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Size, Loading Efficiency, and Cytotoxicity of Albumin-Loaded Chitosan Nanoparticles: An Artificial Neural Networks Study

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ABSTRACT

When designing nanoparticles for drug delivery, many variables such as size, loading efficiency, and cytotoxicity should be considered. Usually, smaller particles are preferred in drug delivery because of longer blood circulation time and their ability to escape from immune system, whereas smaller nanoparticles often show increased toxicity. Determination of parameters which affect size of particles and factors such as loading efficiency and cytotoxicity could be very helpful in designing drug delivery systems. In this work, albumin (as a protein drug model)–loaded chitosan nanoparticles were prepared by polyelectrolyte complexation method. Simultaneously, effects of 4 independent variables including chitosan and albumin concentrations, pH, and reaction time were determined on 3 dependent variables (i.e., size, loading efficiency, and cytotoxicity) by artificial neural networks. Results showed that concentrations of initial materials are the most important factors which may affect the dependent variables. A drop in the concentrations decreases the size directly, but they simultaneously decrease loading efficiency and increase cytotoxicity. Therefore, an optimization of the independent variables is required to obtain the most useful preparation.

Introduction

Chitosan (Cs) is a carbohydrate-based polymer which is commercially produced by deacetylation of chitin under basic conditions. Because of unique properties of Cs such as biocompatibility, biodegradability, and antibacterial activity, nanoparticles (NPs) and microparticles of Cs have gained lots of popularity for drug delivery.[1,2] Cs NPs could be prepared via numerous methods including ion gelation, self-assembly, and polyelectrolyte complexation (PEC).[3] Albumin (Alb) is the main protein of mammalian blood and has an important role in moving small molecules in blood and keeping oncotic pressure (i.e., osmotic pressure which is exerted by plasma proteins, in a blood vessel). Alb is usually used as a model for protein drugs to investigate the effects of different parameters or synthesis methods on proteins.[4,5]

Some very important pharmacokinetic and pharmacodynamic properties of drug-loaded NPs depend on their size, cytotoxicity, and loading efficiency, 3 factors which may be manipulated during preparation procedure.[6] Circulation time, body distribution, immune system response, and plasma clarification time of NPs depend on size of the particles.[7] Loading efficiency is another important factor for drug delivery purposes. Particles with higher loading capacity are desired in efficient delivery systems.[8] Using NPs *in vivo* may be altered by their toxicity profile. More toxic NPs are not usually preferred for drug delivery systems.[9] Therefore, determination of parameters which affect size, cytotoxicity, and loading efficiency of NPs is a critical step in developing a nano-based preparation for clinical applications.

Physicochemical and pharmacokinetic properties of NPs usually depend on preparation methods and nature of initial materials. Effects of preparation methods on size, shape, and surface charge of poly(lactic-co-glycolic acid) NPs have been previously shown.[10] Also, it is shown that long-term stability and loading capacity of lipid NPs are changed when differing the preparation method.[11] PEC method uses noncovalent interactions to make NPs. This method has minimum effects on physiochemical properties of initial materials.[12] Therefore, it is successfully used for preparation of protein- and peptide- loaded NPs. For example, Cs NPs (200-300 nm) are loaded with heparin as a model of protein drug delivery.[13] Also, insulin/Cs NPs that are prepared with PEC method are successfully used as an oral drug delivery system.[14,15]

PEC method could be altered by several variables such as concentration of initial materials, pH, reaction time, and temperature.[12-14] Therefore, determination of relations between

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independent parameters (e.g., concentration of initial materials, time of reaction, and pH) and dependent variables (e.g., size, cytotoxicity, and loading efficiency) in NPs which are made by PEC method could be very helpful in designing protein delivery systems. Effects of preparation methods, initial materials concentration, pH, temperature, and reaction time on size and loading efficiency of Cs-loaded heparin[16] and insulin[17] NPs are evaluated in 1-factor-at-a-time experimental approaches. Processing parameters (i.e., Cs concentration, pH, sonication time, and amplitude) in relation with particles' size of Cs NPs are investigated by artificial neural networks (ANNs).[18,19] ANNs are established methods to study nonlinear phenomena (as commonly observed in preparation of NPs).[20-22] For instance, ANNs have been used to determine the effect of independent variables on size of triblock poly (lactide)–poly (ethylene glycol)–poly (lactide) (PLA–PEG–PLA)[23] and silver[24] NPs.

In 2007, NPs of Cs and tripolyphosphate (TPP) carrying Alb as a model protein were prepared by ion gelation method. Limited number of experiments were performed to study the effect of Cs concentration on particle size and zeta potential of the NPs. Encapsulation efficiency was also investigated in relation with Cs concentration, Alb concentration, and Cs/TPP ratio.[25] However, the limited coverage of factorial space along with lack of providing a model to study the outputs necessitates further investigations in this field. In this work, we used ANNs to provide extensive understanding about parameters ruling size, cytotoxicity, and encapsulation efficiency of Cs/Alb NPs as a model protein-loaded nanoparticulate system. Results are expected to provide a better view of possible relationships between independent parameters (concentration of ingredients, reaction time, and pH) and their effects on the outputs. This could be helpful in designing polymer/proteins NPs for drug delivery purpose.

Material and Methods

Materials

Cs with molecular weight of 500 kDa and deacetylation degree (DD) of 78% was purchased from Eastar Holding Group Co., Ltd (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (Pen/Strep) were purchased from Gibco (Waltham, MA). Mrc-5 cell line (human fetal lung fibroblast cells) was purchased from Pasteur Institute (Tehran, Iran). MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], Coomassie brilliant blue (G-250), bovine serum albumin (BSA), acetic acid, isopropanol, methanol, phosphoric acid, and other chemical materials were purchased from Merck Co. (Darmstadt, Germany) and used for the study without further purification.

Methods

NPs Preparation

Cs/Alb NPs were prepared via PEC method.[26] For preparation of NPs, different concentrations of BSA were added dropwise to Cs solution at desired value under stirring. Thirty NP samples were prepared for ANN modeling with different concentrations of Cs (i.e., 0.5-2 mg/mL) and BSA (i.e., 0.5-2.0 mg/mL) having pH values of 4.7-6.2. All samples were stirred during preparation for 0.5-2.0 hours at 2500 rpm.

Size Determination

Size of NPs was evaluated by photon correlation spectroscopy (Scatteroscope I; K-ONE Ltd., Seoul, Korea) at room temperature using 633-nm laser beam on 1 mL of each sample. All samples were used without any dilution.

Loading Efficiency Assay

Prepared NPs were centrifuged at 15,000 *g* for 30 minutes at 4°C for loading efficiency studies. Afterward, Alb content in supernatant was evaluated by Bradford assay, and loading capacity was calculated by Equation 1.[27] For Bradford assay, 5 mg of G-250 was dissolved in 50-mL ethanol; then, 100 mL of 85% phosphoric acid was added to dissolve dye and mixed well. Subsequently, distilled water was added to solution up to 1000 mL. For evaluation of protein content in supernatant of centrifuged NPs, 200 μl of samples were added to 800 μl of dye solution and incubated for 15 minutes in darkness. Then, absorption of samples was read at 595 nm against dye solution as blank by spectrophotometer (Cecil CE8020; Cecil Instruments, Cambridge, UK).

$$\text{Loading efficiency}\,\% = \left[\frac{\text{mass of protein used} - \text{protein in the suspension}}{\text{mass of protein used}}\right] \times 100 \quad (1)$$

Cell Toxicity Assay

The Mrc-5 cell line was selected as a model of cell line to evaluate NPs cytotoxicity using MTT assay test.[28] Briefly, cells were cultured in DMEM medium with 10% FBS and 1% Pen/Strep and incubated for 24 hours at 37°C. Subsequently, cells were counted and diluted to reach 7500 cells/well and added to desired well of 96-well plate. NPs (100 μg/mL) were exposed to cells and incubated for 24 hours at 37°C. Then, 10 μL of MTT reagent (10 mg/mL) was added to each well and incubated for 3 hours. Afterward, 100-μL isopropanol was added to each well to dissolve Formazan crystals, followed by 2-hour dark incubation. Absorbance of each well was then recorded at 570 nm using microplate spectrophotometer (Epoch, Biotek, USA) against culture medium without NPs as blank. Viability percent was calculated using Equation 2. All experiment runs were performed in triplicate.

$$\text{Viability}\,\% = \frac{\text{Samples Absorbance} - \text{Blank Absorbance}}{\text{Control Absorbance} - \text{Blank Absorbance}} \times 100 \quad (2)$$

ANN Study

ANNs are common approach for determining relationship between input and output parameters. ANNs mimic mammalian neural network (brain process) and are usually used to find nonlinear relationships between parameters. Nowadays, many commercial software are available for ANN applications. In our work, relationship of input parameters, including Cs and Alb concentrations as well as time of reaction (stirring time), and output parameters (i.e., size, loading efficiency, and cytotoxicity) was studied with INForm v4.02 (Intelligensys, London, UK).