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Versatality of Chitosan: A Short Review

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ABSTRACT

Natural polymers are being investigated with renewed enthusiasm as it has tremendous unexploited potential. Chitosan, a linear polysaccharide, extracted from the shells of sea crustaceans forms a versatile macromolecule that has been reviewed extensively for innumerable applications. Although, this co-polymer of glucosamine and N-acetylglucosamine can be modified easily to acquire neo-physicochemical properties such as mucoadhesivity, antimicrobial, antitumor activities and permeation-enhancer activities, still there is an urgent need to further evaluate its modifications as nanoparticles and their functionalization. This cationic polymer possesses some complimentary biological characteristics as it is biodegradable by proteases, nontoxic, and forms a formidable candidate for drug delivery. The present review summarizes the derivatisations of chitosan polymer for possible pharmaceutical and biomedical applications. Thiolated polymers are now gaining considerable interest as it attributes strong mucoadhesivity and enhanced permeation effect (EPR) to the polymer. Applications of chitosan nanoparticles for gene delivery and as adjuvants for vaccine delivery have also been enumerated. We further explore the numerous methods for the preparation of chitosan nanoparticles.

Keywords: *Chitosan, Nanoparticles, Modifications, Adjuvant, Therapeutic Applications.*

INTRODUCTION

Chitosan has emerged as a useful drug delivery carrier owing to its polycationic nature, biodegradability, biocompatibility and its mucoadhesive properties [1]. Its ease of physical and chemical modification further enhances its properties [2]. Moreover, the amino groups that is present in the main backbone of chitosan makes its surface positively charged, which is a prerequisite for the formation of nano/microparticles. Modulating the molecular weight coupled with the degree of deacetylation, chitosan can be altered in order to obtain different physico-mechanical properties. The elemental composition of the chitosan polymer is carbon (44.11%), hydrogen (6.84%) and nitrogen (7.97 %). The viscosity average molecular weight of chitosan is ~$5.3x10^5$ Daltons.

A variety of biocompatible polyanionic substances such as sulfate, citrate, and tripolyphosphate (TPP) [3] are frequently being used to facilitate the formation of nanoparticles with reduced toxicities [4]. The present communication deals with the modifications of chitosan and methods for the preparation of biologically compatible nano/microparticles.

properties of chitosan- physicochemical and biological:

1. Physicochemical properties:

Chitosan, a glycos-aminoglycans (GAGs) is colorless, odorless and semi-crystalline flaky polymer [3] that is isolated from the exoskeleton of arthropods, insects, and some fungi. Chitin is composed of 2-acetamido-2-deoxy-β-D-glucose units linked by a β-(1-4) linkage, whereas chitosan is a copolymer of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) [5]. It mimics cellulose, with just the hydroxyl (OH) group at the C-2 position replaced by the acetamide group. Chitosan is obtained by the deacetylation of chitin through a series of enzymatic and chemical treatments [6].

Relative proportions of N-acetyl-D-glucosamine and D-glucosamine residues are responsible for determining some of the major properties of chitosan [7]. The alkali treatment used for deacetylation may determine the average molecular weight (Mw) and degree of deacetylation (DD) [3], are the two most exploited physicochemical properties of chitosan. Commercially, chitosan is usually available with DD of 70% to 90% and Mw between 100 and 1000kDa [8]. The solubility, viscosity and elasticity may also affect the Mw of chitosan. The primary amino group in the structure of chitosan renders it highly basic. In alkaline or neutral medium, chitosan is insoluble in water due to lack of protonation of the free amino groups, whereas, in acidic pH, its solubility increases owing to protonation of free amino groups resulting in a positively charged molecule. The three reactive functional groups (primary amino at C-2, primary and secondary hydroxyl groups at C-3 and C-6 respectively of chitosan are illustrated in **Fig.1** [3, 9, 10].

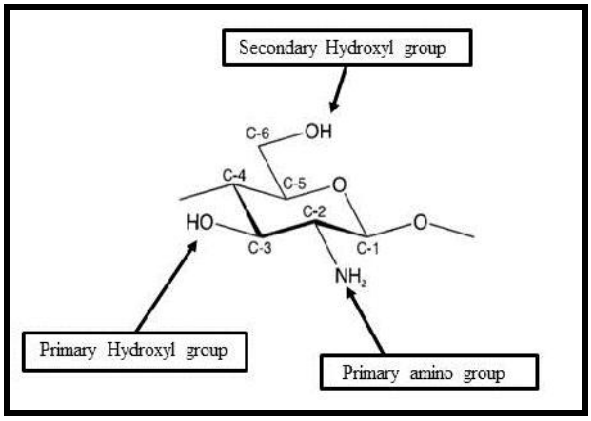

Fig. 1. The reactive functional groups of chitosan: primary amino group at C-2, primary hydroxyl group at C-3 and secondary hydroxyl group at C-6

2. Biological properties:

Modifications of the reactive groups such as (hydroxyl and amino) groups [10] lead to significant changes in the pharmaceutical, physiochemical and biological properties of the native chitosan polymer. The insoluble chitosan molecules are rendered water soluble by 'sulfonation', which also increases the buffering capacity of the molecule [11]. Chitosan gels that are formed at acidic pH, may be used as controlled drug delivery system [9].

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Using a cross-linking agent may possibly help in decreasing the solubility of chitosan, but it adversely affects its swelling property. A linear relationship between viscosity of chitosan solution with its concentration has been reported too [12].

The *in vivo* biodegradability of Chitosan by several proteases leads to the release of non-toxic oligosaccharides that can be expended in metabolic pathways or simply excreted from the body [13]. There are several reports suggesting cholesterol reduction by chitosan [14]. Clinical tests of chitosan have proved that chitosan-based biomaterials do not report spectacular allergic reactions following injection, implantation, or ingestion in the human body [15].

Okamoto et al have evaluated the analgesic effects of chitosan in inflammation model in mice and concluded that the main analgesic effect of chitosan is due to the absorption of proton ions released at the inflammatory site [16]. Derivatives of chitosan have been reported to have anti-cancerous activity *in vitro* as they can directly inhibit cell growth by inducing apoptosis *in vitro* [17]. Several reports also suggest that chitosan and its sulphated oligomers exhibit anticoagulant activity [18]. The anticoagulant activity of chitosan may be related to the positive charge as the membranes of red blood cells are negatively charged [19, 20]. Mucoadhesion and permeation enhancement properties of thiolated chitosans are being used to improve oral bioavailability [21, 22]. Deacetylated chitosan has a significant scavenging capacity against different radical species due to the chelation of metal ions, therefore, it is considered as a potential natural antioxidant for stabilizing lipid containing foods to prolong shelf life [23].

Methods for the Preparation of Chitosan Nanoparticles:

Chitosan nanoparticles were first described in 1994 [24], and were prepared by emulsification and cross-linking method. Thereafter, the original formulation has been modified and extensively used in pharmaceutical applications [25-27]. A variety of hydrophilic and hydrophobic drugs can be loaded into the chitosan nanoparticles during as well as after the preparation of the nanoparticles. The loading efficiency of the drug may depend on its physic chemical characteristics and the preparation method [28, 29].

Nanoparticles are inevitably preferred over the native polymer due to its neo-characteristics as size and increased surface volume. Since, the chitosan polymer can be moulded into chitosan nanoparticles that are smaller in size, less immunogenic, we summarizes a few methods for the preparation of chitosan nanoparticles and highlight their potential as a component of gene and drug delivery systems. Chitosan-heparin nanoparticles were successfully applied to the oral delivery of heparin as they enhanced the absorption of heparin in the gastro-intestinal tract, resulting in effective anti-coagulation after oral administration in rats [30]. The mucoadhesivity of chitosan-TBA (Thiobarbituric acid) nanoparticles was twice as high as that of unmodified chitosan nanoparticles [31]. Besides, showing enhanced mucoadhesivity and cell penetration properties, nanoparticles made of thiolated chitosans have appeared highly effective as gene delivery systems [32]. We discuss suitable methods for the preparation of chitosan nanoparticles, namely, polyelectrolyte complexation [25], emulsification, emulsion-droplet coalescence [33], desolvation [34], reverse micellar [35], emulsion solvent [36] and ionic gelation [37] method along with illustrations (**Fig 2**).

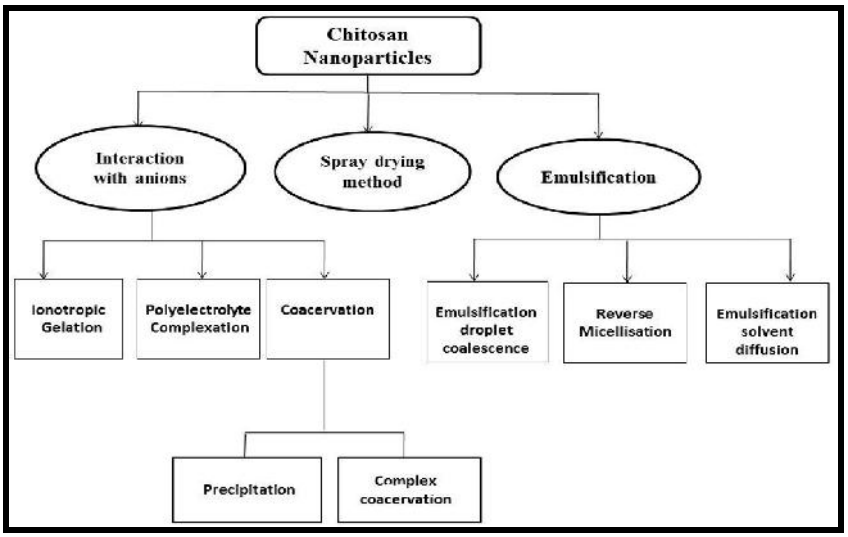

Fig. 2. Methods for the preparation of Chitosan Nanoparticles

1. Ionotropic gelation:

Ionotropic gelation is the most conventional method that involves the electrostatic interaction between the chitosan chain and the negatively charged group of a polyanion like Tripolyphosphate (TPP). Chitosan nanoparticles prepared by ionotropic gelation were first reported by Calvo, et al. [25]. Thereafter, chitosan-TPP conjugates were accidently prepared by Bodmeier, Oh, & Pramar, who intended to prepare the chitosan beads [38]. **(Figure 3)**.

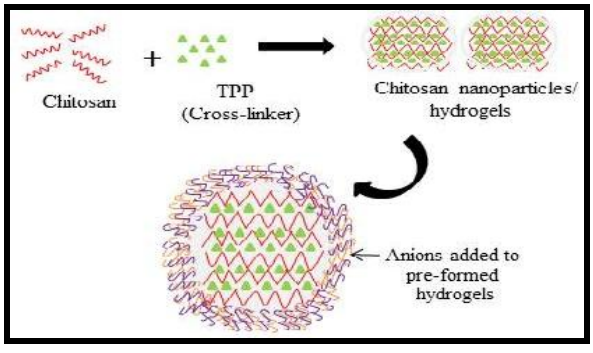

Fig. 3. Ionotropic Gelation Method

The physical cross linking with TPP during the formation of chitosan nanoparticles facilitates the formation of nanoparticles. TPP-cross linked chitosan nanoparticles have been extensively used to deliver various small molecular drugs. Janes, et al. successfully entrapped doxorubicin (Dox) into the chitosan nanoparticles during ionotropic gelation of the chitosan with TPP [39]. The size and surface charge of the nanoparticles can further be modified by varying the ratio of chitosan and the polyanion used [26]. This method is preferred as it uses simple and mild conditions in the aqueous environment without using harsh organic solvents. In this process, chemical cross linking of drug is best avoided as it may interfere with the efficacy of the drug. Physical entrapment maintains the integrity of the drug, which is an added advantage [40]. Chitosan gels can also be formed using this method when the polyanions (such as phosphate, sulphate, citrate etc.) and the amine groups undergo a high degree of protonation [9]. Moreover, the fast gelling ability of TPP coupled with its non-toxicity, has enabled its usage in the food and detergent industry [41, 42]. The Chitosan-TPP conjugate is highly pH dependent achieving a stable complexation between pH 3 and 5.5, thereby, limiting its utility and stability [43-45].

2. Polyelectrolyte Complexation technique:

The quality of hydrogels formed during ionic gelation process can be enhanced by Polyelectrolyte Complexation (PEC). Another polyelectrolyte is added to the pre-formed complex to stabilize their mechanical strength and permeability. It has been

demonstrated that chitosan can form PECs with various polyanions such as sodium alginate [46], carrageenan [47], glucomannan [48], dextran sulphate [49], chrondoitin sulphate [50] heparin [51, 52] carboxymethyl cellulose [53] poly-Y-glutamic acid [54], cyclodextrins [55] sodium lauryl sulphate [56].

PECs prepared by electrostatic interactions between oppositely charged polyions, have been used widely as carrier systems for drug and gene delivery [57, 58]. The complex formation and the physical properties of PECs can be modulated by the degree of ionization of the chitosan and anionic counterparts, pH, temperature, charge distribution over the polymer chain, ionic strength, time of interaction, and concentration of the polymeric solutions [46]. Although, PEC are similar in preparation, nanoparticles produced by PEC are much larger in size and are directly proportional to the amount of polymer added [59-61].

3. Emulsification:

Emulsification is the term used for two or more totally or partially immiscible liquids with or without the addition of a surface active reagent. Two general types of emulsions are **Direct emulsion** i.e. o/w (Oil in water) and **Reverse emulsion** i.e. w/o (Water in oil) [62, 63]. **(Fig 4).**

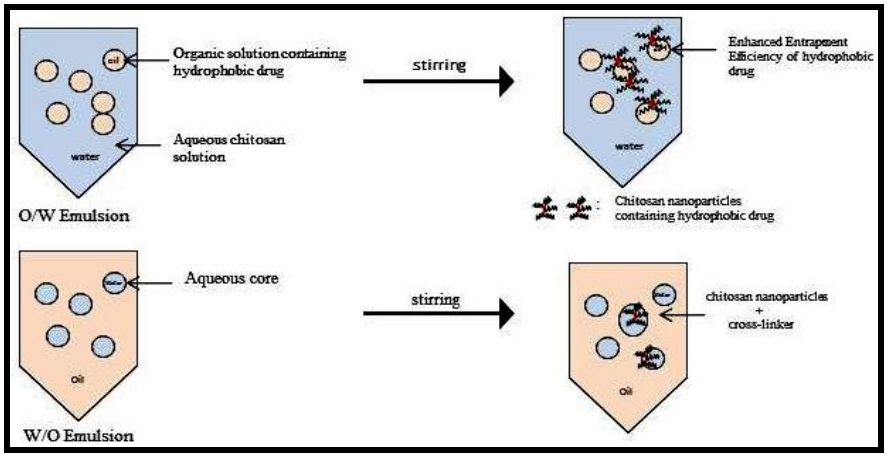

Fig. 4. Direct and Reverse emulsion

3.1. Emulsification solvent diffusion:

Niwa, et al. developed the solvent diffusion method that was based on the partial miscibility of an organic solvent with water [63]. El-Shabouri used an organic phase (such as acetone or methylene chloride) containing the hydrophobic drug that was injected into the chitosan solution with a stabilizing agent (e.g. poloxamer or lecithin) under continuous stirring to further improvise the original method [64]. This was later subjected to high pressure homogenization that led to the formation of an o/w emulsion. Dilution of the emulsion helps in the complete diffusion of the organic solvent that facilitates precipitation of the polymer, thereby forming the nanoparticles. This method enables enhanced entrapment efficiency of hydrophobic drugs. However, the excess use of organic solvents coupled with harsh processing conditions and high shear forces during the preparation of nanoparticles, limits their applicability in biological models.

3.2. Emulsion – droplet coalescence:

Emulsion-droplet coalescence is a unique method involving the principle of both emulsion cross-linking and precipitation to prepare microparticles as first reported by Tokumitsu, et al. [65]. Later, Tokumitsu, et al. reported the preparation of chitosan nanoparticles where a mixture of two emulsions with an equal outer phase promotes random collision between the droplets resulting in the coalescence that leads to uniformly formed droplets, where the drug can be entrapped within the nanoparticles. Using this technique, both chitosan and NaOH solution were emulsified into the same oil phase to prepare two emulsions. The two emulsions were further mixed and stirred at high speed, to form chitosan nanoparticles, that can be loaded with drugs for biological applications. The same authors successfully entrapped gadolinium in chitosan nanoparticles to treat cancer as a part of neutron-capture therapy. Interestingly, size of the particles so obtained was inversely proportional to the degree of deacetylation (DD) thereby, optimizing the entrapment efficiency [65]. The encapsulation efficiency (~70%) of 5-FU in chitosan nanoparticles was not affected by varying the concentration of the chitosan polymer; hence, this was apparently a reliable and defined technique [66].

4. Reverse Micellization:

Conventional micelles are formed in o/w environment where as reverse micelles are formed as w/o droplets [67]. The nanoparticles prepared by Reverse Micellization/Microemulsion have an aqueous core that may subsequently be cross-linked with glutaraldehyde (GTA) [68]. In this method, a lipophilic surfactant was dissolved in an organic solvent to form a w/o micro-emulsion. The organic phase coupled with the aqueous phase consisting of chitosan and GTA was mixed under continuous stirring for 24 hrs [68]. Reverse micelles thus formed, could be later extracted by solvent evaporation in order to remove excessive organic solvent [69]. Mitra, et al. encapsulated doxorubicin into cross-linked chitosan nanoparticles formed by reverse micellization and assessed the anti-tumour activity on macrophage tumour cell line (J774A.1) implanted subcutaneously in BALB/c mice that exhibited enhanced tumor regression as compared to the drug *per se* [68]. This method generates ultrafine nanoparticles of less than 100nm unlike other conventional emulsion based methods by adding varied concentrations of glutaraldehyde [68]. However, Sun, Y. et al have used both GTA and TPP as cross-linking agents, and reported that TPP apparently was less toxic [70].

5. Desolvation/ coacervation/precipitation:

5.1. Simple coacervation:

A simple coacervation/phase separation involves the aggregation of molecules that are partially dissolved in the solution, resulting in the formation of micro particles [71] which is illustrated in **Figure 5**. Chitosan microparticles can be prepared in alkaline solution along with precipitation by desolvating/precipitating agents such as sodium sulphate [72] and acetone [73]. Hejazi & Amiji have successfully prepared chitosan microspheres by precipitation method using sodium sulphate to entrap tetracycline for the treatment of infection against *Helicobacter pylori*. Their findings also revealed that low entrapment efficiency (~8%) was observed when the drug was incorporated before the formation of chitosan microspheres, while entrapment efficiency (~68%) may be enhanced by incubating the drug with pre-formed microspheres [74]. This method eliminates the use of toxic GTA unlike the emulsification-cross linking method. However, since strong bases are being used that may also compromise the activity of microparticles, this method needs further standardization. Nowadays, using coacervation technique, chitosan nanoparticles are being synthesized regularly by drop wise addition of previously formed chitosan solution into an alkaline phase containing polyanions or *vice versa* [75]. This leads to the increased insolubility of the chitosan polymer and eventually its precipitation [39, 76] Despite being a simple and mild method, the low entrapment efficiency was a major limiting factor [5] coupled with weak mechanical properties and irregular morphology [77] of the nanoparticles formed, this method has not become popular.

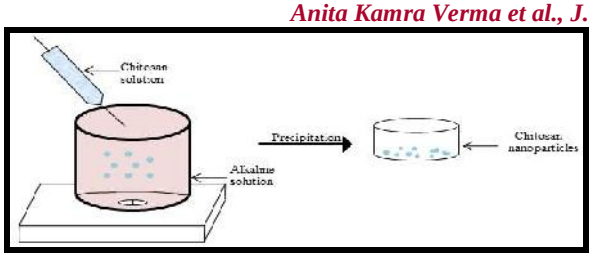

Fig. 5: Simple Coacervation Method

5.2. Complex coacervation:

Complexation between oppositely charged macromolecules is the underlying principles of this method that is similar to ionotropic gelation method as illustrated in **Figure 6**. Chitosan nanoparticles have been prepared by complex coacervation to achieve a high entrapment efficiency. Complex coacervation method involves the formation of chitosan nanoparticles when negatively charged polyelectrolyte such as pDNA solution in sodium sulphate/dextran sulphate is being added to the positively charged polyelectrolyte chitosan solution [78] Chitosan nanoparticles prepared by coacervation entrapping nucleic acid molecules have also been reported for gene delivery [79, 80] Moreover, living cells and labile molecules have also been encapsulated in natural polymers using coacervation in an attempt to protect them from degradation [81].

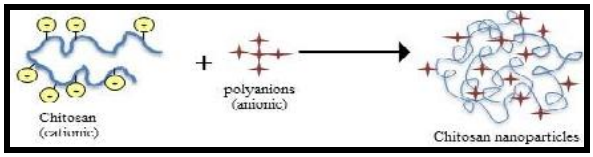

Fig 6. Complex Coacervation Method

6. Spray drying method:

Spray-drying method is effectively being used for production of powders and granules for pharmaceutical and biotech industries [82]. This method gives an advantage of making nanoparticles/microparticles under controlled conditions. Chitosan may be dissolved in an organic solvent (e.g. acetone) followed by dispersion/desolvation of the drug in this solution under continuous vigorous stirring, and atomized in a stream of hot air that leads to formation of free flowing chitosan microparticles due to instantaneous evaporation of the solvent [83]. These microparticles can be later separated by a cyclone separator [84]. He, et al. have successfully reported the encapsulation of cimetidine, famotidine and nizatidine in chitosan microparticles prepared by spray drying method [83]. Similarly, cetylpyridinium chloride, metoclopramide hydrochloride were efficiently encapsulated as anti-infective agents into chitosan microspheres/microparticles by using this method [85, 86].

For the pulmonary delivery of itraconazole (antifungal drug), Jafarinejad, et al. successfully obtained microspheres by spray drying chitosan nanoparticles which were previously prepared by ionic gelation method [87]. However, Tao et. al were able to synthesize WSC-NPs (Water Soluble Chitosan Nanoparticles) using ionic gelation technique and further spray drying for oral administration in rats as a strategy to treat hypercholesterolemia [88]. Other chitosan-based nanoparticles such as chitosan/iron (II, III) oxide nanoparticles have also been reported [89].

Spray flow rate, atomization pressure, the nozzle size, inlet air temperature and extent of cross-linking are the major parameters that facilitate manipulation of the size of nanoparticles [82]. Being relatively cheap and highly reproducible [90, 91] this method has the advantage of preparation under aseptic conditions. The steps for spray drying method are depicted in **Fig 7.**

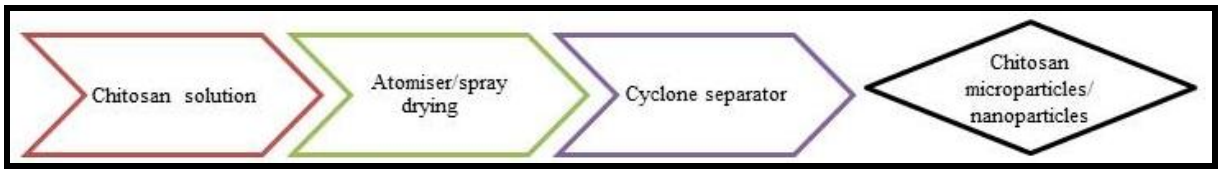

Fig 7. Spraying drying Method

Modifications of Chitosan:

1. Hydrophobic Chitosans:

Covalent attachment of hydrophobic moiety is being done to increase the hydrophobicity of chitosan. Hydrophobic interactions probably enhance the stability of substituted chitosan by reducing the hydration of the matrix, thereby conferring resistance to its degradation by various proteolytic enzymes [92]. The pH-sensitive chitosan can be obtained by the introduction of carboxylic acid groups. Under acidic conditions, the carboxylic groups exist in non-ionized form and are, therefore poorly soluble in water. By contrast, the chitosan polymer is ionized and is considerably more hydrophilic in alkaline conditions. The hydrophobic excipient is expected to increase the mucoadhesivity through hydrophobic interactions and also the permeability by opening the tight junctions [93]. One of the approaches to introduce the hydrophobic groups is being performed by adding long chain acyl chlorides and anhydrides onto the chitosan. Fatty acids have significantly increased the permeability of peptide drugs [94]. They act primarily by disrupting the phospholipid component of the membrane, thereby enhancing permeability. N-acylation of chitosan with various fatty acid chlorides (C_6–C_{16}) resulted in an increased hydrophobicity induced by significant changes in its structural properties. Tein et. al have performed the N-acylation of chitosan with fatty acyl chlorides (C_8–C_{16}) to increase its hydrophobic character and used it as a matrix for drug delivery [95].

2. Hydroxyalkyl Chitosans:

These chitosan derivatives were obtained by reacting chitosan with epoxides [96, 97]. This reaction predominantly takes place at the amino or alcohol groups resulting in the formation of N-hydroxyalkyl or O-hydroxyalkyl chitosans and finds its application in gene delivery [98]. Moreover, it is also proposed that the transfection efficiency in alkylated chitosans was directly proportional to the length of the alkyl side chains [99].

3. Quaternized Chitosan:

The quaternization or methylation in chitosan was performed at the amino groups by introducing various alkyl groups e.g. methyl iodide in alkaline solution of N-methyl pyrolidinone [100]. This renders enhanced solubility in neutral and alkaline environments of the intestine which makes them more efficient than the parent chitosan for absorption across the intestinal epithelium of the jejunum and ileum [101]. Quaternized chitosan derivatives, such as, trimethylated chitosan (TMC) is being prepared by reacting chitosan with trimethyl iodide bond. TMC nano conjugates are successfully used in gene delivery both *in vitro* and *in vivo* [102, 103]. The permeation-enhancing properties of these chitosan derivatives have been attributed to their ionic interactions with the tight junctions and cellular membrane components to increase the paracellular permeation of hydrophilic compounds [101]. TMC nano-conjugates (35% quaternization) give good penetration-enhancement properties and mucoadhesion [104]. An important quaternized derivative, N-hydroxypropyl trimethylammonium chitosan chloride (HTCC), obtained by reacting chitosan with glycidyl trimethylammonium chloride, was found to be mucoadhesive and was preferred for oral insulin delivery [105].

4. Polyethylene glycol (PEG) grafted Chitosan derivatives:

The reaction between chitosan and PEG followed by the reductive alkylation of chitosan's amino group helped in the formation of grafted chitosan nano-conjugates. The introduction of PEG resulted in hydrophilic chitosan nanoparticles that may be used as an anionic drug carrier. However, it has been observed that

PEG-grafted chitosan nano-conjugate works as an efficient drug delivery system than the chitosan nanoparticles [106].

5. Thiolated Chitosan:

Thiolated polymers are now gaining considerable interest as it attributes strong mucoadhesivity and enhanced permeation effect (EPR) to the polymer. Thiolation may be obtained by the derivatization of the primary amino groups of chitosan with coupling reagents such as thioglycolic acid, 2-iminothiolane, cysteine, and thiobutylamidine. The EPR effect of thiolated chitosan nanoparticles have been studied with glutathione (GSH) as permeation mediator tool for inhibiting the ATPase activity of permeability glycoprotein (P-gp) in the intestine [107]. Thiolated chitosan is also known to display *in situ* gelling features due to the pH-dependent formation of inter- as well as intramolecular disulfide bonds thus providing them strong cohesion and stability. The three important thiolated chitosan derivatives are chitosan cysteine conjugates [108] chitosan-4-thio-butyl-amidine (Chitosan TBA) conjugates [109] and chitosan-thioglycolic acid conjugates [110].

In order to couple mucoadhesivity and EPR, attempts have been made to synthesize Trimethyl chitosan–cysteine conjugate (TMC-Cys) that can be used for *in vivo* oral delivery of docetaxel by Saremi, et al. [111]. Some other reported derivatives are mono-N-carboxymethyl chitosan [100]. N-sulphochitosan [112] and trimethylated chitosan [101]. A significant modification based on the immobilization of thiol-bearing chain on the biopolymeric backbone of chitosan [5] has also been demonstrated. Since thiomers are capable of inhibiting efflux pumps such as P-gp, making them essential for oral drug delivery [113].

Biopharmacological Applications of Chitosan:

1. Antibacterial activity:

The antibacterial activity of chitosan [114] and its derivatives also depends on the molecular weight, degree of deacetylation, the type of substituents (cationic) of chitosan coupled with the strain of bacterium used. Tremendous literature supports that Low Molecular Weight (LMW) chitosan derivatives are water soluble and exhibit enhanced antimicrobial potential. Alternately, it is believed that High Molecular Weight (HMW) chitosan conjugates are unable to pass through the cell membranes, thereby, reducing the antibacterial activity. Current literature suggests that even HMW chitosan derivatives are bacteriostatic [115]. Gram negative bacteria, often represented by *E. coli*, having anionic surfaces interact electrostatically with cationic chitosan derivatives, thus leading to membrane disruption of the bacteria. Therefore, many chitosan derivatives are integrated with cationic components (such as ammonium, pyridinium etc.) in order to significantly enhance their antibacterial efficacy. Takahashia et. al have reported the antibacterial activity of chitosan in gram positive bacteria - *S. aureus* where the activity was directly proportional to the positive charge and DD of chitosan [116]. Further, it was observed that the growth of *S. aureus* was arrested by binding of chitosan derivatives to its DNA/RNA [115].

Quanternised chitosan derivatives exhibited pronounced inhibitory activity as compared to chitosan *per se* [117]. Antibacterial activity of another water-soluble chitosan conjugates (N-alkylated disaccharide) against *E. coli* and *S.aureus* was investigated by Tsui-Chu Yang et. al [118]. The disaccharide chitosan derivatives demonstrated decreased antibacterial activity than the parent chitosan at pH 6.0. The derivatives, however, exhibited a higher activity than parent chitosan at pH 7.0. Yadav & Bhise have demonstrated that chitosan showed excellent antibacterial activity against typhoidal bacterial strain. Enhanced efficacy of chitosan nanoparticles was observed against various strains of S*almonella enterica* that showed resistance to some commonly used antibiotics such as chloramphenicol and ciprofloxacin, thereby, validating the application of chitosan as an antimicrobial agent [119].

2. Antifungal activity:

Thio-semicarbazone chitosan derivatives have shown to be fungicidal against many types of fungi (Martinez, et al., 2010) such as *Alternariasolani* (*A. solani*), *Rhizoctoniasolani Kühn* (*R. solani*), *Stemphyliumsolani weber* (*S. solani*), and *Phomopsis asparagi* (*Sacc.*), (*P. asparagi)* [120]. These derivatives showed broad-spectrum antifungal activity that was affected by the concentration of the derivatives and fungal species. *Alternaria* sp., *Penicillium* sp. and *Cladosporium* sp. are some of the fungal species that exhibit growth inhibition by chitosan [121]. However, antifungal effect of formulations prepared from chitosan nanoparticles was found to be significantly effective than the chitosan polymer itself [122].

Chitosan nanoparticles exhibited fungistatic activity in plasticized as well as in non-plasticized films [123] thus, it is being used in food industry as an antifungal additive for food and for making food wraps and films. Extensive studies are required to elucidate a possible mechanism of antifungal action of chitosan nanoparticles on fungi responsible for food spoilage.

***5.3.* Antioxidant activity**

Due to strong hydrogen donating ability of chitosan, the antioxidant scavenging ability of chitosan were further established. Various studies suggest that LMW chitosan and quaternised/methylated derivatives of chitosan depict higher antioxidant activity in comparison to other derivatives [115]. Xue et. al have explored the antioxidant activity of chitosan-oligosaccharide conjugates (OLC), N, O-carboxymethyl chitosan (NOCC) and hydroxyl propylated chitosan (HPC) . Furthermore, it has been observed by the very same authors that chitosan derivatives play a significant role in balancing the antioxidative mechanism of biological systems [124]. Studies clearly implied that chitosan nanoparticles could attenuate H_2O_2-induced stress injury in RAW264.7 cells, exhibiting superior antioxidative activities than the chitosan polymer [125].

4. Anti-tumour activity:

Zhou et. al have reported the anti-tumour activity of chitosan (500mg/ml) was 55% in breast cancer (MCF-7) cell line, being the highest, followed by 29%, 27% and 23% on gastric cancer (BGC-823) cell line, cervical cancer (Hela) cell line and human hepatocarcinoma (SMMC-7721) cell line respectively [126]. The proliferation rate of liver cancer cells (HepG2) were arrested when administered with chitosan thymine conjugate [127]. Ziwei, et al. have prepared and investigated chitosan–silica hollow nanospheres (CS–SiO2 HNPs) that can be used as promising nanocarriers for drug delivery against breast cancer [128]. Furthermore, *in vivo* studies have also confirmed similar results as LMW chitosan and chitosan-oligosaccharide nano-conjugates indicating enhanced anti-tumour activity in S180 tumour bearing mice [129]. Chitosan nanoparticles have been successfully used to treat cancer cells both *in vitro* and *in vivo* (against Sarcoma-180 cells and mouse hepatoma H22 cells) [130]. Thus, it is clearly evident that chitosan nanoparticles/nanoconjugates provide an opportunity to expand the clinical repertoire and can be used as a substitute for the currently available drugs.

5. Drug delivery:

Several reports suggested that chitosan nanoparticles/microparticles have been used for the successful encapsulation of tuberculosis (TB) drugs against *Mycobacterium tuberculosis* [131]. Using chitosan-based microparticles, Ain, Sharma, Khuller & Garg encapsulated tuberculosis drugs and were able to enhance chemotherapeutic efficacy in animal models of tuberculosis [132]. Verma, et al. have successfully prepared chitosan nanoparticles, encapsulating the culture filterate proteins (CFPs) of *Mycobacterium tuberculosis* and evaluated the immune response. Their result suggested the potential role of chitosan nanoparticles as a good adjuvant for TB [1]. Chitosan nanoparticles as drug delivery systems (DDS) are summarized in **Table 1**.

Table No. 1: Chitosan nanoparticles as DDS prepared by various methods for different kinds of drugs

System	Method of preparation	Drug
Chitosan nanoparticles	ionic gelation	insulin, ricin, bovine serum albumin, cyclosporine A, Methotrexate [28, 133]
	coacervation/precipitation	doxorubicin [134]
	emulsion-droplet coalescence	Gadopentetic acid [65]

6. Gene delivery:

Owing to its cationic charge, chitosan can be used as a carrier for drug and gene delivery system which can interact with negatively charged polyanions such as DNA and proteins in order to form polyelectrolyte complexes [136]. Cell toxicity, low transfection rate and anti-inflammatory responses are some of the serious

problems limiting viral carriers. Being positively charged, chitosan has emerged as a dynamic vector for gene delivery. Although, chitosan nanoparticles have lower gene transfection efficiency as compared to the viral gene carriers, transfection efficiency can be modulated based on the pH and molecular weight of the chitosan nanoparticles [136]. Folic acid modified chitosan nanoparticles have been successfully prepared by Mansouri et. al to improve gene tranfection efficiency [137]. Their results revealed that the obtained folic acid-modified chitosan nanoparticles showed reduced cell toxicity and were able to condense DNA effectively acquiring an ideal size and zeta potential. However, chitosan nanoparticles preparing for an ideal gene carrier with improved transfection efficiency is still a challenge. Chitosan nanoparticles as non-viral vector for gene delivery is summarized in **Table 2.**

Table No. 2 Chitosan nanoparticles as non-viral vector for gene delivery

System	Target gene	Disease
Chitosan DNA nanoparticles	Peanut allergen	Peanut allergy [138]
CIN (Chitosan IFN-Y pDNA) nanoparticles	IFN-Y	Allergic asthma [139]
Folate-Chitosan nanoparticles	IL-1Receptor	Rheumatoid Arthritis [140]
Chitosan nanoparticles	TNF-α	Rheumatoid Arthritis [141]

Furthermore, researchers have also successfully prepared multifunctional chitosan nanoparticles to deliver the drugs and gene simultaneously. Wang, et al. have reported chitosan functionalized magnetic graphene (CMG) nanoparticle that successfully delivered the gene, drug and SPIO (Super Paramagnetic Iron Oxide) to the tumor site. SPIO has been used as the contrasting agent in the non-invasive MRI technique to evaluate the anti-tumour activity. They were able to effectively deliver pDNA into A549 lung cancer cells and C42b prostate cancer cells using CMGs coupled with doxorubicin as the anti-cancer drug with enhanced release in A549 lung cancer cells [142].

CONCLUSION

Biocompatability, biodegradability and ease of manipulation indeed makes chitosan a versatile polymer for biomedical applications. The high solubility of chitosan nanoparticles in water makes it an ideal polymer for drug delivery, hence, simple and mild preparation methods can be applied. Besides, ionotropic gelation method, other methods such as emulsification solvent diffusion method, polyelectrolyte complex method, cross-linking method, complex coacervation method, solvent evaporation method and spray drying method are also in use. Although, the biological activity of chitosan and chitosan nanoparticles depend on the DD and Mw of the polymer, different mechanisms have been proposed to explain at the molecular level. Firstly, it has the ability to interact with the cell membrane resulting in cell leakage. Once the chitosan nanoparticles penetrate into the cell, there may be inhibition of protein synthesis resulting in lack of mRNA. Chitosan nanoparticles may also form a coating on the surface of the cell causing acute stress to the cells. Owing to its flexibility, chitosan can be easily modified by coupling with ligands to prepare various formulations, thereby, making it a suitable carrier for sustained/controlled drug delivery and nonviral gene delivery.

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