Chitosan-based nanoparticles by ionotropic gelation

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Introduction

Ionotropic gelation between chitosan, as the polycation of acidic pH, and TPP (penta sodium triphosphate aka tripoly-phosphate) as the anionic oligomeric partner leads to the formation of colloidal systems which can range from approximately 100nm to 2000nm. Depending on the conditions, particles with different properties can be obtained including differences in size, zeta potential, stability at different pH values or loading characteristics [Janes 2001 and therein mentioned literature, review article, Agnihotri 2004 and therein mentioned literature, review article]. Such nano-sized colloidal systems are of great interest as drug or active component delivery systems. The electrostatic interaction is particularly suited for the incorporation of biopharmaceuticals for two reasons: First, the formation process is solely based on the electrostatic interaction of oppositely charged polymers; hence, no chemical modification needs to be applied. Secondly, the incorporation can be achieved in aqueous, physiological conditions [Agnihotri 2004]. Incorporated biopharmaceuticals such as proteins or oligonucleotides may be protected against degradation in *in vivo* applications [Aktas 2005, Gan 2006]. Chitosan-TPP-nanoparticles are mainly characterized by a positive zeta potential. Interaction is therefore strong towards any negative surface charge, or negatively charged oligoanions and polyanions. This type of interaction can be used to obtain strong adhesion and immediate immobilisation on negatively charged surfaces e.g. mucosal tissue [Vila 2003]. On the other hand, interactions with anionic proteins and polymers may stimulate the aggregation or precipitation phenomena, limiting the applicability of some chitosan-TPP nanoparticle formulations. Recently presented studies outlined the stability of chitosan-TPP nanoparticles and particularly, the limitations of this particle stability using model body fluids [Gan 2006]. The work we expose in the present paper is a deeper investigation into the stability of this chitosan-TPP nanoparticle system.

Materials and Methods

Chemicals

Chemicals were purchased at Fluka-Sigma-Aldrich, Switzerland and used without further purification; Chitosan was a middle viscous chitosan (Fluka N°28191) and used after filtration via a 0.8µm filter without further purification. Demineralized water was of pyrogene-free, 0.1µm filtered quality.

Preparation of nanoparticles

Chitosan-TPP nanoparticles were prepared according to Calvo et al. [Calvo 1997]. Preparation conditions and treatments after formation for the herein presented samples are summarized in Table 1.

Infrared Spectra

Infrared spectra were recorded on a Perkin Elmer Spectrum One (Perkin Elmer, Switzerland) infrared system equipped with an ATR probe, spectral resolution of $4cm^{-1}$. Besides offset correction and normalization of absorbance values no further mathematical data treatment on spectra was performed.

Dialysis

A tangential flow system (Vivaflow 200) operated at 2bar was employed; 0.2µm polyethersulfone membrane; volume of filtrate equalled 4times the initial volume.

Filtration and ultracentrifugation
25mm syringe filters Minisart (Sartorius, Germany) of different nominal pore sizes were employed. For ultracentrifugation, a Beckmann ultracentrifuge XL100 with rotor Ti100 and Beckmann polyallomer tubes were used; settings: 50'000rpm, 50min, 25°C.

Table 1: Preparation conditions of chitosan-TPP nanoparticles.

Name	0.1% chitosan (parts)	0.1% TPP (parts)	Additional treatments	Comments
ChTPP	5	1	None	pH 5
ChTPP-D	5	1	ChTPP dialysed	pH 5
ChTPP-PS	5	1	ChTPP-D set to pH 7.4 with NaOH(aq)	Precipitate and supernatant
ChTPP-P	5	1	ChTPP-PS's supernatant decanted and washed with dimin. water	Precipitate

Results and discussion

Chitosan-TPP nanoparticle preparation and characteristics
The chitosan's molar mass, its degree of deacetylation and polanion/polycation ratio all have an effect on nanoparticle size as seen on chitosan-DNA particles [Lavertu 2006]. In the case of chitosan/TPP nanoparticles, decreasing the chitosan to TPP ratio provokes an increasing turbidity, indicating a shift in the size variation of the particles to the larger end of the scale. Adding an excess of TPP (according to our experiments approx. >30%) to a nanoparticle dispersion culminates in a clear flocculation of the nanoparticles, which have a tendency to aggregate once all their surface charges have been negated by excess polyanion. The 5:1 chitosan to TPP ratio was confirmed as a stable and suitable ratio to carry out the following analyses of stability and composition, as done by Calvo et al. [Calvo 1997]. In effect, a 5:1 chitosan to TPP ratio is high enough to observe a colloidal system, but not too high as to drag the zeta potential of the particles too low. Having a sufficiently high zeta potential is extremely important when accounting for the role of nanoparticles as carriers for drugs or proteins; the nanoparticles must be capable of ionically holding active molecules or biomolecules [Aktas 2005, Gan 2006]. Figure 1 shows the consequences of the size distribution on the fractions, after a series of filtrations. Infrared spectra reveal no differences in the chemical nature of each fraction, apart from the last fraction, collected after 0.1µm filtration where no TPP peaks could be detected. This anomaly may originate from small aggregates of secondary material within the chitosan sample. Influence of purity of primary material will be addressed in further studies.

Effect of dialysis on a chitosan-TPP preparation
In Figure 1, dialysed and undialysed samples of chitosan/TPP nanoparticles were submitted to sequential filtration in progressively smaller filters to demonstrate the size dispersion of the particles. What can be noticed from this figure is that a fairly large portion of the nanoparticles are found in the lower end of the size spectrum; approx. 80% of the particles are under 0.45µm in ChTPP and ChTPP-D. The difference in the relative concentration at 0.1µm for ChTPP and ChTPP-D (approx. 40%) is attributable to the dialysis treatment which removes uncomplexed chitosan and the smallest nanoparticles or aggregates which would remove weight from the undialysed sample. Dialysis remains an effective way of removing excess chitosan from nanoparticle preparations, as illustrated by the spectral differences of ChTPP and ChTPP-D in Figure 2: Their chitosan peaks in the 1700-1500cm^{-1} region are of similar intensity due to the normalization, but the predominantly TPP representing peak at 890cm^{-1} is increased for ChTPP-D.

Research into the stability of the nanoparticles should be done with the closest simulation possible of the internal biological environment. In doing so, each step towards the determination of stability must have at least one parameter changed to suit the conditions of an *in vivo* environment. This aspect of reproducing the internal environment of the body is a recurring theme in this paper, which

is undeniably important if prospects of *in vivo* usage of nanoparticles are to be considered.

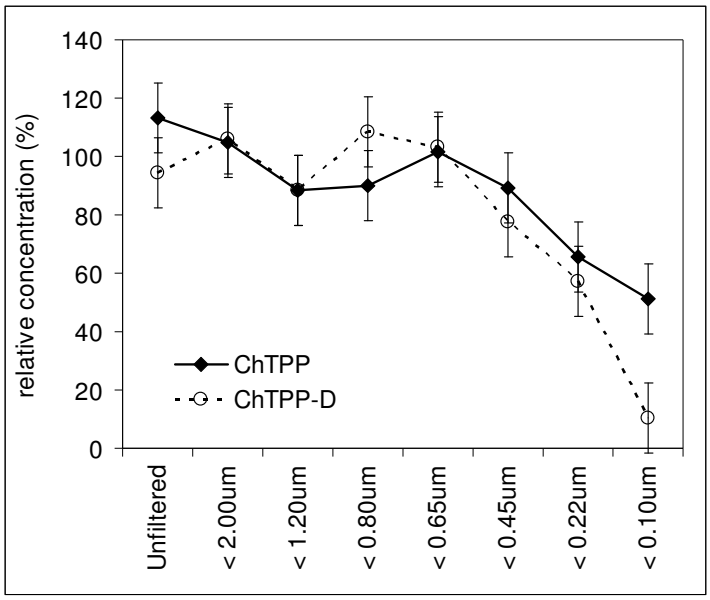

Figure 1: Graph illustrating the relative concentration of nanoparticle fractions, from 2.0µm to 100nm. Two samples were analysed; a dialysed (ChTPP-D) and a non-dialysed (ChTPP).

Stability in 0.9M NaCl solution

In the light of the first argument the ChTPP-D sample was assessed for stability over time and submitted to physiological salinity; keeping the pH values unchanged at pH approx. 5. ChTPP-D was stable after 48h at 37°C and with no noticeable change when salinity was raised to 0.9M NaCl in the sample and left for 48h at 37°C.

Stability at physiological pH

After the post-dialysis stability tests carried out at physiological temperature and salinity, assessing stability at physiological pH (pH 7.4) remained as the other obvious parameter to be investigated. As observed by Gan et al. [Gan 2005] there is an inherent instability of the particles around pH 7. At this pH value the zeta potential is critically low, associated with partial chitosan deprotonation (pK_a 6.2) and particles quickly aggregate to form micrometric complexes. This phenomenon was confirmed when the pH of ChTPP-D was modified to 7.4. Here, precipitation took place practically as soon as the pH neared 7. As shown by Table 1, in one sample the precipitate and supernatant were isolated – then freeze dried (ChTPP-PS) and as for ChTPP-P the supernatant was discarded and the precipitate washed several times with demineralized water before being left to dry. Both samples were analysed via ATR FT-IR spectroscopy. These spectra are seen on the right of Figure 2. The spectrum for ChTPP-P is not a chitosan precipitate, but clearly a complex of our two partners, chitosan and TPP, seen respectively by the 1700-1500cm^{-1} region and the 890cm^{-1} peak. This may be surprising, as chitosan is known to be deprotonated at pH values above its pK_a, so it would be expected to precipitate. However, there are obviously stabilising ionic forces exerted by TPP, strong enough to hold at elevated pH values and maintain the integrity of the chitosan-TPP complex.

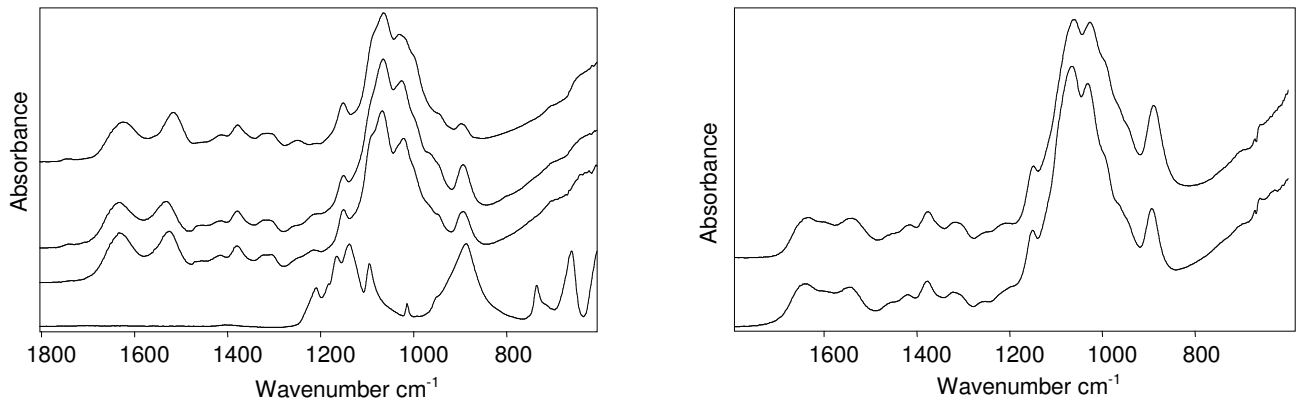

Figure 2: Left: From top to bottom: Chitosan-HCl, ChTPP-D, ChTPP, TPP. Absorbance of chitosan-HCl, ChTPP-D and ChTPP spectra were normalized for equal value of the 1377cm^{-1} peak. Right: From top to bottom: ChTPP-PS, ChTPP-P. Absorbance of spectra were normalised for equal value of the 1377cm^{-1} peak.

Temperature effect on precipitation behaviour
Changing the pH to 7.4 was repeated at 3 different temperatures: 4, 23 and 37°C to observe kinetic effects of this reaction. What came out of this analysis is that nanoparticle stability is heavily dependent on temperature effects. The reaction at 4°C was the most relevant to illustrate this point as the preparation had to be left overnight for full precipitation to be observed, whereas the preparation at 37°C precipitated within minutes.

Stability in serum
A step closer towards an *in vivo* environment involves of using serum as a dispersant for the chitosan-TPP nanoparticles (Fe supplemented calf serum; Sigma C-8056). Elevated pH and the presence of potentially negatively charged proteins unfavour the stability: The nanoparticles tended to precipitate within a few seconds. IR spectra reveal that the precipitate consists of chitosan, TPP and proteins (spectra not presented) underlining the sensitivity of protein-nanoparticle interaction for nanoparticle stability in *in vivo* environments.

Conclusion

The biological properties of chitosan make it an ideal candidate to create nanoparticles for medical applications, although, in order to achieve this, the nanoparticle system needs to be refined to allow an enhanced longevity in the physiological environment. The promising find of the chitosan-TPP precipitate at pH 7.4 suggests that even if the colloidal system is unstable at this pH, the TPP seems to allow the existence of a chitosan-TPP complex in physiological neutrality. The kinetics of this precipitation is strongly determined by temperature and becomes more unfavourable at higher values; therefore care must be taken to carry out experiments at physiological temperatures to ensure a correct assessment of the nanoparticle stability. It is clear, chitosan/TPP nanoparticles remain a tool of great potential, but nonetheless, further research must still be accomplished in order to allow a medical application other than immediate adhesion to e.g. nasal mucosa.

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