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Preparation of thermosensitive magnetic liposome encapsulated recombinant tissue plasminogen activator for targeted thrombolysis

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ABSTRACT

Recombinant tissue plasminogen activator (rtPA) was encapsulated in thermosensitive magnetic liposome (TML) prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, distearolyphosphatidyl ethanolamine-*N*-poly(ethylene glycol) 2000, cholesterol and Fe_3O_4 magnetic nanoparticles by solvent evaporation/sonication and freeze-thaw cycles method. Response surface methodology was proved to be a powerful tool to predict the drug encapsulation efficiency and temperature-sensitive drug release. Validation experiments verified the accuracy of the model that provides a simple and effective method for fabricating TML with controllable encapsulation efficiency and predictable temperature-sensitive drug release behavior. The prepared samples were characterized for physico-chemical properties by dynamic light scattering, transmission electron microscopy, X-ray diffraction and differential scanning calorimetry. Temperature-sensitive release of rtPA could be confirmed from *in vitro* thrombolysis experiments. A thrombolytic drug delivery system using TML could be proposed for magnetic targeted delivery of rtPA to the site of thrombus followed by temperature-triggered controlled drug release in an alternating magnetic field.

1. Introduction

Venous thromboembolism is a silent yet potentially fatal disease that affects millions annually. The best way to improve patient survival and to decrease rate and extent of morbidity is prompt and early treatment of thromboembolism using an effective thrombolytic therapy. Plasminogen activators are used to trigger the dissolution of thrombi (thrombolysis) by catalyzing the conversion of plasminogen to the protease plasmin, which then digests fibrin and lyses the clot. Streptokinase [1,2], urokinase [3,4] and recombinant tissue plasminogen activator (rtPA) [5,6] are plasminogen activators used clinically in the treatment of established thrombus. Since rtPA binds preferentially to plasminogen entrapped in fibrin, it is more effective, safer and useful for thrombolytic therapy [7].

However, plasminogen activators are immunogenic and have short half-lives because of their foreign nature, which requires administration of large doses of rtPA to obtain therapeutic effects and inevitably leads to a significant incidence of hemorrhagic side effects as a consequence [8]. By encapsulating plasminogen activators within novel carrier systems, an increased half-life and decreased immunogenicity might be achieved. Furthermore, targeted delivery of the thrombolytic agent under magnetic guidance for target thrombolysis followed by controlled drug release may reduce the risks of hemorrhage and toxicity associated with systemic administration, thus offering a promising, minimally invasive approach that could decrease the total administered dose of the drug necessary for the treatment and hence its complications [9].

Liposomal vehicle for the delivery of drugs is actively being investigated and has been proposed for thrombus-specific delivery of plasminogen activators to extend the half-life and to reduce hemorrhagic side effects [10,11]. Recent studies showed that thermosensitive liposomes can significantly increase the efficiency of antitumor drug release with hyperthermia, using triggered drug release near the gel-to-liquid crystalline phase transition temperature of the lipids [12]. Upon heating, liquid–solid boundaries start to form in the lipid bilayer and the drug could be released from the internal aqueous compartment or the lipid bilayer [13]. The PEG-derived carriers can reduce the uptake of liposomes by the reticuloendothelial system and are expected to

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prolong the half-lives of plasminogen activators in blood [14]. On the other hand, magnetic liposomes (magnetic nanoparticles encapsulated within liposomes) are widely used to encapsulate drugs and genes [15,16], which could provide an effective magnetic targeted delivery system.

In this work, we propose a more efficient thrombolytic drug delivery system using PEGylated thermosensitive magnetic liposomes (TMLs) for magnetic targeted delivery of rtPA to the site of thrombus followed by temperature-triggered controlled drug release in an alternative magnetic field. rtPA-loaded TMLs were prepared from 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), distearolyphosphatidyl ethanolamine-*N*-poly(ethylene glycol) 2000 (DSPE–PEG_{2000}) and cholesterol by solvent evaporation/sonication and freeze-thaw cycles method. The object was to optimize the formulation of TML–rtPA using response surface methodology (RSM) [17,18]. The experimental parameters or variables considered were the molar percentage of DPPC/lipid, DSPE-PEG/lipid and cholesterol/lipid, and the molar ratio of lipid/magnetic nanoparticle and the results of the experiments were the drug encapsulation efficiency (EE) and the release percentages of rtPA at 37 °C and 43 °C. The effects of the variables were investigated within the context of RSM that incorporates design of experiments and non-linear regression. This approach enables experimental investigation of individual factors and the interactions of factors simultaneously as opposed to one-factor-at-a-time approach. Models of EE and release percentage of rtPA at 37 °C and 43 °C were constructed, which could allow the evaluation of the significance of the parameters and provide the prediction capability for EE and temperature-modulated drug release.

2. Materials and method

2.1. Materials

Fe(II) chloride tetrahydate (99%) and Fe(III) chloride hexahydrate (97%) were purchased from Acros. DPPC was acquired from Avanti Polar Lipids, Inc. (USA). DSPE-PEG_{2000} was obtained from NOF Co. (Japan). Cholesterol and Triton X-100 were purchased from Sigma-Aldrich Co. (USA). Recombinant tissue plasminogen activator (rtPA) was purchased from Boehringer Ingelheim (Germany). All chemicals were of reagent grade and used without further purification.

2.2. Synthesis of iron oxide (Fe_3O_4) magnetic nanoparticles (MNPs)

The chemical co-precipitation method of Fe^{2+} and Fe^{3+} ions (molar ratio of 1:2) by addition of NH_4OH was used for synthesis of Fe_3O_4 magnetic nanoparticles (MNPs) [19]. 0.875g of $FeCl_2{\cdot}4H_2O$ and 2.375g of $FeCl_3{\cdot}6H_2O$ were mixed in 40 ml of double-distilled water (DDI water) and stirred at 400 rpm in a three-neck flask. The temperature was increased to 60 °C in refluxing condition under N_2 atmosphere and kept for 10 min. Five milliliters of 25% NH_4OH was added and pH of the solution was maintained at 10 with vigorous stirring for 60 min. The black-colored colloidal MNPs were recovered by magnetic separation and dialyzed against DDI water (3.5 kDa MWCO) to remove excess of NH_4OH.

2.3. Preparation of thermosensitive magnetic liposomes (TML) and rtPA-encapsulated TML (TML-rtPA)

The TML-rtPA was prepared by the solvent evaporation/freeze-thaw method [20,21]. Lipid mixtures of DPPC, DSPE-PEG_{2000} and cholesterol in various molar composition were dissolved in 1 ml chloroform/methanol solution (2:1, v/v) to form a 10 mM solution. The organic solvent was removed using a rotary evaporator (EYELA N-1200AVF, Japan) at 100 psi and 45 °C with a water bath for 10 min. The solvent was then completely removed in a vacuum oven overnight to from a thin phospholipid film on the wall of the round-bottom flask. The resulting dry film was hydrated with 1 ml of DDI water containing various concentrations of MNP and 0.1 mg/ml rtPA and rotated for 60 min. The solution was removed and sonicated at 200 W for 5 min using a Q500 sonicator (Qsonica, Newton, CT, USA) at 4 °C in a water bath. The sample was flash frozen in liquid nitrogen and thawed completely at room temperature. This freeze-thaw cycle was repeated five times for maximum encapsulation of rtPA. Finally, un-encapsulated rtPA was removed by ultracentrifugation for 30 min at 30,000 rpm and 20 °C.

2.4. Determination of encapsulation efficiency of rtPA in TML-rtPA

The rtPA encapsulation efficiency in TML-rtPA was determined using a Protein Assay Kit from Bio-Rad (USA). The TML-rtPA was disrupted with 1% Triton X-100 solution and diluted with 0.01 M phosphate buffer (pH 7.4) before measurements. The encapsulation efficiency of rtPA was calculated from the following equation,

$$\text{Encapsulation efficiency}(\%) = \frac{W_E}{W_{total}} \times 100 \quad (1)$$

where W_{total} is the amount of rtPA added and W_E is the amount of rtPA encapsulated.

2.5. In vitro release of rtPA

The *in vitro* release behavior of rtPA from TML-rtPA was determined as follows. TML-rtPA (5 mg) was prepared in 1 ml of phosphate buffered saline (PBS, pH 7.4) at 37 °C or 43 °C and shaken at 120 rpm. At predetermined time, the solution was centrifuged at 30,000 rpm for 30 min and 0.5 ml of the supernatant was removed. The solution was supplemented with 0.5 ml of fresh PBS, re-suspended and incubated at the same condition as before. The amount of rtPA in the supernatant was determined by the Protein Assay Kit as described before. The percentage of rtPA released was calculated using the following equation,

$$\text{Release of rtPA}(\%) = \frac{W_{release}}{W_E} \times 100 \quad (2)$$

where W_E is the amount of rtPA encapsulated and $W_{release}$ is the amount of rtPA released.

2.6. Experimental design

The RSM as a generic means for optimization was applied to optimize the formulation of TML-rtPA [22]. The optimization was designed based on a five-level, four-factor central composite rotatable design (CCRD) with a total of 31 experimental runs that combined 16 factorial points, 8 axial points and 7 replicates at the center points. Based on the preliminary experiments and our previous studies, the effect of four formulation independent variables, DPPC/lipid molar percentage (X_1), DSPE-PEG2000/lipid molar percentage (X_2), cholesterol/lipid molar percentage (X_3) and lipid/MNP molar ratio (X_4) were identified as key factors responsible for EE and rtPA release percentage after 60 min at 37 °C and 43 °C. In view of the feasibility of liposome preparation and drug loading ratio for clinical doses, the ranges of the four factors were determined as follows: X_1, 60/92; X_2, 1/5; X_3, 5/33; X_4, 100/1000 (Table 1). Each experimental run was performed in duplicate except at the central point (25–31 runs) of the design. According to the mathematical model, the response surface could be related to the selected variables by a second-order polynomial model as in the following equation,

$$Y_i = \alpha_0 + \sum_i \alpha_i X_i + \sum_i \alpha_{ii} X_i^2 + \sum_{i \neq j} \alpha_{ij} X_i X_j \quad (3)$$

where Y_i represents the predicted responses, X_i and X_j are the coded values of independent variables, α_0 is the intercept coefficient, α_i are