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Delivery of proteins encapsulated in chitosan-tripolyphosphate nanoparticles to human skin melanoma cells

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Graphical abstract

Highlights

- Structurally unrelated proteins can be encapsulated in chitosan-TPP nanoparticles
- Chitosan-TPP nanoparticles delivered proteins to mammalian cells
- Cellular uptake of chitosan-TPP nanoparticles was dependent on pH
Abstract

We have successfully encapsulated two proteins, bovine serum albumin (BSA) and p53, in chitosan-tripolyphosphate (TPP) nanoparticles at various pH values from 5.5 to 6.5 and delivered the particles to human melanoma cells. The particles have diameters ranging from 180 nm to 280 nm and a zeta potential of +15 to +40 mV. Cellular uptake of the particles by human skin melanoma cells was evaluated by: (i) fluorescence microscopy and (ii) gel electrophoresis showing that FITC-labeled BSA and p53 could be recovered in the soluble cell fraction after lysis of the cells. Our data also show that the highest cellular uptake takes place at the lowest pH as the particles have the highest positive charge under these conditions. The method we describe appears to be a general method for delivery of proteins to cells using chitosan-TPP nanoparticles as a drug delivery system, since structurally unrelated proteins such as BSA and p53 with different isoelectrical points can be encapsulated in the chitosan-TPP nanoparticles and be effectively internalized by the cells.

Keywords: Chitosan, nanoparticles, protein, p53, SK-mel 28

Introduction

Chitosan, a biopolymer consisting of β-(1,4)-linked D-glucosamine and N-acetyl-D-glucosamine, is highly popular for preparation of micro- and nanoparticles to be used for delivery of drugs, genes, and food additives to mammalian cells, because the polymer is biocompatible, biodegradable, and mucoadhesive [Dash et al., 2011]. The polycationic nature of chitosan enables the polymer to form stable complexes with negatively charged nucleic acids and has therefore received much attention as a vehicle for the delivery of genes, siRNA and other negatively charged macromolecules to mammalian cells [Leong et al., 1998, Howard et al., 2006, Schrøder et al., 2014]. Recent work has shown that a more efficient delivery and an improved colloid stability can be obtained through the formation of nanoparticles by ionic gelation of the positively charged chitosan with a negatively charged polyanion such as tripolyphosphate (TPP) [Katas et al., 2006, Csaba et al., 2009, Gaspar et al., 2011]. In various formulations, positively charged chitosan-TPP particles may obtain diameters ranging from approximately 60 to hundreds of nanometers [Calvo et al., 1997, Zhang et al., 2004, Gan et al., 2005].
In addition to being a vehicle for delivery of polynucleotides, chitosan-TPP particles can also encapsulate proteins [Xu et al., 2003, Rampino et al., 2013]. Proteins are an important group of biopharmaceuticals utilized in different medical contexts; for example, as antibodies for disease treatment (e.g. cancer) or as activators/inhibitors of various cell-signaling pathways [Tomlinson et al., 2004]. Several proteins have been encapsulated in chitosan-TPP nanoparticles, e.g. bovine serum albumin (BSA), insulin and ovalbumin [Rampino et al., 2013], L-asparaginase [Bahreini et al., 2014] and tetanus toxoid [Siddhapura et al., 2016]. Proteins are encapsulated by participating in the cross-linking network with chitosan and TPP through their charged and polar amino acids, which make stabilizing interactions with both the positive charges of chitosan but also with the negative charges of TPP. Furthermore, proteins may also be physically entrapped when the ionic cross-linking process is induced.

The formation of nanoparticles, and hence their physico-chemical characteristic and colloid stability in addition to the protein encapsulation efficiency, is highly influenced by several factors in the preparation process. Accordingly, size and the deacetylation degree of chitosan, the ratio of TPP and chitosan, pH, ionic strength, temperature and mixing speed may change the particle characteristics [Fan et al., 2012, Gan et al., 2007, Xu et al., 2003]. The isoelectric point (pI) of a protein is the pH at which the protein has a zero net charge. Specifically, interaction of proteins with charged polymers such as chitosan may be dependent on the charge state of the polymer but also the protein. The sensitivity of the particle formation to these factors provides a possibility to modulate, at least in part, the characteristic features of the particles and the protein encapsulation according to the purpose. Furthermore, the protein release profile can be altered due to changes in the particle composition [Gan et al., 2007, Xu et al., 2003].

Some studies already exist describing the formation of chitosan-TPP nanoparticles (prepared both in the presence and the absence of a protein) and their dependency on the fabrication conditions (e.g. Fan et al., 2012, Gan et al., 2007, Rampino et al., 2013, Xu et al., 2003). Increasing the molecular weight of chitosan results in a higher protein encapsulation efficiency [Xu et al., 2003]. The same applies for an increase in the TPP to chitosan ratio [Gan et al., 2007]. Small chitosan molecules may not physically be able to entrap the protein due to their limited length, which results in a low protein encapsulation efficiency [Xu et al., 2003].

Most proteins are not naturally internalized by mammalian cells and they can furthermore be substrates for degradation by various extracellular and intracellular proteases. Nanoparticles facilitate cellular delivery of biomacromolecules and furthermore protect the integrity of their cargo. There is a general therapeutic interest in the delivery of proteins to mammalian cells. For example, systemic delivery of L-asparaginase using nanoparticles could be used to treat various forms of cancer [Wan et al., 2016].
Restoring the activity of the tumor suppressor p53 has also been proposed as a therapeutic strategy to treat various types of cancer [Senzer et al., 2016]. p53 is implicated in cell cycle regulation and induction of cell death in response to various forms of cellular stress such as DNA-damage or oncogene activation; p53 is therefore often referred to as ‘the guardian of the genome’ due to its vital role in preserving the genome integrity [Lane et al., 1992]. p53 is frequently mutated in many types of cancer, which can result in the expression of mutant p53 unable to initiate the apoptotic cascade [Muller et al., 2013]. In this case, delivery of wild type p53 in combination with a chemotherapeutic agent could potentially reactivate the apoptotic function of p53 and hence promote pharmacological induction of cell death, which eventually will result in synergistic killing of the cancer cells. In other cancer forms, which express wild type but functionally inactive p53 such as certain types of human skin melanomas and renal cell carcinomas [Gurova et al., 2004, Houben et al., 2011], delivery of functionally active and stable p53 (e.g. by introducing stabilizing post-translationally modifications) could restore the protein’s apoptotic function.

In the current work, we investigated the ability of chitosan and TPP to form stable nanoparticles encapsulating proteins using BSA as a model protein. We also investigated the colloidal stability, the size and the zeta potential of the nanoparticles in relation to the TPP:glucosamine ratio and pH, and finally evaluated the uptake of the resulting particles in human skin melanoma cells. We show that a positive zeta potential, obtained under mild acidic conditioning pH, favors a higher cellular uptake of the nanoparticles. Finally, we encapsulated the tumor suppressor p53 in chitosan-TPP nanoparticles and successfully delivered the protein to human skin melanoma cells. We present evidence that our method can be used as a general procedure for encapsulating proteins of molecular mass less than 100 kDa, regardless of a negative or neutral net charge, as structurally unrelated proteins such as BSA (pl of 4.7-4.9) and p53 (pl of 6.3) were encapsulated in the nanoparticles and successfully delivered to human skin melanoma cells.

**Materials and Methods**

**Materials**

Chitoceuticals Chitosan 85/100 (Mw 100-250 kD, pharmaceutical grade, deacetylation degree 87.12%) was obtained from Heppe Medical Chitosan (Halle, Germany). Sodium tripolyphosphate pentabasic (TPP) and Albumin from Bovine Serum (BSA) were obtained from Sigma Aldrich (Steinheim, Germany). Albumin from Bovine Serum FITC conjugate and DAPI (4’-6-Diamidino-2-Phenylindole, Dihydroclorid) were purchased from Thermo Fisher Scientific, Life Sciences (Eugene, Oregon USA). BioRad® reagent was obtained from BioRad® Laboratories (München, Germany). Minisart syringe filters (pore size 0.2 µm or 0.45 µm) were obtained from Sartorius Stedium Biotech GmbH (Göttingen, Germany). Skin melanoma 28 (SK-Mel 28) cells were obtained...
from American Type Culture Collection® (Manassas, VA, USA). Dulbecco’s Modified Eagle Medium with GlutaMAX™ (DMEM) was purchased from Life Technologies, Invitrogen (Copenhagen, Denmark), Foetal bovine serum was from Biochrom AG (Berlin, Germany) and Penicillin-Streptomycin from Thermo Fisher Scientific, Life Technologies (Grand Island, New York, USA). Protease inhibitor cocktail was purchased from Roche Diagnostics (Indianapolis, Indiana, USA). All other reagents were obtained from Sigma-Aldrich (Copenhagen, Denmark) unless otherwise stated.

**Preparation of protein-loaded chitosan-TPP nanoparticles**

The nanoparticles were prepared via the ionotropic gelation method [Calvo et al., 1997, Ahmad et al., 2016]. 6.25 mg chitosan 85/100 was dissolved in 5.5 mL 0.1 % (v/v) acetic acid, filtered through a Minisart syringe filter (pore size 0.2 µm) and shaken overnight at 650 rpm on PCTM Thermoshaker (Grant® Instruments LTD, Cambridge, United Kingdom). The pH of the chitosan solution was adjusted to either 5.00, 5.25, 5.50, 5.75 or 6.00 by slow addition of 0.5 M NaOH under vigorous magnetic stirring. De-ionized water was added to give a final volume of 6.25 mL and a chitosan concentration of 1 mg/mL. The chitosan solution was filtered through a Minisart syringe filter (pore size 0.45 µm) to remove insoluble material. A TPP stock solution in de-ionized water with a concentration of 25 mM was prepared, filtered through Minisart syringe filter (pore size 0.45 µm) and diluted to the required concentrations. For the optimization of the preparation conditions BSA was used as a model protein. BSA was dissolved in 50 mM Tris-HCl, 50 mM KCl, 2 mM dithiothreitol (DTT), 5 % glycerol pH 7.5 to a stock protein concentration of 1.5 mg/mL, and filtered through Minisart syringe filter (pore size 0.45 µm). 241 µL of this solution was added to the TPP solution to a final volume of 350 µL to obtain a BSA concentration of 1.0 mg/mL. To optimize the TPP:glucosamine molar ratio the concentration of TPP was varied and the final volume of 350 µL was obtained by the addition of de-ionized water. 1.25 mL of chitosan solution was transferred to 4 mL glass vials (Ø 14.7 mm) and mixed under magnetic stirring (750 rpm) with 300 µL of a TPP/protein solution added by one fast injection [Schröder et al., 2014]. The final concentration of protein (BSA or p53) was 0.2 mg/mL. The ionic gelation was allowed to proceed for one hour under magnetic stirring (750 rpm) at room temperature. Expression and purification of human p53 are described in the Supporting Information.

**Characterization of nanoparticles by dynamic light scattering**

The size (mean hydrodynamic diameter), size distribution (polydispersity index (PDI)) and zeta potential of the chitosan-TPP nanoparticles were determined by dynamic light scattering (DLS) on a DelsaMax Pro (Beckman Coulter, Brea, CA, USA). The autocorrelation function generated from the intensity fluctuations was analyzed using the appertaining software by means of a cumulants analysis method, assuming the
presence of only one population of particles with a Gaussian distribution. The sample temperature was maintained at 25°C during measurement.

**Determination of the protein encapsulation efficiency**

The encapsulation efficiency was determined by (i) the Bradford method (for nanoparticles encapsulating BSA) or (ii) SDS-PAGE analysis (for nanoparticles encapsulating p53). The samples were centrifuged at 25,492×g for one hour at 22°C on Thermo Scientific SL16R (Thermo Fisher Scientific, Roskilde, Denmark) rotor head 75003652. The nanoparticles will upon centrifugation sediment while the free protein will remain in the supernatant.

The encapsulation efficiency was determined according to equation 1, below.

$$EE(\%) = \frac{A-B}{A} \times 100$$

(1)

Here A is the total amount of protein (as measured in the non-centrifuged sample) and B is the amount of free protein in the supernatant after centrifugation.

The protein concentration was measured using the Bradford method (Microassay Procedure using BioRad® reagent) in 96-well plates. Four BSA standards (1 µg, 2 µg, 3 µg and 4 µg BSA) diluted in de-ionized water in a total volume of 160 µL per well were included in each determination to obtain a standard curve. A new standard curve was prepared for each experiment. 15 µL of each nanoparticle sample was diluted in 145 µL water in each of the sample wells. Then, 40 µL BioRad® reagent was added to each well. Empty nanoparticles (i.e. without protein) prepared with a TPP:glucosamine ratio molar ratio of 0.100 were used as base-line correction. The plates were incubated at room temperature for at least 5 minutes and less than one hour. The absorbance was measured at 595 nm in a FLUOstar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany) at room temperature.

To determine the encapsulation efficiency for nanoparticle samples with p53, SDS-PAGE analysis was employed as an alternative to the Bradford method. The polyacrylamide gel consisted of an upper stacking gel (4.8 % acrylamide/bisacrylamide, 0.5 Tris-HCl pH 6.8, 0.01 % sodium dodecyl sulfate (SDS), 0.01 ammonium persulfate (APS) and 0.2 % N,N,N',N'-tetramethylethylenediamine (TEMED)) and a lower separation gel (1.5 M Tris-HCl pH 8.8, 0.1 % SDS, 0.067 % APS, 0.1 % TEMED and 12 % acrylamide/bisacrylamide). Protein samples were diluted in SDS-sample buffer consisting of 0.5 M Tris-HCl pH 6.8, 10 % SDS, 10 mM DTT, 14 % glycerol and 0.025 % bromophenol blue. Samples were denatured by heating (95°C for 3-5 minutes) and centrifuged prior to loading. Samples were loaded alongside a SpectraTM Multicolor Broad Range Protein ladder. The PAGE was conducted in running buffer consisting of 0.1 M Tris-
HCl, 0.1 M Glycine and 0.1 % SDS at 45 mA per gel for approximately 1 hr. The protein bands were visualized by coomassie brilliant blue staining (0.25 % Serva Blue R250, 45 % (v/v) ethanol, 10 % (v/v) acetic acid) for 5 minutes and destained for 10 minutes in coomassie wash (45 % (v/v) ethanol, 10 % (v/v) acetic acid). Until destained, the gel was stored in 10% (v/v) acetic acid.

**Cell culture and treatment**

Skin melanoma 28 (SK-Mel 28) cells were cultivated in Dulbecco’s Modified Eagle Medium with GlutaMAX™ supplemented with 10 % foetal bovine serum and 100 U/mL Penicillin-Streptomycin in culture dishes. The cells were cultured at 37°C under 5 % CO₂ atmosphere. 350,000 SK-Mel 28 cells were seeded in 4 mL medium in 6 cm culture dishes. Cells were treated approximately 24 hours after seeding. The cell medium was aspirated and the cells were washed two times in warm (37°C) Hanks’ balance salt solution (HBSS) modified with sodium bicarbonate, calcium chloride, magnesium sulphate and without phenol red, adjusted to the required pH with HCl (i.e. 5.5, 6.0 or 6.5) and subsequently sterilized by filtration through a Minisart syringe filter (pore size 0.2 μm). Cells were incubated for 5 hours at 37°C, in a humidified atmosphere (in the absence of CO₂) with nanoparticles diluted ten times in HBSS.

**Preparation of whole cell extract**

The nanoparticle suspension was aspirated and the cells were washed three times in cold phosphate buffer saline (PBS). 80 μL harvesting buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 % glycerol, 1 mM dithiothreitol (DTT), 1 mM Na₃VO₄, 10 mM NaF, 30 mM β-glycerophosphate, 10 mM NaF, 100 nm okadic acid, 1 % Triton X-100 and 20 μL/mL protease inhibitor cocktail was added to each petri dish and the cells were harvested by scraping and placed at –80°C. Whole cell extract was obtained by centrifugation at 13,400 x g at 4° C for 20 min. Finally, the supernatant was collected for further analysis.

**Sample preparation for microscopy**

The cells were washed with HBSS adjusted to the required pH, and subsequently twice with PBS. The cells were fixed in 4 % para-formaldehyde in PBS (20 minutes at room temperature). The cells were then washed twice in PBS and stained for 5 min with 300 nM DAPI in PBS. Then, the cells were again washed two times in PBS. The coverslips were rinsed in demineralized water and mounted (in 6 μL 50 % (v/v) glycerol in PBS) on microscope slides.

Confocal images were captured on an inverted multiphoton excitation fluorescence microscope (Zeiss LSM 510 META NLO, Carl Zeiss, Jena, Germany) equipped with a Ti:Sa MaiTai XF-W2S laser (Broadband Mai Tai
with 10 W Millennia pump laser, tuneable excitation range 710–980 nm, Spectra Physics, Mountain View, CA). FITC and DAPI were dually excited at 780 nm. Emission light was captured in two channels; 1) filtered by a 565-615 nm band pass filter (for FITC) and 2) filtered by a 435-485 nm band pass filter (for DAPI), respectively. The emission maximum of FITC is 520 nm, but to avoid green auto-fluorescence, a bandpass filter with a higher threshold of 565-615 nm was found to be optimal. The objective used for experiments with BSA was Zeiss C-Apochromat® 63x water immersion objective, NA 1.2. The objective used for experiments with p53 was Zeiss Plan-Apochromat® 63x oil immersion, NA 1.4. All images were edited in IMAGE J 1.48v, National Institutes of Health, USA.

Results and discussion

Particle size, zeta potential and protein encapsulation efficiency

The uptake of the nanoparticles and hence the delivery of cargo to the cells are highly dependent on the physiochemical properties of the particles (e.g. size and surface charge); a small particle size and a positive surface charge may facilitate the cellular uptake [Rejman et al., 2004]. In addition, the encapsulation efficiency of the cargo (proteins in our case) may depend on the charged state of the particle components, i.e. chitosan and TPP, and of the cargo itself. Also the size and structure of the cargo may influence the efficiency of encapsulation and uptake. Since the charges of chitosan, TPP and BSA are pH-dependent, we first conducted a series of experiments to study the dependency of the size and the surface charge (zeta potential) of the chitosan-TPP particles and the encapsulation efficiency of BSA on pH. As chitosan is only soluble under mild acidic conditions and the polymer will precipitate at neutral pH, all experiments were conducted under mildly acidic condition.

Figure 1 shows plots of the size, the zeta potential and the protein encapsulation efficiency for BSA-loaded chitosan-TPP nanoparticles prepared at different TPP:glucosamine ratios and at different values of pH (of the chitosan solution). Both particle size (Figure 1A) and protein encapsulation efficiency (Figure 1C) increase with increasing TPP:glucosamine ratio. This is most likely a consequence of a higher degree of ionic cross-linking due to the presence of a higher concentration of negative charges because of an increasing TPP concentration. In good correlation with this, increasing the TPP:glucosamine ratio was accompanied by an increase in the turbidity of the nanoparticle suspensions, which is an indicator of a shift towards larger particle sizes and/or a higher level of ionic cross-linking. The presented particle sizes and protein encapsulation efficiencies are in accordance with the findings by Rampino et al., 2013, who report an BSA encapsulation efficiency of 39±9 % in chitosan/TPP nanoparticles prepared at pH 5.5 with 0.2 mg/ml BSA i.e
similar conditions as presented in the current study. Our results furthermore suggests that a higher encapsulation efficiency can be obtained by increasing the pH of the solution.

Precipitation of the particles can be due to neutralization of particle surface charge (zeta potential approaching 0 mV) diminishing the stabilizing repulsive forces between individual particles, or due to cross-linking of individual particles into larger µm-sized unstable aggregates. It was possible to produce colloid stable nanoparticles up to a TPP:glucosamine ratio of 0.100 at pH 6.00; in comparison, it was possible to form colloid stable particles at pH 5.00 up to a molar TPP:glucosamine ratio of 0.175. Increasing the pH will result in fewer protonated amino groups of chitosan available for cross-linking with TPP. The low-molecular weight chitosan used here has an effective pKₐ value of 6.15 (see the Supporting Information Figure S2), i.e. the protonation of glucosamine drops by 40% as the pH is increased from 5.0 to 6.0. Neutralization of the electrical charges, which results in particle aggregation, is therefore reached at a lower concentration of TPP and, therefore, at a lower TPP:glucosamine ratio. Changing the pH seems to have a dual effect on the particle size at low TPP:glucosamine ratios. The particle size increases from pH 5.0 to 5.25 and then decreases as pH is further increased to 6.0.

Decreasing the pH of the chitosan solution from 6.0 to 5.0 resulted in approximately a doubling of the positive zeta potential from +15-20 mV to +35-40 mV as a consequence of an increased protonation of chitosan (see the Supporting Information Figure S2). The zeta potential decreased slightly with an increasing TPP:glucosamine ratio. The decrease in zeta potential is not surprising as increasing the amount of TPP will contribute to a higher density of negative charge, but the decrease (a few mV) is less pronounced than expected, suggesting that most of the TPP is located in the particle matrix and not at the particle surface. The small decrease in the zeta potential could simply be explained by the small increase in the pH and hence deprotonation of chitosan due to the presence of a higher concentration of TPP base. The size range and zeta potentials presented in Figure 1 are in accordance with the physiochemical properties of chitosan/TPP nanoparticles reported by others [Gan et al., 2007; Rampino et al., 2013].

2. Cellular uptake of chitosan-TPP-BSA particles by human skin melanoma cells

To evaluate if the chitosan-TPP particles encapsulating BSA could be internalized by human skin melanoma cells, SK-Mel 28 cells, were incubated for five hours at 37°C with either empty chitosan-TPP nanoparticles, free BSA-FITC or chitosan-TPP nanoparticles encapsulating BSA-FITC as described in the Materials and Methods section. The cellular uptake was evaluated by confocal microscopy and SDS-PAGE analysis of the soluble fraction (supernatant) isolated by centrifugation of the whole cell content after cell lysis. Figure 2A shows confocal microscopy images of SK-Mel 28 cells after five hours of treatment. Large macromolecules such as proteins do not readily cross the cell membrane under normal circumstances. Accordingly, SK-Mel 28
cells did not internalize free BSA-FITC unaided as no fluorescent signal originating from BSA-FITC was detected within the cells by microscopy (Figure 2A). In contrast, cells treated with BSA-FITC encapsulated in chitosan-TPP nanoparticles showed a strong fluorescent signal within the cells (Figure 2A). Subsequent gel electrophoresis confirmed that only BSA-FITC encapsulated in chitosan-TPP nanoparticles was effectively internalized by the SK-Mel 28 cells and was intact upon uptake (Figure 2B).

3. Effect of pH on the cellular uptake of chitosan-TPP particles encapsulating BSA

The protonation of chitosan is highly dependent on pH (see Supporting Information Figure 2A). Table 1 shows the particle characteristic determined after the pH of the final nanoparticle suspension was adjusted to pH 6.5, 6.0 and 5.5, respectively. The size of the nanoparticles increased with decreasing pH in the solution (Table 1). This suggests that the chitosan/TPP nanoparticles, once formed, do not contain a fixed matrix, but are composed of a dynamic ionic network, which can swell upon changes in the composition of the bulk solution. The zeta potential measurements show that the nanoparticles were weakly charged at pH 6.5, but strongly cationic at pH 5.5 (Table 1), which is in agreement with the results presented in Figure S2A. The PDI and visual inspection of the samples confirmed that the particles had a good colloidal stability under the conditions used in Table 1. Increasing the pH to 7 resulted in complete precipitation of particles as the particle surface charge became neutralized. The data in Table 1 does not allow us to determine whether the particles have a genuine isoelectric point (pI, the pH at which the particles carry a net zero net charge) or it is because the surface charge gradually approaches a zero charge as the pH is raised. The latter is illustrated in Figure S3 and is consistent with earlier observations that the tripolyphosphate seems to be located primarily in the particle matrix and not on the surface [Schröder et al., 2014; Huang and Lapitsky, 2011]. Hence, an increase in pH to 7 and beyond is not expected to result in a negative surface charge and hence a negative zeta potential. It should be noted that the pH of buffers used in the following experiment were pH 6.5 or lower to assure colloidal stability of the particles during the conducted experiments.

The polycationic nature of chitosan is the driving force for cellular uptake [Sahay et al., 2010]. It has been reported that the uptake of positively charges nanoparticles were superior compared to negatively charged or neutral particles in eight cell lines tested [Yue et al., 2011]. Furthermore, their results suggest that the positively charged nanoparticles could escape the endocytic pathway; a known ability also for chitosan [Yue et al., 2011]. Accordingly, we investigated the effect of the pH of the uptake medium on the cellular uptake of BSA-containing chitosan-TPP nanoparticles by human skin melanoma cells by (i) conducting SDS-PAGE analysis and measuring the fluorescence intensity of BSA-FITC in the soluble cell fraction and (ii) visualizing the cellular uptake by confocal microscopy. The results are shown in Figures 3 and Figure 4, respectively. Human skin melanoma cells (SK-Mel 28) were incubated for five hours with chitosan-TPP nanoparticles encapsulating BSA-FITC dissolved in uptake medium at pH 5.5, 6.0 and 6.5, respectively. Figure 3A and 3B
show the results of the SDS-PAGE analysis. It is clear that the fluorescence intensity from the soluble cell fraction, indicative of an uptake of FITC-BSA, increases with decreasing pH. This was furthermore confirmed in the confocal microscopy images showing the most intense fluorescence signal was evident for cells exposed to the lowest pH tested i.e. pH 5.5 (Figure 4, montage of Z-stacks, 1 µm between slices). The increasing uptake, due to a decrease in pH, is most likely due to an increasing number of positive charges of chitosan and hence a more positive particle surface charge (Figure S2A in the supporting information): at pH 5.5, 75% of the glucosamine residues of chitosan are protonated, whereas at pH 6.5 only 30% are protonated. The same is apparent from the measurements of the zeta potential of the nanoparticles (Figure 1B). Thus, a positive surface charge of the nanoparticles promotes the cellular uptake of the chitosan-TPP particles.

4. Encapsulation of p53 in chitosan-TPP nanoparticles and their cellular uptake

It could be argued that BSA is especially suited for encapsulation in chitosan-TPP particles because of its low pI (4.7-4.9), which ensures a net negative charge of the protein in the pH region 5-6. Water soluble proteins are large structures, which in a complex manner orientate their hydrophobic structure elements inward and polar/charged structure elements outward and exposed to the surrounding aqueous environment. Chitosan has several functional groups available for non-covalent interactions: primary and secondary hydroxyl groups, the amide of the N-acetyl-D-glucosamine units, the ether of the carbohydrate ring and most importantly the primary amines of the D-glucosamine groups. The potential cross-linking network between charged and polar amino acids of the protein, chitosan and TPP is highly complex. Furthermore, the pI value of a specific protein does not specify the number nor the spatial distribution of charged amino acids and does not account for the polar moieties of the protein surface. Due to the complex 3D structure and high levels of variations between different types of proteins, it may not be possible to predict the efficiency and nature of protein encapsulation solely based on the pI of the proteins. Therefore, we attempted to encapsulate and deliver at therapeutically relevant protein, the tumor suppressor protein p53, which has an pI of 6.3 and therefore has a neutral or slightly positive net charge in the pH region 5-6. In addition, p53 is structurally unrelated to BSA, but the two proteins have compatible molecular masses. p53 was purified as described in the Supporting Information. We successfully encapsulated p53 in chitosan-TPP nanoparticles at pH 6.0 and a TPP:glucosamine ratio of 0.075. The particles had a mean diameter of 268±21 nm and a PDI of 0.238±0.001. The encapsulation efficiency was >90% determined by SDS-PAGE.
p53 was conjugated to FITC to enable visualization of the cellular uptake (described in Supporting Information). SK-Mel 28 were incubated for five hours with FITC-labelled p53 encapsulated in chitosan-TPP nanoparticles, and the uptake was evaluated by confocal microscopy (Figure 5B). For comparison, microscopy images of human skin melanoma cells incubated with free FITC-labeled p53 are shown in Figure 5A. Green fluorescence originating from free p53-FITC was not evenly distributed throughout the cells and was in general not found in close proximity of the nucleus (Figure 5A). This suggest that free p53-FITC was not internalized by the SK-mel 28 cells but was mostly found as aggregates on the cell surface. In contrast, it is evident from the microscopy image in Figure 5B that the chitosan-TPP particles containing p53-FITC are effectively internalized by the cells.

To summarize, we have shown that chitosan-TPP nanoparticles encapsulating BSA and p53 are effectively internalized in SK-Mel 28 cells. The current method may be used as a general platform for delivery of proteins with molecular mass <100 kD to mammalian cells, since it works for structurally unrelated proteins with different electrical charges, such as BSA and p53 explored in this study. Thus, it should also be possible to deliver posttranslationally modified proteins [Appella et al., 2001], i.e. acetylated, sumoylated and phosphorylated forms, of e.g. p53. Consequently, chitosan-TPP nanoparticles containing wild type and post-translationally modified (and stabilized) p53 and relevant chemotherapeutic agents may be used to treat various forms of cancer. Accordingly, cellular delivery of p53 protein may restore the apoptotic function of the tumor suppressor protein and concomitant with chemotherapeutic agents may potentially induce apoptosis in cancer cells. Inhibitors aiming at restoring the intrinsic p53 activity have been developed for the purpose of improving the treatment of skin melanoma e.g. Nutlin-3, SAH-p53-8 and JNJ-7706621 [de Lange et al., 2012; Tseng et al., 2010; Gembarska et al., 2012; Lu et al., 2013]. The success of the aforesaid treatment strategies may be dependent on certain members of p53-regulation to be deregulated in the diseased state. Consequently, treatment success can be limited by the heterogeneity of p53 inactivation. Delivery of active p53 using an appropriate drug delivery system may be an alternative strategy. It will be interesting to get further insight into the mechanism of cellular uptake, protein release from the chitosan-TPP nanoparticles and intracellular trafficking. As illustrated by this work chitosan-TPP nanoparticles are a simple, tunable and biocompatible drug delivery system with many potential applications within health sciences.

**Conclusion**

In the current study, we show that proteins with molecular mass < 100 kDa may be encapsulated in chitosan-TPP particles, which can function as vehicles for delivery of these proteins to human skin melanoma cells. Our results strongly indicate that the chitosan-TPP nanoparticles can effectively facilitate the cellular uptake
of their protein-cargo. Optimal delivery is obtained under mildly acidic conditions (~ pH 5.5) where the particles have a positive surface charge. The cellular uptake decreases significantly at higher pH values.

Acknowledgements

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References


High-level expression of wild-type p53 in melanoma cells is frequently associated with inactivity in p53 reporter gene assays. PLoS ONE 6: e22096


Figure 1. Encapsulation of BSA in chitosan-TPP nanoparticles. A) Particle size, B) zeta potential and C) protein encapsulation efficiency of chitosan-TPP nanoparticles encapsulating BSA at different TPP:glucosamine molar ratios and different pH values. N=3, n=3.
Figure 2. Chitosan-TPP nanoparticles (NP) mediate the uptake of BSA-FITC by SK-Mel 28 cells. SK-Mel 28 cells were incubated for five hours with either HBSS (control), empty nanoparticles, free BSA-FITC or BSA-FITC encapsulated in chitosan-TPP nanoparticles. The pH of the chitosan solution for the preparation of the nanoparticles was 6.0, the molar TPP:glucosamine ratio was 0.100 and the final concentration of BSA-FITC was 0.2 mg/mL. The nanoparticles and 0.2 mg/mL free BSA-FITC were diluted ten times in HBSS buffer pH 5.5. A) Two-photon confocal microscopy images (63x magnification) of treated SK-Mel 28 cells. The cells were fixated and stained with DAPI (blue). B) SDS-PAGE analysis of treated SK-Mel 28 cells showing both the coomassie brilliant blue stained gel and laser scanning of the gel with appropriate excitation and emission wavelengths for FITC. 1 µg BSA-FITC was run alongside the cell extract samples in the gel as a size-control. N=2.
Figure 3. The uptake by SK-Mel 28 cells of BSA-FITC in chitosan-TPP nanoparticle depends on the pH of the uptake medium (HBSS). SK-Mel 28 cells were incubated for five hours with BSA-FITC (green) encapsulated in chitosan-TPP nanoparticles diluted in HBSS buffer adjusted to pH 6.5, 6.0 and 5.5, respectively. A) SDS-PAGE analysis of treated SK-Mel 28 cells showing the laser scanning of the gel with appropriate excitation and emission wavelengths for FITC. B) The relative fluorescence intensity of bands in the gel depicted in A. N=2.
Figure 4. Effect of pH on uptake of chitosan:TPP nanoparticles encapsulating FITC-labeled BSA into human melanoma cells. 1-10: Microscopy images, Z-stacks, 1 µm slices. SK-Mel 28 cells were incubated for five hours with BSA-FITC (green) encapsulated in chitosan-TPP nanoparticles diluted in HBSS buffer adjusted to pH 6.5, 6.0 and 5.5, respectively. The pH of the chitosan solution for the preparation of the nanoparticles was 6.0, the molar TPP:glucosamine ratio was 0.100 and the final concentration of BSA-FITC was 0.2 mg/mL. Two-photon confocal microscopy images (63x magnification) of the SK-Mel 28 cells. The cells were fixated and stained with DAPI (blue). N=2.
Figure 5. Uptake of p53-FITC encapsulated in chitosan-TPP nanoparticles by human skin melanoma cells. SK-Mel 28 cells were incubated for five hours with either free p53-FITC (green) or p53-FITC (green) encapsulated in chitosan-TPP nanoparticles diluted in HBSS buffer adjusted to pH 5.5. The cells were fixated and stained with DAPI (blue). Two-photon confocal microscopy images (63x magnification) of treated SK-Mel 28 cells. A) Cells incubated with free p53-FITC. B) Cells incubated with chitosan-TPP nanoparticles encapsulating p53-FITC. N=2.
Table 1. Determination of mean diameter, zeta potential and PDI of BSA-loaded chitosan-TPP nanoparticles after adjustment of the pH of the nanoparticle suspensions. N=3, n=3.

<table>
<thead>
<tr>
<th>pH</th>
<th>Diameter (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
<th>The true degree of dissociation of chitosan**</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8*</td>
<td>188±3</td>
<td>13.7±0.8</td>
<td>0.222±0.008</td>
<td>0.9</td>
</tr>
<tr>
<td>6.5</td>
<td>202±3</td>
<td>21.5±0.4</td>
<td>0.235±0.004</td>
<td>0.7</td>
</tr>
<tr>
<td>6.0</td>
<td>232±4</td>
<td>29.8±0.6</td>
<td>0.235±0.004</td>
<td>0.4</td>
</tr>
<tr>
<td>5.5</td>
<td>257±7</td>
<td>34.6±1.0</td>
<td>0.235±0.003</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* The initial pH of the final nanoparticle suspension after mixing. The pH of the chitosan solution was 6.0 before mixing with TPP/protein.
** The true degree of dissociation is based on the results presented in Figure S2A.