Gold Nanoparticles as Localization Markers for Direct and Live Imaging of Particle Absorption through a Caco-2 Cell Monolayer Using Dark-Field Microscopy

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Introduction

The application of nanotechnology to food-related sectors is generally referred to as food nanotechnology. One of the main approaches in food nanotechnology is the reduction in size of food materials, since novel properties that could be beneficial in food processing and bioavailability are expected to be achieved upon size reduction. Some particles with diameters in the nano- or micro-meter range, encapsulating proteins, nutrients and functional substances, have enhanced the absorption and bioavailability as well as protected against degradation and instability. In the food industry, liposomes and water-soluble and -insoluble substances, respectively. However, the absorption of nano- and micro-particles are generally assumed to mainly permeate the paracellular route across tight junctions, the inter-cellular boundaries between epithelial cells. To determine the effects of encapsulation of food components on intestinal absorption, therefore, it is important to clarify how such food particles associate with intestinal epithelial cells.

Recently, efforts have been made to reduce the size of food particles containing functional ingredients, since reducing the size is expected to improve intestinal absorption. However, the absorption mechanisms have yet to be fully clarified. Therefore, a microscopy-based method for studying interactions between the particles and intestinal cells is required. We optimized the experimental conditions for observing gold nanoparticles (AuNPs) on the surface of an unfixed Caco-2 cell using dark-field microscopy (DFM). Tight junctions were clearly visible with AuNPs on the cells, producing intense scattered light under DFM. This suggests that AuNPs could be used as localization markers to visualize particle absorption through Caco-2 cells.

Notes

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promising labeling material as a marker for imaging interactions of food particles with Caco-2 cells using DFM.

Experimental

Gold nanoparticles (AuNPs)

Positively charged and negatively charged AuNPs, 30 nm in diameter, were prepared according to the literature.\textsuperscript{14,15}

Cell culture

Caco-2 cells (HTB-37; American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, 1% non-essential amino acids (Invitrogen), 100 U/ml penicillin, and 0.1 mg/ml streptomycin in a humidified atmosphere of 5% CO\textsubscript{2} at 37°C. Cells between passages 35 – 60 were used. Cells were seeded into cell culture inserts (membrane material, polyethylene terephthalate; pore size, 0.4 μm; surface area, 4.2 cm\textsuperscript{2}; Falcon, NJ) coated with type-I collagen. After seeding, the cells were cultured for 10 – 17 d to allow monolayer formation. The integrity of the cell layer was evaluated by measuring the transepithelial electrical resistance (TER) with Millicell-ERS equipment (Corning, Cambridge, MA). Monolayers with a TER > 250 Ω × cm\textsuperscript{2} were used for immunostaining and transepithelial transport experiments.

Immunostaining

Caco-2 cell monolayers were washed with phosphate buffered saline, and fixed in methanol at 0°C for 20 min. Cell monolayers were blocked in 3% bovine serum albumin in Tris-buffered saline, and incubated for 1 h with 1:100 diluted rabbit polyclonal anti ZO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation for 1 h with 1:500 diluted goat Alexa 546-conjugated anti rabbit IgG (Invitrogen). Fluorescence was visualized using a Nikon Eclipse 80i (Nikon, Tokyo).

Dark-field microscopic imaging

For DFM imaging of the interactions between AuNPs and the Caco-2 cell monolayer, the monolayer on the culture insert was washed with Hanks' Balanced Salt Solution (HBSS, pH 6.0, Sigma-Aldrich, St. Louis, MO). Five microliters of a 0.0574 wt% AuNP suspension and 200 μL of HBSS were added to the upper chamber of the insert, and the Caco-2 cell monolayer was incubated at room temperature for 1 h. After washing the cells with HBSS, the upper chamber was filled with 3 mL of HBSS and directly placed onto a glass slide. The resultant specimen was subsequently utilized in DFM imaging.

In DFM, only the light scattered by the structure under study is collected in the detection path, while directly transmitted light is blocked using a dark-field condenser. Dark-field light scattering images were acquired using a Nikon Eclipse 80i with a dark-field condenser, a 100-W halogen lamp and a Canon Power Shot A640 digital camera (Canon, Tokyo).

Results and Discussion

Transportation of food ingredients across Caco-2 cells is conventionally studied using commercially available trans-membranes on which Caco-2 cells are fully grown and successfully differentiated. The culture insert attached to the trans-membrane was removed from the washing buffer and placed directly onto the glass slide (Fig. 1). DFM observations showed that a single monolayer of Caco-2 cells was clearly formed on the trans-membrane when the Caco-2 cells, at an initial cellular concentration ranging from 1.5 × 10\textsuperscript{4} to 2.5 × 10\textsuperscript{5} cells/cm\textsuperscript{2}, were grown for 10 – 17 days (Fig. 2). In contrast, a higher initial concentration of Caco-2 cells produced a multilayer on the trans-membrane, and inhibited clear observation of Caco-2 cells after full growth. This is because a cell multilayer significantly attenuates scattered light, and makes light scattered from out-of-focus cells appear in the field of view.

A Caco-2 monolayer on the trans-membrane is preferred for accurate and quantitative analysis of conventional transport. Indeed, cell culture conditions affect the transport activity, and a Caco-2 cell multilayer on the trans-membrane also disturbs fluorescent imaging.\textsuperscript{16} Therefore, the use of Caco-2 cell monolayers would result in reliable measurements during microscopic imaging as well as conventional transport studies.

We next determined whether the network-like structure of the Caco-2 cell monolayer observed with DFM, as in Fig. 2,
corresponded to tight junctions or not. Tight junctions are properly formed in the differentiated epithelial cells by an assembly of scaffold proteins. ZO-1 is one of the scaffold proteins, and is located beneath the plasma membrane in the tight junction. Therefore, ZO-1 is widely used as a protein marker for localization of tight junctions of successfully differentiated Caco-2 cells. Here, the anti ZO-1 antibody was applied to immunostaining of the Caco-2 cell monolayer on the trans-membrane, since differentiated Caco-2 cells with tight junctions are required for a transport study. Immunostaining showed that ZO-1 was expressed along the tight junctions as is normally observed in differentiated cells, and the fluorescent signals from ZO-1 corresponded to scattered signals observed under DFM (Fig. 3, lower panels). On the other hand, a treatment without the primary antibody did not produce any fluorescence (Fig. 3, upper panels), suggesting that the Caco-2 monolayer did not have intrinsic fluorescence under this experimental condition. In addition, chemical fixing of the cells did not affect how they appeared under DFM. Taken together, these observations indicate that Caco-2 cells on the trans-membrane, at an initial cell concentration of $1.5 \times 10^4 – 2.5 \times 10^5$ cells/cm$^2$, grew as a monolayer with sufficient differentiation, and that the signals observed under DFM corresponded to their tight junctions.

AuNPs with either positive or negative charges on their surface were added to HBSS in the upper chamber, and incubated with the cells. Since metal nanoparticles, such as Au or Ag, exhibit enhanced scattering behavior originating from their localized plasmon resonance (LPR), they would be advantageous for the observation of adsorption onto the cell surfaces using DFM.

In the case of positively charged AuNPs, several red spots

![Fig. 3](image1.png) Correlation of the localization of ZO-1 protein with DFM signals from a Caco-2 cell monolayer. ZO-1 localization was visualized using fluorescence microscopy (FM). Caco-2 cells were treated with (lower panels indicated as “ZO-1”) or without (upper panels indicated as “none”) the anti ZO-1 antibody, and then with Alexa 546-conjugated anti rabbit IgG. The obtained images from both DFM and FM were merged. The scale bars indicate 10 μm.

![Fig. 4](image2.png) Binding of AuNPs to a living Caco-2 cell monolayer. The Caco-2 cell monolayer was imaged with DFM in the presence of positively charged AuNPs (A) or negatively charged AuNPs (D). Signals from AuNPs (B, E) and Caco-2 monolayer (C, F) were individually detected after color separation of the original images (A, D). The scale bars indicate 10 μm.
were observed on the Caco-2 cell monolayer (Fig. 4A). In general, individual spheres of AuNPs are observed as green spots originating from their LPR colors. When metal nanoparticles are closely organized, their LPR peak is shifted towards lower energy.\textsuperscript{18} Thus, red spots indicate that several AuNPs were aggregated and attached to the cell surface. Localizations of AuNPs were simply distinguished from those of the tight junctions by color separation of the original image with RGB information. AuNPs could be obtained by subtracting green from red (Fig. 4B), whereas the tight junctions apparently corresponded to blue (Fig. 4C). On the other hand, negatively charged AuNPs were rarely associated with the cell surface (Figs. 4D – 4F), although negatively charged AuNPs with Brownian motion could be observed in the buffer when the focal plane was above the cell surface. Considering that the plasma membrane of a Caco-2 cell is negatively charged under physiological conditions,\textsuperscript{19} this result suggests that alterations in the surface charge of AuNPs would greatly affect their interaction with cells.

Since AuNPs attached to the cell surface have their own LPR colors, locations of individual AuNPs could be distinguished from the white network of tight junctions in the same field of view under a microscope (Fig. 4). This characteristic of AuNPs would prominently contribute to single-step live-imaging of the interaction between the Caco-2 cell monolayer and AuNPs. In addition, AuNPs of various diameters and charges are available, and could be chosen for the labeling of food particles, which contain functional food ingredients, depending on the size of the particles. Finally, preparations of AuNPs are more cost-effective than using fluorescent molecules. Therefore, these results suggest that AuNPs could be used as localization markers for food particles during their absorption through Caco-2 monolayers, as analyzed by DFM.

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References