

Alcian blue staining to study nanoparticle-cell interactions at transmission electron microscopy

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1. Introduction

Transmission electron microscopy (TEM) is the technique of choice to investigate the distribution of nanoparticles (NPs) inside cells and tissues, providing essential information on their actual spatial relationships with biological structural components. In order to track NPs in the biological environment, they must be unequivocally visualised. This is easily obtained for NPs containing electron dense components such as metal ions, but it may be difficult for NPs made of organic components such as polymers or lipids: in fact, their moderate electron density makes them hardly detectable in the inter- and intracellular space. In previous studies, we solved this problem by labelling chitosan-based and phospholipidic NPs with fluorochromes, and subsequently applying the diaminobenzidine photo-oxidation technique. This method gives rise to a finely granular electron dense product thanks to the reactive oxygen species originating upon fluorochrome irradiation, thus allowing a very precise labelling of NPs at the ultrastructural level [1, 2]. However, in the absence of appropriate fluorochrome labelling or in the presence of a marked background, diaminobenzidine photo-oxidation cannot be applied. We recently faced this problem with hyaluronic acid-based (HA) NPs. As an alternative approach, we set up a novel application of a long-established histochemical technique suitable for revealing glycosaminoglycans, *i.e.*, the critical electrolyte concentration method of Alcian Blue (AB) staining [3].

2. Materials and Methods

NPs made of HA and polyarginine were obtained by ionic gelation technique (Carton et al., Submitted). NPs were characterized in term of size and polydispersity index (PdI) using Dynamic Light Scattering. C2C12 mouse myoblasts were grown in 75 cm² plastic flasks using Dulbecco's modified Eagle medium, at 37°C in a 5% CO₂ humidified atmosphere. When subconfluent, the cells were trypsinized and seeded onto slides in 24-multiwell plates; 24 h later, the cells were treated with NPs for 2 h and 24 h. Untreated cells were used as control. In order to visualise HA NPs at light microscopy, C2C12 cells were fixed with 4% paraformaldehyde, and the AB staining was performed under acid conditions [3]; the nuclei were counterstained with nuclear fast red that also makes the cytoplasm pale pink. Some HA NPs were submitted to the AB staining in suspension in culture medium. For TEM, we applied the protocol proposed by Schofield and coworkers [4], who modified the original AB method intended for light microscopy to discriminate cartilage mucopolysaccharides at the ultrastructural level. Briefly, cells were fixed with 3% glutaraldehyde, stained with AB, post-fixed with OsO₄ and potassium ferrocyanide, dehydrated and embedded in Epon resin. Ultrathin sections were observed without lead staining with a Philips Morgagni TEM equipped with a MegaView II digital camera for image acquisition.

3. Results and Conclusions

HA NPs were about 300 nm in diameter, with a PdI of 0.04. After AB staining, HA NPs were visualized at light microscopy as blue dots both as free NPs in suspension (Figure 1a) and after their internalization in C2C12 cells (Figure 1b). At TEM, AB-stained NPs were easily detectable due to the presence of a granular electron dense product (Figure 2). No reaction product was found on any

cell structural components; moreover, in control cells no staining was observed. Thanks to the AB ultrastructural staining, we were able to describe the very early uptake steps as well as the intracellular fate of HA NPs in C2C12 cells. This result demonstrates that, in the nanomics era, the proper application of traditional histochemical techniques can be crucial to get direct evidence of the nanoparticle-cell interactions.

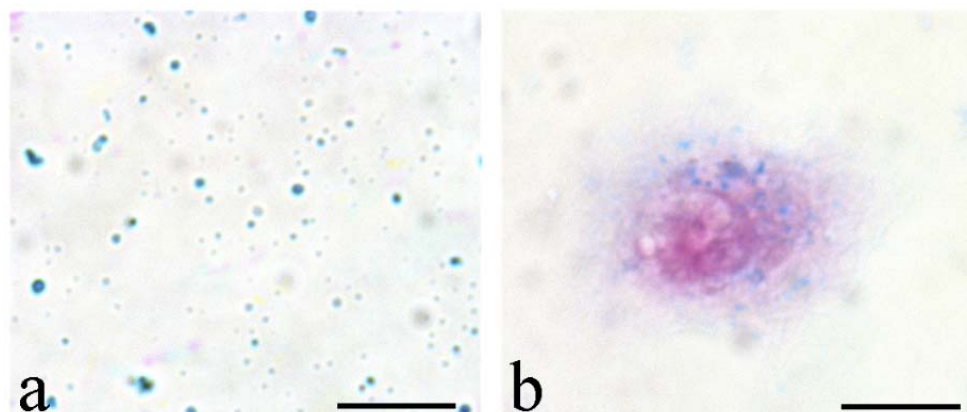


Figure 1. Light microscopy. AB-stained HA NPs suspended in medium (a) and inside a C2C12 myoblast (b). Bars: 20 μ m.

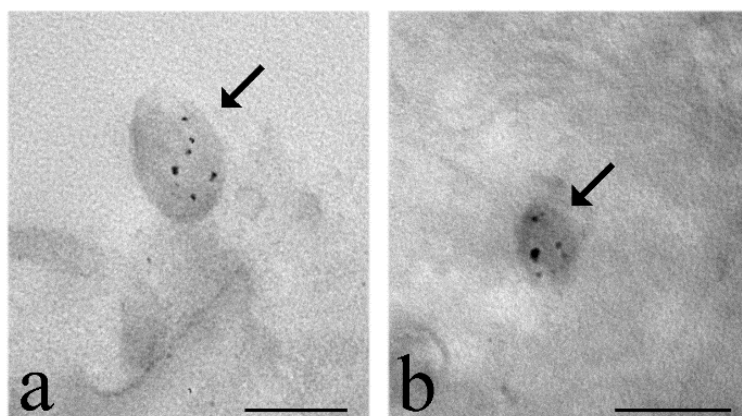


Figure 2. Transmission electron microscopy. AB-labelled HA NPs (arrows) occurring at the cell surface (a) and free in the cytosol (b). Bars: 200 nm.

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