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Conjugation of nattokinase and lumbrukinase with magnetic nanoparticles for the assay of their thrombolytic activities

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ABSTRACT

Two important thrombolytic enzymes, nattokinase (NK) and lumbrukinase (LK), were immobilized onto fine magnetic Fe_3O_4 nanoparticles using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) as the coupling reagent, and their thrombolytic activities were studied. The Fe_3O_4 nanoparticles and NK- and LK-conjugated magnetic nanoparticles were characterized by transmission electron microscopy, Fourier transform infrared spectrophotometry, vibrating sample magnetometry, X-ray diffraction, and UV–vis absorption spectroscopy. Dual kinetic absorbance measurements at 405 and 630 nm were employed to measure their thrombolytic activity. Analysis of protein amount showed that the optimum conditions for NK and LK binding to nanoparticles were respectively at a mass ratio of 2:1:1, 2:1:2 (magnetic nanoparticles:protein:EDC), and pH 6.00. Thrombolytic activity assay showed that the best thrombolytic activity could reach 91.89% for NK–nanoparticle conjugates and 207.74% for LK–nanoparticle conjugates, which are much higher than the pure enzymes (NK, 82.86%; LK, 106.57%).

1. Introduction

Thrombotic events affect many individuals in a number of ways, all of which can cause significant morbidity and mortality [1–5]. According to the report of the World Health Organization, 17 million people die of cardiovascular diseases every year [6]. Previous studies demonstrated that intravascular thrombosis, a blood clot in a blood vessel, is one of the main causes for various thrombotic events, and the major component of blood clots is fibrin formed from fibrinogen during the proteolysis by thrombin [3,7–10]. Meanwhile, fibrin clots can be hydrolyzed by plasmin to avoid thrombosis in blood vessels. In a normal situation, these reactions are kept at a balance. However, once the balanced situation is triggered by some disorders, the clots cannot be hydrolyzed, and thus thrombosis occurs [11]. Based on the working mechanisms, two kinds of thrombolytic agents have been developed and been applied in clinic therapy [4–6,12]: (1) plasminogen activator, such as tissue-type plasminogen activator (t-PA), streptokinase, and urokinase, which activate plasminogen into active plasmin to degrade fibrin; and (2) plasmin-like proteins, such as nattokinase, lumbrukinase, and fibrolase, which directly degrade the fibrin in blood clots.

Of the thrombolytic enzymes mentioned above, nattokinase was reported to not only possess plasminogen activator activity, but also directly digests fibrin through limited proteolysis. Its fibrinolytic activity can be retained in the blood for more than 3 h. It was also reported that nattokinase is less sensitive to the cleavage of fibrinogen, but is more sensitive to the cleavage of cross-linked fibrin compared to plasmin [6,13–17]. Earthworm fibrinolytic enzymes (lumbrukinase) are a group of serine proteases that have strong fibrinolytic and thrombolytic activities [2,18,19]. Therefore, these two enzymes have been regarded as promising agents for thrombosis therapy. However, plasmin in blood has a short half-life, and its expensive price and undesirable side-effects prompt researchers to search for cheaper and safer resources [6]. In addition, all available thrombolytic agents suffer significant shortcomings, including large therapeutic doses, limited efficacy, reocclusion, and bleeding complications, thus discouraging their widespread application [1,5,7,20].

To address these problems and improve therapy effectivity, targeting the delivery of these thrombolytic agents to localized diseases has gained increasing interest over the past decade in the field of nanobiotechnology [21–24]. Among them, magnetite (Fe_3O_4) nanoparticles were found to have extensive applications in this field since the 1960s due to their magnetic and electronic properties [25–29], good biocompatibility, low toxicity, and easy drug-binding characteristics by changing their surface properties [30]. To date, the targeting of drug-bearing magnetic particles to a specific part of the body has been studied using magnetic flu-

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ids, unstable suspensions, and magnetic microspheres [31,32]. All of these demonstrate that Fe_3O_4 nanoparticles are ideal for carrying small molecular weight pharmacologically active substances to a target area. In addition, the resulting enzymes conjugated on the magnetic particles have long-term stability and high enzymatic activity [33–35].

In earlier studies, an attempt was made to link streptokinase, a thrombolytic agent, directly to magnetic particles using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) as the coupling agent. The immobilized streptokinase was assayed *in vitro* by lysing the standard fibrin clot. The magnetic properties of the particle–streptokinase congener allow the treatment to be focused to the exact location where the clot was located, thus reducing the amount of enzyme required and, in turn, reducing the risk of eliciting an immune response [24,28]. However, the dynamic process of the formation and lysis of clots have not yet been studied. In particular, the action of nattokinase and lumbrukinase has not been reported to date according to our best knowledge.

In the present study, an attempt was made to link the protein molecules NK and LK directly to magnetic particles using EDC as the coupling agent. Different values of pH and mass ratios of magnetic particles (MNPs) to protein and to EDC were studied in order to establish the optimum conditions for immobilization. Meanwhile, systemic assay of the coagulation and thrombolytic capacities was further investigated, which not only showed the whole process of thrombosis, but also the process of thrombolysis under different conditions.

2. Materials and methods

2.1. Materials

The fibrinogen and thrombin used in this work were obtained from Sigma–Aldrich (St. Louis, MO). The BCA™ Protein Assay Kit was purchased from Pierce Biotechnology (Rockford, IL). The NK (20,000 FU/mg) was obtained from Tianyi Biotech, Ltd. (Xi'an, China), while the LK (16,000 U/mg) was received from Guoyuan Biotech, Ltd. (Shanghai, China). The 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (EDC) was purchased from GL Biochem, Ltd. (Shanghai, China). All solvents and other chemicals were purchased from local commercial suppliers and were of analytical reagent grade, unless otherwise stated. All solutions were prepared using distilled and deionized water.

2.2. Preparation of magnetic Fe_3O_4 nanoparticles

Magnetic Fe_3O_4 nanoparticles were prepared by coprecipitating ferric and ferrous salts in an alkaline solution [25,26,35]. 3 mL $FeCl_3$ (2 M dissolved in 2 M HCl) was first added to 10.33 mL double distilled water, and 2 mL Na_2SO_3 (1 M) was then added dropwisely within 1 min under magnetic stirring. Just after mixing the solutions, the color of the solution changed from light yellow to red, indicating complex ions formed between the Fe^{3+} and SO_3^{2-}. When the solution returned to its original color, 80 mL $NH_3{\cdot}H_2O$ solution (0.85 M) was added under vigorous stirring. A black precipitate quickly formed, which was allowed to crystallize completely for another 30 min under magnetic stirring. The precipitate was washed with deoxygenated water by magnetic decantation until the pH value of the suspension was less than 7.5. After sealing, the prepared Fe_3O_4 was stored in a refrigerator prior to use.

2.3. Conjugation of NK and LK onto magnetic Fe_3O_4 nanoparticles

The conjugation of NK and LK onto magnetic particles was performed using a protocol reported previously [28]. To determine the optimum conditions for NK and LK immobilization, also, the coupling reactions were carried out under different conditions, including the pH value of the reaction mixture and the proportion of magnetic nanoparticles (MNPs) to protein to EDC (MNPs:protein:EDC). All steps were performed at room temperature and in a laminar flow hood to maintain sterility of all reagents. Caution was used in handling biological materials.

For a given value of pH 5.14 and a mass ratio of 2:1:2 (MNPs:NK:EDC), 0.5 mL of NK solution (20 mg/mL, pH 5.14 in 0.003 M sodium and potassium phosphate buffer) was added into 1 mL of EDC solution (20 mg/mL dissolved in the same buffer). Then, 1 mL of magnetic particles (20 mg/mL in the same buffer, pH 5.14) was added to the mixture. After shaking for 24 h in a shaker incubator, the protein-conjugated nanoparticles were separated with a magnet, and were stored in a refrigerator at 4 °C until use. All of the other experiments for coupling condition optimization were also performed following the same procedures described above.

2.4. Analysis of the amount of protein drugs bound onto the nanoparticles

To analyze the amount of protein drugs (NK and LK) bound onto the magnetic nanoparticles, the concentrations of each protein drug in the initial solutions and in the reaction supernatants after immobilization were determined with the BCA™ Protein Assay Reagent Kit using a microplate reader (Bio-Rad Model 680, USA). The amount of protein drug attached onto the magnetic nanoparticles was then calculated as

$$D = \frac{C_i V_i - C_f V_f}{W}$$

where D is the amount of protein drug (NK or LK) bound onto magnetic nanoparticles (mg/mg), C_i and C_f are the concentrations of the initial and final protein drug in the reaction medium (mg/mL), respectively, and V_i and V_f are the volumes of the reaction medium (mL). Herein, W is the weight of the magnetic nanoparticles (mg).

2.5. Characterization methods

The size of Fe_3O_4 nanoparticles and protein-conjugated nanoparticles were characterized by transmission electron microscopy (TEM, JEM-3010, with EDS of Oxford INCA and CCD Camera of Gatan 894, Japan). The crystal structure was characterized by an X-ray diffractometer (XRD, Philips D/Max-2500, Holland) using a monochromatized X-ray beam with nickel-filtered Cu Kα radiation. Magnetic measurements of Fe_3O_4 nanoparticles and protein-conjugated nanoparticles were carried out on a vibrating sample magnetometer (VSM, LAKESHORE-7304, USA) by changing H between +4000 and −4000 Oe. The FT-IR spectra of protein-conjugated nanoparticles were recorded using Fourier transform infrared spectroscopy (Nicolet NEXUS 670, USA).

2.6. Activity measurement

For each drug protein-conjugated nanoparticle sample analyzed for thrombolytic activity, 100 μL of fibrinogen (3 mg/mL) was added into two wells of 96-well Elisa plates (Jet Biofil). After warmed for 3 min at 37 °C, 0.72 μL of thrombin (140 units/mL) was mixed in each of the two wells. Then, the plate was immediately placed in an eight-channel microplate reader (Bio-Rad 680) for dual kinetic absorbance measurements at 405 and 630 nm. Data were recorded continuously at a 60 s interval for 2 h. After that, 100 μL of pure NK or LK and particle–NK or –LK congener solutions (0.2 mg/mL) were added into the mixture, respectively. Followed by 1 s mixing step, the absorbance of the reaction mixtures were then continuously recorded for another 2 h under the same conditions. Each experiment was repeated three times. Blank controls were