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Intracellular trafficking and cellular uptake mechanism of mPEG-PLGA-PLL and mPEG-PLGA-PLL-Gal nanoparticles for targeted delivery to hepatomas

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ABSTRACT

The lysosomal escape of nanoparticles is crucial to enhancing their delivery and therapeutic efficiency. Here, we report the cellular uptake mechanism, lysosomal escape, and organelle morphology effect of monomethoxy (polyethylene glycol)-poly (D,L-lactide-co-glycolide)-poly (L-lysine) (mPEG-PLGA-PLL, PEAL) and 4-O-beta-D-Galactopyranosyl-D-gluconic acid (Gal)-modified PEAL (PEAL-Gal) for intracellular delivery to HepG2, Huh7, and PLC hepatoma cells. These results indicate that PEAL is taken up by clathrin-mediated endocytosis of HepG2, Huh7 and PLC cells. For PEAL-Gal, sialic acid receptor-mediated endocytosis and clathrin-mediated endocytosis are the primary uptake pathways in HepG2 cells, respectively, whereas PEAL-Gal is internalized by sag vesicle- and clathrin-mediated endocytosis in Huh7 cells. In the case of PLC cells, clathrin-mediated endocytosis and sialic acid receptor play a primary role in the uptake of PEAL-Gal. TEM results verify that PEAL and PEAL-Gal lead to a different influence on organelle morphology of HepG2, Huh7 and PLC cells. In addition, the results of intracellular distribution reveal that PEAL and PEAL-Gal are less entrapped in the lysosomes of HepG2 and Huh7 cells, demonstrating that they effectively escape from lysosomes and contribute to enhance the efficiency of intracellular delivery and tumor therapy. *In vivo* tumor targeting image results demonstrate that PEAL-Gal specifically delivers Rhodamine B (Rb) to the tumor tissue of mice with HepG2, Huh7, and PLC hepatomas and remains at a high concentration in tumor tissue until 48 h, properties that will greatly contribute to enhanced antitumor efficiency.

1. Introduction

Nanoparticles show promising application prospects in tumor diagnosis and therapy due to their unique properties, including low drug systemic toxicity and specific targeting to tumors [1–4]. However, their successful application in tumor diagnosis and therapy requires them to effectively enter cells that is crucial for the nanoparticles to effectively execute their diagnostic or therapeutic functions *in vivo* [5,6]. Therefore, clarifying the interaction of nanoparticles with cells, such as the mechanism of cellular uptake and the intracellular trafficking pathway of nanoparticles within cells, is essential [7,8]. Studies have been performed to illustrate the intracellular transport pathway of nanoparticles in cells [9–11], however, it is noteworthy that the intracellular trafficking and uptake mechanism of nanoparticles are different in different cell lines. For instance, hepatocellular carcinoma patients may exhibit different clinical symptoms due to differences in hepatoma cell properties [12]. Thus, systematic studies aimed at investigating intracellular trafficking and cellular uptake mechanisms of nanoparticles in different cell lines are consequently highly indispensable for investigating and promoting the application of nanoparticles [13,14]. For this, we prepared the monomethoxy (polyethylene glycol)-poly (D,L-lactide-co-glycolide)-poly (L-lysine) nanopartilces (mPEG-PLGA-PLL, PEAL) and 4-O-beta-D-Galactopyranosyl-D-gluconic acid (Gal)-modified PEAL (PEAL-Gal). Subsequently, systemic research on the intracellular trafficking pathway and cellular uptake mechanism of PEAL and PEAL-Gal in HepG2, Huh7, and PLC hepatoma cells was carried out. The main aim was to further illustrate the properties of PEAL and PEAL-Gal in different hepatoma cells and promote their clinical application.

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2. Materials and methods

2.1. Materials

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-*t*-butoxycarbonyl-L-phenylalanine (Boc-L-Phe) were purchased from GL Biochem Co., Ltd. (Shanghai, China). N^{ϵ}-(Z)-L-lysine-*N*-carboxyanhydride (NCA) was synthesized and purified as described by Dorman et al. [15]. Rhodamine B (Rb), fluorescein isothiocyanate (FITC), paraformaldehyde (PFA), sodium azide (SA), nystatin, chlorpromazine, genistein, phorbol 12-myristate 13-acetate (PMA), and amiloride were purchased from Sigma–Aldrich Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS), DMEM high-glucose cell culture media, and 0.25% EDTA trypsin were purchased from Biowest SAS (Nuaillé, France). LysoTracker red probe was purchased from Invitrogen (California, USA). MTT reagent was purchased from Amresco LLC (OH, USA). Dicyclohexylcarbodiimide (DCC), N-Hydroxysuccinimide (NHS), 4-dimethylaminopyridine (DMAP), and 4-O-beta-D-Galactopyranosyl-D-gluconic acid (Gal) were purchased from Acros Organics Company (Geel, Belgium). 5-Ethynyl-2′-deoxyuridine (EdU) was purchased from RiboBio Co (Guangzhou, China). Other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

HepG2, Huh7 and PLC hepatoma cell lines (HepG2, Huh7, and PLC cells) obtained from the Shanghai Cancer Institute were grown in DMEM medium (Paisley, UK) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified environment containing 5% CO_2.

Nude female Balb/c mice (4–6 weeks old; body weight: 14 ± 2.1 g) were purchased from the Shanghai Cancer Institute (Shanghai, China). All animal procedures were performed according to the research protocol approved by the Animal Care and Use Committee of Shanghai Cancer Institute.

2.2. Synthesis and characterization of the copolymer

PEAL and PEAL-Gal copolymers (Mw = 12,000, PEAL: Gal = 1:1, mol:mol) were synthesized according to our previous protocol [16]. Briefly, the following synthesis procedures were performed: 1) synthesis of the hydroxyl-terminated mPEG-PLGA through ring-opening polymerization (ROP) of D,L-lactide and glycolide, which was initiated by mPEG and catalyzed by Sn(OCt)2; 2) synthesis of Boc-L-Phe end-capped mPEG-PLGA through the hydroxyl end-group of the mPEG-PLGA copolymer, which was converted to Boc-L-Phe; 3) synthesis of amino-terminated mPEG-PLGA through the removal of the t-Butoxycarbonyl end-group from the Boc-L-Phe end-capped mPEG-PLGA; 4) synthesis of mPEG-PLGA-poly(N^{ϵ}-(Z)-L-lysine) through ROP of the initiated NCA using the amino-terminated mPEG-PLGA; 5) synthesis of amino-terminated PEAL through the removal of the N^{ϵ}-(carbonylbenzoxy) end-group of the mPEG-PLGA poly(N^{ϵ}-(Z)-L-lysine) block copolymer; and 6) synthesis of PEAL-Gal by conjugating Gal to the amino-terminated PEAL using EDC and NHS.

2.3. Preparation and physicochemical characterization of the nanoparticles

The fluorescence probe Rb was encapsulated into the interior of the nanoparticles to track the intracellular transport pathway [17]. Briefly, 20 μL of Rb solution (2 mg/mL) was emulsified in 200 μL of PEAL or PEAL-Gal copolymer solution (20 mg/mL) by sonification (300 W, 10 s × 4). Additionally, 2 mL of F68 aqueous solution (1 mg/mL) was added and sonicated (400 W, 10 s × 4). The resultant emulsions were then removed with dichloromethane through rotary evaporation to obtain Rb/PEAL or Rb/PEAL-Gal.

Size distribution and zeta potential of Rb/PEAL and Rb/PEAL-Gal were determined using Nicomp-380ZLS dynamic light scattering (DLS) (California, USA). Transmission electron microscopy (TEM) images were captured using H-800 transmission electron microscopy (Hitachi, Japan) operated at an acceleration voltage of 200 kV.

2.4. Encapsulation efficiency determination

2.4.1. Standard curve

Various concentrations of Rb aqueous solution were separately dissolved in a 10-mL volumetric flask to obtain 0.2, 0.5, 1, 2, 5, 10, and 20 μg/mL solutions. The solutions were then assayed at a wavelength of 550 nm using a TU-1901 ultraviolet–visible spectrophotometer from Beijing Purkinje General Instrument Co., Ltd. (Beijing, China). The standard curve was established by performing linear regression between the concentration and absorbed light value.

2.4.2. Encapsulation efficiency

The encapsulation efficiency was defined as the ratio of the amount of Rb encapsulated in the PEAL-Gal to the total amount of Rb initially used. Briefly, 2 mL of Rb/PEAL-Gal solution was added to a millipore centrifuge tube (molecular weight cutoff: 3500) and centrifuged (7000 rpm × 30 min) at 4 °C. The liquid in the tube was then determined at the wavelength of 550 nm using an ultraviolet–visible spectrophotometer.

2.5. Concentration of nanoparticles available for cellular uptake

The cytotoxicities of nanoparticles against HepG2, Huh7, and PLC cells were determined using MTT assay. Briefly, HepG2, Huh7, and PLC cells (5×10^4 cells/well) were separately seeded in 96-well plates and incubated for 24 h. Various concentrations of PEAL or PEAL-Gal (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, and 1 mg/mL) were separately added and incubated for 24 h. Thereafter, 20 μL of MTT (5 mg/mL) was added and incubated for an additional 4 h. The culture medium was removed and replaced with 100 μL of DMSO. The absorbance was measured at the wavelength of 490 nm using a 680 model microplate reader from Bio-Rad Laboratories (California, USA).

2.6. Cell proliferation imaging

Cell proliferation was assayed using an EdU cell proliferation imaging kit. Briefly, HepG2, Huh7, and PLC cells (4×10^3 cells/well) were separately seeded in 96-well plates and incubated for 24 h. Various concentrations of PEAL or PEAL-Gal (0.05, 0.5, and 5 mg/mL) were added and incubated for 24 h. The cells were treated with 50 μM EdU culture medium for 2 h and washed twice (5 min/once) with PBS, followed by incubation with 4% PFA for 30 min. After removing the solution from the wells, 50 μL of glycine (2 mg/mL) was added and incubated for 5 min. Next, 100 μL of PBS solution containing 0.5% Triton X-100 was added and incubated for 10 min. Thereafter, 100 μL of Apollo® staining reaction liquid was added and incubated for 30 min. Subsequently, the cells were incubated with 100 μL of Hoechst 33342 for 30 min, washed three times (5 min/once), and imaged using an IX-51 fluorescence microscope from Olympus Optical Company, Ltd (Tokyo, Japan). Cell proliferation was then calculated using Image-Pro Plus 7.0 software from Media Cybemetics, Inc. (Rockville, USA).

2.7. Influence of nanoparticles on organelle morphology

The influence of PEAL and PEAL-Gal on organelle morphology was evaluated using TEM [18]. Briefly, HepG2, Huh7, and PLC cells (6×10^6 cells/well) were separately seeded in 10-cm plates and incubated for 24 h. Next, 0.5 mg/mL of PEAL or PEAL-Gal was added and incubated for 24 h. The cells were then washed three times with PBS, trypsinized, harvested, and resuspended in 500 μL of stationary liquid containing 4% paraformaldehyde and 5% glutaraldehyde. The cells were embedded in agar gel, which was then cut into 1-mm slices. Each slice was again fixed using osmic acid, dehydrated, embedded, and imaged using TEM.

2.8. Tomographic scan of cellular uptake

Tomographic scan of cellular uptake were determined using confocal laser scanning microscope (CLSM). Briefly, HepG2, Huh7, and PLC cells (5×10^3 cells/dish) were separately seeded in a confocal dish and incubated for 24 h. Rb/PEAL or Rb/PEAL-Gal (0.2 mg/mL) was added and incubated for 3 h. The cells were then fixed with 4% PFA for 30 min, and the tomographic scan was performed at an excitation wavelength of 540 nm and an emission wavelength of 625 nm with a FV1000 CLSM from Olympus Corporation (Osaka, Japan).

2.9. Cellular uptake mechanism

2.9.1. Time dependence of cellular uptake

HepG2, Huh7, and PLC cells (5×10^4 cells/well) were separately seeded in 6-well plates and incubated for 24 h. Rb/PEAL or Rb/PEAL-Gal solution (0.2 mg/mL) was added and incubated for 0.5, 1, 2, or 4 h. The cells were then washed 3 times with PBS, trypsinized, harvested, and resuspended in 200 μL of PBS. Subsequently, the mean fluorescence intensity (MFI) of cellular uptake was measured using a FACScan flow cytometer from Becton Dickinson (New York, USA) operated at an excitation wavelength of 540 nm and an emission wavelength of 625 nm.

2.9.2. Concentration dependence of cellular uptake

HepG2, Huh7, and PLC cells (5×10^4 cells/well) were separately seeded in 6-well plates and incubated for 24 h. Different concentrations of Rb/PEAL or Rb/PEAL-Gal (0.4, 1, 1.6, and 2 mg/mL) were added and incubated for 2 h. The cells were then washed 3 times with PBS, trypsinized, harvested, and resuspended in 200 μL of PBS. Subsequently, the MFI of cellular uptake was measured using a FACScan flow cytometer operated at an excitation wavelength of 540 nm and an emission wavelength of 625 nm.

2.9.3. Energy dependence of cellular uptake

HepG2, Huh7, and PLC cells (5×10^4 cells/well) were separately seeded in 6-well plates and incubated for 24 h. The cells were incubated at 4 °C or with SA (1 mg/mL) for 1 h, respectively. Rb/PEAL and Rb/PEAL-Gal were added and incubated for an additional 2 h, and the cells were then washed three times with PBS, trypsinized, harvested, and resuspended in 200 μL of PBS. The MFI of cellular uptake was measured using a FACScan flow cytometer operated at an excitation wavelength of 540 nm and an emission wavelength of 625 nm.

2.10. Cellular trafficking pathway

2.10.1. Cytotoxicity of cellular uptake inhibitors

The cytotoxicities of cellular uptake inhibitors against HepG2, Huh7, and PLC cells were determined using the MTT assay. Briefly, HepG2, Huh7, and PLC cells (2×10^4 cells/well) were separately seeded in 96-well plates and incubated for