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Nanocapsule assemblies as effective enzyme delivery systems against hyperuricemia

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Abstract

The uricase nanocapsule assemblies (UNAs) were developed as effective delivery systems against hyperuricemia following parenteral enzyme therapy. UNAs were characterized in terms of micromorphology, size, catalytic activity, stability, and enzymatic kinetics. The pharmacokinetics/pharmacodynamics in rats after intravenous or subcutaneous injection was investigated. Immunogenicity, hemolysis and stimulation were determined. UNA was composed of many nanocapsules, and thus had higher loading efficiencies and stabilities than a single nanocapsule. The uricase molecules entrapped inside nanocapsules were separated from the circulating bloodstream to retain catalytic activities for a longer time than free uricase. UNAs increased the bioavailability and uric acid-lowering efficacy of uricase, while the immunogenicity and hemolysis rate of uricase were decreased. The superior properties of UNAs might be ascribed to the favorable conformational changes of uricase. Nanocapsule assemblies appeared to be able to deliver uricase effectively. This study also highlighted the importance of suitable systems for therapeutic enzyme delivery.
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Key words: Nanocapsule assemblies; Enzyme delivery systems; Uricase; Enzymatic therapy; Hyperuricemia

Enzymatic therapy has become an important means of treating diseases, such as myocardial infarction (which can be treated with tissue plasminogen activator, t-PA), severe combined immunodeficiency disease (adenosine deaminase, ADA) and hyperuricemia (uricase, UC).[1] There have been a few delivery vehicles which are developed to increase the therapeutic efficacy of enzymes. For example, t-PA has been loaded into echogenic liposomes,[2] poly (lactic-co-glycolic acid) nanoparticles,[3] or magnetic nanoparticles[4] to improve the treatment effects, slowly lyse fibrin clots or target vascular diseases, respectively. ADA has been conjugated to polyethylene glycol to achieve sustained enzymatic activity and enhanced therapy efficacy.[5]

Hyperuricemia and its related diseases (e.g., gout, cardio-metabolic and hypertension)[6] are getting more attention due to their increased prevalence. According to public health statistics, there are more than 100 million Chinese patients with hyperuricemia.[7] UC is a uric acid catabolic enzyme produced by various microorganisms and animals but not by humans.[8] In clinical practice, UC has effects that are superior to those of chemical drugs (such as the first-line drug allopurinol and its substitute febuxostat) for lowering uric acid levels. In addition to UC, recombinant UC[9] and PEGylated recombinant UC[10,11] have been approved for clinical applications. Other formulations employed to deliver UC include biohybrid hydrogel,[12] liposomes[13] and polypyrrole-ferrocenium film.[14] However, none of these formulations meet all of the requirements for an ideal UC delivery system, which mainly includes high and long-lasting activity and low immunogenicities.

This study aimed to develop novel UC nanocapsule assemblies (UNAs) as effective delivery systems against hyperuricemia following parenteral enzyme therapy. UNAs have the above-mentioned required properties for UC delivery. A UNA is composed of many nanocapsules (Supplementary Figure S1), giving it a much higher UC-loading efficiency than a single nanocapsule. Additionally, UNAs may be much more stable than single nanocapsules, just as the multi-layered colloidal aphrons consisting of many droplets are more stable than a single

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droplet.[15] The UC molecules entrapped inside various nanocapsules are separated from the circulating bloodstream to make them less affected by external environmental factors, and thus are able to keep their catalytic activities for a longer time, a trait similar to one of the liposomes prepared by our laboratory.[13,16] Furthermore, because the large protein UC protein molecule (tetramers of 35 kD subunits) has been encapsulated inside of nanocapsules mainly made of biodegradable pharmaceutical excipients that are generally recognized as safe (GRAS) and approved by many national Food and Drug Administrations, UNAs have very low immunogenicity.

Methods

Preparation of the UNAs

Phospholipid (60.0 mg), cholesterol (46.2 mg) and triolein (66.2 mg) were dissolved in 3 mL of diethyl ether, and the resulting solution was added with 3 mL of 3% glucose solution (containing 5% poloxamer 188, 22.5% poloxamer 407 and 9.8 units of UC) and vortex-mixed for 8-10 min to obtain a water-in-oil emulsion. Next, 3 mL of the emulsion was quickly injected into 10 mL of 4% glucose solution (containing 40 mmol/L L-lysine), and the mixture was blended in a homogenizer at 6,000 rpm for 24 s to obtain water-in-oil-in-water multiple emulsions. The emulsions were evaporated for 10 min under hypobaric conditions to get rid of diethyl ether. Then, the uniformly dispersed systems (UNAs) were formed.

Principle characteristics of the UNAs

The morphologies of the UNAs were observed with natural light, while the micromorphologies of UNAs labeled with FITC were examined by a laser microdissection system (Leica AS LMD, Wetzlar, Germany) with a laser light at 497 nm. The average particle sizes of the UNAs were obtained by measuring the diameters of five hundred particles.[17] The zeta potentials of the UNAs were determined by dynamic light scattering (Zeta-Sizer Nano ZS90 instrument, Malven Instruments Ltd., UK). The sample was prepared by diluting the above preparation with 9 times the volume of corresponding buffer. The entrapment efficiency of the UNAs was examined using gel filtration chromatography combined with a Coomassie brilliant blue method reported previously.[18,19]

Micromorphology changes and structural estimation

UNAs were added into 5 volumes of pH 7.4 PBS, and the mixture was placed at 37 °C and continuously stirred at 10 rpm for 48 h. The micromorphology and structural changes of the UNAs were observed using microscopy at predetermined times.

The interactions of UC molecules with vesicle membranes were determined according to the FITC fluorescence method reported previously.[19] Briefly, one portion of FITC was added into 49 portions of UC or blank UNA, and then the mixtures were incubated in the darkness for 5 min and detected using a fluorescence spectrophotometer (F-2500, Shimadzu). The excitation wavelength was set at 480 nm. The effects of UC were examined by incubating UC with blank UNA at 25 °C for 1 h before adding the FITC solution.

External factor effects on uricase activity

At first, the hypothermal effects on UC activity were investigated. The hypothermal stability was investigated by incubating UNAs at 4 °C for 21 d. The remaining activities were assessed at predetermined times. The electrical conductivities of the UNAs were determined at 25 °C with an electric conductivity analyzer (DDS-307A; Shanghai Yidian Scientific Instrument Co., Ltd, Shanghai, China). Fluorescence changes of UC were determined using a fluorescence spectrophotometer at 280 nm.[20] One part chloroform was added to two parts of UNA, and the mixture was centrifuged at 3,000 rpm for 5 min. The supernatant was collected and tested as described above. The ultraviolet absorbance change of UC was determined using a Shimadzu UV-2600 spectrophotometer. The UNAs were treated with chloroform and tested as described above. PAGE electrophoresis was performed according to a previously described method.[21] Briefly, equal amounts of free UC and UNAs were spotted and subjected to a Bio-Rad electrophoresis system. Then, the gel was stained with Coomassie brilliant blue R-250 dye solution for 20 min, and then destained with a Commassie blue staining destaining solution for 2 d. SDS-PAGE was performed in a similar manner, except that the samples were heated in a boiling water bath for 5 min before they were spotted; 10% SDS was added into both the separation gel and the spacer gel.

Secondly, the thermal effects on UC activity were investigated. The optimal temperature of UNA was investigated within the temperature range of 20-70 °C by determining the UC activity according to the method we previously reported.[13] The thermal stability was determined by placing UNA in a water bath at 55 °C for 5 h to determine the remaining activities. The fluorescence change of UC induced by heat treatment was determined according to the fluorescence method that we previously reported.[19]

Thirdly, the acidity-alkalinity effects on UC activity were investigated. The optimal pH of UNA was investigated within the pH range of 7.0-9.5 by determining the UC activity according to the method that we previously reported.[13] The effects of pH were determined by placing UNA into buffers with pH values ranging from 5.0 to 9.5 for 40 min and determining the remaining activity.

Lastly, the proteolytic enzyme effects on UC activity were investigated. UNA was treated with trypsin at 37 °C and the remaining activities were determined at specific times.

Enzymatic kinetics of uric acid oxidation

The *in vitro* kinetic parameters of UC were determined by catalyzing the oxidation of uric acid solutions at different concentrations at 25 °C and were analyzed by the Lineweaver–Burk plotting equation.[18]

All experiments were approved by the Laboratory Animal Committee, Chongqing Medical University. All the procedures followed were in accordance with institutional guidelines. The pharmacokinetics in rats was carried out according to the following protocol: thirty SD rats (200 ± 20 g) were

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