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The transport mechanisms of polymer nanoparticles in Caco-2 epithelial cells

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ABSTRACT

As the primary physiological barrier, intestinal epithelial cells regulate the transportation of oral therapeutic agents including nanomedicines which significantly improves the bioavailability of many drugs. However, currently there seems in the lack of comprehensive understanding on nanoparticle transport in intestinal epithelial cells as well as the mechanisms related. So, in an attempt to illustrate the profile of nanoparticle transport in intestinal epithelial cells, Caco-2 cells and polymer nanoparticles (PNs) were used as the models to explore the whole transport process including endocytosis, intracellular trafficking, exocytosis and transcytosis. Via various techniques, the transport pathways of PNs in Caco-2 cells and their mechanisms were clarified. Firstly, the transport was characterized by its non-specificity. The co-mediation of clathrin, lipid raft/caveolae and macropinocytosis as well as the co-involvement of different proteins like actins, protein tyrosine kinase (PTK) and cyclooxygenase (COX) were found in the endocytosis of PNs. The endocytosed PNs could transport to apical early endosome (AEE) and then from AEE to lysososmes via AEE/late endosome (LE)/lysosome pathway, as well as to recycling endosome compartment (REC) or endoplasmic reticulum (ER) through AEE/REC and AEE/ER pathways, respectively. Both ER/Golgi and Golgi/REC/plasma membrane (PM) pathways were involved in the exocytosis of PNs. The transcytosis of PNs across the cell monolayer was very low with a ratio less than 0.5%, due to complicated reasons. Secondly, the transport was evidenced by its partial energy-dependency. Beside the energy-dependent transport mediated by some proteins, quantitative study demonstrated the obvious internalization as well as surface binding of PNs at both 37 °C and 4 °C, but significantly higher at 37 °C. Interestingly, the consistency between surface binding and internalization at each temperature was found, suggesting that cell binding was the precondition and key step for the following endocytosis. The involvement of both energy dependent and independent mechanism was also observed in the exocytosis and transcytosis process of PNs. Finally, there were opposite mechanisms found between the exocytosis and endocytosis of PNs, including the regulation role of lipid raft/caveolae, COX and Golgi complex, which also contributed to the fact of "easy entry and hard across" for PNs. Overall, this study depicts a clear picture of nanoparticle transport in Caco-2 epithelial cells characterized by non-specificity, partial energy-dependency and low transcytosis.

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1. Introduction

Intestinal tract is the prime physiological barrier for the oral absorption of multiple drugs. Over the last decade, the application of nanotechnology in drug delivery has significantly changed the traditional comprehension on oral formulations. Via the utilization of various nanotechnology-based systems including polymer nanoparticles (PNs), nanomedicines effectively enhance the bioavailability of loaded drugs and avoid the negative effect of intestine environment [1]. Especially for the water insoluble chemical medicines, nanotechnology greatly improves their solubility and permeability in biomembrane [2], showing the huge potential for the application of nanotechnology in oral pharmaceutical preparations.

Nowadays, the interaction between nanomedicines and cells attracts great attention of scientists in the field of pharmacy, biology and material science. Generally, many studies on nanomedicines are focused on the tumor cells, especially on the cell uptake and intercellular trafficking [3], due to the interesting in tumor therapy. Nevertheless, a comprehensive understanding on

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the whole transcytosis process and mechanism of nanomedicines across intestinal tract are very insufficient, although they are vital for the development of oral nano-drug as well as the awareness of possible nanotoxicity through gastrointestinal tract.

As the component of intestine tract, multiple types of intestine cells including epithelial cells and M cells in Peyer's patchs are reported to absorb nanomedicines in different ways [4,5]. Because epithelial cells account the overwhelming majority of intestine cells, they were chosen as the research subject in this study, although both types of cells need investigation. In the transport study of nanomedicines through intestinal epithelial cells, Caco-2 cell line is generally accepted as the classical cell model owing to its homology and similar morphology with intestinal epithelial cells [6]. With regard to the oral nanomedicines, PNs are most frequently chosen as drug carrier for the oral drug because of easy preparation and low cost. Typically, PLGA nanoparticles have been used to load drugs with different solubility and hydrophilicity for the improvement of oral bioavailability. However, there is little study on the detailed transcellular mechanism of PLGA nanoparticles in intestinal epithelial cells like Caco-2 cells.

The transcellular transport of nanomedicines through epithelial cells includes various processes such as cell surface binding, endocytosis, intracellular trafficking, exocytosis and transcytosis, and most of them relates to the subcellular structures in cells [7]. Nanomedicies may also transport through opened cell junctions, which is defined as paracytosis [8]. Additionally, nanocarriers may transport in cells via different pathways, which may be specific or non-specific and energy-dependent or independent [9]. As a result, the transport of nanomedicines in cells including epithelial cells may be complicated and diverse, remaining many mysteries to unravel.

With the purpose of depicting a relative comprehensive picture of nanoparticle transport in intestinal epithelial cells, various transport pathways, mechanisms and key characteristics were carefully investigated here. Caco-2 cells were used as the cell model, while PLGA nanoparticles without any surface modifications were utilized as the representative of oral nanomedicines to eliminate the effect of chemical modification on the nano-bio interaction [10]. It has been concluded previously that the size, surface charge and hydrophilicity of nanoparticle as well as the type of cells all influence the transport of nanoparticles in some phase such as cellular uptake [11], so all these factors are fixed here. Our studies were conducted at different transport processes, respectively, including cell surface binding, endocytosis, intracellular trafficking, exocytosis and transcytosis, with various quantitative and qualitative techniques. As the result, a panorama of nanoparticle transport in intestinal epithelial cells was obtained.

2. Materials and methods

2.1. Materials

Caco-2 cell line was purchased from National Platform of Experimental Cell Resources for Sci-Tech (Beijing, China). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Poly (D, L-lactic-co-glycolic acid) (PLGA, 50:50, Av. MW 15000) was supplied by Shandong Daigang Institute of Medical Instrument. LysoTracker probe, ERTracker probe, MitoTracker probe, Rhodamine-phalloidine, Texas Red-conjugated goat anti-rabbit IgG, Texas Red-conjugated goat anti-mouse IgG, and Hoechst 33258 were all obtained from Invitrogen (Eugene, Oregon, USA). Rabbit anti-Rab5, mouse anti-Rab7 and Rabbit anti-Rab11 antibodys were purchased from Abcam (Cambridge, MA, USA). Coumarin-6 (C6), Poloxamer 188 (Pluronic F68), methyl-β-cyclodextran (MβCD), nystatin, 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), genistein, Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Brefeldin A (BFA), monensin (MON) and indomethacin were achieved from Beyotime (Haimen, Jiangsu, China). Phenylarsine oxide (PAO) was purchased from Acros (New Jersey, USA). Penicillin, streptomycin, trypsin, RIPA lysis buffer, EDTA, phosphate buffer solution (PBS), were obtained from Mcgene Co (Beijing, China). All chemical reagents utilized in study were analytical grade.

2.2. Preparation and characterization of PNs

PLGA nanoparticles were fabricated by the classical emulsion solvent evaporation method. First, 5 mg PLGA 50/50 was added into 2 ml acetone to form the organic phase dispersion. The water phase was then prepared by dissolving 0.5 mg poloxamer 188 (Pluronic F68) in 5 ml distilled water. Under continuously stirring, the organic phase was slowly dropped into F68 contained water to achieve the initial emulsion. Subsequently, the organic solvent acetone that existed in emulsion was eliminated by the rotary evaporator under 60 °C condition. After the centrifugation at 10000 g for 20 min to remove the dissolved F68 in solution and re-dispersion in medium, the PNs were finally prepared. To further trace the intracellular behavior of PNs, the coumarin-6 (C6) loaded PNs (C6-PNs) were also fabricated by the same approach. During the preparation of C6-PNs, the mass ratio of coumarin-6 to PLGA was defined as 1:1000. The finally obtained C6-PNs dispersion was further centrifuged at 10000 g for three times to eliminate the free coumarin-6 in solution.

The particle size and surface charge of PNs were detected by the dynamic light scattering (DLS) analyzer (Malvern, Zetasizer Nano ZS). Before the measurement, PNs were dispersed in mediums with different ion strengths. Because of the necessary for comparison, all measurements of nanoparticle dispersions were regulated to the same condition. The reference of material was set as polystyrene latex. Keeping the detection temperature as 25 °C and dispersant viscosity as 0.8872 cP, 1 mg/ml PNs in various mediums were placed in sizing cuvette and zeta cell for measurement.

The morphology and absolute particle size of PNs was further detected by transmission electron microscopy (TEM). After negative stained by 2% phosphotungstic acid, 20 μl distilled water containing 10 μg/ml PNs was dropped on a copper grid and monitored by a transmission electron microscopy (JEM1230, JEOL, Japan).

2.3. In vitro coumarin-6 leakage detection from C6-PNs

To evaluate the efficiency of coumarin-6 as the marker of PNs, the leakage of coumarin-6 from C6-PNs was detected as the similar method with *in vitro* release measurement. Serum-free medium (SFM) with pH7.2 and pH5.5 values were both utilized as the leakage mediums. Firstly, 1 mg/ml newly prepared C6-PNs were placed in dialysis bag with 2 ml volume. After tightly sealed with two ends, the dialysis bag was then located in 38 ml different mediums. Under the sustaining shaking condition with 100 rpm at 37 °C, 1 ml leakage medium was aspirated at certain intervals for the subsequent coumarin-6 examination and the same volume of fresh medium was supplemented meanwhile. The concentrations of coumarin-6 in samples were measured by a high performance liquid chromatography (HPLC) system (Shimadzu, Japan) equipped with a fluorescence detector (Model RF-10AXL) and a pump (Model LC-10AT). The column temperature was set as 35 °C and the mobile phase composition was regulated as 95:5 (methanol: water, *v*/*v*). In the excitation at 467 nm and emission at 502 nm situation, 20 μl sample was injected into HPLC with a flow rate of 1 ml/min and the peak for C6 was achieved at the retention time of 5 min.

2.4. Caco-2 cell culture

Human colon carcinoma Caco-2 cells were grown in a culture flask containing Dulbecco modified Eagle's minimal essential medium (DMEM, 25 mM glucose) supplemented with 10% (*v*/*v*) fetal bovine serum, 1% (*v*/*v*) non-essential amino-acid, 1% (*v*/*v*) L-glutamine, 100 UI/ml penicillin and 100 μg/ml streptomycin. Then the culture flask was placed in a constant temperature incubator at 37 °C with 5% CO_2 supply. After the proliferation of 6 days, Caco-2 cells were digested with 0.25% trypsin/0.02% EDTA and seeded in a 12-well sterile plate with 1×10^5/ml to detect the cellular uptake of PNs.

For the establishment of *in vitro* transcellular model, Caco-2 cells were cultured on a polycarbonate membrane (Transwell, 12-well, CORNING) with numbers of pores in 3 μm diameter. 500 μl DMEM was added into the upper compartment of transwell insert and the medium volume required in bottom plate well was regulated as 1.5 ml. During the long-term culture of 21 days, mediums in both upper and basilar compartments were changed every other day and the transepithelial electrical resistance (TEER) was measured meanwhile by epithelial volt-Ω m (Millicell ERS-2, Millipore) to monitor the integrity of cell monolayer. In the end of the Caco-2 cell culture in transwell plates for 21 days, the insert wells with TEER value above 500 Ω cm^2 were selected for the subsequent transcellular detection of PNs.

2.5. Cytotoxicity study and LDH release assay

Cell counting kit-8 (CCK-8) assay was utilized here to evaluate the influence of PNs on cell viability. Caco-2 cells were seeded in 96-well plate at 5×10^4/ml in 200 μl culture medium under 37 °C and 5% CO_2. Before measurement, cells were firstly incubated with 1 mg/ml PNs in SFM for 6 h at 37 °C. Then, nanoparticle dispersion in each well was replaced by 200 μl CCK-8 solution as the instruction and cells were further incubated for another 2 h. Finally, the absorbance of cell medium in each well was measured at 450 nm via multiskan FC (Thermo Scientific, USA).

Whether PNs make damage to cell membrane was detection by lactate dehydrogenase (LDH) assay kit (Beyotime, Haimen, Jiangsu, China). In brief, Caco-2 cells

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