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Chitosan Based Nanomaterials in Plant Growth and Protection

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Abstract

The aim of this brief is to explain the synthesis, properties and potential uses of chitosan-based nanomaterials in plant protection and growth. Precise assessment of chitosan-based nanomaterials' synthesis and properties are mentioned. A description of various factors which affect their synthesis and characters has been elucidated. Biological activities like antimicrobial activity (e.g. antifungal-antibacterial activity) including the mode of action have been discussed. In addition, the effect of chitosan-based nanomaterials in plant growth is also pointed out. Authors summarize the plant protection and growth regulatory applications of chitosan nanomaterials. Current and possible utilization of chitosan nanomaterials in plant nutrition, abiotic stress management and post-harvest application is also highlighted. The authors have highlighted their own research views and concluded the recent and future prospects of chitosan nanomaterials' applications in plant protection and growth.

Keywords

Preface

The term 'Nanotechnology' comprises the understanding of the fundamental physics, chemistry, biology, and technology of nanometer scale objects. In recent years, it has become one of the most important and exciting forefront fields in science. It shows great promise for providing us in the near future with many breakthroughs that will change the direction of technological advances in a wide range of applications. Of late, it has become a popular and well-accepted term and a central pillar in many nationally and internationally funded research programs. Considerable funding has been allocated and invested in the development of this discipline in many countries such as USA, Korea, Japan, Australia, and the European Union.

Life is organized at the level of cells, but it is well known that natural cellular events, interactions, and processes take place at the subcellular scale and at the molecular level. This is the reason why nanotechnology is meant to play a key leading role in developing tools able to identify, measure, and study such events at the nanometric level, as well as in contributing to the disclosure of unknown biological interactions and mechanisms.

Taking into consideration the above facts and views and given the importance and potential impact of nanotechnology in agriculture, this brief document aims to provide an overview of synthesis, properties, and potential uses of chitosan-based nanomaterials in plant growth and protection. Precise assessment of chitosan-based nanomaterials' synthesis and properties are mentioned. The topics presented here were chosen based on the maturity of understanding of the subjects, their potential for applications, and/or the number of already existing applications. Many of the chapters discuss present and future possibilities. General references are included for those who wish to further pursue some of the areas in which this technology is moving ahead. The book is divided into five chapters. The book starts with the introduction, importance, and the potential capacity of bulk and nanochitosan in the agriculture sector. Second chapter provides full description of methodologies followed for the synthesis and characterization of chitosan-based nanomaterials with state-of-the-art figures. The other chapters are dedicated to the biological activities of chitosan-based nanomaterials.

This mini book provides established scientists, junior researchers, and students involved in nanotechnology research with a sound foundation of a wide variety of approaches in agriculture sector. We would like to thank PhD Scholars of Nano research facility lab, Ram Chandra Choudhary, Kumaraswamy R.V., Manju Kumari Choudhary, Sarita Kumari and Swati for their contributions to this book. We sincerely acknowledge Dr Ramesh Raliya, Research Scientist, Department of Energy, Environmental and Chemical Engineering, Washington University in St. USA for valuable suggestions.

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Chapter 1
Introduction

Recent advancements in the synthesis of various nanomaterials of different sizes, shapes and functions have established nanotechnology as an indispensable technology for agriculture (Khot et al. 2012; Saharan et al. 2014). Materials which show unique properties linked to their size (ranging from 1 to 1000 nm at least in one dimension) are considered nanoparticles and deemed under nanotechnology (Buzea and Robbie 2007). Nanomaterials have some high value properties like high surface-to-volume ratio, more molecules/reactive groups on surface; prefer nano-encapsulation and are independent of gravity. These unique properties of nanomaterials offer a vital role in agriculture, especially in plant growth and protection. It is predicted that in coming decades the progress in agriculture will be expedited by nanotechnology. Though nanotechnology is less explored in agriculture in general, but substantial research has been done in crop protection (Park et al. 2006; Jo et al. 2009; Kim et al. 2012; Nair et al. 2010; Sharon et al. 2010; Ghormade et al. 2010; He et al. 1999; Lamsal et al. 2011; Kim et al. 2012; Perez-de-Luque et al. 2012; Jayaseelan et al. 2013; Wani and Ahmad 2013; Saharan et al. 2013).

Among the various nanomaterials, metal-based nanoparticles have been intensely used in agriculture as manifested by accumulation of publications (Park et al. 2006; Panacek et al. 2009; Nair et al. 2010). But, reports on phytotoxicity of metal nanomaterials have raised conscientious situation (Lin and Xing 2008; Kumari et al. 2009; Sharon et al. 2010; Dimkpa et al. 2012; Khot et al. 2012). It has led to accelerated cumbersome research to delve the level of toxicity and the faith of metal nanoparticles in crop plants (Thuesombat et al. 2014). Research has been initiated to investigate the biopolymers for use in crop protection through nanotechnology (Shukla et al. 2013; Saharan et al. 2014). The utilization of biogenic components like chitosan biopolymer from living organisms has created a reliable place in biological

sciences due to its biocompatible and biodegradable nature. Its additional high value properties like easy modulation and availability of functional groups mark chitosan a highly researchable biopolymer in various fields (Hadwiger, 2013).

1.1 Bulk and Nano-Chitosan

Chitosan, a biopolymer of glucosamine and N-acetyl glucosamine residues, is a de-acetylated product of chitin. It is available in abundance with economical cost from the waste exoskeleton of crab and shrimp (Hadrami et al. 2010; Badawy and Rabea, 2011). Chitosan is well-recognized biomaterial in agriculture and successfully applied in many crops (Lerouge, 1990; Reddy et al. 1999; Bittelli et al. 2001; Sharathchandra et al. 2004; Dzung et al. 2011; Zeng et al. 2012). It is known for boosting defense response in plants and also possesses antimicrobial properties (Reddy et al. 1999; Sharathchandra et al. 2004; Rinaudo, 2006; Guo et al. 2006; Hadrami et al. 2010; Liu et al. 2010; Meng et al. 2010; Yin et al. 2010; Zeng et al. 2012; Ma et al. 2013a, b; Chen et al. 2014). With the advancement of nanotechnology, chitosan-based nanomaterials are being largely adapted for their exploration in plants along with other nanomaterials (Table 1.1). Increasing number of publications on chitosan nanoparticles in plant growth and protection

Table 1.1 Use of nanomaterials for crop protection and growth

Purpose	Material	Finding	References
Pesticide	Silica nanoparticles	Pesticide loading	Liu et al. (2007)
Antifungal activity	Silica silver nanoparticle	Effective against powdery mildew of pumpkin	Park et al. (2006)
Abiotic stress	Chitosan and SOD	Synthesis and characterization	Celik and Akbuga (2007)
Antifungal activity	Ag nanoparticle	Effective against *Candida albicans*	
Antifungal activity	Ag nanoparticle	Effective against powdery mildew disease of cucumber and pumpkin	Lamsal et al. (2011)
Antifungal activity	ZnO nanoparticle	Effective against *Compylobacter jejuni*	Xie et al. (2011)
Pesticide runoff	Cu-chitosan nano-composite	Removal of agricultural pesticide	Jaiswal et al. (2012)
Antifungal activity	Ag nanoparticle	Effective against different phytopathogenic fungai	Kim et al. (2012)
Antimicrobial activity	Copper(II) with chitosan solutions	Effective against *Fusarium graminearum*	Brunel et al. (2013)

(continued)

Table 1.1 (continued)

Purpose	Material	Finding	References
Antifungal activity	Chitosan-based nanoparticles	Effective against *A. alternata, M. phaseolina* and *R. solani*	Saharan et al. (2013)
Plant growth	Chitosan nanoparticles	Biophysical characteristics and growth of Robusta coffee	Van et al. (2013)
Postharvest loss	Chitosan/nano-silica	Physicochemical effect on fruit	Shi et al. (2013)
Antifungal activity	Au nanoparticle	Effective against *Candida albicans*	Wani and Ahmad (2013)

infers vast potential of nanochitosan over bulk chitosan (Table 1.1). This is because nanochitosan as compared to bulk chitosan has superior physico-chemical characteristics that provide enhanced biological activities (Saharan et al. 2013; Van et al. 2013). Hence, there is a pressing need to explore chitosan biopolymer not only for its novel antimicrobial and plant defense booster property but also for its role in plant growth to combat future challenges under global warming (Saharan et al. 2014).

Chapter 2
Synthesis of Chitosan-Based Nanomaterials

2.1 Emulsion Cross-linking

Basic concept of this method is to convert the material into microscopic droplets through emulsion and further stabilize the particles into nano size by cross-linking. Water-in-oil (w/o) emulsion is prepared under mechanical stirring by mixing chitosan aqueous solution into oil. A suitable surfactant is used to achieve the stable emulsion. The stable emulsion is cross-linked by using an appropriate cross-linking agent such as glutaraldehyde, formaldehyde, genipin, glyoxal, sulfuric acid, poloxamer, etc. For efficient cross-linking, overnight stirring of the reaction should be performed. Stirring rate is based on the volume of the reaction and desired physical properties of nanoformulation. A translucent solution obtained from overnight stirring is then filtered and washed repeatedly with n-hexane followed by alcohol to remove oil. The surfactant can be removed by precipitation with appropriate salts like $CaCl_2$ followed by centrifugation. Resulted precipitates can then be lyophilized to get a fine dry nanomaterial (Akbuga and Durmaz 1994). Method is explained in Fig. 2.1.

By this technique, a narrow size distribution of nanoparticles can be obtained by varying the amount of cross linker and chitosan. Size of the nanoparticles also depends on the size of droplets formed in emulsion. Hence, before cross-linking, the size of the droplets can also be controlled by adjusting the oil, water and surfactant amount. Stirring speed during cross-linking also plays an important role in the size distribution and nature of distribution of particles. This method is tedious and time-consuming due to emulsion phase. Removal of surfactant and oil is also a wearisome step along with unkind shearing force and complexity in washing step. In addition to above-mentioned drawbacks, there are some benefits like high loading efficiency, controlled release with improved bioavailability and easy to control particle size (Agnihotri et al. 2004).
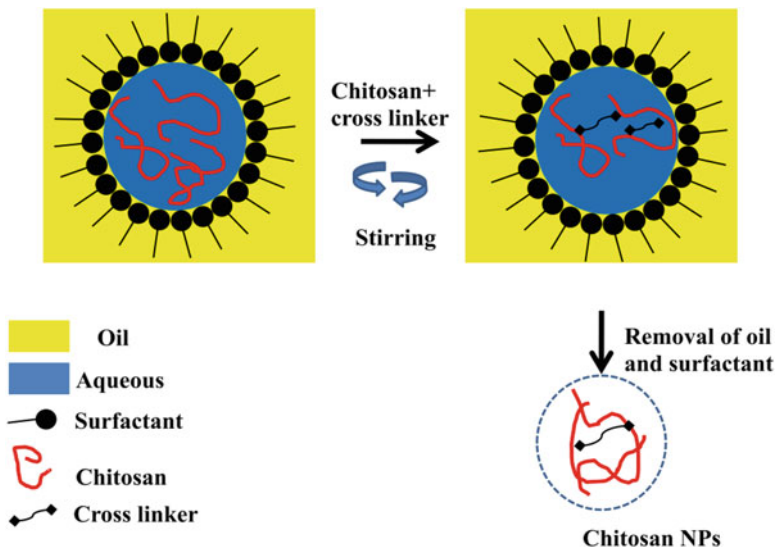
Fig. 2.1 Emulsion cross-linking

2.2   Emulsion-Droplet Coalescence Method

This method utilizes the principles of emulsion and precipitation without cross-linking (Tokumitsu et al. 1999; Anto and Annadurai 2012). First, a stable emulsion is established through aqueous solution of chitosan, oil and active ingredient. In the same way, a stable emulsion of chitosan aqueous solution with NaOH is also prepared. Both emulsions are mixed under high speed stirring to induce random collisions between droplets and subsequent precipitation of chitosan droplets gives small size particles (Fig. 2.2). This method also has same demerits as explained in emulsion cross-linking methods. However, this method does not use cross linker; hence, amino groups of chitosan are available to bind with active ingredients. Therefore, by using this method, it is possible to achieve higher loading efficiency and smaller particle size as compared to the emulsion cross-linking method.

2.3   Ionic Gelation Method

This method is most accepted method for the synthesis of stable, non-toxic and organic solvent free chitosan nanoparticles. The synthesis of chitosan NPs is based on electrostatic interactions between positively charged amino groups ($-NH_3^+$) of chitosan and negatively charged cross linkers (Saharan et al. 2013; Saharan et al. 2015). In this procedure, chitosan is dissolved in week acidic aqueous solution and mix systematically into aqueous solution of negatively charged cross linkers. In this
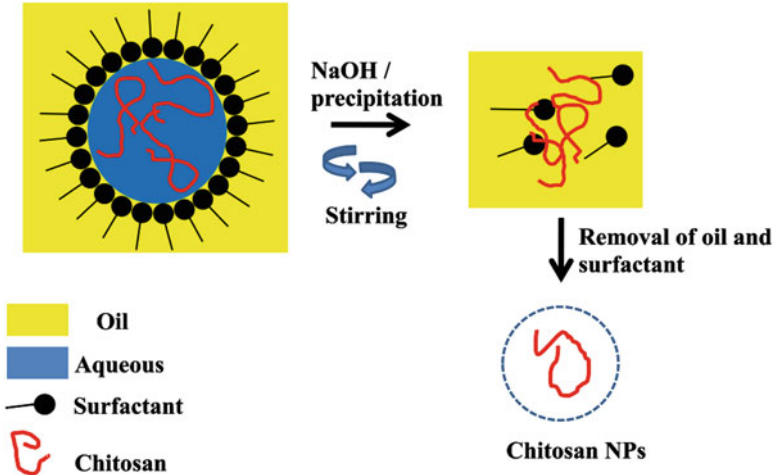
Fig. 2.2 Emulsion-droplet coalescence method
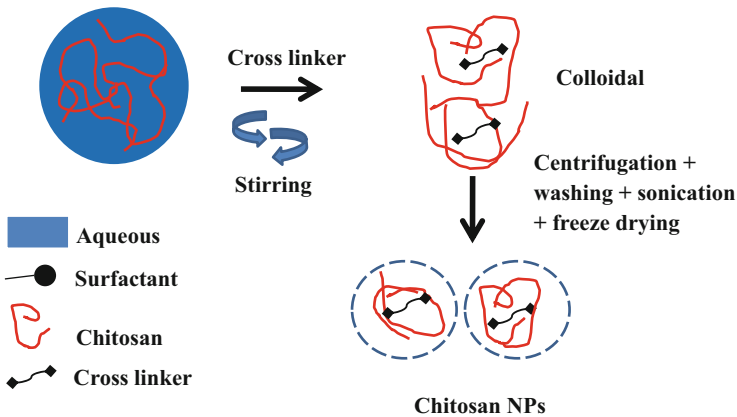
Fig. 2.3 Ionic gelation method

method chemical cross-linking is avoided and a reversible physical cross-linking by electrostatic interaction is performed. Tripolyphosphate (TPP), a polyanion, has become very popular because of its non-toxic property. TPP is added drop wise under steady stirring to acidic chitosan solution. Chitosan undergoes ionic gelation and precipitates to form particles (Fig. 2.3). It is important to understand the volume and concentration ratio of chitosan and TPP as it affect the size and surface charge of particles. In addition, the deacetylation of chitosan is also crucial for particles formation, more is the percentage of deacetylated chitosan, more is the electrostatic interaction. Stirring rate during gelation is also important as it provides equal probability of interaction between opposite charges. This method demonstrates some striking features like easygoing procedure, no chemical cross-

linking, minimum toxic effects of chemicals, no additional steps for removal of oil and surfactant, etc. which make it more reliable and accurate (Jaiswal et al. 2012).

2.4   Reverse Micellar Method

Basic principle of this method is to first establish thermodynamically stable, monodisperse, isotropic reverse micelles. To initiate reverse micellar method, a suitable surfactant and organic solvent is mixed to prepare translucent, fine microscopic micelles droplets solution. For nanoparticles' synthesis, acidic aqueous solutions of chitosan along with active ingredient to be encapsulated are added to isotropic reverse micelles with constant vortexing to avoid any turbidity. In the next step, a cross-linking agent is added to this ultra-fine transparent solution with constant stirring. Subsequent cross-linking is achieved by overnight stirring which leads to the formation of nanoscale size chitosan particles. Dry material is obtained by evaporating organic solvent and surfactant is further removed through suitable salt precipitation. The mixture is then subjected to centrifugation and nanoparticles containing supernatant is further used (Fig. 2.4). Monodisperse, thermodynamically stable, narrow size particles are the most vital aspects of reverse miceller method. However, this method is very comprehensive in making nanoparticles in the range of 50–100 nm as compared to other methods where size range exceeded 200 nm.
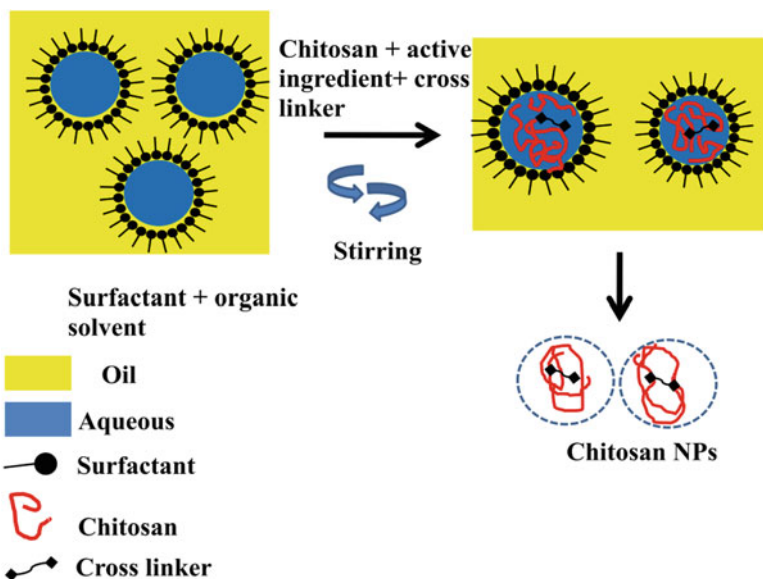
Fig. 2.4 Reverse micellar method

This method has tedious and laborious steps and needs an accuracy in performing the reaction (Brunel et al. 2008).

2.5   Sieving Method

It is still a challenge to produce stable and mono-dispersed chitosan nanoparticles for achieving desirable results. Methods invented till date are not accurate for the synthesis of desirable size of nanoparticles. Owing to this obstacle, filtering of nanomaterials through desirable mesh size was invented (Agnihotri and Aminabhavi 2004). Here, the synthesized chitosan materials are passed through the sieve having fixed size. This leads to the accumulation of filtered material with higher percentages of mono-dispersed nanoparticles (Fig. 2.5).

2.6   Spray Drying

Spray drying method is used for the synthesis of dry powder, granules and pellets of chitosan nano/microparticles. This is an atomized procedure for rapid and mass synthesis of chitosan nanomaterials. In this method, acidic aqueous chitosan solution along with cross linker and active ingredient is passed through a nozzle with hot air stream. Compressed air induces the cross-linking and size reduction of
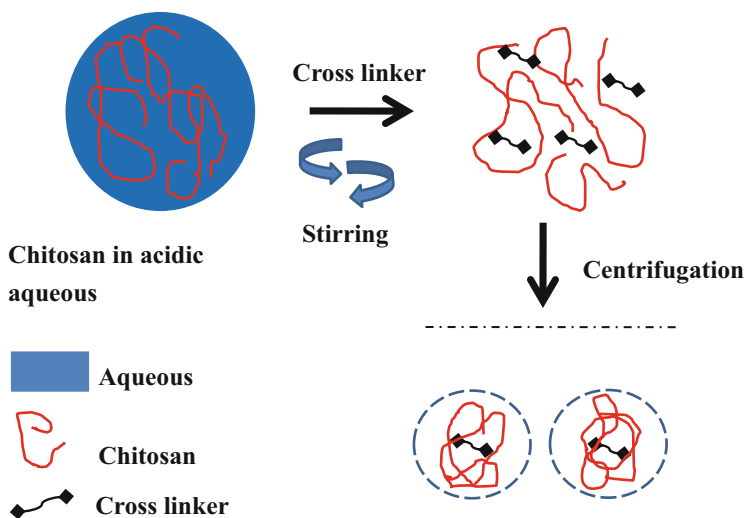
Fig. 2.5 Sieving method
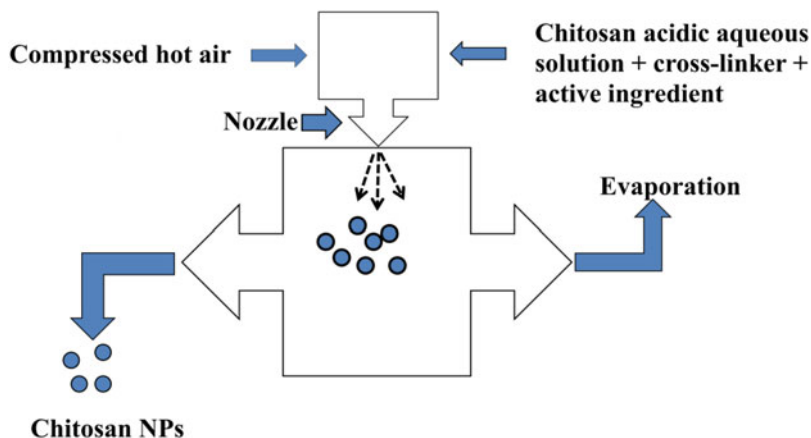
Fig. 2.6 Spray drying method

chitosan. During this process, the solvent is also evaporated and dry form of chitosan is received at the end of the reaction. Size of nozzle, velocity of compressed air, its temperature and degrees of cross-linking of chitosan are some of the vital factors which can amend the size of the chitosan NPs (He et al. 1999). This method is fast with single step, inexpensive and can be used to synthesize particles with or without cross-linking (Fig. 2.6). The encapsulation can also be performed in the same step with higher encapsulation efficiency (Table 2.1).

2.7 Factor Affecting Chitosan Nanomaterial Synthesis

As discussed in previous sections, different methods have been developed to prepare chitosan NPs. These methods have their own advantages and disadvantages based on where they are applied and what kind of physico-chemical features are required. In view of the serious issue regarding increasing toxic chemical load on ecosystem, novel strategies to develop eco-friendly, biodegradable, nontoxic nanoformulation for protection and growth of crop plants are in prime priority (Saharan et al. 2013, 2015). Therefore, more emphasis is on those methods in which stable, controllable, highly active, non-toxic, organic solvent free and easy synthesis of chitosan nanoparticles could be achieved. Amongst the various methods, ionic gelation technique has attracted considerable attention because this process is non-toxic, organic solvent free, uses minimum chemicals, easy in mass synthesis, convenient and controllable (Berger et al. 2004; Agnihotri et al. 2004). In ionic gelation, ionic interactions are ensued between the positively charged primary amino groups of chitosan and the negatively charged groups of sodium tripolyphosphate (TPP). A polyanionic sodium tripolyphosphate (TPP) is the most widely used non-toxic cross-linking agent (Shu and Zhu 2002). This physical cross-linking between chitosan

Table 2.1 Synthesis of chitosan-based nanoparticles: advantages and disadvantages (Agnihotri et al. 2004; Kashyap et al. 2015)

Methods	Advantages	Disadvantages
Emulsion cross-linking	1. Easy control of particle size	1. Need to remove oil and surfactant
	2. Good loading efficiency	2. Cross-linker can react with active ingredient
	3. Good stability of nanomaterials	3. Purification of nanoparticle is tedious process
Emulsion-droplet coalescence method	1. Higher encapsulation efficiency	1. Excess alkali induces more precipitation and leads to increased particle size
	2. No cross-linker leads to higher Zeta potential	2. Due to absence of cross-linker nanoparticles stability decreases
	3. No reactivity of cross-linker to active ingredient	
Ionic gelation method	1. Reduced the chemical side effect	1. Partial size, distribution and stability strongly affected by degree of deacetylation, MW of chitosan and molar ratio of chitosan and TPP
	2. Better control on physico-chemical feature of nanoparticles	
	3. Easy and fast	
Reverse Micellar Method	1. Stable and smaller and monodispersed nanoparticles with suitable polydispersity index	1. Cumbersome procedure
		2. Chance of side effect of reaction components (solvent, surfactant, etc.)
Seiving method	1. Very simple and rapid procedure	1. Need specialized sieve with particular size for desirable size of nanoparticles
	2. Easy mass synthesis	
Spray drying method	1. Mechanized method for mass production	1. Size of nanoparticles depends on nozzle size, flow rate and temperature of air
		2. Temperature sensitive component could not be encapsulation

and TPP evades two things – no use of chemical cross-linkers and avoids the emulsifying agents, both of which are often toxic for biological system. Due to a number of advantages, ionic gelation method is extensively researched amongst the others methods. A number of valuable informative reviews are available for specific details of various factors which affect the synthesis of chitosan-based nanoparticles (Kalotia and Bohidar 2010). Most vital thing related to chitosan NPs synthesis is the production of custom-made nanoparticles for precision applications. Hence, it is imperative to focus on the study of various factors which affect the physico-chemical characters (size, dispersity, zeta-potential, polydispersity index and solubility). Followings are some of the vital factors which affect the physico-chemical characters of chitosan-based nanomaterials.

2.7.1 Concentration of Chitosan and TPP

It is well understood that concentrations of both components affect the synthesis process and physico-chemical features of the chitosan NPs. Before getting into the details, it is important to know the different interaction systems existing in the chitosan solution. In acidic conditions, amino groups of chitosan get protonated and a strong electrostatic repulsion occurs between chitosan molecules which keeps them apart and prevents their agglomeration (Mi et al. 1999; Shu and Zhu 2002). On the other hand, an interchain hydrogen bonding (intermolecular interaction) between chitosan molecules tend to bring them together. Hence, the size of the chitosan NPs is chiefly affected by these two interactions during synthesis process (Qun and Ajun 2006). If electrostatic repulsion is more dominant, the particle size remains small, and if interchain hydrogen bond is stronger, the size of chitosan NPs becomes larger. Various research groups have concluded that certain ranges of concentrations (2.0–1.5 mg /ml and 1.0–0.5 mg /ml) of chitosan and TPP is generally able to synthesize the nanoscale particles (Calvo et al. 1997; Gan et al. 2005; Fan et al. 2012; Jaiswal et al. 2012; Saharan et al. 2015; Kashyap et al. 2015). With increase in chitosan above the critical concentration, the interchain hydrogen bonds become stronger and abundant chitosan molecules mixed up in the cross-linking making the particle size larger (Fig. 2.7). In addition to this, at higher concentrations of TPP,
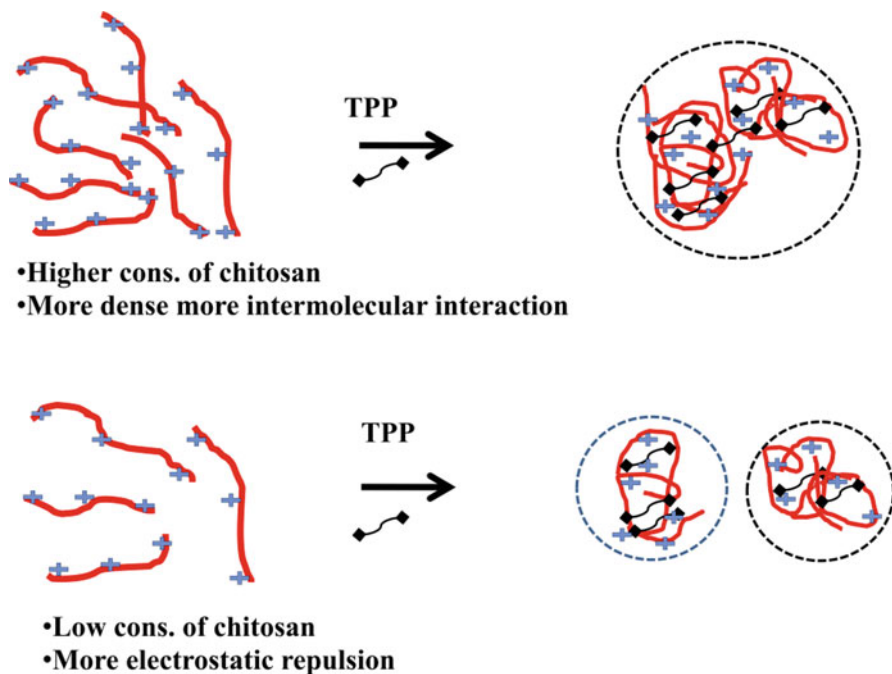

Fig. 2.7 Effect of chitosan concentration on chitosan nanoparticles

zeta-potential of the NPs decreases because TPP occupies most of the positively charged amino groups. Decrease in zeta potential also affects the stability of the NPs as electrostatic repulsion between chitosan particles reduces and particles start to aggregates and large particles appear in the reaction (Fig. 2.7). Therefore, the concentrations of chitosan and TPP play a crucial role in NPs synthesis. Further research has revealed that TPP concentration should mostly be kept equal or lower to chitosan to prevent the excess intermolecular cross-linking. All together, the stable chitosan NPs can only be synthesized at certain concentrations of chitosan and TPP (Kashyap et al. 2015).

2.7.2 Effect of the Mass Ratio of Chitosan and TPP

Effect of the mass ratio of chitosan and TPP can easily be understood by experiment done by Fan et al (2012). They mixed different volumes of TPP (0.5 ml/ml) in 10 ml of chitosan solution (0.5 ml/ml) and found that increasing TPP volume from 2.5 to 3.5 ml (mass ratio decreasing from 4.0:1 to 2.9:1) first steadily decreased the size of NPs from 172 to 133 nm and then increased to 237 nm. The zeta potential also decreased linearly (from $+39$ to $+26$ mV) due to the neutralization of protonated amino groups by TPP anions. During chitosan TPP cross-linking, researchers observed that the reaction solution showed clear appearance without visible opalescence which indicates that inadequate volume of TPP is unable to make cross-linked structure. Increasing the volume of TPP, the particle size decreased due to cross-linking in chitosan and compact structure appears and solution becomes opalescent. As TPP volume continued to increase, chitosan molecules got fully cross-linked and excess TPP got involved in intermolecular cross-linking resulting in larger particle size (Fig. 2.8). With further addition TPP in the reaction mixture, the electrostatic repulsion becomes very less due to neutralization of amino groups by TPP and larger particles start to precipitate. Overall the optimum mass ratio of chitosan and TPP has not been recommended because different research groups used different types of chitosan with respect to MW and degree of deacetylation. However, in majority of the experiments, the mass ratio of chitosan and TPP was kept from 2.5:1 to 5:1 to get chitosan NPs (Fan et al. 2012; Jaiswal et al. 2012; Saharan et al. 2015; Kashyap et al. 2015).

2.7.3 Effect of pH of the Reaction Solution

The pH of the reaction solution has very prominent role on synthesis process and quality of nanoparticles. Chitosan is insoluble at neutral and alkaline pH and easily solubilize in low concentration of acidic environment. In acetic medium, H^+ ions protonate the amino groups of chitosan, conferring a high charge density. Therefore, the surface charge density of chitosan molecules is strongly dependent
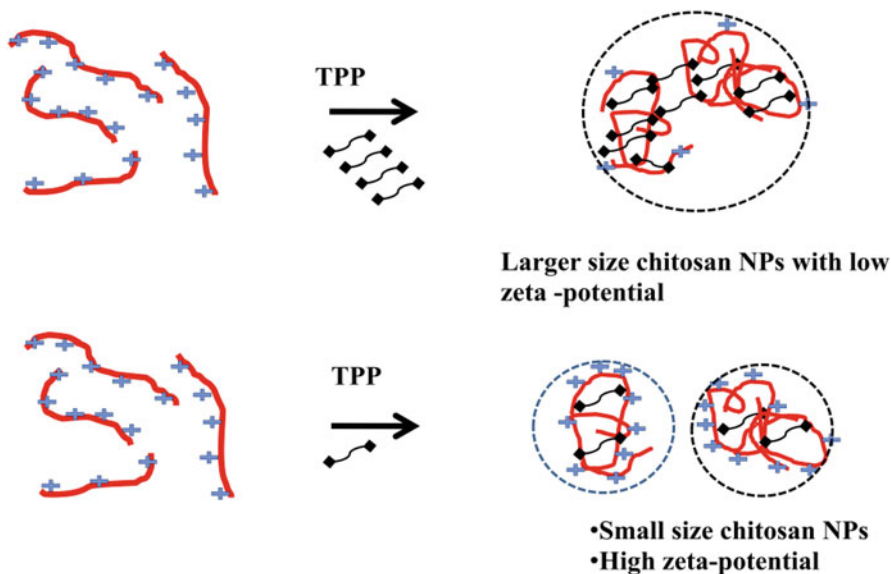
Fig. 2.8 Effect of TPP concentration on chitosan NPs

on pH of the solution. Hence the change in pH of the solution will have strong impact on cross-linking process during reaction. Gan et al. (2005) have explained the effect of pH on the size and zeta-potential of chitosan nanoparticles. It was observed that particle size and zeta-potential are very sensitive to the changing pH of the aqueous solution. During the cross-linking reaction of chitosan and TPP (mass ratio 4:1), with a gradual increase in pH above 5.5, a sharp increase in particles size and precipitation was noticed. It is suggested that with gradual increase in pH from 5.5, the degree of protonation decreases due to increase in OH$^-$ ions which leads to decreased electrostatic repulsion between the particles thereby increasing the probability of particle aggregation. When pH is steadily decreased below 5.0, it is not easier to achieve proper cross-linking by TPP, and as a result there is absence of compact chitosan nanostructures in the highly repulsive conditions in chitosan solution. Variation of pH also influences the zeta-potential of nanoparticles. It steadily lowers down as pH of the reaction mixture is increased. This is due to the neutralization of amino groups of chitosan. When pH of the reaction mixture gets down from 5.0, a sharp increase in zeta-potential is noticed due to the protonation of chitosan (Figs. 2.9, and 2.10). It is clearly evident that a pH around 4.5–5.5 is ideal to get lower average size of particles with net positive zeta-potential (Fan et al. 2012).
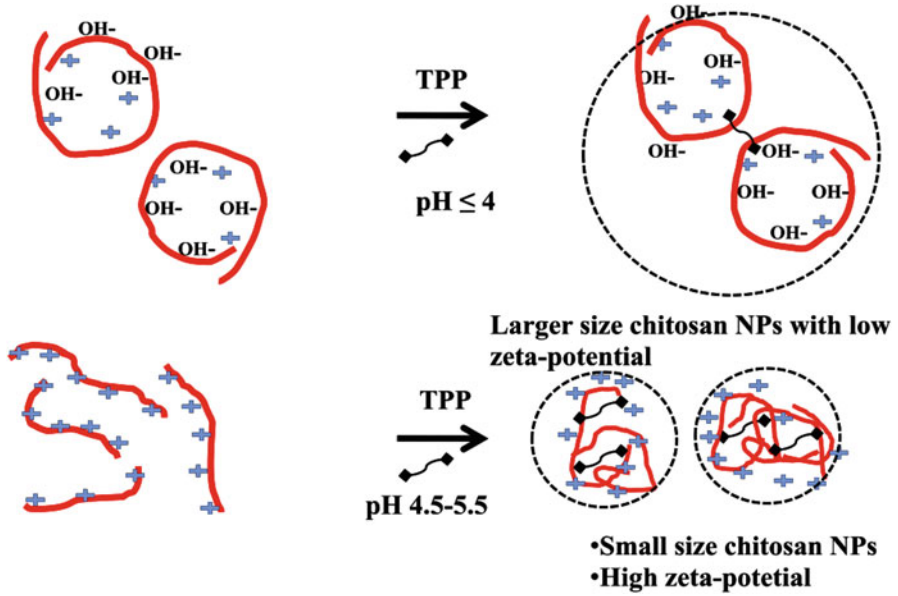
Fig. 2.9 Effect of pH on chitosan NPs
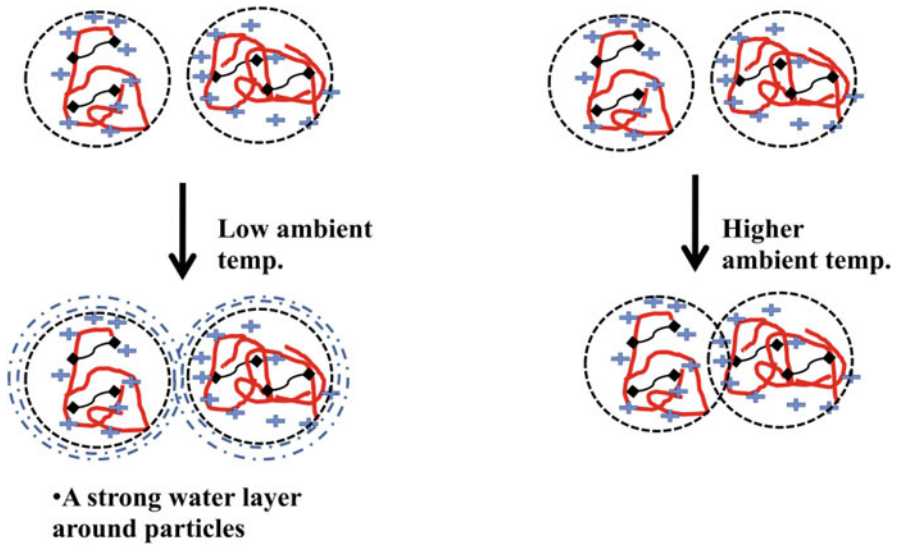
Fig. 2.10 Effect of temperature on NPs synthesis

2.7.4 Effect of the Concentration of Acetic Acid

Acetic acid is primarily used to dissolve chitosan in aqueous. Fan et al. (2012) did experiment to investigate the effect of different concentrations of acetic acid (0, 0.1, 0.2, 0.5 and 0.8 mg/ml) to dissolve chitosan (0.50 mg/ml in aqueous) for formation of chitosan NPs. At lower acetic acid concentration (0.1 mg/ml), a two centric (bimodal) particle distribution around 164 nm and 850 nm size was observed. This indicates varying interaction of TPP and chitosan due to insufficient cross-linking. Further, increasing concentration to 0.2 mg/ml exhibited monodispersed nanoparticle in reaction solution. As we discussed that protonation of chitosan is imperative for cross-linking with TPP to form nanoparticles, thus acetic acid increases the H^+ ions concentration that makes the solution conductive (high protonation) and TPP cross-linked to chitosan to form nanoparticles. In case of lower concentration of acetic acid, lesser number of H^+ ions are available for protonation of amino groups, thus an insufficient number of interaction sites are available for TPP. Albeit at lower concentration of acid, the cross-linking is not uniform to obtain monodispersed nanoparticles. Increasing the acetic acid concentration from 0.5 mg/ml to 0.8 mg/ml increases the amount of smaller particles but also increases the number of larger particles which results in a decrease in the monodispersity of the particles. To prepare chitosan nanoparticles at higher acetic acid concentration, NaOH is added to reaction mixture to bring pH to 4.7–4.8. At this conductive pH, nanoparticles could be synthesized in unimodal fashion at higher acetic acid concentration. However, higher concentration of acetic acid indirectly causes an increase in counter ions (CH_3COO-), which shields the cross-linking points to be accessed by TPP. Thus the intramolecular electrostatic repulsive forces decreases, which make the molecules contracted (Cho et al. 2006; Tsaih and Chen 1997). Contracted conformations of chitosans are not in proximity to accumulate more nanoparticles as compared to their extended conformation. Thus a greater number of smaller nanoparticles are formed. On the other hand, shielding effect of counter ions will lower down the electrostatic repulsion between particles and reduce the thickness of surface hydration layer on the particles. As a result, a number of particles start to aggregate resulting in a wider particle size distribution. Small size monodispersed chitosan nanoparticle synthesis can't be possible at too higher and too lower concentration of acetic acid. Optimum concentration of acetic acid needs to be standardized.

2.7.5 Effect of Temperature of Chitosan Solution

Temperature of the cross-linking reaction also has role in amending the monodispersity and size of particles. During the synthesis process, flexibility, rigidness and hydrogen-bonded hydration layer in chitosan molecules are directly influenced by temperature (Chen and Tsaih 1998; Colic et al. 1998). Narrow unimodal small-sized

chitosan nanoparticles could be obtained at low ambient temperature (0–4°C). It is noticed that as the temperature of chitosan solution decreases, there is an increase in specific volume of chitosan molecule and a decrease in chitosan chain flexibility. The hydrogen bond between the polar groups of chitosan and water molecules increase speedily which build a hydration shield around the particles which prevent the particle aggregation. Under low ambient temperature, the rigidness of the chitosan is high which is favorable for the stabilization of the nanoparticle structures (Fig. 2.10).

2.7.6   Effect of the Stirring on Cross-Linking

Stirring during the ionic interactions between amino group and TPP is recommended to evade particle aggregation and to achieve narrow size distribution. It is manifested that stirring of cross-linking reaction endows equal chance to functional group of chitosan and TPP to link in symmetry. Even though stirring speed comprehensively narrows down the size distribution, increasing the speed from 200 to 600 rpm significantly lessen the size of nanoparticles. This happens because stirring at particular rate does not allow the excess chitosan and TPP to participate in cross-linking and also prevent intermolecular cross-linking. In spite of this, excess stirring with higher RPM may wipe out repulsive forces between the particles and induces bigger size particles synthesis. Albeit, stirring at higher RPM may disturb the symmetrical interactions of chitosan and TPP.

2.8   Characterization Techniques

After the synthesis of nanoparticles, it is most important to measure various key characters like mean size, distribution, charge density (zeta-potential), polydispersity index (PDI), chemical bonding, shape and topography of the particles. Elemental analysis is another key technique to find out the elements involved in NPs makeup. Chitosan nanomaterials have been used for the encapsulation of various other materials which could be characterized by atomic absorbance spectrophotometer and other *in vitro* release kinetics. The state-of-the-art techniques can be used to infer physico-chemical characterization of chitosan nanoparticles.

2.8.1   Particle Size, Zeta-Potential and Polydispersity Index

Dynamic light scattering (DLS) is a technique used to measure the size of particles in suspension. Nanoscale-sized particles are governed by electromagnetic forces and gravitational forces are insignificant. The nanoparticles in suspension follow the
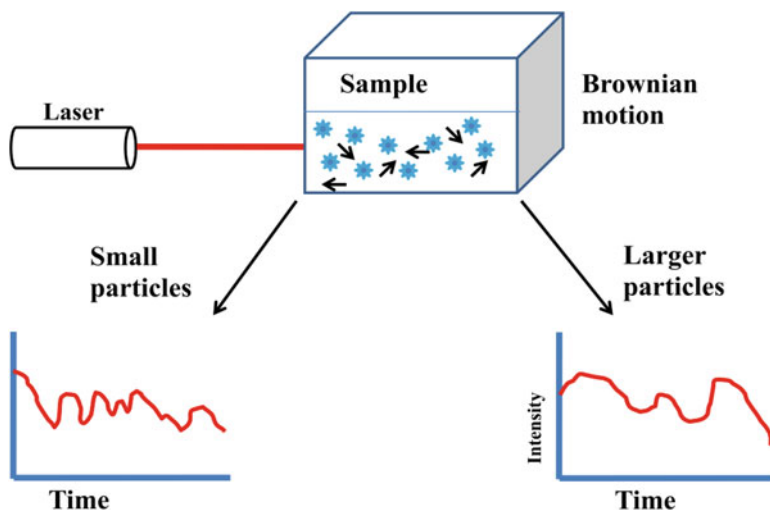
Fig. 2.11 Brownian motion, particle size and intensity fluctuations

Brownian motion caused by collision of solvent molecules. The technique of DLS measures the speed of particles undergoing Brownian motion which is influenced by particle size, viscosity and temperature. The smaller particles show rapid Brownian motion as compared to larger particles. The velocity of Brownian motion is defined by the translational diffusion coefficient (D) which can be converted into particle size using the Stokes-Einstein equation (where d_H = hydrodynamic diameter, k = Boltzmann's constant, T = absolute temperature, η = viscosity and D = diffusion coefficient)

$$d_H = \frac{kT}{3\pi\eta D}$$

DLS estimates the time-dependent fluctuations in the scattering intensity to determine the translation diffusion coefficient (D) and subsequent hydrodynamic size. The rate of intensity fluctuation is dependent upon the size of the particles (Fig. 2.11). The mean size measured by the DLS is the mean hydrodynamic diameter. Hydrodynamic diameter is diameter of particle along with the layer of water molecules or ions or media components on the surface (Fig. 2.12). Chitosan nanoparticles having amino groups provide a net positive charge. The cationic nature of chitosan nanoparticles has more probability to interact with media components particularly to anionic substances. These interactions affect its diffusion in the medium and subsequently module hydrodynamic diameter. PDI gives a value which indicates the size distribution of particles. Its value range from 0 to 1, value 1 or above signify highly polydispersed particles and less than 1 indicates monodispersed particles. Chitosan nanoparticles display variability in
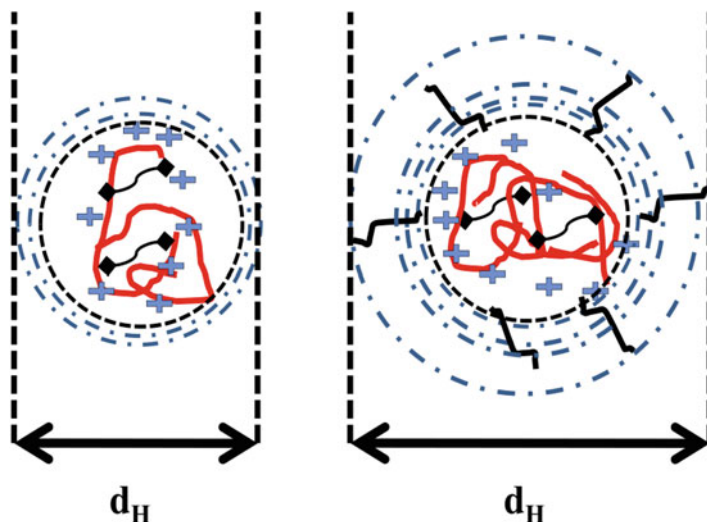
Fig. 2.12 Hydrodynamic diameter

PDI values in different experiments due to different mass ratio and concentrations of chitosan and TPP during particles preparation. Chitosan-based nanoparticles have been synthesized by various groups using various methods. The mean size of the chitosan nanoparticles ranges from 50 to 750 nm depending on various factors and different encapsulation ingredients. Chitosan-based nanomaterials have been used to encapsulate various drugs, micronutrients, biological molecules and metals. The information regarding the use of chitosan nanomaterials in plant is limited. Most of the available information deal with pure chitosan nanoparticles or encapsulation of metals salts and/or other micro/macro nutrients. Therefore the size and particle distributions are diverging. It is manifested that being a polymer, the size and shape of the chitosan nanoparticles are variable and not confined. The other pivotal character is the surface charge also known as zeta-potential. Its value for chitosan nanoparticles always remain positive due to amino groups. It is very crucial to have higher value of zeta-potential for many things. First is the stability of nanoparticles, higher the positive charge on the particles, higher is the repulsion between particles. Hence the particles remain apart and no agglomeration occur. Second is the biological activity of the particles, higher the zeta-potential, higher is the biological activity. Being positively charged, chitosan have higher affinity towards biological membranes and other anions. The values of zeta-potential in chitosan nanoparticles vary from +20 to +90 mV, and it is evident that higher zeta-potential is more valuable in biological system. Other decisive factor is PDI which represents the homogeneity of the particle distribution. A low PDI value indicates the monodispersed nature of chitosan nanoparticles. The chitosan nanoparticles are usually synthesized by cross-linking with TPP. The further functionalization

of particles is done by other components. The interactions of various components within the particles are found out by Fourier transformation infrared spectroscopy (FTIR). In FTIR, IR rays are passed through the particle sample where some of the infrared radiations are absorbed by the bond(s) existing in the sample and some of it are passed out (transmitted). The resulting spectrum represents the molecular absorption and transmission and creates a molecular fingerprint of the particles. A specific absorption at particular vibration indicates a specific bond energy. A plot of % transmittance (E) versus vibration frequency in wavenumbers (cm^{-1}) can easily map the interactions in chitosan nanoparticles. In chitosan nanoparticles, FTIR clearly showed specific peaks to denote amide -$CONH_2$, anhydrous glucosidic and primary amine (-NH_2) groups. Further addition of functional component to particles can easily be found out by redistribution of vibration frequency (Fig. 2.13).
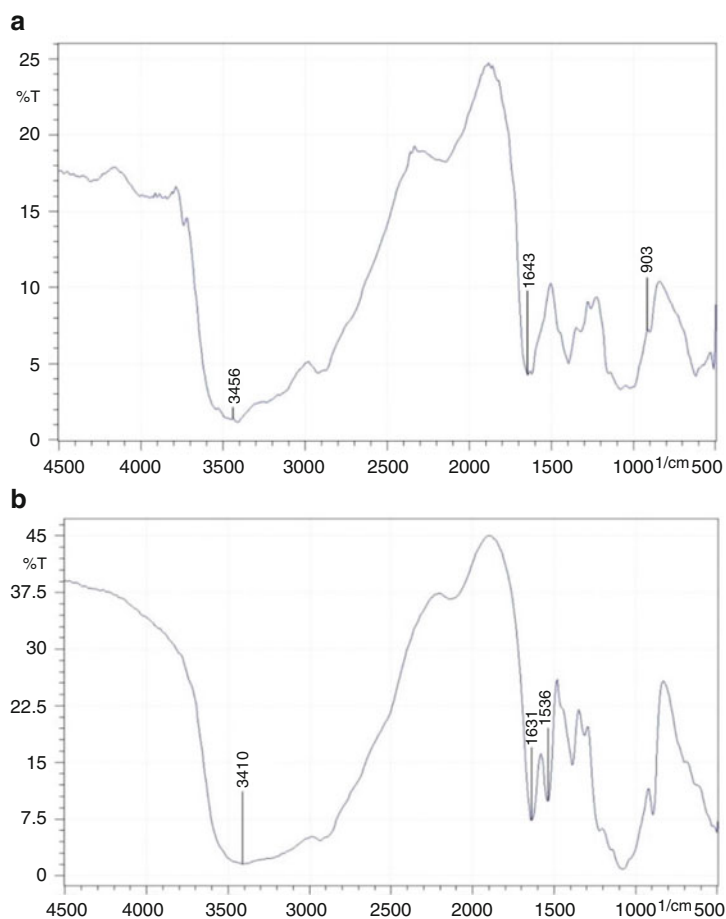
Fig. 2.13 FTIR spectra. (**a**) Bulk chitosan, and (**b**) Nanochitosan

   The scanning electron microscope (SEM) uses a focused beam of high energy electrons to generate an array of signals at the surface of particles. The signals derived from sample-electron interactions provide information about the external morphology (texture), chemistry and crystal structures. A two-dimensional image is generated that provides size, shape, surface morphology and elemental analysis data. Chitosan is not conductive in nature and to make it conductive a layer of metal is applied by sputter coating on it. To collect the backscatter electrons (BSC), a separate EDS (Energy dispersive X-ray spectroscopy) analyser is used. EDS analysis provides the quantitative data of elements inside the samples. Size, shape and topography also play decisive role in the application of chitosan nanoparticles. The analysis of size shape and topography is done by transmission and scanning electron microscopy. The TEM study reveals the internal structure of the particles whereas SEM gives surface topography. TEM and SEM are also used to verify the size of the nanomaterials. Various groups have illustrated different shapes and topography of the chitosan nanoparticles varying from ovule shape to highly porous (Fig. 2.14).
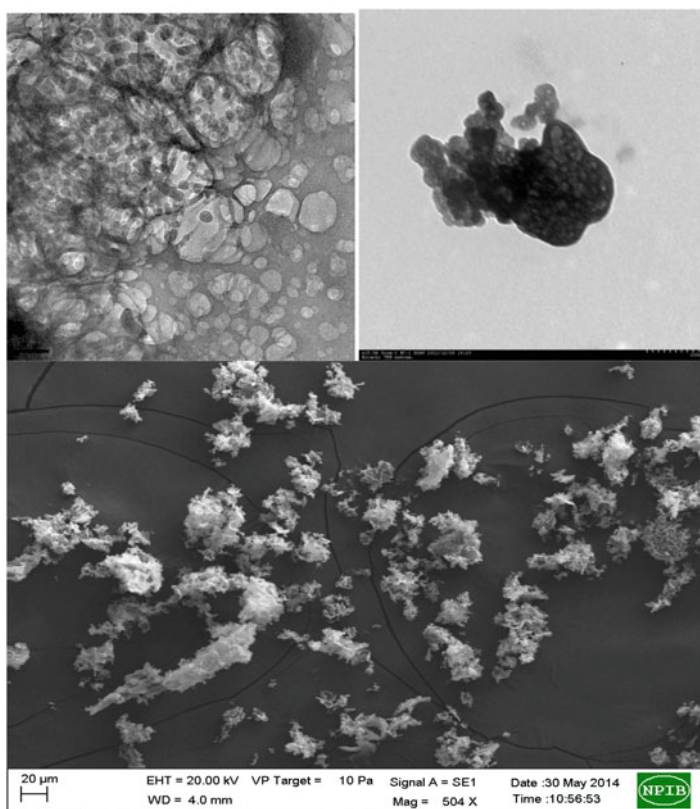
Fig. 2.14 Chitosan NPs TEM and SEM Micrograph

Chapter 3
Properties and Types of Chitosan-Based Nanomaterials

In the recent years, numerous natural as well as synthetic polymers have been examined for agricultural, biotechnological, medical and pharmaceutical applications (Kadajji and Betageri 2011). The basic advantage of these polymers used in plants or animals is that they don't have any toxic effects on environment. Among such polymers, chitosan is a linear homo-polymer of glucosamine and N-acetyl glucosamine units linked by β (1–4) glycosidic linkage (Rajan and Raj 2013). As such chitosan is not present in nature and thus it can't be extracted from naturally occurring resources. Indeed, chitosan is the deacetylated product of natural chitin; the second most abundant polysaccharide in nature. Due to unique characteristics, such as non-toxic, biocompatible, safe and biodegradable, it is globally used as an antibacterial, antifungal and adhesive agent either blended with other polymers or alone (Dutta et al. 2004).

Nanochitosan is a bioactive and eco-friendly natural material with excellent physicochemical properties, and it is extensively used for formulating carrier in various fields including drug delivery, antibacterial and tissue engineering (Yue et al. 2011). Additionally in textiles industry, nanochitosan is used for improving the strength and washability and to confer antibacterial effect (Huang et al. 2009). Because of high surface to volume ratio, polymeric NPs widely improve the loading capacity of the selected molecules. Chitosan is poorly soluble in water and highly soluble in acidic condition, hence free amino groups contribute positive charge on entire chain. Polymeric chitosan-based nanoparticles can be prepared using many methods including ionic gelation, emulsion cross-linking, spray drying and complex coacervation. Among these, ionic gelation technique is fast and easy to carry out (Ing et al. 2012).

Formation of nanoparticles occurs spontaneously through the mechanism of intra- and intermolecular cross linking using polyanionic agent such as tripolyphosphate (TPP) and glutaraldehyde under continuous stirring condition at constant temperature (Ing et al. 2012). Initially sodium sulphate was used as precipitation

agent to cross link the free amino group of chitosan. In 1999, Tian and Groves obtained 600–800 nm nanochitosan particles by improving this technique.

3.1 Physico-chemical Characters

Chitin is a linear homopolysaccharide composed of N-acetylglucosamine, a glucose derivate, and it is a characteristics component of exoskeleton of crustaceans and cell walls of fungi (Fig. 3.1). It is second most abundant polysaccharide next to cellulose. On partial alkaline deacetylation chitin gives rise to chitosan which as such is not found in environment. Chitosan is a linear, semi-crystalline, cationic, non-toxic and biodegradable polymer of (1,4)-2-acetamido-2-deoxy-b-D-glucan (N-acetyl D-glucosamine/Glc-NAc) and (1–4)-2-amino-2-deoxyb-D-glucan (D-glucosamine/GlcN) units linked by β 1,4 linkage (Aranaz et al. 2009). It has unique biological, physical properties and defined chemical structure that can be chemically and enzymatically modified and make it advantageous over other similar biomolecules (Fig. 3.2).

3.1.1 Degree of Deacetylation

The degree of deacetylation refers to the ratio of Glc-NAc to GlcN structural units. Chitin, upon enzymatic and chemical hydrolysis under alkaline condition
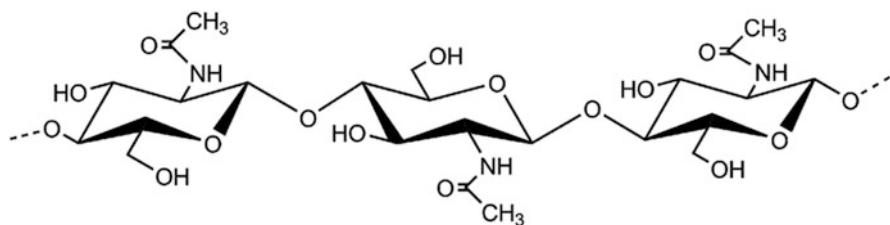
Fig. 3.1 Chemical structure of chitin
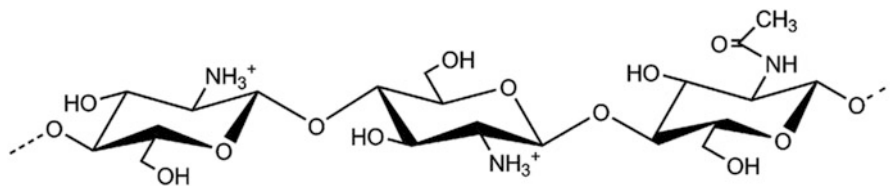
Fig. 3.2 Chemical structure of chitosan

gives chitosan by deacetylation process (Croisier and Jerome 2013). When the degree of deacetylation (DD; indication of the number of amino groups along the chains) is less than 50 %, it is said to be chitosan and it represents a group of partially and fully deacetylated chitin. Depending on source and preparation, the DD varies from 60 to 100 % and molecular weight from 300 to 1000kDa. Using specific enzyme or reagent such as hydrogen peroxide, chitosan oligomer can be prepared by degradation of chitosan (Croisier and Jerome 2013). Several techniques including IR-spectroscopy, 1H NMR spectroscopy, pH-potentiometric titration, colloidal titration and enzymatic degradation are available for determination of DD, among them, IR, NMR spectroscopy and pH-potentiometric titration are most commonly used. Molecular mass can be obtained from viscometry or by size exclusion chromatography.

3.2 Chitosan Depolymerization

High viscosity and poor solubility of chitosan at neutral pH limits its use and therefore, instead of chitosan, low molecular weight chitosan and oligomers play an important role in some specific applications. Chitosan depolymerization can be carried out through chemical, physical and enzymatical mode. Chemical depolymerization is mainly carried out by acid hydrolysis (HCl) or by oxidative reaction using HNO_2. By attacking HNO_2 on amine group of D-glucosamine and subsequent cleavage of glycosidic linkage, it produces small oligomers. Using radiation such as Co-60 gamma rays, depolymerization produces oligomers like dimers, trimers and tetramers with low yields. In enzymatic depolymerization, several enzymes such as chitinase, chitosanase and gluconase and some non-specific enzymes including lipase, amylase, cellulase and lysozyme have been used to produce water-soluble, low-molecular weight chitosan (Aranaz et al. 2009).

3.2.1 Surface Charge and Functional Modification

The unique properties of nanoparticles such as small size, high surface area and surface charge have attracted attention through their capability of breaking biological barriers, carrier of biomolecules, accumulation at the target region and cell membrane penetration. Among all the existing nanoparticles, chitosan-based ones provide peculiar advantage such as biodegradability, biocompatibility and low cost. Therefore, it has been used in target delivery of drugs, plasmid DNA delivery and topologic immunization.

The reactivity of primary amine groups at the C2-position and/or hydroxyl groups at the C-6 position of glucosamine units takes great advantage when

chitosan is subjected to derivatization to change the physico-chemical, biological and pharmaceuticals properties of the parent chitosan (Zambito 2013).

The weak solubility in aqueous medium, stability and adsorption capacities of chitosan polymer can be improved in acidic medium with certain chemical and physical modifications. Several chemical modifications can be done to modify properties so as to maximize the polymer processability, antimicrobial activity and ability to interact with other substances which may be exploited in specific applications. Among the several existing chemical modifications, the most common is cross-linking process in which the adsorption capacity of chitosan is found to be increased with the addition of cross-linkers such as glutaraldehyde, dextran sulfate, 1,1,3,3-tetramethoxypropane, glyoxal and polyanion tripolyphosphate (TPP) (Anitha et al. 2012). The electrostatic interactions of positively charged primary amino group of chitosan and the negatively charged counterion TPP make intermolecular covalent bridges between polymer chains, which has non-toxic and multivalent properties (Vijayalakshmi et al. 2014). In glutaraldehyde, the presence of active aldehyde groups allows easy cross-linking with chitosan molecules (Fig. 3.3).

3.2.2   Stability and Viscosity Behaviour

The biodegradability of chitosan is affected by its particle size; hence the stability behaviour of nanochitosan dispersion should be taken into consideration during its application. In general, the stability in terms of change in viscosity of chitosan is governed by its molecular weight, which can be improved with decrease in molecular weight. It has been found that nanochitosan dispersion obtained from
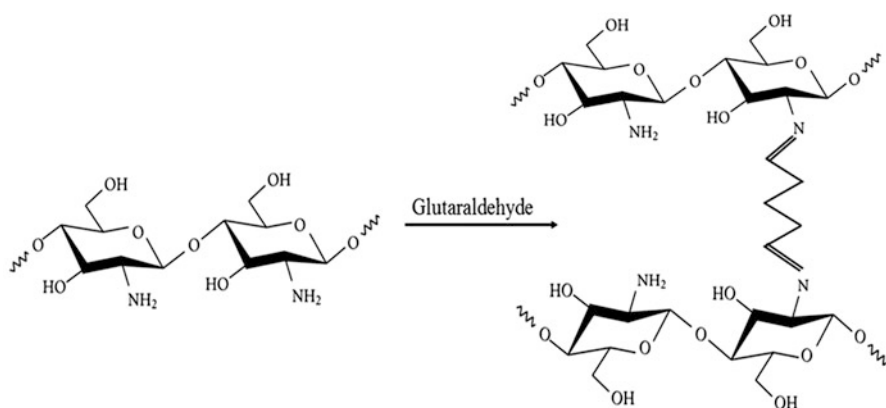

Fig. 3.3 Interaction of chitosan with glutaraldehyde and ionic cross-linking

high molecular weight is more stable and less susceptible than the nanochitosan dispersion obtained from low molecular weight chitosan.

At the molecular level, the viscosity of polymer solution is a direct measure of the chain length and molecular weight. The viscosity of nanochitosan dispersion decreases with decrease in particle size and the effect of larger particle is more significant than the smaller one. It is because large particle size offers resistance to flow of liquid thereby gives more viscosity and vice versa (Chattopadhyay and Inamdar 2012). Higher molecular weight chitosan scales down to nano size to a greater extent than the low molecular weight chitosan because large size chitosan molecules can accommodate more amount of solvent, occupy large hydrodynamic volume in solution and hence compress to greater extant by intramolecular cross-linking. On the other hand, in low molecular weight chitosan, extant of swelling is comparatively lesser and therefore lesser is the tendency to compress.

The incorporation of cross-linking agent such as TPP results in alteration of physico-chemical parameters namely nanoparticle size, zeta-potential and production yield which finally affect its functional properties (Rodrigues et al. 2012). Atomic force microscopy (AFM), X-ray diffraction (XRD), FTIR analysis and DLS is used to determine the physico-chemical properties of chitosan based nanoparticles (Qi et al. 2004). Chemical composition can be studied using 1H NMR; therefore it may be used to determine the degree of acetylation of chitin. Transmission electron microscopy (TEM) and infrared (IR) spectroscopy are widely used analytical techniques to study the chemical structure of chitin and chitosan (Trung and Bao 2015). The measurements of nanoparticle size and zeta-potential are performed by photon correlation spectroscopy, FTIR and laser doppler anemometry.

Zeta-potential is the potential difference between the surfaces of particles in suspension. The chitosan-based nanomaterials have positive zeta-potentials because the amino and hydroxyl groups of chitosan form hydrogen bonds with the hydroxyl groups of water. With the addition of TPP to chitosan solution, zeta-potential gradually decreases and at a certain level the positive charge on the chitosan particle gets neutralized by the negative charge of phosphate group. This gradual decrease is due to the cross-linking between the phosphate groups of TPP and amino groups of chitosan. For proper cross-linking, the mass ratio of low molecular weight chitosan (LWCS) and TPP should approximately be 5:2 ($\zeta = 7.8$) and it decreases with the molecular weight of LWCS (Yang et al. 2010).

Chitosan is a weak base insoluble at neutral and alkaline pH values. It is soluble in acidic medium and has an average amine group density of 0.837 per disaccharide unit (Gan et al. 2005). Chitosan-based materials are strongly dependent on pH of solution that indicates that these materials are pH responsive and hence provides opportunities to modulate formulation and properties of these materials.

Generally, opalescence is observed initially after mixing of chitosan and cross-linking agent like TPP that indicates the rapid cross-linking reaction. It is reported that increasing the solution temperature decreases the ratio of radius of gyration of chitosan, reduces the hydrogen bonded hydration water of chitosan, increases chitosan chain flexibility and decreases the specific volume of chitosan molecule.

These approaches ultimately decrease particle size by formation of compact structure during cross-linking process. Low temperature serves to gradually stabilize the structure of the particle by causing chitosan molecule to become stiff faster (Fan et al. 2012).

3.3   Types of Chitosan Based Nanomaterials: Chitosan Nanocomposites

3.3.1   Chitosan-Metal Nanocomposites

In crop plants, chitosan-based nanomaterials have been under various levels of research from laboratory to controlled conditions of polyhouse. Currently pure chitosan nanomaterials without any blending of other components are being tested for promotion of plant growth and disease prevention. Among the chitosan nanomaterials, composites of chitosan have a great scope in agriculture to abate the deficiency of micronutrients and disease protections. Composite of chitosan refers to a complex of nano-sized chitosan which encompasses one or more other component(s). The aim of fabrication of chitosan nanocomposites is to enhance its physico-biological activities for more pronounced effects. Chitosan biopolymer has native property of chelating with metal ions *viz.* Cu, Zn etc. Numerous studies have established the fact that chitosan-metal nanocomplexes are biologically more valuable as compared to sole/bulk chitosan and metals (Wang et al. 2004; Du et al. 2009; Saharan et al. 2013; Shukla et al. 2013). The indispensable uses of Cu and Zn in cellular system makes these micronutrients important for agriculture. Based on this, two most important chitosan-based nanomaterials have been synthesized for possible applications in crop plants.

3.3.1.1   Chitosan-Zn Nanocomposite

Zinc essentiality was established in 1869 in plants as a micronutrient. With the advancement in nanotechnology, the concept of slow release of micronutrients for long-term effect has emerged. Zn has been targeted to encapsulate into chitosan nanoparticles for enhancing its availability and effectiveness to biological system. Wang et al. (2004) synthesized five different chitosan-Zn complexes with different zinc content. Chitosan (30 mg) was dissolved in 30 ml of 1 % acetic acid and different amounts of $ZnSO_4 7H_2O$ (molar ratio of 4:1, 2:1, 1:1, 0.5:1, 0.25:1 compared to chitosan) was added into the solution. The reaction mixture was kept at 80°C for 3 h under continuous stirring. The white precipitate was obtained by addition of acetone followed by filtration. After washing with ethanol, the dried powder was obtained under vacuum. X-ray diffraction and FTIR study revealed various interactions of chitosan-TPP-Zn complex. Two models have been classified
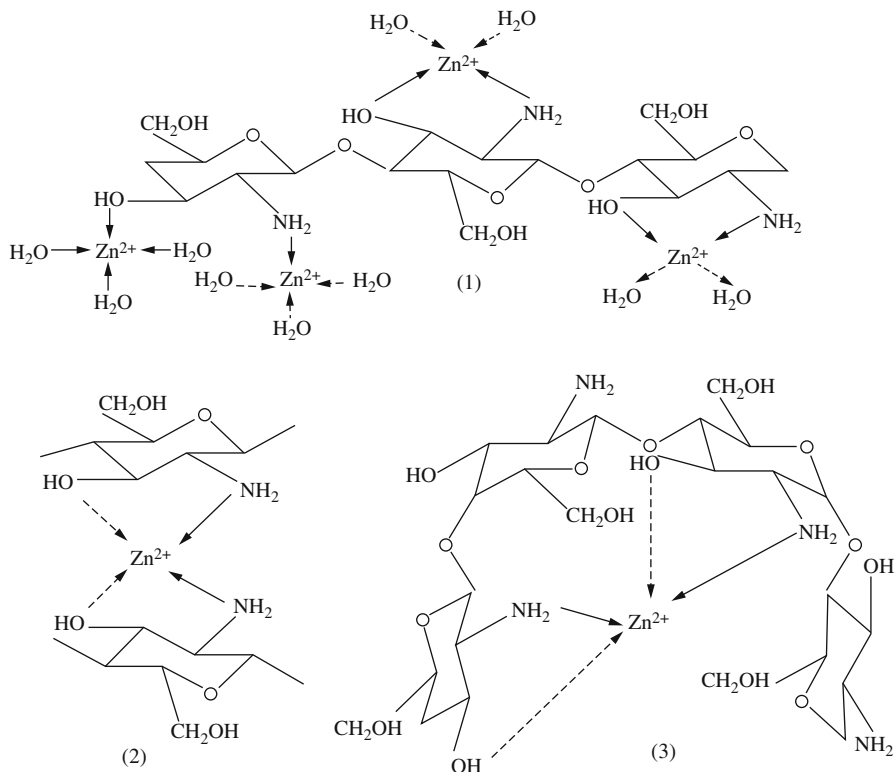
Fig. 3.4 Interaction of Zn with chitosan (Adopted from Wang et al. 2004)

to sort out the structure of chitosan and metal ions: (1) metal ions bound to one amino group of chitosan known as pendant pattern; (2) metal ions bound to two or more amino and hydroxyl groups of one or more chitosan chains as a bridge known as bridge pattern (Fig. 3.4).

Du et al. (2009) synthesized chitosan-Zn nanocomposite by ionic gelation method. To sum up, chitosan (0.3 % w/v) was dissolved in 1 % (v/v) acetic acid. TPP (1% w/v) was added into chitosan solution with a ratio of 1:25. The mixture was sonicated at 1.5 kW for 30 min under constant stirring. The sonicated mixture was subsequently centrifuged at 12,000 g for 10 min. The precipitated material was re-suspended in water, centrifuged again and freeze dried. Chitosan nanoparticles loaded with Zn^{2+} were obtained by adding metal ion solution ($ZnSO_4$) into the chitosan nano-suspensions (0.3 %, w/v) to make a final concentration of 120 μg/ml. The mean particle size was measured 210.9 nm with a zeta-potential of +86.65 mV. It was concluded that increasing the Zn ions in chitosan-Zn complex improved the zeta-potential which further contributes to improved antibacterial activity. In our laboratory, Zn-chitosan nanoparticles were synthesized by ionic gelation method (unpublished data). The developed nanoparticles have been evaluated further for

Size Distribution by Intensity

		Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 354.0	Peak 1:	323.5	100.0	102.4
Pdl: 0.344	Peak 2:	0.000	0.0	0.000
Intercept: 0.925	Peak 3:	0.000	0.0	0.000
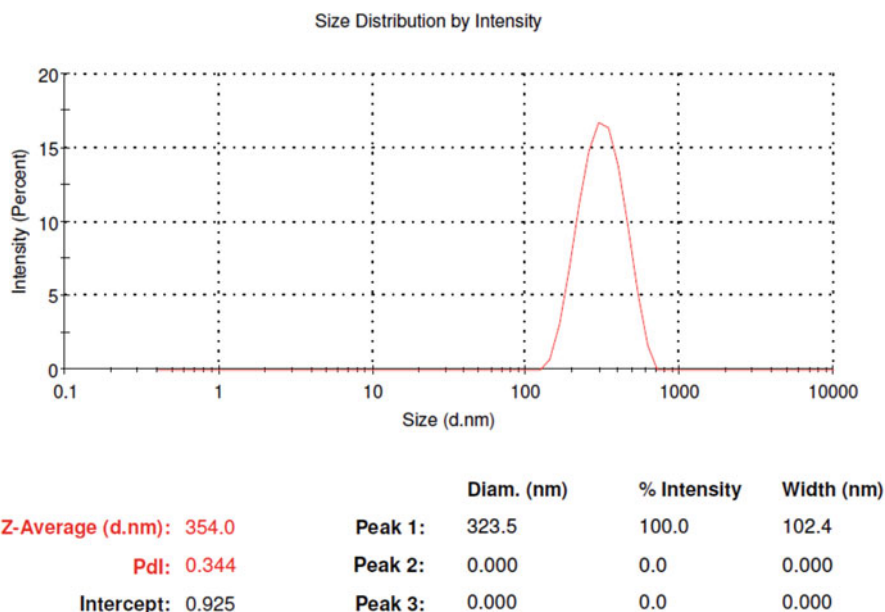
Fig. 3.5 Particle size distribution of Zn-chitosan nanocomposite and PDI value

their effect on plant pathogenic fungi and seedling growth of maize crop through seed germination test. The synthesized nanoparticles avail mean size of 354 nm with PDI value of 0.34 (Fig. 3.5).

3.3.1.2   Chitosan-Cu Nanocomposite

Copper is used in copper-based fungicides for disease prevention and acts as a crucial micronutrient for plants. Hence, Cu is most studied metal in agriculture. However, excessive use of Cu could be toxic to plant growth and exert residual effect in environment. In deliberation of its importance, copper-based agrochemicals are approved in organic farming. Furthermore, continuous application of Cu may increase its concentration in biosphere which could exceed the specified limit of Cu in the environment (Deluisa et al. 1996). Due to its crucial role in agriculture, various Cu-formulations have been developed to enhance its bioavailability and activity without crossing the toxic level. In view of this, the concept of developing chitosan-based Cu nanocomposites has been initiated (Jaiswal et al. 2012, Saharan et al. 2013 and 2015). The major advantages of nanocomposites include steady and slow exposure, minimum drifting in the surrounding environment and effective absorbent for pesticides. Jaiswal et al. (2012) fabricated chitosan-Cu nanocomposite by adding copper sulfate (3.0 g) in chitosan solution (1 g in 100 ml of 5 % acetic acid) (v/v) followed by addition of NaOH. Further the precipitated material was

Fig. 3.6 TEM study of
Cu-chitosan nanoparticles
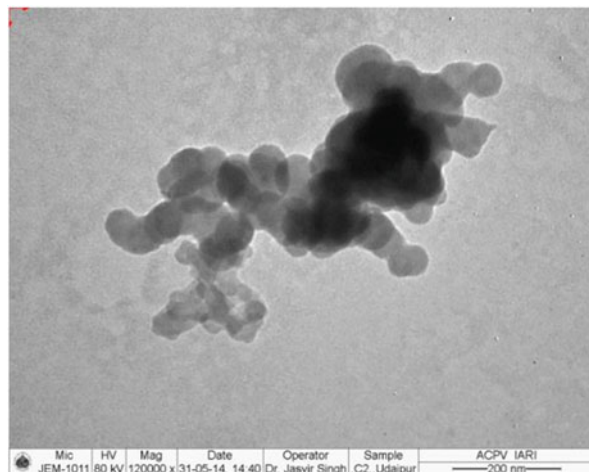
dried in oven at 80°C overnight. The particle size of this composite was found
to be 700–750 nm. Prepared Cu-chitosan nanocomposite was used to remove
pesticides from water resources. Study has revealed the crucial role of doped copper
in chitosan for absorption of parathion and methyl parathion residues. We have
undertaken an extensive study to synthesize Cu-chitosan nanocomposite at mass
level by adopting the ionic cross-linking method (Saharan et al. 2013 and 2015).
The main aim of this study was to assess the antifungal and plant growth promotory
effects of Cu-chitosan nanoparticles. The 0.01 % $CuSO_4$ solution was added into
the formulation and using double beam Atomic Absorption Spectrophotometer
(AAS) the amount of entrapped Cu into chitosan was determined. Extended
studies revealed that Cu-chitosan nanocomposite showed narrow hydrodynamic
diameter (374 ± 8.2 nm) with lower polydispersity index 0.33 (PDI) which denotes
the monodisperse nanoformulation. Under TEM study, mean physical diameter
of 150 nm was recorded (Fig. 3.6). Cu was found to encapsulate into highly
porous network of chitosan nanocomposite as revealed by TEM study. SEM analysis
further confirmed the porous surface of Cu-chitosan nanocomposite. EDX analysis
elucidates the higher encapsulation of Cu in porous area of chitosan nanomaterials.
Chitosan Cu nanonetwork is evident through a higher Cu accumulation in porous
area which supports the ion-exchange resins and surface chelating mechanism
(Fig. 3.7) (Saharan et al. 2015). Cu chitosan nanoparticles exhibited +22.6 mV
zeta-potential that indicates substantial stability through electrostatic repulsion of
positively charged nanoparticles. In addition, positive value of the zeta-potential
provides more electrostatic interaction with biological membranes and thereby
exhibits better antifungal activity (Saharan et al. 2013). This study concluded that
high value of zeta-potential of Cu-chitosan nanocomposite is vital to achieve strong
antifungal activity. Cu-chitosan nanocomposite (at 0.10 and 0.12 % concentration)
considerably hampered fungal mycelial growth and spore germination. In pot
experiments, these nanoparticles were very effective in disease control in tomato
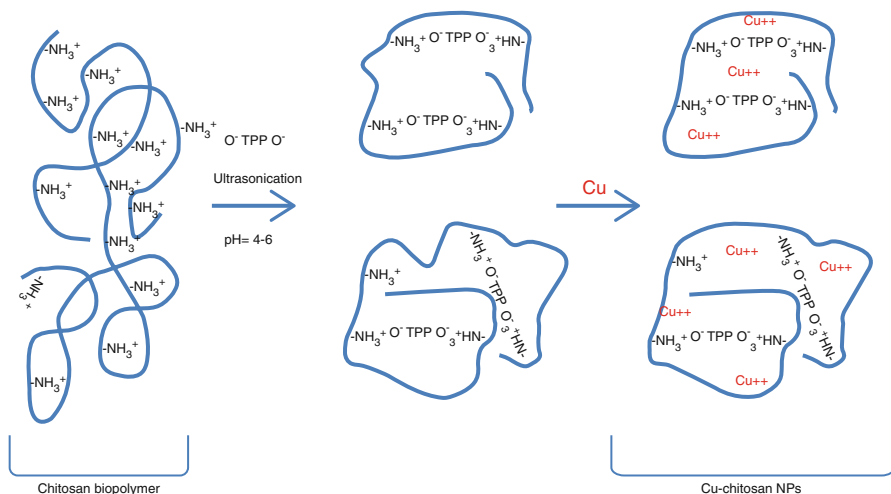
Fig. 3.7 Hypothetical model of ionic cross-linking reaction of chitosan, TPP and Cu (Saharan et al. 2015)

(Saharan et al. 2015). Results in pot experiments were more effective as compared to Petri plate (Saharan et al. 2013, 2015). These results could be explained by the strong elicitor activity of chitosan biopolymer in plant defense. Chitosan present in Cu-chitosan nanoparticles enhanced enzymes activities involved in plant defense. In addition, acidic environment created by the fungi (as they produce different types of acids) in infected plant provokes the break-up of nanostructure and releases Cu ions (Brunel et al. 2013). The released Cu ions produce reactive hydroxyl radicals to eradicate fungi (Borkow and Gabbay 2005). These studies strongly hypothesize a cumulative effect of chitosan elicitors to induce defense enzymes and direct Cu ions toxicity towards fungi. Potential toxicity of nanocomposites in plants is very vital (Lin et al. 2009; Kumari et al. 2009; Dimkpa et al. 2012) and therefore, it is essential to estimate the toxicity of nanomaterials. Cu-chitosan nanocomposites did not show any growth inhibitory effects on seedling growth of tomato and maize. The observed boost in seed germination and subsequent growth was due to the enhanced activities of starch and protein degrading enzymes (unpublished data).

Chapter 4
Biological Activities of Chitosan-Based Nanomaterials

Chitosan is naturally occurring biopolymer derived from fully or partially deacetylated chitin. From the last 151 years, ever since the discovery of chitosan, considerable progress has been made in understanding and exploitation of its potentiality. Due to the unique biological properties such as antimicrobial activity, biodegradability, biocompatibility, metal complexation and non-toxicity, chitosan has gained attention with potential applications in agriculture, food, pharmaceutical and textile industries (Marquez et al 2013). In fact, a number of commercial applications of chitosan take advantage of antimicrobial and bio-stimulating properties. Chitosan has been reported to induce innate immune response in plants against a broad spectrum of microbial species including fungi, bacteria and viruses. Further, polycationic and chelating properties of chitosan towards various organic and inorganic compounds make it a suitable biopolymer for bio-fabrication and controlled releasing formulations (CRFs) of agrochemicals. These physico-chemical and biological properties have been attracted in the field of agriculture (Kong et al. 2010; Liu et al. 2013).

Chitosan as signalling molecule enhances crop production by inducing and regulating defensive, symbiotic, growth and developmental processes in plants (Dzung et al. 2011). It is reported to activate more than 20 pathogen-related genes like β-glucanase, chitinase, etc. and metabolism-related genes such as auxin, cytokines, etc. (Hadwiger et al. 2002; Guo et al. 2006). These biological activities of chitosan enhance germination, photosynthesis, nutrient uptake and develop resistance against abiotic stress. The application of chitosan acts as an anti-transpirant agent which induces the production of jasmonic acid and ABA signaling which in turn leads to closure of stomata thereby checking the loss of water (Iriti and Faoro 2008; Sharp 2013).

As advancement in the technical application in agriculture, chitosan-based nanoparticles provide wide applications in contrasts to bulk. Bulk chitosan polymer is insoluble in aqueous media and has relatively low biostimulant actions thus limit its prospective in agriculture (Saharan et al. 2013). Chitosan-based nanoparticles

imbues flexibility in biological activities due to altered physico-chemical characteristics *viz*. size, surface area, active functional groups and higher encapsulation proficiency with or without blending with other compounds (Saharan et al. 2015).

4.1   Antimicrobial Activity

Chitosan introduces antibacterial, antifungal and antiviral activities and can be used as biocide for plant protection and growth (Xing et al. 2015; Van et al. 2013). A number of studies have been performed on the antimicrobial activity of chitosan and its derivatives against plant pathogens and has been exploited in commercial applications due to its broad spectrum nature (Badawy and Rebea 2011). The antimicrobial activity of chitosan has been acknowledged for more than 30 years but its mode of action still remains ambiguous (Marquez et al. 2013). Its antimicrobial activity closely correlates with its polycationic structure. As environmental pH is below the pKa of chitosan and its derivatives, the electrostatic interactions between the polycationic structure and the predominantly anionic components of the microorganisms' surface (such as lipopolysaccharide and cell surface proteins) play a primary role in antimicrobial activity (Kong et al. 2010). Antimicrobial activity of chitosan also depends on MW, DA, pH of chitosan and also on the nature of organism. In general, yeasts and moulds are more sensitive followed by Gram-positive and Gram-negative bacteria (Badawy and Rebea 2011).

4.1.1   Antibacterial and Antifungal Activity

Since 1979, chitosan is exhibiting broad spectrum activities against both Gram-positive and Gram-negative bacteria (Xing et al. 2015). The exact mechanism of the antibacterial activities of chitosan and its derivatives are still not fully clear. It is known that its antimicrobial activity is influenced by a number of factors that act orderly and in independent fashions (Kong et al. 2010; Hadwiger 2013). On the basis of chitosan's interactions with pathogens or host, the mechanism of antimicrobial activity can be explained in two ways.

(i) Direct activity – Here, the chitosan directly interacts with pathogen either by metal-chelation or by electrostatic interactions between the negative residues on cell surfaces of pathogens. These interactions lead to disabled plasma membrane, increased cell wall permeability and leakage of intercellular substances. The low molecular weight chitosan interferes in gene expression which leads to disrupted metabolic activities eventually killing the pathogens (Helander et al. 2001; Raafat et al. 2008). In additions, the deposition of chitosan onto microbial surface prevents nutrient flow and blocks the metabolic excretions (Helander et al. 2001).

(ii) Indirect activity (Plant cellular responses) – Here, chitosan acts as the revitalizator of elicitors and enhances various defense reactions in plant cellular system. Elicitation of plant defense response includes pathogen related proteins, hypersensitive responses, production of phytoalexins, accumulation of cellular secondary metabolites and reinforcement of cell wall against the pathogen attack (Kong et al. 2010; Katiyar et al. 2015).

4.1.1.1 Mechanism of Direct Antimicrobial Activity

Chitosan's antibacterial activity closely correlates with the polycationic nature and cell surface characteristics of bacteria. Cell surface hydrophobicity is another crucial factor in the interactions between bacteria and chitosan surface for adhesion. Adhesion of chitosan on negatively charged phospholipid of bacterial cell membrane induces changes in the bacterial membrane permeability (Chen and Cooper 2002). The increased membrane permeability leads to destabilization of cell membrane and leakage of intracellular substances ultimately leading to the death of bacteria (Kong et al. 2010). Further interactions may denature membrane proteins and initiate penetration of chitosan molecule into the bacteria.

The surfaces of pathogens are structurally complex, chemically heterogeneous and contain several types of polymers such as lipopolysaccharide (LPS), mycolic acids, lipoteichoic acids (LTA), capsular polysaccharides and proteins. These polymers are considerably involved in interactions with chitosan surface leading to strong attachment through short-range, non-covalent, weak interactions such as hydrogen bonding. The Gram-negative bacterial cell wall possesses an outer membrane lipopolysaccharide (LPS) which imparts a strong negative charge to the bacterium with a hydrophilic surface. The presence of anionic groups such as phosphate and carboxyl in the inner core of the LPS molecules contributes to the electrostatic interactions with polycationic chitosan (Helander et al. 1997). The cell wall of Gram-positive bacteria is comprised of peptidoglycan (PG) and teichoic acid (TA). The TA and PG, being rich in the phosphate group, contributes stout anionic properties (Raafat et al. 2008). Similar negatively charged compounds such as proteins and glycoproteins in the fungal or viral cell surfaces are also targeted by the chitosan. Thus, chitosan's potentiality to interact with the polyanionic structures of microbial cell membranes leads to destabilization of cell membrane and leakage of proteinaceous and other intracellular contents (Sudarshan et al. 1992).

Replacement of Mg^{2+} and Ca^{2+} present in the cell wall of bacteria also disrupts the integrity of cell wall. Once the cell looses its protection of cell wall, cell membrane is unguarded. The exposed cell membrane without cell wall starts to loose integrity and consequently collapses (Kong et al. 2008).

A parallel evidence of mode of action of chitosan has been demonstrated based on the interactions with DNA or RNA. Low molecular weight chitosan can penetrate cell wall and interacts with cellular DNA of fungi and bacteria which consequently inhibits RNA and protein synthesis (Sudarshan et al. 1992). The interaction of chitosan with DNA or mRNA directly regulates the metabolisms

and reproduction which could ultimately lead to death of microorganisms. The interactions of chitosan with genetic material is facilitated by negatively charged phosphate group of DNA and RNA and amino groups (positive charge) of chitosan. Fluorescence micrographs and gel-retardation experiments evidence the blocking of transcription of DNA in bacteria (Liu et al. 2001; Xing et al. 2009). These findings enlightened that disruption of protein synthesis and membrane destabilizations are primary and secondary mode of antimicrobial action of chitosan (Marquez et al 2013).

4.1.1.2   Mechanism of Indirect Antimicrobial Activity

Chitosan acts as elicitor and inducer of innate immunity with high affinity towards the plant transmembrane receptors. Plant possesses transmembrane pattern recognition receptors (PRRs) which are able to interact with pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) (Iriti and Faoro 2009; Hadrami et al. 2010). Polysaccharides such as chitosan and glucans secreted by the pathogens can be effective PAMPs/MAMPs. Through PRRs, chitosan can easily trigger a panel of defense responses (Amborabe et al. 2008). In *Arabidopsis*, receptor kinase CERK1 binds with chitosan and its homodimerization leads to the activation of immune signalling (Chen and Xu 2005). Chitosan induces a cascade of biochemical and molecular changes which includes DNA damage, chromatin alterations, increase in cytosolic Ca^{2+}, activation of MAP-kinases, oxidative burst, callose deposition, increase in pathogenesis-related (PR) gene mRNA, PR protein synthesis, phytoalexins accumulation, hypersensitive response (HR), synthesis of jasmonic acid (JA), abscisic acid (ABA) and accumulation of hydrogen peroxide in plants (Hadwiger et al. 2002).

Chitosan binding to DNA competes with histone sensitive DNA sites allowing DNA polymerase complexes to continue to transcribe through the open reading frames of PR genes. Hence, chitosan biopolymer stimulates the defense system of plant (Schwabish and Struhl 2004). It may cause alterations of DNA helical structure, single strand cleavage and removal of H_2A and H_2B histones (Weake and Workman 2008). The induction of systemic acquired resistance by chitosan has been reported. Chitosan rapidly induces the pathogenesis related and defense-related enzymes such as phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, catalase, superoxide dismutase, glucanase, chitinase etc. The induced enzyme activity by chitosan participates in ROS scavenging system (Ahmad et al. 2013). The lignification and suberization processes are also triggered by chitosan which provide mechanical strength and thus create barriers for invading pathogen.

However, the biological activity of chitosan as a general elicitor remains tied to its physico-chemical properties such as molecular weight, deacetylation degree and viscosity. These properties can make the difference between cytotoxicity due to higher concentrations and the priming of resistance using appropriate molecular weight, deacetylation degree, viscosity and concentration.

4.1.2   Antiviral Activity

Till today, many studies have provided conclusive evidences that chitosan can inhibit the viral infections (Katiyar et al. 2015). Although the mechanism of antiviral activity still remains obscure, it has been reported that chitosan activates the systemic acquired resistance such as programmed cell death and hypersensitive responses (Iriti et al. 2006). Chitosan also induces the callose biosynthesis by enhancing ABA synthesis. Callose is a component of plasmodesmata and membrane-lined channels and contributes to the regulation of plant symplastic permeability through changes in plasmodesmata opening, following to it synthesis or hydrolysis by β-1,3-glucane synthase and β-1,3-glucanase, respectively. Consequently, the spread of virus through cell-to-cell is impaired either by callose deposition around the channels or by inhibition of callose degrading enzymes. Thus, it is most likely that chitosan's antiviral activity mostly resides in the induction of programmed cell death (PCD) while callose deposition would slow down cell-to-cell movement of the virus from the first infected cells. This would allow PCD program to be mounted before excessive spreading of the infection (Iriti and Faoro 2008).

4.2   Antimicrobial Activity of Nanochitosan

Chitosan-based nanoformulations are more efficient due to their improved physico-chemical and biological characteristics. Nanoparticles exhibit high surface area, higher solubility and biostimulating activity in comparison to its bulk form. Chitosan nanoparticles express more affinity towards pathogen's outer membrane and thus easily enter into the pathogens' cell (Van et al. 2013). Interactions between positively charged nanochitosan molecules and the polyanionic structures of microbial cell membranes lead to destabilization of cell membrane. This induces the leakage of intracellular contents and eventually causes death of pathogens. In addition, nanochitosan could penetrate into cell and bind to negatively charged molecules such as DNA and mRNA blocking the gene expression. These interactions lead to arresting of cellular metabolism.

Nanochitosan shows high potentiality to bind biological membranes of plants and induces various signaling pathways related to defense-related protein. Thus, nanochitosan upregulates the activities of PAL, PPO, POX, CAT, SOD and PR related proteins. It also induces the hypersensitive-related reactions in plants to avoid or eliminate the invaded pathogen from the cell (Fig. 4.1; Chandra et al. 2015).

In recent studies, chitosan nanomaterials exhibited porous network structure (Jaiswal et al. 2012; Saharan et al. 2015). Porous nanochitosan proved high encapsulation efficiency of metal ions. Chitosan biopolymers get easily dissolved in acidic environment. Encapsulation of metal or other drugs into chitosan can act as slow releasing and long-lasting complex in biological systems. The Cu-chitosan nanocomplex can effectively be used against plant pathogenic fungi since fungal
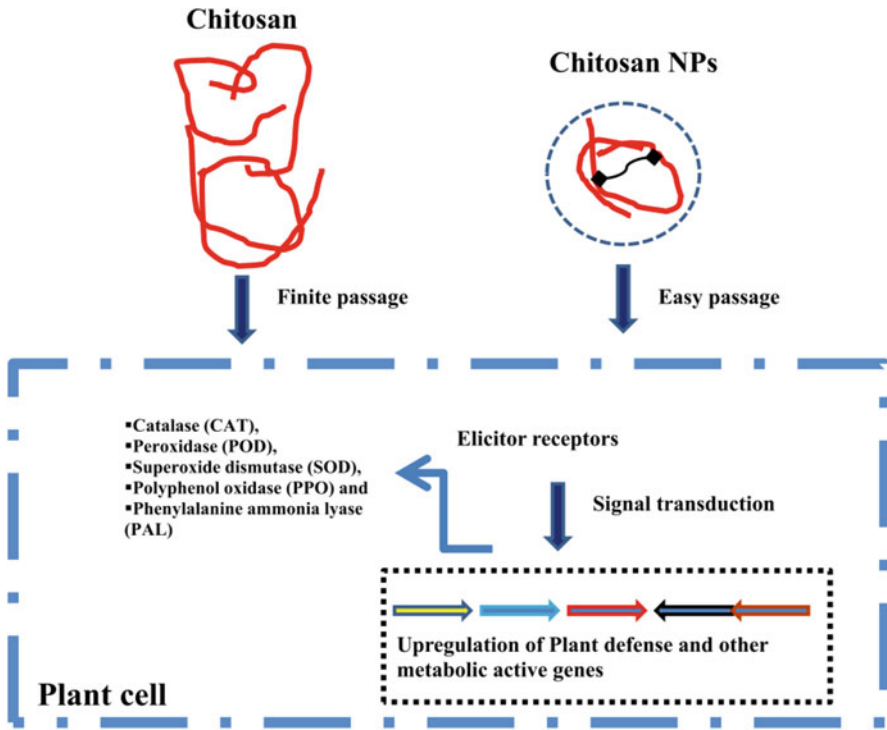
Fig. 4.1 Chitosan nanoparticles induce various defense related and other genes in plants

infection makes the plant microenvironment acidic. Cu-chitosan has proven high antifungal activity by inhibiting spore and mycelium formation of *Alternaria alternate*, *Macrophomina phaseolina* and *Rhizoctonia solani* (Saharan et al. 2013 and 2015.).

4.3 Plant Growth and Development

Advancement in chitosan research has initiated various applications in agriculture. Biostimulating activity and protection from pathogens are some of the major characteristics of chitosan which could be exploited in agriculture. Chitosan is reported to enhance seed germination capacity, root length, seedling height, seed vigour index (SVI) and increases root activity. It also improves physiological responses such as chlorophyll synthesis, photosynthesis and chloroplast enlargement. In addition, it directly affects plant nutrition by improving soil fertility, enhancing nitrogen fixation and increasing mineral uptake (Dzung et al. 2011; Van et al. 2013; Katiyar

et al. 2015). The effects of chitosan and its derivatives have been verified in large groups of crops including cereals, pulses and horticultural (Dzung et al. 2011; Van et al. 2013; Katiyar et al. 2015; Manjunatha et al. 2008 and Sui et al. 2002).

4.3.1   Promotion of Seedling Growth and Development

Seed germination and establishment of seedling is an important phenomenon in agriculture. The effect of chitosan on seedling vigour and plant establishment is well documented in various plants species (Katiyar et al. 2015). Chitosan has excellent film-forming property making it an easy agent to form a semipermeable film on the seed surface which can maintain the seed moisture and absorb soil moisture which thus can promote seed germination. Additionally, it restrains the seed respiration by preventing oxygen entery, restricting loss of CO_2 and maintaining high concentration of CO_2 within the film (Furbank et al. 2004).

Chitosan can also increase soluble sugar content and enhances the activity of protease to increase free amino acid content (Zeng et al. 2012). Researchers have reported that chitosan increases shoot length, root length, shoot dry weight and relative water content under salinity stress. Similar results were reported by Ma et al. (2014) who reported that wheat seeds treated with chitosan showed higher growth than control under salinity stress. The chitosan oligosaccharides act on plants in a manner similar to phytohormone in the regulation of morphogenesis, growth and development (Cote and Hahn 1994). It can effectively increase IAA concentration and promote cell division in protoplasts grown in the absence of auxin, cytokinin and activates the expression of AX 11, a gene related in auxin signalling (Guo et al. 2006). It is suggested that chitosan triggers the defensive mechanisms in plants, stimulates root growth and induces enzymes such as chitinases, pectinases and glucanases (Hien 2004). Chitosan promotes plant growth through increasing the availability and uptake of water and essential nutrients by adjusting cell osmotic pressure (Guan et al. 2009).

There are 61 genes out of 2375 in *Arabidopsis* having three-folds higher degree of transcription in plants treated with chitosan in comparison to the control (Ramonell et al. 2002). Effect of chitoligosaccharides on gene expression in rice also shows that 166 genes are considerably induced and 93 genes are repressed (Akimoto-Tomiyama et al. 2003).

4.3.2   Physiological Response

Chitosan stimulates various physiological aspects of growth and development. It has been reported to significantly enhance photosynthesis, chloroplast enlargement and photosynthetic pigments like chlorophyll a, b and carotenoids (Van et al.

2013). The content of leaf water reflects the water status of plant. Chitosan coating can improve the leaf water content of seedlings and acts as an antitranspirant agent. The induction of a variety of genes and transcriptional activation of jasmonic acid and ABA by the chitosan nanoparticles play a vital role in water use by the plants (Iriti and Faoro 2008). The experimental results showed that chitosan significantly increased the concentration of chlorophyll which illustrate that chitosan can enhance the photosynthesis performance and thereby the yield (Dzung et al. 2011). Application of chitosan nanoparticles also increased the number of stomata as well as increased stomatal conductance. Due to polycationic nature, it induced the ABA level which resulted in higher conductance and CO_2 accumulation into the cell. Increased stomatal conductance and photosynthetic pigments have pronounced effect on the yield (Bittelli et al. 2001; Van et al. 2013).

In drought stress, chitosan could effectively promote the development of root system in wheat and strengthen the capability of water absorption so as to enhance the drought resistance of wheat seedlings. Application of chitosan could also increase the activities of various defense enzymes like SOD, POD and CAT thereby strengthening the clearing ability of reactive oxygen species and slowing down the damage to plasma membrane (Zeng and Luo 2012).

4.3.3   Plant Nutrient Uptake

The nutrient uptake by the plants increases with the application of bulk as well as nanochitosan. Chitosan and all its derivatives have high nitrogen content with thermal and chemical stability. Plants can access nitrogen via microbial breakdown and release inorganic nitrogen or directly take up the monomers as organic nitrogen. The application of chitosan increases nutrient uptake and stimulates the growth of coffee seedling. The uptake of mineral nutrients such as nitrogen, potassium, phosphorus, calcium and magnesium was significantly increased in plants treated with chitosan. Particularly, the increased content of magnesium and total nitrogen contributes to increased chlorophyll content as compared to control (Dzung et al. 2011).

Chitosan oligosaccharides are molecular signals to induce and regulate not only the defensive but also the symbiotic, developmental and growth processes in plants (Dzung 2011). Lipo-chitooligosaccharides (LCOs) play an important role in the symbiotic nitrogen fixation and induce NOD gene that regulates the formation of nodules in leguminous plants (Sharp 2013). Induction of NOD genes in the *Rhizobium* bacteria clearly indicated that chitosan has potentiality to increase nitrogen fixation in leguminous plants (Staehelin et al. 2000).

The functional groups of chitosan (hydroxyl and amino groups) allow the formation of complexes with ions of copper, zinc, iron and others. This makes chitosan a sustainable alternative to synthetic chelating agents. The cationic property of chitosan also makes it a suitable medium for supplying additional essential

nutrients. Chitosan boosts the soil anionic exchange capacity (CAE) and thus reduces the leaching of anionic nutrient fertilizers such as nitrates and phosphates from the soils. In addition to the controlled release of nutrients, chitosan polymers have also been successfully used to improve the delivery of certain pesticides to crops to improve their efficacy and reduce environmental impact (Hadwiger and McBride 2006; Sharp 2013).

4.4 Plant Growth and Developmental Activity of Chitosan Nanoparticles

Due to the unique physico-chemical characteristics of chitosan nanomaterial, the application of chitosan nanoparticles has gained increased interests among the researchers. However, a few reports are available on the utilization of chitosan nanomaterials in plant growth and development. The role of bulk chitosan towards plant growth and development has been limited due to its poor solubility and stability in aqueous medium. In addition, bulk chitosan is a polymer which exists in larger size and unable to interact with the biological system efficiently. Chitosan nanomaterials, in contrast, have proved greater stability and bioactivity as compared to bulk.

The nanochitosan notably increased the levels of nutrients uptake, photosynthetic pigments and seedling growth. High opening and high conductance of stomata has been induced by the application of chitosan nanoparticles through increasing osmotic pressure of stomatal cells (Van et al. 2013). Saharan et al. (2015) recently reported the growth promoting effect of Cu-chitosan nanoparticles on tomato seedlings. Further experiments on maize seedlings revealed that Cu-chitosan nanocomplex induced the amylase and protease activity in seed and mobilizes the stored food which ultimately helps in seed germination (unpublished data). These findings conclude and give superior idea about the chitosan nanoparticles' potentiality towards plant growth and developments.

Chitosan biopolymer based nanoparticles have significant plant growth stimulatory activity. There is great scope for the utilization of chitosan-based nanoparticles in agriculture due to their polycationic nature and amenability to biofabrication or chemical modifications. These properties make them suitable candidate to increase the production and productivity in agriculture. However, much work has to be done regarding the mode of action of chitosan-based nanoparticles. Most of the proposed mechanisms explain the general modes of action. There is an urgent need to exploit chitosan-based nanoparticles alone or in conjugation with various organic and inorganic compounds. Chitosan-based nanomaterials have biostimulating activity in plants to strengthen their defense systems and also act as controlled releasing complex for agrochemicals which ultimately reduce the risk of toxicity of agrochemical in the food chains.

Chapter 5
Current and Future Prospects of Chitosan-Based Nanomaterials in Plant Protection and Growth

Today, agriculture sector is facing various hurdles because of change in climate, urbanization, diminishing arable land, death of natural resources, etc. Hence, it has become important to increase crop production to feed the ever-growing world population. Despite the significant annual increase in input, yield has stagnated and even declined in some cases. So, different approaches have been exploited to improve the yields of various crops. The conventional approaches are inefficient, in many cases, in narrowing down the gap between potential and average yield (Mondal et al. 2013). There is enormous scope for researchers to develop an efficient and eco-friendly production technology based on the innovative techniques to increase seedling vigour and plant establishment against the biotic and abiotic stresses (Siddiqui and Whaibi 2014).

Due to the complexity of diseases and mutating pathogens, it is very difficult to manage the diseases with single control measure (Kulkarni and Anahosur 2011). Hence, efforts are needed to explore the feasibility of combination of various control measures for integrated management (Trivedi et al. 2002). The application of chemical pesticides such as systemic and non-systemic biocides provides primary means for controlling the plant pathogens (Badawy and Rabea 2011). Conventionally, pathogens and pests are controlled by the application of ∼3 million tons pesticide per annum worldwide, of which ∼90 % get run-off (Pimentel and Burgess 2014). Moreover, the indiscriminate use of such compounds has faced major obstacles such as pesticide residues (bioaccumulation) and proliferation of resistance in pathogens. Hence, there is growing emphasis on eco-friendly and alternative novel biocides which are natural and have multiple target sites on disease causing microorganisms so that development of resistance can be delayed (Rabea et al. 2003). Blending of nanoscience with biogenic substances has set up a novel field of research known as nano-biotechnology. In future, nano-biotechnological approaches would be the alternative to existing technologies for management of plant diseases (Saharan et al. 2013). Nanotechnology plays an important role in modern agriculture to address global challenges such as climate change, severity of plant diseases and the limited

availability of important plant nutrients (Parisi et al. 2015). These challenges can be solved with the development of nanomaterials for the treatment and detection of diseases, to enhance the ability of plants to absorb nutrients, etc. (Puoci et al. 2008). A variety of nanomaterials, mainly metal-based nanomaterials, have been studied by several groups of independent researchers in various crops for plant growth (Wang et al. 2012; Yang et al. 2015). Polymer-based nano-formulations have recently received the greatest attention with the key objectives of developing less harmful, plant growth promoting and protective agents of biodegradable and natural origin (Kah and Hofmann 2014). Many natural polymers extracted either from micro-organisms or crabs shells possess strong elicitor effects in plant. In the recent years, numerous biopolymers such as starch, cellulose, alginate, chitin, chitosan and carbohydrates have been used for the development of new materials with environmental sustainability and desirable functionality (Babu et al. 2013). Due to unique characteristics such as non-toxic, biocompatible, safe and biodegradable, amino-polysaccharide chitosan has been exploited globally as an antibacterial, antifungal agent either alone or blended with other biomaterials (Dutta et al. 2004). The ability of chitosan to chelate metal ions such as Cu^{+2} and Zn^{+2} could make perfect composite based on the principles of nanotechnologies (Hardy et al. 2004; Wang et al. 2004; Saharan et al. 2015). Chitosan-based copper nanomaterials have been used as antifungal, antibacterial as well as plant growth promoting agents (Hardy et al. 2004; Saharan et al. 2015). Similarly zinc-chitosan complex showed a wide spectrum of antimicrobial activity (Wang et al. 2004). Various studies have shown that chitosan biopolymer enhances the efficiency of plants to reduce the deleterious effects of unfavorable conditions by affecting various physiological responses like plant immunity, defense mechanisms which involve enzymes such as phenylalanine ammonium lyase, polyphenol oxidase, tyrosine ammonium lyase and antioxidative enzymes SOD, CAT and POD (superoxide dismutase, catalase and peroxidase) (Yin et al. 2008; Ma et al. 2014; Katiyar et al. 2015). Researchers have also depicted the positive effect of chitosan on growth and development of seedling (Batool and Asghar 2013).

5.1   Plant Nutrition, Biotic and Abiotic Stresses and Post-Harvest Management

Chitosan is a well-recognized biomaterial in agriculture and successfully applied in many crops for enhancing seed germination, lengthening shoot-root, ameliorating abiotic stress, increasing chlorophyll content, enhancing nitrogen fixation and increasing nutrient uptake (Reddy et al. 1999; Nge et al. 2006; Dzung et al. 2011; Zeng et al. 2012). It is known for boosting the defense response in plants (Guo et al. 2006; Hadrami et al. 2010; Liu et al. 2010; Meng et al. 2010; Yin et al. 2010; Zeng et al. 2012; Ma et al. 2013a, b; Chen et al. 2014). Bulk chitosan has been precisely demonstrated to induce plant defenses in tomato, cucumber, chilli,

strawberry fruits, etc. It can set-off plant immunity by rousing hydrogen peroxide (H_2O_2), nitric oxide (NO) pathways, triggering defense-related gene expression, etc. It has been suggested that chitosan could enhance growth and yield in soybean. Seed soaked with chitosan exhibited increased germination rate, length and weight of hypocotyls and radicle in rapeseed. It is also found beneficial in enhancing the germination rate of cucumber, chilli, pumpkin and cabbage. Seed priming with chitosan enhances seed germination and seedling vigor in pearl millet. Further, it is also noticed that seed priming with acidic chitosan solutions improves maize vigor. Similarly, rice seedlings treated with chitosan showed induced defense responses against rice blast pathogen. Moreover, chitosan also stimulated the growth and yield of rice along with reinforcing the defense response. Recently, chitosan coating has emerged as an ideal alternative to chemically synthesized pesticides. In post-harvest technology to prevent undesirable losses, chitosan biopolymer has become a promising coating agent to fruits and vegetable because of its antimicrobial and defense elicitor activity. Recent studies have indicated that pre-harvest spray of chitosan on strawberry plants showed reduced fungal infection on fruit. Coating of chitosan on fruits and vegetables induces various defense enzymes including pheny-lalanine ammonia lyase (PAL), chitinase and glucanase. Introduction of chitosan nano-film composites made up of chitosan and other antimicrobial agents could be more effective in preventing post-harvest losses of fruits and vegetables. Chitosan can also help to maintain the safety of edible products. The protection of fresh-cut broccoli against *E. coli* and *Listeria monocytogenes* was assisted by chitosan along with bioactive components such as bee pollen and extracts from propolis and pomegranate. From the above points, it is clear that the chitosan products are more effective and can be used in a numbers of ways to reduce disease levels and enhance crop productivity in an eco-friendly and sustainable manner. Nano-chitosan has not been exploited copiously in plant nutrition and biotic and abiotic stress management. Applications of bulk chitosan have been well documented and thus various conclusions can be drawn from various valuable characters of bulk chitosan to be included in nano-chitosan for higher outputs in plant growth and protection. With the advancement of nanotechnology, chitosan-based nanomaterials are being largely adopted for their exploration in plants (Shukla et al. 2013). Increasing number of publications on chitosan nanoparticles in plant growth and protection infers vast potential of nanochitosan over bulk chitosan (Shukla et al. 2013). Nanochitosan as compared to bulk chitosan has superior physico-chemical characteristics that provide enhanced biological activities (Saharan et al. 2013; Van et al. 2013). Hence, there is a pressing need to explore chitosan biopolymer not only for its novel antimicrobial property but also for its plant defense booster property to combat future challenges of mutating pathogenic population under global warming (Saharan et al. 2014). Various chitosan-based nanoparticles have been developed and amongst them Cu and Zn-chitosan nanoparticles exhibit significant plant growth promoting and antimicrobial activity (Qi et al. 2004; Wang et al. 2004; Du et al. 2009; Brunel et al. 2013; Jaiswal et al. 2012; Saharan et al. 2015). But these nanomaterials have not been much exploited and in-depth study is needed to extract their potential in agriculture (Qi et al. 2004; Saharan et al. 2013). Zn and Cu have traditionally

been used as a major component of agrochemicals. But because of increased use at high dose, their levels have exceeded the recommended limits of heavy metals in soil. Therefore, newer strategies need to be developed and executed to overcome the problems of high dose of agrochemicals and to prevent their run-off. *In vitro* antimicrobial activities of chitosan-Zn complexes (nano/micro) have been evaluated against various species of bacteria and fungi (Wang et al. 2004; Du et al. 2009). The complexes showed 2–16 times higher antimicrobial activities as compared to chitosan and zinc sulfate. Chitosan-Zn was found effective against *E. coli* and *Corynebacterium* with a MIC value of 0.000313 % (CS–Zn w/v). The antimicrobial activity depends on the content of Zn ions and value of zeta-potential. Zinc is an important micronutrient for plant growth and is absorbed by plants through diffusion and specific transporters in the form of divalent ions. Zn moves through xylem and phloem and contributes in several bioactivities in various parts of plant. Absorption of Zn from soil is highly dependent on soil chemistry. Presence of high amount of phosphorus, excess Cu and higher pH (alkaline calcareous soils) can impede the bioavailability of Zn to plants. As a result, Zn deficiency is more frequently seen in the agriculture field in spite of the fact that most soils contain significant amount of Zn. The application and interactions of chitosan-Zn complex have not been fully addressed especially in agriculture sector. Past studies on the chelation of Zn ions with chitosan have mainly been focused on antimicrobial activities (Jeon and Kim 2000; Wang et al 2004). It is predicted that divalent form of Zn in chitosan-Zn complex contributes to higher biological activities. Our results have demonstrated that chitosan-Zn nanocomposites are more effective as compared to bulk chitosan and $ZnSO_4$ in percentage of seed germination, shoot-root length, root number and seed vigor index of maize. Our laboratory has further evaluated the interaction of Zn-chitosan nanoparticles on enzymes responsible for seedling growth *viz*. protease and α-amylase. Zn-chitosan nanomaterials significantly enhanced the protease activity at 3rd day of seed germination whereas α-amylase activity was maximum at 5th day. Chitosan-Zn nanoparticle clearly showed growth promoting effect on maize seedling. However, the antifungal activity of chitosan-Zn was not much promising in our experiments (with maximum 30 % inhibition against *Fusarium verticillioides* and *Curvularia* sp.) as compared to other chitosan-metal nanocomposites (unpublished data). Copper is very important indispensable metal for plant growth. It is a key element in proteins involved in photosynthetic electron transport chain, mitochondrial respiration, oxidative stress responses, hormone signaling, etc. Cu^{+2} acts as cofactors in enzymes like superoxide dismutase, cytochrome c oxidase, amino acid oxidase, laccase, plastocyanin and polyphenol oxidase (Mazhoudi et al. 1997). It is also crucial in signaling of transcription, protein trafficking and oxidative phosphorylation. Therefore, plants require Cu ions as a vital micronutrient for growth and development (Xiong et al. 2006). Its deficiency as well as excess can induce abnormal plant growth (Fernande and Henriques 1991). Hence, for vigor plant growth, Cu ions need to be supplied from outside. In addition, Cu-doped chitosan complex acts as a potential adsorbent for various agro-materials especially pesticides. Jaiswal et al. (2012) has developed porous Cu-chitosan nanocomposite (size ranging from 700 to 750 nm) of higher surface area (20 m^2 g-1 and a pore

volume of 0.11 cc g^{-1}). The developed Cu-chitosan nanocomposite has significant adsorption capacity (322.6 ± 3.5 mg g^{-1}). FTIR results revealed that malathion (a pesticide) formed complex with copper dithionate. The developed porous Cu-chitosan nano-composite totally removed malathion in the agricultural run-off. In another study, laboratory synthesized Cu-chitosan nanoparticles showed growth promotory effect on tomato seed germination, seedling length, fresh and dry weight (Saharan et al. 2015). At 0.12 % concentration, Cu-chitosan nanoparticles caused 70.5 and 73.5 % inhibition of mycelia growth and 61.5 and 83.0 % inhibition of spore germination in *A. Solani* and *F. oxysporum*, respectively, in *in vitro*. A concentration of 0.12 % of Cu-chitosan nanoparticles was found significantly effective in percentage efficacy of disease control (PEDC) in tomato plants against early blight and *Fusarium* wilt. In general, Cu-chitosan nanoparticles showed growth promotory and antifungal potential. This could lead to open up the possibility of applying Cu-chitosan nanoparticles at field level (Saharan et al. 2015).

5.2   Future Prospects

Use of chitosan-based nanoparticles in agriculture field is still in a budding phase. Significant outcomes have been reported in *in vitro* and a few *in vivo* studies in plant growth and protection by chitosan-based nanomaterials. Use of nanochitosan for delivery of agrochemicals (pesticides, micronutrients, fertilizers and plant growth hormones) would be the most promising field in coming times for nanotechnology application in agriculture. Various studies have concluded that a slow and steady release of agrochemicals (or other encapsulated materials) defiantly exerts a profound effect in crop plants without increasing the agrochemicals doses. Additionally, nano-encapsulated agrochemicals with the phenomenon of slow release can reduce the environmental hazard. Moreover, the slow release of agrochemicals also lessen cross-talk phenomenon where burst of higher dose of agrochemicals may show overturned effect on plant growth due to synthesis of cellular components which abolish the effect of applied agrochemicals. Further, a smart delivery system could be developed by chitosan nanomaterials for crops where agrochemicals encapsulated in nano-chitosan are released through external stimulation. This could be understood by the fact that chitosan materials get dissolved under acidic pH. During microbial infection, the micro-environment of infected tissues becomes acidic and encapsulated materials get released which start to act on pathogens (Fig. 5.1). This can be a most advanced way of development of smart delivery system for pesticides' application in agriculture. Despite nano-chitosan based delivery system, the elicitor property of chitosan biopolymer can also be further improved by converting the chitosan into nano-chitosan so that it may have alleviated effect on plant defense system. Being nanoparticles, they can easily transfer deep into the plant system and become systemic in nature. It has been demonstrated that chitosan nanoparticles considerably improve innate immune
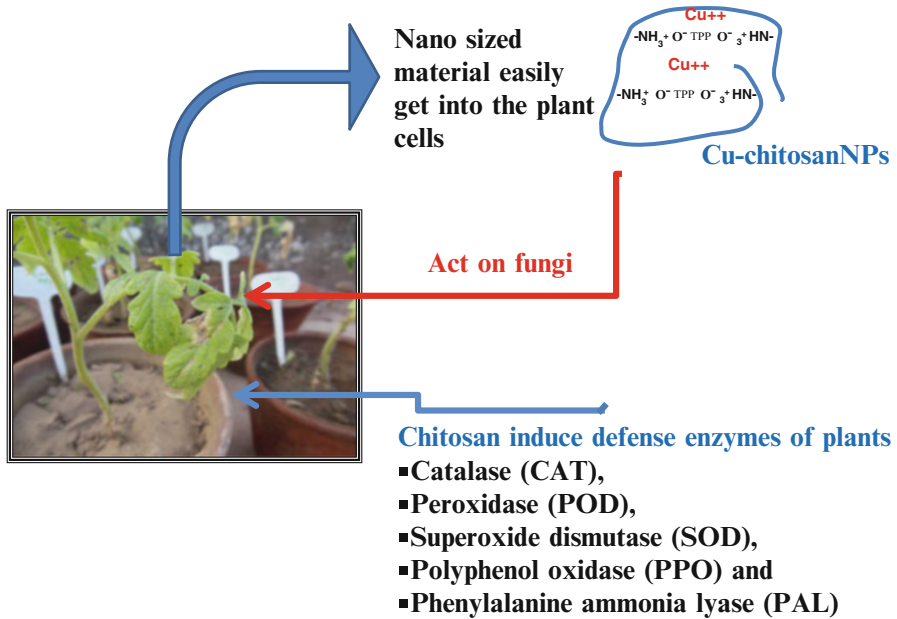
Fig. 5.1 Smart delivery system of chitosan nanomaterial

response in plants through activation of defense enzymes and up-regulation of defense related genes like phenolics and antioxidant enzymes. Taken into account, chitosan nanoparticles may be better promising disease controling agents as compared to bulk chitosan in crop. Further, there is a great opportunity of developing chitosan-based nano-bioconjugates (NBCs). NBC can be defined as a nano-size complex of two or more biomolecules or an encapsulated nanocomplex of two or more biomolecules which having high surface area with unique biological activity. The involvement of two or more compounds in a single complex can perform multiple tasks in plants. Various metals like copper, zinc, manganese, selenium and other biologically active compounds like salicylic acid, jasmonic acid, plant secondary metabolites and essential oils (thymol, neem oil) are targets which could be efficiently used in chitosan NBCs for applications in plants. Therefore, chitosan-based nanoparticles wield dual role in plants by exciting its defense system and delivering the active ingredients systemically for growth. The vague information of molecular interactions of chitosan in plants prevents its full exploitation in agriculture field and entails further research at molecular level.

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