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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 variates 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.

Abbreviations

BCs:	bioactive compounds
CM-CS:	carboxymethyl chitosan
CMI:	carboxymethyl inulin
CS:	chitosan
CS-NCs:	chitosan-based nanocarriers
DS:	dextrane sulphate
HAP:	hydroxyapatite
mV:	milliVolts
NCs:	nanocarriers
nm:	nanometer
PCL:	polycaprolactone
PLGA:	poly (lactic-co-glycolic acid)
PO-500:	hexaglycerin penta ester
PVL:	Poly (Vinyl Alcohol)
TPP:	pentasodium tripolyphosphate

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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

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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 (ζ-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; Mastrogiovanni *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 β-1,4-linked 2-amino-2-deoxy-β-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,

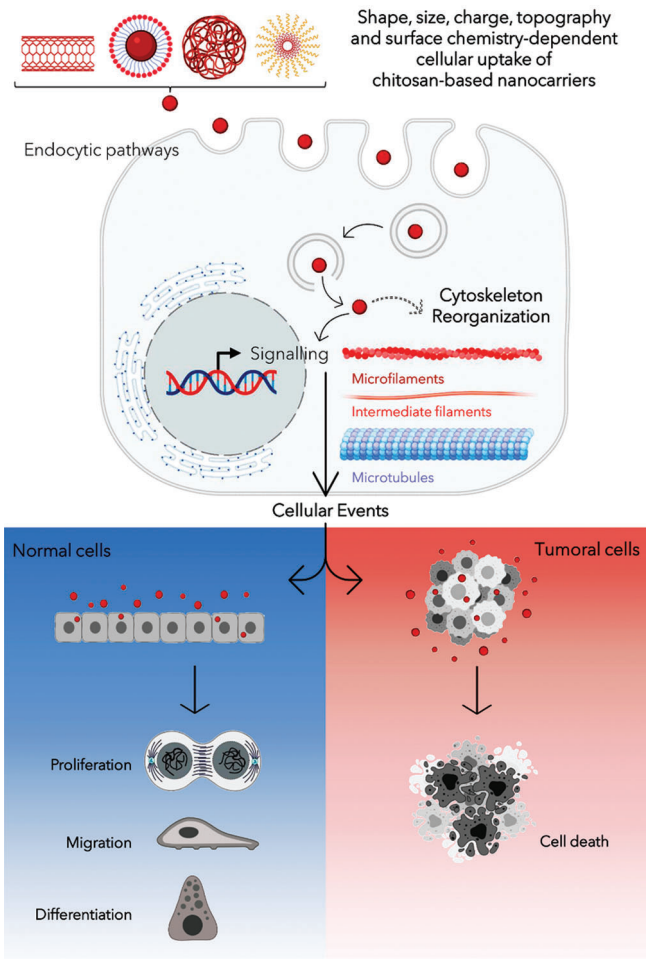

FIGURE 1. Schematic diagram summarizing the interaction among chitosan-based nanocarriers (CS-NCs) and mammalian cells. Created with *BioRender* platform.

and biological effects. After the cellular uptake of CS-NCs, transient effects could be observed in the cytoskeletal organization, along with the propagation of intracellular signals (Fig. 1). When CS-NCs do not affect cell viability, as mostly occurs in normal cells, they can occasionally trigger biological responses, e.g., cell migration. The latter could be the case observed in macrophages (Coya *et al.*, 2019) and lymphocytes (Lin *et al.*, 2019). Those cells, due to their activity in immunity, exhibit asymmetrical changes in their morphology (polarization) and mobility (chemotaxis), whose events are regulated by microtubules and microfilaments (Billadeau *et al.*, 2007; Gomez and Billadeau, 2008). Notably, it has been reported that CS-NCs can stimulate T-cell maturation and proliferation (Malik *et al.*, 2018). Also, the macrophage nitric oxide (NO) production and chemotaxis were assigned to the N-acetylglucosamine unit present in the CS structure rather than to the glucosamine residue. Furthermore, the CS-induced immune stimulatory response resulted to be highly specific since other glycosaminoglycans, such as N-acetyl-o-mannosamine and N-acetyl-p-galactosamine, had no effects on NO production. Similar stimulatory effects were detected on lymphocytes (Peluso *et al.*, 1994).

The "Cell Death Nomenclature Committee" (Kroemer *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; Taranejoo *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

TABLE 1

Impact of CS-NCs on cytoskeleton dynamics

Type of CS-NC	Physicochemical properties of CS-NC mean size ζ-potential	Biological model *In vitro* (normal or tumoral cell lines) *In vivo* (animals)	Major outcomes	References
CS + TPP	289 nm +35.9 mV	*IOBA-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	*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	*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.	L929 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)	*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 issues. Is it really possible to modulate endocytosis to favor therapeutic administration through the use of CS-NCs by any particular route? would be the arising question. Thus, the use of specific pharmacological inhibitors or key receptor gene silencing of each endocytic pathway could be explored. In this sense, these tools could add further knowledge to the understanding of the interactions between CS-NC and target cells. Either way, despite it being somewhat complex to draw general conclusions, it is evident that CS-NCs can be a useful vehicle for drug and BCs delivery. Likewise, the lack of studies on this topic reveals a vacant area that must be rigorously explored for the successful implementation of NCs in biomedical applications.

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