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Article

The Study of the Interaction of Chitosan NPs with Epithelial Cultures: A Simplified Scheme of Wound Healing at a Cellular Level

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Abstract: Chitosan is naturally considered an excellent biomaterial for wound healing treatment. In the present work, the synthesis and physicochemical characterization of three different average sizes of CsNPs were achieved (15, 50 and 125 nanometers) with the purpose of studying the in vitro interaction with Madin-Darby canine kidney (MDCK) cells. From our results we found that CsNPs have a crystalline structure with a diameter size less than 15 nm. In cell viability we observed that the three average sizes of CsNPs were not toxic at a concentration of 400 µg/ml. Moreover, it was found on wound healing assays that 125 nm CsNPs induced the best results for cell migration. Besides, proteomic analysis showed the expression and overexpression of proteins induced by the presence of CsNPs in MDCK cells.

Keywords: biomaterials; CsNPs; cell migration; proteins

1. Introduction

Wound healing is complex and involves countless biological processes like migration and proliferation of several kinds of cells, triggering endless studies. To promote a faster wound healing, biomaterials capable of optimizing cell migration are being developed. Among the prominent materials are biopolymers, mainly of natural origin like collagen, alginate, cellulose, gelatin and chitosan. During the wound healing process, cell migration is essential, for epithelium (such as MDCK), this type of massive cell movement must be collective, based on cell-extracellular matrix interface proteins involved in cell adhesion processes. It is well known that the dissociation of cell-cell and cell-extracellular matrix junctions promotes the migration mechanisms within cellular systems, through the modulation of different proteins such as E-cadherin, integrin, galectin-1 and 3 [1], transglutaminase 2 and α -actinin-1, in addition to aquaporin-5 [2] and the Rac subfamily [3]. The aim of this work was to characterize chitosan nanoparticles of defined sizes and evaluate the participation of nanoparticulated chitosan on the enhancement of MDCK migration during wound closure. Chitosan (CS) is mainly obtained from the alkaline deacetylation of chitin (poly N-acetyl β 1, 4 glucosamine), generating a random distribution of acetylated and deacetylated units. This cationic polysaccharide is composed of D-glucosamine (2-amino-2-deoxy- β -D-glucopyranose) and N-acetylglucosamine (2-acetamide-2-deoxy- β -D-glucopyranose), linked by β glucosidic bonds (1-4). In its chemical structure, chitosan has two functional groups, the amino group ($-NH_2$) and hydroxyl group (OH), which determine the degree of deacetylation and its molecular weight. Due to the presence of amino and hydroxyl groups in the polymer chain, the molecular structure of chitosan can be modified, allowing applications for drug delivery [4], regeneration and wound healing [5], as

mucoadhesive [6,7], as anticancer [8], as procoagulant [9], as antimicrobial [10] and as anti-inflammatory. As a nanocomposite, chitosan offers varied and very interesting advantages as mentioned above, but further research still needs to be done to elucidate its nanotoxicity and the skin healing-regeneration process at the cellular level. Regarding the use of Madin Darby canine kidney (MDCK) derived epithelial cells, this model has proven to be useful on forming monolayers due to its ability to assemble both tight and adherent junctions, as well as inducing cell polarity and showing collective motility.

To date, each of the published studies is based on the early epithelialization of the wound as a result of using chitosan-based mesoscopic dressings or scaffolds, demonstrating that the dressing provides a scaffolding structure conducive to rapid healing [11]. Here we show the effect of the coexistence addition of CsNPs to the cell culture, on cell toxicity and migration, together with the overexpression of a 57 kDa protein.

2. Results and Discussion

2.1. Particle Formation: CsNPs

For CsNPs formation, chitosan was combined with sodium tripolyphosphate (TPP), which reacts directly with functional groups that are present in the chitosan molecule, such as amine and hydroxide, forming inter and intramolecular bonds with the phosphate groups of TPP (oxygen and phosphorous atoms) [12]. Three average sizes of particles were obtained: 15, 50, 125 nm respectively, (Supplementary Figure S1 and S2 provide a grain analysis and micrographs of the corresponding sizes of 15 and 50 nm). Figure 1a shows an AFM micrograph, where spherical nanoparticles with an average size of 125 nm can be observed.

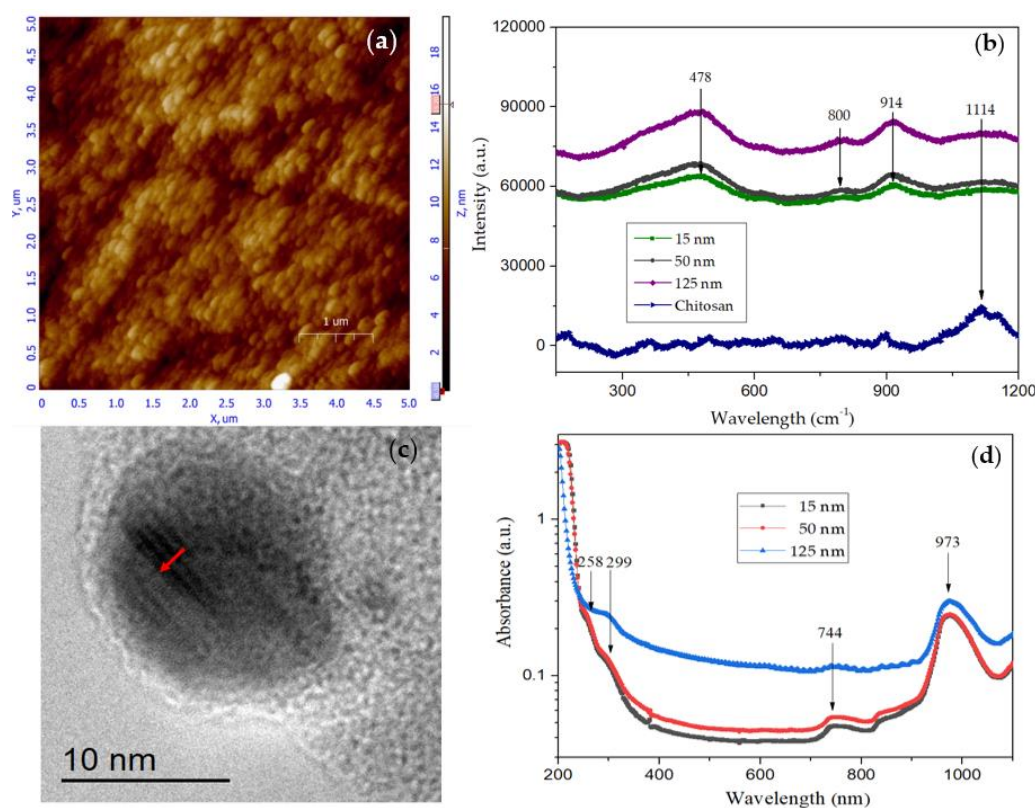


Figure 1. (a) AFM micrograph of 5 μ M CsNPs with average size of 125 nm, after 30 min stirring. (b) Raman spectra from raw chitosan without any processing, and different size CsNPs. (c) TEM image of CsNPs, crystalline planes indicated as parallel to the red arrow. (d) UV-vis spectrum of the plasmon due to the presence of the 15, 50 and 125 nm CsNPs.

2.2. Raman Spectroscopy

To verify that the CsNPs formation was successful we studied the possible modification of vibrational modes of the CS molecule after the synthesis of the nanoparticles. We performed an optical characterization by Raman spectroscopy, which allowed to observe the vibrational information of the molecules and the influence of the substrate. It can be clearly observed that the Raman spectrum for CsNPs in Figure 1b shows several peaks at 479, 800, 914 and 1114 cm^{-1} which become more intense as the grain size increases. The bulk chitosan only has one intense peak at 1114 cm^{-1} , this can be due to the loss of liberty grades when the size and dimensions of the chitosan are reduced from bulk to nanoparticles. It is clear that bulk chitosan should be an amorphous material and CsNPs could have some crystalline degree that increases the observed vibrational degrees of freedom.

2.3. UV-VIS Spectrpscopy

The UV-VIS experiments were performed to prove the formation of CsNPs. Figure 1d shows the experimental absorbance of the three preparations and two little broad shoulders centered at 258 and 299 nm were observed and one of them was already reported and could be associated with the formation of CsNPs [13]. Some other broad peaks at 744 and 973 nm were registered and could be understand as an absorption caused by a partial aggregation of CsNPs [14].

2.4. Transmission Electronic Microscopy (TEM)

By using TEM, we found that chitosan nanostructured can be a crystalline material as shown in Figure 1c. CsNPs present two patterns of parallel lines (parallel to the red arrow) typical of crystalline arrangements; the interplane distance is 2.2 Å and was calculated using Digital Micrograph software. TEM images of these CsNPs showed the crystalline planes due to a periodic and continuous arrangement of its atoms when CS is modified at the nanometric scale. The pattern of crystalline planes is observed only in one direction, which strongly suggests the formation of a single crystal, due to the lack of grain boundaries within the nanoparticles.

2.5. Cell Viability Assay

Even though chitosan is widely reported as biocompatible, most of the literature presents studies with microfiber and objects so much bigger than nanoparticles. Thus, we consider it important to evaluate the cell viability when chitosan is in contact with cells and became as small as a nanoparticle, little enough to pass through the cell membrane. Results in Figure 2 show the quantitative analysis of the cell viability of canine kidney (MDCK) epithelia comparing the control groups with those treated with CsNPs of three average diameter sizes (15, 50, and 125 nm). The concentration of CsNPs showed an effect on the cell viability compared to untreated cells. Graphs in Figure 2 show a notable change in the decrease in viability for the concentration of 400 $\mu\text{g/ml}$. This change in viability is also related to the size of the nanoparticle, maximum impact is shown for 15 and 50 nm at 24 h (upper graph), and 125 nm at 48 h (lower graph). It is important to note that CsNPs at this high concentration do not suggest a toxic effect on cells, and ANOVA analysis performed by GraphPad prism reveals those certain concentrations of CsNPs for the different size distributions: 15, 50 and 125 nm resulted statistically significant ($p < 0.05$). Analysis was performed by grouping nanoparticle size and incubation time.

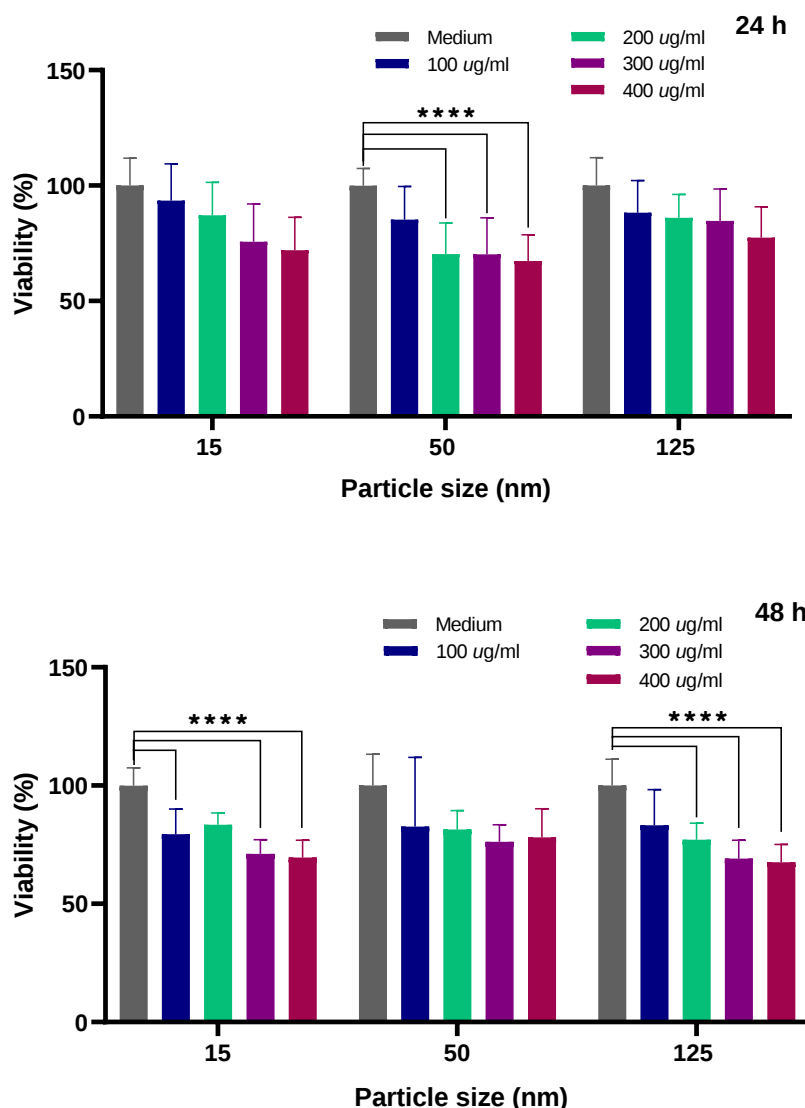


Figure 2. Percentage of cell viability of MDCK cell after treatments with CsNPs for 24 and 48 h at 37 °C. The results shown in the graphs are pooled data from three biological replicates of each nanoparticles size (15, 50, and 125 nm) and concentrations. Statistical analysis was performed with a significance level of $p < 0.05$.

2.6. Wound Closure Assay

From wound closure assays, it was determined that CsNPs of 125 nm size induced cell migration. Figure 3a shows the width of each wound in μm (area without cells) of the control and treatments with CsNPs at 0, 4 and 8 h respectively is presented. The closure of the wound occurred at 8 h after the addition to the monolayer of the CsNPs, while the control condition still presents 45 % of empty area. In addition, figure 3b shows, by immunofluorescence, the wound healing process, which was almost complete at 8 h, when CsNPs at concentrations of 300 and 400 $\mu\text{g/ml}$ were added. Supplementary Figures S3 and S4 show representative figures of bright field and immunofluorescence of the wound closure assay.

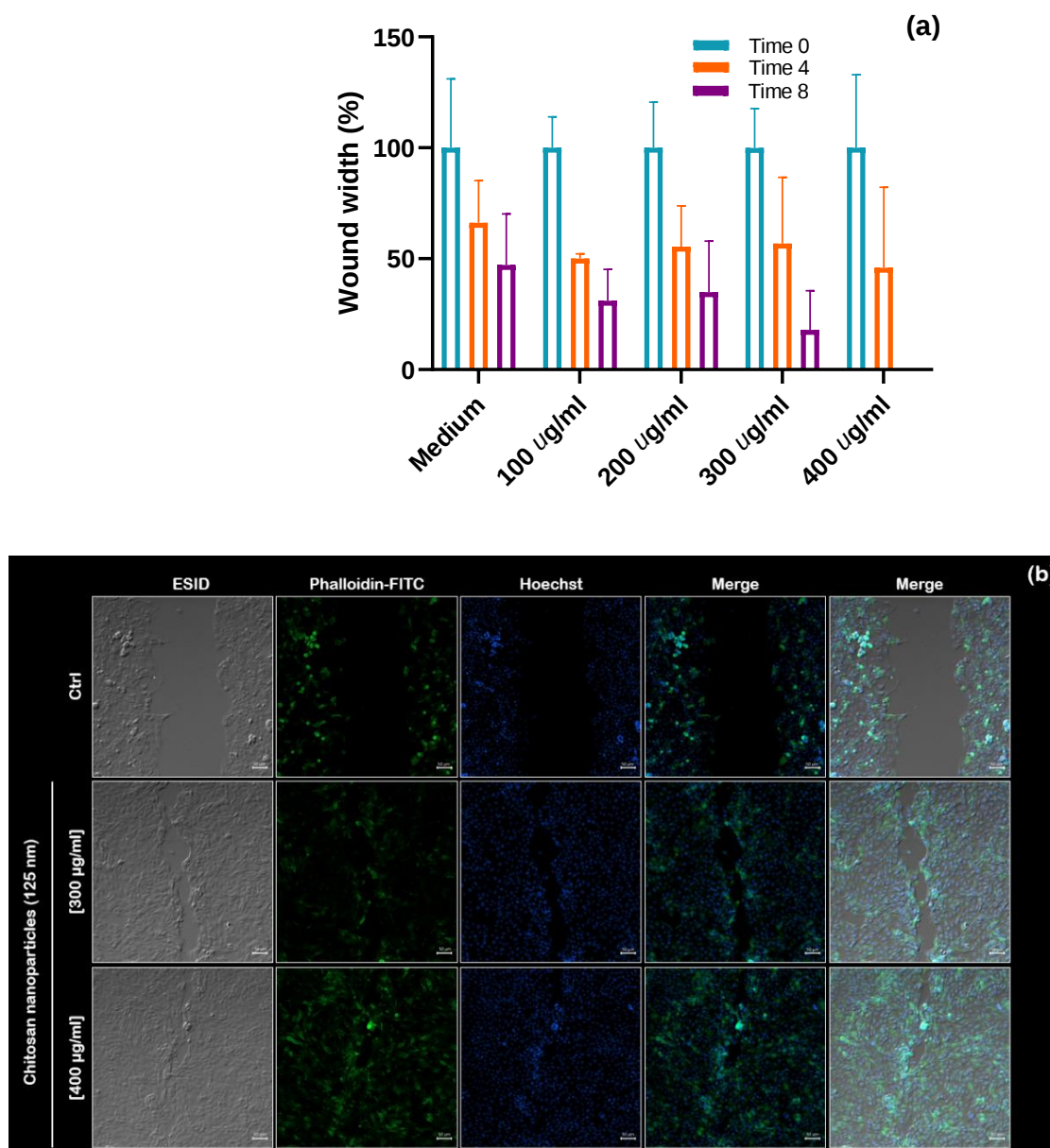


Figure 3. Cell migration induced by CsNPs: (a) Graph of wound closure kinetics at 0, 4, and 8 h, and (b) images obtained by confocal microscopy (50 μm) of cells stained with Phalloidin coupled to FITC to visualize the actin cytoskeleton and with Hoechst to stain cell nuclei, with their respective merge image; also, the bright field images (ESID) and the merge of the coupling between ESID, cytoskeleton and nucleus of the wound closure assay.

2.7. Effect of CsNPs on Protein Expression

To determine if there was a change in the protein expression pattern as a result of the interaction of CsNPs with the MDCK cells, a protein total extract was analyzed by SDS-PAGE. As shown in Figure 4, there is a very visible thicker band approximately of 57 kDa, for the CsNPs conditions (200 and 400 $\mu\text{g/ml}$), although it is more intense in lane 2. It is considered that this observed overexpression could be mainly due to the CsNPs and the incubation time. Mass spectrometry analysis showed that several proteins were overexpressed in the cells treated with CsNPs in comparison with the cells without treatment (Supplementary Table 2). Analysis of these results suggests that the increase in some proteins could be directly related to the biological processes of migration and wound healing; however, this proposal needs further confirmation.

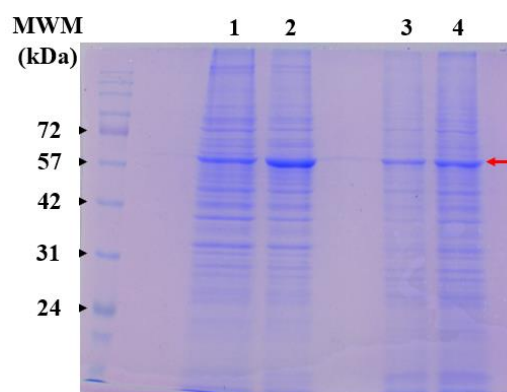


Figure 4. Commassie blue staining of a 10% SDS-PAGE of CTL (lanes 1 and 3), and treatments with 125 nm CsNPs at 200 µg/ml at 8 h (lane 2), and 400 µg/ml at 48 h (lane 4).

2.8. Proteomic Analysis and Bioinformatics

Quantitative proteomic analysis of MDCK cells and MDCK plus CsNPs cultures were performed and a total 2188 proteins on the samples, of which we removed false discoveries, were identified. After that, found proteins were listed according to the highest amount (femtomole, fmol) in the samples treated by CsNPs.

From the analysis of the raw data of mass spectrums, we found that cells exposed to CsNPs express the following proteins: polyglutamine-binding protein 1, NADH dehydrogenase 1 beta subcomplex subunit 11_ mitochondrial, moesin, WAP four-disulfide core domain 8, calcineurin like phosphoesterase domain containing 1, and cell division cycle and apoptosis regulator 1, which are absent within the control sample. Despite the fact that some proteins have a femtomole concentration below our cutting criteria we found, among them, cell division cycle and apoptosis 1 (CCAR1), and moesin (MSN) which participate in the cell migration process. As results of bioinformatic analysis on development NCBI gene database [15] indicates that CAAR1 is involved in the positive regulation of cell migration [16], positive regulation of cell population proliferation, and positive regulation of apoptotic process. CCAR1 is a gene that acts as a coactivator of the CAR enhancers [17], of p160 family, and p53. MSN has also been reported to be involved in cell migration [18,19], cell motility, and T cell migration [20]; it is a protein complex formed by MSN, ezrin (EZR), and radixin (RDX), that connects the actin cytoskeleton to the plasma membrane. MSN interacts with Na⁺/H⁺ exchange regulatory cofactor NHE-RF (SLC9A3R1), CD44 antigen (CD44), actin cytoplasmic 1 (ACTB), talin-1 (TLN1), and CD81 antigen (CD81), a group of proteins responsible for the formation of the cytoskeleton.

Subsequently, a Functional Annotation Chart was obtained from NIH's tool (David bioinformatics) (Supplementary Figure S5), and a list of the highest femtomole protein concentration was provided with a cut edge of 10 times over the control's concentration, Supplementary Table S3. It is important to notice that biological process (BP) like microtubule cytoskeleton organization which contains 5 proteins enlisted as follow: APC regulator of WNT signalling pathway 2 (APC2), cingulin (CGN), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), microtubule associated protein 4 (MAP4) and tubulin alpha 1b (TUBA1B). With respect to the BP actin cytoskeleton organization, we found: CXADR Ig-like cell adhesion molecule (CXADR), annexin A1 (ANXA1), dystrophin (DMD), filamin C (FLNC), and spectrin beta, non-erythrocytic 1 (SPTBN1). These BP were founded as result of bioinformatic analysis of the proteomic processed data from samples of CsNPs interaction with epithelial cells. Moreover, same set of the proteins were employed at string database in order to obtain the protein-protein interactions (Supplementary Figure S6). It is important to notice that several coincidences were found between the two bioinformatics software tools employed, where the most important proteins in the case of cytoskeleton organization were: GAPDH and ANXA1, due to their interaction with each other and with galectin-3 (LGALS3).

3. Materials and Methods

3.1. Reagents

The materials that were employed in this work were: chitosan of low molecular weight (Sigma Aldrich, Cat. No. 448869), acetic acid (JTBaker, Cat. No.), and sodium tripolyphosphate (TPP) (Sigma Aldrich, Cat. No. 238503), for the synthesis of CsNPs. For the cell culture: DMEM (Dulbecco's Modified Eagle Medium GIBCO, Cat. No. 11965092), FBS (fetal bovine serum CORNING, Cat. No. 16000044) and non-essential amino acids mixture (GIBCO, Cat. No. 1974074). Finally, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Cat. No. 2060695) for cell viability and Alexa Fluor™ 488 Phalloidin (Thermo Fisher Scientific, Cat. No. A12379), and Hoechst (Thermo Fisher Scientific, Cat. No. 33258) for immunofluorescence.

3.2. Synthesis of CsNPs

The molecular weight of no processed chitosan was determined by liquid chromatography with a diode array detector (HPLC-DAD) Perkin Elmer-200, obtaining a molecular weight of 164,518.02 Da. CsNPs were obtained by employing ionotropic gelation [21], as a first step, 1 mg/ml of CS was dissolved in 1% (v/v) acetic acid solution for 4 h at 55 °C. The self-assemble of the CS nanoparticles was obtained by stirring the solution with sodium tripolyphosphate (TPP). During chemical synthesis, temperature, stirring time, and volume of the chitosan solution were varied to determine the size of the nanoparticles; see Supplementary Table 1, nanoparticle synthesis parameters. CsNPs were centrifuged at 14000 rpm for 15 min, and resuspended in milli Q water in order to avoid additional toxicity due to residues of acetic acid. Finally, CsNPs were filtered by a 0.22 µm membrane.

3.2. Optical and Morphological Characterization of CsNPs

Optical properties were determined by UV-vis spectroscopy in order to prove the presence of nanoparticles through the rise of absorbance similar to the plasmon resonance behavior at metallic NPs. The particle size and morphology of CsNPs were observed by atomic force microscopy (AFM) (NTMDT model Solver Next 1100) in a semi-contact top mode, a gain of 0.21, and a cantilever frequency of 8 kHz. For transmission electronic microscopy study (TEM, JOEL-JEM-2010, Tokyo, Japan), an aliquot of CsNPs was placed onto a copper grid and dried. An acceleration voltage of 200 kV was applied for TEM visualization with a 256 pixels resolution.

All samples were aliquoted from a colloidal suspension of CsNPs with a nominal concentration of 1 µg/µl. For the obtention of a homogenous sample, the colloidal suspension was vortexed. For AFM and Raman spectroscopy an aliquot of 8 µl was left for 15 min on a substrate (wafer and glass) until dry (1 h at room temperature). For UV-Vis, due to the geometry of the quartz cell, a 3 ml volume was required. Thus, variations on concentration of the sample due to inhomogeneity at micrometric levels should be expected. Sample measurements by Raman spectroscopy were done with a Hiroba Jovin Lab Raman equipment, with an excitation line of He-Ne working at 632.8 nm and 20 mW of nominal power of the laser. UV-Vis was developed on a Perkin Elmer lambda 25 system, this equipment has a spectral range of 200-1100 nm.

3.3. Cell Culture

Madin-Darbi canine kidney Cells (donated by Dr. Abigail Betanzos, CINVESTAV), were cultured in Dulbecco's modified Eagle's Medium (DMEM) that was supplemented with 5% fetal bovine serum (FBS) and non-essential amino acids at 1 %, at 37 °C, 5% of CO₂, and humidity.

3.4. Cell Viability Assay

Cell viability was determined by the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay. Cells (1 × 10⁴) were plated in 96 wells culture plates and incubated with DMEM medium containing four different concentrations of CsNPs (100, 200, 300, and

400 µg/ml) during 24 and 48 h for three nanoparticle average sizes (15, 50 and 125 nanometers). Then 20 µl aliquot of MTT solution (5 mg/ml) was added to each well, and plates were incubated for additional 4 h at 37 °C. Subsequently the medium was aspirated and 100 µl of acid isopropanol were added to each well. Optical density was measured at a wavelength of 595 nm in a BIO-RAD 680 ELISA microplate reader to obtain the absorbance. The experiment was performed in triplicate with three biological replicates for data accuracy.

3.5. Scratch Wound Healing

Cells (4×10^5) were plated in 24 well plates and cultured overnight. Then, cells were treated with mitomycin (10 µg/ml) for 2 h, prior to the nanoparticle's treatment. The cells reached 90% confluence, and a vertical scratch was done on the monolayer with a SPLScar commercial Scratcher-0.5 (SPL Lifesciences, Cat. No. 201924). Each well was treated with 100, 200, 300, or 400 µg/mL of CsNPs; the wells were observed under a microscope and each well was photographed, including the control, before incubating for 4 h intervals, until the wound closure process was completed.

3.6. Immunofluorescence

Cells (6×10^4) were plated on coverslips, and the same procedure described in the previous assay was performed for the wound closure assay. After this, cells were fixed with 4% paraformaldehyde at 37 °C, then stained with phalloidin-FITC (1:250) at 37 °C and Hoechst (1:1000) at room temperature for 15 min. Coverslips were mounted on slides with VectaShield. Confocal microscopy was performed with a Carl Zeiss LSM900 (Carl Zeiss, Jena, Germany) microscope, using a 10x objective. Images were processed with ZEN 2.3 Pro Software.

3.7. Protein Extraction

Total protein extracts of MDCK cells were obtained from cell lysis with RIPA buffer containing 50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 % NP-40, 1 mM EDTA, and Complete protease inhibitor cocktail 1X. The extract was sonicated and centrifuged at 14000 rpm for 15 min, followed by protein quantification by the Lowry method. Sample buffer containing β-mercaptoethanol was added to the proteins and they were heated to boiling in a water bath for 5 min. The samples were loaded onto a 10% acrylamide gel and run for approximately 2 h at 120 V, after that the gel was stained with Coomassie blue solution.

3.8. Mass Spectrometry and Bioinformatics Analysis

Quantitative proteomic analysis was performed in the Genomic, Proteomic and Metabolomic Unit (UGPM), using a mass spectrometer (WATERS-SYNAPT G2-SI). 50 µg of protein suspended in RIPA buffer were precipitated using MeOH/Chloroform and enzymatically digest using iST Sample Preparation Kit (PreOmics, Munich, GER). Then, 50 µL of Lyse reagent was added to protein pellets, placed in a heating block during 10 min, 95°C at 1000 rpm. Samples were sonicated during 20 cycles using BioRuptor Pico (Diagenode, Liège, BEL) 30 sec ON/30 sec OFF per cycle. Protein samples were digested using 50 µl of a Lys-C/Trypsin mix (Digest reagent) and heating at 37 °C during 2 hrs. Resulting peptides were cleaned in an iST cartridge using a Wash 1 buffer to eliminate hydrophobic contaminants and Wash 2 buffer to eliminate hydrophilic contaminants molecules; afterward, peptides were eluted using Elute reagent and evaporated to dryness in a SpeedVac (TermoFisher Scientific, Waltham, USA), then, peptides were resuspended with LC-Load reagent and normalized at a concentration of 1 µg/µl, adding an aliquot of alcohol dehydrogenase 1 (ADH1) from *Saccharomyces cerevisiae* (Uniprot accessionP00330; 1 pmol/µl) as internal standard in order to obtain a final concentration of 25 fmol/µl. Bioinformatics analysis of the biological processes and the protein protein interactions were performed by David and string.

5. Conclusions

We succeeded in the synthesis and physicochemical characterization of CsNPs, that were obtained by ionotropic gelation. The nanoparticles of three different average sizes showed “low” cytotoxicity when interacted with MDCK cells. Moreover, cell migration was observed during the closure of scratch assays when CsNPs of 125 nm were added. Furthermore, from confocal microscopy we can infer that even without scaffold the MDCK cells start a 3D change of phenotype (filopodia formation through actin filaments).

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1: AFM micrographs of the corresponding sizes of 15 and 50nm; Figure S2: Grain analysis of CsNPs; Figure S3: Brightfield images of monolayer wound closure; Figure S4: Immunofluorescence images of monolayer wound healing; Figure S5: Biological processes present in protein enrichment; Figure S6: Protein-protein interaction (PPI) network; Table S1: Nanoparticle synthesis parameters; Table S2: Overexpressed proteins in the presence of CsNPs; Table S3: Selection of overexpressed proteins for bioinformatic analysis.

Author Contributions: Conceptualization, L.G. and S.G.; methodology, L.G., P.T. and S.G.; investigation, L.G. and J.O.; resources, P.T. and S.G.; writing—original draft preparation, L.G.; writing—review and editing, L.G., P.T. and S.G.; supervision, P.T. and S.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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