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Leading opinion

Elucidating the molecular mechanism for the intracellular trafficking and fate of block copolymer micelles and their components

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ABSTRACT

Block copolymer micelles have shown promise for the intracellular delivery of chemotherapeutic agents, proteins, and nucleic acids. Understanding the mechanism of their intracellular trafficking and fate, including the extracellular efflux of the polymers, will help improve their efficacy and minimize their safety risks. In this Leading Opinion paper, we discuss the molecular mechanism of block copolymer micelle trafficking, from intracellular uptake to extracellular efflux, on the basis of studies with HeLa cells. By using FRET (fluorescence resonance energy transfer) with confocal microscopy, we found that, following their intracellular transport via endocytosis, the micelles dissociated into their polymeric components in late endosomes and/or lysosomes. Furthermore, we confirmed that the intrinsic proteins NPC1 and ORP2 are involved in the intermembrane transfer of polymers from the endosome to the plasma membrane via the ER (endoplasmic reticulum) by using knockdown experiments with siRNAs. After the polymers were transported to the plasma membrane with the aid of ORP2, they were extruded into the cell medium via ABC transporter, ABCB1. Experiments with ABCB1-expressing vesicles indicated that the polymer itself, and not the fluorescent compounds, was recognized by the transporter. These findings, and the analysis of related mechanisms, provide valuable information that should help minimize the potential risks associated with the intracellular accumulation of block copolymer micelles and to improve their therapeutic efficacy.

1. Introduction

Drug delivery systems that use nanometer-sized carriers show promise for the targeted transfer of chemotherapeutic agents, proteins, and nucleic acids to tissues or organs. Nanomaterials have been extensively studied as drug carriers, and some formulations for cancer treatment have been applied clinically [1–3]. Block copolymer micelles have recently received considerable attention as targetable carrier systems [4–7]. The formulation of block copolymer micelles can alter the pharmacokinetic characteristics such as the volume of distribution, clearance, half-life, and tissue distribution of the active substances included [8–10]. Moreover, finely tuning the design of the block copolymers can increase their longevity in the bloodstream and allow the controlled release of the drugs, which consequently improves the pharmacodynamics of the drugs and/or avoids systemic toxicity.

The development of these drug carriers for the cellular uptake of therapeutic proteins and nucleic acids is of particular interest. Because nucleic acids, proteins, and peptides are not taken up into cells via passive diffusion, their intracellular uptake by nanocarriers is a key to targeting the delivery of these compounds at the cellular or organelle level. Specifically, the incorporation of these compounds into nanocarriers will improve the efficiency of their intracellular uptake or delivery to specific organelle, thereby ensuring their therapeutic effects. Furthermore, clarifying the intracellular trafficking mechanisms may also facilitate the

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discovery of new drug delivery strategies, such as targeting to specific cell organelles. Thus, to improve the efficiency of the intracellular uptake of these compounds, it is essential to understand the detailed mechanism of their trafficking including the fate of the micelles and their component polymers after their uptake via endocytosis. To this end, the use of covalently bound fluorescent reagents as probes has gradually shed light on the internalization pathways and intracellular localizations of polymeric nanoparticulate carriers [11–13].

In parallel, the safety of these carriers must be investigated. To ensure that these materials are safe, it is essential to know whether the components of the carriers are accumulated inside the cell or undergo sequestration (i.e., metabolism or efflux). The potential long-term effects of these novel polymers when used as nanosized particles have not yet been determined. To address this issue, we investigated the intracellular fate of polymer micelles conjugated with doxorubicin (Dox) in HeLa cells [14]. We demonstrated that Dox is endocytosed and localized to the endoplasmic reticulum, and that an ABC transporter, ABCB1, is involved in the efflux of the polymer from these cells. However, many factors remain unknown, for example, where do the micelles dissociate into their constituent polymers after internalization? What are the molecular mechanisms involved in trafficking to each organelle and in the efflux of polymers or micelles? Moreover, the trafficking phenomenon we found previously was limited to the case of Dox-conjugated polymers. Questions remain regarding the trafficking of other block copolymers, for example, those conjugated with different compounds or those with different poly(ethylene glycol) (PEG) lengths.

In the present study, we constructed three micelles by using three block copolymers (doxorubicin, Nile Red, and DBD (4-(*N*,*N*-dimethylsulfamoyl)-2,1,3-benzoxadiazole)) with different poly(ethylene glycol) (PEG) lengths (Mw 5000 or 12,000), detailed descriptions of which can be found in Section 3.1. To investigate the structural integrity of the micelles inside the cells, fluorescence resonance energy transfer (FRET) micelles were also constructed by using two types of polymers, that is, polymers with covalently bound Nile Red and polymers with covalently bound DBD. The trafficking of the micelles and their components, from intracellular uptake to extracellular efflux, was evaluated and the intrinsic molecules involved in the trafficking process were identified.

2. Materials and methods

2.1. Materials

Poly(ethylene glycol)–poly(aspartate) (PEG–P(Asp)) block copolymers with conjugated Dox were synthesized by Nippon Kayaku Co., Ltd. (Tokyo, Japan) [15]. Dextran (Dextran Texas Red, Molecular weight 10,000), polystyrene particles (FluoSphere Red, average particle size; 40.1 nm), Dulbecco's modified Eagle's Medium (DMEM), RPMI-1640, penicillin/streptomycin, and Opti-MEM I were purchased from Life Technologies (Brooklyn, NY, USA). Fetal bovine serum (FBS) was obtained from Nichirei Biosciences (Tokyo, Japan). Fluorescently labeled amorphous silica particles (Sicastar RedF, average particle size; 45.5 nm) were obtained from Micromod Partikeltechnologie (Rostock, Germany). Isolated mammalian cell membranes containing human ABCB1, for vesicle transport assays, (SB-MDR1-K-VT) were purchased from SOLVO Biotechnology (Hungary). All chemicals used in this study were of the highest purity available. HeLa cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in DMEM. The medium was supplemented with 10% FBS, 100 U/mL penicillin/streptomycin. Cells were grown in a humidified incubator at 37 °C/5% CO_2.

2.2. Synthesis of the DBD-conjugated polymer and Nile Red-conjugated polymer

Poly(ethylene glycol)–poly(aspartate) block copolymer (PEG–P(Asp)) was obtained as described previously [15]. The degree of polymerization of PEG–P(Asp) was determined to be 35–45 by neutralization titration (Supplementary Fig. 1-1).

For the synthesis of DBD-conjugated polymer, PEG–P(Asp) (100 mg) and dimethylaminopyridine (23 mg, 0.8 equiv. for COOH) were dissolved in DMF (1.5 mL) and then DBD-ED (13 mg, 0.2 equiv.) and 4-phenyl-1-butanol (25 μL, 0.7 equiv.) were added. Diisopropylcarbodiimide (36 μL, 1.0 equiv.) was added to the solution and stirred at room temperature for 5 h. Diisopropylcarbodiimide (36 μL, 1.0 equiv.) was added again and the solution was stirred for 18 h. The reaction mixture was then dropped into a mixture of ethyl acetate and hexane (1:3). The resulting precipitate was filtered, washed with the mixture of ethyl acetate and hexane (1:3), and dried under vacuum to obtain the DBD-conjugated polymer (99 mg) as a powder. ^{1}H NMR spectra in DMSO-d_6 and the assignment are shown in Supplementary Fig. 1-2.

For the synthesis of the Nile Red-conjugated polymer, PEG–P(Asp) (100 mg) and dimethylaminopyridine (28 mg, 1.0 equiv.) were dissolved in DMF (1.5 mL) and then Nile-Red (8.8 mg, 0.1 equiv.) and 4-phenyl-1-butanol (36 μL, 1.0 equiv.) were added. Diisopropylcarbodiimide (36 μL, 1.0 equiv.) was added to the solution, which was then stirred at room temperature for 18 h. The reaction mixture was dropped into a mixture of ethyl acetate and hexane (1:3). The resulting precipitate was filtered, washed with the mixture of ethyl acetate and hexane (1:3), and dried under vacuum to obtain the Nile Red-conjugated polymer (109 mg) as a powder. ^{1}H NMR spectra in DMSO-d_6 and the assignment are shown in Supplementary Fig. 1-3.

2.3. Physicochemical properties of block copolymer micelles

The particle size and polydispersity index (PDI) of the block copolymer micelles were determined with a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK).

2.4. Evaluation of the intracellular trafficking of block copolymer micelles

To quantify the intracellular uptake of the polymers, we used a final polymer concentration of 50 μg/mL in this study. HeLa cells (5×10^4) were seeded onto 6-well plates in medium containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for 24 h at 37 °C/5% CO_2, the cells were exposed to 50 μg/mL micelles in culture medium. After incubation for pre-determined durations, the incubation medium was replaced with Hanks' balanced salt solution (HBSS). The cells were trypsinized with 0.25% trypsin–ethylenediamine tetraacetic acid (EDTA) (Life Technologies), washed with HBSS three times, and suspended in lysis buffer (1.0% Triton X-100 in HBSS). The cell suspension was then shaken and centrifuged (15,000× *g*, 4 °C, 10 min). The fluorescence intensity of the resultant supernatant was measured on a fluorescence spectrophotometer (F-7000; Hitachi High-Technologies, Tokyo, Japan) using 440 nm excitation and 580 nm emission for DBD-conjugated polymers, 580 nm excitation and 640 nm emission for Nile Red-conjugated polymers, and 470 nm excitation and 590 nm emission for Dox-conjugated polymers. The fluorescence intensity was normalized with respect to the protein content of the cells. The protein concentration was determined by using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.5. Confocal microscopy

To observe the colocalization of block copolymer micelles with the intracellular compartment, specific intracellular compartment components were labeled by using fluorescent dyes. All dyes for confocal microscopy were purchased from Life Technologies and used in accordance with the manufacturer's instructions. Endosomes were labeled with transferrin conjugated to Alexa Fluor 488 or Alexa Fluor 594, and lysosomes were labeled with LysoTracker Green DND-26 or LysoTracker Red DND-99. The ER was labeled with ER-Tracker Green or ER-Tracker Red, and the Golgi apparatus was labeled with Bodipy-FL C5-ceramide or Bodipy-TR C5-ceramide complexed to BSA. Confocal microscopy was performed as previously described [14]. Briefly, cells (1.0×10^5) were plated on 35-mm glass-bottom dishes coated with poly-L-lysine (Matsunami Glass, Osaka, Japan) in medium containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for 24 h, cells were exposed to 50 μg/mL micelles in culture medium. At a pre-determined time after addition of the micelles, cells were washed and kept in HBSS for imaging with a confocal microscope (Carl Zeiss LSM 510; Carl Zeiss Microscopy GmbH, Germany). Pseudocolor luminescent images were captured using LSM Image Browser (Carl Zeiss Microscopy GmbH, Germany).

An FRET experiment was performed using FRET micelles composed of DBD-conjugated polymers and Nile Red polymers (9:1, w:w). Cells were exposed to 50 μg/mL FRET micelles in culture medium. Two hours after addition of the micelles, cells were washed and kept in medium without micelles and then imaged with a confocal microscope at 2, 10, and 24 h after addition of the micelles.

2.6. Endocytosis inhibition and Golgi destruction

To investigate the mechanism of endocytosis of the prepared micelles, 10 μg/mL chlorpromazine (a clathrin-mediated endocytosis inhibitor), 150 μM genistein or 2.0 mM methyl-β-cyclodextrin (MβCD) (caveolae-mediated endocytosis inhibitors), or 50 μM 5-(N-ethyl-N-isopropyl) amiloride (a macropinocytosis inhibitor) were used [16,17]. Each endocytosis inhibitor was added to the culture medium 30 min before the addition of the micelles. To inhibit ER-to-Golgi transfer, to investigate whether ER-to-Golgi transfer is involved in the intracellular trafficking of micelles or polymers, cells were incubated in medium containing 1 μg/mL brefeldin A 30 min before the addition of the micelles [18].

2.7. Small interfering RNA (siRNA) transfer

To clarify which intrinsic proteins are involved in the intracellular trafficking and efflux of the polymers, the expression of specific proteins was down-regulated by