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Effects of particle uptake, encapsulation, and localization in cancer cells on intracellular applications

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ABSTRACT

Endocytosis is a normal process in living cells, often used to internalize drug-containing particles and probes for intracellular mechanics. The cell type, and especially malignancy, may affect particle internalization and transport. Specifically, membrane-encapsulation following internalization can affect particle interaction with the cell interior. Hence, particle-tracking measurements that reveal intracellular mechanics and dynamics require determination of effects of encapsulation. Here, we compare closely related, breast-cancer cell lines with high- and low-metastatic potential (MP) and benign, control cells. We evaluate time-dependent particle internalization, localization with endocytotic-pathway organelles, and membrane encapsulation at 2, 6, 24, and 48 h after initial cell exposure to particles. High MP cells internalize particles more rapidly and in larger amounts than low MP and benign cells. Moreover, while only cells at the edge of two-dimensional colonies of benign cells internalized particles, all cancer cells uniformly internalize particles. Particles mostly colocalize with late endosomes (>80%), yet surprisingly, overall membrane encapsulation decreases with time, indicating release into the cytoplasm; encapsulation at 48 h is <35% in all three cell types. We discuss implications to drug delivery and show that encapsulation does not significantly affect intracellular particle-tracking experiments, showing the applicability of endocytosis.

1. Introduction

Increasing numbers of biomedical applications utilize nanoparticles internalized into living cells. Such particles have been used as vehicles for drug delivery [1–5], as sensors for imaging and diagnostics purposes [6,7], and as probes to measure intracellular mechanics [8–11]. In many of those assays, internalization of particles into cells is accomplished by the spontaneous mechanism of endocytosis. While endocytosis is a natural process in cells, there is typically no control over amounts of internalized particles or their localization within the cells. More precise control of particle amounts and localization can be obtained using other internalization methods, such as microinjection [12] and ballistic injection [13,14]. Those internalization methods, however, also require specialized equipment and are invasive and typically damaging to the cells. Hence, endocytosis often remains a favored method, easily employed and minimally perturbing. Thus, to be able to rely on endocytosis for mechanical measurements, careful characterization is required to determine time-dependent amounts of internalized particles, their intracellular localization, and interactions with carrier organelles of the endocytotic pathway. That can affect mechanical measurements and optimal utilization of endocytosis for therapeutic purposes, and may reveal novel strategies for cancer therapy.

Fluid, molecules, and particles are naturally internalized when a sac, called an endosome, pinches off the cell membrane, engulfing and internalizing external objects [15]; that process may or may not be receptor mediated. From those early endosomes, cargo is typically transported into late endosomes that serve as a sorting station. Following sorting, cargo may be delivered into lysosomes for degradation or into the Golgi and endoplasmic reticulum (ER) for protein-relate processes. The cargo can also be released into the cell cytoplasm or discharged from the cell entirely (i.e. exocytosis); the encompassing vesicle is then recycled back into the plasma membrane. Those routes are normally used by cells for uptake of proteins and other macromolecules.

Synthetic particles have also been shown to undergo endocytosis, depending on their size, chemistry, and also cell type and activity. The effects of particle parameters such as size, shape, charge, and surface chemistry on endocytosis [16–22] and interaction with the cell interior [23] have been studied extensively. However, very few works have considered cell related parameters, such as cell type and cell cycle stage [24,25]. Cell malignancy and metastatic potential (invasiveness) affect particle endocytosis [26], which has implications in drug delivery applications. Particle internalization has been shown to be slower in malignant breast cells than into their benign

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counterparts, yet more particles ultimately entered the malignant cells [27]. In addition, internalization of particles and their co-localization with lysosomes was faster in invasive, cancerous breast-cancer cells as compared to malignant (cancerous, yet non-invasive) breast tissue cells [28].

Here, we evaluate the time-dependent amounts of internalized 200-nm diameter particles and their membrane-encapsulation within endocytotic organelles, comparing benign, low metastatic potential (MP) and high MP epithelial breast cells. We note differences related to growth patterns and cell–cell interactions in two-dimensional (2D) culture. In addition, we determine the time-dependent endocytotic pathway of the particles by quantifying encapsulation in early and late endosomes, lysosomes, endoplasmic reticulum (ER), and the Golgi. Uptake and encapsulation dynamics were evaluated by determining colocalization of particles into each of the organelles at 2, 6, 24, and 48 h after exposure to particles. Our work shows uniform internalization of large numbers of particles into all the cancer cells, differing from the benign cells evaluated here. In addition, we show that particles gradually lose membrane encapsulation, and demonstrate that particle tracking experiments, to evaluate intracellular mechanics and dynamics, may be carried out after endocytosis.

2. Materials and methods

2.1. Cell culture

We have used three human, epithelial, breast cell lines: high metastatic potential (high MP), MDA-MB-231 (HTB-26, ATCC Manassas, VA), low MP, MDA-MB-468 (HTB-132, ATCC), and as control, a benign cell line MCF-10A (CRL-10317, ATCC). Benign cells were kindly provided by Prof. Israel Vlodavsky from the Faculty of Medicine, Technion-Israel Institute of Technology.

Cells were cultured and maintained in a humidified incubator at 37 °C, 5% CO_2 and were used at passages 10–30 from stock. High and low MP cell lines were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, ThermoFisher Scientific, Waltham, MA) and 1 vol.% of L-glutamine, sodium pyruvate and penicillin–streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). Benign cells were grown in DMEM/Ham's F-12 medium supplemented with 5% horse serum (Hyclone, Waltham, MA), 1 vol.% of L-glutamine and penicillin/streptomycin, 10 μg/ml insulin, 10 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, and 100 ng/ml cholera toxin; experiments were run with FBS containing media.

2.2. Flow cytometry

Fluorescent (Ex/Em 505/515), carboxylate-modified polystyrene particles, 200-nm in diameter (Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA) were added to the media of adhered cells, plated the day before. The same amount of particles was added to each plate (3000 particles/cell) to allow comparison of amounts of particles internalized between different cell lines. Cells were washed, to remove any unincorporated particles and then harvested using trypsin–EDTA at 2, 6, 24 and 48 h following particle addition to the media. The relative amount of particles inside each cell was estimated through measurement of fluorescence levels, where more particles induce proportionately stronger fluorescence. Measurements were performed in a BD LSR-II analyzer (BD Biosciences, Franklin Lakes, NJ), and included 10,000 cells compared to control cells without particles at each particle-exposure time ($n \geq 3$).

2.3. Sample preparation for imaging

Cells were seeded on number 1.5 thickness cover-glasses (Menzel-Gläser, Germany), incubated overnight to allow attachment, and only then particles were added; seeded cell numbers were similar between the lines, but smaller amounts of the faster proliferating benign cells were used to maintain comparable cell numbers during the experiment. Fluorescent 200-nm diameter particles (same as in flow cytometry) were added to the media and internalized by endocytosis. Cells were incubated with particles for different times prior to cell fixation: 2, 6, 24, and 48 h. The number of particles added to cells was in large excess, a few thousands to tens of thousands of particles/cell, and was separately optimized for imaging in each cell line and time point to reduce formation of clusters; the average numbers were 3000 particles/cell. In the benign cells, culture media was replaced with fresh media containing fetal bovine serum after cell adhesion, and only then particles were added, as horse serum caused large particle aggregates to form.

2.4. Fixation and staining

The staining protocol was optimized for each organelle and cell line. Cells were fixed with cold methanol or with 3.2% paraformaldehyde and permeabilized with 0.5% triton. Blocking for non-specific binding was done with 1% gelatin or 1% BSA (Sigma, St. Louis, MO). Early and late endosomes were labeled with the primary antibodies EEA-1 and CD-MPR (Santa Cruz Biotechnologies, Santa Cruz, CA), respectively. Lysosomes, the Golgi system, and the endoplasmic reticulum were labeled with the primary antibodies LAMP1, giantin (both Abcam, Cambridge, MA), and by AF18 (Thermo Scientific, Rockford, IL), respectively. Secondary antibodies were: Fluorescein Donkey anti-goat IgG for early endosomes, Cy5 Donkey anti-rabbit IgG for late endosomes and the Golgi system, Cy5 Donkey anti-goat IgG for lysosomes and Cy3 goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA and R&D Systems, Minneapolis, MN). During secondary antibody staining, cell nuclei were labeled with 4′-6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO).

2.5. Imaging

Imaging was performed on the spectral-imaging Zeiss LSM700 or Zeiss LSM510 confocal systems mounted, respectively, on motorized Axio Observer Z1 or Axio Imager Z1 microscopes. In both systems a 63x/1.4NA oil objective was used. Image thickness was 1 μm, corresponding to 1 airy unit of the longest wavelength used in the system.

2.6. Particle–organelle colocalization

Encapsulation of particles in organelles was determined using an automated, custom-written module in MATLAB 2010b (Mathworks, Natick, MA) that identified fluorescent particle colocalization with a stained organelle. Shortly, images of fluorescent particles and organelle stains are overlaid and colocalization is detected by color. Organelle stains images were transformed to green pseudocolor, particle images were transformed to red, and the module searches for areas of yellow overlap. Measurements were also manually verified to eliminate any erroneously detected colocalization that can occur due to background staining in cells. In each image, we obtain the total number of cells, particles, and encapsulated particles (see Table S1). We evaluated a total of 150–800 cells for each condition and cell type in several experiments; in the ER only 80–150 cells were evaluated, as negligible amounts of particles were internalized.

2.7. Particle tracking in cells

Experiments were performed in previous works [8,9]. In short, particle motion was recorded at 60 frames-per-second using a XR/TURBO-120EX CCD camera (Stanford Photonics, Palo Alto, CA), at a final magnification of 99 nm/pixel, obtained using an inverted, epi-fluorescence microscope (IX81 Olympus, Japan) with a 100x/1.4NA