Journal of Pharmaceutical Sciences xxx (2016) 1–8

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Research Article

One-Step Self-Assembling Method to Prepare Dual-Functional Transferrin Nanoparticles for Antitumor Drug Delivery

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ARTICLE INFO

Article history:
Received 22 July 2015
Revised 1 December 2015
Accepted 8 December 2015

Keywords:
nanotechnology
drug delivery systems
protein folding/refolding
proteins
self-assembly
solubility
targeted drug delivery

ABSTRACT

Protein-based nanoparticles hold great promise in both preclinical and clinical practices due to their high biocompatibility and biodegradability. However, the complicated preparations often denature proteins which subsequently diminish their bioactivity. To overcome these drawbacks, we developed a one-step self-assembling method for preparing protein-based nanoparticles. Transferrin (Tf), a targeting protein, was mixed with 2-mercaptoethanol to break disulfide bonds. Using this method, Tf-PTX-NPs (paclitaxel-loaded Tf nanoparticles) could be readily obtained. Tf-PTX-NPs were round and their diameter could be controlled in the range of 5-200 nm. The bioactivity of Tf to its receptor after forming nanoparticles was also confirmed *in vitro*. Tf-PTX-NPs also could inhibit the tumor growth to some extent in a mice tumor xenograft model. Therefore, using this self-assembling method, we fabricated this antitumor Tf-based nanoparticle, in which Tf acted as both the targeting moiety and drug carrier.

Introduction

Polymeric nanoparticles have brought new opportunities as a new platform for delivering therapeutics or diagnostic agents. Previously, many drug delivery systems with proven therapeutics failed in clinic because of their poor solubility, bioavailability, or therapeutic effectiveness. The use of polymeric nanoparticle is a promising strategy to overcome these challenges. The nano-size allows the access of carrier to biological matrixes of similar size (e.g., tumor tissue). An example of such a formulation is Genexol-PM (Samyang Corporation), a micellar paclitaxel (PTX) formulation consisting of polyethylene glycol and poly(D,L-lactic acid).[1] Preclinical *in vivo* studies with Genexol-PM demonstrated a 3-fold increase in the maximal tolerance dose and a significantly increased antitumor efficacy compared with free PTX.[2] However, side effects still exist, such as peripheral sensory neuropathy, arthralgia, or hypersensitivity reactions.[3] The lack of active targeting in polymeric nanoparticles might cause such nonspecific toxicity and therefore limit their further clinical uses.

Targeting polymeric nanoparticles have thus been devised in an attempt to reduce side effects, improve specificity, and enhance tumor accumulation. This strategy mainly involves coupling of polymeric nanoparticles with monoclonal antibodies, folate, transferrin (Tf), luteinizing hormone-releasing hormone, epidermal growth factor, and so forth.[4] Folate-conjugated polymeric nanoparticle is such an example. Folate behaves as a ligand having high affinity for its receptor, folate-binding protein, that is overexpressed on the cell surface of many tumor types.[5] The folate-conjugated polymeric nanoparticle can hence be directed to the tumor cells in the body and internalized in the target cells via receptor-mediated endocytosis. However, so far no polymeric nanoparticle has been approved by Food and Drug Administration for intravenous drug delivery, most likely due to the potential side effects of polymers. Additionally, the manufacturing complexity of targeting polymeric nanoparticles may also create a significant hurdle for generic drug companies to develop equivalent therapeutics.[6]

Proteins such as gelatin,[7] casein,[8] and albumin[9,10] have been widely studied for delivering drugs to human body owing to their high biocompatibility and biodegradability. Albumin is one of the

This article contains supplementary material available from the authors by request or via the Internet at http://dx.doi.org/10.1016/j.xphs.2015.12.007.

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http://dx.doi.org/10.1016/j.xphs.2015.12.007
0022-3549/

most abundant plasma proteins. It keeps stable in the pH range of 4-9 and soluble in 40% ethanol.[11-13] These properties make albumin an ideal carrier for drug delivery. The successful protein-based nanoparticle is Abraxane™, which is PTX-loaded albumin nanoparticle with a mean particle size of approximately 130 nm and which was approved by the Food and Drug Administration for the treatment of breast cancer in 2005.[13] Because the mixed solvent Cremophor EL/ethanol used in Taxol™ was replaced by albumin, the solvent-related hypersensitivity reactions were significantly reduced.[14] In preclinical and clinical practices, Abraxane showed higher tolerance and efficacy than Taxol™.[13-16]

Besides albumin, Tf is another ideal carrier for drug delivery. It is responsible for the transport of iron into the cells by binding to Tf receptor (TfR). TfR overexpression is confirmed in many human tumor types including breast cancer and prostate cancer.[17,18] In the past few decades, Tf-TfR–mediated drug delivery has been widely used for targeting delivery of drugs to tumor.[19,20] A case in hand is the recently announced completion of phase I tolerability evaluation of transferrin-siRNA nanoparticles (CALAA-01),[21] a Tf-targeted RNAi nanotherapeutic for delivering siRNA to reduce the expression of the M2 subunit of ribonucleotide reductase (R2) for solid tumor therapy. Results showed that Tf could specifically deliver cargoes to tumor cells after systemic administration and could reduce the nonspecific effects. Therefore, using Tf as drug carrier might show great promise because it could function in 2 ways: (1) encapsulate small molecules and (2) recognize and target tumor cells.

In the past several years, many methods of preparing protein-based drug nanocarrier have been reported. Taking albumin nanoparticles as an example, the preparation methods include desolvation-crosslinking[22-24] and emulsification.[25] For the conventional desolvation-crosslinking method, ethanol is commonly used to remove hydrated sheath of molecules, which are forced to form unstable nanoparticles. Then glutaraldehyde irreversibly cross-linked the amino groups of so that the nanoparticles could form stably. For the emulsification preparation method, it mainly includes dissolving a hydrophobic drug into organic solution and emulsifying them into protein aqueous solution by a homogenizer. After evaporating the organic solution, drug-loaded nanoparticles would form. However, both the desolvation-crosslinking and emulsification methods are not suitable to prepare dual-functional Tf nanoparticles because glutaraldehyde and high pressure homogenization would denature Tf and thus lose their targeting effects.

Previously, we reported a self-assembly method to prepare protein nanoparticles.[26-29] In the method, disulfide bonds and compact structures of albumin were temporarily broken down to expose the drug binding sites. PTX was then added to formed nanoparticles. However, albumin is a nontargeting protein which cannot specifically deliver drugs to tumor. In this study, we are aiming to use a targeting protein, Tf, as both drug carrier and targeting moiety (dual-functional) to enhance the therapeutic effects. Because no glutaraldehyde and high pressure homogenization was involved in this preparation method, the Tf bioactivity would not be affected. By using this method, a high drug-loading efficiency could be achieved. The binding activity of formed Tf nanoparticles to its receptor was determined by cell binding assay and flow cytometry. The targeting effects and antitumor efficacy were also evaluated.

Materials and Methods

Materials

Human Tf, Coumarin-6 (Cou6) was obtained from Sigma-Aldrich Chemical Co. PTX was purchased from Hongdoushan Co. Ltd. (Jiangsu, China). Taxol™ was obtained from Jiangsu Province Hospital (Nanjing, China). Human breast cancer cell line (MCF-7) was purchased from Shanghai Institute of Cell Biology (Shanghai, China).

Preparation and Characterization of PTX-Loaded Tf Nanoparticles

PTX-loaded Tf nanoparticles (Tf-PTX-NPs) were prepared via a molecular switch method as described previously.[26] Twenty milligrams of Tf was dispersed in 10 mL of distilled water (37°C) with constant stirring at a speed of 600rpm. Then 50 μL of β-mercaptoethanol (β-ME) was added. After 10 min stirring, PTX (10 mg/mL, dissolved in ethanol) was added into the protein solution drop by drop to form Tf nanoparticles. Then the nanoparticle solution was ultrafiltrated (ultrafiltration membrane MW 30 KDa, Millipore) for 5 times to completely remove unbound PTX and β-ME. Finally, the concentrated solution was freeze-dried to obtain Tf-PTX-NPs for further use.

To illustrate the physical character of formed nanoparticles, we selected the Tf nanoparticles with a proportion of 12:100 (PTX:Tf, wt/wt) for analysis. The morphology of nanoparticles was analyzed by scanning electron microscope (SEM). Samples were coated in a cathodic evaporator with a fine gold layer and observed under S-3400N II SEM (Hitachi, Japan). Nanoparticles were also observed under a transmission electron microscope (TEM, EM-200CX, JEOL, Japan). Nanoparticles were dried on a copper grid coated with amorphous carbon and observed at 200kV. Size distribution of Tf-PTX-NPs were measured by dynamic light scattering (DLS) using a Brookhaven 90 plus system (Brookhaven Instruments Corporation). The solid-state of PTX in Tf -NPs was studied using powder X-ray diffraction (XRD, ARL, Switzerland). The XRD patterns for pure PTX, Tf and physical mixture of PTX and Tf (12:100) were also observed for comparison. All samples were ground into fine powder before it was measured. To evaluate physical stability, Tf-NPs-PTX (30 mg) was dissolved in phosphate-buffered saline (PBS) (7.4), 5% glucose at 25°C, and also in fetal blood serum (10%) at 37°C. At different time intervals, the nanoparticle size distribution was recorded by DLS. In addition, Taxol™ and Tf-NPs-PTX were both diluted with 2-mL fetal blood serum to inspect their compatibility with serum. Both Taxol™ and Tf-NPs used here contained 6 mg/mL PTX. Then, we evaluated the chemical stability of PTX in nanoparticles at different times (0 h and 48 h) after nanoparticles prepared by related substances analysis using high-performance liquid chromatography assay.

Characterization of Nanoparticle Formation

To demonstrate how PTX molecules interact with Tf, synchronous fluorescence was used to evaluate the interaction between PTX and protein molecules. The fluorescence of tyrosine and tryptophan can be detected simultaneously by fixing the scanning wavelength intervals $\Delta\lambda$ ($\Delta\lambda = \lambda\text{emission} - \lambda\text{excitation}$) as 15 nm or 60 nm, respectively. β-ME was added into Tf aqueous solution (2 mg/mL) which was then stirred at 37°C for 10 min. Subsequently, different amount of 10 mg/mL PTX ethanol solution (0%, 4%, 8%, 12%, 16%, 20%, 24%, 28%, and 32%, PTX:Tf, wt/wt) was gradually added, and the resulted solution was tested by synchronous fluorescent detection and DLS. In control group, the corresponding amount of ethanol was gradually added.

Free Tf Blocks Tf-PTX-NPs Binding Activity

MCF-7 cells were seeded into 6-well plates (Corning) at 1.0×10^5 cells/well and attached for 24 h before use. Competitive binding effects of endocytosis was studied by preincubating the cells with excess free Tf (400 μg/mL, 20:1 = free Tf:Tf-PTX-NPs, 100 μg/mL,