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Ultrastructural cytochemistry is a valuable tool for tracking nanoparticles and monitoring drug delivery in single cells

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In recent years, nanoparticles (NPs) have become a popular subject in biomedical investigations due to their unique properties, such as small size and easy functionalizable high surface area, which facilitate their passage through biological barriers, penetration of plasma cell membrane and accumulation in the target sites, making them an extraordinary tool for drug delivery, device-based therapy, tissue engineering and medical imaging.

Among the numerous biomaterials used to manufacture NPs, the chitin-derived polysaccharide, chitosan is considered of particular interest in the field of pharmaceutics and biomaterials due to many advantageous features: chitosan may be obtained from natural products, it is enzymatically degradable (although mostly by enzymes that are not ubiquitously expressed by mammalian cells), it can be easily functionalized and/or complexed and, above all, it has a relatively low toxicity, in comparison with most polycations [1].

Chitosan NPs are especially promising as drug delivery carriers since they are able to protect the encapsulated molecules and/or improve their bioavailability by modifying their pharmacokinetics. In particular, chitosan NPs proved to be suitable for delivering molecules characterised by low stability, such as peptides, proteins, oligonucleotides and plasmids [2]. In fact, these NPs can establish mucoadhesive interactions and increase membrane permeability thus facilitating cell uptake [3,4]: NP uptake appears to occur predominantly by adsorptive endocytosis, but also clathrin- and caveolin-mediated pathways as well as clathrin- and caveolin-independent endocytosis may be involved [5,6]. NPs are also able to escape endosomes, thus protecting the incorporated drugs from the enzymatic degradation in the lysosomes and ensuring their controlled release in the intracellular milieu [7,8]. In addition, chitosan NPs proved to cross the blood brain barrier [9,10], opening a new way for drug delivery to the brain.

Understanding the intracellular location of NPs with respect to their uptake mechanism and monitoring the release of the loaded molecules is therefore essential in designing drug delivery strategies.

In our studies, diaminobenzidine (DAB) photoconversion was applied to correlate fluorescence and transmission electron microscopy for investigating the intracellular fate of chitosan NPs in a neuronal cell line. This technique allowed us to easily visualize chitosan NPs at transmission electron microscopy. In fact, in conventionally stained samples, chitosan NPs appeared as roundish moderately electron-dense structures hardly distinguishable in the cytosol or inside the lysosomal compartments but, after DAB photoconversion, the NPs were labelled with homogeneously distributed, dark, finely granular reaction product which made them unequivocally recognizable. NPs were mostly found within electron-lucent vacuoles, and were ubiquitously distributed in the cytoplasm, from the cell periphery (sometimes just beneath the plasma membrane) to the perinuclear region (often very close to the nuclear envelope); some NPs were also found to be free in the cytosol. After long incubation times (8 to 24 hours) the NPs were observed to accumulate in perinuclear position, but never inside the cell nucleus. Moreover, many NPs were found inside multivesicular or residual bodies: their morphology was often severely altered in either organelle, so they were only recognizable from the dark reaction product.

Subsequently, neuronal cells were administered chitosan NPs loaded with D-Ala2-D-Leu5-enkephalin (DADLE), a syntethic opiod able to induce reversible hypometabolizing effects [11] that has been extensively studied for its potential use in biomedicine, i.e., for preservation of explanted organs [12], neuroprotection [13] and anti-tumour treatments [14]. However, DADLE has a short plasmatic half-life (a few minutes) and it is unable to cross the blood brain barrier, thereby making systemic administration inefficient. Encapsulating DADLE in chitosan NPs would allow the blood brain barrier to

be crossed, the peptide would be protected from enzymatic degradation, and functionalization of NP surface would even allow specific targeting. Ultrastructural immunocytochemistry and morphology were used to test the efficacy of chitosan NPs in DADLE delivery and to evaluate the distribution of DADLE molecules in the various cellular compartments as well as their effects on transcriptional activity and cellular organelles.

Our results demonstrated that DADLE-loaded chitosan NPs internalized by neuronal cells still contain and release DADLE 24h after their withdrawal from the culture medium, thus maintaining low transcriptional activity. When administered as a free molecule, 24h after removal from the culture medium DADLE was hardly detectable inside the cells and its hypometabolizing effects were fully reversed [15]; therefore, encapsulation in chitosan NPs enables prolonged DADLE delivery and effect. Moreover, endocytosed chitosan NPs apparently release DADLE at low rate, as no opioid was found in chitosan NPs after 24h in aqueous solution [7].

Immunoelectron microscopy proved to be a powerful tool for tracking NP-carried molecules inside the cell over time also providing evidence of the drug subcellular targeting. The intracellular distribution of DADLE immunolabelling was similar after adding free [15] or chitosan NP-carried (present study) DADLE; in particular, the opioid accumulated in the nucleus. The ability of chitosan NPs to escape endosomes and accumulate in the perinuclear region likely facilitates DADLE translocation to the nucleus, where it specifically binds perichromatin fibrils and the nucleolar dense fibrillar component, i.e. the sites of transcription and early splicing of pre-mRNA and pre-rRNA, respectively [16]. Although the functional role of DADLE in these sites remains unknown, the immunolabelling for phosphorylated (i.e., activated) polymerase II decreased at the same sites, indicating a reduced transcriptional activity which in turn suggests a general reduction of cellular metabolism. Accordingly, an increase of mitochondrial granules, which often form under hypometabolic conditions [15] was found following DADLE exposure. However, no other structural alteration in cell organelles was found.

In conclusion, our results demonstrate that chitosan NPs are valuable tools to deliver the hypometabolizing opioid DADLE to neuronal cells, paving the way to *in vivo* experiments aimed at elucidating whether DADLE-loaded chitosan NPs may efficiently deliver the opioid to the central nervous system.

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