Impact of chitosan-based nanocarriers on cytoskeleton dynamics: Current status and challenges

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Abstract: Chitosan-based nanocarriers (CS-NCs) show a promising role in improving drugs and bioactive compounds delivery for therapy. However, the effects exerted by CS-NCs at the cellular level, including their recognition and uptake, have not been fully investigated yet. Many factors, including size, shape, concentration, and surface chemistry of CS-NCs, play an important role in determining the types of intracellular signals triggered. The mechanism of uptake and the involvement of the cytoskeleton during the CS-NCs endocytosis varies among the different cell types as well as further effects observed inside cells. In the present work, we discuss the effects induced by CS-NCs per se on the cytoskeleton, a key component in cell architecture and physiology. The focus of this report is made on tumoral and normal biological models in which CS-NCs could differentially affect the cell cytoskeleton. The recent years reports regarding the impact of CS-NCs on cytoskeleton dynamics and the current techniques for its evaluation are summarized and discussed. Understanding mechanisms underlying cytoskeletal impact after cell exposure to CS-NCs is critical for the design of safest value-added formulations in the biomedical field. Furthermore, this revision points out some interesting aspects of cytoskeletal changes and cell death encompassing anti-tumoral effects.

Introduction

Bioactive compounds (BCs) are substances that have biological activity, related to its capability to modulate one or more metabolic processes (Angiolillo et al., 2015). BCs are present as natural constituents in food, providing health benefits beyond the basic nutritional value of the product (DeFelice, 1992). These compounds can be applied for pharmaceutical or nutraceutical purposes (Sansone et al., 2019). Nutraceuticals, a term invented in 1989 by Stephan DeFelice, reflect their existence in the human diet and their biological activity.

Nanomedicine is a rapidly expanding area among life sciences which is mostly dominated by the production of nanocarriers (NCs) for drugs and BCs protection (Ahmad et al., 2021). The nanodelivery system has several advantages, such as overcoming the limitations and problems that comprise conventional pharmaceutical agents, older formulations, and delivery systems (Bayda et al., 2020; Buosi et al., 2020). The assessment of NCs biocompatibility is closely related to cytotoxicity and adverse effects they can potentially induce on target cells. Also, the control of the internalization mechanisms has become a challenge in the design process of the physicochemical characteristics of the NCs to be used (Rennick et al., 2021). Into this framework, the...
cellular uptake of NCs has been investigated in terms of the cellular and/or tissue environment, the dynamics of the cytoskeleton, and the capacity to activate specific cellular pathways during the internalization process (Donahue et al., 2019; Sousa de Almeida et al., 2021; Vtyurina et al., 2021).

In the present point of view, the focus was put on the impact of CS-NCs on cytoskeletal dynamics when experimenting in different biological systems (normal and tumoral cells). We summarized and discussed recent years reports addressing this topic, by adding information about type of CS-NCs employed, biological models used and main outcomes. The current techniques used for cytoskeleton analysis after CS-NCs exposure and vacancy areas for research were reviewed.

**Nanocarriers Uptake, Trafficking Routes, and Cytoskeletal Organization**

Increasing research in NCs used for biomedical applications raises a concern about how they can interact with cells beyond their therapeutic purposes. In this line, there are four points with crucial implications for their biological impact. Firstly, NCs cellular uptake and intracellular trafficking routes depend on the intrinsic physicochemical properties, like size, surface functionalization, charge, stiffness, and topography shape as well as the colloidal stability ($\zeta$-potential). Secondly, the route of administration (e.g., intraocular, intravenous, intramuscular inhalation, topical, oral). Thirdly, the cell/tissue target, and the specific cell uptake route employed to internalize NCs in the process of endocytosis (Sousa de Almeida et al., 2021). Particularly, the primary cellular mechanisms of uptake are (a) clathrin-dependent (b) caveolin-dependent (c) clathrin- and caveolin-independent (d) macropinocytosis and (e) phagocytosis (Donahue et al., 2019; Rennick et al., 2021). The NCs uptake rate depends on the triggered endocytosis mechanism. Notably, the same type of NCs could be internalized by different endocytosis mechanisms according with the cellular type, physiological state, variations in the cell membrane curvature, presence, or absence of specific receptor sites as well as surfactant molecules that prevent serum proteins from binding to the surface of NCs efficiently (Rennick et al., 2021; Sousa de Almeida et al., 2021). Fourthly, the involvement of the cytoskeleton during the endocytosis, which is a highly dynamic network of microfilaments, intermediate filaments, and microtubules. This 3D network ensures cell shape maintenance, strength, and structural integrity as well as a rail for active transport mechanisms (Shahzad et al., 2020; Mastrogiavanni et al., 2020). Moreover, the cytoskeleton allows the transmission of mechanical signals, being a key structure within the internal environment for cell survival, differentiation, migration, and proliferation (Finkenstaedt-Quinn et al., 2016). Cell types constituting the different tissues do not use the same intracellular trafficking routes for the same kind of NCs. In other words, the involvement of the cytoskeleton dynamics fluctuates among the different cell types (Gilleron et al., 2013).

**Chitosan-Based NCs Uptake and Its Impact on the Cytoskeleton Dynamics**

Chitosan (CS) is a linear polysaccharide whose structure is comprised of $\beta$-1,4-linked 2-amino-2-deoxy-$\beta$-D-glucose (deacetylated D-glucosamine) and N-acetyl-D-glucosamine units (di Santo et al., 2020b). The substantial attention of CS in the pharmaceutical and biomedical fields resides in its biodegradable, biocompatible and non-toxic features (Prudkin-Silva et al., 2020). Also, CS’s amine groups are responsible for its cationic nature, controlled drug release, muco-adhesion, permeation enhancement, etc. As a result, CS is one of the most employed polysaccharides in the drug delivery design strategies for administration of BCS (di Santo et al., 2020a; Dubashynskaya et al., 2020; Prudkin-Silva et al., 2020).

The interactions between CS-NCs and cellular membranes produce a reversible structural rearrangement of the binding proteins, encompassing a specific rearrangement of the cytoskeletal F-actin and tight-binding proteins. Such ability allows CS and CS-NCs to enhance mucosal absorption of drugs through all routes of administration, contributing to an increased bioavailability (Zhao et al., 2018). We found interesting to note that CS observations could be extended to other polymers with similar characteristics, such as PEI (polyethylenimine) and PLL (poly-l-lysine), whose molecules can also enter cells via endocytosis and affect the cytoskeleton functionality. In fact, CS, PEI and PLL are widely used in transfection tests due to their low cytotoxicity and cationic nature (Wang et al., 2019). Particularly, due to latter characteristics, the mechanism of PEI-mediated gene delivery has been well demonstrated in many literature studies. As a polycation, PEI will spontaneously adhere to and condense exogenous DNA to form spherical complexes that cells readily uptake. These complexes can interact with the cell membrane and, consequently, be endocytosed. Once into the cell, vesicle movement depends on the cytoskeleton (Grosse et al., 2007), and PEI/DNA complexes also travel along the cytoskeletal network up to the nucleus (Zhang et al., 2011). Thus, the functioning of the cytoskeleton is affected by this transport showing a parallelism with CS-NCs effects.

The information collected and offered in this report summarizes the employment of CS-NCs for pharmaceutical and biomedical ends in both in vitro and in vivo models, for which cytoskeletal effects have been reported. The lack of consensus on the cytoskeletal alterations that is described in Table 1 could be related with the wide variability in methodological approaches for NCs design. Furthermore, the concentration of proteins in culture medium determines whether the NCs would be adsorbed, and these interactions regulate the promotion or inhibition of their internalization. Depending on the type of endocytosis, specific internalization pathways ensure differential trafficking of NCs inside cells (Yameen et al., 2014). The scheme shown in Fig. 1 refers to a generalized case without specifying a particular route of endocytosis. After their release into the cytoplasm, NCs give rise to a signal cascade that culminates in the triggering of a major cellular event. The latter differs whether the cellular context is a physiological or a tumor environment. Those cascades may or may not be accompanied by the cytoskeleton network reorganization depending on the physicochemical characteristics of the CS-NCs delivered to the target cell type. Additionally, dose, concentration and/or exposure time of the NCs are critical variables for both, the internalization,
lymphocytes (Lin et al., 2008) formulates guidelines for the definition and interpretation of morphological, biochemical, and functional perspectives observed in different cell death. The most robust guide was reported by Galluzzi et al. (2018) and one-year later, an update was published by Tang et al. (2019). Nowadays, cell death is categorized into two types: accidental cell death and programmed cell death. At the same time, the latter is subclassified in apoptosis, necroptosis, lysosome-dependent cell death, autophagy-dependent cell death, ferroptosis, pyroptosis, netosis, parthanatos, entosis, anoikis, mitotic death, oxeiptosis, autosis, alkaliptosis. Whatever the type of cell death, these guidelines describe the rearrangements that cytoskeletal dynamics could undergo (Kroemer et al., 2008; Galluzzi et al., 2018; Ren et al., 2021). Although the absence of cytotoxicity is a general feature of CS-NCs, in some specific systems, such as tumoral cells, death pathways are triggered (Venkatesan et al., 2011; Taranjeoo et al., 2016; Zamproni et al., 2020) (Fig. 1). Thus, the information presented in Table 1 opens some questions: Could CS-NCs differentially affect the tumoral cell cytoskeleton by specific molecular signaling pathways? or is merely a consequence of the chosen experimental conditions, i.e., dose, exposure time, materials combined with CS? The answers to those questions could be found in the report of Abedian et al. (2019), in which, low and high molecular weights (MW) CS resulted biocompatible with normal foreskin-derived fibroblasts. However, both types of CS exhibited growth inhibitory effects against tumoral cell lines, such as HeLa, MCF-7 and Saos-2. As a result of the difference in the mechanism of cytotoxicity between fibroblasts and cancer cell lines, authors hypothesized that the latter have greater cell membrane negative charges than normal cells, which may be more attractive to the positively charged amino groups on CS molecules. In this way, CS could affect tumoral cells directly through interaction with the plasma membrane or extracellularly through a specific receptor, or through endocytosis. Whatever the form, the polysaccharide could alter the cell membrane through electrostatic interaction, leading to the secretion of inflammatory cytokines, such as IL-6 and IL-8 (Abedian et al., 2019). In agreement, Ivanova and Yaneva (2020) highlight two aspects in relation to the anticancer and the immunostimulatory properties of CS:

a) The redox regulatory mechanisms of CS could explain its anticancer activity. That is to say, the initiation of intracellular ROS rise in cancer cells could be closely associated to the activation of intracellular calcium signaling that lead to enhancement of the human defense system and consequently, the apoptotic cell death.

b) The activated human immune system has great potential to destroy cancer cells without being toxic to the healthy tissue and organs. Taken together, the use of CS-NCs could be a cancer treatment option with fewer side effects. However, it is necessary to deeply understand the molecular mechanism that explains the differential response, including in addition in vivo experiments (Abedian et al., 2019).

Visualization techniques are another key issue in the aim to understand the cytoskeletal dynamics. Depending on whether samples are fixed or living cells different information can be acquired from the assay (McKayed and Simpson, 2013; Finkenstaedt-Quinn et al., 2016). There are specific microscopic techniques which require high resolution fluorescent imaging methods that maintain a
| Type of CS-NC | Physicochemical properties of CS-NC | Biological model | Major outcomes | References |
|--------------|------------------------------------|-----------------|---------------|------------|
| CS + TPP    | 289 nm +35.9 mV ζ-potential | IODA-NHC Normal. Human conjunctival cells | No changes were detected in the actin filaments distribution. | Enríquez de Salamanca et al. (2006) |
| CS + HAP    | 144 nm -1.8 mV ζ-potential | HCT-15 Tumoral. Human colon adenocarcinoma cells | The elongated shape actin filaments network exhibited alterations. | Venkatesan et al. (2011) |
| CM-CS + PO-500 | 219 nm +39 mV ζ-potential | HKC Normal. Human proximal tubular epithelial cells | No changes were detected in actin filaments distribution. | Yue et al. (2011) |
| Nanotubes: CS + collagen | 40–60 nm N.A. | 1.929 Normal. Mouse fibroblast cells | Cytoskeleton did not display the typical fibroblastic morphology. Cells were mostly rounded. | Zhao et al. (2014) |
| CS + lecithin | 150–200 nm N.A. | MDCK-C7 Normal. Canine kidney epithelial cells | Cell migration that correlated with moderate cytoskeleton alterations. Actin filaments redistribution and reduction. | Kaiser et al. (2015) |
| CS + CMI | 70 nm +20 mV (pH < 7.6) N.A. | MDA-MB-231 Tumoral. Human breast adenocarcinoma cells NIH3T3 Normal. Mouse fibroblasts | The cytomorphology and the tubulin cytoskeleton were not altered. | Merli et al. (2016) |
| CS + TPP | 93 nm +14 mV (pH 7.4) +55 mV (pH 3) | SW48 Tumoral. Human colon adenocarcinoma cells | Changes in microfilament contents (F-actin). | Taranejoo et al. (2016) |
| CS + TPP | N.A. | MCF 10A Normal. Human mammary epithelial cells | No negative influence on the cytoskeleton. | Catalano (2017) |
| CS + Oleic Acid + Span 85 + Tween 20 | 104 nm +21 mV | Human macrophages differentiated from CD14+ monocyte | CS-NC were internalized via an actin cytoskeleton-dependent process. | De Matteis et al. (2016); Coya et al. (2019) |
| CS + TPP | 50 nm +28 mV (water), -4 mV (culture media with serum) | HGF Normal. Human gingival fibroblasts | No visible changes in cytoskeleton arrangement. | Martin et al. (2019) |
| CS + TPP | 113 nm +54 mV | Human Vγ9Vδ2 T-lymphocytes | No changes in the α-tubulin and β-actin expression levels. α-tubulin cytoskeleton rearrangement was detected. | Lin et al. (2019) |
| CS-DS | 186 nm -25 mV | N2a Tumoral. Murine neuroblastoma cells | Neurotoxicity correlates with changes in β-tubulin distribution. | Zamproni et al. (2020) |
| CS + PLGA | 254 nm +22 mV | HUVEC Normal. Human umbilical vein endothelial cells | No changes were detected in the actin filaments organization and expression levels. | Jin et al. (2021) |
| 3-layer nanofiber: CS+ PCL/ PVL/ CS + PCL | 103 nm N.A. | Skin of male Wistar rats | The NF has no effect on alpha-smooth muscle actin mRNA expression. | Mirmajidi et al. (2021) |

Note: N.A. = not available data.
perfect focus and optimal cellular growth conditions. Confocal laser scanning microscopy (CLSM) is the most widely used technique since z-section imaging of samples can be performed. However, many reports showed images acquired with epifluorescence equipment, which has less resolution. On the other hand, atomic force microscopy (AFM) allows the simultaneous measurement of local cell elasticity and living or fixed cell topography with high spatial resolution and force sensitivity (Finkenstaedt-Quinn et al., 2016). The combination of AFM and CLSM has become a common strategy to investigate the correlation between cytoskeletal rearrangement and mechanical response or morphology changes, especially in living cells, as shown by Lin et al. (2019). Moreover, Correlative Light and Electron Microscopy (CLEM) has become a highly fashionable method (Svitkina, 2019). It bridges the spectrum of dyes and probes which in turn enable the localization of molecules of interest within living cells by fluorescence microscopy with the cellular ultrastructure from electron microscopy (de Boer et al., 2015). The different CLEM methods have played an active role in developing strategies to capture and study dynamic events at high-resolution in *in vitro* and *in vivo* models (Jin et al., 2021). Despite the improved spatial resolution of CLSM, cytoskeletal structures smaller than the “Abbe diffraction limit” cannot be detected. In this sense, super resolution fluorescence imaging like stochastic optical reconstruction microscopy (STORM), photoactivated localization microscopy (PALM), and stimulated emission depletion fluorescence microscopy (STED), could resolved those limitations (Finkenstaedt-Quinn et al., 2016).

## Conclusions and Future Perspectives

NCs delivery systems have the potential to improve the treatment of various diseases. The cellular uptake and trafficking of NCs are critical processes to understand how they reach the site of action. In this context, there are few reports related to the internalization process of CS-NC and its cellular impact; hence, the aim of this contribution was to recapitulate their effect on the cytoskeleton dynamics.

Greater understanding of the uptake process can potentially be obtained to take advantage of cellular biological mechanisms for more efficient delivery of CS-NCs. Studies comparing cytoskeletal distribution, cell functions, and cytotoxicity with the same type of CS-NCs assessed in a variety of cell lines is needed to clarify these functions, and cytotoxicity with the same type of CS-NCs. Studies comparing cytoskeletal distribution, cell functions, and cytotoxicity with the same type of CS-NCs administrated jointly with epi-fluorescence equipment, which has less resolution. On the other hand, atomic force microscopy (AFM) allows the simultaneous measurement of local cell elasticity and living or fixed cell topography with high spatial resolution and force sensitivity (Finkenstaedt-Quinn et al., 2016). The combination of AFM and CLSM has become a common strategy to investigate the correlation between cytoskeletal rearrangement and mechanical response or morphology changes, especially in living cells, as shown by Lin et al. (2019). Moreover, Correlative Light and Electron Microscopy (CLEM) has become a highly fashionable method (Svitkina, 2019). It bridges the spectrum of dyes and probes which in turn enable the localization of molecules of interest within living cells by fluorescence microscopy with the cellular ultrastructure from electron microscopy (de Boer et al., 2015). The different CLEM methods have played an active role in developing strategies to capture and study dynamic events at high-resolution in *in vitro* and *in vivo* models (Jin et al., 2021). Despite the improved spatial resolution of CLSM, cytoskeletal structures smaller than the “Abbe diffraction limit” cannot be detected. In this sense, super resolution fluorescence imaging like stochastic optical reconstruction microscopy (STORM), photoactivated localization microscopy (PALM), and stimulated emission depletion fluorescence microscopy (STED), could resolved those limitations (Finkenstaedt-Quinn et al., 2016).

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## Authors' Contribution

The authors confirm contribution to the paper as follows: M.C.D.S. and A.P. DR wrote the first draft. O.E.P critically reviewed the draft. All authors reviewed and approved the final version of the manuscript.

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## Conflicts of Interest

The authors declare that they have no conflicts of interest to report regarding the present study.

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