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Freeze Dried Chitosan/ Poly-ε-Caprolactone and Poly-ε-Caprolactone Nanoparticles: Evaluation of their Potential as DNA and Antigen Delivery Systems

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Abstract

Nanoparticles prepared from natural or synthetic polymers have shown potential for antigen and DNA vaccine delivery to mucosal surfaces. The purpose of this research was to prepare chitosan/poly-ε-caprolactone (Chitosan/PCL) nanoparticles and PCL nanoparticles and evaluate their potential as DNA and protein/vaccine delivery systems. Both preparation methods resulted in particles of low cytotoxicity and sizes suggested to be ideal to be taken up by cells (199 ± 62 nm and 165 ± 35 nm, respectively for chitosan/PCL and PCL nanoparticles). However, Chitosan/PCL nanoparticles offered considerable advantages over PCL nanoparticles as antigen and DNA delivery system. Namely, higher loading efficacies for model antigens studied (myoglobin, BSA, ovalbumin, lactalbumin, α-casein and lysozyme), much higher uptake by A549 cells, great ability to form stable complexes, which protect DNA from nucleases. However, in spite of good DNA and protein loading capabilities, Chitosan/PCL nanoparticles showed much better qualities as a protein delivery system since the rate of cells transfected were not very high.

Keywords: Polymeric nanoparticles; Chitosan; Poly-ε-caprolactone; DNA vaccine; Recombinant antigens

Introduction

In developing countries traditional vaccines are mostly administered by injection, which potentially results in a significant transmission of viral infections, due to the reuse of material and unsafe injection practices. Developing needle free vaccination methods to render the administration of vaccines safer is therefore a priority [1]. The nasal mucosa has been considered an administration route due to its relatively large surface area, permeable endothelium, high total blood flow, avoidance of first pass metabolism, avoidance of harsh environmental conditions of the gastrointestinal tract, and ease of administration due to its accessibility [2]. Nevertheless, when a vaccine is administered mucosally it encounters the same host defense barriers as do microbial pathogens and other foreign macromolecules. They are diluted in mucosal secretions, retained and cleared in mucus gels, attacked by proteases and nucleases and barred by the epithelial barrier, which leads to a poor and limited contact of the formulations with the nasal mucosal epithelium itself [2,3]. Even so, this region presents advantages that are worth exploiting for nasal immunization, for instance the high amount of lymphoid tissue in the nasal passages and the potential eliciting of both mucosal and systemic immune responses [2].

Protein based vaccines are widely used and present good efficacy for the prevention of a wide range of infections, although only antibody mediated immune responses (humoral responses) are generated and periodic booster injections are often required. Nevertheless, the immune response induced is not indicated for the clearance of intracellular pathogens requiring the generation of cytotoxic T lymphocytes, which might be crucial in the protection against some diseases that currently have no prophylactic treatment available [4-6].

DNA vaccines can produce a coordinated activation of both humoral and cell mediated responses that result from the intracellular synthesis of the encoded antigen within the host's cells. The encoding sequence may be translated into antigen protein sequences by antigen presenting cells (APC) and then processed and presented by the major histocompatibility complex (MHC) class I, making it recognizable by the receptor of cytotoxic CD8+ T cells; or it may be expressed in other cells, being released into the extracellular space and then captured by APC, processed and presented by MHC class II and recognized by the CD4+ receptors of T helper cells, which facilitate humoral as well as cellular responses [4,7-9]. DNA vaccines also present no potential for the antigen reverse to virulence, the initiation of long lasting immunity, the possibility of widespread use even in immunocompromised individuals, are inexpensive, versatile, extremely stable and relatively easy to produce [7,10]. The main obstacle concerning DNA vaccination lies with the necessary intracellular delivery of the encoding sequence. Several factors make this task a difficult one to accomplish: the protection of DNA plasmid from degradation, low clearance from the interstitial space, transport through the extracellular matrix to the surface of target cells, internalization by target cells, escape from the endosomal/ phagosomal compartment and, once in the cytoplasm, translocation of DNA into the nucleus, transcription and then translation into the protein antigen [4].

Encapsulation or complexation of DNA with a biomaterial can significantly enhance DNA stability, cellular uptake of DNA and ultimately protein expression [4]. Polymeric nanoparticles (NPs) have shown the potential to deliver DNA vaccines, as they are able to

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protect DNA from extracellular degradation, can accommodate larger size plasmids, viruses and immunostimulatory agents simultaneously, possess the ability to offer a phagocytosis-based passive targeting to APC and the ability to be conjugated with appropriate functionalities to enhance cellular targeting and uptake [10]. The ability to co-deliver an immunopotentiator that for instance acts by binding to specific receptors is an essential feature as the immunogenicity and transfection efficiency of DNA vaccines is low, especially in humans [8,10,11].

Chitosan is a cationic polymer consisting of β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) monomers that can be obtained by deacetylation of chitin [12]. It has been considered as a non-toxic, biodegradable and biocompatible polymer [13], and extensive research has been directed towards its use in medical applications such as drug and vaccine delivery [14-17]. Chitosan is also known to be mucoadhesive and its ability to stimulate cells of the immune system has been shown in many studies [18]. When applied to cells, the positively charged polyplexes will mediate transfection via a multistage process that includes cationic binding to the negatively charged cell membrane, which facilitates entrance into the cytoplasm. However, a high density of positive charges leads to an increased cytotoxicity [10]. Moreover, the results of mucosal DNA vaccination studies using chitosan nanoparticles as vectors already described in the scientific literature are in general not very encouraging. The strong interaction between chitosan and pDNA in complexes may not allow for a subsequent dissociation of the complex and unpacking of the DNA, which is necessary for gene expression and may therefore be a possible reason for the low transfection efficiency reported [19,20]. Current knowledge suggests a balanced and moderate interaction between the carrier and the pDNA as one of the key factors to successful therapeutics (extensively reviewed elsewhere [19]).

The introduction of a second biodegradable polymer during the preparation of the chitosan particles, e.g., the hydrophobic polymer poly-ε-caprolactone (PCL), will allow obtaining more amphiphilic particles of modified chitosan-DNA interaction in the complexes and therefore may improve gene expression. Other advantages are present when synthetic polymers such as PCL are considered for DNA delivery applications. Their chemical composition, total molecular weight and block length ratios can easily be adjustedto allow to control the size and morphology of the polymeric carriers [10]. PCL was also chosen due to its higher hydrophobicity, which could further enhance uptake of nanoparticles by the nasal mucosal immune system(NALT) and there *in vitro* stability, lower costs, and allow for the safe eliminationof PCL metabolites, lactic and glycolic acid [21].

The purpose of this research was to optimize the preparation of chitosan/PCL nanoparticles and simple PCL nanoparticles in order to obtain two potential freeze dried vaccine delivery systems. Their properties were studied and compared to determine the potential of antigen loading and delivery, DNA loading and delivery, as well as transfection efficiency.

Materials and Methods

Chitosan purification

Chitosan (ChitoClear™) was purchased from PrimexBioChemicals AS (Avaldsnes, Norway). According to the provider's specifications, the degree of deacetylation was 95 % (titration method) and the viscosity 8 cP (measured in 1 % solutions in 1 % acetic acid). The polymer was purified by a technique adapted from [22]. 1 g of chitosan was suspended in 10 mL NaOH 1 M solution. This suspension was heated to between 40-50 °C under continuous magnetic stirring for 3 h. After this time, at room temperature, it was filtered using a Buchner funnel. Insoluble chitosan on the filter was washed with water and then recovered to be dissolved in 200 mL 1 % acetic acid solution and stirred for 1 h at room temperature. The chitosan solution was then filtered through a 0.45 µm filter and 1M NaOH solution was used to adjust the pH value of the filtrate to pH 8.0. The precipitate was then washed with deionized water through 3 consecutive 30 min centrifugations at 4500 x g. The precipitate was recovered and freeze dried.

Preparation of the Delivery Systems

Nanoparticle production method: The procedure for the preparation of chitosan/PCL particles in our laboratory resulted from the adaptation of different techniques described in the literature, with special consideration to the one described by Bilensoy [23] based on the nanoprecipitation technique patented by Fessi [24]. Briefly, an aqueous phase of acetic acid containing 0.1 % chitosan and 5 % Tween™ 80 was placed under a high speed homogenizer (homogenizer Ystral X120, Ballrechten-Dottingen, Germany). The organic phase, consisting of 0.2 % PCL (Sigma Aldrich Quimica SA, Alcobendas, Madrid) diluted in acetone, was added dropwise to the first solution at a ratio of 1:3 (V/V) to give a final volume of 18 mL. Agitation was continued for 1 min after the complete mixture of both phases. At this point, the particle suspension was formed and was placed under magnetic swirl for 45 min to achieve maturation. Finally, the organic phase was removed by evaporating acetone with a nitrogen flux in a water bath (40°C maximum). The nanoparticles suspended in the original medium were isolated, resuspended and concentrated in other diluents by centrifugation at 16000 x g, for 75 min at 4°C. To achieve minimal aggregation of the particles after the centrifugation, a 200 µL glycerol bed for each 18 mL batch is recommended. Another methodology for isolating the particles is by dialysis of the original medium against water for 48 h, using Spectra®Por cellulose ester dialysis membrane, MWCO 300.000 (Spectrum laboratories, Inc., Rancho Dominguez, CA, USA). Then, to the resulting solution trehalose (Sigma Aldrich Corp., MO, USA) was added to a final concentration of 2.5 %, so that a freeze-drying process (FreezeZone 6, Labconco Corporation, Kansas City, MO, USA) was successfully achieved maintaining the particle original properties.

PCL particles were produced using the methodology described above, by replacing the 0.1 % chitosan solution by a simple acetic acid solution with 5 % of Tween 80.

Model proteins were adsorbed to nanoparticles, previously resuspended in buffer, by simple incubation with slight agitation at room temperature at variable protein:nanoparticles ratios and incubation times as described below.

Production of Nanoparticle-DNA complexes: Plasmid DNA (pCMVluc) encoding luciferase was amplified in *E. coli* strain DH 5α and purified using QIAGEN Plasmid Giga kit (QIAGEN, Hilden, Germany). The purified pDNA was dissolved in MilliQ water and its concentration and purity assessed by UV spectrophotometry by measuring the absorbance at 260/280 nm. Nanoparticle-DNA (NP-DNA) complexes were prepared by mixing equal volumes of a nanoparticle suspension in phosphate buffer (PB) pH 5.7 (several concentrations) with a 100 µL/mL luciferase plasmid solution during an incubation time of 30 min. NP-DNA complexes with surface-adsorbed protein were prepared by adsorbing human serum albumin (HSA, 96 % fraction V, Sigma Aldrich Corp., MO, USA), to the nanoparticles, through simple incubation (several ratios tested). The

resulting suspension was mixed with an equal volume of 100 µL/mL luciferase plasmid solution during an incubation time of 30 min at room temperature.

Characterization of the Delivery Systems

Surface appearance: Cryo Scanning Electron Microscopy (CryoSEM) was performed on a FE-CryoSEM/EDS, JEOL JSM 6301F (CEMUP - Materials Centre of the University of Porto, Portugal). Particles were prepared as described above, acetone was evaporated with a nitrogen flux and a 48 hour dialysis accomplished. After the dialysis samples were treated with liquid nitrogen, fractured and then observed.

Size and Zeta potential measurements: Delsa™ Nano C particle analyzer (Beckman Coulter) was used to measure the particle size by Dynamic Light Scattering (DLS), and their zeta potential by electrophoretic light scattering (ELS). For the size, analyses were performed at 25°C and scattered light collected at a 165° angle. Particle suspensions were characterized in the production medium, after centrifugation and after the resuspension of the freeze dried particles.

An MPT-2 autotitrator coupled to a Zeta Sizer Nano ZS (Malvern Instruments, Ltd., Worcestershire, UK) was used to measure the zeta potential and intensity of the particles versus a wide range of pH values of the suspension medium. The nanoparticles, freeze-dried in the presence of trehalose were resuspended in water and placed in the sample tube connected to a clear disposable cuvette. The assay was performed automatically at 25°C and the titrants used were NaOH 0.25 M, HCl 0.25 M and HCl 0.01 M. pH ranged from 2 to 11 during approximately 6 hours, and the final volume of added titrants was approximately 0.5 mL.

Protein adsorption studies: Bovine serum albumin (BSA, 96 % fraction V), ovalbumin (98 %), myoglobin from equine skeletal muscle (95 %-100 %), α-casein (>70 %), lysozyme (≥ 80 %), lactalbumin from bovine milk (≈ 80 %) (Sigma Aldrich Corp., MO, USA) were incubated with fresh nanoparticles centrifuged and resuspended in phosphate buffer (PB) pH 7.4. Chitosan/PCL and PCL nanoparticles were used at a Protein: NP ratio of 1.5:1 and 1.6:1, respectively. The incubation was extended for 3 h maximum and at different times, aliquots of the particle suspension were centrifuged at 16000 x g for 30 min and the supernatant collected for non-bound protein quantification. Biocinchoninic acid (BCA) protein assay was performed in microplates (Pierce Chemical Company, Rockford, IL, USA).

The percentage of loading efficacy (% LE) and the percentage of loading capacity (% LC) of the nanoparticles was calculated using the following equations (eq.1 and eq. 2, respectively):

$$LE(\%)=\frac{(\text{total amount of protein}(\mu g/mL)-\text{non bound protein}(\mu g/mL))}{\text{total amount of protein}(\mu g/mL)}\times 100 \quad \text{(eq. 1)}$$

$$LC(\%)=\frac{(\text{total amount of protein}(\mu g/mL)-\text{non bound protein}(\mu g/mL))}{\text{weight of the particles}(\mu g/mL)}\times 100 \quad \text{(eq. 2)}$$

Cytotoxicity of the nanoparticles: A549 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were cultured at 37°C and 5 % CO_2, in Nutrient mixture F12 Ham (Sigma Aldrich Corp., MO, USA) with 10 % FBS supplemented with 1 % Pen Strep (Live Technologies Corporation, Paisley, UK) having a final pH of 7.2 to 7.4. Subcultures were performed by detaching the cells with trypsin (Live Technologies Corporation, Paisley, UK). Cytotoxicity assays were performed after 18 h incubation of 100 µL of a A549 cell suspension seeded in a 96-well plate at a density of 10^5 cells/mL. Serial dilutions of the nanoparticles, freeze-dried with 5 % of trehalose, were prepared in serum-free F12 Ham's at a concentration range between 0.29 µg/mL and 300 µg/mL. Prior to the addition of the nanoparticles, the medium was removed and 100 µL of new medium was added. 100 µL of each sample was added and allowed to incubate with the cells for 24 h, at 37°C and 5 % CO_2. After 24 h, an MTT cytotoxicity assay was performed (MTT reagent, Sigma Aldrich Corp., MO, USA). The relative cell viability (%) related to control (cells in culture medium without nanoparticles) was calculated by the following equation (eq. 3):

$$\%\text{cell viability}=\frac{\text{OD sample}(540nm)-\text{OD sample}(630nm)}{\text{OD control}(540nm)-\text{OD control}(630nm)}\times 100 \quad \text{(eq. 3)}$$

Cytotoxicity assays were performed also with complexes following the incubation time as for transfection assays (described below).

DNA complexation assay / Gel retardation assay: To evaluate the complexation of the DNA with the nanoparticles an electrophoresis in agarose gel was performed. Samples were diluted with PB pH 5.7 at a ratio of 1:4 and 10 µL of each resulting sample was added to 2 µL of a loading buffer containing bromophenol blue to monitor the run. 6 µL of each blend were placed in individual wells in a 1 % agarose gel, stained with 1 % ethidium bromide for the electrophoresis run (horizontal DNA electrophoresis System, Bio-Rad, Hercules, CA, USA). The electrophoresis was set to 45 min at 100 V. The control was pLuciferase solution at 12.5 µg/mL. Data analysis was performed in a UV transilluminator (UVITEC Cambridge, Cambridge, UK).

DNA protection assay /DNase I assay: Different DNA complex formulations were incubated with several concentrations of a DNaseI solution (Sigma Aldrich Corp., MO, USA) for 15 minutes at 37°C. DNase I was maintained in a buffer solution with 50 Mm Tris-HCL, 10 mM $MnCl_2$ and 50 µg/mL BSA. The reaction was stopped by using an EDTA 0.5 M solution (1 µL/unit of DNase I). Controls using inactivated DNase I were performed after its inactivation with EDTA, at the same theoretical concentrations for 15 minutes at 37°C. To evaluate DNase I activity on the DNA complexed with the particles, an electrophoresis in agarose gel was performed as described above.

***In vitro* uptake studies:** To perform uptake studies with simple Chitosan/PCL nanoparticles, chitosan was labeled with fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to a protocol described previously with some modifications [25]. Briefly, 35 mL of dehydrate methanol containing 25 mg of FITC was mixed with 25 mL of a 1 % w/v chitosan in 0.1 M of acetic acid and incubated for 3 hours, at room temperature in the dark. FITC labeled chitosan was then precipitated with 0.2 M NaOH to pH 10, and centrifuged for 30 minutes at 4500 x g. The resulting pellet was washed with a mixture of methanol and water (70:30, v/v) three times. Labeled chitosan was resuspended in 15 mL of 0.1 M acetic acid solution and stirred overnight. Polymer solution was dialyzed in 2.5 L of distilled water for 3 days under darkness, before freeze-drying. The resulting powder was used to prepare 0.1 % chitosan solution used in the nanoparticle production method as described above.

The formulations analyzed for uptake by the cells consisted of FITC-labeled nanoparticles, FITC-labeled nanoparticle-DNA complexes and FITC-labeled nanoparticles adsorbed with protein.

For flow cytometry studies A549 cells were seeded on glass coverslips on 48 well plates at a density of 5 x 10^4 cells/well and cultured at 37 °C in 5 % CO_2 for 48 hours. The medium was then replaced with serum free medium and cells were incubated with different formulations for 4 hours. Following the uptake period, medium containing nanoparticles

was removed and cells were washed and trypsinized with 50 µL of Trypsin-EDTA. The cells of six wells were collected into one tube and the medium replaced with 300 µL of PBS pH 7.4. Cells were kept at 4°C until analysis. 1.5 µL propidium iodide solution (PI) 50 µg/mL (Sigma Aldrich Corp., MO, USA) was added to the samples prior to analysis by an BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA). The mean fluorescence data for a population of 20000 cells were collected and results processed by CellQuestModfit LT software.

For confocal laser scanning microscopy (CLSM) studies, A549 cells were seeded on glass coverslips in 12 well plates at a density of 1.2 x 10^5 cells/well and cultured at 37°C in 5 % CO_2 overnight. After approximately 16 h incubation, the medium was replaced with serum free medium and cells were incubated with different formulations for 4 h. Following the uptake period, medium containing nanoparticles was removed, cells washed with phosphate buffer saline (PBS) pH 7.4 and fixed with 4 % paraformaldehyde in PBS for 15 min at 37°C. Plasma membrane and cell nucleus of the pre-fixed cells were labeled with image-It™ LIVE Plasma membrane and nuclear labeling kit (Live Technologies Corporation, Paisley, UK), according to manufacturer's instructions. Nucleus were stained with a cell permeable nucleic acid (Hoechst 33342), and their plasma membranes stained with cell impermeable Alexa Fluor 594 wheat germ agglutinin, that binds selectively to N-acetylglucosamine and N-acetylneuraminic (sialic) acid residues on the cell membrane [26]. After labeling, cells were washed twice with PBS and coverslips mounted on microscope slides with DAKO mounting medium, and examined under an inverted laser scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Oberkochen, Germany) equipped with imaging software (LSM 510 software, Carl Zeiss).

Transfection studies: Transfection studies were performed in order to assess the suitability of the complexes (DNA:Chitosan/PCL particles) to efficiently mediate gene transfer. A549 cells were seeded in a volume of 500 µL at a density of 5 x 10^4 cells/well on 48-well plates, and incubated for 48 hours, 37°C and with 5 % CO_2, prior to the transfection assay. After this period, the cells were adherent and the F12 Ham´s medium was replaced for serum free F12 medium or complete F12 medium. Different ratios of NP:HSA:DNA were prepared and added to cells based on the previous DNA complexation and protection assays, ensuring a volume containing 1 µg of pDNA per well. The incubation of complexes with the cells lasted for 4 hours at 37°C. A solution of pDNA was used as negative control. After incubation, the medium was replaced by F12 Ham's medium and cells were cultured for another 48 hours in the incubator to allow gene expression. To determine the transfection efficiency of the complexes, after the incubation time, the culture medium was removed, the cells washed with PBS pH 7.4 and the adherent cells lysed with a solution of 0.1 % Triton X-100 in PBS (100 µL/well). The resulting lysate was the centrifuged and the supernatant used to quantify the luciferase expression, using 50 µL, placed in a white 96-well plate. The samples in the 96-well plate were analyzed in aLmax II 384 Luminometer (Molecular Devices, Sunnyvale, CA, USA), where at 37°C, 100 µL of D-Luciferin sodium salt solution and 100 µL of ATP(Sigma Aldrich Corp., MO, USA) were added to the samples and immediately read for the luminescence emitted. To normalize the luminescence values, the total protein content of the samples was also measured from the resulting supernatant with the BCA protein assay described above. Luminescence values were expressed in Relative Light Units (RLU)/mg of protein present in the 50 µL of sample.

Statistical analysis

Results are expressed as mean values ± standard deviation (SD). Data analyses and determination of significance ($p<0.05$) were determined using SPSS software (IBM Corporation, New York, NY, USA) for protein adsorption studies and Graph Pad software (GraphPad Software, Inc., La Jolla, CA, USA) for all other data.

Results

Characteristics of the nanoparticles

The chitosan purification process did not induce any modification in the acetylation degree as confirmed by Fourier Transform Infrared Spectroscopy (FTIR) (data not shown).The precipitation technique allowed us to efficiently produce two different types of nanoparticles: Chitosan/PCL nanoparticles and PCL nanoparticles. To determine the efficiency of the process, after maturation and acetone evaporation, particles were centrifuged and successively freeze-dried. The yield of Chitosan/PCL nanoparticle preparation was 61 % and that of PCL nanoparticles 57 %. This yield was calculated considering only the initial mass of PCL used for preparation and the final mass of particles. Yields are relatively low due to the formation of small nanoparticle species that are eliminated during the purification process by centrifugation. Purification of particles by dialysis generally results in higher yields and should be preferred.

Particle size was measured right after preparation in the original medium and after the two particle isolation/purification steps tested, during the development and optimization of the particle preparation method. Method 1 - centrifugation and method 2- dialysis; in this latter case, the size was not measured immediately after the dialysis, but was measured after the subsequent step, the lyophilization. As clearly

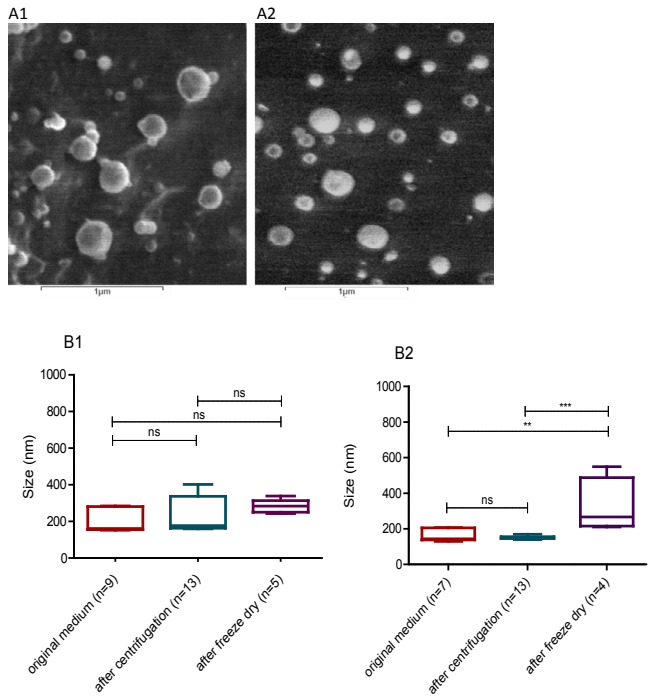

Figure 1: A) Nanoparticles observed by CryoSEM after a 48 hours dialysis against water. (A1) Chitosan/PCL nanoparticles; (A2) PCL nanoparticles. Length of scale bar: 1 µm. B) Particles size distribution on the original medium and after re-suspension in water after two different isolation procedures. Box and whiskers graphics showing maximum, minimum, median and 75th and 25th percentiles (ns: no statistical difference; ** $p<0.05$; *** $p<0.01$). (B1) Chitosan/PCL particles; (B2) PCL particles.

shown in Figure 1B, both particle isolation methods, applied in order to eliminate unreacted compounds, did not result in any alteration of the size of the Chitosan/PCL particles (Figure 1B1). Therefore, the sizes obtained during the three phases of the preparation of the particles were 199.5 ± 62.0 nm (PI 0.162), 235.7 ± 96.4 nm (PI 0.180), 282.5 ± 36.7 nm (PI 0.248), respectively for size measured in the original medium (immediately after production) and size measured after the isolation methods, centrifugation and dialysis. PCL particles were of similar size in the original medium and after centrifugation using a glycerol bed, showing values of 165.3 ± 35.1 nm (PI 0.152) and 151.2 ± 8.3 nm (PI 0.098), respectively. Nevertheless, after freeze-drying of the dialyzed PCL particles, the size increased significantly to a medium size of 323.0 ± 155.7 nm and a polydispersity index (PI) of 0.205, which also reflects poor reproducibility of the freeze-drying process. The results also indicate that the concentration of trehalose used appears to be adequate for the Chitosan/PCL particles and probably needs to be optimized for the PCL particles. Finally, it was also possible to conclude that the inclusion of chitosan into PCL particles did neither alter their size (statistic treatment not shown), nor their morphology. In fact, the morphology of the particles, evaluated by Cryo Scanning Electron Microscopy (Figure 1A), revealed small, round shaped nanoparticles for both chitosan/PCL and PCL formulations. The scale bar in the image allowed us to confirm sizes of around 250 nm at a low polydispersity index (PI).

Zeta potential depends on the ionic strength, pH and ion type of the medium in which particles are suspended [27]. Chitosan/PCL and PCL nanoparticles, when suspended in phosphate buffer (0.1 M; pH 7.4) showed similar values for zeta potential with no statistical significant differences (-10.1 ± 3.6 mV and -11.9 ± 3.1 mV, respectively; $p<0.05$).

In order to study surface properties under different conditions for both nanoparticle species a zeta potential titration over a pH range from 2 to 11 was performed. Zeta potential and size data were recorded. We observed two different zeta potential titration curves corresponding to each nanoparticle type. As illustrated in Figure 2, Chitosan/PCL nanoparticles are highly positively charged from pH 2 to pH 6,

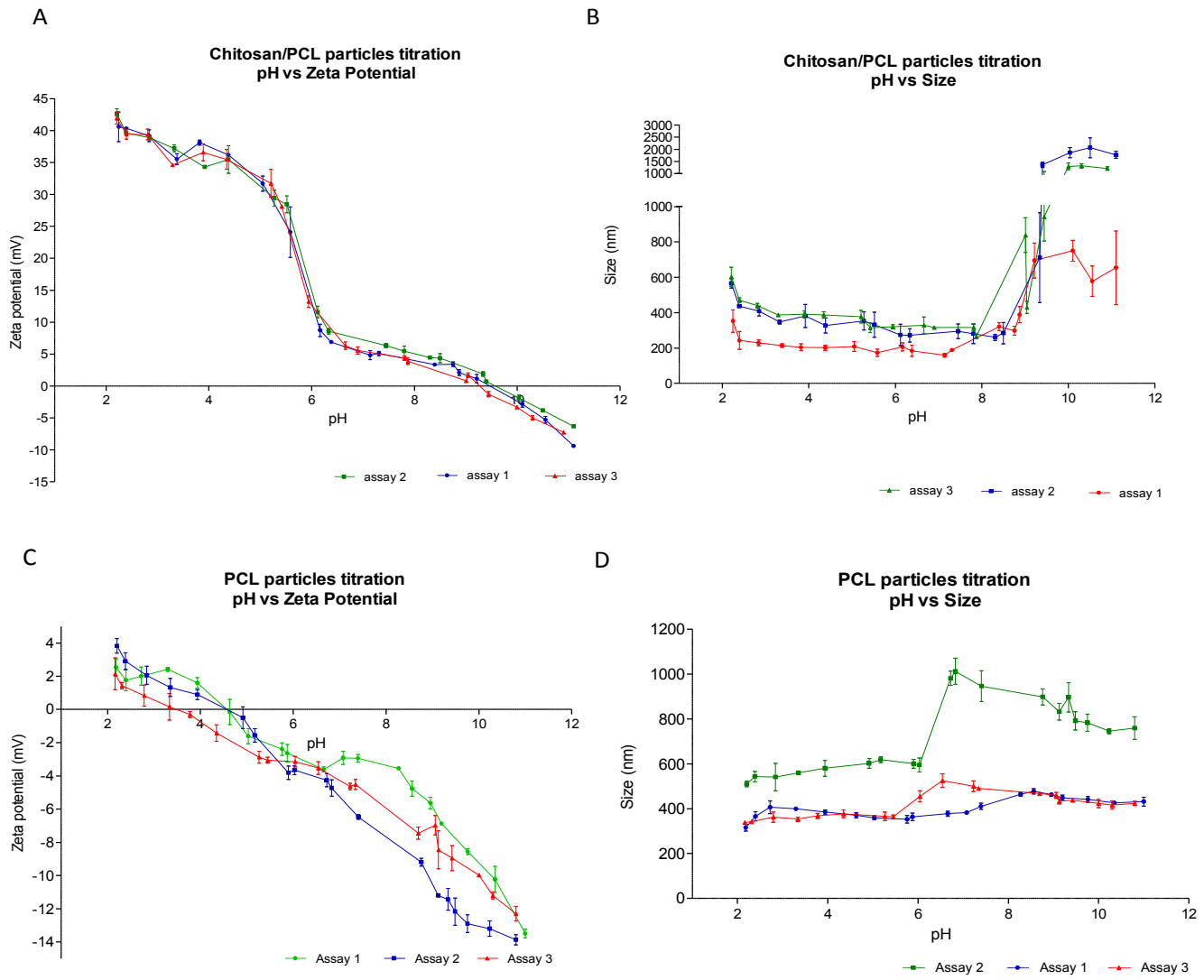

Figure 2: Titration of lyophilized Chitosan/PCL and PCL nanoparticles zeta potential and size according to the pH of the medium. pH changed from 2 to 11 following alkalization with NaOH 0,25 M. A) Chitosan/PCL nanoparticles zeta potential according to pH; B) Chitosan/PCL nanoparticles size according to pH; C) PCL nanoparticles zeta potential according to pH; D) PCL nanoparticles size according to pH.

slightly positive from pH 6 to pH 9 and negatively charged at higher pH values (Figure 2A). PCL nanoparticles presented slightly positive (almost zero) zeta potential values between pH values from 2 to 4.5 and negative values up to pH 11 (Figure 2C). These zeta potential titration curves were expected and indicated the presence of chitosan adsorbed to the surface of the Chitosan/PCL particles. In fact, the zeta potential titration curve of chitosan particles are similar (data not shown) at low pH values, so it is possible to conclude that chitosan is contributing to the positive charge of the particles observed at low pH values of the curve, which is absent in PCL nanoparticles. The zeta potential titration curves also allow the determination of the isoelectric point of the particles, corresponding to the pH value at which the zeta potential is zero. This property is especially important since it gives an indication at which pH value the particles are usually unstable (isoelectric point ± 2 pH units) [28]. Instable particles tend to flocculate or agglomerate, which may be incorrectly viewed as a simple increase of particle size. In fact, observing graphs B and D in Figure 2, a high increase in size occurred when the isoelectric point was achieved, approximately at pH 9 for the Chitosan/PCL particles and larger sizes were maintained for the pH values above that point. PCL nanoparticles also showed the same tendency, although the increase in size varied between batches, and was in general a minor variation. It may therefore be concluded that the incorporation of chitosan in Chitosan/PCL nanoparticles decreased particle stability at pH values above 9.

Both Delivery Systems Have High Protein Loading Capacities

The adsorption of antigens to the particle surface has been recognized as a very convenient method to load particles. One of the reasons is because the process can be performed in water or in buffer maintaining the bioactivity of the biomolecules. The inclusion of a hydrophilic polymer, chitosan, into PCL particles certainly modifies the surface properties of these particles and thus the adsorption of antigens. With the intention to study the differences between the two particle species, six model antigens (proteins) with different iso-electric points were used to perform the adsorption studies. A fixed concentration of particles was incubated with 500 µg/mL of protein solution resulting in a ratio of 1:1.5 Chitosan/PCL particles:protein and a ratio of 1:1.6 PCL particles:protein. The incubation time lasted for a maximum of 3 h, and loading efficacy and loading capacity of the particles were assessed at different times. The results are shown in Figures 3A and 3B, allowing a comparison between the two nanoparticle formulations.

Chitosan/PCL nanoparticles showed higher loading efficacies than PCL nanoparticles for 5 of the 6 proteins studied. The only protein that showed similar adsorption was myoglobin. Nevertheless, for Chitosan/PCL nanoparticles the loading efficacies were superior to 50 %, only with the exception of lysozyme, the protein with the highest isoelectric point. PCL nanoparticles presented loading efficacies of less than 50 % for almost all proteins assayed.

The normalization between loading efficacy values and the nanoparticle concentration for the chitosan/PCL and PCL formulations in each case allowed for a more realistic comparison between the two delivery systems. As the concentrations of both particle species are only slightly different, loading capacity results show the same profile as those for loading efficacy. Nevertheless, it is noteworthy that Chitosan/PCL nanoparticle loading capacity exceeded 100 %, confirming the superiority of these nanoparticles as a protein delivery system as compared to PCL nanoparticles.

Both Freeze-Dry Delivery Systems Have Low Cytotoxicity

The effects of freeze dried Chitosan/PCL and PCL nanoparticles on A549 cells were investigated by performing the MTT viability assay. To determine the concentrations suitable for subsequent *in vitro* studies at minimal toxicity, a serial dilution for each nanoparticle suspension was prepared. These nanoparticle formulations contain a high amount of trehalose to prevent agglomeration during the process of resuspension of the particles in the culture medium, however, previous cytotoxicity studies at high concentrations of trehalose were performed and no decrease in cell viability was observed (data not shown). As illustrated in Figure 4, Chitosan/PCL nanoparticles and PCL nanoparticles present a similar profile, with concentrations superior to 18.8 µg/mL per well resulting in significant toxicity (cellular viability below 50 %).

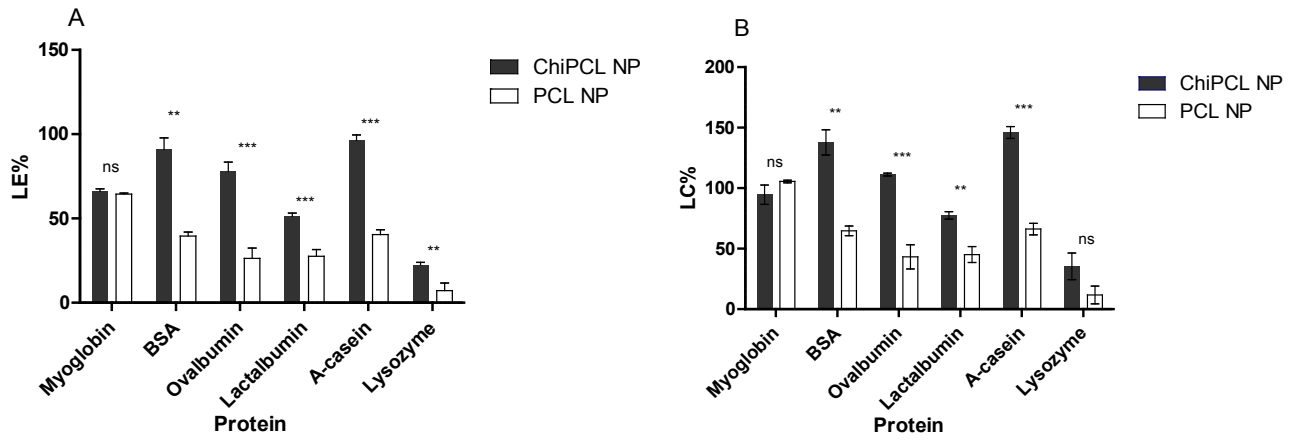

Figure 3: Loading efficacy (LE) and loading capacity (LC) results of freshly prepared nanoparticles, isolated by centrifugation.(A) Maximum LE (%) observed on ChiPCL (blue bars) and PCL (green bars) particles for different proteins; (B) Maximum LC (%) observed on ChiPCL (blue bars) and PCL (green bars) particles for different proteins (**p<0.01, ***p<0.001, ns p>0.05).

Only Chitosan/PCL Particles are able to form Complexes with DNA

As already stated, our main goal was not only to optimize particle preparation methods but also to evaluate the suitability of the particles to deliver both, DNA and protein antigens. For the studies with NP-DNA complexes, freeze-dried nanoparticles were preferred instead of freshly centrifuged ones due to increased stability of the former [29].

Therefore, the suitability of particles to efficiently complex DNA was assessed through an agarose electrophoresis assay. In the initial experiments Chitosan/PCL and PCL nanoparticles were compared, using large amounts of nanoparticles to complex lesser amounts of DNA. Immediately differences were found between the two formulations. Using ratios from 20:1 to 5:1 (NP:DNA) chitosan/PCL nanoparticles revealed total efficiency in complexing DNA, resulting in no free DNA in the agarose gel (Figure 5 A, wells a to d). PCL nanoparticles were not able to efficiently complex DNA, which is possible to infer from the migration of free DNA verified in wells f to i (Figure 5A).

In order to assess the suitability of particles to efficiently deliver pDNA and to promote gene transfer, the particles were modified. Human serum albumin (HSA) has been studied for its capacity of increasing transfection efficacy of lipoplexes and polyethyleneimine complexes [30], and therefore HSA was previously adsorbed to particles at a ratio of 1:1 (NP:HSA) immediately before the complexation with pDNA. The binding capacity of the modified nanoparticles was assessed as described for unmodified nanoparticles. In this case it was decided to use only lower NP:pDNA ratios in order to reduce potential toxic particle concentrations in future cell uptake studies. The results were similar to what was observed before. Chitosan/PCL nanoparticles adsorbed with HSA presented total complexation activity, except for the lowest NP: pDNA ratio (0.6:1) and in contrast, PCL particles confirmed once more their unsuitability to form complexes with DNA (Figure 5 B).

The successful NP: pDNA complexes presented here were very stable maintaining pDNA adsorbed as freshly formed complexes when incubated in different culture media for 1 h at 37°C (data not shown). Therefore, complexes prepared with chitosan/PCL nanoparticles were subsequently used in subsequent studies. In contrast, PCL nanoparticles, which showed their relative inferiority, were not used in the majority of the subsequent studies. So, considering these earliest comparative studies, it can be concluded that PCL nanoparticles did not present suitable properties for DNA delivery system and inclusion of chitosan into these particles have increased its capacity to complex DNA. Therefore, additional methods were performed with the aim to evaluate if complexes are able to protect DNA from nucleases and if, effectively, complexes would facilitate the transfection.

The protection achieved by complexation with chitosan/PCL particles was assessed by agarose gel electrophoresis. A NP:pDNA ratio of 2.5:1 was used for the complex preparation and protection of complexed DNA submitted to different concentrations of DNase I was evaluated. The highest concentrations of DNase I tested (lane a and c, Figure 6A) were able to degrade pDNA when complexed with the nanoparticles. Parallel experiments using pre-inactivated DNase I showed no degradation of pDNA. When using 1.25 x 10^{-2} U DNase I/µg DNA or lower concentrations the nanoparticulate system was able to efficiently protect the plasmid (lane g, i and k, Figure 6A).

Considering 1.25 ×10^{-2} U DNase I/µg DNA to be the highest concentration of DNase I that is not able to degrade DNA protected by Chitosan/PCL nanoparticles, a new protection assay was performed using a lower NP:pDNA ratio and HSA adsorbed to the surface of the

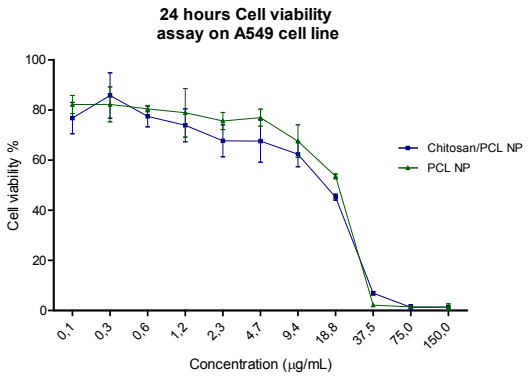

Figure 4: Cytotoxicity induced by different concentration of Chitosan/PCL (blue line) and PCL (green line) nanoparticles on A549 cell line. Nanoparticles concentration refers to the dilution on the well and the incubation was maintained for 24 hours. Error bars represent standard deviation of the mean, n=4.

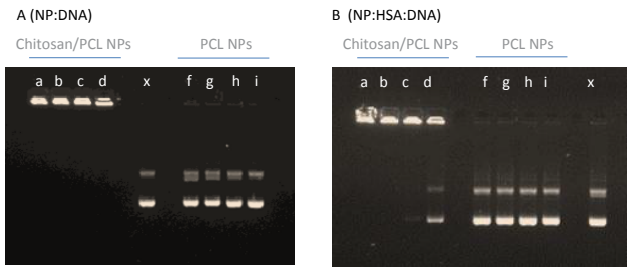

Figure 5: Electrophoresis in agarose gel illustrating the capability of plasmid DNA immobilization by Chitosan/PCL (lane a,b,c,d) and PCL nanoparticles (lane f,g,h,i). A) Different ratios Nanoparticles:DNA : a and f) 20:1 NP:pDNA; b and g) 15:1 NP:pDNA; c and h) 10:1 NP:pDNA; d and i) 5:1 NP:pDNA; control presented on lane x (free DNA plasmid). B) Different ratios Nanoparticles:HSA:DNA : a and x) 2.5:2.5:1 NP:HSA:pDNA; b and g) 1.875:1.875:1 NP:HSA:pDNA; c and h) 1.25:1.25:1 NP:HSA:pDNA; d and i) 0.6:0.6:1 NP:HSA:pDNA; control presented on lane x (free DNA plasmid).

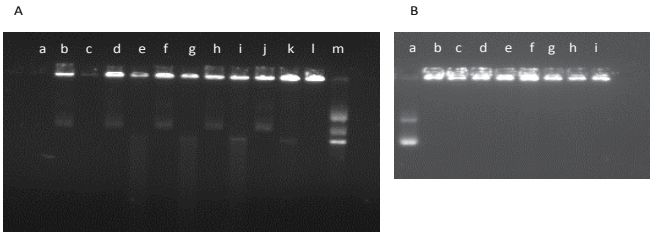

Figure 6: Electrophoresis in agarose gel illustrating the capability of plasmid DNA protection by Chitosan/PCL nanoparticles. A) Protection revealed by a ratio of 2.5:1 NP:pDNA when submitted to diferent concentrations of DNase I: a) 0.25 U DNase I/µg DNA; c) 3.125X10^{-2} U DNase I/µg DNA; e) 1.25X10^{-2} U DNase I/µg DNA; g) 6.25X10^{-3} U DNase I/µg DNA; i) 1.25X10^{-3} U DNase I/µg DNA; k) 1.25X10^{-4} U DNase I/µg DNA; b, d, f, h, j, l are the respective controls with inactivated DNase I). Control presented on lane m (naked DNA plasmid). B) Protection revealed when different ratios NP:HSA:pDNA are submitted to a concentration of 1.25X10^{-2} U DNase I/µg DNA: b) 1.25:0.1:1 NP:HSA:pDNA; d) 1.25:0.075:1 NP:HSA:pDNA; f) 1.25:0.05:1 NP:HSA:pDNA; h) 1.25:0.025:1 NP:HSA:pDNA; c, e, g, i are the respective controls with inactivated DNase I. Control presented on lane A (naked DNA plasmid).

particles. This assay revealed the potential of chitosan to deliver pDNA into the cells and all NP:HSA:pDNA ratios tested protected pDNA from degradation (Figure 6 B).

Enhanced uptake of chitosan/PCL NP by A549 cells

The particulate delivery systems should allow to concentrate and protect biomolecules against degradation during administration. This property is especially important when the administration is performed by one of the mucosal routes where physiological barriers restrict to the entrance of foreign species, like microorganisms, dust, allergens or particulate delivery systems. Moreover, particles potentially increase the cell internalization of the bioactive molecules, which is important for its function. In particular, protein antigens should be taken up by antigen presenting cells (APC's) to be processed and DNA vaccines to express the antigen. Therefore, our first studies to evaluate cell uptake and intracellular localization of the nanoparticles were performed using A549 cells and later visualized by confocal laser scanning microscopy (CLSM). The A549 cell line is a well-characterized human lung carcinoma cell line utilized for a variety of scientific studies, including respiratory immunotoxicity test, protein expression and apoptosis, etc and was the cell line available in our laboratory at the time of these preliminary comparative experiments. Images of individual cells (Figure 7A1 and 7A2) confirmed that chitosan/PCL particles were extensively internalized by A549 cells and were localized in the cytoplasm (Figure 7A1). In contrast, confocal cell images of PCL particle uptake studies showed (Figure 7A2) that PCL nanoparticles were only marginally internalized by these cells. In case of PCL particles were stained with bovine serum albumin-FITC by adsorption, as we previously observed that PCL particles are able to adsorb on its surface diverse model vaccines, although with a lower loading capacity when compared with the chitosan/PCL particles. However, we found that PCL particles are not capable of transporting protein into cells. This fact was decisive to finally conclude that PCL nanoparticles are of reduced interest as antigen delivery systems. Therefore, the quantitative analysis of particle internalization was made solely with Chitosan/PCL particles.

Flow cytometry analysis of particle uptake was performed in order to achieve quantifiable results of the internalization of the Chitosan/PCL nanoparticles. Nanoparticles loaded with protein and nanoparticles loaded with pDNA (Figure 7 B) were also used. Initially, non-loaded nanoparticles were tested at 4 different concentrations (1000, 500, 250 and 100 μg/mL). The uptake was successful in 98.5 ± 1.9 % and 95.6 ± 1.6 % of the cells for the 2 higher concentrations, respectively, and it decreased to 31.27 ± 5.3 % when concentration reached 250 μg/mL. At the same time, the cytotoxicity of the uptake was assessed with propidium iodide staining and it was verified that although the uptake of the 1000 and 500 μg/mL suspension had no statistical difference, the use of less nanoparticles decreased cytotoxicity from 75.0 ± 4.7 % to 44.5 ± 8.8 %.

The same study was performed with protein adsorbed at the surface of the nanoparticles, using a 1:1 ratio of NP:protein. The results for the formulations with 1000 and 500 μg/mL nanoparticles presented an uptake of 92.8 ± 6.1 % and 95.9 ± 1.9 % of the cells, respectively. When the concentration was 250 μg/mL, the uptake decreased to 63.1 ± 28.3 %, an increase over the non-loaded nanoparticles. The cytotoxicity results showed the same tendency as for non-loaded nanoparticles. Therefore, the percentage of cell viability is directly related to particle uptake.

When Chitosan/PCL nanoparticles were complexed with pDNA, the uptake decreased significantly. The nanoparticle concentrations tested were the same as before, and the amount of pDNA was constant, generating NP:DNA ratios of 1:0.1, 1:0.2, 1:0.4 and 1:1. The results showed an uptake of 33.4 ± 13.6 % for the highest concentration added to cells (1000 μg/mL) and almost no uptake for the others. Most probably, in the first group we also had some NPs, stained with fluorescein, not associated with DNA. So, we must not exclude that some particles that entered into cell were the above mentioned particles. Consequently, we can conclude that chitosan/PCL nanoparticles have excellent characteristics to be taken up by cells and to transport model antigens into cells. However, same particles seem do not have the same ability when associated with DNA. We believed that this is because DNA confers a negative charge to particles limiting the interaction of the delivery system with the cell membrane and consequently, their internalization.

Modest improvement of transfection by association of DNA with particles

The results obtained with PCL nanoparticles (complexes NP:DNA) in the transfection studies were disappointing (data not shown), with measurements similar to the ones obtained with naked DNA. These results were anticipated following the results obtained previously. Therefore, the results shown in this report correspond to transfection studies performed with the more promising complexes DNA:chitosan/PCL nanoparticles selected in previous studies.

The objective of the experiment was to study the influence of different NP:DNA ratios, the presence of HSA and its different concentrations in the complexes, and the presence of fetal bovine serum at a concentration of 10 % in Ham's F12 culture medium. The results illustrated in Figure 8 are representative of the best results obtained until now. As predictable, naked DNA included as a negative control on the experiments did not produce any luminescence signal. The association of the nanoparticles with DNA improved the transfection rates with the best results observed with NP: DNA ratios of 2.5:1 and 1.25:1 in serum-free cell medium. In order to improve these results, increasing amounts of human serum albumin were added prior to complex formation with DNA. The results are shown in Figure 8. From the analysis of the results we concluded that the inclusion of the protein in complexes did lead to better results under serum free medium conditions.

Comparing the results with the positive control we can conclude that complexes, able to protect DNA, allowed cell transfection, however, at rates lower than observed for the positive control. Therefore, further experiments are in progress to definitively evaluate the utility of the chitosan-PCL particles as a gene delivery vector.

Discussion

The differences observed in our transfection studies are partially in accordance with published data. When nanoparticle-DNA complexes are incubated with cells in a serum containing culture medium, transgene expression tends to be higher than under serum free conditions. On the other hand, nanoparticle-DNA complexes with HSA showed slightly better transfection results when serum free conditions were established.

In order to explore the causes that led to low transfection, viability assays with Chitosan/PCL nanoparticle-DNA complexes were performed. When applying the same concentrations and conditions as for the transfection studies the results showed a percentage of cell survival of around 70 % (data not shown). Considering the 48 h of the transfection assay, including the 4 h incubation with the particles, this

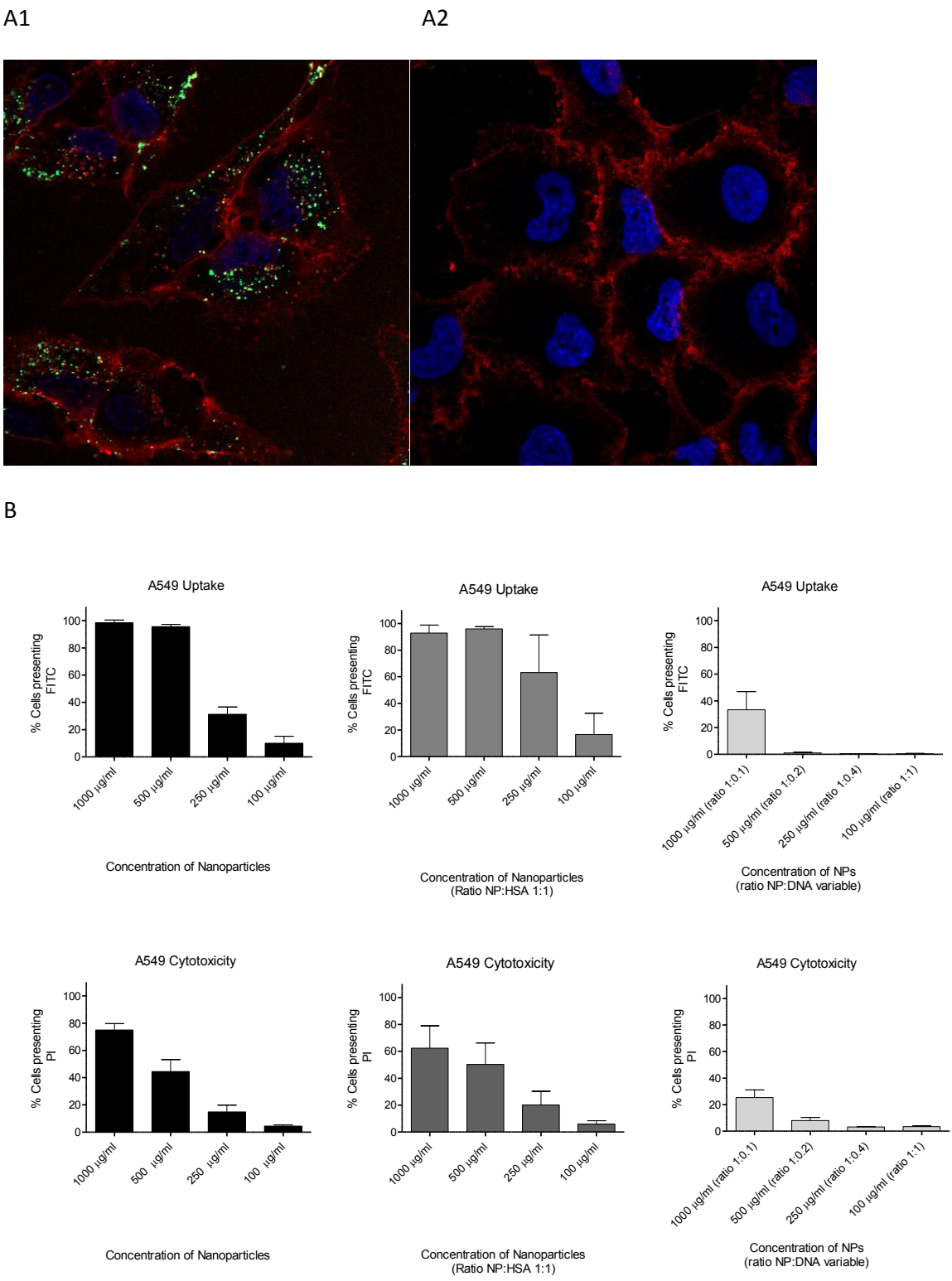

Figure 7: A) Uptake and cytotoxicity studies of NPs on A549 cells after 4 hours incubation. Cells were stained with Alexa Fluor 594 (blue fluorescence – nucleus) and with Hoechst 33342 (red fluorescence – plasmatic membrane). Images representing two different assays performed in duplicate. A1) Chitosan/PCL nanoparticles, produced with Chitosan covalently linked to FITC. A2) PCL nanoparticles adsorbed with BSA covalently linked to FITC. B) Flow cytometry analysis of the uptake (FITC) and cytotoxicity (PI) on A549 cells after 4 hours incubation. 1) Simple Chitosan/PCL nanoparticles. 2) Chitosan/PCL nanoparticles adsorbed with HSA. 3) Chitosan/PCL nanoparticles complexed with pDNA. Variations on the concentration of nanoparticles ranged from 1000 to 100 µg/mL. (Error bars represent standard deviation of the mean, n=3).

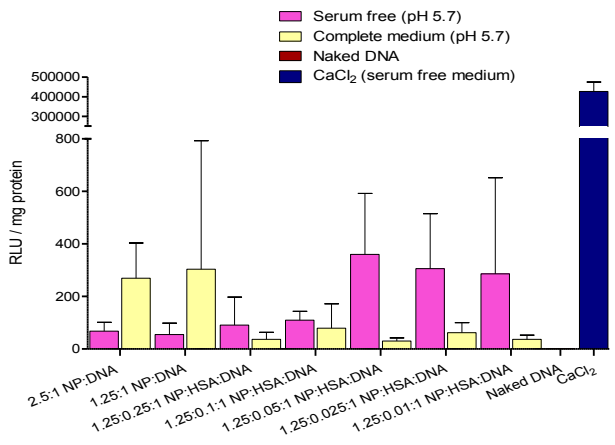

Figure 8: Transfection efficiency of Chitosan/PCL nanoparticles. After an exhausting study evaluating the ratio NP:DNA that led to better transfection results 2.5:1 and 1.25:1 where selected. Trying to improve those results HSA was adsorbed to the surface of the nanoparticles at different concentrations previous to the complexation with DNA at the ratio 1.25:1 NP:DNA. The influence of the serum in the transfection media was also assessed. Results wer compared with naked DNA and CaCl2 (positive control). The results are representative of 4 different assays for NP:DNA and 2 different assays for NP:HSA:DNA . Transfection is presented in Relative light units / µg protein (Error bars represent standard deviation of the mean, n=3).

result was expected, and the observed cytotoxicity is not considered to be the cause for low transfection results.

As was reported for the uptake studies, lower zeta potential values may have caused low internalization rates of the complexes. In addition, the increasing size of the complexes compared to non-loaded nanoparticles may play a role. In fact, the complexes, when suspended in F12 Ham's medium (in the presence or absence of serum), presented sizes of around 1 µm, a large size for the complex to be taken up extensively by endocytosis [31]. The complexes had a low nanoparticles:DNA ratio, therefore the contribution of the negative charge of the DNA present at the nanoparticle surface was superior to the positive charges of chitosan, which lead to an overall negative zeta potential for the complexes. When suspended in F12 Ham's medium, complexes presented zeta potentials below –30 mV. The electrostatic interaction between the complexes and the cell membrane of target cells is not favorable under these conditions.

Uptake studies revealed the internalization of complexes containing luciferase plasmid and the successive expression of the reporter gene. Published data refer that the transfection efficiency achieved by DNA/chitosan complexes depend on several factors, such as the degree of deacetylation (DDA) and molecular weight (MW) of chitosan, pH of the medium, protein interactions, charge ratio of chitosan to DNA, cell type, nanoparticle size, interactions with cells, preparation techniques of chitosan/nucleic acid particles and routes of administration [20,32]. Nevertheless, although there is a consensus on the multitude of factors, there is controversy on which affect the practical results. For instance when we consider the MW, Sato et al. [33] conclude that chitosan of a molecular weight of 10 KDa to 50 KDa is an excellent gene transfer reagent as compared to higher MW chitosan. Lavertu et al.[34] found a correlation between MW and DDA, and concluded that maximum transgene expression occurred at DDA:MW values that run along a diagonal from high DDA/low MW to low DDA/high MW. The interaction with proteins can also have several interpretations as far as transfection efficiency is concerned. The presence of HSA in lipoplexes can be associated with the binding to nonspecific cell receptors, which mediate endocytosis, resulting in transfection enhancement by facilitating the escape of DNA from the endolysosomal pathway [35]. On the other hand, transfection efficiency can be decreased due to the interaction of the DNA complexes with serum proteins present in the culture medium [35,36]. Due to this phenomenon, *in vitro* transfection studies are frequently performed under serum-free conditions, which is not the best model for results extrapolation and also cause the serum deprived cells to grow slower, diminishing transgene expression [32,35]. Some promising results, however, have been published referring that chitosan can be used for transfection studies in the presence of serum once chitosan does not experience the inhibitory effects generated by the medium serum, and rather benefits from the increased cell metabolism [32,33].

According to our studies, poly-ε-caprolactone (PCL) nanoparticles are not a good gene delivery system. The addition of a cationic polymer, such as chitosan to PCL appears to be a viable strategy; however, additional work has to be done in order to increase transfection rates.

To our knowledge, this is the first time that the preparation of DNA:Chitosan/poly-ε-caprolactone nanoparticles are described and evaluated as a gene delivery system. A study published by Jochen [37] describes the preparation of poly-ε-caprolactone nanoparticles by an emulsion-diffusion-evaporation method using a blend of poly-(vinyl alcohol) and trimethylchitosans with varying degrees of quaternization. The inclusion of the two stabilizers forced authors to adopt a more complex and time-consuming method in order to obtain particles. Particles obtained by these authors appeared to have size and zeta potential values similar to the ones detected in this study. The transfection results showed that the complexes or the conditions of the transfection assay needed to be improved. Complexes were superior to naked DNA, however, inferior to the transfection results obtained by the positive control and not comparable with our results since they were presented as fluorescence units.

Conclusions

Chitosan/PCL nanoparticles presented advantages over PCL nanoparticles in terms of protein loading and pDNA complexation. These particles were shown to be stable in a freeze-dried formulation, suitable for antigen loading, antigen transport and delivery to epithelial cells *in vitro*. These new nanoparticles may be considered as an antigen delivery system in terms of stability of the nanoparticles and antigen adsorption efficiency, so work is scheduled in our laboratory aiming at *in vivo* evaluation of chitosan/PCL nanoparticles as a vaccine (recombinant protein/antigen) adjuvant.

Although DNA: chitosan/PCL NPs were shown to be very stable and capable to protect DNA from nucleases, more *in vitro* work has to be done before starting immunization studies using these nanoparticles as DNA vaccine delivery systems.

Acknowledgments

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