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Preparation and characterization of chitosan nanoparticles for gene delivery

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Master degree in Biotechnology

Abstract: The possibility of using gene-based therapy for treatment of both genetic and acquired diseases such as infections, degenerative disorders or cancer, has grown exponentially, mostly due to the development of several methods for delivering genes to mammalian cells (viral and non-viral vectors).

In this study, chitosan polymer was used as non-viral vector for delivery of plasmid pVAX1GFP to mammalian Chinese hamster ovary cell line, being the final goal the expression of green fluorescent protein in the nucleus of these cells.

Chitosan and plasmid DNA nanoparticles were prepared by self-assembly at amine to phosphate ratios of 5, 10, 20, 50 and 70, although complexation was not totally achieved for lower ones (N/P=5 and 10). Different molecular weight (60–220kDa) and glycol chitosans were tested, in order to determine which one provides the highest transfection efficiency.

Two lipids, a cholesterol derivate and a type of lecithin, were used to modify chitosan molecule in order to determine if their presence enhances the delivery process.

Characterization of nanoparticles size (102 to 2094nm), zeta-potential (-36 to 40mV) and polydispersion index (0.21 to 0.89) was performed in order to select the most suitable formulations for cell delivery. Also, sonication of chitosan particles and medium filtration were important processes to obtain stable and homogeneous nanoparticles, features mostly achieved in low molecular weight chitosan complexes (60-120kDa).

High transfection efficiencies were only obtained in positive control (pDNA + lipofectamine).

Keywords: gene therapy; non-viral vectors; chitosan; nanoparticles; oral delivery

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Introduction

The use of nanoparticles as non-viral delivery system for bioactive components has gained wide attention in recent years due to their small size, revolutionary controlled release, protection to the bioactive compounds against environmental stress, for instance the adverse gastrointestinal (GI) environment, enabling easy transport and internalization through intestinal epithelial cells, in turn improving the pharmacokinetics, bioavailability and therapeutic efficacy after administration (Mukhopadhyay *et al.* 2012). Another huge advantage of using nanoparticles in this area is the fact that they can be administered orally, a non-invasive procedure, thus avoiding the use of needles that must be kept sterile and administered by trained personnel, which in some cases is not easy, especially in developing countries. These particles can be synthesized by a variety of methods, most of them using chemicals deemed to be non-biocompatible and, therefore, not suitable for use in medical or food applications. To overcome the problem associated with toxicity issues of using non-biocompatible chemicals, complexation of polyelectrolytes (PEs) through the "bottom-up" approach is perhaps one of the simplest methods to create nanoparticles without the need to use any organic solvents or cross-linking agents. This method allows nanostructures to be generated from individual atoms or molecules that are capable of self-assembly (Chen *et al.,* 2011). Self-assembly of chitosan and plasmid DNA pVAX1GFP was herein studied. Chitosan is a linear, natural copolymer of β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine (NAG) whose molecular structure comprises a linear backbone linked through glycosidic bonds. The basic amine groups of this polysaccharide can be protonated and, thus, positively charged in most physiological fluids. This characteristic allows the interaction with anionic compounds, such as pDNA, through electrostatic interactions and the positively charged nanoparticles formed are able to interact with the negatively charged mammalian cell membrane, thus enhancing the pDNA delivery process. Also, chitosan is widely used as non-viral vector due to its biodegradability, bioavailability, mucoadhesion and antibacterial properties (Kim *et al*., 2007). Chitosan chemical modifications with two different lipids, a cholesterol derivative and a modified type of lecithin, were also tested in order to determine if their presence enhance the transfection process, since interaction with cell membrane should be facilitated (Channarong *et al*., 2011). Several tests were performed with chitosan particles in suspension, before self-assembly with previously purified and concentrated pVAX1GFP, to determine their diameter, net charge and time stability. The effect of sonication, buffer filtration and sample concentration was herein studied. Diameter, net charge (zeta potential), polydispersion index (Pdi) and time stability of self-assembled nanoparticles were also assessed to determine which nanoparticles were suitable for cell delivery. Small diameters, positive zeta potential values, low Pdi values (high homogeneity) and time stable nanoparticles were then used in transfection assays with Chinese Hamster Ovary (CHO) cells. Several transfection parameters were finally studied, such as transfection and incubation periods and cell confluency percentage, in order to determine their effect on green fluorescent protein expression in cell nucleus.

Materials and Methods

Materials

High purity chitosans with different molecular weights (low molecular weight (LMW) 60–120kDa); medium molecular weight (MMW) 110–150kDa); high molecular weight (HMW) 140–220kDa) as well as high purity glycol chitosan, chitosan oligosaccharide lactate (COL) (≈ 5kDa), cholesteryl chloroformate (CHOL) (97%) and L-A-lysophosphatidylcholine type 1 powder (modified lecithin ML) were purchased from Sigma-Aldrich® (St. Louis, USA). COL and CHOL were used as received.

Plasmid DNA replication, purification and concentration

The plasmid vector pVAX1GFP having 3697 bp was used (Azzoni *et al.* 2007). Cells were cultured in 2000mL Erlenmeyer flasks at 37ºC and 250 rpm with 250 µL of kanamycin stock solution and the cell culture progressed for approximately 6h before the stationary growth phase was reached ($O.D._{600nm}$ ≈4). Plasmid recovery was achieved by alkaline lysis, followed by a precipitation step with 16.8mL of 100% (v/v) 2-propanol, per 250mL of cultured cells (Raiado-Pereira *et al.* 2013). pVAX1GFP purification was performed by hydrophobic interaction phenyl membrane chromatography (Sartorius AG) and supercoiled fractions were collected. After purification, desalting and concentration steps were made by centrifugation of pDNA solution, during 5min at 4000rpm and 4ºC in a 2mL Amicon® Ultra centrifugal filters (Milipore, Ireland) bearing a 50kDa Ultracel® cellulose membrane, to a final volume of 0.3mL. Concentration was then measured on NanoVuePlus equipment (General Electric Healthcare, UK). Typical purified pDNA concentration values obtained were around 250ng/µL.

Preparation of chitosan/pDNA nanoparticles

5mg of each chitosan (LMW, MMW, HMW and glycol) were dissolved in 5mL filtered/non-filtered 50mM acetic acid solution with pH ≈ 3.0, overnight at 50ºC in a hybridization oven/shaker (General Electric Healthcare, UK) and were/weren't sonicated during 5min at 50W with a pulse of 5sec and 10sec between pulses, in a sonifier sonoplus (Bandelin, Berlin Germany). The goal was to study the effect of both filtration and sonication in chitosan particles properties (diameter size and zeta potential) as well as on their stability with time. Self-assembly of chitosan nanoparticles with pVAX1GFP was made by simple and quick mixing of both solutions in 1.5mL test tubes, followed by 30s vortex and incubation for 30min at room temperature to enhance electrostatic interactions between the polyelectrolytes. The final volume of the mixture in each preparation was limited to below 500µL, in order to yield uniform nanoparticles. Different chitosan amine to pDNA phosphate charge ratios (N/P ratios = 5, 10, 20, 50 and 70) were tested and the pDNA volumes added were fully dependent on those ratios, as well as on pDNA concentrations, fixed at 75ng/µL. The chitosan volume was fixed in 100µL.

Preparation of chitosan/cholesterol-derivative/pDNA nanoparticles

The procedure of Maity and Jana, 2011 was followed. 60mg (0.012mM) of COL was dissolved in 2 mL of dimethylsulfoxide (DMSO) at room temperature with gentle mixing. Then triethylamine solution (120µL of triethylamine dissolved in 0.5mL of dichloromethane) was added and stirred for 30min at room temperature. Next, 17mg (≈0.038mM) of CHOL were dissolved in 0.5mL of dichloromethane and added to it for covalent conjugation and the reaction was continued for 3 days at room temperature with gentle stirring. Product was precipitated by centrifugation (10min, room temperature, 4000rpm) and washed with dichloromethane 4 to 5 times, with centrifugation steps (20min, room temperature, 4000rpm) between each wash step. The dried product was then dispersed in water by sonication (5min, 50W, 5s pulse, 10s between pulses) and the pH was adjusted to 3.0 with HCl 1M buffer solution. The dissolution of conjugate was obtained by placing it overnight at 50ºC in a hybridizer oven with gentle stirring. The solubilized conjugate was then placed at 4ºC until characterization and complexation with pVAX1GFP. Complexation assays of chitosan-cholesterol conjugate with the referred pDNA vector were performed as previously stated for the other chitosans, except that only N/P ratios of 10 and 50 were tested, before their characterization in Zetasizer Nano equipment.

Preparation of LMW chitosan/lecithin/pDNA nanoparticles

Both L-A-Lysophosphatidylcholine type 1 powder (modified lecithin ML) and LMW chitosan were separately dissolved in a previously filtered, with 0,45µm membrane, 50mM acetic acid solution with pH ≈ 3.0, to a final concentration of 1mg/mL, overnight, at 50ºC and with gentle stirring in a hybridizer oven. The reaction of positively charged chitosan with anionic ML was performed by simple

mixing the two solutions in a molar mixing ratio of 4.9 chitosans per ML molecule, followed by 30s vortex and incubation overnight at room temperature, to promote electrostatic interactions between the polyelectrolytes. The complexation of LMW chitosan/ML conjugate with pVAX1GFP was performed as previously stated for the other chitosans, except that only N/P ratios of 10 and 50 were tested, before their characterization in Zetasizer Nano equipment (Chuah *et al*., 2009)

Diameter, zeta potential and polydispersion index characterization of prepared nanoparticles

The size and zeta potential of chitosan nanoparticles and of all the formulations tested were measured in a Zetasizer Nano ZS (Malvern, UK). Particle size measurements were made in disposable cuvettes at 37°C to mimic transfection conditions, by non-invasive back scatter, with dynamic light scattering detected at an angle of 173°. One mL samples were used and 1:10 dilutions were performed (900µL buffer + 100µL sample), to avoid multiple scattering phenomenon. In chitosan suspensions the buffer used was 50mM acetic acid pH ≈ 3.0 and for complex preparations a mixture of 50mM acetic acid pH ≈ 3.0 and 10mM Tris-HCl pH ≈ 8.0 was used in the same volume ratio of chitosan per pDNA molecule (N/P ratio dependent). The effective hydrodynamic diameter was calculated from the diffusion coefficient by the Stokes-Einstein equation using the method of cumulants. Zeta potential measurements were also performed at 37°C using a combination of laser Doppler velocimetry and phase analysis light scattering (PALS). The measured electrophoretic mobilities were converted into zeta potential values using the Smoluchowski approximation. Zeta potential and hydrodynamic diameters are expressed as the mean standard deviation of at least two independent measurements (Zetasizer Nano Series User Manual, 2004).

In vitro transfection of CHO cells with prepared nanoparticles

In vitro expansion of CHO cells was performed in a T-75 culture flask with 10 mL of Dulbecco´s Modified Eagle Medium (GIBCO High Glucose, +Pyruvate, +Glutamine, -HCO3-) supplemented with 10% Fetal Bovine Serum (FBS from GIBCO, heat inactivated) and 1% penicillin and streptomycin (PenStrep from GIBCO). CHO cells were cultured at a concentration of $1.5x10^6$ cells/mL and incubated in 5% CO2 at 37ºC and seeded 24 h prior to transfection into a 24-well tissue culture plate at a density of $\approx 10^5$ cells/mL, in order to obtain a confluence of 80-90% on the day of transfection. At the time of transfection, cells were washed with 500 µL of PBS, to remove dead ones and toxins. The amount of polyplexes, which is dependent on N/P ratio tested, was added to each well. DMEM without FBS and antibiotics was added to a final volume of 500µL and incubated with the cells for a certain period of time (1h, 3h or 6h). The transfection experiments were performed in duplicate. Cells transfected with Lipofectamine 2000™-DNA complexes were used as positive controls of the transfection. The transfection efficiency was evaluated using Flow Cytometry analysis after 24h and 48h of transgene expression. Cells were washed with 500 µl PBS and trypsinized for 5 min at 37ºC. Complete medium was added to stop the action of trypsin and the cells were centrifuged (1500g, 25ºC, 5min). Supernatant was discarded and cell pellet was carefully washed with a small amount of PBS, to remove any traces of trypsin. Pellet was then resuspended in 1mL of ice-cold PBS supplemented with 4% FBS and kept on ice until analysis. The equipment used was a FACScan Scalibur (Becton-Dickinson, NJ, USA), which recorded the forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1) in each run. Data were analyzed and green fluorescence intensity corresponding to GFP expression level was generated with CellQuest Pro Software © (Becton Dickinson, NJ, USA).

Results and Discussion

Characterization of chitosan particles in acidic solution

Effect of chitosan sonication (C + D) and buffer filtration (E + F) on diameter and zeta potential values and stability of different chitosan molecules (LMW, MMW, HMW and Glycol) in 50mM acetic

acid pH ≈ 3.0 were studied in order to determine which were suitable for complexation with pVAX1GFP.

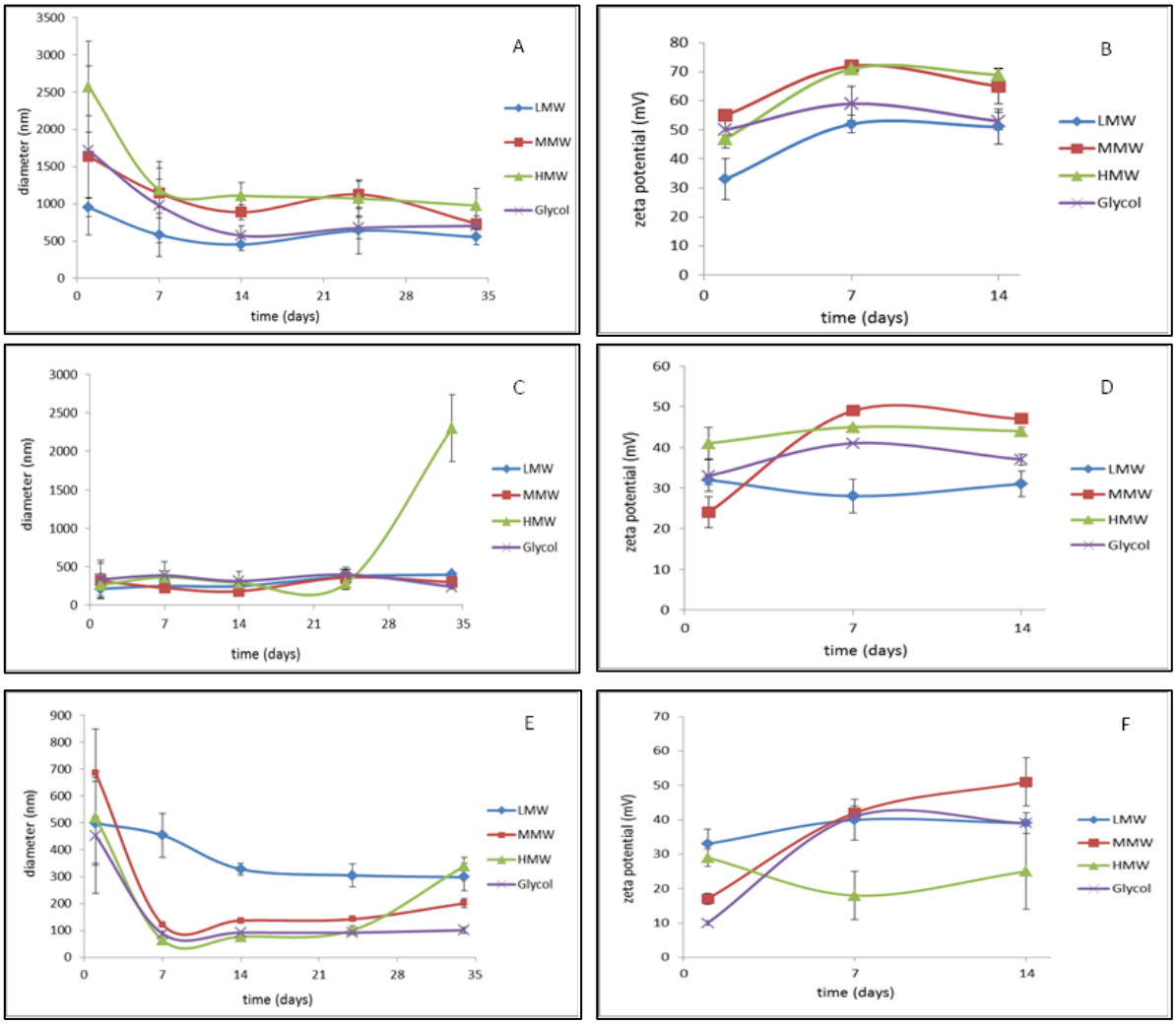

Figure 1 – Effect of sonication (C+D) and buffer filtration (E+F) on chitosan particles diameter (A+C+E) and zeta potential (B+D+F) values and time stability.

Analysing Figure 1, it is notorious that sonication plays an important role in physical stability of chitosan particles in suspension. Both diameter and zeta potential values of sonicated sample (C and D) show less oscillation along time, when compared to those values of non-sonicated sample (A and B). This is due to the application of ultrasound energies that promotes a shear on chitosan molecule, disperse it in the surrounding medium, thus avoiding aggregation or sedimentation phenomena, that would increase their diameter. However, the diameter of high molecular weight chitosan particles increases almost 10-fold at 34 days after preparation (Figure 1 C). The stability of these particles is affected by aggregation phenomenon that starts to occur about one month after their preparation and bioavailability of those same particles is then reduced (Floris *et al.,* 2013). The diameter stability of LMW, MMW and glycol chitosan particles was only obtained 14 days after their preparation, while for HMW chitosan particles this stability was not achieved as it might be noticed by diameter oscillation represented in Figure 1 E. Stability of LMW chitosan particles in acetic acid buffer showed to be the one that is less influenced by buffer filtration process, probably due to the lower number of repeated units (n) in chitosan molecules. Filtration of buffer solution in which the particles will be suspended is of extreme importance for their physical stability along time, because the presence of dust residues negatively affects the DLS and EM measurements.

Characterization of chitosan/pDNA nanoparticles

Suitable chitosan particles in solution were then complexed with pVAX1GFP. Regarding molecular weight, LMW chitosan was the one that showed smaller diameters and higher diameter and zeta potential stability along time, important parameters to achieve an efficient transfection process. Glycol chitosan, which showed small diameters and relatively high stability along time, was also chosen to determine if this chemical modification in chitosan structure had any influence on complexation efficiency. HMW chitosan was also tested.

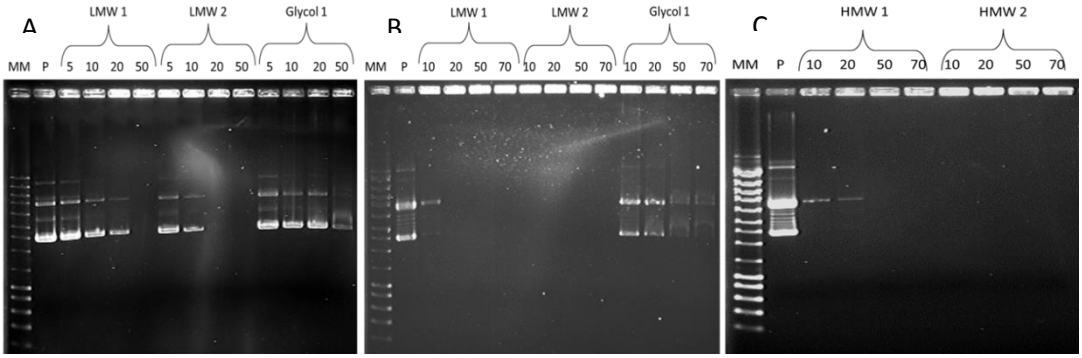

Figure 2 - 1% agarose gel of polyplexes. 2µL of loading buffer were added to 10µL of sample. Electrophoresis ran for 1h30 at 120V and ethidium bromide was used to stain the samples. MM – molecular marker NZYladderIII (6µL); P – pVAX1GFP (negative control); 5, 10, 20, 50 and 70 are the N/P ratios tested; LMW1 – low molecular weight chitosan sonicated; LMW 2 – low molecular weight chitosan non-sonicated Glycol1 – glycol chitosan sonicated; HMW1 – high molecular weight chitosan sonicated; HMW2 – high molecular weight chitosan non-sonicated. A – non-freshly prepared chitosan particles in suspension; B and C – freshly prepared chitosan particles in suspension

Freshly prepared chitosan particles in suspension give rise to higher complexation efficiency with pDNA, since no aggregates were formed yet. This is confirmed by pDNA migration patterns in gels A and B. However, in glycol chitosan this complexation was not 100% achieved for every N/P ratios tested, because additional OH groups in chitosan molecule probably interfere and make the interaction between pDNA and this natural cationic polymer more difficult. Regarding HMW chitosan complexes, the complexation efficiency with pDNA is almost total, meaning that the molecular weight of chitosan does not have much influence on this efficiency.

Diameter and zeta potential stability of chitosan/pDNA nanoparticles was also evaluated for 3 days (Figure 3).

Analysing LMW1 chitosan complexes diameter and zeta potential stability was not achieved only in N/P 50 polyplexes, which indicates that in these nanoparticles the physical stability is not linear with the N/P ratio, since N/P 10, 20 and 70 showed to be relatively stable in 50mM acetic acid pH 3.0 plus 10mM Tris-HCl pH 8.0 buffer. Regarding LMW2 chitosan polyplexes diameter stability was achieved in all the tested nanoparticles, although only N/P 20 and 50 showed zeta potential stability during the 3 days of measurement.

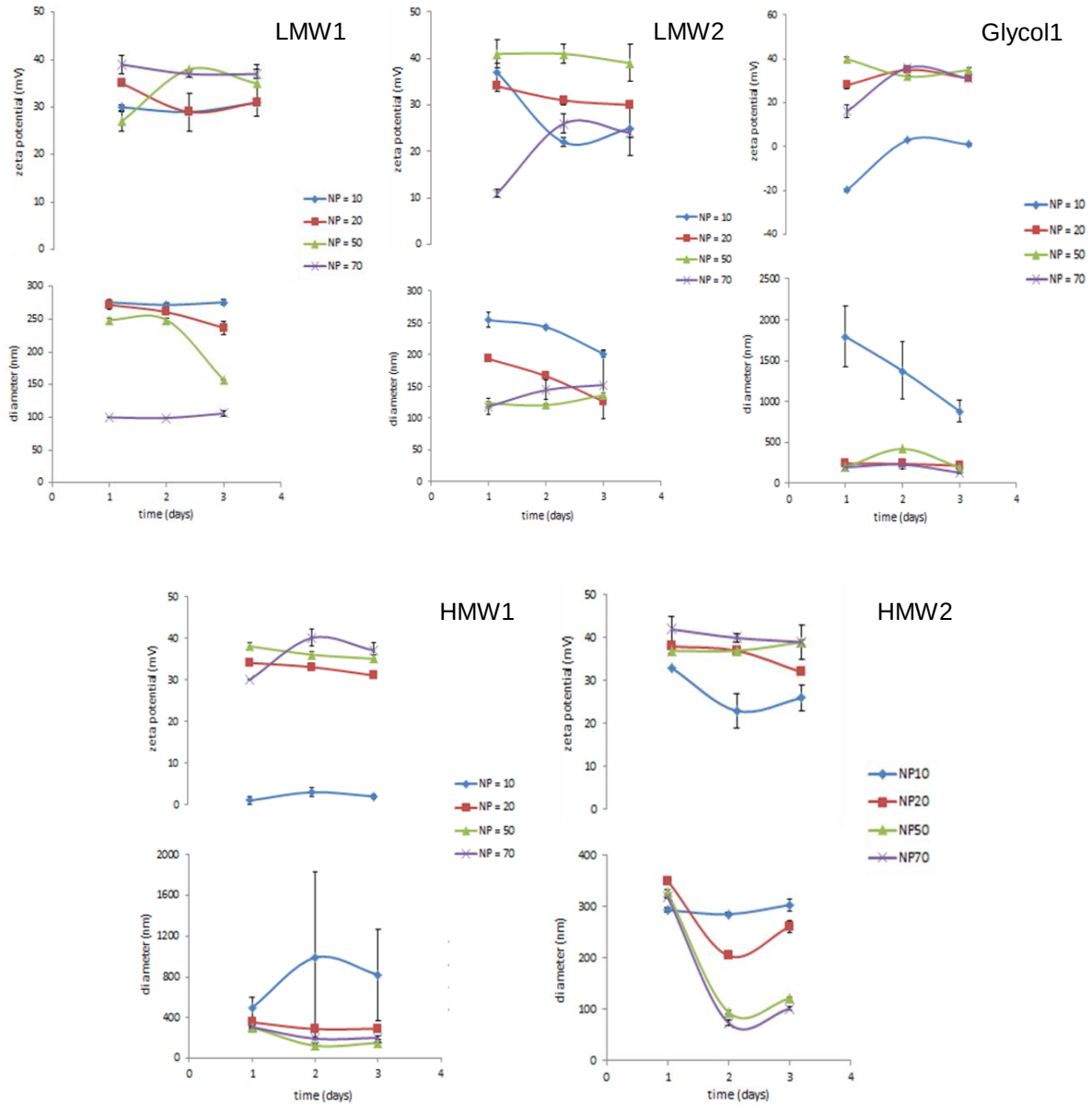

Figure 3 – Diameter and zeta potential stability of different chitosan molecules complexed with pVAX1GFP at different amine to phosphate ratios (N/P = 10, 20, 50 and 70). Mean values of at least two measurements. Measurements performed for 3 consecutive days.

For glycol chitosan complexes, it is visible that N/P10 nanoparticles were not stable in surrounding buffer, both in diameter and zeta potential. Also, negative values of net charge were present, meaning that pDNA encapsulation by chitosan particles was not totally achieved (see agarose gel in Figure 2) and negative pDNA molecules stayed on complex surface, resulting in the referred negative values, incompatible with interaction with negatively charged cell membrane. Regarding HMW1 chitosan complexes, N/P10 particles showed to be unstable in the surrounding media and surface charge values were nearly zero. Complexation with pDNA was not total and the amount of chitosan positive charges was similar to the amount of pDNA negative charges, resulting in null zeta potential values. For the rest of N/P ratio nanoparticles, diameter and zeta potential stability was achieved, with relatively similar values between them (values between 200nm for N/P50 and 350nm for N/P20 nanoparticles). Sonication of chitosan particles before complexation assay also had an effect on polyplexes physical stability. Comparing HMW1 and HMW2 (sonication vs non-sonication) it is notorious that this process influenced positively the diameter stability of N/P20, 50 and 70 and

negatively the N/P10 complex in suspension. The decreasing size of N/P20, 50 and 70 HMW2 nanoparticles might be related with the fact that complexation only occurred on the second measurement day, resulting in smaller particles. For all chitosans tested, nanoparticles with N/P ratio of 10 were the most stable during this test. Also, Pdi values (results not shown) showed that these were the more homogeneous samples, compared with other N/P ratio nanoparticles herein tested. It was also verified that diameter values are smaller than the ones obtained when chitosan particles are prepared 15 days before complexation, which is an important feature for future transfection assays. Aggregation and sedimentation phenomena were then avoided.

Characterization of lipid/chitosan/pDNA nanoparticles

The use of lipids, namely in the form of liposomes, has been widely investigated for use as non-viral vectors for cell delivery of therapeutic agents, pDNA included. In this study, a cholesterol derivative (cholesteryl chloroformate, 97%) (CHOL) and a modified lecithin (ML) (L-A_lysophosphatidylcholine type I from egg yolk) were used, individually, to coat chitosan particles and determine if their presence influences the efficiency of transfection, after complexation of these conjugates with pVAX1GFP.

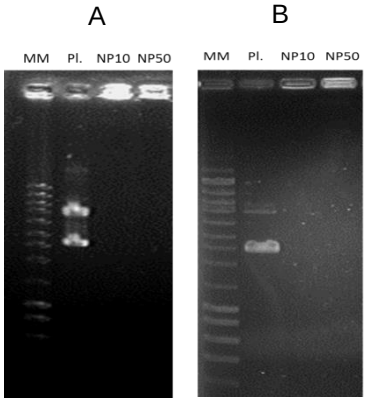

Figure 4 - 1% agarose gel of lipopolyplexes. 2µL of loading buffer were added to 10µL of sample. Electrophoresis ran for 1h30 at 120V and ethidium bromide was used to stain the samples. MM – molecular marker NZYladderIII (6µL); Pl. – pVAX1GFP 75ng/ µL (negative control); A – CHI/CHOL conjugate complexed with pDNA at N/P10 and 50 ratios; B – CHI/ML conjugate complexed with pDNA at N/P10 and 50 ratios.

Complexation was achieved in both conjugates at both N/P ratios tested, since no pDNA migration is observed in the gel, when compared to the negative control (plasmid itself).

Diameter, polydispersion index and zeta potential characterization of formed nanoparticles as well as the controls are represented on Table1.

Table 1 - Diameter mean Pdi and zeta potential values of chitosan oligo lactate (control), CHI/CHOL conjugate and N/P10 and 50 complexes of conjugate and pDNA. Diameter (Z-average), mean Pdi and zeta potential values of LMW1 CHI, modified lecithin coated chitosan (conjugate control), and N/P10 and 50 complexes of conjugate and pDNA; ± SD

Sample	Diameter (nm)	Mean Pdi	Zeta potential
COL	649 ± 29	0.80 ± 0.02	36 ± 0.1
CHOL	702 ± 51	0.57 ± 0.03	23 ± 1.5
COL/CHOL/pDNA NP10	242 ± 15	0.29 ± 0.02	16 ± 0.7
COL/CHOL/pDNA NP50	231 ± 48	0.28 ± 0.05	21 ± 1.7
LMW1 CHI	315 ± 64	0.40 ± 0.01	34 ± 3.0
CHI/ML	2557 ± 1050	0.90 ± 0.20	32 ± 2.0
CHI/ML/pDNA NP10	378 ± 27	0.30 ± 0.05	13 ± 0.8
CHI/ML/pDNA NP50	458 ± 15	0.30 ± 0.04	16 ± 1.0

Diameter values increased when both conjugates were performed, compared to respective chitosan used (control), since cholesterol derivative and modified lecithin would remain at chitosan molecule surface. The electrostatic interaction between the two components resulted in a decrease of zeta potential value, since less chitosan positive charges will be available at conjugate surface. When complexed with pVAX1GFP, electrostatic interaction of this bio-component with COL/CHOL and CHI/ML conjugates would promote a molecule condensation, resulting in lower diameter values for both N/P ratios tested. Zeta potential values remained positive but decreased a little when compared to reaction test, since less chitosan positive charges are available after complexation. Also, more homogeneous nanoparticles were formed since their Pdi values decreased for about 0.3, meaning that complexes with pDNA are more stable than lipid/chitosan conjugate.

Results from N/P10 and N/P50 nanoparticles were relatively similar. However, higher zeta potential values were observed for N/P50 complex due to the lower content in pDNA present, compared to the other ratio tested. However, when analysing the correlograms of the samples (results not shown), it was verified that these nanoparticles were not totally stable due to the presence of some aggregates. It is important, in future works, to use freshly prepared samples before characterization in Zetasizer equipment. Furthermore, several parameters of these reactions, such as the time of reaction and chitosan preparation, should be studied. Also, to obtain more detailed information about the topology, dynamics and three-dimensional structure of COL/CHOL and CHI/ML conjugates, NMR spectroscopy is a powerful tool, which should be used in future studies. Transmission electron microscopy (TEM) or scanning electron microscopy (SEM) may also be used.

Transfection of produced nanoparticles to animal cells

The selection of chitosan nanoparticles, to be used in transfection assays, was made in order to obtain the maximum efficiency of this delivery process, i.e. high percentage of transfected cells, resulting from the expression of GFP in cell nucleus. Criteria used in this selection process include small diameter sizes (nano – range), positive net charges (zeta potential values), complex stability along the time and nanoparticles that were as homogeneous as possible (Pdi values less than 0.30). Several transfection parameters were also studied (Figure 5).

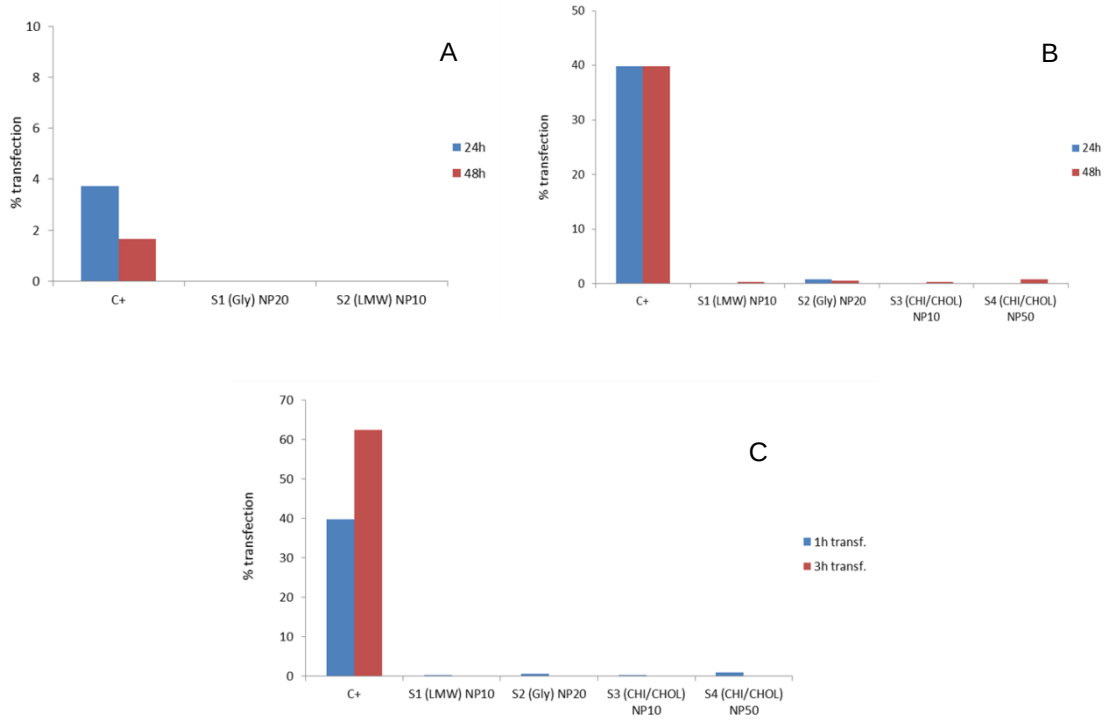

Figure 5 – % of transfected CHO cells with selected chitosan nanoparticles. A – 100% cell confluency (1h of transfection; 24h and 48h of incubation); B – 80% cell confluency (1h of transfection; 24h and 48h of incubation); C – 80% cell confluency (1h and 3h of transfection; 48h of incubation); Cell autofluorescence discounted from the results obtained

Sonicated LMW CHI complexed with pVAX1GFP at N/P ratio of 10 and sonicated Glycol CHI complexed with pVAX1GFP at N/P ratio of 20 were the firstly chosen nanoparticles to be introduced into CHO cells. Negative control composed only for CHO cells and positive control, in which pVAX1GFP was complexed with the transfection reagent Lipofectamine 2000 and then placed in contact with the referred cells, were also tested.

Transfection % was significantly higher in experiments B and C, compared to the ones obtained in experiment A. This fact might be related with the 100% cell confluency used in A, in which were present too many cells, resulting in contact inhibition thus making cells resistant to uptake foreign DNA. GFP expression levels were extremely low when chitosan nanoparticles were used as a vector, for both B and C experiments, even at 80% cell confluency. Only in positive control those values were relatively high, resulting in transfection efficiencies of 40% to 60%, for 1h and 3h of transfection, respectively. Lipofectamine2000 is an established complex with transfection % normally higher that 30% when GFP is used as reporter gene. It is composed of cationic lipidic subunits that form liposomes in aqueous environments, allowing the entrapment of pharmaceutical ingredients, pDNA included. The presence of liposomes in the formulations should facilitate the interaction with the cell membrane, enhancing their uptake. However, the presence of cholesterol derivative in chitosan nanoparticles surface did not influenced transfection efficiency, meaning that the nanoparticles are not being uptaken by the cells or are being degraded inside them, moreover in the lysosome. In future studies, it should be interesting to perform a microporation assay, in order to understand which is the origin of these low transfection values.

Another important parameter that was determined was the pH of the nanoparticles, which was around 7.3 for all the tested complexes. Typical set-points for pH in CHO cell culture are in the range between 6.8 and 7.4 (Zhou *et al*., 2010). If the nanoparticles were outside this range, viability and metabolic activity of the cells, as well as recombinant protein productivity and quality would be affected. The appearance of the cells was also verified, before and after the contact with the nanoparticles, in an optical microscope. No significant differences were observed, meaning that the tested complexes were not extremely toxic to the cells, which could cause morphological changes or even apoptosis. Elongated form was maintained and the nucleus was sometimes visible. Another experiment was performed using 6h of transfection, but the results remained inconclusive.

More research has to be made to achieve higher transfection efficiencies, especially in the optimization of polyplex and lipopolyplex formulations and their quantity (mass) used, as well as on transfection parameters such as to test other transfection and incubation periods.

Conclusions and future work

The main goal of this study, which was to obtain high transfection efficiencies with chitosan nanoparticles in CHO cells, was not achieved. However, some important conclusions were made during this project regarding the preparation and characterization of the referred particles. Sonication and buffer filtration showed to be important processes to obtain stable, homogeneous chitosan particles in suspension with positive net charges and nanometer range diameters. Low molecular weight chitosan were the one that presented more regularly these important characteristics. Self-assembly demonstrated to be an efficient complexation method for chitosan particles in suspension and previously purified pVAX1GFP. Freshly prepared particles are important to avoid aggregation and sedimentation phenomena, meaning that these complexes are not stable for a long period of time, even at 4ºC. Regarding amine to phosphate ratios (N/P), no significant conclusions were made, since the quality of the polyplexes/lipopolyplexes is variable from chitosan to chitosan and from lipid to lipid. In this study, to calculate the mass of molecules used in complexation assays, chitosan's mass was fixed and pDNA mass was calculated based on the molecular mass and N/P ratio used in each formulation. For future studies, the mass of pDNA should be fixed and chitosan's mass should be calculated from the MM and N/P ratio used, in order to determine if this factor has influence on transfection efficiency. Further work on creating homogenous, large-scale preparations of polyplexes and lipopolyplexes with known characteristics other than particle size distribution and zeta potential

will be necessary, to enable progress in this area. Transmission electron microscopy (TEM) or scanning electron microscopy (SEM) should also be performed, in order to observe the morphology of the nanoparticles. Nuclear magnetic resonance spectroscopy (NMR) should also be taken into account to study in detail the dynamics of these reactions. Regarding the transfection assays, 100% cell confluency should not be used in order to avoid cell to cell contact inhibition. With the "positive" results obtained for the positive control with the transfection reagent Lipofectamine 2000, it is clear that "negative" results obtained with chitosan nanoparticles are not derived from pVAX1GFP quality, quantity and stability. However, pDNA quantity used in each formulation was different. In positive control, 1µg of pDNA was used to complex with Lipofectamine 2000 while its mass on chitosan formulations varied according to N/P ratio used (from 2µg to 28µg). In future works, pDNA mass needs to be optimised in order to enhance the transfection process. At 3h of transfection, the efficiency of this process for pDNA complexed with Lipofectamine showed to be higher than at 1h of transfection, for an incubation period of 48h. However, for chitosan polyplexes and lipopolyplexes tested no ideal periods were assessed. In a near future it should be interesting to realize a microporation assay in order to verify if these low transfection efficiencies are related with the interaction of the nanoparticles with the cell membrane or if they are been degraded inside the cell, mainly in liposomes. Confocal microscopy could also be helpful to understand the location of those chitosan nanoparticles inside the cell because both pDNA and chitosan would be marked with different fluorescent dies.

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