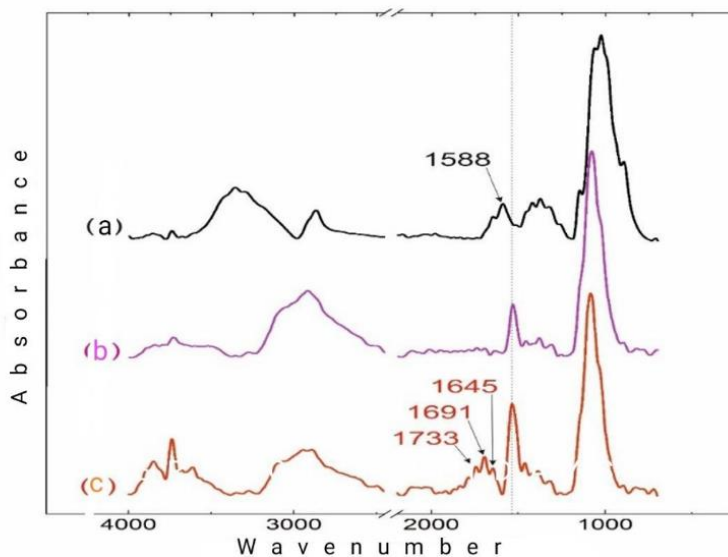
Fig. 6. IR spectra of the original chitosan powder (a), chitosan nanoparticles without the addition of additional substances (b) and with the addition of etoposide (c). The dotted line shows the δ(N-H) band position of the polysaccharide after nanoparticle formation

Based on the obtained infrared spectroscopy data, we can assume that etoposide is incorporated into the mesh structure of polysaccharide nanoparticles, but the electrostatic interactions of its functional groups ($-NH_3^+$) are virtually absent and binding occurs by a different mechanism. This should be verified in further experiments by tuning chemical conditions supporting electrostatic stronger interactions between etoposide and chitosan-based nano-carrier.

3 Conclusions

As a result of our work, we optimized the technology for the synthesis of chitosan nanoparticles by ionic gelation under physiological conditions at pH 6.3. Aggregation of chitosan nanoparticles was prevented by immobilization with bovine serum albumin. Deposition of the chemotherapeutic agent etoposide in chitosan nanoparticles has been proven and confirmed by IR spectra, although further experiments are needed to tune conditions for stronger interactions. This could be implemented by adjusting the pH or using a reversible crosslinker.

The obtained chitosan nanoparticles can become promising nanomaterials for biomedical applications.

References

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