

METHOD FOR DETECTION OF POLYCATIONIC NANOPARTICLES LOADED WITH DNA IN EUKARYOTIC CELLS

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ABSTRACT

Development of new delivery systems of biological macromolecules in eukaryotic cells is a scientific issue intensively studied in recent decades. Most of the methods used determine the general cytotoxicity or availability of product as a result of transfection, without considering the presence and behavior of the nanoparticles themselves in cells. This requires the development of methods proving effective penetration and movement of nanoparticles into cells. We propose a modified method by which one can trace the penetration of nanoparticles loaded with DNA in different cell cultures.

Key words: *Nanoparticles, HepG2 cells, acridine orange staining, lysosomes*

Introduction

Several types of chronic diseases are linked to long term exposure to particulate matter [1]. On the other hand, many new technologies in different fields used nanoparticles. They can be applied not only in traditional gene therapy applications, but also for delivery of siRNA for gene silencing. Polycations are promising non-viral vectors for nucleic acid delivery, which can form nanosized polyplexes with nucleic acid molecules via electrostatic interactions. Various cationic polymers have been studied as DNA carriers [2, 3]. Most studies on nanosized particles determine the general cytotoxicity or availability of product as a result of transfection, without considering the presence and behavior of the nanoparticles in cells. An important stage after cytotoxicity assessment is adequate evaluation of the internalization and transfection efficiency.

The nanosized materials from environmental pollution are extremely stable and cause extensive cellular damage, but can be easily visualized by conventional microscopy techniques. On the opposite, nanoparticles for medical uses have to be unstable into the target cells to deliver carried biological macromolecules. The purpose of this study was to optimize the available methods for visualization of polycationic nanoparticles by photon microscopy to allow simultaneous evaluation of the way of penetration of particles into the cell and its further behavior. Here we compared several classic microscopic methods and offered an optimized method for fluorescent microscopy by supravital staining with acridine orange.

Materials and methods

Cell cultures

We used two human cancer cell lines – hepatocarcinoma HepG2 and lung carcinoma epithelial cells A549. Cells were grown in 25 cm² "CELLSTAR[®]" flasks, at standard conditions in humidified atmosphere with 5% CO₂, at 37°C, in DMEM, supplemented with 10% FBS (BioWhittaker[™]) and 1% (v/v) antibiotic-antimycotic solution (penicillin 100 U/ml, streptomycin 100 µg/ml and amphotericin B 0,25 µg/ml, BioWhittaker[™]). Cells were plated on sterile cover slips in 24-wells plates „Nunk[™]”.

Nanoparticles

Nanoparticles containing polyethylene glycol and poly(L-lysine (stable for up to 10 days; mentioned below as polyA) and hyperbranched or comb-like polyethylenimine (stable for 24 hours,

mentioned below as polyB) were used as DNA carriers. Both probes have an average diameter of about 120 – 250 nm. Salmon sperm DNA (D-1626, 2000 bp, corresponding to M_w of 1.32×10^6 Da) was purchased from Sigma-Aldrich.

Staining protocol

24 hours after plating, cells were incubated for 6 hours with nanoparticles in concentration, which ensures $2 \mu\text{g}$ DNA per 1×10^6 . Methylene blue staining for evaluation of cell morphology was performed after fixation of cells with phosphate buffered formalin for 20 min and several washes in PBS. Cells were stained with 1% Methylene blue in PBS for 5 min, dehydrated and mounted with Canada balsam. Cell morphology was observed under light microscope Olympus CX21. For fluorescent microscopy several fluorochromes were selected and used following the instructions of manufacturers (Table 1). Slides were mounted with PBS and immediately observed under fluorescent microscope Nikon EZ-C1.

Fluorochrome	Concentration	Incubation time	Reference
SybrGreen®	dilution 1:1 000	1 min	Noble and Fuhrman [4]
DAPI	$2 \mu\text{g/ml}$	1 min	Cold Spring Harb Protoc [5]
Hoechst 33342	$2 \mu\text{g/ml}$	1 min	Invitrogen [6]
Acridine orange	$5 \mu\text{g/ml}$	5 min	See below

Results and discussion

The appropriate fluorochrome must meet the following requirements: 1) to be non-toxic for the cells; 2) to allow specific staining of cellular components and/or nanoparticles; and 3) to bind DNA without electrostatic interactions. The last requirement is critical because the polycationic nanoparticles are formed on the basis of such interactions between the polymer used and DNA. Positively charged nitrogen atoms of the polymer facilitate the effective binding and condensation of the negatively charged DNA [7].

As a first step we attempted to stain the DNA with intercalating fluorochromes (SybrGreen®, DAPI and Hoechst 3345) before formation of nanoparticles. Unfortunately, SybrGreen® was toxic for the living cells and the fluorescence of DAPI and Hoechst was quenched probably by the polymer (data not shown). Results from staining of previously formed nanoparticles with the same fluorochromes are presented on Fig. 1.

Additionally, we stained both types of nanoparticles and pure plasmid DNA with acridine orange. The stable polyA nanoparticles did not stain, while some of unstable polyB particles were slightly fluorescent (Fig. 1). However, we detected this signal about 10 hours after preparation.

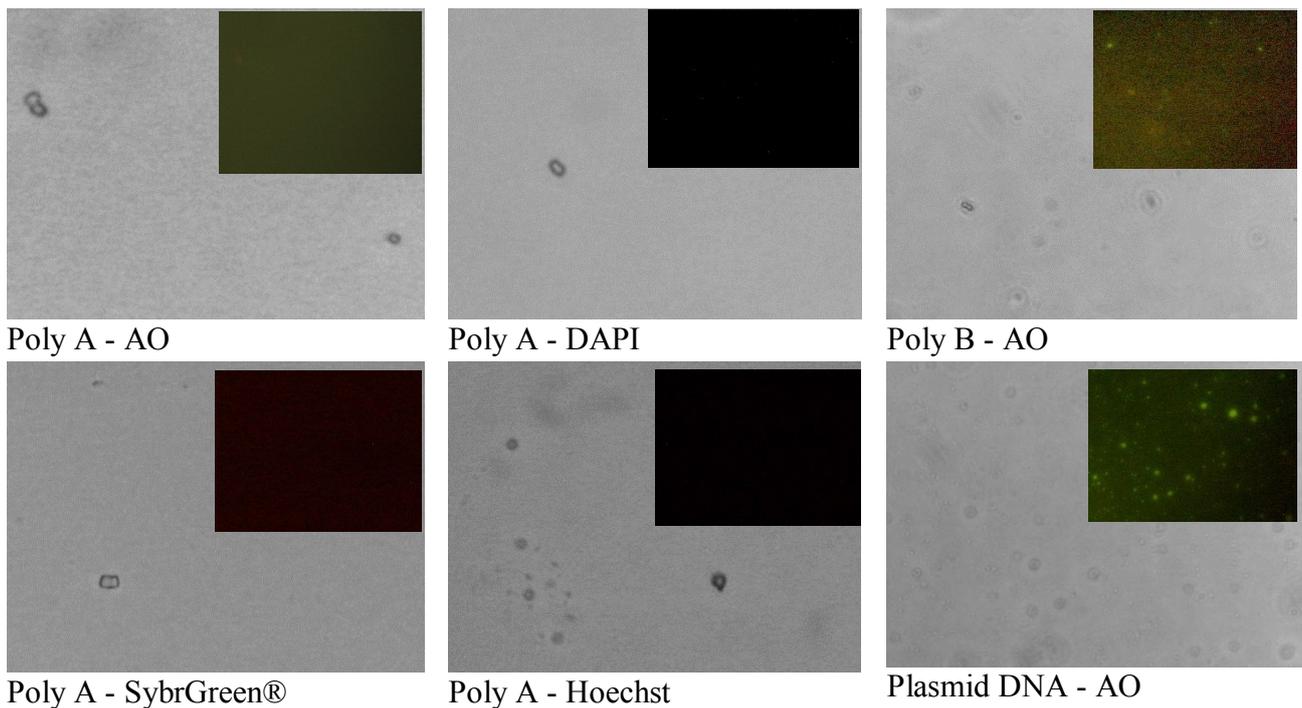


Fig.1 Nanoparticles (polyA and polyB) and plasmid DNA, stained with the DNA binding dyes: AO - acridine orange, DAPI, Hoechst 33342, SybrGreen® (phase contrast and images with corresponding excitation filter of fluorescent microscope - inserts).

Next step was to choose appropriate conditions to stain cells incubated with the nanoparticles in concentration, which ensures $2 \mu\text{g DNA per } 1 \times 10^6$. We applied this DNA concentration to compare the transfection efficiency with a recent transfection protocol as Effectene Transfection Reagent (QIAGEN). Using Methylene blue staining, we were able to detect internalized nanoparticles after 6 hours of incubation (Fig. 2). This method required fixation of the cells before staining and thus it is difficult to follow the process of internalization. Furthermore, the same samples of fixed cells were stained with SybrGreen® and we did not see any fluorescent particles, except micronuclei in few control cells (marked with star in Fig. 2).

Promising data were obtained by acridine orange staining of living unfixed cells simultaneously with incubation with nanoparticles. Acridine orange is a metachromatic dye and fluoresce differently under different conditions. Upon intercalation between the bases of DNA green fluorescence is obtained, while the electrostatic binding to RNA results in a yellow fluorescence. Following exposure to acidic environment in lysosomes acridine orange is ionized and form clusters with red fluorescence [8]. Several groups used this property of the acridine orange to evaluate in vivo lysosome integrity, but the protocols and resulting images are quite different. For flow cytometry analysis of cell vitality, acridine orange is applied at concentration 1-2 $\mu\text{g/ml}$ for 30 min and the final results are pale green cells with bright red lysosomes when observed at 488 nm without washing. According Fröhlich E. et al., supravital staining of lysosomes in cell culture were performed with 1 μM acridine orange (which corresponds to 0.265 $\mu\text{g/ml}$) in PBS for 30 min at 37°C and, after rinsing of the slides, fluorescence was determined with two different filters 485 nm/520 nm and 584 nm/612 nm [1]. Tseng et al., stained lysosomes in retinal pigment epithelial cells with 5 μM acridine orange (about 1.3 $\mu\text{g/ml}$) for 30 min and fixed them before the observation [9].

All of these protocols require relatively long incubation and are focused on obtaining bright lysosomes and pale cells fluorescence. We intend to optimize the method to get more intense staining of cells and to allow visualization of any changes in the cell periphery caused by adsorption and internalization of nanoparticles. Another obstacle has been the use of a dye dissolved in a buffer solution which has a lower osmotic strength than natural or culture medium for the cells and could affect the penetration of nanoparticles. A classical study for the uptake of biologically active substances including acridine orange by isolated lysosomes [10] reveal that the maximum is about 5-10 min at 0 and 37°C, so we focused on shorter incubation with acridine orange diluted in culture medium. Best results were obtained using 5 µg/ml acridine orange in cell culture medium for 5 min before observation (Fig. 2, in the middle). Up to 15 min after incubation, PolyA nanoparticles were detected on cell periphery as pits during adsorption (data not shown), but after 30 minutes penetration occurs and particles were seen as dark dots (shown with white arrow) closed to lysosomes (red clusters) in the cells.

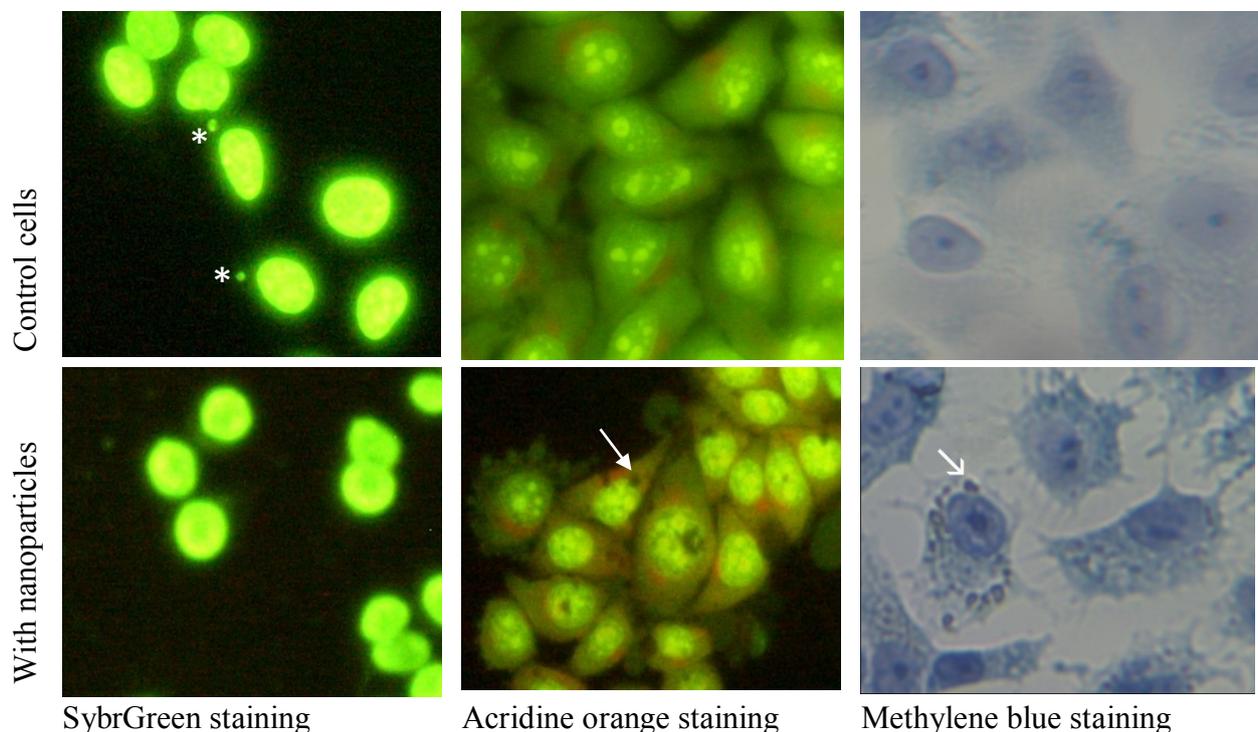


Fig. 2 HepG2 and A549 cells – control and incubated 6 hours with nanoparticles polyA, stained with the DNA binding dyes: AO - acridine orange, SybrGreen® (with corresponding excitation filter of fluorescent microscope, magnification 400x) and methylene blue (light microscope, 400 x). Some micronuclei are seen in control cells (stars). Nanoparticles are indicated with arrows.

Conclusions: Using a modified acridine orange staining of living cells for 5 min we are able to detect and follow internalization of polycationic nanoparticles in the cells.

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