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Mechanisms of transcellular transport of wheat germ agglutinin-functionalized polymeric nanoparticles in Caco-2 cells

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ABSTRACT

Transcellular transport is essential for transmucosal and plasma-to-tissue drug delivery by nanoparticles, whereas its fundamental pathways have not been fully clarified. In this study, an in-depth investigation was conducted into the intracellular itinerary and the transcytosis pathway of wheat germ agglutinin-functionalized nanoparticles (WGA-NP) with various polymer architectures in the Caco-2 cell model. GFP-Rabs, Rab4, Rab5, Rab7, Rab11, GTPases served as key regulators of vesicular transport, and their mutants were transfected to Caco-2 cells respectively to determine the cellular itinerary of WGA-NP and the role of Rabs therein. Transcytosis inhibition experiments indicated that transcellular transport of WGA-NP (PEG_{3000}-PLA_{40000} formulation) happened in a cytoskeleton-dependent manner and majorly by means of clathrin-mediated mechanism. Intracellular transport, especially the endolysosome pathway was found largely contribute to the transcytosis of WGA-NP. WGA-NP with shorter surface PEG length (2000) resulted in higher cellular association and more colocalization with the clathrin-mediated transport pathway, while that with longer surface PEG length (5000) avoided the clathrin-mediated transport pathway but achieved higher transcytosis after 4 h incubation. WGA-NP with PLGA as the core materials obtained elevated lysosome escape and enhanced transcytosis after 2 h incubation. These findings provided important evidence for the role of polymer architectures in modulating cellular transport of functionalized nanocarriers, and would be helpful in improving carrier design to enhance drug delivery.

1. Introduction

Development of nanoparticulate drug delivery systems (DDS) has provided a promising strategy for obtaining desirable biopharmaceutic and pharmacokinetic properties for medicines and become one of the most important areas of nanomedicine [1–3]. Enhanced drug solubility/stability, prolonged systemic circulation and target-specific accumulation have been achieved by the application of nanotechnology [4]. However, numerous biological barriers exist to protect human body from invasion of foreign particles [5], among which cellular barrier is the most studied one [6]. It is believed that successful delivery of drugs by nanocarriers largely depends on their safe and efficient cellular transport. Endocytosis mechanisms for various nanoparticulate DDS have been extensively investigated to optimize efficient intracellular drug delivery [7,8]. However, pathways utilized for the transcellular transport of nanoparticles, a critical process for transmucosal and plasma-to-tissue drug delivery has less been clarified [9–11] and remains a clear and immediate need for improved carrier design and enhanced drug delivery.

Development of functionalized nanocarriers obtained by attaching ligands such as antibodies, glycoproteins, peptides and carbohydrates to the surface of nanocarriers for enhanced and selective delivery of medicines to target areas was now a particular interest in pharmaceutical sciences. Lectins, proteins or glycoproteins of nonimmunological origin, recognizing sugar molecule specifically and therefore capable of binding glycosylated membrane components, have been developed to conjugate with nanocarriers for efficient drug delivery [12,13]. Wheat germ

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agglutinin (WGA), a lectin from *Triticum vulgare*, specifically binding to N-acetyl-D-glucosamine and sialic acid which is abundantly observed in both intestine [12] and nasal cavity [14], has been exploited to functionalize nanoparticles for improved bioavailability and site-specific drug delivery [15–18]. Previous studies in our lab and others have indicated that cellular uptake of WGA-functionalized nanocarriers began with binding of WGA to its receptor at the cell surface; the subsequent endocytosis happened by means of both clathrin- and caveolae-mediated mechanisms [19,20]. However, to date a fundamental understanding about the mechanism of transcellular transport of WGA-functionalized nanocarriers as well as its modulation by the polymer architectures is still lack, and remains a significant obstacle to nanocarrier optimization.

The aim of this study was to characterize the mechanism of transcellular transport of WGA-modified nanoparticles (WGA-NP), as a model of functionalized nanocarriers, for improved carrier design and enhanced transcellular drug delivery. For this purpose, quantum dots (QDs), fluorescent semiconductor nanocrystals characterized by high extinction coefficients, reduced photobleaching rates and narrow size-tunable emission spectra [21], and a highly hydrophobic fluorescent probe – coumarin-6, were used as the fluorescent probes. The fluorescent probe was encapsulated into poly (ethylene glycol) – poly (lactic acid) (PEG-PLA) or PEG – poly (lactic-co-glycolic acid) (PEG-PLGA) nanoparticles via an emulsion/solvent evaporation technique. The obtained nanoparticles were functionalized with WGA via a maleimide-mediated covalent binding procedure. Particle size distribution, zeta potential, internal structure of the obtained WGA-NP and the probing ability of the fluorescent probes were determined. Caco-2 cell, human colon carcinoma cell with WGA-binding sugars extensively expressed on the cell surface [18], was used as the cell model. Plasmids GFP-Rab4, Rab5, Rab7, Rab11, GTPases serve as key regulators of vesicular membrane transport and locate at distinct intracellular organelles, and their mutants (Rab4 S22N, Rab5 S34N, Rab7 Q67L and Rab7 T22N), were transfected to Caco-2 cells respectively to determine the cellular itinerary of WGA-NP and the role of Rabs therein. Transcytosis inhibition experiments were performed to clarify the mechanism of transcellular transport of WGA-NP. Permeability of WGA-NP across Caco-2 monolayer with stable Rab5 gene silencing was also determined to evaluate the role of Rab5 in WGA-NP transcytosis. For nanocarrier optimization, effects of polymer architectures such as surface PEG length and core material on the cellular itinerary and transcytosis of WGA-NP was evaluated, and the related mechanisms were discussed.

2. Materials and methods

2.1. Materials

Methoxy-PEG-PLA/PLGA$_{40000}$ and maleimide-PEG-PLA/PLGA$_{40000}$ block copolymers were synthesized by ring opening polymerization of LA or LA/GA (75:25) initiated by methoxy-PEG (2000, 3000 or 5000 Da) and maleimide-PEG (2000, 3000 or 5000 Da), respectively, using stannous octoate as the catalyst according to the method described previously [16]. High-quality, hydrophobic tri-n-octylphosphine oxide (TOPO)-QDs (7.2 nm, E_m 607 nm) were prepared at elevated temperatures in TOPO as reported previously [22]. WGA was obtained from Vector Laboratories; 2-iminothiolane hydrochloride (2-IT), from Sigma; 5,5-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), from Acros (Belgium); Coumarin-6, from Sigma–Aldrich (St. Louis, MO, USA); Dulbecco's Modified Eagle Medium (DMEM) (high glucose) and fetal bovine serum (FBS) from Gibco (Invitrogen, USA). NaN_3 was kindly provided by Department of Medicinal Chemistry, School of Pharmacy, Fudan University, and chlorpromazine by Shanghai Mental Health Center. Filipin was purchased from Fluka (Germany), and monensin, nocodazole, cytochalasin D and Brefeldin A from *Penicillium brefeldianum* (BFA) from Sigma (USA).

Plasmid construction: GFP-Rab4, GFP-Rab5, GFP-Rab7 and GFP-Rab11 were constructed by PCR from human universal cDNA library as described previously [23]. All mutant Rab GTPases plasmids were constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). pGPU6/GFP/Neo siRNA expression vectors for Rab5 were obtained from Gene Pharma (Shanghai, China). The sequence integrity of the DNA constructs was confirmed by DNA sequencing.

2.2. Nanoparticles preparation and characterization

2.2.1. Preparation of WGA-conjugated QDs/coumarin-6-loaded PEG-PLA/PLGA nanoparticles

QDs/coumarin-6-loaded PEG-PLA/PLGA nanoparticles (NP) were prepared with a blend of methoxy-PEG-PLA/PLGA and maleimide-PEG-PLA/PLGA using an emulsion/solvent evaporation technique [16]. Briefly, 9 mg methoxy-PEG-PLA/PLGA and 1 mg maleimide-PEG-PLA/PLGA were dissolved in 0.5 ml of dichloromethane containing 2×10^{-6} mol/l of QDs or 1–10 μg of coumarin-6. The solution was then emulsified by sonication (220–280 W, 30 s) on ice in a 2 ml of 1% aqueous sodium cholate solution using probe sonicator (Ningbo Scientz Biotechnology Co. Ltd., China). In order to achieve similar particle size for the formulations, sonication power at 220, 240 and 280 W was used for the preparation of WGA-NP with the matrix of PEG$_{2000}$-PLA/PLGA, PEG$_{3000}$-PLA and PEG$_{5000}$-PLA/PLGA, respectively. The emulsion obtained was diluted into 8 ml of a 0.5% aqueous sodium cholate solution under moderate magnetic stirring. Five minutes later, dichloromethane was evaporated under low pressure at 30 °C using Büchi rotavapor R-200 (Büchi, Germany). The nanoparticles were centrifuged at 21,000 g for 45 min using TJ-25 centrifuge (Beckman Counter, USA.) equipped with an A-14 rotor. With supernatant discarded, the obtained nanoparticles were resuspended in distilled water and subjected to a 1.5 × 20 cm sepharose CL-4B column for removing the unentrapped QDs/coumarin-6. WGA was conjugated to the surface of nanoparticles via a maleimide-thiol coupling reaction as described previously [16].

2.2.2. Characterization of WGA-NP

Morphological examination of WGA-NP was performed via transmission electron microscopy (TEM) (Philips CM120) following negative staining with sodium phosphotungstate solution. Particle size and zeta potential of the obtained nanoparticles was determined via dynamic light scattering (DLS) analysis using Zeta Potential/Particle Sizer NICOMPTM 380 ZLS (Santa Barbara, California, USA.) with He–Ne lamp at 632.8 nm, respectively. Freeze-fracture electron microscopy analysis was performed under a JEOL 2010 microscope (JEOL, Japan) to visualize the internal structure of the QDs-loaded WGA-NP (WGA-QDs-NP).

WGA level on the nanoparticle surface was determined with a bicinchoninic acid (BCA) protein assay kit using unmodified nanoparticles at the same concentration as the blank. Hemagglutination test was performed to determine whether QDs/coumarin-6 was an appropriate probe to WGA-NP. Briefly, QDs/coumarin-6-loaded NP and WGA-NP (10 mg/ml, 50 μl) were incubated with 4% (v/v) suspension of fresh rat blood in 0.9% NaCl (200 μl) at 37 °C for 30 min and 24 h, respectively. After that, the mixtures were observed under a fluorescence microscope (Olympus IX71) with images taken and colored with Image-Pro Plus.

2.3. Cell experiments

2.3.1. Cell culture and transfection

Caco-2 cells were obtained from American Type Culture Collection (Rochville, MD, USA) and grown in a 5% CO_2 humidified atmosphere at 37 °C in DMEM medium supplemented with 10% FBS, 1% non-essential amino acids, 100 units/ml penicillin and 100 μg/ml streptomycin.

Transfection of Caco-2 cells was performed with the application of lipofectamine™ 2000 reagent (LF2000) (Invitrogen, USA) following the manufacturer's instruction [24]. Caco-2 cells were seeded in 24-well plates at the density of 5×10^4 cells/well and allowed to culture for 24 h before transfection. For each well, 0.8–1.5 μg (1–1.5 μg optimal) of DNA and 3 μl of LF2000 were firstly diluted into 50 μl of OPTI-MEM® Reduced Serum Medium (Invitrogen, USA), respectively, incubated for 10 min, and then mixed and further incubated at room temperature for 25 min to allow the formation of DNA-LF2000 complexes. The resulted complexes (100 μl) were added into each well and the cells were further cultured at 37 °C for 24–48 h before analysis.

To obtain a population of Caco-2 cells with stable Rab5 gene silencing, the cells were transfected with a Rab5 siRNA constructed-in GPU6/GFP/Neo expression vector, allowed to growth for 48 h, and then treated with 900 μg/ml G418 for a week and 300 μg/ml G418 thereafter. The gene silencing efficiency was determined by Realtime PCR and western blot as described previously [25].

2.3.2. Intracellular distribution of WGA-NP and the role of Rab GTPases in cellular internalization of WGA-NP

For charactering the intracellular distribution of WGA-NP, Caco-2 cells were plated onto multiple glass-bottom tissue culture plates (MatTek, Ashland, MA) and transfected with GFP-Rab4, Rab5, Rab7 and Rab11, respectively, using LF2000 as the transfection agent. Thirty-six hours later, WGA-QDs-NP (200 μg/ml nanoparticles prepared from PEG$_{3000}$-PLA$_{40000}$, containing 20 pM QDs) was added to the transfected cells and incubated for 2 h. After that, the cells were subjected to fixation, washing and examination. Colocalization between WGA-QDs-NP and GFP-Rab4, 5, 7 and Rab11 were determined under a confocal microscope (Zeiss LSM 510) and analyzed with Imaris 7.1 (Biplane).