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The utilization of pathogen-like cellular trafficking by single chain block copolymer☆

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ABSTRACT

Amphiphilic triblock copolymer, poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide), Pluronic® P85, is unexpectedly shown to utilize sophisticated cellular trafficking mechanisms and enter brain microvessel endothelial cells and primary neurons that are poorly penetrable. Though caveolae serve as a primary entry site for the copolymer single chains, in cells devoid of caveolae, the copolymer can still exploit caveolae- and clathrin-independent routes. This parallels the copolymer's trafficking itinerary with that of biological pathogens. The similarity is reinforced since both bypass early endosomes/lysosomes and transport to the endoplasmic reticulum. The copolymer finally reaches the mitochondrion that serves as its final destination. Notably, it also succeeds to gain entry in brain microvessel endothelial cells through caveolae and in primary neurons through caveolae- and clathrin-independent pathway. In neurons the copolymer accumulates in the cell body followed by anterograde trafficking towards the axons/dendrites. Overall, dissecting the trafficking of a synthetic polymer in multiple cell types triggers development of novel delivery systems that can selectively target intracellular compartments and provide entry in cells currently considered impenetrable.

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1. Introduction

Millions of years of evolution shaped the ability of viruses and bacteria pathogens to invade mammalian cells. In doing so, these pathogens are employing sophisticated intracellular trafficking mechanisms that allow them to avoid lysosomal degradation. Most recently caveolae/lipid raft-based endocytosis has gained tremendous attention as a portal of entry of many viruses like SV40, as well as bacterial pathogens like Cholera toxin [1]. The high infectiveness of these pathogens to a broad spectrum of cells is believed to be due to the presence of high affinity receptors concentrated in the caveolae/lipid raft structures. Sophisticated structural arrangement is therefore believed to be crucial for the ability of these pathogens to enter cells. Surprisingly, in this study we demonstrate that a synthetic polymer with a very simple structure – poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) (PEO-PPO-PEO, also known as Pluronic® or poloxamer), can transport into the cells using exactly the same pathways as the biological pathogens.

Pluronic® block copolymers are very interesting molecules that have attracted attention due to their remarkably broad spectrum of useful pharmacological activities [2]. Examples include the ability of Pluronics to sensitize multidrug-resistant (MDR) tumors [3], activate nuclear import and transcription of DNA [4] and stimulate immunity [5]. These effects are all believed to be related to the interactions of the block copolymer chains with the plasma membrane. An ability of one copolymer, Pluronic® P85 (P85), to localize with caveolae and employ this pathway for cellular entry has been recently shown [6]. In this study we further examine the initial, intermediate and later stages of trafficking of P85, and compare it to Cholera toxin B (CTB), which can employ multiple pathways, such as caveolae as well as caveolae- and clathrin-independent endocytosis, for cellular entry [7]. Along, with the brain microvessel endothelial cells that have functional caveolae we also use cells devoid of caveolae, such as confluent epithelial cells (caveolae absent at the apical side), caveolae-deficient fibroblasts and neurons. The ability of Pluronic® block copolymers to

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employ multiple pathways and enter the blood brain barrier (BBB) and neuronal cells is of considerable interest for the potential use of these molecules in the development of novel agents for central nervous system (CNS) drug delivery.

2. Material and methods

2.1. Materials

P85, EO_{26}-PO_{40}-EO_{26} (lot # WPAU-549B) was kindly provided by BASF Corp. Alexa 488-or 594-labeled CTB, Alexa 488- or 647- labeled Tf, DAPI (4′,6-diamidino-2-phenylindole), ERtracker™, Mitotracker™, Lysotracker™, Lipofectamine™ 2000, Organelle Lights™ Endosome-GFP and Cellular Lights™ Actin-GFP were purchased from Invitrogen Inc (Carlsbad, CA). Methyl-β-cyclodextrin (MBCD), sucrose, tetramethylrhodamine B isothiocyanate (TRITC) or fluorescein isothiocyanate (FITC) was purchased from Sigma–Aldrich, (St. Louis, MO). Bovine serum albumin (BSA) was purchased from Fisher Scientific (Waltham, MA). DRAQ-5 was purchased from Biostatus Limited (UK). K44A-GFP dynamin mutant was a kind gift from Dr. Steve Caplan lab at the University of Nebraska Medical Center.

2.2. Labeling of P85

Anhydrous Pluronic P85 (2 g) was activated with CDI (213 mg) in 10 ml of acetonitrile for 2 h at 37 °C and reacted with ethylenediamine (313 mg) in 20 ml ethanol for 12 h at room temperature. The reaction mixture was dialyzed in a 2-kD cutoff membrane against 15% ethanol for 72 h (change ethanol twice) and lyophilized. TRITC or FITC was dissolved in dimethylformamide (DMF) (2.6 mg/mL) with 0.5% (v/v) diisoproplethylamine and 520 μL of this solution was added to amino-Pluronic (11.7 mg, 2.54 μM). After 3 days on a rocker at 37 °C the DMF was removed in a stream of air and the residual taken up in 400 μL methanol. Free dye was removed by gel filtration (Sephadex LH20 in methanol) until no band of the free dye was observed. The methanol was removed in a stream of air and the product (8.1 mg) freeze-dried from water.

2.3. Cell lines

Madin–Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified eagle's medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS), penicillin/streptomycin as described elsewhere. All tissue material media was obtained from Gibco Life Technologies, Inc. (Grand Island, NY). MDCK cells were used 48 h after plating. 3T3 mouse embryonic fibroblasts (MEFs), KO (knock out) cell line (homozygous for a disruption of the caveolin-1 gene Cav-1 −/−, ATCC CRL-2753) and 3T3 MEFs WT (wild type, Cav-1 +/+) cell line (ATCC CRL-2752) were maintained in DMEM, containing 10% heat inactivated FBS and 0.01% penicillin/streptomycin.

2.4. Primary culture of cortical neurons and brain microvessel endothelial cells

Primary culture of cortical neurons was prepared as described below. In brief, cortices were isolated from fetal (embryonic 18 days old) rat and then incubated with trypsin for 20 min at 37 °C. This enzymatic reaction was stopped by adding 200 μl/ml of fetal bovine serum along with DNAase and incubated at 4 °C for 30 min. Cells were dissociated by gentle trituration via polished glass pasteur pipettes, washed 3 times with the DMEM media and then plated at a total density of 2×10^5 cells per well of a 6-well plate having a coverslip coated with poly-D-lysine. Complete neurobasal medium was used for plating the neurons, which consists of Neurobasal/B-27/Dextrose/Pen-strep/Glutamine (Invitrogen Inc Carlsbad, CA). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. After 72 h, 40% of the media was replaced by fresh medium having 10 μM of Ara-C (Sigma Aldrich, St Louis, MO) so as to prevent glia proliferation. The culture medium was changed every 3 days for the remainder of the experiment. All experiments were performed on cultured cells at days 7–10. Primary culture of brain microvessel endothelial cells isolated from the gray matter of the cerebral cortex of bovine brain was isolated and maintained as previously described [8].

2.5. FACS analysis

Brain microvessel endothelial cells (5×10^4 cells/well in 24 well plates) were exposed to 0.001% FITC-P85 alone in the presence or absence of cells pre-treated for 30 min with inhibitors of endocytosis (5 mM MBCD, or 0.45 M sucrose) and then the same inhibitors were also present during subsequent incubation with the copolymer. In select experiments Cav KO and WT cells were treated with 0.001% TRITC-P85. Cells were washed, trypsinized and resuspended in phosphate-buffered and saline/1% BSA. The mean fluorescence intensity was analyzed using Becton Dickinson FACStarPlus flow cytometery operating under Lysis II (San Jose, CA) equipped with an argon ion laser. Data were acquired in linear mode and visualized in logarithmic mode. Data from 10,000 events were gated using forward and side scatter parameters to exclude debris and dead cells. All experiments were conducted thrice and measurements were conducted in triplicates and data presented as means +/− SEM. Statistical comparisons between groups were made using Student's *t*-test.

2.6. Confocal analysis on live cells

MEF 3T3 KO cells (1×10^6) were plated in Lab-Tek™ chambered coverglass (Fischer Scientific, Waltham, MA) and after two days (37 °C, 5% CO_2) were exposed for 30 min to 0.001% TRITC-P85. In select experiments cells were treated in the presence of Alexa 488-CTB (5 μg/ml) or Alexa 488-Tf (5 μg/ml), washed and kept in complete media for imaging using the confocal microscope. Brain microvessel endothelial cells were exposed to 0.001% FITC-P85 for 30 min in the presence or absence of 5 μg/ml Alexa 594-CTB or 5 μg/ml Alexa 647-Tf. In another experiment, MDCK cells were treated with Lysotracker™ green (100 nM) or ERTracker™ green (5 μg/ml) or Mitotracker™ green (1 μg/ml) for 10 min, cells were washed and incubated with TRITC-P85 for 30 min and imaged under confocal microscope. Cortical neurons grown on coverslips were treated with 0.001% TRITC-P85 for 1 h. In select experiments the neurons were treated with 0.001% TRITC-P85 in the presence of CTB (5 μg/ml). Cells were washed and coverslips transferred to Bioptech dishes (Fischer Scientific, Waltham, MA) for live cell confocal imaging.

2.7. Immunocytochemistry on fixed cells

Brain microvessel endothelial cells were pre-treated with DMEM without FBS for 30 min, and then treated with 0.001% FITC-P85 for 1 h, washed and fixed with 4% paraformaldehyde. Rabbit anti-caveolin-1–Cy3 antibody (Sigma Aldrich, St Louis, MO) (1:100) and mouse anti-clathrin antibody (Affinity Bio-reagents, Golden, CO) (1:10) were incubated in blocking buffer overnight at 4 °C. For detection of anti-clathrin antibody, mouse-specific IgG antibody (1:250) conjugated to Alexa 568 (Invitrogen Inc, Carlsbad, CA) was added to cells for 1 h at 37 °C. Cells were examined under confocal microscope. In a separate experiment primary cortical neuronal cells were pre-treated with DMEM without FBS for 30 min, and then treated with 0.001% TRITC-P85 for 1 h, washed and fixed with 4% paraformaldehyde.Cells were then exposed to mouse anti-MAP-2 antibody (Millipore, Billerica, MA) (1:100) in blocking buffer for overnight at 4 °C. For detection of anti-MAP-2, mouse-specific IgG secondary antibody (1:250) conjugated to Alexa 488 (Invitrogen Inc, Carlsbad, CA.) was added to cells and visualized using confocal microscopy.

2.8. Cell transfection

MDCK cells were transfected using Organelle Lights™ Endosome-GFP and Organelle Lights™ Actin-GFP kit. This kit contains an Organelle Lights™ reagent which is baculovirus, a efficient delivery system that contains a gene sequence which encodes for Rab5a (targeting sequence, an early endosome marker) or actin fused to a GFP (fluorescent protein) already incorporated into the viral genome and the kit also has an enhancer solution for increased expression of the chimera. Briefly, cells were plated, allowed to attach, and treated with Organelle Lights reagent at room temperature in the dark for 2–4 h. The reagent was aspirated and cells were incubated in DMEM containing 1X enhancer for 2 h followed by washing and addition of complete medium. The transfected cells were treated with TRITC-P85 and exposed for 30 min, washed, and imaged using confocal microscopy. In select experiments MDCK cells were transfected with plasmid DNA (1 μg/ml) of K44A dynamin mutant using Lipofectamine™ 2000, with the transfection protocol as previously described [9].

3. Results

3.1. Internalization and intracellular trafficking of pluronic®

P85 molecules entered cells through vesicular trafficking and remained in distinctive vesicular structures in MDCK cells (Fig. 1A, Movie 1). The observed pathways did not depend on the fluorescent label used (TRITC or FITC). Previously, we reported that the initial entry of this copolymer's single chains (unimers) is based on caveolae-mediated endocytosis [6]. However, to our surprise, the copolymer uptake also proceeded in the confluent MDCK cells, which are devoid of functional caveolae endocytosis pathway at the apical side [10]. Therefore, to further tackle the entry mechanism, we transfected MDCK cells with a dynamin mutant $K44A^{dyn}$, this inhibits both caveolae-and clathrin-dependent endocytosis [11,12]. Interestingly, P85 unimers were able to enter cells expressing $K44A^{dyn}$, which suggests that they can use a dynamin-independent pathway (Fig. 1B). The internalization of Tf in such cells was completely abolished (data not shown). We also observed significant colocalization of P85-containing vesicles with the actin