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Ultra-small and anionic starch nanospheres: Formation and vitro thrombolytic behavior study

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ABSTRACT

This paper is considered as the first report on the investigation of nattokinase (NK) release from anionic starch nanospheres. The ultra-small and anionic starch nanospheres were prepared by the method of reverse micro-emulsion crosslinking in this work. Starch nanospheres were characterized through Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM) and dynamic light scattering (DLS). Effects of preparation conditions on particle size were studied. The cytotoxicity, biodegradable and vitro thrombolytic behaviors of nattokinase (NK) loaded anionic starch nanospheres were also studied. The results showed that the anionic starch nanospheres are non-toxic, biocompatible and biodegradable. Moreover, the anionic starch nanospheres can protect NK from fast biodegradation hence prolongs the circulation in vivo and can reduce the risk of acute hemorrhage complication by decreasing the thrombolysis rate.

1. Introduction

As a source of stored energy, starch is a naturally occurring polysaccharide produced by many plants and composed of two different components linear amylose and branched amylopectin. Normally, amylose account for 20–30% and amylopectin account for 70–80% of starch, respectively, which depend on the species of source. Due to many attractive properties of starch, such as economy, abundant, renewable, innocuity, biodegradability, biocompatibility and the capacity to bind with a wide variety of molecules via physical and chemical interaction (Li & Ni, 2004; Polaczek, Malenki, & Tomasik, 2000), starch can be used as biocompatible materials (Jobling, 2004; Song, Zhao, Dong, & Deng, 2009). Whereas, the big size of native starch particles restricts their applications in many field (Angellier, Molina-Boisseau, & Dufresne, 2005; Kristo & Biliaderis, 2007; Valodkar & Thakore, 2011), especially in medical field (Chen et al., 2008; Simi & Emilia Abraham, 2007; Thielemans, Belgacem, & Dufresne, 2006). Starch nanoparticles have many advantages as a delivery material, such as convenience of modifying surface properties, controlling particle size and release of pharmacologically active agents, less toxic, to achieve the targeting action of the drug at the therapeutically optimal rate and dose regimen. Recently, a widely summarized review on starch nanoparticle preparation, characterization, and applications has been published (Le Corre, Bras, & Dufresne, 2010). Starch nanoparticles have been prepared via reactive extrusion, regioselective modification and precipitation (Angellier et al., 2005; Kim & Lim, 2009; Ma, Peter, & Yu, 2008; Song, Thio, & Deng, 2011), respectively. The starch nanoparticles display a spherical or oval shape, with diameters in the range of 10–200 nm.

Recently, thrombotic diseases does harm to the life and health of human severely, especially, heart and cerebral thrombolic disease is one of the most important and common diseases that are harmful to the health of aged people. Herein, the studies of thrombolytic drugs have been paid attention in the world. Currently, nattokinase (NK) that can dissolve fibrin directly is high efficient, secure and economic, thus became the focus of the studies of thrombolytic drugs. Although NK possess a high fibrinolytic efficiency, an increased blood drug concentration of the free NK will cause serious side-effects such as hemorrhage (Brekenfeld et al., 2007). On the other hand, protein is flimsy in vivo due to fast clearance by enzymes like proteinase (Erdoğan, Özer, & Bilgili, 2005). To find a way to minimize side effects of NK, improve their stability and lengthen their circulation time for the treatment of thrombotic diseases holds great promise. Ultra-small systems are more ideal for the thrombolysis therapy because drugs have to be delivered to the thrombus through the narrow residual blood flow channels around the arterial obstruction (Francis, Blinc, Lee, & Cox, 1995; Jin et al., 2012). Therefore, the development of new biodegradable and biocompatible nanometer-sized drug carriers is still urgent. These

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nanoparticles have given much highlight as advanced vehicle of different kinds of drugs due to their advantages in terms of drug protection, transport and delivery (Panyam & Labhasetwar, 2003; Ravi Kumar, Bakowsky, & Lehr, 2004). With ultra-small size and biocompatibility, the nanoparticles can be transported through the blood circulation to remote body sites. These characteristics may favor the therapeutic applications in the delivery of thrombolitics for longer circulation time.

The objective of the current work is to prepare anionic starch nanospheres to explore the potential thrombolytic application of nanosphere-loaded NK. With negative charges, the nanospheres could not only enhance loading capacity of NK, but also can improve its stability, lengthen its circulation time and minimize the effect of hemorrhage. The anionic starch nano-spheres were prepared to load NK to overcome the above disadvantages. The nanoparticles were obtained by using inverse micro-emulsion crosslinking. The effects of preparing conditions, such as oil–water ratio, starch amount, surfactant content and crosslinking agent on starch nanospheres size were systematically studied. Besides, the morphology, cytotoxicity, biodegradability, and vitro thrombolytic behaviors of NK-loaded anionic starch nanospheres were also studied.

2. Experimental

2.1. Materials and methods

Soluble starch was purchased from Zhejiang linghu Chemical Reagent Factory, chemical purity grade. Span 80 and liquid paraffin were purchased from Fine Chemical Industry Institute of Tianjin Guangfu, chemical purity grade. Phosphoric trichloride ($POCl_3$) was of analytical purity grade and was provided by Shanghai Chemical Reagent Company of Chinese Medical Group. Sodium hydroxide (NaOH) was purchased from Xi'an Chemical Plant. NK was provided by Shanghai Shenggong Bioengineering Technology Service Co., Ltd. The other reagents were of analytical purity grade and used without further purification.

2.2. Preparation of starch-nanoparticles

Preparation of aqueous phase (W phase): 10 mg/mL of soluble starch solution was prepared and hydrolyzed in boiling water bath for 10 min, then ultrasonicated for 30 min, followed by adjusting the pH to 10 with 1 mol/L NaOH solution. Preparation of oil phase (O phase): 0.5 g of Span 80 was dissolved in 100 mL of paraffin in a beaker and stirred for 30 min in a 30 °C thermostated water bath. Then 10 mL of W phase was added dropwise into 100 mL of O phase under mechanical agitation of 1000 r/m. After 30 min, 30 μL of $POCl_3$ was added. The anionic starch nanospheres formed after 4 h. The emulsion was centrifuged and purified with distilled water and ethanol alternatively for several times. The obtained starch nanospheres was air-dried and suspended in alcohol, respectively, for further analysis and utilization.

2.3. FTIR analysis

IR absorption spectra of the samples were taken on a Fourier transform infrared (FTIR) spectrometer (Nicolet 670 FTIR, USA) over the region from 4000 to 400 cm^{-1}. The exhaustively dehydrated starch nanospheres were thoroughly ground with KBr powder prior to analysis, and pellets were prepared by compression under vacuum.

2.4. Starch-nanoparticle morphology by scanning electron microscope (SEM) images

The starch particles immersed in anhydrous alcohol were ultrasonicated for 15 min, followed by dropping the dispersions onto aluminum stubs and sputter-coated with gold for 300 s fractured, and the exterior morphology was examined with scanning electron microscope (SEM) (JSM-5600 LV SEM, Japan).

2.5. Transmission electron microscope (TEM) analysis

The morphologies and particle size of anionic starch nanospheres were characterized by a JEM-1200 EX/S transmission electron microscope (TEM). Before examination, the particles were dispersed in anhydrous alcohol and ultrasonicated for 15 min, and then deposited on a copper grid covered with a perforated carbon film.

2.6. Analysis of Z-potential

For *Z*-potential measurements, starch nanospheres ethanol dispersions with concentration of 1 g/L were ultrasonicated for 15 min prior to being transferred into dish. Zetasizer (2004, Malvern Instruments, U.K.) was used for measuring the distribution of the electrophoretic mobility of particles, the analyzer calculates the *Z*-potential from electrophoretic mobility using the Henry equation and Smolukowsky approximation. The isoelectric point corresponds to the point where potential value is zero, and all charges of particles are neutralized (Tan, Koopal, Weng, Vanriemsdijk, & Norde, 2008). Each sample was measured in triplicate at 20 °C.

2.7. Particle size measurements

Particle size of the obtained particles was recorded by a thermally controlled dynamic light scattering (DLS) at the scattering angle of 90° in alcohol using 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation) at room temperature. The particle size was determined as the maximum of the peak obtained in the CONTIN histogram. Further, the experimental data proved to give monomodal CONTIN histogram analyzed by the cumulants method giving access to some information on the *z*-average diffusion coefficient of the system. About 10 mg of starch particles were transferred into 10 mL anhydrous alcohol and ultrasonicated for 15 min to avoid the agglomeration of particles during measurements. The final particle diameter was calculated from a mean of at least three measurements.

2.8. Cytotoxicity assay

Cytotoxicity essay of anionic starch nanospheres was performed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay using HeLa cells which grown in DMEM with 10% of fetal bovine serum (FBS) and were cultured in humidified atmosphere (5% CO_2, 37 °C). Then the cells were seeded into 96-well plates at a density of 7×10^3 cells/well, followed by incubating the plates at 37 °C for 4 h. Then, the original medium was replaced with 100 μL of fresh medium. Solution (10 μL) of nanospheres was sterilized by autoclave, and were injected into the 96-well plates, mixed, and incubated at 37 °C for 24 h. Next, the MTT reagent (10 μL in PBS, 5 mg/mL) was added to each well for further 4 h. Then, the medium was removed and 100 μL of DMSO was added to dissolve the formed formazan crystals. Untreated cells which were taken as control had 100% viability, and the cytotoxicity of nanoparticles was defined as the relative viability (%) which correlates