International Journal of Pharmaceutics 420 (2011) 223–230

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Effect of chitosan malate on viability and cytoskeletal structures morphology of Caco-2 cells

Claudia Juliano[a,*], Grazia Galleri[b], Therese Klemetsrud[c], Jan Karlsen[c], Paolo Giunchedi[a]

[a] Dipartimento di Scienze del Farmaco, University of Sassari, Via Muroni 23/A, 07100 Sassari, Italy
[b] Porto Conte Ricerche Srl, Loc. Tramariglio, Alghero, Italy
[c] Farmasøytisk Institutt, Det Matematisk-naturvitenskapelige Fakultet, University of Oslo, Oslo, Norway

ARTICLE INFO

Article history:
Received 16 May 2011
Received in revised form 17 August 2011
Accepted 24 August 2011
Available online 1 September 2011

Keywords:
Chitosan malate
Caco-2 cells
Cytotoxicity
Cell cytoskeleton
Tubulin
Actin

ABSTRACT

The aim of this work was to evaluate the effects of the treatment with chitosan malate (CM) on viability of Caco-2 cells and on the morphology and the integrity of their cytoskeletal structures (microtubules, microfilaments). Cytotoxicity of CM, both as a solution and as microparticles obtained by spray drying, was evaluated by using the reduction of MTT reagent; microtubule and microfilaments organization of Caco-2 cells treated with CM solution was examined with immunofluorescence techniques in monolayers fixed with the glutaraldehyde–borohydride method.

CM as a solution displayed a concentration-dependent cytotoxicity towards Caco-2 cells, with viability percentages of 5 ± 2%, 7 ± 3% and 31 ± 15% at 15, 10 and 5 mg/mL, respectively, while at 2.5 mg/mL or less cell viability was 90% or higher. CM microparticles also produced a remarkable cytotoxic effect (cell viability 84 ± 17%, 16 ± 8% and 5 ± 6% after treatment with 1, 5 and 10 mg CM per well, respectively), resulting more toxic than CM solution.

Microtubules pattern of Caco-2 cells, which is a network regularly arranged around the nucleus, appeared deeply modified by CM treatment in a concentration-dependent way, with progressive microtubule changes in length and spatial disposition and deposition of fluorescent aggregates at the periphery of the cells. Furthermore, after treatment with 5–15 mg/mL CM, remarkable alterations of actin organization were observed, with a progressive disruption of the network of stress fibers and the appearance of actin aggregates inside the cell cytoplasm.

In conclusion, viability and the cytoskeletal pattern of Caco-2 cells are modified by treatment with CM at high concentrations, which might be locally reached *in vivo* after application of drug-loaded chitosan microparticles onto mucosal cells.

1. Introduction

Chitosan is a cationic polysaccharide obtained on an industrial scale by partial alkaline or enzymatic N-deacetylation of chitin, the most abundant polymer found in nature after cellulose; it is a linear eteropolymer composed of glucosamine and *N*-acetylglucosamine units linked through 1–4 glycosidic bonds, and can be found in a variety of forms, differing in average molecular weight and degree of deacetylation.

Chitosan has been employed over the last years in cosmetics, pharmaceutical and food industry, as well as in agriculture and biotechnology (Ravi Kumar, 2000), due to its favourable biological properties and its versatility. In particular, this polymer stands out by its high biocompatibility (Hirano et al., 1990) and biodegradability (Muzzarelli, 1997), as well as by its multiple biological activities (Xia et al., 2011), such as mucoadhesive (Lehr et al., 1992), antibacterial (Je and Kim, 2006; Friedman and Juneja, 2010), antifungal (Seyfarth et al., 2008), antiviral (Chirkov, 2002), antioxidative (Friedman and Juneja, 2010) and wound-healing properties (Ueno et al., 2001), and has been proposed for formation of extracellular matrices in a wide range of tissue engineering applications (Di Martino et al., 2005; Kim et al., 2008; Hao et al., 2010; Wang et al., 2010). In addition, chitosan has been reported to exhibit some interesting physico-chemical features, such as an excellent film forming ability (Domard and Domard, 2001), metal chelation (An et al., 2001) and the improvement of molecular transport across biological barriers (Kotzé et al., 1999; He et al., 2009). The unusual combination of the biological activities and physico-chemical properties of this polymer led to the development of novel or improved pharmaceutical and biomedical applications, including the development of peroral, nasal and transdermal drug delivery systems, gene delivery, the enhancement of vaccine effects and

* Corresponding author. Tel.: +39 079 228735; fax: +39 079 228715.
E-mail address: julianoc@uniss.it (C. Juliano).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.08.039

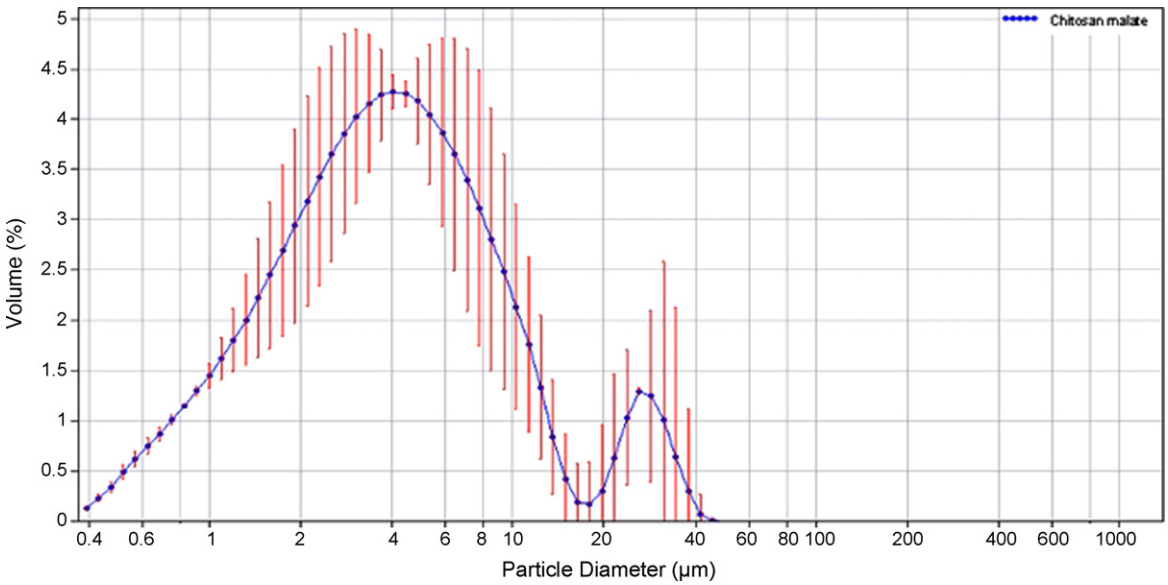

Fig. 1. Particle size distribution of CM microparticles.

bone tissue engineering. Moreover, chitosan has been approved for dietary applications in Japan, Finland and Italy, and is used as a nutritional supplement to reduce cholesterolemia and fat absorption in the intestine and to promote weight loss (Muzzarelli, 2000); however, the mechanisms of these effects are still not fully understood (Furda, 2000), and results of long term trials indicate that the effect of chitosan on body weight is minimal and unlikely to be of clinical significance (Mhurchu et al., 2005).

Although chitosan is widely described in the literature as a safe, biocompatible and non toxic polymer (Kean and Thanou, 2010), there are some reports that a number of soluble chitosans (chitosan hydrochloride, hydroglutamate, hydrolactate and glycol chitosan) show some degree of concentration- and molecular weight-dependent cytotoxicity and erythrocyte lysis (Carreño-Gómez and Duncan, 1997). Moreover, contradictory data have been reported about the influence of chitosan on actin cytoskeletal structures: some authors found that chitosan does not affect microfilament network organization in Caco-2 cells (Dodane et al., 1999; Smith et al., 2004), while other researchers demonstrated a decreased fluorescence intensity of actin staining in Caco-2 actin microfilaments after treatment with this polymer (Artursson et al., 1994; Ranaldi et al., 2002); furthermore, Dodane et al. (1999) observed in the same cells shortening of the basolateral actin filaments and appearance of aggregates of the same protein after chitosan treatment. Therefore, further investigation on cytotoxicity of chitosans and their interaction with cell cytoskeleton is desirable.

In the present contribution, we studied the concentration-dependent cytotoxicity of chitosan malate, a hydrosoluble salt of this polymer, on the human carcinoma cell line Caco-2, a well-characterized *in vitro* transport model system largely used for the testing of chitosan penetration effects across biological membranes; chitosan malate was tested both as solution and as microparticles obtained by spray-drying. We also investigated the effects of the polymer on the morphology of tubulin and actin cytoskeletal structures of these cells.

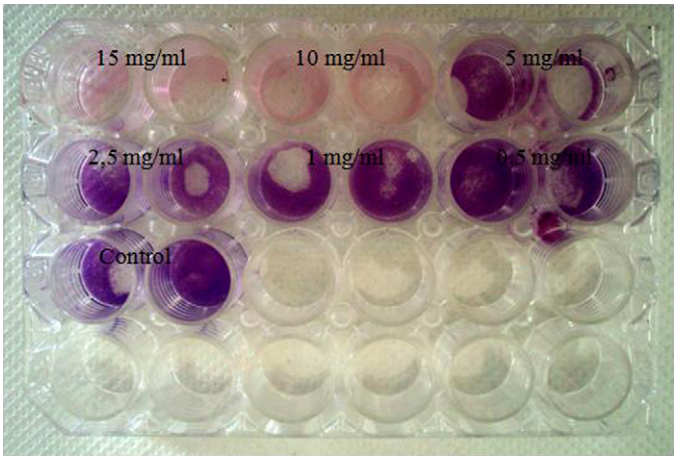

Fig. 2. Caco-2 cell monolayers stained with Giemsa after treatment with different concentrations of CM.

2. Materials and methods

2.1. Materials

Chitosan malate (CM; batch 203-490-14 SM, degree of acetylation > 70%, MW 190–310 kDa) was obtained from FMC BioPolymer AS (Drammen, Norway). Phosphate Buffered Saline tablets (PBS, Dulbecco A; pH 7.3) was purchased from Oxoid (Basingstoke, England). All other solvents and chemicals were of analytical grade.

2.2. Cell cultures

Caco-2 cells, kindly donated by Prof. Maria Cristina Bonferoni (Dipartimento di Chimica Farmaceutica, Università di Pavia, Italy) at the 20th passage, were grown in T-25 flasks at 37 °C in 5% CO_2, in a complete culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, 4 mM L-glutamine and 110 mg/L sodium pyruvate (Invitrogen Gibco) containing 10% heat-inactivated Foetal Bovine Serum (FBS; Invitrogen Gibco), 1% MEM non-essential amino acids solution (MEM NEEA; Invitrogen Gibco) and 1% penicillin–streptomycin solution (10,000 units/mL Penicillin G and 10,000 µg/mL of streptomycin sulphate; Invitrogen Gibco). Cells were allowed to reach 70–80% confluence, detached with 0.25% trypsin/0.02% sodium EDTA solution (Sigma) and split 1:3–1:6 (i.e., seeded at a density of $2–4 \times 10^5$ cells/cm^2). When required, cells were frozen at −80 °C in freezing medium (FBS + 10% DMSO).

2.3. Preparation of chitosan malate solution

CM solution was prepared just before each experiment and eventual residues were discarded. CM was dissolved in sterile conditions at 15 mg/mL in complete culture medium under magnetic stirring; since the resulting solution was quite acidic, its pH value was adjusted at 5.6 with sterile NaOH solution (above pH 5.6 chitosan precipitates).

2.4. Preparation of chitosan malate microparticles

CM microparticles were produced by the spray-drying method. The polymer was dissolved at 1% (w/v) in ultra-pure sterile water (MilliQ) under magnetic stirring at room temperature. The solution was spray-dried through a 0.5 mm nozzle using a Büchi Mini Spray Dryer B-191 (co-current flow type; Büchi, Flawil, Switzerland). The conditions of the spray-drying process were: inlet air temperature 125 °C, outlet air temperature 71–84 °C, pump ratio 8%, aspirator ratio 80%, flow control 500 L/h and feed rate 2.5 mL/min. The recovered microparticles were stored in a desiccator at room temperature until further use.

2.5. Characterization of chitosan malate microparticles

The yield of production was calculated as the percent weight of microparticles obtained with respect to the initial polymer amount in the spray-dried solution.

An amount of CM microparticles corresponding to about 3 mg were suspended in 1 mL of isopropyl alcohol and sonicated for 10 s in an ultrasonic bath, and their particle size was determined by laser diffraction (Coulter LS 100 Q Laser Sizer, Beckman Coulter, Miami, FL, USA). The average particle size was expressed as volume–surface diameter, d_{vs} (μm) (Edmundson, 1967) (Fig. 1).

2.6. In vitro cytotoxicity assays

Preliminary assays were performed to define the range of CM concentrations suitable for cytotoxicity testing. Caco-2 cells were seeded at a density of 2×10^5 cells/well in 2 mL of complete medium, and after 3 days they were treated with different CM concentrations; after 24 h of incubation, monolayers were washed 5 times with PBS, then fixed for 5 min with 1 mL of methanol and finally stained with Giemsa solution (azure eosin methylene blue; Sigma). This test allowed to determine 0.05–15 mg/mL as a useful range of CM concentrations for further quantitative assays (Fig. 2).

Cell viability was quantitatively assessed by using the reduction of the MTT reagent (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma), as described by Shi et al. (2006). In order to evaluate the cytotoxicity of CM in solution, Caco-2 were seeded at the density of 2×10^5 cells per well in 2 mL of complete medium. After 3 days, the medium was replaced with 2 mL of fresh medium containing suitable volumes of the CM solution previously described (polymer concentrations ranging from 0.05 mg/mL to 15 mg/mL); control experiments were carried out using the complete medium alone as a non-toxic control, 1% (v/v) Triton X-100 (Sigma) as a toxic control, and complete medium acidified to 5.6 with hydrochloric acid as an acidic control, to match the pH of the highest (15 mg/mL) chitosan sample. Cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h; at the end of exposure, medium was removed from each well and replaced with 710 μL of complete medium and 142 μL of MTT solution (5 mg/mL in PBS). After 3 h of incubation at 37 °C in 5% CO_2, the medium was removed and the formazan crystals formed were solubilized in 710 μL of DMSO for 15 min; solutions were then centrifuged at 13,000 rpm for 4 min to remove cell debris and their absorbance measured at 570 nm in a Lambda3 Perkin Elmer spectrophotometer. The MTT assay was performed three times and in each experiment each concentration was tested in triplicate. Cell viability was expressed as percentage of the non-toxic control (Fig. 3).

Some experiments were performed to assess the reversibility of CM cytotoxic effect. To this purpose, cells were treated for 24 h with different concentrations of CM (ranging from 1 to 15 mg/mL) as described previously. After the incubation, the medium containing CM was removed by washing the cell monolayer several times with PBS and replaced with fresh medium; cells were thereafter incubated for other 24 h and MTT test performed as described above (Fig. 3).

To assay the effect of CM microparticles on cell viability, Caco-2 were seeded as previously described; after 3 days, the medium was removed and replaced with 0.5 mL of complete medium after two washings with sterile PBS. Different amounts of CM microparticles, ranging from 1 to 15 mg, were then added to each well; to make the microparticles quickly sediment and attach to cell monolayers, microplates were centrifuged for 15 min at 2000 rpm. After centrifugation, 1.5 mL of complete medium were added slowly to each well; cells were incubated at 37 °C in a 5% CO_2 atmosphere for 24 h and MTT test was performed as previously described. Assays were

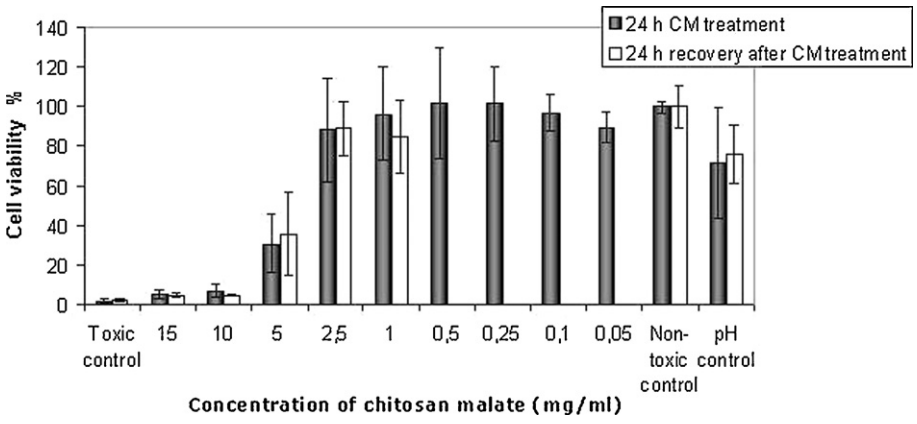

Fig. 3. Cell viability after 24 h treatment with CM solution (black) and cell viability 24 h after the end of the treatment with CM (white). Cell viability is expressed as percentage of the non-toxic control. Results displayed are the mean values of three experiments. Data are shown ± standard deviation. Toxic control = treatment with Triton X-100, Non-toxic control = normal growth conditions (medium), pH control = medium with the resembling pH of the solution of 15 mg/mL chitosan malate used in the experiment.

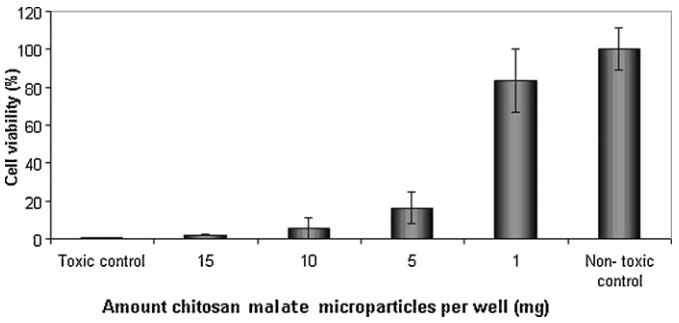

Fig. 4. Cell viability after 24 h treatment with CM microparticles. Cell viability is expressed as percentage of the non-toxic control. Results displayed are the mean values of three experiments. Data are shown ± standard deviation. Toxic control = treatment with Triton X-100, Non-toxic control = normal growth conditions (medium).

performed three times and in each experiment each concentration was tested in triplicate. Results are shown in Fig. 4.

2.7. Immunofluorescence

Analysis of the cytoskeletal structures of Caco-2 cells treated with CM in solution was performed by an indirect immunofluorescence technique. A medium control and an acidic control were included in each experiment.

Fixation was carried out according to Mattana et al. (1992): cells were seeded at a density of 1×10^5 per well on sterile coverslips in complete medium, and after 3 days of incubation they were treated with different CM concentrations, ranging from 0.25 to 15 mg/mL; after 24 h of incubation, cells were extensively washed with PBS, permeabilized with 0.5% Triton X-100, 0.25% glutaraldehyde in cytoskeleton buffer (pH 6.1; Small, 1981) for 2 min, and then fixed in 1% glutaraldehyde in the same buffer for 10 min. Cells were finally washed several times with sodium borohydride solution (0.5 mg/mL) freshly prepared in TBS (10 mM Tris–HCl, 155 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.6) to remove aldehyde traces, and transferred in fresh TBS until the immunostaining.

To visualize tubulin, Caco-2 were incubated at 35 °C for 45 min in a moist chamber with a monoclonal anti-β-tubulin antibody in mouse (clone TUB 2.1; Sigma) diluted 1:50 in TBS; after extensive washing with the same buffer, cell monolayers were incubated at 35 °C for 45 min with Alexa Fluor® 488 goat anti-mouse (Invitrogen), diluted 1:100 in TBS, as a secondary antibody. At the end of the incubation, coverslips were washed several times in TBS and mounted in Gelvatol (Monsanto Corp., Springfield, M.A., U.S.A.) prepared as described by Rodriguez and Deinhardt (1960); mounting medium was allowed to polymerise at 4 °C before microscopic observation.

For filamentous actin (F-actin) labelling, cells fixed according to the abovementioned protocol were stained by using TRITC–phalloidin (Sigma) diluted 1:1000 in TBS (15 min, 37 °C). To perform a double staining of tubulin and F-actin, fixed cells were co-incubated with TRITC–phalloidin and the secondary antibody in the previously described conditions.

Recovery after treatment was investigated by treating the cells with CM concentrations ranging from 1 to 15 mg/mL for 24 h, thereafter washing cell monolayers 7 times with TBS followed by incubation with complete medium for another 24 h. After this incubation, cells were fixed and stained for tubulin and actin as described. The data from at least three independent experiments monitoring 50 cells per experiment and per every condition are presented.

Slides were observed by an Olympus IX71 fluorescence-inverted microscope at 100× magnification oil immersion. Images were collected by using a View II Image camera coupled to the software "ANALYSIS".

3. Results

3.1. Characterization of CM microparticles

The yield of production of CM microparticles was 56.7%; their size distribution was obtained by using laser diffraction measurements. Particle size analysis shows that microparticles have a mean volume surface diameter of 2.6 ± 0.1 μm and a median of 3.8 ± 0.4 μm. The particle size distribution results narrow but bimodal, as shown in Fig. 1; the curve bimodality indicates the presence of microparticle aggregates in the batch produced.

3.2. Effect of CM solution on Caco-2 cells viability

The effects of 24-h incubation with increasing concentrations of CM on Caco-2 cells viability are reported in Fig. 3. The results show that CM affects cell viability in a dose-dependent manner. CM was found to be non-toxic at concentrations from 0.05 to 2.5 mg/mL, giving percentages of cell viability similar to that of positive control. At higher concentrations of CM, cell viability gradually decreased, resulting to be 31 ± 15% and 7 ± 3% after treatment with 5 mg/mL and 10 mg/mL, respectively, and reaching the minimum value of 5 ± 2% at 15 mg/mL; at this CM concentration, cell mortality resulted near to that of the toxic control (2 ± 1%). Acidic pH values affected the cell viability of the Caco-2 cells and might to some extent explain the cytotoxicity seen when treating the Caco-2 cells with high CM concentrations. However, at the lowest pH value reached in the experiments, 5.6, cell viability was of 75 ± 12%; therefore, the effect of low pH values can account only very partially for the almost complete cell death seen after treatment with CM at the same pH.

In order to determine whether CM-induced cytotoxicity was reversible, separate experiments were performed, where the cells, pre-treated for 24 h with concentrations of CM ranging from 1 to 15 mg/mL, were washed and incubated for 24 with fresh medium before MTT assay. The results of these recovery experiments, displayed in Fig. 3, show that cell viability values obtained were comparable to that before recovery; therefore, cytotoxic effect of CM does not seem to be reversible.

3.3. Effect of chitosan malate microparticles on Caco-2 cells viability

CM microparticles also affect Caco-2 viability in a dose-dependent manner, as shown in Fig. 4. After treatment with 1, 5 and 10 mg CM per well (growth surface 1.9 cm^2; medium volume 2 mL), cell viability was 84 ± 17%, 16 ± 8% and 5 ± 6%, respectively. Although data on cell viability after treatment with CM in solution and CM microparticles are not completely comparable, they imply that CM as microparticles is more toxic compared to CM as a solution. Since CM microparticles did not dissolve in the culture medium but formed a film above the cell monolayer, the strong reduction of cell viability observed is probably caused by local, high concentrations of CM reached above the cells; in addition, due to the experimental conditions, pH adjustment could not take place, so that the high toxicity observed with the CM microparticles can also in part be explained by the low pH in the proximity of the cell monolayer.

3.4. Effect of chitosan malate on cytoskeleton of Caco-2 cells

Normal distribution of actin and tubulin in Caco-2 cells under normal cultivation conditions is displayed on the top row of Fig. 5.

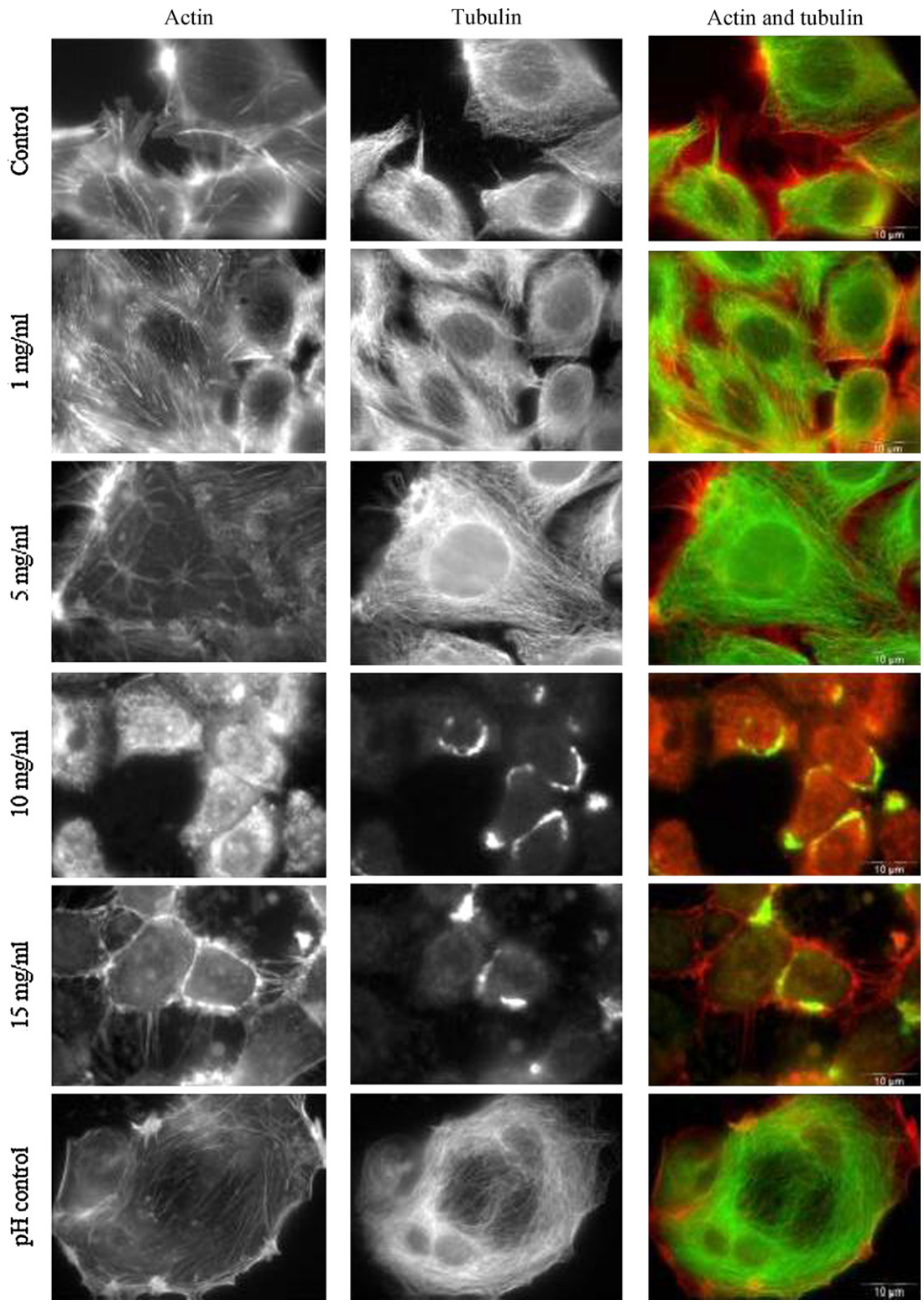

Fig. 5. Immunofluorescent staining of β-tubulin and F-actin of Caco-2 cells after 24 h treatment with chitosan malate solution. In the merged pictures actin is displayed in red, and tubulin in green. Magnification is the same in all pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Cells showed a normal microtubule arborisation; moreover, they exhibited a regular distribution of actin microfilaments over the cell monolayer; the filamentous biopolymers of F-actin appeared abundant and organised into stress fibers arranged in a parallel alignment and in elongated and extended filopodia. Treatment with CM at as low concentrations as 1 mg/mL does not seem to affect the architecture of the cytoskeletal structures examined, whereas severe alterations were observed after exposition at higher CM concentrations. In particular, after treatment with 5 mg/mL CM, the actin stress fibers did no longer display the ordered parallel alignment as in the control, but they showed an irregular arrangement, caused by a strong microfilament depolymerisation; as a

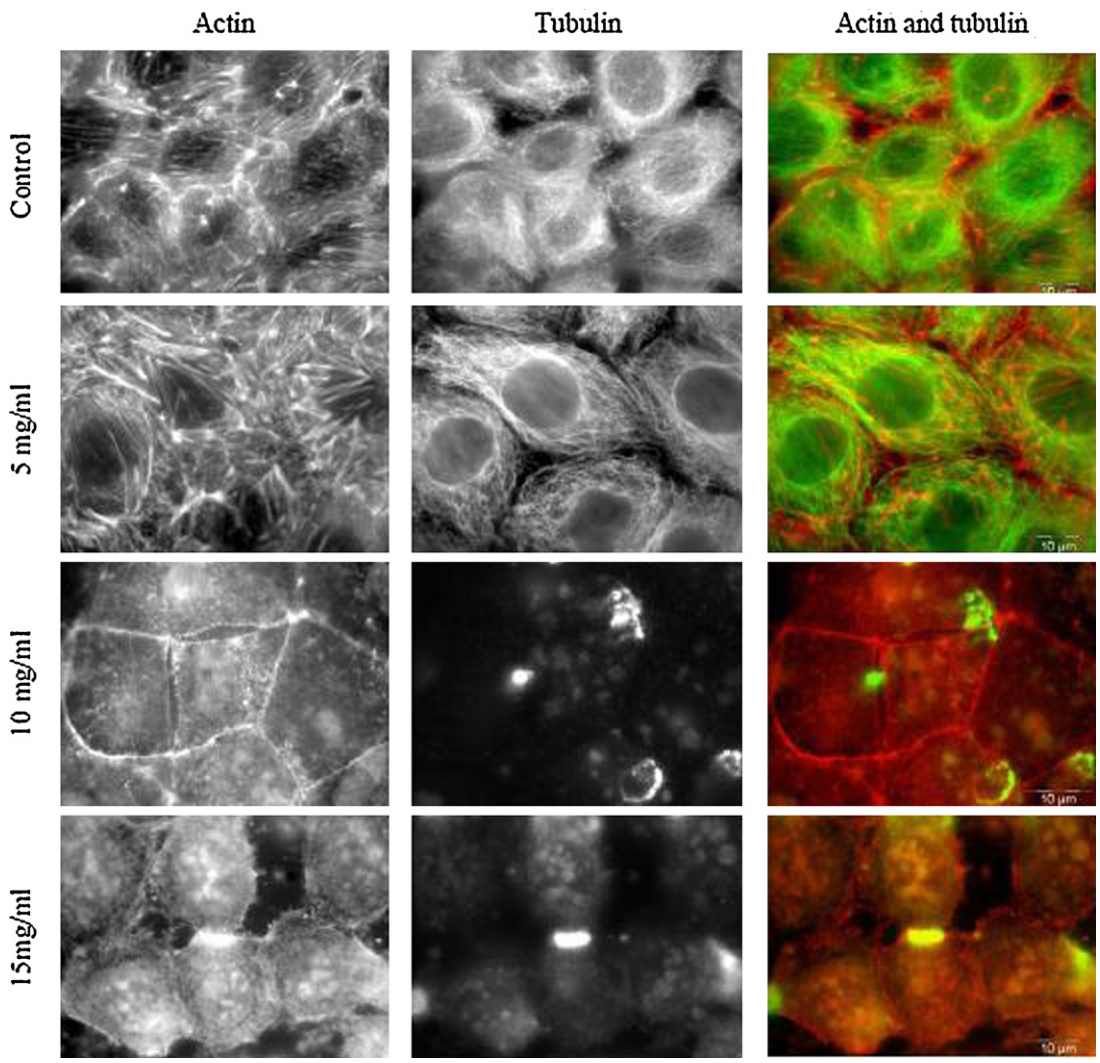

Fig. 6. Immunofluorescent staining of β-tubulin and F-actin of Caco-2 cells treated with chitosan malate after recovery. In the merged pictures actin is displayed in red, and tubulin in green. Magnification is the same in all pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

consequence, at the cell edges there were signs of retraction of filopodia, with cell shape more rounded. The radial distribution of tubulin appeared to be similar to the control, but a higher fluorescent signal was evident around the nucleus, probably due to a microtubule retraction from the cell periphery towards the microtubule-organizing centre (MTOC). After treatment of cells with 10 mg/mL CM, cells appeared less spreaded, and the cytoskeletal architecture was destroyed. A diffuse distribution of aggregates of actin was present in the cell cytoplasm. Microtubules were no more visible in the cytosol, while tubulin appeared mostly localized close to the plasma membrane, where it seemed to collapse and deposit as plaques. At 15 mg/mL CM, the microtubules arborisation appeared the same as observed at 10 mg/mL; as far as actin is concerned, microfilament network was totally destroyed and the cytoskeletal protein showed a more evident thickening just below plasma membrane. Finally, as shown in Fig. 5 (bottom row), cells incubated with medium adjusted at the same pH value as 15 mg/mL CM (5.6) did not display any alteration of cytoskeletal architecture: the distribution and arrangement of actin and tubulin was the same as the control, demonstrating that acidic pH values in our experiments did not affect cytoskeletal architecture, as stated above with regard to cell viability. Recovery experiment showed a partial rearrangement of the cytoskeleton after 24 h since the end of treatment with CM solutions of 5 mg/mL and 10 mg/mL, while the rearrangement of the cell cytoskeleton observed after treatment with 15 mg/mL appeared irreversible (Fig. 6).

4. Discussion

Over the last two decades, chitosan attracted the attention of pharmaceutical researchers because of some interesting properties, such as mucoadhesivity and enhancement of mucosal epithelial permeability, that account for its use as a biomaterial for tissue engineering, in wound healing and as an excipient for controlled drug delivery (Dash et al., 2011). Moreover, chitosan possesses various biological activities, such as the hypocholesterolemic, immuno-enhancing and antimicrobial effects, which account for some potential applications (Ueno et al., 2001; Alsarra, 2009); in some countries it is approved as a nutritional supplement and is used to reduce fat absorption as a mean for controlling weight; edible chitosan biofilm has also been prepared for food storage utilizing its antimicrobial activity (Dutta et al., 2009).

Chitosan is widely regarded as being a biocompatible, biodegradable and non-toxic polymer; clinical tests carried out in order to boost the development of biomedical chitosan-based applications did not report any inflammatory or allergic reactions

following administration of this polymer to humans, although chitosan uptake, distribution and toxicity studies are very few. Moreover, it should be born in mind that the term "chitosan" encompasses a large group of structurally different chemical entities that may exhibit different biodistribution, biodegradation and toxicological profiles. Data about *in vitro* and *in vivo* toxicity of chitosan and chitosan derivatives have been recently reviewed by Kean and Thanou (2010). In general, chitosans were found to show little cytotoxicity against different cell lines, and toxic effect were found to be related to deacetylation degree, molecular weight and charge density of the polymer; the concentration range tested was quite different, and IC_{50} values of chitosans and chitosan derivatives ranged between 0.21 and >10 mg/mL (Kean and Thanou, 2010). Our experiments demonstrated that chitosan malate in solution at high concentrations was quite toxic to Caco-2 cells, with viability percentages of $5 \pm 2\%$, $7 \pm 3\%$ and $31 \pm 15\%$ at 15, 10 and 5 mg/mL, respectively. CM concentrations used in our cytotoxicity experiments are quite higher than those reported in the literature. Our choice was justified by the observation that, for biomedical applications, chitosan and its derivatives are often formulated as micro- or nano-particles (Van der Lubben et al., 2001; Guliyeva et al., 2006; Ko et al., 2002; Kang et al., 2009); when these particles are applied onto the delivery tissue, very high polymer concentration could be reached locally, with possible toxic effects. Actually, when we compared the level of toxicity of CM microparticles with that one of the polymer aqueous solution, it was evident from the results that microparticles are quite more toxic to Caco-2 cells; for instance, viability of cells treated with 10 mg microparticles in 2 mL (in theory corresponding to 5 mg/mL) was $5 \pm 6\%$, therefore corresponding to viability displayed by cells treated with CM in solution at 10 mg/mL ($7 \pm 3\%$). This finding should not to be unexpected, because it is obvious that the application of CM microspheres directly onto the cell monolayer can produce high polymer localized concentrations; moreover, in the experiments with CM microparticles, the pH of the medium is probably quite more acidic if compared with the experiments based on CM solution, whose pH value was adjusted at 5.6. In this regard, acidic pH values affect mildly the viability of the Caco-2 cells and might to some extent explain the cytotoxicity seen when treating the Caco-2 cells with CM solution. However, the lowest pH value in the experiments, 5.6, showed a cell viability of $75 \pm 12\%$; for comparison, cell viability after treatment with 15 mg/mL CM was $5 \pm 2\%$. Therefore, the effect of low pH values can account only very partially for the almost complete cell death seen after treatment with chitosan malate at comparable pH.

The typical cytoskeletal organization of a cell includes actin microfilaments, vimentin intermediate filaments and tubulin microtubules. These filamentous structure create the three-dimensional network bound to a huge number of cytoskeleton-associated proteins, that simultaneously cross-link the filaments and are involved in many vital cellular processes of life. The cytoskeleton is now recognized as an important cell component: it is a scaffolding determining cell shape and providing mechanical support, controls cell locomotion, and takes part to signal transduction and cell trafficking; moreover it plays an important role in fundamental events such as cell proliferation, differentiation and eventually death. For these reasons, we extended our investigation to detect the effect of CM on the main cytoskeletal structures of Caco-2 cells, microtubules and microfilaments; while some authors investigated the effect of chitosan on actin architecture, to our knowledge this is the first time that the effect of a chitosan on cell microtubules is described.

Several reports have shown that chitosan is able to induce a reversible opening of tight junctions in different cell models, including the Caco-2 cell line (Dodane et al., 1999; Smith et al., 2004). Several agents that increase the tight junction permeability of intestinal epithelial cells are also known to alter the actin cytoskeleton. Dodane et al. (1999) reported that treatment with chitosan 0.1% (w/v) for short contact times (30–60 min) induced redistribution of polymeric actin, F-actin. They observed a focal condensation of actin at areas of cell-to-cell contacts and a shortening of stress fibers; however, the actin network was not modified. Although these authors could not explain the mechanism by which chitosan induced these actin changes, they excluded an interference with the assembly of monomeric G-actin to F-actin. These results were consistent with a study by Smith et al. (2004), who also observed changes in actin architecture after Caco-2 treatment with 0.5% chitosan glutamate for 1 h; no alterations were found in the actin network, but the authors observed a slight increase in perinuclear actin localization and some truncation of basolateral microfilaments, without changes in their distribution. Changes in F-actin distribution were also reported by Artursson et al. (1994) and Ranaldi et al. (2002) referred that treatment with 0.005% chitosan for 1 h resulted in a loss of staining for F-actin and alterations in its distribution. Our results demonstrating the severe alteration of actin architecture are in agreement with the observations by Dodane et al. (1999); moreover, we also demonstrated that actin depolymerisation caused by high CM concentrations (10–15 mg/mL) is not reversible.

Microtubules pattern of Caco-2 cells, which is a network regularly arranged around the nucleus, appeared deeply modified by CM treatment in a concentration-dependent way, with progressive microtubule changes in length and spatial disposition and deposition of fluorescent aggregates at the periphery of the cells. Recovery experiments showed the original arborisation of tubulin appeared irreversibly altered by treatment with the highest CM concentrations (10–15 mg/mL).

5. Conclusions

At present chitosan represents a resource with great biomedical potential, and many future therapeutic and biological applications of this polymer can be expected in the near future. Chitosan is a generally regarded safe and non toxic material; however, many factors potentially able to affect its toxicity (average molecular weight, degree of deacetylation, salt form, derivatization) should be taken into account whenever this polymer is applied in drug delivery or tissue engineering. Our experiments demonstrated that viability and the cytoskeletal architecture of Caco-2 cells are deeply modified by treatment with CM at high concentrations, which might be locally reached *in vivo* after application of drug-loaded chitosan microparticles onto mucosal cells, although data so far collected do not allow to speculate about the mechanism underlying these effects. It appears therefore that further work is needed in order to fully establish the safety of chitosan for biomedical applications.

Declaration of interest

The authors report no declarations of interest.

Acknowledgements

We thank Prof. Maria Cristina Bonferoni (Dipartimento di Chimica Farmaceutica, Università di Pavia, Italy) for Caco-2 cells. Financial support from the Italian Ministry of University and Research (MIUR) is gratefully acknowledged.

References

Alsarra, I.A., 2009. Chitosan topical gel formulation in the management of burn wounds. Int. J. Biol. Macromol. 45, 16–21.
An, H.K., Park, B.Y., Kim, D.S., 2001. Crab shell for the removal of heavy metals from aqueous solutions. Water Res. 35, 3551–3556.

Artursson, P., Lindmark, T., Davis, S., Illum, L., 1994. Effect of chitosan on the permeability of monolayers of intestinal epithelial cells (Caco-2). Pharm. Res. 11, 1358–1364.
Carreño-Gómez, B., Duncan, R., 1997. Evaluation of the biological properties of soluble chitosan and chitosan microspheres. Int. J. Pharm. 148, 231–240.
Chirkov, S.N., 2002. The antiviral activity of chitosan. Appl. Biochem. Microbiol. 38, 1–8.
Dash, M., Chiellini, F., Ottembrite, R.M., Chiellini, E., 2011. Chitosan – a versatile semisynthetic polymer in biomedical applications. Prog. Polym. Sci. 36, 981–1014.
Di Martino, A., Sittinger, M., Risbud, M.V., 2005. Chitosan: a versatile polymer for orthopaedic tissue-engineering. Biomaterials 26, 5983–5990.
Dodane, V., Amin Khan, M., Mervin, J., 1999. Effect of chitosan on Caco-2 epithelial permeability and structure. Int. J. Pharm. 182, 21–32.
Domard, A., Domard, M., 2001. Chitosan: structure–properties relationship and biomedical applications. In: Severian, D. (Ed.), Polymeric Biomaterials. Marcel Dekker Inc., New York, pp. 187–212.
Dutta, P.K., Tripathi, S., Mehrota, G.K., Dutta, J., 2009. Perspectives for chitosan based antimicrobial films in food applications. Food Chem. 114, 1173–1182.
Edmundson, I.C., 1967. In: Bean, H.S., Carless, J.E., Beckett, A.H. (Eds.), Advances in Pharmaceutical Sciences, vol. 2. Academic Press, London, p. 950.
Friedman, M., Juneja, V.K., 2010. Review of antimicrobial and antioxidative activities of chitosans in food. J. Food Protect. 73, 1737–1761.
Furda, I., 2000. Reduction of absorption of dietary lipids and cholesterol by chitosan and its derivatives and special formulation. In: Muzzarelli, R.A.A. (Ed.), Chitosan per Os: From Dietary Supplement to Drug Carrier. Atec, Grottammare (Italy), pp. 41–63.
Guliyeva, Ü., Öner, F., Özsoy, Ş., Haziroğlu, R., 2006. Chitosan microparticles containing plasmid DNA as potential oral gene delivery system. Eur. Pharm. Biopharm. 62, 17–25.
Hao, T., Wen, N., Cao, J.K., Wang, H.B., Lü, S.H., Liu, T., Lin, Q.X., Duan, C.M., Wang, C.Y., 2010. The support of matrix accumulation and the promotion of sheep articular cartilage defects repair in vivo by chitosan hydrogels. Osteoarthr. Cartil. 18, 257–265.
He, W., Guo, X., Xiao, L., Feng, M., 2009. Study on the mechanisms of chitosan and derivatives used as transdermal penetration enhancers. Int. J. Pharm. 382, 234–243.
Hirano, S., Seino, H., Akiyama, Y., Nonaka, I., 1990. Chitosan: a biocompatible material for oral and intravenous administrations. In: Gebelein, C.G., Dunn, R.L. (Eds.), Progress in Biomedical Polymers. Plenum Press, New York, pp. 283–290.
Je, J.-Y., Kim, S.-K., 2006. Chitosan derivatives killed bacteria by disrupting the outer and inner membrane. J. Agric. Food Chem. 54, 6629–6633.
Kang, M.L., Cho, C.S., Yoo, H.S., 2009. Application of chitosan microspheres for nasal delivery of vaccines. Biotechnol. Adv. 27, 857–865.
Kean, T., Thanou, M., 2010. Biodegradation, biodistribution and toxicity of chitosan. Adv. Drug Deliv. Rev. 62, 3–11.
Kim, I.Y., Seo, S.J., Moon, H.S., Yoo, M.K., Park, I.Y., Kim, B.C., Cho, C.S., 2008. Chitosan and its derivatives for tissue engineering applications. Biotechnol. Adv. 26, 1–21.
Ko, J.A., Park, H.J., Hwang, S.J., Park, J.B., Lee, J.S., 2002. Preparation and characterization of chitosan microparticles intended for controlled drug delivery. Int. J. Pharm. 249, 165–174.
Kotzé, A.F., Luessen, H.L., Thanou, M., Verhoef, J.C., de Boer, A.G., Junginger, H.E., Lehr, C.M., 1999. Chitosan and chitosan derivatives as absorption enhancers for peptide drugs across mucosal epithelia. In: Mathiowitz, E., Chickering, D.E., Lehr, C.M. (Eds.), Bioadhesive Drug Delivery Systems. Marcel Dekker Inc., New York, pp. 341–385.
Lehr, C.M., Bouwstra, J.A., Schacht, E.H., Junginger, H.E., 1992. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. Int. J. Pharm. 78, 43–48.
Mattana, A., Juliano, C., Bennardini, F., Mignano, M., Marceddu, S., Picci, V., Di Simplicio, P., Franconi, F., 1992. Hydrogen peroxide-induced cytotoxicity in cultured epithelial cells (WISH): a functional and morphological study. Toxicol. In Vitro 6, 533–541.
Mhurchu, C.N., Dunshea-Mooij, C., Bennet, D., Rodgers, A., 2005. Effect of chitosan on weight loss in overweight and obese individuals: a systematic review of randomized controlled trials. Obes. Rev. 6, 35–42.
Muzzarelli, R.A.A., 1997. Human enzymatic activities related to the therapeutic administration of chitin derivatives. Cell Mol. Life Sci. 53, 131–140.
Muzzarelli, R.A.A., 2000. Recent results in the oral administration of chitosan. In: Muzzarelli, R.A.A. (Ed.), Chitosan per Os: From Dietary Supplement to Drug Carrier. Atec, Grottammare (Italy), pp. 3–40.
Ranaldi, G., Marigliano, I., Vespignani, I., Perozzi, G., Sambuy, Y., 2002. The effect of chitosan and other polycations on tight junction permeability in the human intestinal Caco-2 cell line. J. Nutr. Biochem. 13, 157–167.
Ravi Kumar, M.N.V., 2000. A review of chitin and chitosan applications. React. Funct. Polym. 46, 1–27.
Rodriguez, J., Deinhardt, F., 1960. Preparation of a semipermanent mounting medium for fluorescent antibody studies. Virology 12, 316–317.
Seyfarth, F., Schliemann, S., Elsner, P., Hipler, U.-C., 2008. Antifungal effect of high- and low-molecular-weight chitosan hydrochloride, carboxymethyl chitosan, chitosan oligosaccharide and *N*-acetyl-D-glucosamine against *Candida albicans*, *Candida krusei* and *Candida glabrata*. Int. J. Pharm., 139–148.
Shi, Z., Neoh, K.G., Kang, E.T., Wang, W., 2006. Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles. Biomaterials 27, 2440–2449.
Small, J.V., 1981. Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshwork. J. Cell Biol. 91, 695–705.
Smith, J., Wood, E., Dornish, M., 2004. Effect of chitosan on epithelial cell tight junctions. Pharm. Res. 21, 43–49.
Ueno, H., Mori, T., Fujinaga, T., 2001. Topical formulations and wound healing applications of chitosan. Adv. Drug Deliv. Rev. 52, 105–115.
Van der Lubben, I.M., Verhoef, J.C., Borchard, G., Junginger, H.E., 2001. Chitosan for mucosal vaccination. Adv. Drug Deliv. Rev. 52, 129–144.
Wang, G., Ao, Q., Gong, K., Wang, A., Zheng, L., Gong, Y., Zhang, X., 2010. The effect of topology of chitosan biomaterials on the differentiation and proliferation of neural stem cells. Acta Biomater. 6, 3630–3639.
Xia, W., Liu, P., Zhang, J., Chen, J., 2011. Biological activities of chitosan and chitooligosaccharides. Food Hydrocolloids 25, 170–179.