Chapter 17
Chitosan Nanoparticles: An Overview on Preparation, Characterization and Biomedical Applications

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Abstract Chitosan (CS) and Chitosan nanoparticles (CNPs) have multifaceted applications in medicine, agriculture, pharmaceutics, tissue engineering, waste water treatment and food industries. CS is recognized as a less or non-toxic, biocompatible polymer by US Food and Drug Administration (FDA) for wound dressing as well as in dietary application. The properties of CS have upgraded by making their nanoparticles. Due to their exceptional properties including nanosize with large surface area to volume ratio, presence of reactive groups ($-NH_2$ and $-OH$), cationic nature (NH_3^+), bioadhesivity, biocompatibility, bioavailability and biodegradable nature; CNPs are explored in many ways in biomedical filed as an antimicrobial agent, wound healing agent, scaffolds for tissue engineering, anti-tumour agent in cancer therapy, carriers for gene and drug delivery, etc. In this chapter we highlight on CNPs preparation, characterization and certain important biomedical applications.

Keywords Chitosan (CS) · Chitosan nanoparticles (CNPs) · Scanning electron microscopy (SEM) · Transmission electron microscopy (TEM) · Tripolyphosphate (TPP)

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Abbreviations

AFM	Atomic force microscopy
CNPs	Chitosan nanoparticles
CS	Chitosan
DA	Degree of acetylation
DD	Degree of deacetylation
DLS	Dynamic light scattering
ELS	Electrophoretic light scattering
FTIR	Fourier-transform infrared spectroscopy
HMW	High molecular weight
IBV	Infectious bronchitis virus
LMW	Low molecular weight
MMW	Medium molecular weight
nAg	Silver nanoparticles
nCu	Copper nanoparticles
NDV	New castle disease virus
NPs	Nanoparticles
$nTiO_2$	Titanium dioxide nanoparticles
nZnO	Zinc oxide nanoparticles
PDI	Polydispersity index
PEC	Polyelectrolyte complex
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
TPP	Tripolyphosphate
XRD	X-ray diffraction

17.1 Introduction

Marine products have been in the forefront of natural materials used in the therapeutic applications against several human diseases (Venugopal 2008). Majority of marine products are derived from exoskeletons of crustaceans such as crabs, shrimps, krills and lobsters. Chitin (polysaccharide) is one of the biopolymers majorly extracts from shells of crustaceans, cell walls of fungi and certain insects. Since chitin is insoluble in many common solvents, it is not widely used for fabrication of products or a food commodity (Tsigos et al. 2000; Crini 2006). The deacetylated chitin, i.e., chitosan has remarkable properties including biocompatibility, biodegradability, mucoadhesive, non-antigenic, non-toxic, solubility in weak acids, cationic nature, and hence, used in many biomedical applications (Gupta et al. 2019; Onsosyen and Skaugrud 1990; Felt et al. 1998; Han et al. 1999; Zhang and Zhang 2002; Kmiec et al. 2017; Guo and DiPietro 2010). The United States Food and Drug Administration (USFDA) has recognized chitosan as a GRAS (Generally

Recognized as Safe) material, and approved to use in food, agriculture and biomedicine (Kumar et al. 2019). For the last two decades, extensive studies have been conducted on fabrication and application of chitosan based nanocomposites in various fields including medicine, pharmaceutics and agriculture. Most of the research studies revealed that chitosan nanoparticles have superior physiochemical and biocompatible properties over CS, and have significant attraction in tissue engineering, biomedicine, drug delivery and cancer therapy. Due to small size, CNPs possess larger surface area to volume ratio, and hence, used as delivery vehicles for anti-cancer drugs, anti-inflammatory drugs, vaccines, antibiotics, peptides (arginine-glycine-aspartate; RGD), aptamers, folate, glycoproteins, polysaccharides, genes, growth factors, etc. (Jayasuriya 2017). However, due to the larger surface area and charge, CNPs can be readily absorb impurities from the medium, and hence, to overcome this limitation, selection of appropriate method for their fabrication and further characterization is an important key parameter. This chapter describes various methods and their principles for fabrication and characterization of CNPs for biomedical applications.

17.1.1 Chitin

Chitin is the most abundant linear biopolymer and structural polysaccharide widely occurring in the nature after cellulose. It is found in the exoskeletons of crustaceans, cell walls of fungi and in certain invertebrates. Various crystalline allomorphs of chitin are α, β and γ, which differ in orientation of microfibrils. The α-forms are more abundant and stable than β and γ forms. The α-forms are mainly present in cell walls of fungi, shells of crustaceans and arthropods, whereas β and γ forms are found in in squid pens, *Ptinus* beetles and Loligo squids (Jang et al. 2004; Carlstrom 1957). Insolubility of chitin in solvents such as water, organic solvents and basic solutions is due to the generation of intra- and intermolecular hydrogen bonds with acetyl, amino and hydroxyl groups of its polysaccharide chain. Insolubility of chitin affects the production of chitin based products. Crustacean's wastes from fish processing industries are the main source of chitin.

17.1.2 Chitosan

Chitosan is a natural linear polysaccharide synthesized by deacetylation of chitin. It is composed of D-glucosamine and N- acetyl D-glucosamine sub-units. The polycationic nature of chitosan is remarkable as most of the polysaccharides in the acidic solutions are either neutral (or) negatively charged. Because of this specific property, it forms electrostatic complexes with negatively charged polymers, lipids, proteins and DNA (Venkatesan and Kim 2010; Pavinatto et al. 2010; Madihally and Matthew 1999; Takahashi et al. 1990; Kim et al. 2007). CS has exceptional

properties such as non-toxicity, low allergenicity, biocompatibility, hydrophilicity, antimicrobial activity, bioactivity (Kumar et al. 2004; Gällstedt and Hedenqvist 2006; Pillai et al. 2009), and hence, it is widely used in various applications include, among other, wound healing (Chandy and Sharma 1990), waste water treatment (Onsosyen and Skaugrud 1990; Kumar et al. 2019), drug carrier (Felt et al. 1998), treatment for obesity (Han et al. 1999), and as a scaffolds for tissue engineering (Zhang and Zhang 2002).

CS is insoluble in aqueous solutions, however, it is soluble in dilute aqueous acidic solutions such as acetic acid (pH $<$ 6.3). CS solubility decreases as pH increases. At lower pHs, CS becomes protonated and shows stronger antimicrobial activity. Solubility of CS in aqueous solutions can be improved by chemical modifications, such as quaternization of nitrogen atoms of the amino groups (Goy et al. 2009).

17.2 Chitin to Chitosan Nanoparticles

Fabrication of CNPs from exoskeletons of crustaceans or composite chitin involves many methodologies including, extraction of pure chitin, chitin deacetylation for chitosan, preparation and characterization of CNPs. Further, these CNPs can be used for various biomedical applications and drug delivery. Overall strategies for CNPs fabrication are represented in the pictorial diagram (Fig. 17.1)

Marine bio-wastes such as shells of crustaceans used as a principal raw material for industrial production of chitin. The exoskeleton of crustaceans is composed of chitin (15–40%), proteins (20–40%), calcium carbonate (20–50%), pigments and lipids (Yan and Chen 2015). This composite chitin is much harder than the pure chitin. Two main extraction methods including conventional chemical extraction and biological extraction were followed to extract the pure chitin from composite chitin in industrial processing. Further, the chitin is converted to chitosan by deacetylation (chemical or enzymatic deacetylation). Chemical extraction of chitin is suitable for large scale production, however, it has many disadvantages like high energy consumption, high environmental pollution and difficulty in recovering waste products (pigments and proteins) (Gortari and Hours 2013; Cheung et al. 2015; Manni et al. 2010). Proper washing, drying, grinding and sieving up to 1 mm size of shells of crustaceans are the common initial steps in both extraction methods.

17.2.1 Chemical Extraction

In this extraction method, the composite chitin under goes demineralization, deproteinization and decoloration/bleaching to produce pure chitin. Demineralization majorly involves separation of minerals such as calcium carbonate and calcium phosphate by concentrated/diluted acid (HCl, HNO_3, H_2SO_4 and CH_3COOH)

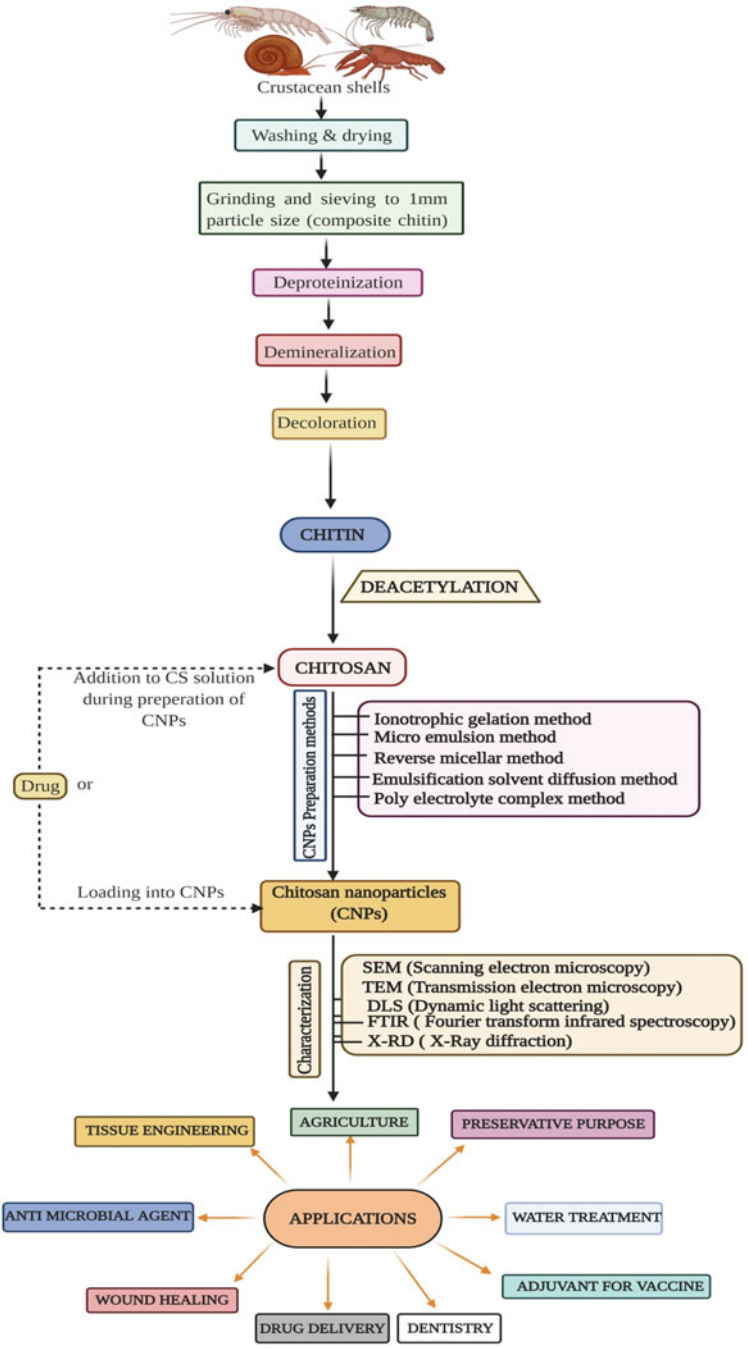

Fig. 17.1 Diagram representing overall fabrication methodologies of CNPs from chitin and their applications

treatment at room temperature. Then the alkaline (NaOH/KOH) treatment at high temperature (100°C to 120°C) separates proteins (deproteinization). Order of these two methods is interchangeable based on the source and proposed use of chitin. The third phase involves separation of pigments such as carotenoids (mainly astaxanthin and its esters) by NaOCl, H_2O_2 or $KMnO_4$ treatment. The fine white powder of pure chitin is subsequently deacetylates and converts into chitosan by alkaline treatment (25–50% NaOH) at high temperature (80°–140°).

17.2.2 Biological Extraction

In this method, demineralization and subsequent deproteinization are carried out by means of microbial (bacteria: *Lactobacillus* sp. *Pseudomonas* sp. *Bacillus* sp./Fungi, *Aspergillus* sp.) fermentation and proteolytic enzymes (chymotrypsin, trypsin alcalase, pepsin, papain, devolvase and pancreatin) treatment, respectively. Demineralization must be the prior step to deproteinization, since the minerals can inhibit the activity of proteases. The resultant pure chitin is then deacetylated by chemical (NaOH)/enzyme (chitin deacetylase) treatment to convert into chitosan.

17.3 Preparation of Chitosan Nanoparticles

Nanoparticles (NPs) are small solid colloidal particles ranging from 10 to 1000 nm, provide large surface area to volume ratio and unique physiochemical properties that allows them in enormous applications (Du et al. 2009; Prasad et al. 2016, 2017). NPs shows more specialized characteristics compared to their bulk materials, because, as the size decreases, the percentage of surface atoms increases (Gupta et al. 2007). Studies have revealed that CNPs can be acquired unique physiochemical and biological properties than to their bulk CS form. Therefore, CNPs are widely used in drug delivery, tissue engineering and other biomedical fields.

CNPs were first synthesized in 1994 by Ohya and co-workers through emulsification and cross-linking method for intravenous delivery of anti-cancer drug 5-fluorouracil (Grenha 2012). Since then, many methods have been employed for synthesis of CNPs, these include, Ionotropic gelation, microemulsion, emulsification solvent diffusion, polyelectrolyte complex and reverse micellar method (Tiyaboonchai 2003). All these methods comprise bottom-up fabrication processes, which involves the assembly of molecules in solution to form defined structures (Chan and kwok 2011).

17.3.1 Ionotropic Gelation Method

This method is relatively simple and mild, and conducted at aqueous conditions without the use of any organic solvent (Fig. 17.2). It was first reported by Calvo et al. (1997). The main strategy of this method is to establish electrostatic interactions between the cationic chitosan polymer and polyanion like tripolyphosphate (TPP) or sodium sulphate with or without stabilizing agent such as poloxamer. In this method, firstly, chitosan is dissolved in acetic acid aqueous solution to become cationic polymer and further the solution is allowed to react with polyanions. It leads to the formation of CNPs under constant stirring at room temperature. Physiochemical properties of nanoparticles (size and surface charge) could be modulated by changing the ratio of chitosan and TPP, and the pH value of the solution (Calvo et al. 1997).

17.3.2 Microemulsion Method

In this method, Chitosan nanoparticles are prepared using surfactant, for example, AOT (sodium bis (2-ethylhexyl) sulfosuccinate) and a cross linker, glutaraldehyde. Initially, surfactant/hexane mixture is prepared by dissolving surfactant into n-hexane, thereafter, the chitosan solution and glutaraldehyde are added into the above mixture by continuous stirring at room temperature. Overnight stirring allows establishing cross link between free amine group of chitosan and glutaraldehyde.

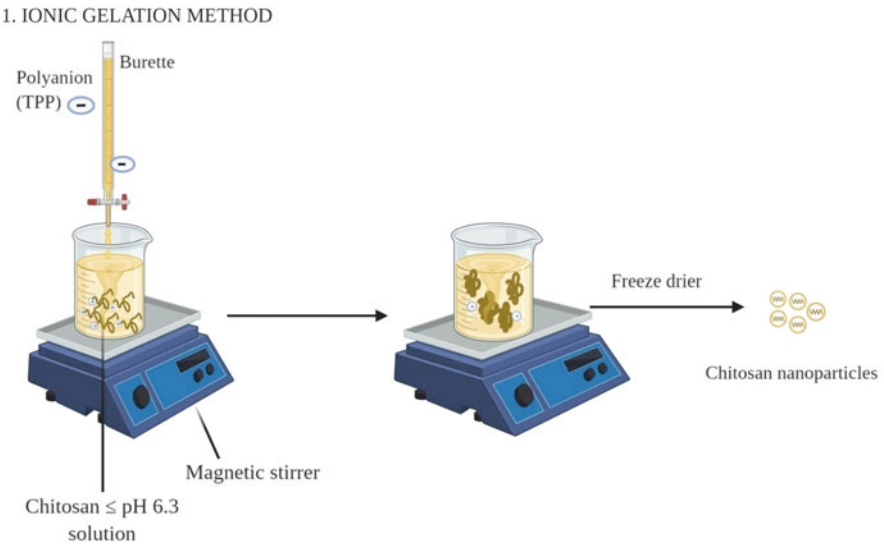

Fig. 17.2 Schematic diagram representation of Ionotropic gelation method

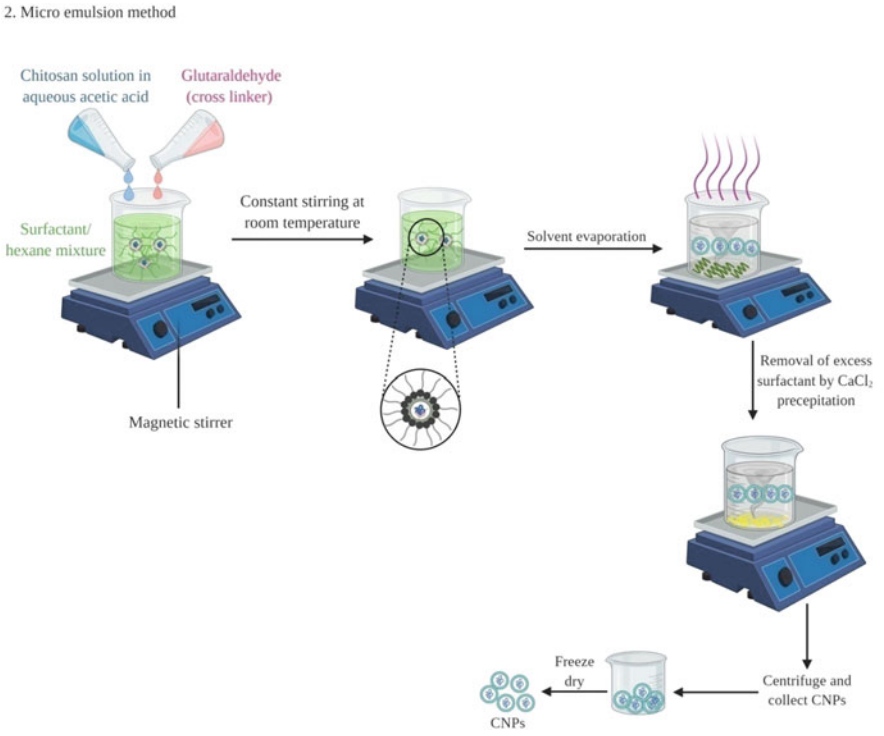

Fig. 17.3 Schematic diagram representation of microemulsion method

Further, the organic solvent and excess surfactant are removed by evaporation under low pressure and precipitation with $CaCl_2$ followed by centrifugation, respectively. The resultant nanoparticle suspension is then dialyzed and lyophilized (Maitra et al. 1997). In this method less than 100 nm sized nanoparticles can be produced, and further the size could be altered by varying the concentration of glutaraldehyde (Sailaja et al. 2011).Usage of glutaraldehyde (toxic agent) and cumbersome process could be the disadvantages of this method (Fig. 17.3).

17.3.3 Reverse Micellar Method

This method was reported by Brunel et al. (2008), it is an adoption of microemulsion method. The method is free from addition of cross linker and toxic organic solvents. In brief, the surfactant is dissolved in organic solvent, to which added chitosan aqueous solution under constant stirring to obtain reverse micelles. By this method extremely thin nanoparticles can be formed (Fig. 17.4).

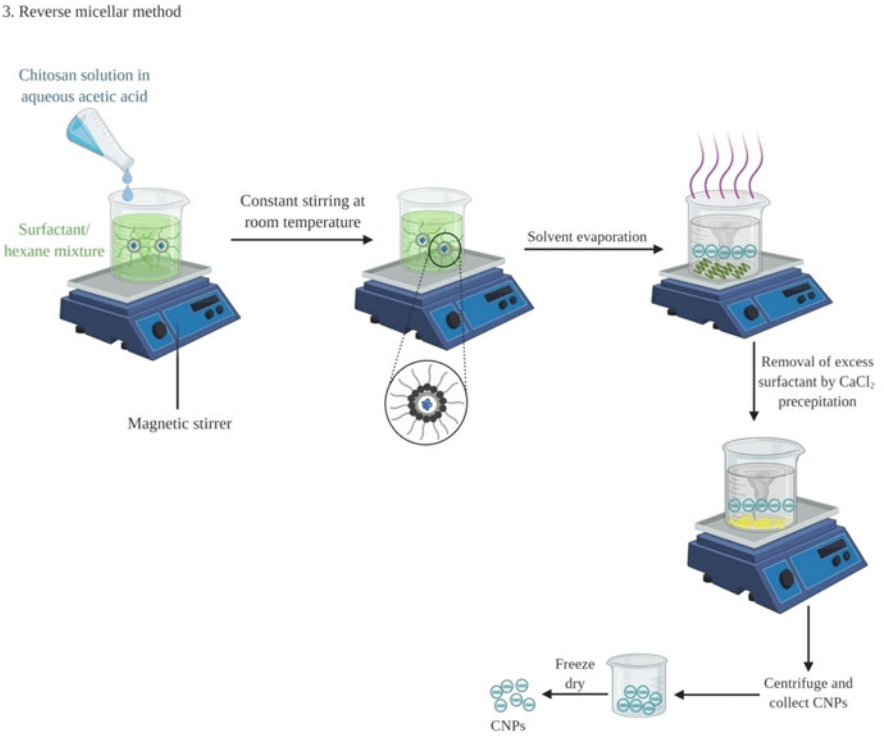

Fig. 17.4 Schematic diagram representation of reverse micellar method

17.3.4 Emulsification Solvent Diffusion Method

This method was first discovered for the fabrication of poly D, L-lactide/glycolide (PLGA) nanoparticles (Niwa et al. 1993), and later it was adapted to prepare chitosan nanoparticles by El-Shabouri (El-Shabouri 2002). In this method, an organic phase (e.g., methylene chloride and acetone) was injected into chitosan solution containing stabilizing agent (e.g., poloxamer and lecithin) under high shearing force, followed by high-pressure homogenization (Fig. 17.5). The resultant emulsion is then diluted with more water to overcome the organic solvent miscibility in water. Polymer precipitation occurs upon the diffusion of organic solvent into water, which subsequently leads to the formation of NPs. High percentage of hydrophobic drug entrapment could be achieved by this approach, however, use of organic solvents and high shearing forces are again the major drawbacks of this method (Mohammed et al. 2017).

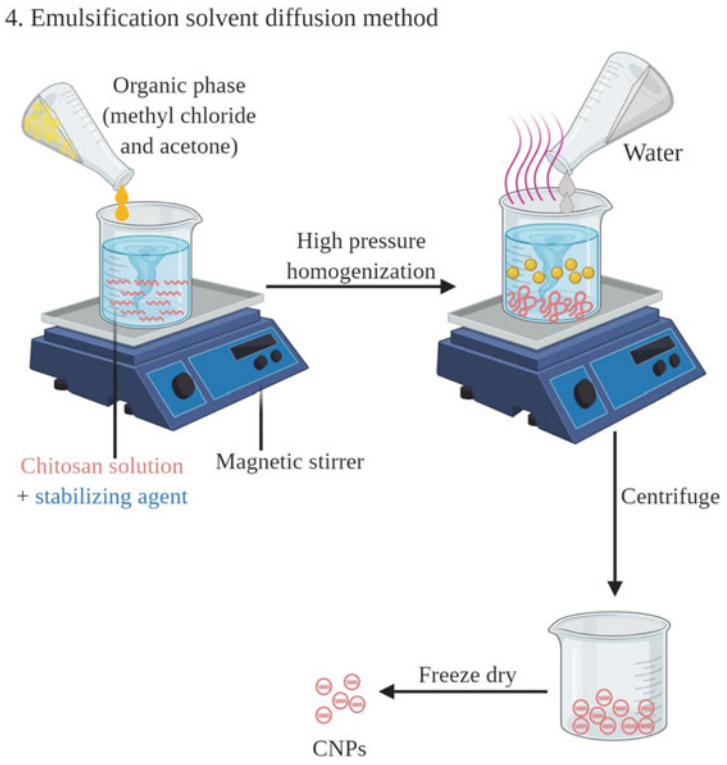

Fig. 17.5 Schematic diagram representation of emulsification solvent diffusion method

17.3.5 Polyelectrolyte Complex Method (PEC)

It is quite simple and not requires any catalysts/ initiators or toxic organic solvents for preparation of nanoparticles. PECs are self-assembled NPs, resulted from electrostatic interactions between cationic polymer and polyanions. The cationic chitosan polymer (pH $<$ 6.0) is spontaneously associated with polyanions of chondroitin sulphate and hyaluronate (Denuzier et al. 1998), dextran sulphate (Chen et al. 2003; Chen et al. 2007), carboxymethyl cellulose (Ichikawa et al. 2005), heparin (Liu et al. 2001; Tan Tang et al. 2011), and DNA (Erbacher et al. 1998) in solutions to form PECs (Fig. 17.6).

17.4 Characterization of Chitosan Nanoparticles

Characterization of NPs tells about their physiochemical properties include, among others, particle size and size distribution, surface morphology and surface charge, etc. Particle morphology and surface characteristics can be studied by Scanning

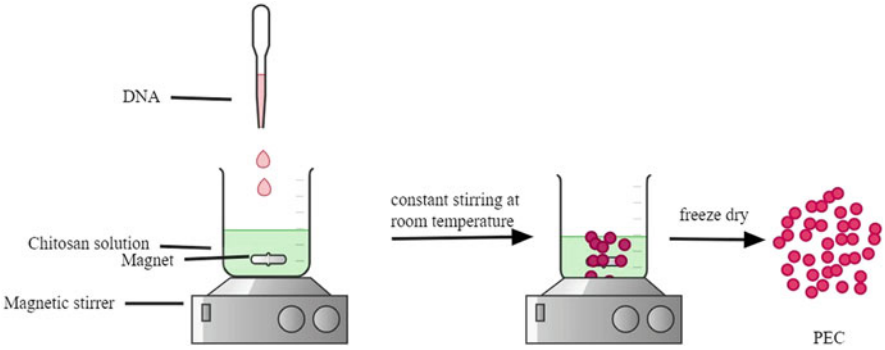

Fig. 17.6 Schematic diagram representation of formation of PEC

electron microscopy (SEM), Transmission electron microscopy (TEM) and Atomic force microscopy (AFM). Under these microscopic studies, most often spherical shaped CNPs are observed, however, some authors also reported a mixture of globular, toroids, rod-like particles (Danielsen et al. 2004; MacLaughlin et al. 1998; Köping-Höggård et al. 2001; Huang et al. 2005). The instrument, Zetasizer provides other particle characteristics using Dynamic light scattering (DLS), poly-dispersity index (PDI) and zeta potential (ζ).

17.4.1 Scanning Electron Microscopy (SEM)

SEM is one of the microscopic techniques to study the surface morphology and size of the nanoparticles. In principle, a high energy beam of electrons scan over the surface of nanoparticles and emit signals such as low-energy secondary electrons, backscattered electrons and X-rays, etc., that are detected by a detector and generates their three dimensional image. Samples to be studied under SEM are mounted on a metal stub and coated with a thin film of gold or other conducting material under vacuum. In our laboratory we have prepared chitosan nanoparticles by ionic gelation method found to have spherical shape with 141 nm in size Fig. 17.7 (Unpublished data). Spherical chitosan nanoparticles with an average size of 200 nm have observed under SEM (Jingou et al. 2011).

17.4.2 Transmission Electron Microscopy (TEM)

The size and surface morphology of nanoparticles can be studied by using TEM. It has much higher spatial resolution over SEM and it provides 2-dimensional image of the sample. The working principle of TEM is, when a high-energy electron beam pass through the sample, generates transmitted and diffracted electron beams. The

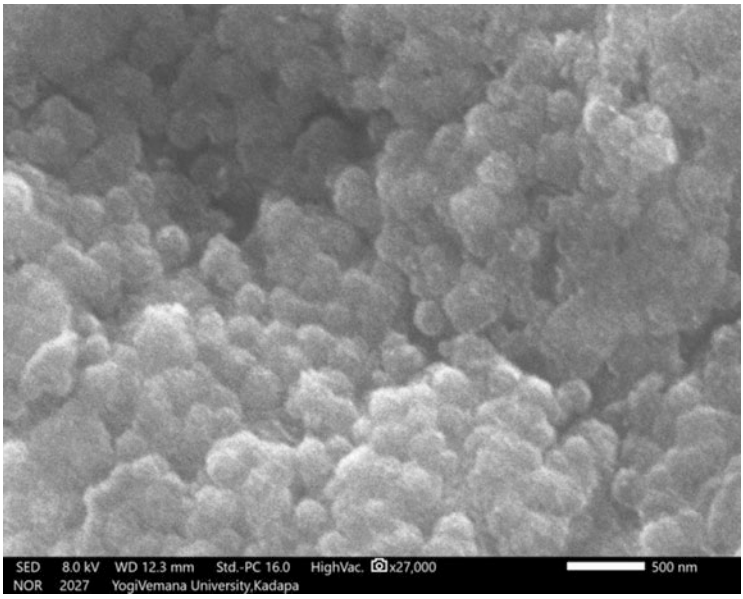

Fig. 17.7 SEM image of CNPs prepared by Ionotropic gelation method

interference between the two beams forms an image on the fluorescent screen which is coupled with a charge-coupled device (CCD) detector. Samples are to be examined under TEM, dispersed on carbon coated copper grid and allowed to dry at room temperature. TEM images of chitosan nanoparticles showed an average size distribution of 25–30 nm (Phan et al. 2019). Deng et al. (2006) observed, lysosome loaded CNPs as spherical structures with 50–280 nm in diameter. In a study, polymeric CNPs are resulted from reverse micellar method, which are spherical in shape with smooth surface and narrow size distribution of about 90 nm (Manchanda and Nimesh 2010).

17.4.3 Zetasizer

The instrument Zetasizer can be used to study the particle characteristics such as particle size, size distribution, surface charge, etc., using Dynamic light scattering (DLS), polydispersed index (PDI) and zeta potential (ζ).

17.4.3.1 Dynamic Light Scattering

Dynamic light scattering (DLS), also known as Photon correlation spectroscopy or Quasi-Elastic light scattering is one of the popular light scattering analytical

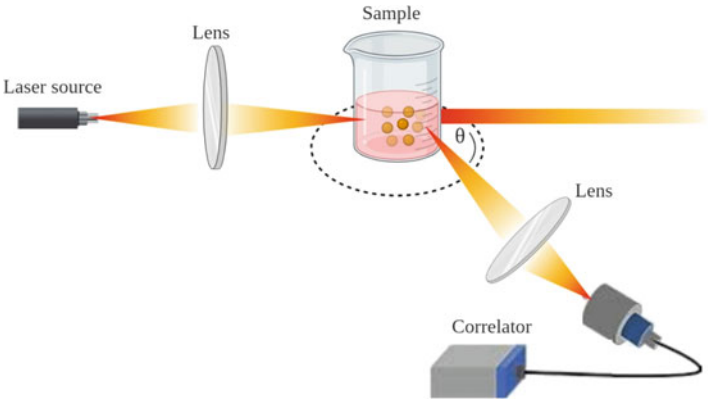

Fig. 17.8 Diagram representing dynamic light scattering principle

techniques used to study size distribution of nanoparticles or submicron particles include, among others, micelles, polymers, emulsions and proteins. The basic principle is simple: particles (in solution) to be studied are illuminated with monochromatic light beam, which in turn generates fluctuations in scattered light intensity are subsequently detected at a known scattered angle (θ) by a photo detector (Fig. 17.8). When a particle sample is dispersed in a solution, particles move randomly (Brownian motion) due to the collision of the solvent molecules around them (Choudhary et al. 2017a). Smaller particles move with greater velocity than the larger particles, hence, the distance between the particles is constantly varying. When moving particles in solution are exposed to a single frequency laser beam over a period of time, generates time dependent fluctuations in scattered light intensity depending on their sizes. These time dependent fluctuations can be related to particle speed by autocorrelation function. The autocorrelation function is used to determine the diffusion coefficient. The Stokes–Einstein equation can be used to convert the diffusion coefficient to the hydrodynamic diameter (Barth 1984). The size of CNPs in water measured using DLS ranges from 40 to 374 nm with an average size of ~ 250 nm (Saharan et al. 2013, 2015; Choudhary et al. 2017a, b). The CNPs obtained from ionotropic gelation method have shown size ranges from 68–75 nm by DLS (Fig. 17.9) (unpublished data).

17.4.3.2 Polydispersity Index Value (PDI)

The polydispersity index value (PDI) is a measure of the heterogeneity of a sample based on size. It means, the particles exhibit either monodispersed or polydispersed distribution in solutions, which can be studied by the instrument that use dynamic light scattering (DLS). According to the International standards organizations (ISOs), the PDI value < 0.05 represents that the particles are monodispersed in nature, whereas the value > 0.7 indicates larger polydispersed particle distribution.

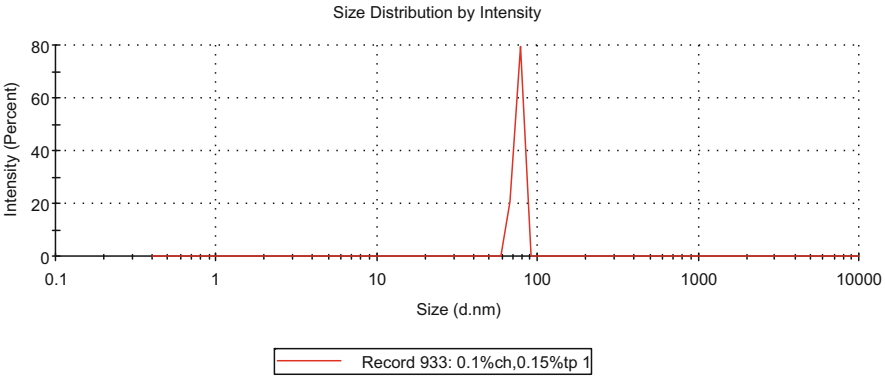

Fig. 17.9 Size distribution of CNPs

The PDI value of chitosan nanoparticles varies from 0.1 to 0.4 which indicates uniformity and stability of particles in suspension. Othman et al. (2018) reported that Chitosan nanoparticles loaded with L-Ascorbic Acid and Thymoquinone (CNP-LAA-TQs) had PDI values of 0.207 ± 0.013. PDI values of Methotrexate-chitosan–polyanion NPs, i.e. MTX-DCH-PAM-18Na NPs and MTX-DCH-PAM-18K NPs ranging from 0.238 to 0.485 and 0.247 to 0.339, respectively (Ciro et al. 2020).

17.4.3.3 Zeta Potential (ζ)

Zeta potential is an important parameter which tells about degree of electrostatic repulsion between charged groups present on particle surface (Saharan et al. 2013, 2015). It is an indicator of the stability of colloidal dispersion, which can be detected by electrophoretic light scattering (ELS). Formation of aggregation is not observed when the particles in the suspension have either high negative or positive zeta potentials as they tend to repel each other, whereas aggregation is observed at low zeta potentials due to less repulsion forces of particles. In general, nanoparticles with zeta potentials of $> +30$ to < -30 have high stability (Kumar et al. 2017). The zeta potential value of nanoparticles is affected by surface chemistry, particle concentration, size of particle, pH of the medium, temperature, solvent, and ionic strength (Mudalige et al. 2019). The optimised zeta potential values for stability of colloidal dispersion were given in the Table 17.1 (Kumar et al. 2017). In different studies, the zeta potential values of CNPs ranged between +21 mV and +50 mV, indicating CNPs are highly stable (Rampino et al. 2013; Li et al. 2018; Kheiri et al. 2017; Ali et al. 2011).

Table 17.1 The optimized zeta potential values for stability of colloidal dispersion (Kumar et al. 2017)

Zeta potential (mV)	Stability behaviour of colloidal Dispersion
0 to ±5	Rapid coagulation or flocculation
±10 to ±30	Incipient instability
±30 to ±40	Moderate stability
±40 to ±60	Good stability
>61	Excellent stability

17.4.4 Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR is one of the absorption spectroscopic techniques widely used in nanoparticles characterization. It measures all of the infrared frequencies simultaneously, rather than individually, and provides sufficient information about the functional groups of a compound. When a sample is illuminated with infrared radiation, it absorbs and transmits certain amount of radiation, from which a detector generates interpretable spectrum that provide structural insights of the sample. The spectrum consists stretching (symmetric and asymmetric stretching) and bending (scissoring, rocking, wagging and twisting) vibrations. Stretching vibrations changes the bond length, whereas bending vibrations change the angle between two bonds of the molecules. In FTIR spectra of chitosan nanoparticles, the peak at 3447 cm^{-1} is attributed to $–NH_2$ and –OH groups stretching vibration. These peaks shift hypsochromically to 1639 and 1557 cm^{-1} in the FTIR spectra of CN which is caused by the interaction between NH_3^+ groups of chitosan and phosphate groups of TPP. The peaks at 1657 cm^{-1} and 1598 cm^{-1} are attributed to the $CONH_2$ and NH_2 groups, respectively (Lustriane et al. 2018; Qi and Xu 2004; Bhumkar and Pokharkar 2006; Sarkar et al. 2013). Similar FTIR spectra have been obtained for CNPs in our laboratory (Fig. 17.10).

17.5 Biomedical Applications

17.5.1 Antimicrobial Activity

Chitosan is a versatile biopolymer has many biomedical applications. Its effective role on microbes majorly depends on its molecular weight, pH and degree of deacetylation (DD). Many studies have revealed antimicrobial activity of chitosan against bacteria, fungi and yeasts; however, exact mode of action is still not fully understood. As of now, the following possible theories have somehow made an attempt to explain the mode of antimicrobial action of chitosan.

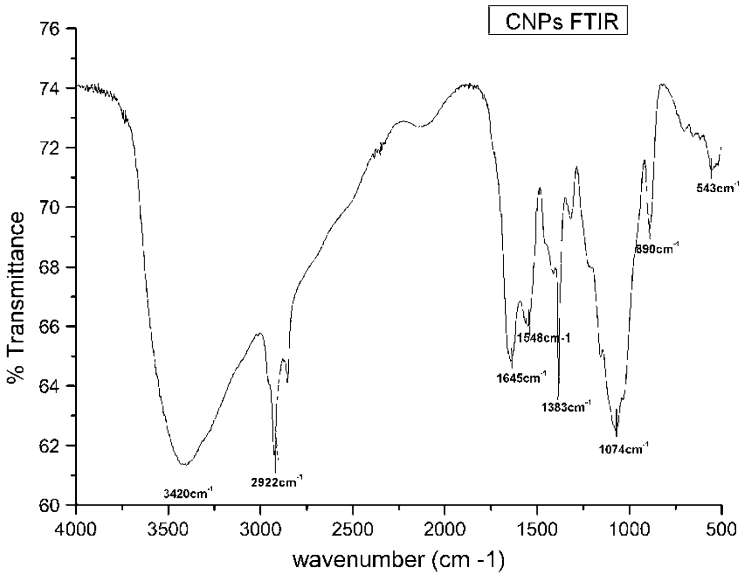

Fig. 17.10 FTIR spectrum of CNPs, shown specific peak at 3420 cm^{-1} due to the overlap of O-H and N-H stretching, similarly, peaks at 1645 cm^{-1}, 1548 cm^{-1} and 1383 cm^{-1} are attributed to the CO-NH_2, NH_2, C-H groups, respectively (unpublished data)

17.5.1.1 Polycationic Nature of Chitosan

As per this hypothesis antimicrobial activity presumably depends on the alteration of membrane permeability of microbes such as bacteria, fungi and viruses. The cell walls of Gram-positive bacteria is largely composed of peptidoglycan layer with certain composition of teichoic acids (wall teichoic acid and lipoteichoic acid), which gives negative charge to the bacterial surface, while the Gram-negative bacteria cell wall outer membrane possess lipopolysacharides (LPS), which provides surface negative charge. In acidic aqueous solutions, the NH_2 groups at C2 position of chitosan protonates to yield NH_3^+, which in turn forms electrostatic interactions with negatively charged groups (mostly phosphate groups of teichoic acids and LPS) located on the bacterial cell surfaces, leads to enhance the cell wall/ cell membrane permeability followed by leakage of intracellular constituents and death of the cell (Fig. 17.11) (Tsai and Su 1999; Aziz et al. 2014, 2015, 2016; Inamuddin et al. 2021).

In a study, in vitro assays, killing kinetics, cellular leakage measurements, membrane potential estimation, electron microscopy and transcriptional response analysis have given a speculation that antimicrobial activity of chitosan (LMW) is due to electrostatic binding of protonated amine groups with negatively charged teichoic acids (predominantly with lipoteichoic acids) of bacterial cell wall of Gram-positive bacteria *Staphylococcus aureus* (Raafat et al. 2008).

In several studies, CS shown more bactericidal effect in Gram +ve bacteria (*Listeria monocytogenes*, *Bacillus megaterium*, *B. cereus*, *S. aureus*, *Lactobacillus*

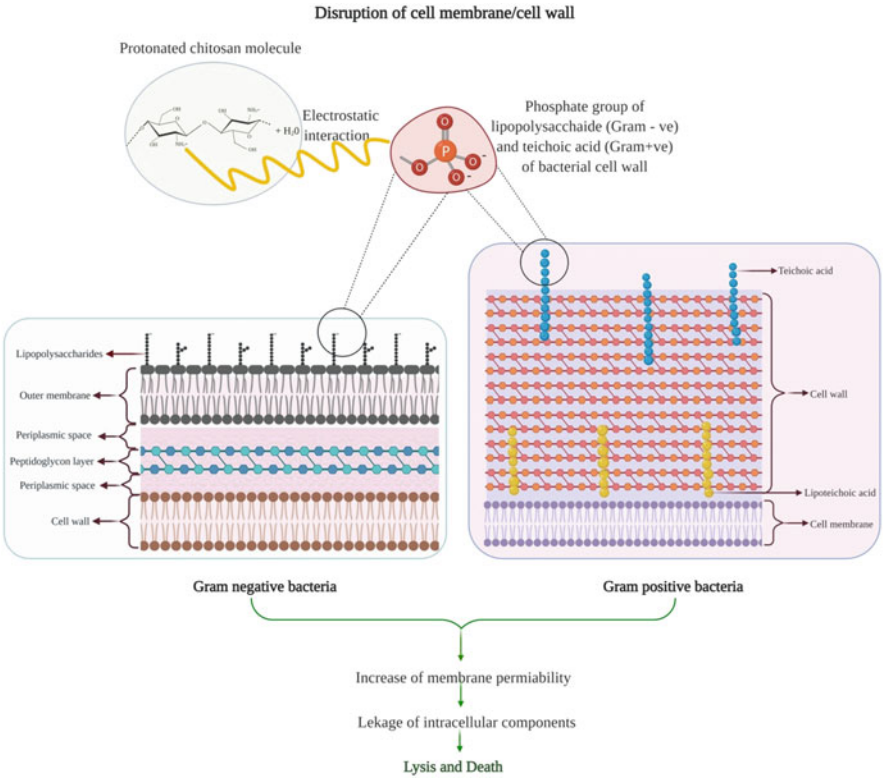

Fig. 17.11 Antimicrobial effect of CS by Polycationic nature

plantarum, *L. brevis*, *L. bulgaricus*) than in Gram –ve bacteria (*E. coli*, *Psedomonas fluorescens*, *Salmonella typhimurium*, *Vibrio parahaemolyticus*) (No et al. 2002).

Similar mechanisms might also be applicable to fungal pathogens due to presence of phospholipids in their membrane. At low pH, the protonated amine groups of chitosan interact with the negatively charged phosphate groups of carbohydrate side chains of fungal cell wall proteins and decrease the negative charge that leads to alteration of important metabolic pathways (Ing et al. 2012; Pena et al. 2013). In a study, LMW CS showed antifungal activity against the pathogenic yeast *Candida albicans* by decreasing the cell surface negative charge. Sialic acid, a constituent of cell wall glycoprotein of *C. albicans*, which provides negative charge to the cell wall (Soares et al. 2000; Tronchin et al. 2008). Positively charged groups of chitosan increase *E.coli* membrane permeability and lysis of membranes (Li et al. 2015).

17.5.1.2 Chitosan Interaction with Nucleic Acid

Low molecular weight (LMW), micro and nano size chitosan particles can able to penetrate into the cytoplasm of microorganisms and bind to the negatively charged biomolecules such as DNA and RNA by electrostatic interactions and subsequently effect on the downstream mechanisms such as transcription and translation (Fig. 17.12) (Jarmila and Vavrikova 2011; Sudarshan et al. 1992).

17.5.1.3 Chelating Nature of Chitosan

Divalent metal ions are prerequisite for microbial growth, enzymatic functions, membrane integrity and other (Varma et al. 2004; Hosseinnejad and Jafari 2016; Rabea et al. 2003; Chien et al. 2016; Matica et al. 2019; Kong et al. 2008). Chitosan acts as chelating agent for metal ions, at lower pH (below 6.0) its amine groups (NH_2) become protonated ($-NH_3^+$) and compete with divalent ions for electrostatic binding to phosphate groups of teichoic acid and lipopolysaccharide (LPS) of Gram +ve and Gram −ve bacteria, respectively (Fig. 17.13). On other hand, chitosan at higher pH value (above pKa 6.3) also chelates many metal ions. Less availability of essential metal ions leads to enhancement of cell wall permeability and sensitive to several chemicals or antibiotics (Clifton et al. 2015).

17.5.1.4 Cell Surface Blocking Nature

High molecular weight (HMW) chitosan molecules deposit as dense polymer layer on cell surface of microbes and became a barrier to uptake of essential nutrients, minerals and oxygen (aerobic microbes) as well as excretion of their metabolic products, leads to death of cells (Fig. 17.14) (Yuan et al. 2016; Devlieghere et al. 2004).

17.5.2 Factors Affecting the Antimicrobial Activity of Chitosan

The main factors that affect antimicrobial activity of chitosan and its derivatives are pH, temperature, molecular weight and degree of acetylation.

Higher antimicrobial activity is observed at low pH, i.e. <6.3 while the inhibitory efficiency is decreased with increased pH (Muzzarelli 1996; Helander et al. 2001).

Temperature may affect the chitosan viscosity, molecular weight and antimicrobial activity during storage (No et al. 2006). In a study, it was observed that CS was remain stable and showed antimicrobial activity against *Listeria monocytogenes*,

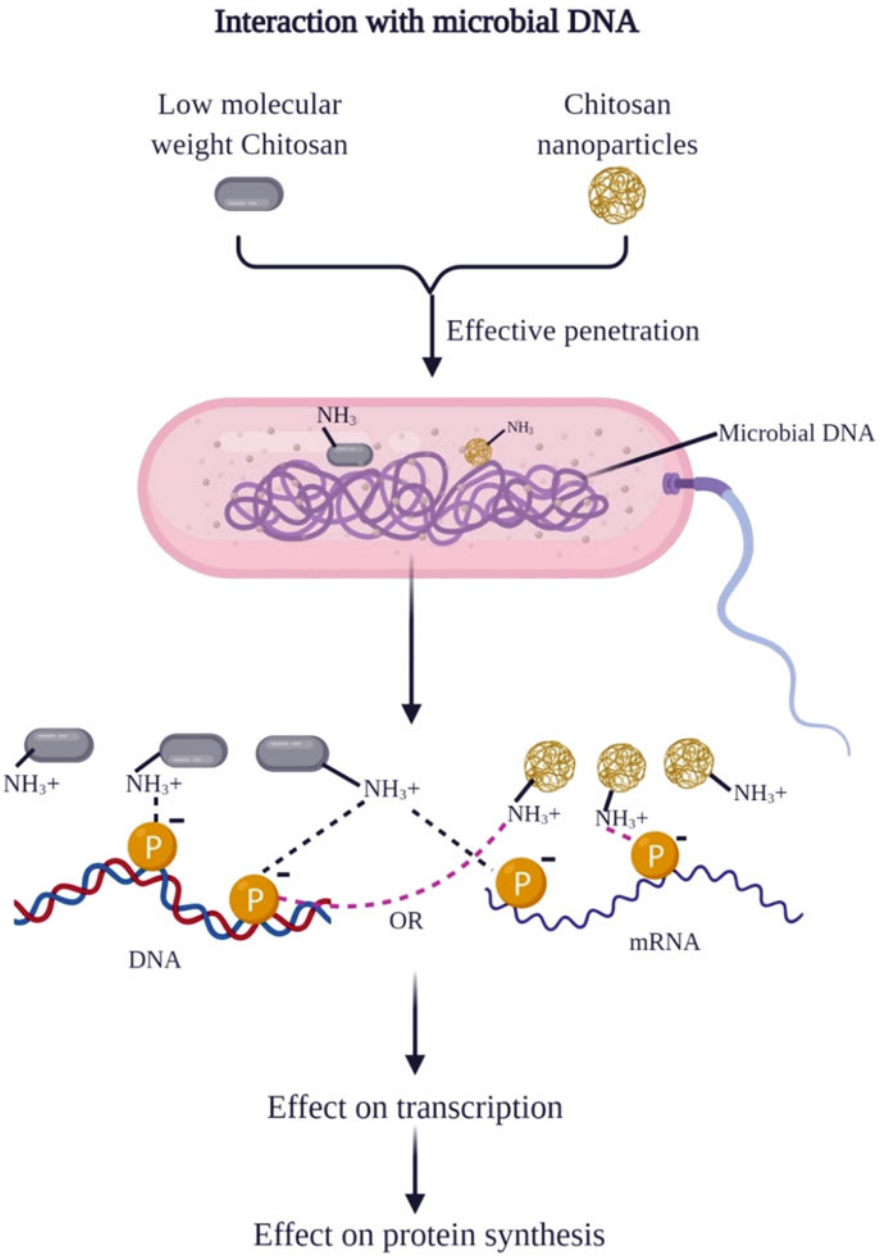

Fig. 17.12 Antimicrobial effect of CS and CNPs by polycationic nature

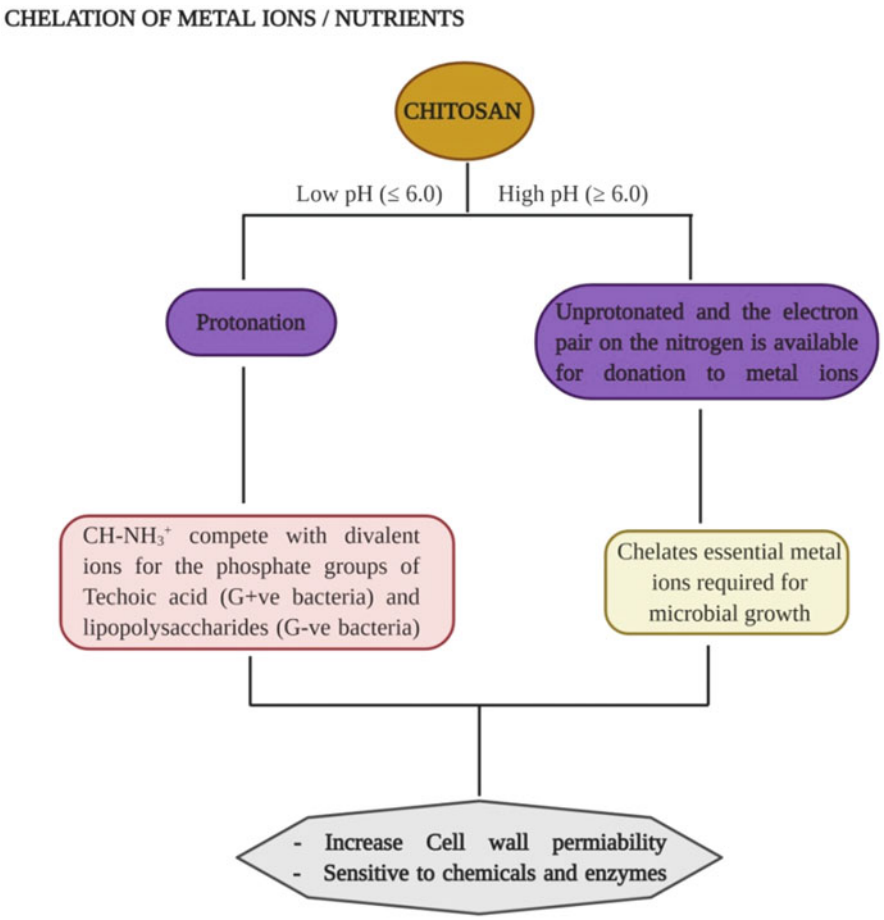

Fig. 17.13 Antimicrobial effect of CS by chelating nature

Salmonella enteritidis, *Staphylococcus aureus* and *E.coli* even after 15 weeks storage at 4°C than 25°C (No et al. 2006).

Molecular weight and degree of acetylation will affect the chitosan antimicrobial activity. Based on the molecular weight, CS is often classified as high molecular weight (HMW, 64.8 kDa to 375 kDa), medium molecular weight (MMW, 250 kDa to 310 kDa), low molecular weight (LMW, 10 kDa to 150 kDa) (Matica et al. 2019). Relation between antimicrobial activity and molecular weight depends on the type of microorganisms. High molecular weight (HMW) chitosan accumulate on the surface of bacterial membrane and inhibit the nutrient transport, resulting in cell death (Li et al. 2010), whereas low molecular weight (LMW) chitosan could pierce into the bacterial surface membranes and bind with DNA, thus blocking mRNA and protein synthesis. (Kulikov et al. 2015). When chitosan molecular weight is

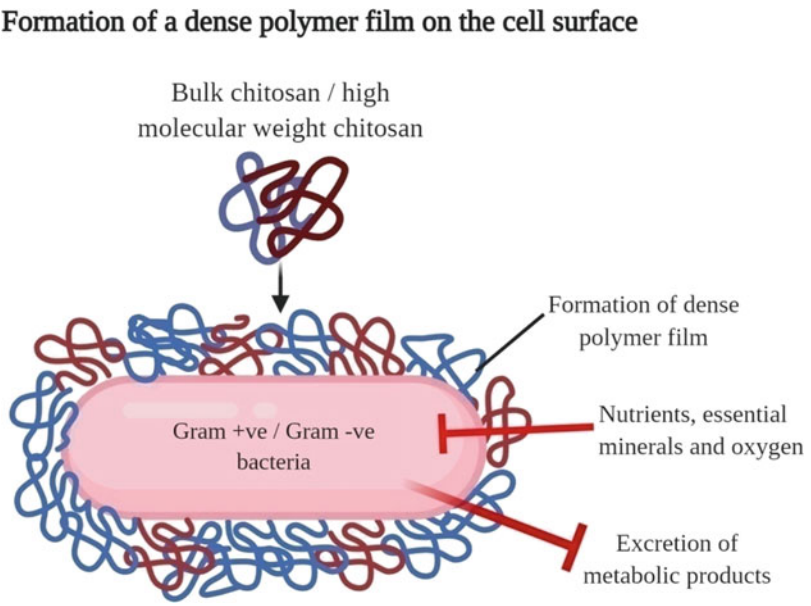

Fig. 17.14 Antimicrobial effect of CS by cell surface blocking nature

decreased bactericidal effect of Gram-negative bacteria was enhanced (Younes et al. 2014).

In the same way the degree of acetylation also influence the chitosan antimicrobial activity. Chitosan antimicrobial activity was increased with the decrease in the degree of acetylation (Goy et al. 2009). More positively charged cations of chitosan are associated with the degree of acetylation (Kong et al. 2008). Highest antibacterial activity against *S. aureus* and *E. coli* was observed at 30–40% of degree of acetylation (Takahashi et al. 2008). Chitosan with a higher degree of deacetylation have more cationic positive amino free groups which influence the antimicrobial activity.

17.5.3 Wound Healing Activity of Chitosan Nanoparticles

Chitosan plays vital role in wound healing because of its antimicrobial, haemostatic, film forming and analgesic and anti-inflammatory properties (Gupta et al. 2019). Chitosans have the similar structure of glycosaminoglycans (GAGs) which are constituents of extra cellular matrix (ECM), hence, used in skin tissue engineering (Chen et al. 2008). Chitosan is biocompatible and non-toxic to living cells and tissues and has been proved in vitro with different types of cells like fibroblasts, keratinocytes, hepatocytes, myocardial and endothelial cells (Dash et al. 2011). Wound healing is a natural response to injury, patients with non-healing disorders

due to factors like age, sex hormones, stress diabetes, obesity, alcoholism, smoking and nutrition makes them stressed and inconvenient or discomfort (Guo and DiPietro 2010).

General programmed phases for wound healing are haemostasis, inflammation, proliferation and remodelling. Many factors include age, sex hormones, infection, oxygenation, medication, nutrition, alcoholism, smoking, diabetes, stress and obesity involved in wound healing by disturbing the programmed phases leads to delay in wound healing (Guo and DiPietro 2010). Impaired healing of wounds enters in to pathological inflammation which leads to chronic wounds like ulcers, diabetes mellitus and venous stasis disease. Non-healing wounds result in immense healthcare expenditures. Thus many studies lead to therapeutics that promote tissue repair, improve impaired wound healing and at the same time inexpensive easily available to sufferers. Chitosan is a natural therapeutic that is easily available and promote tissue repair and improve wound healing. Chitosan reduce the inflammatory phase and accelerates proliferate phase for wound healing fastly (Liu et al. 2018). Chitosan can easily mould into desired hydrogels (Ahmadi et al. 2015), sponges (Huang et al. 2015), membranes (Mi et al. 2001) and films without hazardous chemicals. Hydrogels moistens the infected area by storing high capacity of water (Hoffman 2012). Sponges give prefect matrixes to most wound healing areas due to its open porosity and swelling properties (Mori et al. 2016). Membranes fabricate the three-dimensional matrices with high surface-volume ratio for nutrient supply and cell proliferation. Films should be resistant to pathogenic bacteria in biomedical applications (Zhang et al. 2015).

Chitosan based antimicrobial wound dressing can be incorporated with antibiotics (ciprofloxacin, gentamicin, sulfadiazine or tetracycline), metallic antimicrobial nanoparticles (e.g. nAg, nCu, nZnO and $nTiO_2$) and natural compounds and extracts (honey, *Aloe vera, Juglena regia,* etc.) or fabricated alone with native molecules (Simões et al. 2018; Yang et al. 2016; Ahmadi et al. 2015; Huang et al. 2015; Mi et al. 2001; Coma et al. 2002).

Chitosan is associated with antibiotics to evoke the antimicrobial effect by interfering with bacterial metabolic pathways (Bermingham and Derrick 2002) bacterial structure, cell wall biosynthesis (Patrick 2003), protein synthesis (Hong et al. 2014). Genotoxic, oxidative and cytotoxic effects with metallic nanoparticles can be reduced by using chitosan based biomaterials as carriers (Travan et al. 2009).

In recent studies silver nanoparticles (nAg) owed much interest as a potent antimicrobial agent and in clinical studies in wound dressing to nAg coated medical equipment (Madhumathi et al. 2010). nAg is the metallic nanoparticle showed broad inhibitory activity against many antibiotic resistant bacteria (Zewde et al. 2016).

CNPs are used as stabilizing materials as they are having more permeability towards aqueous solution and its mechanical strength, biofilm formation, liable to chemical modifications and cost-effectiveness (Javid et al. 2013). CNPs and its derivatives make it possible for versatile applications in the medical fields in blood clotting, wounds healing and skin tissue engineering, skin burns, blood lipid cholesterol control, membrane and scaffolds, surgical sutures, etc. (Baghdan et al. 2018; Li et al. 2018; Mohebbi et al. 2019; Gupta et al. 2019).

In the recent studies CS-polyvinyl alcohol (PVA)-silver nanoparticles were used to provoke the wound healing process as it is involved in the wound healing dressing (Hajji et al. 2019).

In another in vivo study on albino rats CS-nanosilver dressings showed enormous and best wound healing activities when compared with intra dermal injection of mesenchymal stem cells injections (Ghannam et al. 2018).

Chitosan-entrapped metallic nanoparticles are safe to use to living cells and have the properties of anti-bacterial effects; interacts with cell wall composition and inhibits the membrane of mitochondrial organelle; enhance the mechanical support and provoke the regrowth of granulation tissue. Chitosan/sodium alginate-Cu (hydrogel), Chitosan-nAu(film), Quaternized chitosan-nAg (film), Chitosan/algetic acid-nZnO (sponge), Chitosan/ECM/n-TiO_2, Chitosan/gelatine-nFe_3O_4 (composite)are the chitosan based nanoparticles used in the recent studies against the microbial activity of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* (Wichai et al. 2019; Rahimi et al. 2019; Regiel-Futyra et al. 2015; Bal-Ozturk et al. 2019; Cai et al. 2016; Woo et al. 2015).

17.5.4 Chitosan Based Nanoparticles in Vaccine Delivery

As the nanoparticles size is small they are easily incorporated into antigen presenting cells and are used as adjuvant in vaccines (Kreuter 1995). Properties of chitosan like polycationic, non-reactivity and high affinity for metals make it to be used as carrier molecule. As the size of pathogens met during proliferation of immune system is almost same that of nanoparticle carrier it is easily taken up by the antigen presenting cells (Xiang et al. 2006). Nanoparticle carriers provoke the mucosal uptake of vaccines and stimulate the mucosal immune response (IgA). Chitosan nanoparticle enhance the mucosal administration because of its tight binding with mucin, small size and the opening ability of tight junctions between epithelial cells.

Intra nasal administration of chitosan nanoparticle encapsulated mucosal vaccines against influenza, diphtheria, pertussis and hepatitis B virus (Illum et al. 2001; Pawar and Jaganathan 2014) stimulate the production of significant IgG and IgA responses in mice. Delated clearance time is also observed in the nasal mucosa with this vaccine and provokes both mucosal and humoral immune response (Pawar and Jaganathan 2014). Oral chitosan nanoparticles vaccine loaded with tetanus toxoid have also activated mucosal and humoral immunity (Barhate et al. 2014; Harde et al. 2014).

Mucoadhesive and osmotic properties of chitosan helps in adsorption and passage of protein peptides through the nasal epithelium (Dodane et al. 1992) and passage of macro molecules across the mucosal barrier (Huang et al. 2016). In recent studies glucomannan modified chitosan nanoparticles maintained biological activity of mediator molecules and blocked antigens. Glucuronidation based chitosan nanoparticles effectively induced systemic serum IgG, mucosal secretary IgA, cell mediated immune responses of IL-2 and IFN- γ (Harde et al. 2014).

N-2HACC and N,O-Carboxy methyl chitosan (CMC) is a vaccine adjuvant for New castle disease virus (NDV) and Infectious bronchitis virus (IBV). Nanoparticles containing NDV/IBV can enhance the proliferation of lymphocytes (Zhao et al. 2017) and induce intranasal inoculation of IgG and IgA antibodies. Chitosan nanoparticles along with plasmid DNA enhance antigen specific immune response (TaO et al. 2013). Some research studies have done on intranasal DNA vaccination (Torrieridramard et al. 2011).

Chitosan's immune stimulatory, mucoadhesive, negative zeta potential, poly cationic, non-reactive properties made it to use as adjuvant carrier for vaccines in the nanoparticle plat form.

17.5.5 CNPs in Drug Delivery

Chitosan is used as one of the important natural polymers with vast applications in drug delivery because of its solubility in the aqueous medium and its cationic amino groups function (Bellich et al. 2016). CNPs in the drug delivery are used to overcome the side effects of drugs, to maintain control rate of drug delivery and to ensure correctly the only targeted area is treated (Teare et al. 1995; Ewart et al. 2019).

The nanoparticles can pierce in to the infected cell (or) tissue due to the presence of larger junctions of epithelial cells. This piercing is of two types—passive targeting and active targeting. In actively targeting drug carrier system is conjugated to a tissue (or) specific cell ligand, whereas in passive targeting due to leaky junctions a nanoparticle reaches the target organ site (Varshosaz and Farzan 2015).

Desirable nanoparticles drug delivery system should reach, identify, bind and deliver its load to specific tissues and avoid drug induced harm to healthy tissues. Targeting ligands on the surface of nanoparticles should be in the form of peptides, antibodies, designed proteins, small molecules and nucleic acids (Liu et al. 2009; Friedman et al. 2013). Drugs which are encapsulated with chitosan nanoparticles can improve their absorption and bio-availability and allowing themselves to deliver gene and protein drugs and are effectively protected from enzyme degradation in vivo (Senapati et al. 2018). Cationic charges of chitosan when interact with anionic charges of nucleic acid molecules form poly electrolyte complex (PEC), this complex protects the nucleic acids from nuclease degradation (MacLaughlin et al. 1998). Bio distribution of chitosan can differ depending on the surface charge, size, molecular weight and hydrophobic nature of chitosan and its derivatives (He et al. 2010). Elimination of chitosan after the drug delivery is through renal clearance because of its solubility and low molecular size.

Mucosal membrane surfaces are common and easy routes for delivering drugs into the body system. Macromolecular drugs such as peptides and proteins are unable to cross the mucosal barriers as they are degraded by enzymes before reaching blood flow. To solve this problem, nanostructures based mucoadhesive polysaccharide chitosan is used (Amidi et al. 2010). Biotin (Vitamin H) has high

Table 17.2 Drug fabricated Chitosan nanoparticles for cancer therapy

S. No	Compound incorporated with CNPs	Treatment	References
1	5-fluorouracil (5-FU) encapsulated chitosan nanoparticles	Cancer therapy	Tıglı Aydın and Pulat (2012)
2	Photosensitizer tetraphenylchlorin Chitosan nanoparticles (TPC−CS NPs) loaded with mertansine (MRT) or cabazitaxel (CBZ)	Breast cancer cell lines	Pandya et al. (2020)
3	Copper-loaded chitosan nanoparticles (Cu-CNPs)	Osteosarcoma cancer	Jw and Liao (2017)
4	α-santalol functionalized chitosan nanoparticles (Sn-CNPs)	Breast cancer	Zhang et al. (2020)
5	Chitosan-PLGA based catechin hydrate nanoparticles (CS-CTH-PLGA-NPs)	Lung cancer	Ahmad et al. (2020)
6	Quercetin Loaded Chitosan Nanoparticles (Qu-CS NPs)	Colorectal cancer	Rashedi et al. (2019)
7	Curcumin-loaded Chitosan nanoparticles (Cu-CNPs)	Cancer therapy	Le et al. (2013)
8	Arg-Gly-Asp (RGD) peptide-labelled chitosan nanoparticles loaded with SiRNA (RGD-CH-NPs)	Ovarian cancer	Han et al. (2010)
9	Biotinylated chitosan nanoparticles (bio-CNPs)	Liver cancer	Cheng et al. (2017)
10	Alginic acid-coated chitosan nanoparticles (A.C. NPs)	Breast cancer	Liu et al. (2013)
11	Hyaluronic acid (HA)-decorated glycol chitosan (GC) nanoparticle conjugated to doxorubicin (DOX) and co-loaded celecoxib (CXB) (HA-GC-DOX/CXB)	Lung cancer	Lee et al. (2020)
12	Folate-Chitosan Nanoparticles Loaded with Ursolic Acid (FA-CS-UA-NPs)	Breast cancer	Jin et al. (2016)
13	Folic acid-conjugated temozolomide (TMZ)-loaded chitosan nanoparticles (CSTMZ-FLA-NP)	Lung cancer	Li et al. (2017)
14	Niclosamide loaded chitosan nanoparticles (Nic-Chi Np's)	Breast cancer Lung cancer	Naqvi et al. (2017)
15	Ketorolac-loaded chitosan nanoparticles	Cancer therapy	Venu et al. (2018)
16	Gemcitabine loaded fucoidan/chitosan nanoparticles	Breast cancer	Oliveira et al. (2018)

affinity for streptavidin and is used for conjugation with nanoparticles (Pramanik et al. 2016). It was reported certain tumor cells overexpress folate receptors than to normal cells, hence, folic acid (vitamin B9) used for targeting in several cancers treatments due to its immense affinity for folate receptors (Zhao et al. 2008). Similarly, the smart targeting of nanoparticles with specific carbohydrates, short peptides, antibodies and small molecules have been designed and studied (Friedman et al. 2013). Some of the recent studies on various cancers have revealed that CNPs have exceptional role in targeted drug delivery (Table 17.2).

17.6 Conclusion

Chitosan is an inexpensive biopolymer extracted from chitin, shows remarkable properties such as biocompatibility, biodegradability, non-antigenic, mucoadhesive, biological activity, cationic nature and low toxicity. A stupendous research has been done on chitosan and its functionalised derivatives to intend in wound healing, tissue engineering, drug delivery, antimicrobial and anti-tumour activity, anti-diabetic and a cholesterol reducing activity. Nanoparticles bio-fabrication transmits desirable functional characteristics to chitosan. Several effective methods are used for fabrication and characterization of CNPs. CNPs provide larger surface area volume to ratio, and their size ranges easily penetrate in to the cells and hence, used as a potential vehicle for delivery of several molecules including drugs, antibiotics, vaccines, genes, peptides, etc., and scaffolds for tissue engineering. However, all these CNPs based therapeutics are in preclinical stages and further extensive studies are required to reveal the safety and effectiveness for their applications.

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