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A biophysical model of intracellular distribution and perinuclear accumulation of particulate matter

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ABSTRACT

We have measured in human alveolar cells the cytoplasmic distribution of the fluorophore coumarin-6 carried by Solid Lipid Nanoparticles (SLNs) and observed a perinuclear accumulation of the fluorescence that can be described by a single exponential growth along an ideal line joining the plasma membrane to the nuclear border and by a sigmoidal relationship as a function of time. Intracellular distribution was affected by lowering the temperature from 37 to 4 °C and by the disruption of cytoskeleton by cytochalasin D, but it was minimally perturbed by the inhibition of ATP dependent molecular motors. A biophysical model was developed for an accumulation of loaded particles against a diffusion gradient based on a mean field interaction energy, whose origin we ascribe to the actin structure of the cytoskeleton. The estimated value for the load diffusion coefficient was four and two orders of magnitude less than that of free coumarin-6 and of SLNs in aqueous solutions, respectively, suggesting that the load moves within the cell cytoplasm in a form still reminiscent of the nanocarrier structure.

1. Introduction

The uptake of extracellular material across the plasma membrane of eukaryotic cells is well described and characterized by a variety of mechanisms going from the passive diffusion to the active transport [1]. The former, defined as a concentration gradient-driven mass transport of a compound, is considered the dominant route [1]. The latter, including the endocytosis mediated processes, is in charge of taking up larger molecules and molecular complexes.

Once entered the cells, the intracellular traffic and distribution of substances that do not follow the endocytotic pathway are related to aspecific movements or microtubule-based active mechanisms [2,3]. Furthermore, exogenous material of various origin tends to accumulate in the perinuclear region after cellular uptake [2,4–9]. In a recent study involving human alveolar epithelial cell line, we confirmed this observation also for Solid Lipid Nanoparticles, SLNs, loaded with the fluorophore coumarin-6 (c-SLNs) and we hypothesized a pathway for their uptake [10].

Here we want to analyze, from a biophysical point of view, the cytoplasmic distribution of the fluorophore carried by the SLNs, and the way it is affected by the incubation temperature, by the state of the cytoskeleton and the efficiency of the molecular motors. We focused our efforts on the perinuclear accumulation of coumarin-6, taken here as a model hydrophobic compound and develop a biophysical model of the fluorophore distribution in the cell that assumes an interaction energy between the fluorophore carried by the SLNs and cellular components. This interaction energy counterbalances the free thermal diffusion of the molecules within the cytoplasm and could be specifically related to the cytoskeleton or to active transport mechanisms such as those related to the molecular motors. The modeling of the perinuclear accumulation of nanoparticulate that we outline here, may be of potential interest for the biomedical use of nanoparticulate structures, in particular for the delivery of drugs or genetic material in the nuclear region.

2. Materials and methods

2.1. Nanoparticles

SLNs were produced by NANOVECTOR, by choosing tripalmitin as lipidic matrix. SLNs have been loaded with coumarin-6 (c-SLNs) to allow their visualization by means of fluorescence microscopy (λ_{exc} = 450 nm, λ_{em} = 505 nm). The molar ratio coumarin-6:tripalmitin was about 80 (NANOVECTOR, personal communication). The average

Abbreviations: a.u., Arbitrary units; cyto D, Cytochalasin D; c-SLNs, (coumarin-6) loaded in the SLNs; EL, Emission Line; min, Minutes; NPs, Nanoparticles; SLNs, Solid Lipid Nanoparticles; ROIs, Regions of Interest.

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diameter of heat sterilized c-SLNs dispersion, determined by Dynamic Light Scattering (Brookhaven 90Plus system, Brookhaven Instruments Corp.), was 116 ± 15 nm, and remained essentially stable (≤130 nm) either when the particles were dispersed in buffer or in cell culture medium supplemented with 1% FBS. The Z potential of the SLNs was −24 ± 2 mV (Brookhaven 90Plus system, Brookhaven Instruments Corp).

In experiments where coumarin-6 was used as free drug, a stock solution of 20 μg/ml in DMSO was prepared and diluted at the desired working concentration.

2.2. Cell culture

A30 cells [11] were grown on Petri dishes in DMEM medium supplied with 10% FBS, 1% of L-Glutamine and 1% of Penicillin/Streptomycin and incubated in a controlled environment at 37 °C with 5% CO_2. During the experiments the cells were incubated with medium supplemented with 1% FBS to prevent NPs aggregation.

2.3. Effect of temperature on NPs intracellular distribution

We estimated the cytoplasmic concentration of coumarin-6 by monitoring the fluorescence distribution in cells exposed to c-SLNs (0.01 mg/ml) for 45 min either at 37 °C or at 4 °C. In the latter case the cells were pre-cooled for 30 min at 4 °C. At the end of the incubation period, cells were immediately fixed in paraformaldehyde 4% in PBS.

2.4. Image acquisition

The experimental set up consisted of a wide field NIKON Eclipse FN1 equipped with a 63X objective, numerical aperture 1.2, motorized table (Prior Scientific, Rockland, MA, USA), Photometric coolSNAP ES Digital Camera and Metamorph (Molecular Device, Downington, PA, USA) software for image acquisition and analysis. We used fields of view that encompassed at least 5 cells for analysis. The fluorescence signal form the c-SLNs was discriminated by a band pass filter ($\lambda_{em} = 505$ nm) and the images were averaged over an exposure time of 400 ms

Starting from the top of the cell, 40 Z planes were acquired along the Z axis at 0.5 μm inter-distance. We typically considered the overall fluorescence acquired in the wide field. In specific cases, we used the fluorescence estimated on a Z plane corresponding to the mid cell height, obtained through a deconvolution process (AutoDeblur, Media Cybernetics, Bethesda, MA, USA).

2.5. Drugs treatment

The role of cytoskeleton in affecting fluorescence cytoplasmic distribution was addressed by treating cells, previously exposed to c-SLNs (0.01 mg/ml for 45 min at 37 °C) and analyzing the perinuclear fluorescence that these cells displayed when incubated with cytochalasin D (CytoD) at a concentration of 2 μM for 1 h at 37 °C. In other experiments, before the exposure to the NPs, cellular energy stores were depleted by a pre-treatment for 1 h at 37 °C with 0.2 mM sodium azide (NaN_3) or 50 mM 2-deoxy-D-glucose (DDG) [12]. After this pre-treatment, the cells were incubated also with c-SLN (0.01 mg/ml) for additional 45 min at 37 °C. At the end of the incubation time the intracellular fluorescence distribution was monitored and the images were acquired and analyzed as described. Nuclei were labeled with DAPI 1 μM.

2.6. Immunocytochemistry

Cells plated on glass coverslips were fixed with paraformaldehyde 4% in PBS at room temperature for 20 min, washed three times in PBS, LS (Low Salt PBS) and in HS (High Salt PBS) respectively and then permeabilized with digitonin 0.01% in GDB for 30 min. Fixed and permeabilized cells were incubated with phalloidin conjugated with Texas RED (dil 1:200. Molecular Probes) for actin filaments staining or primary antibody antimyosin (dil 1:500, Covance) diluted in GDB at RT for 2 h. After washing with HS for three times, cells labeled for myosin were incubated with Alexa conjugated secondary antibody diluted in GDB (1:100). Cells were then washed with HS AND LS three times respectively. DAPI was used to stain cell nuclei at a concentration of 1 μM in PBS for 5 min. Coverslips were mounted with glycerol.

2.7. Statistical analysis

Statistical analysis was carried out by *t*-test and the significance level was set at $p < 0.001$ (indicated by symbol * or # in the figures).

3. Results

The intracellular fluorescence distribution of the fluorophores loaded in the c-SLNs was measured on monolayers of human lung epithelial A30 cells, very similar to the more studied A549,[11] an important component of the blood-air barrier and a sensitive site for particulate-body interaction. The analysis of the coumarin-6 fluorescence distribution was performed by selecting, on individual cells, a line (emission line, EL) running from the plasma to the nuclear membrane (Fig. 1, image) and four cytoplasmic regions of interest (10 pixel square ROIs) positioned along such EL at 25%, 50% and 75% of the total length. The location of the plasma membrane was obtained by saturating the color levels to half the maximum color depth in the images. We verified that this allowed us to locate the cell membrane by comparing with images in which the actin filaments were specifically labeled. Since the width of the cell cytoplasm varies among the cells we needed to define a normalized distance along the EL in order to compare and average the fluorophore distributions measured on different cells. Therefore in the following we will refer to the distance along the EL as $x = 100\ (r - r_N)/(r_C - r_N)$, where r_N, r_C and r are the positions of the nuclear and the cell membrane and the selected ROI along the EL (x = 100% indicates the nuclear membrane).

The cells were exposed to an estimated concentration of 1.8×10^{10} c-SLNs/ml, corresponding to a tripalmitin concentration ≅ 0.01 mg/ml, one order of magnitude lower than the toxic concentration (0.2 mg/ml) estimated by LDH release assay and MTT test [10].

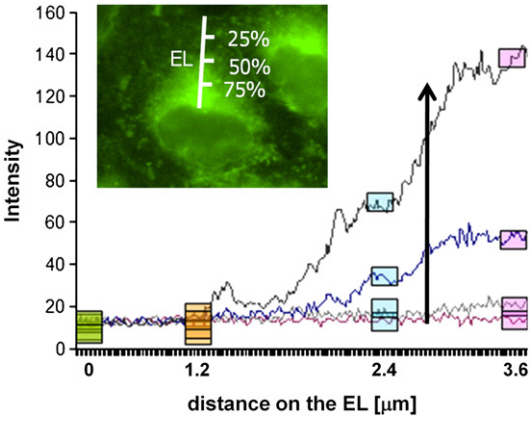

Fig. 1. Image: an Emission Line (EL) is drawn on a representative cell image acquired after 30 min of c-SLNs incubation ($\lambda_{exc} = 488$ nm, integration time 400 ms). The EL runs from the cell to the perinuclear region and four different ROIs (at 0, 1, 2.4, 3.6 μm along the EL) on which the fluorescence analysis was carried out, are marked on the image. Plot: we report the representative traces of the fluorescent signal collected on a single cell along the EL and recorded at increasing incubation time from bottom to top (0, 9, 15 and 30 min). The signal is shown in arbitrary units.