A review on factors affecting chitosan nanoparticles formation

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Abstract. Chitosan has been widely used as an excipient in the pharmaceutical industries, due to its biodegradable, biocompatible, low toxicity, and mucoadhesive properties. Chitosan nanoparticles have been extensively investigated for delivery of drugs, herbal products, proteins or peptides and genes. The particle size of chitosan nanoparticles has an important effect on their properties in its pharmaceutical application. Smaller particle size can entrap higher concentration of therapeutic agents, improve drug stability and its bioavailability, and provide sustained delivery. Although diverse efforts have been made to obtain the chitosan nanoparticles and its potential pharmaceutical applications, optimization of the fabricating conditions and the comprehensive properties of the resultant chitosan nanoparticles is still an ongoing important study subject. In this review, we will describe several factors that affect chitosan nanoparticle formation specifically in ionic gelation method, such as chitosan characteristic, i.e. degree of deacetylation, molecular weight, and the ratio of chitosan-crosslinker, type and concentration of crosslinker, mixing procedure, and condition. We will also give an overview of the characterization process of the chitosan nanoparticles.

1. Introduction

Chitosan is an amino polysaccharide biopolymer derive from N-deacetylation processes of chitin [1] and consist of β-1,4-linked glucosamine and N-acetylg glucosamine residues [2]. Chitosan has diverse biological activities, such as antimicrobial [3-5], antioxidant [6,7], anticancer [8,9], anti-inflammation [10,11], immune stimulant [12] and wound healing properties [13]. These activities are assume due to the free amino groups in its molecule which had positive charge at acidic pH, hence it can interact easily with polyanionic molecules, such as proteins, DNA, or phospholipids [2]. Chitosan had renewable, sustainable, nontoxic [14], biodegradable and biocompatible characteristic [15-17]. It can encapsulate various compounds, either hydrophobic or hydrophilic [18]. Therefore, chitosan is widely developed as potential drugs carrier.

The development of chitosan nanoparticles (CNPs) for active pharmaceutical ingredients (APIs) encapsulation has been developed rapidly. CNPs is a potential drug carrier, as it can controls drug release, improves the solubility and stability of the drugs, enhances efficacy, and reduces toxicity. The small particle size makes it easier to pass through biological barriers and improve drug targeting to enhance the efficacy of the drug. CNPs had positive charge in its surface and mucoadhesive properties that allow the particles to adhere on the mucus membranes and release the drug molecules [19]. CNPs also enhance the penetration of the APIs through the epithelium by opening the tight junctions, which will facilitates paracellular and transcellular transport [20].
This review will give an overview regarding parameters that affect the production of CNPs and its characterization, since the fabrication of nanosized particles is pivotal for the successful administration of the APIs. It will focus especially on the production of CNPs through ionic gelation process.

2. Factors affecting chitosan nanoparticle formation

CNPs can be prepared by several methods, i.e., ionic gelation, emulsification cross-linking, reverse micellization, spray-drying, and nanoprecipitation [21-23], modified ionic gelation with radical polymerization, emulsion droplet coalescence, emulsion solvent diffusion and desolvation [15]. Among them, ionic gelation is the most common method used in CNPs production, because of its ease of formation, fast, low cost [21], and absence of organic solvent [24].

In ionic gelation method, the CNPs are formed (Figure 1) through complex formation of the positively charged amino groups in the chitosan molecule and the negatively charged groups of polyanion or crosslinker [25]. The amino group of chitosan will be protonated to form -NH$_3^+$ when dissolve in aqueous acidic solution. When the cationic charges chitosan added with polyanionic solution dropwise under constant stirring, it will form spherical hydrogel particles known as CNPs [21]. Parameters that reported affect the formation of CNPs in the ionic gelation method are molecular weight (MW), degree of deacetylation (DDA) and concentration of the chitosan, the type and amount of crosslinking agent, mixing procedure, and the mixing condition, such as pH and temperature.

![Figure 1. The illustration of CNPs Synthesis through ionic gelation using TPP as crosslinker.](image)

2.1. Chitosan

DDA and MW of chitosan affect the properties and functionality of CNPs [26]. DDA value correlate with the amount of free amino group present in the chitosan [27]. The free amino group will be interacting with the anion groups in the crosslinker to form CNPs. Chitosan with high MW and DDA reported to form CNPs with lower drug release rate [28], while chitosan with a low DDA tend to degrade faster, hence they had faster drug release profile. One of factor that affecting chitosan degradation is variation of the acetamide distribution in the molecule [29].

Chitosan has linear nonbranched structure, with MW varied from 300 to over 1000 kDa. In acid solution, chitosan with higher MW had higher viscosity and behaves as a pseudoplastic material [30]. Xu and Du [31] reported that chitosan with higher MW had greater encapsulation capacity, because the molecule had longer chains which can entrap more drug molecule. CNPs produce using high MW chitosan also had lower drug release rate.

The DDA value of chitosan affecting the particle size [30, 31], zeta potential [30], encapsulation efficiency and release rate of CNPs [31]. The DDA value indicate the percentage of the deacetylated amino groups in the chitosan molecules, and it is increased as the function of the percentage of amino groups to acetylated groups. The density of the positive charge in the CNPs surface is correlate with DDA value and it is affecting the zeta potential. The potency of the chitosan molecules to cross-link with TPP was higher when the positive charge increase, which eventually led to smaller CNPs [25, 30,
Chitosan which had higher DDA value with same MW provides more compact CNPs due to the greater number of ammonium groups interact with polyanion, hence it had lower CNPs permeability which results in slow release rate. Higher DDA value also reported to increased encapsulation efficiency [31].

Sreekumar et al. [2] reported that chitosan concentration also had impact on the average of hydrodynamic diameter of the CNPs at a molar ratio of 1.5 and this effect is stronger when the chitosan had high DDA value. But when a lower molar ratio was used, i.e. 1, the influence of chitosan concentration on the average of the CNPs diameter is stronger when a low DDA chitosan is used. It also showed that chitosan with low (10%) or high (60%) DDA had less control over the CNPs production process. Hence, the CNPs production with average diameters can be controlled by simply changing the chitosan and TPP concentration to get constant chitosan-TPP ratio, if the DDA value of the chitosan was in the range of 20–50%.

2.2. Crosslinker

Chitosan is a linear amino-polysaccharide consisting of 2-deoxy-d-glucosamine and 2-deoxy-N-acetyl-d-glucosamine units linked by glycosidic β-(1→4) bonds [32]. It has amine and hydroxyl groups as the active sites to form linkages comprise of Schiff base formation, amide, or ester bonding, which lead to the formation of chitosan hydrogels [33]. Chitosan has pH sensitive behavior due to the amine groups in its molecule. Hence, chitosan dissolves and depolymerizes in acidic solution. The depolymerization is a major downside for controlled oral delivery, but it can be circumvent using crosslinking agents.

Cross-linking affects polymer properties such as dimensional stability, mechanical properties, chemical stability, swelling, aqueous permeability, and solubility [34, 35]. Crosslinkers that are used in the formation of CNPs via ionic gelation method called polyanions. The anion groups of crosslinker have electrostatic interaction with the amine group of the chitosan, which will weaken the chitosan surface charge and reduce its solubility leading to the spontaneous CNPs formation. These polyanions can be classified into three groups (a) low MW ions, i.e., sodium pyrophosphate [SPP], tripolyphosphate, tetrapolyphosphate, octapolyphosphate, and hexametaphosphate; (b) hydrophobic, i.e., sodium alginate and k-carrageenan; and (c) high MW ions, i.e., octyl, dodecyl, hexadecyl, and cetyl stearyl sulfate [30]. The polyanions type reported to affect the production time of the CNPs, particle size, polydispersity index (PDI), drug loading capacity, stability, mucoadhesiveness, and drug release profile.

It was reported that the polyanions variation, i.e. MW and binding site, affects the size, PDI, and loading capacities of the CNPs. Thandapani et al. [23] compared two type of crosslinkers, i.e., sodium tripolyphosphate (STPP) and sodium hexametaphosphate (SHMP), in the preparation of CNPs. It showed that CNPs-STPP had smaller particle size compare to CNPs-SHMP[1,23]. It was because SHMP had six negative charges in neutral and slightly basic solution which present readily available interaction with amino groups of chitosan compare to STPP. SHMP also had stronger ionic complexation and higher interaction with chitosan because it had more binding sites [23]. It was also assumed that HMP is core-ionotropic crosslinker, while TPP acts in the surface. Hence, TPP yields electrostatic interaction to the loose cationic chitosan layers directly underneath CNPs surfaces leading to the subsidence of the particle size, while the core layers of the chitosan will be denser and harder to compress by HMP electrostatic effect thus it forms bigger CNPs. In comparison, polyphosphoric acid (PPA) that work as both core/shell crosslinker had smaller CNPs compare to CNPs-TPP and CNPs-HMP. Triwulandari et al. [36] reported that CNPs-STTP had the smallest particle size compared to CNPs-alginate, CNPs-carrageenan, and CNPs-sodium dodecyl sulfate (SDS). CNPs-SDS had the largest particle size because of the influence of the MW of the polyanion, while STTP has the smallest MW. It was also reported that the PDI value of the CNPs formed using different polyanions show similar result, except for particles formed using SDS, which had higher tendency to aggregate. Saeed et al. [1] and Sang et al. [37] reported that CNPs prepared using polyphosphoric acid (PPA), hexametaphosphate (HMP), and TPP had different encapsulation efficiency. CNPs-PPA and CNPs-HMP have higher encapsulation efficiency compared to CNPs-TPP when used to encapsulate model drugs, i.e. methylene blue and doxorubicin. It was also reported that CNPs-PPA and CNPs-HMP had slower drug release rate compare to CNPs-TPP.
Crosslinker MW also affect the time needed in cross-linking process. CNPs-sulfate and CNPs-citrate had faster reaction compare to CNPs-TPP, it was assumed due to its smaller molecular size. But CNPs-TPP had stronger mechanical strength than that of CNPs-sulfate or CNPs-citrate. CNPs-sulfate and CNPs-citrate swelled and segregated in the simulated gastric fluid (SGF), it led to the release of drug molecules which is completed in 5 hours. While in simulated intestinal fluid (SIF), they kept in the shrink stage, therefore the drug released profile was slower. In comparison, CNPs-TPP swelling and drug release was reported to be insensitive toward media pH [38].

2.3. Mixing procedure
Colloidal systems have tendency to aggregate because of the attraction force between colloidal particles may surpass the electrostatic repulsion energy [39]. The formation of aggregates can greatly obstruct the redispersion process of the lyophilized samples and affecting the particle size [40, 41]. The homogeneity of the mixture and turbulent force in the suspension is directly correlate with stirring process [42]. But high stirring rate could cause a less uniform energy distribution that lead to a bigger particle size [43].

Several articles reported that the increasing stirring speed resulted in to the formation of smaller particle size [23,25, 44]. It was assumed that the increasing shearing force accelerates the dispersion of TPP in chitosan solution and resulted in monodisperse particles. Thandapani et al. [23] reported that increasing stirring speed from 200 rpm to 600 rpm drastically reduced the particle size of the CNPs from 210 to 50.75 nm for CNPs-TPP and 333 to 122.4 nm for CNPs-SHMP. Stirring speed above 800 rpm showed no significant change in the particle size, while agitation speed below 400 rpm resulted in bigger CNPs size. But higher stirring speed, around 1000 rpm, caused particle aggregation because intense stirring could destroy the repulsive force between particles [44].

There are several alternatives of chitosan-TPP mixing method, i.e., dropwise addition, single-shot mixing and dilution. Among them, dilution method showed the most noticeable particle size gradual increase, whereas dropwise addition and single shot mixing induced particle size sharp increase. Dropwise addition showed the biggest abrupt increase. The probable reasons for these results was still unclear. The dilution method assumed to offers the most precise control over the formation of particle size with the average size. The PDI-values of CNPs sample from three mixing methods were not different significantly, i.e., 0.19 for the dilution method, 0.17 for the single shot and 0.13 for the dropwise addition methods [45].

2.4. Mixing condition
Shu and Zhu [46] reported that the electrostatic interaction between the polyanions and chitosan during the formation of CNPs was depend on the pH of the mixing solution. The electrostatic interactions only occur in certain pH region which correspond with the anion’s natural characteristics, for examples 1.0–7.5 for CNPs-sulphate, 4.5–7.5 for CNPs-citrate and 1.9–7.5 for CNPs-TPP.

Liu and Gao [47] reported that pH also affecting particle size and zeta potential of CNPs. The particle size growing rapidly from pH 1 to 3.5 and then decreased slowly from pH 3.5 to 5.5. The effect of pH to zeta potential was similar, with a slightly higher transition at pH 4, the zeta potential value increased from pH 1 to 4, then reduced slowly from pH 4 to 5.5. It was because in acidic condition the amine groups of chitosan were protonated that lead to strong charge repulsion and molecule extension [48,49]. In low pH condition, TPP molecule also got protonated and had low charging density. Hence, the chitosan molecules cannot crosslink sufficiently with TPP to form stable CNPs in acidic condition. It was concluded that the stable CNPs with a suitable size was form at pH 3.5. At higher pH value, the charging degree of TPP molecule enhanced and neutralize the chitosan charge to a greater extent, which lead to the shrinkage of CNPs size. At pH value of 4.5 to 6.0, the CNPs had tendency to decrease its particle size and zeta potential, thus form relatively unstable particles. It suggested that the chitosan positive surface charge is shielding because of reorganization of the molecule structure or/and adsorption of other negative charged ions at low pH.
Torres et al. [49] reported that the CNPs protein adsorption capacity was depends on the solution pH. The BSA adsorption capacity of CNPs-glutaraldehyde decreased with the increase of pH, from 6 to 7.5, because of the electrostatic interaction. The optimum adsorption capacity was reported at its isoelectric point, pH about 4.8, because at this pH value, the substrate has a high charge density which will increase the adsorption capacity. The lysozyme adsorption capacity also reaching maximum at around the isoelectric point of the lysozyme. This result was different with Katas et al. [50], which reported that it showed optimum binding capacity at above its the isoelectric point, although the crosslinker used also different. Katas et al. [50] reported that the entrapment efficiency (EE) of BSA in the CNPs-dextran was affected by solution pH and the optimum pH was at 5.5. When protein dissolved at pH above its isoelectric point, it tends to negatively charge and had ionic interaction with positively charged amino groups which favors the entrapment of BSA into the CNPs. Thus, further research is needed to confirm these findings.

Several studies reported that the CNPs formation also being affected by temperature [23, 51, 44, 52] Gomathi reported that when CNPs-SHMP was produce using stirring at 600 rpm at the concentration of 5mg/ml it obtained the smallest CNPs when produced at ambient temperature (25°C) compare to higher temperature range (40, 50, 60 °C). CNPs obtained in higher temperature were easier to agglomerate as the result of the increased size and yield. Kamat et al, 2016 also reported that lower temperature produced smaller particle size. CNPs was smaller when produce at 4 °C with chitosan concentration of 0.2 and 0.4 mg/mL, while when the concentration 0.8 mg/mL the particle size was not different significantly when it was compare to CNPs produced at 27 °C and 35 °C. CNPs produced at 4 °C were smaller (60–80nm) and with narrower size distribution, compare to the one produced at 35 °C which had larger size (130–190nm) and broader size distribution, it was also easy to agglomerated.

Tsai reported that mixing procedure also had effect to the optimum temperature to obtain smallest CNPs. In the CNPs production by mechanical shearing at 1000 rpm, the best temperature to produce the smallest CNPs were at 45 °C (145 nm), followed by 4 °C (150 nm), and 25 °C (163 nm). But it may be because the larger CNPs was removed during centrifugation thus the smaller CNPs remained than the other CNPs form in the temperature of 4 °C or from 25 °C ones. Meanwhile in ultrasonic radiation method, the CNPs average diameter decreased as the function of the increasing temperature. The viscosity of the solution decreasing as the temperature increase thus aid the sporadic cavitation effect wield on the chitosan molecules producing smaller MW fragments.

Report from Gomathi and Tsai were contradicted with the finding by Fan. Fan reported that CNPs produced from 0.5 mg/ml low MW chitosan solution with agitation at 700 rpm, the increasing temperature from 10 °C to 60 °C produced lower CNPs size, but when the temperature was higher than 60 °C the decrease of particle size was negligible. The homogeneity also getting better with the increasing temperature. It showed that when the temperature was 10 °C the PDI value was 0.112 and it get lower than 0.05 at 25 °C and 70 °C. Thus, further study is needed to confirm the effect of several factor at once on the formation of CNPs.

3. Characterization of chitosan nanoparticles
The characterization of CNPs is an important step to curb the evaluation of the particle’s property. Several parameters that commonly used in CNPs characterization are particle size and surface charge, morphological and surface characteristics, encapsulation efficiency, loading capacity, and in vitro release profile (Table 1). These parameter could give insight to the stability, physicochemical performance, and also cell uptake of the CNPs [53, 54]

Particle size can be evaluated using non-imaging and/or imaging-based technique, the option depends on the predicted size and population of the CNPs. Non imaging technique is based on light scattering, either Static Light Scattering (SLS) and/or Dynamic Light Scattering (DLS). These methods are fast, precise, sensitive, and utilisable for a wide range particle size measurement [55], [55], but the downside of these technique is they cannot provide information about particle morphology and the sample need to be meticulously prepared. It is advised to be complement non-imaging techniques with imaging technique and vice versa [56]. Imaging technique based on electron microscopy can be used to evaluate...
CNPs shape and morphology, beside its particle size [57, 58]. There are two commonly used electron microscope used for this analysis, i.e., scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM gives image of the sample surface, while TEM gives better resolution and image of the inner structure of the CNPs. The CNPs surface and morphology obtained from imaging techniques can be used as one of parameter to deduce the dissolution behavior or in vivo responses [59]. But the electron microscopy technique come with several disadvantages such as longer preparation time, expensive, cannot provide information about the particle size distribution and destructive sample preparation.

Table 1. Assessment techniques for physicochemical characterization of CNPs.

| Characterization technique          | Parameters                                                                 |
|-----------------------------------|-----------------------------------------------------------------------------|
| Imaging based technique           |                                                                            |
| SEM                               | CNPs size, size distribution, surface structure, stability                  |
| TEM                               | CNPs size and size monodispersity, shape and inner structure, stability     |
| Non-imaging technique             |                                                                            |
| DLS                               | CNPs size and size distribution                                            |
| SLS                               | CNPs size, mass-weighted particle size, particle shape and structure        |
| Spectroscopy based technique      |                                                                            |
| UV-Vis spectroscopy               | Optical properties, size, concentration, agglomeration state, hints on CNPs shape |
| Raman spectroscopy                | Determine the interactions between the API and its excipients              |
| FTIR                              | Determine surface composition, ligand binding, the interactions between the API and its excipients |
| Zeta potential                    | Surface charge                                                             |
| Thermal analysis                  |                                                                            |
| DSC                               | Identify modification and physical qualities of the materials              |
| TGA                               | Determine the precise nature of the thermal transitions of the samples     |
| X-Ray technique                   |                                                                            |
| XRD                               | Determine the crystallinity of the sample, Size (structural properties); the atomic and molecular level structure of the material |

The CNPs surface charge gives an overview about the physical stability and redispersibility of the formulation as well as their in vivo performance. It can predict the formulation long-term storage stability profile. Zeta potentials is the net charge of the particles, which reflects the electrostatic repulsion or attraction degree between charged particles. High zeta potential value, above ± 30 mV, indicates a stable dispersion system because of the resistance of the particles to aggregate or high repulsion forces [60]. Zeta potential also can be used to study the interaction of CNPs with the biological system.

The efficiency of the CNPs preparation and dose calculation can be done through the determination of several parameter such as CNPs yield, encapsulation efficiency (EE), and loading capacity (LC). The drug content of the CNPs should be determined using a suitable analytical method, either based on spectrophotometry or chromatography method. Then the drug EE, LC, and nanoparticles yield are determined by the following equations:

\[
EE \, (\%) = \frac{Amount \, of \, drug \, in \, definite \, mass \, of \, the \, prepared \, CNP}{Theoretical \, amount \, of \, drug \, in \, the \, same \, mass} \times 100
\]  

\[
LC \, (\%) = \frac{Amount \, of \, drug \, in \, definite \, mass \, of \, the \, prepared \, CNP}{Total \, mass \, of \, the \, CNP} \times 100
\]
Differential scanning calorimetry (DSC) evaluate the modification of the material structure in pharmaceutical formulation based on thermal analysis as a function of temperature. It measures the heat flow, endothermic and exothermic events, i.e., heat uptake or heat emission. It also can be used to determine the physical qualities of the materials. While thermogravimetric analysis (TGA) measures sample mass during the heating process and used to determine the precise nature of the thermal transitions. It is a very convenient method for discrimination of hydrates or substance decomposition from its melting point, polymorphic transitions, and loss of water. It can also be used to determine temperature-dependent weight measurement [56].

Spectroscopic methods, such as X-ray diffraction (XRD), infrared and Raman also often used in CNPs characterization. XRD give information about the atomic and molecular level structure of the material. It also can characterize crystalline size, shape, and lattice distortion of the material. But for liquid sample, it needs to be prepared in the dry state to eliminate the moisture. Both IR and Raman are vibrational spectroscopic techniques. IR spectroscopies operate in IR wavelength region, while Raman is in the visible, near IR or near-ultraviolet (UV) wavelengths. IR can be used to determine the interactions between the API and its excipients, based on the ability of the molecules to absorb certain frequencies, either the transition energy of the vibrating bond or functional group in the molecule. Raman technique detect the vibration, rotation and other low-frequency transitions of the materials [56].

In vitro drug release test determines the profile of the drug released from CNPs in the simulation media. The release mechanism generally depends on the location of the drug molecules in the CNPs, whether in the encapsulated in the particle core or adsorbed in the CNPs surface. The encapsulated drug needs to diffuse from chitosan matrix or released after the degradation and erosion of the chitosan to determine its release profile [55]. Other parameter that also affecting drug release profile are chitosan MW and DDA, CNPs particle size, cross-linking degree of chitosan-polyanion, other excipients in the formulation, polarity, pH, and enzymes in the dissolution media [21, 61].

4. Conclusion

The most common method for CNPs production is ionotropic gelation, because of its simplicity, rapid, and lower cost. The CNPs hydrogel production through ion gelation method is based on ion interaction between the cationic groups of chitosan and the anionic groups of the crosslinker. Parameters that affecting the CNPs formation are chitosan MW, DDA and concentration, the type and amount of crosslinking agent, mixing procedure, and production condition, such as pH and temperature. The quality of the CNPs is evaluated based on several parameters, such as particle size, zeta potential, morphological and surface characteristics, encapsulation and loading efficiency, and drug release profile.

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