International Journal of Pharmaceutics 500 (2016) 42–53

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

The transport mechanism of integrin $\alpha_v\beta_3$ receptor targeting nanoparticles in Caco-2 cells

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ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 23 December 2015
Accepted 14 January 2016
Available online 16 January 2016

Keywords:
Transport mechanism
Nanoparticles
FQSIYPpIK
Oral delivery
Protein and peptide drugs
Epithelium

ABSTRACT

As for the existence of epithelium barrier, accelerating the transport remains huge challenges for orally delivered protein and peptide drugs into blood circulation. Modifying nanopaticles (NPs) with targeting peptides can enhance the intestinal absorption of loaded macromolecular drugs. However, the transport process, which mainly means how the NPs pass through the apical membrane and the basolateral side and then enter into blood circulation, is needed comprehensive investigation. In this study, we systemically studied the transport mechanisms in Caco-2 cell model of trimethyl chitosan based NPs (TMC NPs) before and after modification of FQS, an integrin $\alpha_v\beta_3$ receptor targeting peptide. Our results showed FQS peptide mediated multiple endocytosis pathways and could activate integrin $\alpha_v\beta_3$ receptor by interacting with FAK and Src-family kinases to induce receptor-mediated endocytosis of the NPs. Then, both endocytosed NPs could transport from early endosome to lysososmes via late endosomes/lysosome pathway, as well as to recycling endosomes and Golgi apparatus through early endosome/recycling endosomes and Golgi apparatus/recycling endosomes/plasma membrane pathways, respectively. After FQS peptide modification, the endocytosis subpathways of NPs have been changed, and more pathways are involved in exocytosis process for FQS-modified NPs compared with non-modified NPs. Our study indicated the ligand modification could enhance the uptake and transport by altering some pathways in whole transport process of NPs.

1. Introduction

Oral administration of protein and peptide drugs is considered to be a preferable choice for patients owing to the good compliance and superior safety (Delie and Blanco-Prieto, 2005). To improve the bioavailability, nanoparticles (NPs) have been developed in the recent decades because of their protective effect against enzymatic and hydrolytic degradation (des Rieux et al., 2006). Since NPs need to resist the intestinal epithelium, a major absorption barrier, the oral absorption of encapsulated drugs typically remains low, despite some promising results obtained (Rieux et al., 2013). Obviously, how to conquer the epithelium barrier and accelerate the transport of drugs into blood circulation are still the great challenges for orally administered protein and peptide drugs in clinic.

The transport process across the intestinal epithelial cells includes paracellular transport and transcelluar transport. The former is a pathway in which substances pass through the intercellular spaces between epithelial cells (Ballard et al., 1995). The transcytosis of NPs involves endocytosis, intracellular trafficking and exocytosis. Endocytosis, a mechanism common to enter cells in the first step, internalizes macromolecules or foreign particles and retains them in transport vesicles that traffic along the endolysosomal scaffold (Bareford and Swaan, 2007). An array of vesicular internalization mechanisms exist such as macropinocytosis, clathrin-mediated and caveolae-mediated endocytosis. It was reported that the physicochemical properties of the particles could influence their uptake. For example, the smaller particles had a higher amount of uptake (He et al., 2012). Up to now, the NP surface conjugating a ligand, which favors cell–NP interactions and then enhances NP internalization, is deemed to be a good strategy to enhance NP uptake by overcoming epithelial barrier (Yun et al., 2013). Also, the mechanism of uptake will affect subsequent intracellular trafficking and other cellular events (Bareford and Swaan, 2007).

In fact, the ultimate goal of using oral protein and peptide drugs loaded NPs is that drugs can enter into the blood circulation. Hence, NPs need to undergo a transport process from the apical membrane to the basolateral side. Namely, NPs may enter into

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http://dx.doi.org/10.1016/j.ijpharm.2016.01.028
0378-5173/

cytoplasma via the endocytosis, intracellularly transport, and finally exit from apical and basolateral membrane via exocytosis. However, current studies on the transport mechanism are mostly focused on the uptake pathway (Sahay et al., 2010). Therefore, the systemic investigation including the cellular uptake, NP trafficking inside the cells and the exocytosis of the delivered drugs from basolateral membrane into blood circulation during the transport across the epithelial barrier, is very important.

FQSIYPpIK (FQS) peptide, identified from PNA-encoded peptide library, exhibits high affinity to integrin $\alpha_v\beta_3$ receptor (Svensen et al., 2011). Our group has recently developed an insulin-loaded FQS-modified NP. The results showed that FQS-NPs significantly enhanced intracellular uptake and transport in Caco-2 cells model. The oral bioavailability of modified NPs with a prominent hypoglycemic response was 25-fold and 1.42-fold higher than that of free insulin and unmodified NPs respectively (Liu et al., 2015). However, the molecular mechanisms of their endocytosis, intracellular trafficking and exocytosis are kept unknown.

Therefore, in the present study, the mechanism of cellular transport of FQS peptide modified TMC NPs was investigated. FQS peptide was conjugated to the TMC materials (FQS-TMC), and then the cationic TMC/FQS-TMC was cross-linked with sodium tripolyphosphate (TPP) and insulin to prepare TMC/FQS-TMC NPs. Caco-2 cell, a human colon carcinoma cell which integrin $\alpha_v\beta_3$ receptor was extensively expressed on the surface, was used as the cell model (Hsu et al., 2013). The transport mechanisms of the NPs including endocytosis, intracellular trafficking, exocytosis and paracytosis were explored.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree >90% and molecular weight of 400 kDa) was provided by AK Biotech Co., Ltd. (Shandong, China). Porcine insulin was purchased from Wanbang Bio-Chemical Co., Ltd. (Jiangsu, China). FQSIYPpIK peptide was chemically synthesized by Chinese Peptide Co., Ltd. (Hangzhou, China). 1-[3-(Dimethylamino)propyl]-3-ethylaarbodiimide hydrochloride (EDC·HCl) was gained from Meapeo Co., Ltd. (Shanghai, China). *N*-Hydroxysuccinimide (NHS), *N*-methylpyrrolidone, iodomethane and acetonitrile were all obtained from Kelong chemical Co., Ltd. (Chengdu, China). Fluorescein isothiocyanate (FITC), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), filipin, chlorpromazine and nocodazole were all purchased from Sigma–Aldrich (St. Louis, MO). NVP-TAE226, PP2 and rottlerin were all purchased from Selleck Chemicals (Shanghai, China). Rhodamine-phalloidine, Lyso-Tracker Red, Mito-Tracker Red and mouse anti-claudin-4 antibody were all purchased from Invitrogen (Carlsbad, CA). ER-Tracker Red, Golgi-Tracker Red, brefeldin A, monensin and LY294002 were all acquired from Beyotime (Haimen, Jiangsu, China). Amiloride, mouse anti-integrin $\alpha_v\beta_3$, rabbit anti-ZO-1, rabbit anti-Rab5, mouse anti-Rab7 and rabbit anti-Rab11 antibodys were all obtained from Abcam (Cambridge, MA, USA). All chemical regents utilized in study were analytic grade.

2.2. Synthesis of TMC and FQS-***TMC***

N-Trimethyl chitosan chloride (TMC) was obtained by methylation the amine groups of chitosan (CS) with methyl iodide (CHI_3) in sodium hydroxide (NaOH) using *N*-methylpyrrolidone (NMP) as solvents. The reaction proceeded for 45 min at 60 °C. After that, the product was purified by dialysis (MW cut off 8000–14,000 Da) and then lyophilized (Free Zone 2.5 L, LABCONCO Inc., USA) (Jin et al., 2012a). The degree of quaternization (DQ) was calculated from the integration of ^{1}H NMR (Sieval et al., 1998). The obtained TMC was conjugated with FQS peptide via amide bond formed among the residual primary amino groups on TMC and carboxyl groups on FQS peptide. Briefly, TMC (0.25 mmol) was dissolved in 10 mL of water, in which EDC·HCl (0.3 mmol), NHS (0.3 mmol), FQS peptide (0.05 mmol) were added. The reaction was conducted at ambient temperature for 3 days in dark. Then, the product was dialyzed (MW cut off 8000–14,000 Da), lyophilized and stored at 4 °C. The obtained TMC-FQS was identified by ^{1}H NMR and the content of the conjugated peptide was determined through the amino acid detection (835-50, Hitachi Co., Tokyo, Japan) (Liu et al., 2015).

2.3. The preparation and characterization of NPs

Insulin loaded TMC nanoparticles (TMC NPs) as well as the ligand modified NPs (FQS-TMC NPs) were prepared by cross-linking TMC or TMC-FQS with TPP (Sadeghi et al., 2008). Briefly, TMC or TMS-FQS aqueous solution was mixed with aqueous solution of insulin under magnetic stirring at room temperature. By slowly dropping TPP aqueous solution, the mixture was stirred at 500 rmp for 10 min, yielding an opalescent suspension. The suspension was ultracentrifuged at 14,000 rpm (30 min, 4 °C). The supernatant was discarded, and the obtained NPs were resuspended for further use. Fluorescence-labeled NPs were prepared with the same method using FITC-labeled insulin (FITC-insulin). FITC-insulin was synthesized according to the previous reported procedure (Due et al., 1985).

The NPs were characterized for the particle size, polydispersity index (PDI) and zeta potential with a Malvern Zetasize NanoZS90 (Malvern Instruments Ltd., U.K.). To evaluate the encapsulated efficiency (EE%) and drug loading efficiency (DL%), NP suspensions were ultracentrifuged at 14,000 rpm for 20 min at 4 °C. After centrifugation, the amount of insulin in supernatant was measured by a reverse-phase high-performance liquid chromatography (RP-HPLC) method (Agilent 1200 series) (Jin et al., 2012b). In addition, the amount of free FITC-insulin in the supernatant of fluorescence-labeled NPs was measured by Varioskan Flash Multimode Reader (Thermo Fisher Scientific). The excitation and emission wavelengths were set at 488 and 516 nm, respectively. The EE% and DL% of NPs were determined as follows:

$$\mathrm{EE}\,(\%) = \frac{\text{total amount of insulin} - \text{free insulin}}{\text{total amount of insulin}} \times 100$$

$$\mathrm{DL}\,(\%) = \frac{\text{total amount of insulin} - \text{free insulin}}{\text{NPs weight}} \times 100$$

2.4. Stability assay

The obtained NPs and the free insulin solution were suspended in simulated intestinal medium with trypsin, further incubated at 100 rpm at 37 °C. The equal amount of all incubating solutions was taken out at particular time intervals (0, 0.5, 1, 2, 3 and 4), and the degradation was then immediately stopped by adding of ice-cold 0.1 M hydrochloric acid (HCl) solution. Samples were centrifuged at 14,000 rpm at 4 °C, and then the amount of insulin in supernatant was measured by RP-HPLC method (Zhang et al., 2014).

2.5. Caco-2 cells culture

Human colon carcinoma Caco-2 cells were gained from Institute of Biochemistry and Cell Biology (Shanghai, China). They were grown in a culture flask containing Dulbecco modified Eagle's minimal essential medium (DMEM, Gibco, NY, USA) supplemented