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AN OVERVIEW ON VARIOUS MODIFICATIONS OF CHITOSAN AND IT'S APPLICATIONS

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ABSTRACT: Chitosan is a natural polysaccharide which is widely exploited for its various applications. It has received much attention as a functional biopolymer especially in pharmaceutics and medicine because of its better biodegradability, biocompatibility, versatility and availability. The unique biological applications of chitosan are attributed to these specific properties. The chemical and biological modification of chitosan is mainly done to increase its solubility in aqueous solutions and absorbability in the *in vivo* system. A variety of chemical modifications are employed to modify this carbohydrate polymer. The modifications of chitosan are influenced by several factors such as molecular weight of chitosan, viscosity, reaction conditions etc. The selective biological applications include antimicrobial, hypocholesterolemic, antitumor, anti-inflammatory, antioxidant, angiotensin-I-converting enzyme (ACE) inhibition, excluding toxins from the intestines, reducing heavy-metal poisoning in humans, mucoadhesive haemostatic, analgesic, radio-protective properties, preventing tooth decay and tooth diseases and immunity enhancing activities. It is also widely useful in biomedical industries and food industries. The present paper reviews the current trend of investigation on various useful modifications on chitosan and their applications in various fields.

INTRODUCTION: Natural polysaccharides are being utilized more and more in the markets for the reason that they show biodegradability, biocompatibility, versatility, and are found plentiful in nature. The diversity of natural polysaccharides provides the chemist with a broad spectrum of raw materials that can be used in many biological applications.

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Chitin and Chitosan are important among such polysaccharides.The history of chitin and chitosan compounds dates back from 18th century itself that is, in 1811, Prof. Henri Braconnot, isolated fibrous substances from mushroom and found it insoluble in aqueous acidic solution.

A decade later in 1823, Ojer named it 'Chitin' from Greek 'khiton" meaning "envelope" present in certain insects. In 1894, Hope Seyle named it as 'chitosan'. In 1930 to 1940, this biopolymer of glucosamine gained much interest in the field of medicine [1]. Chitin is the second richest polysaccharide of animal origin found in nature and it is characterized by its fibrous structure.

Chitin is the most important constituent of the exoskeletons of crustaceans and insects as well as of cell walls of some bacteria and fungi.

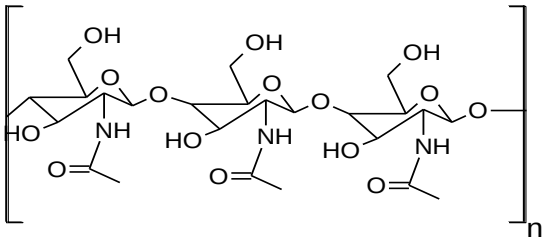
The main industrial sources of chitin are the shell waste of shrimps, lobsters, and crabs. In the world, several million tons of chitin is harvested per annum and hence this biopolymer represents an economical and readily accessible source. Chitin is often considered as a derivative of cellulose. Like cellulose, it is also a glucose-based unbranched polysaccharide; the difference is that in chitin instead of hydroxyl group an acetamido group is at the C-2 position [2].

Chitosan, A major derivative of the chitin, is a linear polymer of β-1,4-linked 2-amino-2-deoxy β –D-glucopyranose. There are many advantages for chitosan over chitin. The main advantages of chitosan are that it is readily soluble in diluted acetic acid, whereas chitin gets dissolved in highly toxic solvents such as lithium chloride and dimethylacetamide [3].

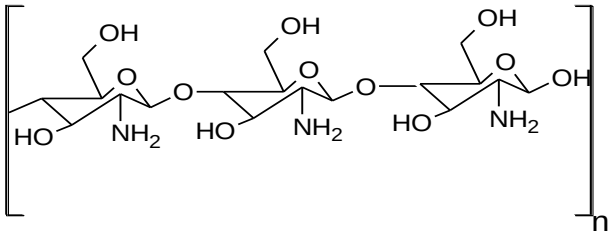
Chitosan gained curiosity of researchers not only because of its various properties but also due to its unique biological applications such as antimicrobial, hypocholesterolemic, antitumor, anti-inflammatory, antioxidant, angiotensin-I-converting enzyme (ACE) inhibition, excluding toxins from the intestines, reducing heavy-metal poisoning in humans, mucoadhesive haemostatic, analgesic, radio-protective properties, preventing tooth decay and tooth diseases and immunity enhancing activities [4].

It is also widely used in biomedical industries for enzyme immobilization and purification, in chemical plants for wastewater treatment and in food industries for food formulations as binding, gelling, thickening and stabilizing agent [5]. In this present review, we aim to have a general overview of chitosan, its manufacturing, chemical modification and applications.

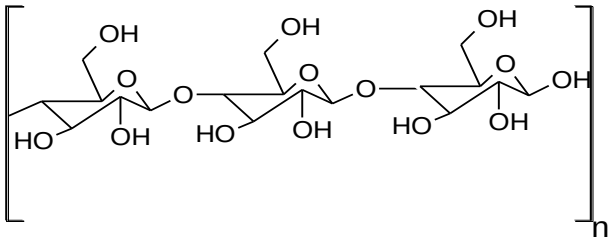
Chemical structure of Cellulose, Chitin and Chitosan: As it is mentioned earlier that chitin is similar to cellulose. The similarity exists both in chemical structure and in biological function. It exists as a structural polymer. In **figure 1**, the crystalline structure of chitin has been shown to be similar to cellulose because of the similarity in the arrangements of inter and intra chain hydrogen bonding [6].



CHITIN



CHITOSAN



CELLULOSE

FIGURE 1: STRUCTURES OF CHITIN, CHITOSAN AND CELLULOSE

Manufacturing of Chitin and Chitosan: The natural sources of chitin are found to be squid, fungi, insects and some algae. A large quantity of chitin is manufactured from the exoskeleton of crustacean sources (shrimp, crab, lobster, and crayfish) and from the shells of mollusks. It is mainly isolated by chemical procedure, which involves separation of proteins by treating with alkali and minerals such as calcium carbonate and calcium phosphate by treatment with acid.

At first the shells are deproteinized by treatment with (3-5 %) aqueous sodium hydroxide solution. The resulting product is neutralized and calcium is separated by treatment with (3-5 %) aqueous Hydrochloric acid solution at room temperature to precipitate chitin. Then chitin is dried and deacetylated to give chitosan by treatment with 40-50 % aqueous sodium hydroxide solution at moderate temperature 110^{o}C (100-120^{o}C) and the precipitate is washed with water.

The crude sample is dissolved in acid (2% acetic acid) and the insoluble material is then removed. The resulting clear supernatant solution is neutralized with aqueous sodium hydroxide to give a white precipitate of chitosan. It is further purified dried and ground to a fine uniform powder or flocks (**fig. 2**) [7].

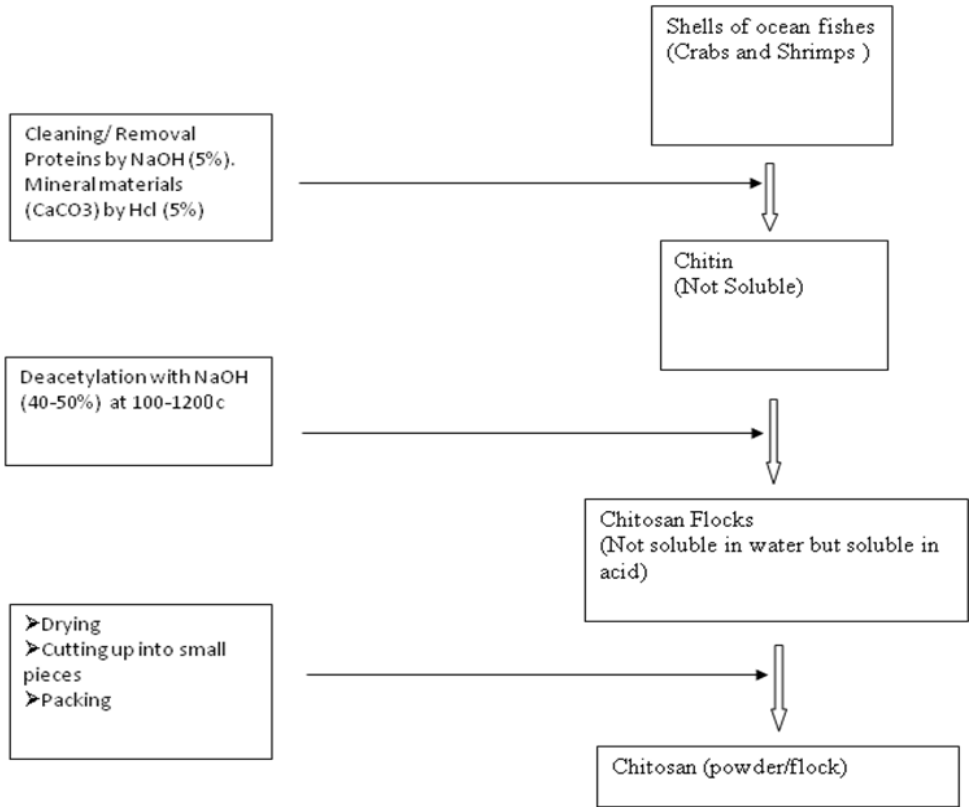


FIGURE 2: MANUFACTRURING OF CHITIN AND CHITOSAN

Properties of Chitin and Chitosan: Chitin and chitosan are highly basic polysaccharides, as against most of the naturally occurring polysaccharides like cellulose, dextran, pectin, alginic acid, agar, agarose and carrageenans, which are neutral or acidic in nature. Their unique properties include polyoxy salt formation, ability to form films, chelate metal ions and optical structural characteristics [8]. In cellulose the β (1-4) glycosidic linkage is there which is same as that in chitin and chitosan, and the similarity exist in both structure and biological activity, but the characteristic properties of chitin and chitosan is not at all same as cellulose. So here we can discuss about the properties of chitin and chitosan individually.

Chitin: Chitin is measured as a suitable constructive substance for the reason that it exhibits brilliant properties such as biocompatibility, biodegradability, non-toxicity, and adsorption properties, but this bio- polymer exhibits a restriction in processibility due to troubles related to its solubility. The chitin is having highly ordered crystalline structure. This is due to the fact that extensive hydrogen bonding is occurring between the hydroxyl groups and the N-acetamido groups in the repeating units. Intramolecular hydrogen bonds between C_3-OH hydroxyl and C_5-O ring oxygen across each β(1→4)-glycosidic linkage limit chitin units to the low-energy chair conformation, which results in a strong, rigid and linear polymer structure. This prevents the polymer from dissolving completely in common organic solvents (DMSO, DMF, DCM, and NMP) and aqueous solvents [9].

Chitin has been reported to show solubility in concentrated acidic solvents such as H_3PO_4, HCl, H_2SO_4, and amide/LiCl systems (e.g. N,N-dimethylacetamide/LiCl and N-methyl-2-pyrrolidone/LiCl). Because of the number of problems associated with solubility of chitin in aqueous and organic solvents, chemical modification of chitin to generate new bio-functional resources is of primary interest, where such modification would not change the fundamental skeleton of the polymer.

The very significant and widely accepted procedure includes alkaline N-deacetylation of the N-acetamido functional groups of chitin to give a compound of multi-functionality. Modification of the N-acetamido groups by N-deacetylation results in functional amines that can undergo nucleophilic substitution reactions [10]. Chitin undergoes alkaline deacetylation in the presence of sodium hydroxide to give chitosan, whereas in the presence of hydrochloric acid chitin undergoes hydrolysis to yield glucosamine (**figure 3**).

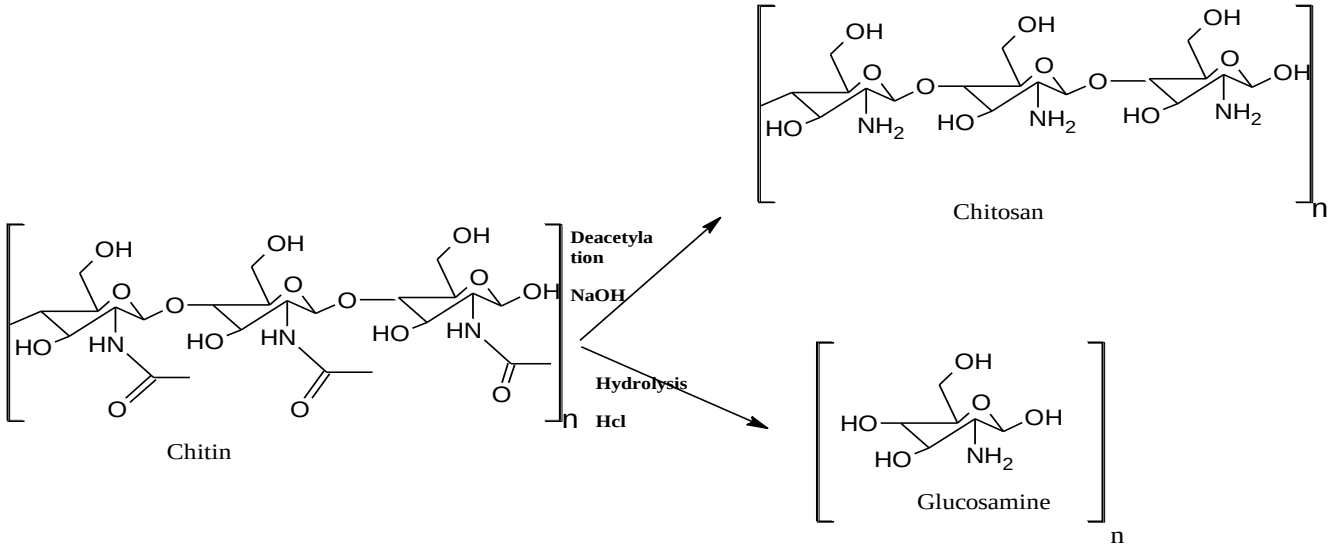


FIGURE 3: N-DEACETYLATION AND HYDROLYSIS OF CHITIN

Chitosan: As chitin is feebly soluble in aqueous solution and organic solvents, the practical applications of chitin are very less whereas chitosan as a mock alternative of chitin, is more suitable for useful bioapplications. Even though chitosan is insoluble at neutral and alkaline pH, it can form water-soluble salts with inorganic and organic acids including glutamic, hydrochloric, lactic and acetic acids [11]. The most generally used is 1% acetic acid solution at about pH 4.0 as a reference.

Chitosan is also soluble in 1 % hydrochloric acid but insoluble in sulfuric and phosphoric acids. Solubility of chitosan in inorganic acids is quite restricted. Upon dissolution in acidic media, the amino groups of the polymer become protonated which gives positive charge to the molecule [12]. With the action of glycosidases such as chitosanase, chitinase, lysozyme and cellulase the chitosan is found to be biodegradable. The chitosan is having exceptional biocompatibility and commendable biodegradability with safety and low toxicity. It is currently utilised intensively for its applications in several fields such as pharmacy, biomedicine, agriculture, food industry and biotechnology [13].

Chitosan is mainly characterised by its molecular weight and degree of deacetylation (DD). Deacetylation involves the elimination of acetyl groups from the molecular scaffold of chitin, which gives chitosan. Chitosan have highly reactive amino group ($-NH_2$) which makes the degree of deacetylation an important property in chitosan production and characterisation. Deacetylation is also affecting the biodegradability and immunological activity of chitosan [14].

Commercially available chitosan is > 85% deacetylated**.** Mainly the degree of deacetylation can be employed to distinguish between chitin and chitosan because it determines the content of free amino groups in the two polysaccharides**.** For the determination of the degree of deacetylation various methods have been reported, which include ninhydrin test, linear potentiometric titration, near-infrared spectroscopy, nuclear magnetic resonance spectroscopy, hydrogen bromide titrimetry, infrared spectroscopy and first derivative UV-spectrophotometry [15]. Chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material sources and the method of preparation.

Till now there is no specific standard to describe the molecular weight of chitosan but it is known that the range of low molecular weight chitosan is < 50 kDa, medium molecular weight chitosan is 50-100 kDa, and high molecular weight chitosan is <150 kDa respectively [16].

Biodegradation is a normal natural process by which organic chemicals in the environment are transformed to simpler compounds, mineralized and redistributed through elemental cycles such as carbon, nitrogen and sulphur cycles [17]. Biodegradation also plays a major role in the metabolic fate of chitosan in the body. Mainly two types of degradation are done that is chemical as well as enzymatic degradation. Chemical degradation is referred to acid catalyzed degradation such as in the stomach. Enzymatically, chitosan can be degraded by enzymes able to hydrolyse glucosamine-glucosamine, glucosamine –N acetyl- glucosamine and N-acetyl-glucosamine-N-acetylglucosamine linkages.

Even though depolymerisation through oxidation–reduction reaction and free radical degradation of chitosan have been reported these are unlikely to play an important role in the *in vivo* degradation of chitosan.

Chitosan is known to be degraded in vertebrates predominantly by lysozyme and by certain bacterial enzymes in the colon. The enzymatic behaviour of various chitosans is investigated by observing changes in the viscosity of chitosan solution in the presence of lysozyme. It was found that chitosan with a low DD tended to be degraded more rapidly [18].

Chemical modifications of Chitosan: As shown in **Fig. 4,** Chitosan contains three main types of reactive functional groups, an amino/acetamido group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively. The main reason for the differences between the chitin and chitosan is the amino group content in them. This will make great difference in structures and physicochemical properties. It is also attributed to their chelation, flocculation, biological functions and applications.

Chemical modification of chitin and chitosan to generate new biofunctional compounds is of key interest because such practice would not alter the fundamental skeleton of chitin and chitosan. Also it would keep the unique physicochemical and biochemical properties depending on the nature of the group introduced [19, 20].

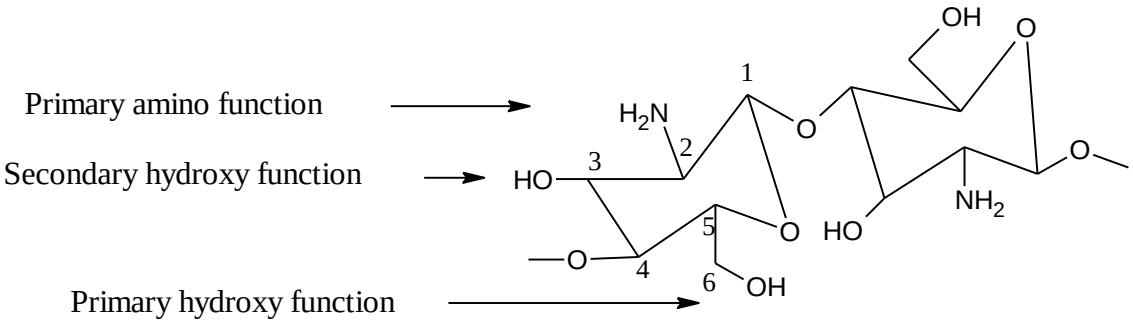


FIGURE 4: FUNCTIONAL GROUPS IN CHITOSAN

Chemical modification of chitosan provides a new way for developing new derivatives having promising biological activities and physiochemical properties. As discussed earlier chitosan has a primary amino group, and a primary and secondary free hydroxyl groups, the strong functionality of chitosan (two hydroxyl groups (C-3, C-6) and one primary amine group (C-2) per-repeat unit) gives it a considerable chance of chemical modification.[21] Mainly the chitin contains 5-8 % of nitrogens depending on the degree of deacetylation, these nitrogens are the primary aliphatic amino groups present in the chitosan.

The reaction undergone by the chitosan is classically same as that of amines, which involves N-acylation and schiff base formation. The N-acylation is mainly done by acid chlorides or acid anhydrides which introduces the amido group to the chitosan nitrogen. This intermediate is very suitable for further attachment of side chains.

The attached side groups on chitosan provide versatile resources with specific functionality alter biological properties or modify physical properties.

In an attempt to advance solubility of chitosan in physiological media, to improve its biological properties and to widen its applications, a variety of derivatives of chitosan have been synthesised. The chemical modification of chitosan includes the preparation of various derivatives of chitosan. Without disturbing the overall effects of chitosan, one can chemically modify this versatile bio-polymer since it provides functional groups as primary amine and primary as well as secondary hydroxyl groups in its monomers.

The important examples of modified chitosans that hold prominent places in research are

1. Quarternised Chitosan
2. N Alkyl Chitosan
3. Carboxy Alkyl Chitosan
4. Acyl Chitosan
5. Thiolated Chitosan
6. Sulfated Chitosan
7. Phosphorylated Chitosan

1. **Quaternized chitosan derivatives:** Chemical modification of chitosan by introducing the quaternary ammonium functionality into the polymer backbone gained attention due to its improved antimicrobial activity (**fig. 5**). As reported by Thanou *et al* (2000), the preparation of quaternary salts of chitosan mainly involves the methylation of chitosan with methyl iodide in the presence of strong base.

   The product can be isolated by precipitation with ethanol and centrifugation. The next step is the reductive methylation, to yield the final products trimethyl chitosan iodide having required degree of substitution. The product is then precipitated by adding ethanol and can be isolated by centrifugation. The purification of the final product involves the exchange of the counter ion iodide with chloride.

The product is dissolved in sodium chloride containing aqueous solutions and reprecipitated by ethanol, again isolated by centrifugation and thoroughly washed with ethanol and diethyl ether [22]. Another method for quaternisation of chitosan is by way of reacting with betaine in the presence of the coupling reagent 2-ethoxy-1-ethoxycarbonyl-1, 2-dihydroquinoline (EEDQ) in aqueous media at pH 5.5 ± 0.5. This reaction results in preparation of N-(trimethylammonio) acetyl/chitosan chloride and its amphiphilic derivatives. The degree of quaternization increases with increasing EEDQ/chitosan ratio and is partly followed by N-ethoxy carbonylation. That side-product formation can be slowed down by increasing betaine/EEDQ ratio.[23]

The quaternized chitosan derivatives can also be prepared by microwave irradiation. Chitosan is dispersed in isopropyl alcohol and the PH of solution was adjusted to basic. EPTMAC (Epoxypropyl trimethyl ammonium chloride), the intermediate in the preparation of quaternised derivatives, was dissolved in water and added to chitosan suspension at 80^{o}C. After reaction for 50 min at 80^{o}C, the reaction mixture was precipitated by acetone, dialyzed, and finally freeze-dried at 50^{o}C to obtain quaternized chitosan [24].

The advantage of quaternary salts over the parent chitosan is accredited to its permanent positive charge and the synergetic effect of the introduced alkyl moiety. Also, the solubility of chitosan at physiological pH is low; while for the quaternary salts the solubility is high both in acid and basic conditions. The main applications of these quaternary derivatives are they have enhanced antibacterial potential. The mechanism of antibacterial activity of chitosan and its derivatives is still not known. But, it is proposed that the positive charge density of quaternized chitosan absorbed on to the negatively charged cell surface of bacteria leads to the leakage of proteinaceous and other intracellular constituents [25].

Thus, the modification by quaternization proved effective for preparation of new antimicrobial drugs with high potential.

The quarternised chitosan derivatives can be used as cutotoxic agenta, antibacterial as well as antifungal agents.

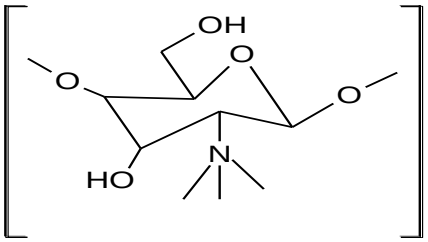


FIGURE 5: STRUCTURE OF N-QUATERNISED CHITOSAN DERIVATIVES

2. **N-alkyl chitosan derivatives:** The primary amino groups of chitosan undergo a Schiff reaction with aldehydes and ketones, to yield the equivalent aldimines and ketimines, which can be then converted to an N-alkyl derivative by reduction with sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaBH_3CN$), among other reducing agents (**figure 6**) [26, 27].

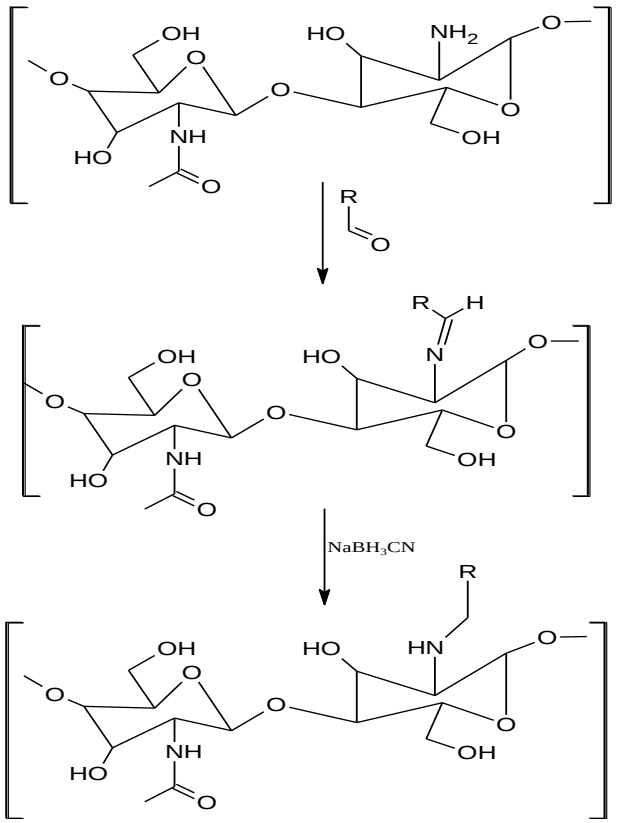


FIGURE 6: PREPARATION OF N-ALKYL CHITOSAN DERIVATIVES

Modification of chitosan with butanal, hexanal, octanal or decanal aldehydes are very useful to prepare a biocompatible and biodegradable hydrophobic chitosan membrane that can replace Nafion® for electrode coatings in both sensor and fuel cell applications [28, 29]. The other applications of N-alkyl chitosan involves antimicrobial, additive in paper making, for hydrophobization of cellulose fibres and paper surfaces, simultaneously with dry strength improvement; improvement and control of retention and drainage of the paper stock; adsorption of the sticky contaminants from process water and in drug delivery [30].

The N-alkylation reactions of chitosan can be carried out by halogen displacement reaction also, that is by adding chitosan to isopropanol /4 N sodium hydroxide solution and stirring at 70^{o}C for 30 min (**fig. 7**). The alkyl halide was added dropwise to the mixture and allowed to react for 4 h, and then the reaction mixture was centrifuged. The obtained precipitate was washed with ethanol and then dried at vacuum to obtain the alkylated chitosan derivatives [31].

3. **Carboxy Alkyl chitosan derivatives:** In order to overcome chitosan's inadequate solubility in its effectiveness as absorption enhancer at neutral pH values such as those found in the intestinal tract, chitosan derivatives have been developed as carboxy alkylated derivatives and have been found to be most investigated one as they are amphoteric in nature. The presence of both carboxyl groups and amino groups in CM-chitosan macromolecules elicits special physicochemical and biophysical properties. It is interesting for pharmaceutical applications because of their novel properties, especially for controlled or sustained drug-delivering systems. Normally novel carboxy alkyl chitosan derivatives are prepared by reacting parent chitosan with monohalocarboxylic acid by using different reaction conditions.

Both N- and O-carboxylation are done on the chitosan scaffold. By using different reaction conditions the controlled selectivity of the reaction can be obtained. The carboxy alkylation is mainly affected by reaction time, reaction temperature and the presence of catalytic base [32]. N-carboxymethylation of chitosan is affected through Schiff base formation from the free amino group of chitosan with an aldehyde or keto group and the successive reduction with cyanoborohydride or sodium borohydride [33].

O-Carboxymethylation was carried out by stirring chitosan (5 g) in 20% NaOH (w/v, 100 ml) for 15 min. to which monochloroacetic acid (15 g) was added dropwise and the reaction was continued for 2 h at 40±2°C with stirring. Then the reaction mixture was neutralized with 10 % acetic acid /HCl then was poured into an excess of 70% methanol / ethanol to precipitate O-carboxymethyl chitosan [34]. N-carboxy methylated chitosan has a wide range of biomedical applications such as to increase the permeation and adsorption of low molecular weight heparin, an anionic polysaccharide across intestinal epithelia, wound dressings, artificial bone and skin, antibacterial, antifungal, antioxidant, apoptosis inhibitor and blood anticoagulants, due to its unique chemical, physical, and biological properties, especially its excellent biocompatibility [35-37]. The pharmaceutical applications include wound healing, tissue engineering in drug delivery of anti-inflammatory, anti-cancer, proteins peptides and vaccines, in gene therapy, bio imaging and in green chemistry [38].

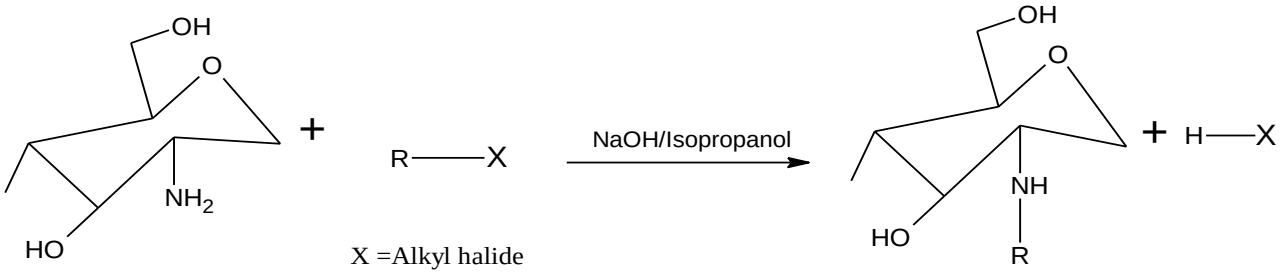


FIGURE 7: N-ALKYLATION OF CHITOSAN BY HALOGEN DISPLACEMENT

4. **Acyl Chitosan:** The acylation on the chitosan scaffold can be done in the amino group, hydroxyl group, or on both the groups. The fully acylated chitosan can be prepared by using pyridine and chloroform as reaction medium. To a mixture of chitosan, pyridine and chloroform in large excess of acyl chloride, after 8 hours of reflux reaction one fold excess of acyl chloride is again added. The reaction mixture is kept as such until it becomes clear and then poured into methanol to precipitate the product [39-40]. The *N*-acylated chitosans are characterised by increased hydrophobic character and thus produce important changes in the structural features**.** The main mechanism of *N*-acylation is the addition elimination reaction. The *N*-acylation of chitosan with anhydrides in a mixture of aqueous acetic acid and methanol at room temperature that proceeds selectively at the *N*-amino functional groups, have also been reported [41]. Min *et al* (1995) carried out the acylation with various cyclic acid anhydrides and showed that the substituents greatly reduced the normal regularity of intermolecular hydrogen bonding of chitosan, which resulted in derivatives with very good solubility in water [42].

Synthesis of N acylated chitosan derivative:

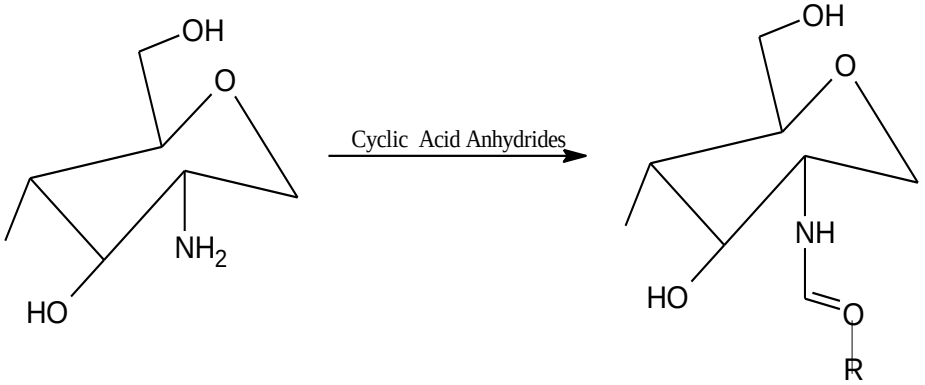


FIGURE 8: SYNTHESIS OF N ACYLATED CHITOSAN DERIVATIVE

The N-acylation can be carried out by dissolving chitosan in aqueous acetic acid followed by addition of acetic anhydride (**fig. 8**). After stirring at ambient temperature for 5 h, sodium hydroxide is added to pH 8–9 in order to stop the reaction. Then the reaction mixture was dialyzed against deionized water for 2 days to remove any micro ions and lyophilized. The acetylated chitosan was then treated with methanolic potassium hydroxide for 5 h at room temperature and repeatedly washed with methanol using a centrifuge. Finally, it is dissolved in deionized water and lyophilized [43]. The half N-acylated chitosan thus obtained had a random distribution of the N-acetyl groups, and the lower the molecular weight, the higher the water solubility [44].

Shigehiro H. *et al* (2002) carried out the syntheses of novel water soluble N-saturated fatty acyl derivatives of chitosan. In this, the chitosan was reacted with propionic, butyric, pentanoic, hexanoic, and octanoic anhydride and the longer chain acid anhydrides like decanoic, lauric, myristic, palmitic and stearic anhydrides. From the study they found that the length of the chain and degree of substitution had greater effect on chitosan solubility. They reported that the shorter the chain and low to moderate degree of substitution, the derivatives exhibit solubility in water. But moving to higher degree of substitution, the derivatives display very little to no solubility in water. The longer fatty acyl derivatives (**fig. 9**) of chitosan were insoluble in water regardless of the degree of substitution, due to an increase in hydrophobicity [45].

Synthesis of Fatty acid derivatives of Chitosan:

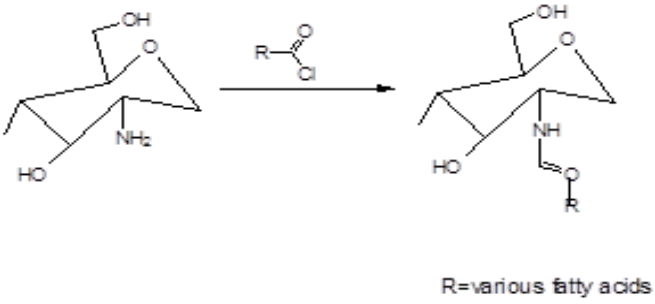


FIGURE 9: SYNTHESIS OF FATTY ACID DERIVATIVES OF CHITOSAN

The amino group of chitosan is more reactive than the hydroxyl group, so the protection of amino group is necessary before performing the reactions on the hydroxyl group. Phthalic anhydride and BOC are commonly used for the protection of amino group, of which phthalic anhydride is more preferred for the reaction (**figure 10**).

Protection of Amino group with phthalic anhydride:

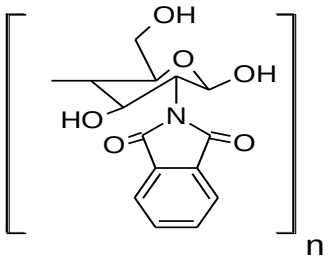


FIGURE 10: PROTECTION OF AMINO GROUP WITH PHTHALIC ANHYDRIDE

The O-acylation reaction is an important reaction. The selective O-acylation is done by using sulphuric acid as catalyst. Chitosan after dispersing in distilled water the acid derivative and sulphuric acid was added and stirred at 80^{o}C for 4 h. The pH was adjusted to 7 with sodium bicarbonate and the compound was precipitated in acetone. Then the precipitate was extracted with acetone for 2 days and dried to give the O acylated chitosan [46].

5. **Thiolated chitosan derivatives:** The chemical modification of chitosan by thiolation is mainly for the improvement of its muco-adhesive properties. The introduction of thiol group into the chitosan scaffold can be done by reacting chitosan with various reagents; common ones are thioglycollic acid (TGA) and glutathione (GSH).

    a. **Thiolation with thioglycollic acid:** To the solution of chitosan in 1% acetic acid ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was added. Then TGA was added and the pH was adjusted to 5.0 with sodium hydroxide. To eliminate the unbound TGA and to isolate the polymer conjugates, the reaction mixture was dialyzed against 5 mM HCl five times (molecular weight cut-off 10 kDa) over a period of three days in the dark, then two times against 5 mM HCl containing 1.0 % NaCl to reduce ionic uninteractions between the cationic polymer and the anionic sulfhydryl compound (**fig. 11**) [47].

Synthesis of Thiolated chitosan derivatives:

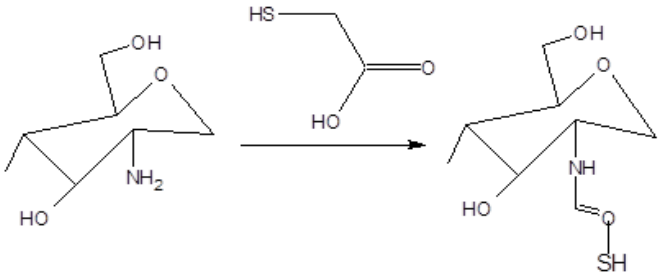


FIGURE 11: SYNTHESIS OF THIOLATED CHITOSAN DERIVATIVES BY THIOGLYCOLIC ACID

b. **Thiolation with glutathione:** As shown in **figure 12**, the chitosan hydrated in HCl was dissolved in demineralized water and the pH of the solution was adjusted to 6.0 with sodium hydroxide. Subsequently, reduced glutathione in demineralized water was supplemented over solution under continuous stirring. Then 1-ethyl-3-(3-dimethyl amino-propyl) carbodiimide (EDAC) in demineralized water was added in a final concentration of 200 mM. Thereafter, N-hydroxy succinimide (NHS) dissolved in demineralised water was also added into the reaction mixture under vigorous stirring in a final concentration of 200 mM. The pH was readjusted to 6.0 with sodium hydroxide (5 M). The reaction mixture was incubated for 15 hours at room temperature with continuous stirring. In order to eliminate unreacted reagents, the resulting polymer conjugate should be dialyzed first against 5 mM HCl, twice against 5 mM HCl containing 1% NaCl, and finally twice against 1 mM HCl. Controls were prepared in the similar way but devoid of EDAC and NHS. Finally, the frozen aqueous polymer solutions were lyophilized [48, 49].

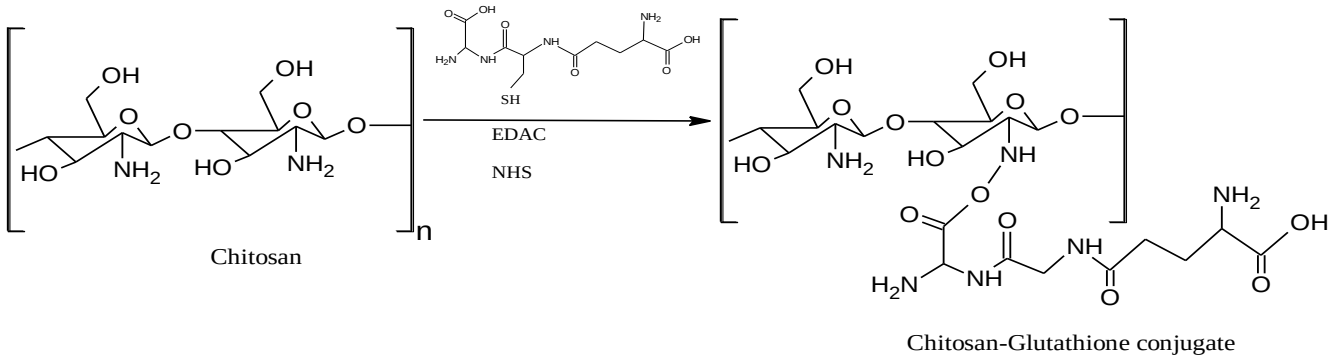


FIGURE 12: SYNTHESIS OF THIOLATED CHITOSAN DERIVATIVES BY GLUTATHIONE

The chemical modification of chitosan by thiolation reaction exhibits a very useful property that is, the compound prepared by this method can prolong the stability and the adhesion of different dosage forms on various mucosal tissues compared to well-established polymers. Therefore based on these properties thiolated chitosan derivatives represent a promising type of new mucoadhesive polymers [50].

6. **Sulfated chitosan derivatives:** Chemical modification of the amino and hydroxyl groups in chitin and chitosan with sulfate by site specific reactions can create products which have high impact in pharmaceutical applications. This is mainly because of the structural similarities of sulfated chitin and chitosan with that of the natural blood anticoagulant heparin, demonstrate bio-molecular mechanism of anticoagulant activity, antisclerotic and antiviral activities (**fig. 13**) [51-53]. Sulfonation reactions of multi-functional polysaccharides are unavoidably followed by the look of structural heterogeneity in polymer chains. This gives rise to indecision but on the other hand, some of the structures that come out from random allocation of modified groups along the chain can disclose new description of biological functions.

When chitosan is sulfated, a structural diversity of products is obtained, which may be related to the various reactivities of the three functional groups of the parent polymer, leading to different degrees of completion in the individual groups [54-60]. Sulfated chitosan is a water-soluble anionic chitosan derivative with antiviral, anticoagulant, antimicrobial and osteogenic activity.

This derivative also blocks human malignant melanoma cell adhesion and shows anti-obesity effect by the promotion of anti-adipogenesis inhibition. In addition, sulfated chitosan has low cytotoxicity [61-64].

For sulfation of polysaccharides like chitosan, a choice of methods which involve the combinations of sulfating agents and reaction media, have been anticipated. The fact that most of the polysaccharides are insoluble or only slightly soluble in the organic solvents used as the reaction medium in the conventional sulfation procedure, the reaction is performed in a heterogeneous medium which is the main complexity with the sulfation reaction.

As a result, it can be supposed that the constitution of the products is heterogeneous [65]. But the sulfation is carried out in homogenous condition also by the reaction of polymer with sulfur trioxide-pyridine complex in 5% LiCl/DMAc solvent system.

Sulfation at room temperature was regio-selective for the C-6 hydroxyl position with the degree of sulfation. When the reaction temperature was elevated, sulfation at the C-3 hydroxyl position also occurred. The extent of sulfation at the C-3 position was a function of the concentration of sulfating reagent, reaction time and temperature [66]. There are several methods for the preparation of sulphated chitosans of which two important general methods are postulated here.

Method 1: Sulfated chitosan was obtained by adding the sulphating complex. The sulfating complex was obtained by dropwise addition of $HClSO_3$ with stirring to previously cooled (4^oC) DMF. The reaction mixture was stirred until the solution reached to room temperature. Chitosan was added to DMF and stirred for 12 hours at room temperature. The excess of solvent was eliminated by filtration to give a solvated chitosan. The solvated polysaccharide was added to the sulfating complex and the reaction was run at room temperature for 5 hours with stirring. The final mixture was neutralized by 20 % (m/v) NaOH and precipitated with methanol in an ice bath. The precipitate was dissolved in water and the solution dialyzed against distilled water for three days with two water changes per day. The product was recovered by lyophilisation [67].

Method 2: According to Zhou *et al* (2009), sulfation is carried out by preparing the sulfating complex by previous method but modifying the amount of reagents. In the sulphating complex the amounts used were: $HClSO_3$ (5.0 ml instead of 4.5 ml) and DMF (50 ml instead of 30 ml). For the solvation, 2.5 g chitosan (in contrast to 2.0 g in Method 1) was added to the same volume of DMF plus 2 ml formic acid. The sulfating complex was then added and the reaction was run at room temperature for three hours with stirring. The product was precipitated with 700 ml ethanol, filtered under vacuum, washed with ethanol and dried with hot air.

The precipitate was dissolved in water and the pH adjusted to 7.0 with 20 % (w/v) NaOH. The undissolved material was removed by filtration, and the solution dialyzed against water for three days then lyophilized [68]. The degree of sulfation at the C-6 position can be estimated by elemental analyses. The anticoagulant activity of the prepared sulphated chitins is linked directly to the degree of sulfation. The higher the degree of substitution yielded, the better is its anticoagulant activity.

General structure of Chitosan sulphate:

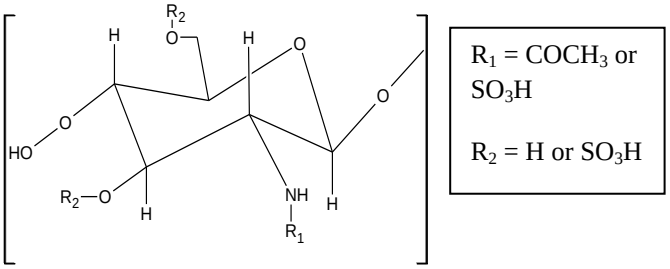


FIGURE 13: GENERAL STRUCTURE OF CHITOSAN SULPHATE

7. **Phosphorylated chitosan derivatives:** The modification by phosphorylation of chitosan seems to be of exciting interest for orthopaedic applications, due to the cation-exchange properties of phosphate functionalities. Phosphate groups attach calcium ions, which may then persuade the formation of a calcium phosphate layer known to endorse the osteo-conduction of polymer-based implants [69].

There are quite a lot of methods for the preparation of phosphorylated chitosan derivatives; one of them includes phosphorylation by phosphorous pentoxide and methane sulfonic acid. To the mixture of chitosan in methane sulfonic acid phosphorous pentoxide is added and the mixture was stirred at 0-5^{o}C for 2-3 hours.

The product was precipitated by ether and then centrifuged. The residue was then washed with ether, acetone and then dried. Methane sulphonic acid (**fig. 14**) works as an efficient catalyst for the esterification reaction [70].

Synthesis of Phosphorylated chitosan derivatives:

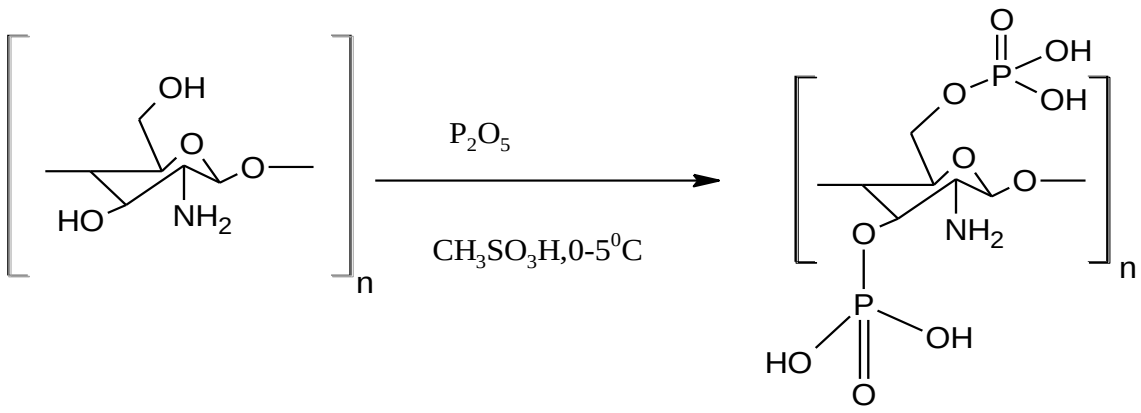
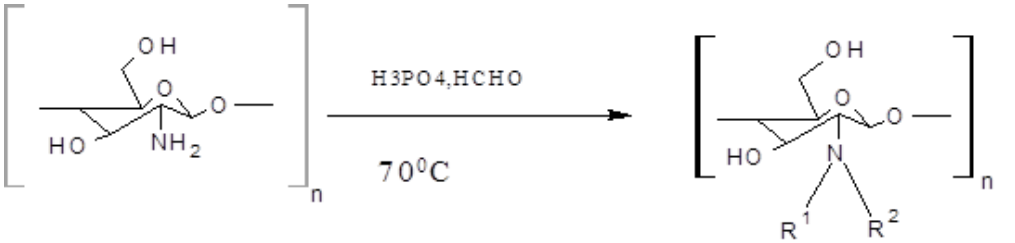


FIGURE 14: SYNTHESIS OF PHOSPHORYLATED CHITOSAN DERIVATIVES

N-methylene phosphoric acid chitosan is another derivative which is having wide variety of applications. It is prepared by mixing phosphoric acid and water, to which chitosan was added dropwise by stirring for one hour. Then, the temperature of the reaction vessel was raised to 70^{o}C and formaldehyde (36.5 %) was added drop-wise over one hour with reflux. Heating was extended for six hours. The clear pale yellow solution was dialyzed against demineralized water or until the pH of water was raised to 6.8. Finally, the solution was frozen and freeze-dried (**fig. 15**) [71].

Synthesis of N-methylene phosphorylated chitosan derivatives:



$R^1 = H, R^2 = CH_2PO_3H_2$

$R^1 = R^2 = CH_2PO_3H_2$

FIGURE 15: SYNTHESIS OF N-METHYLENE PHOSPHORYLATED CHITOSAN DERIVATIVES

Heterocyclic derivatives of Chitosan: The works done so far in the derivatisation of chitosan by introduction of heterocyclic ring in the chitosan scaffold revealed that it have greater effect on the pharmacological activity of chitosan. Kumar et al (2009) reported that the free amino group at C-2 position was more reactive towards electrophiles than hydroxyl groups at C-3 and C-6 in the 2-amino- 2-deoxy-d-glucopyranose units of chitosan. To introduce alkyl and aryl groups selectively at the amino group of chitosan, reductive amination reaction was the most reliable reaction. As shown in **fig. 16,** Chitosan was treated with aliphatic or aromatic aldehydes to produce the corresponding biopolymeric Schiff base, which upon reduction by sodium cyanoborohydride produced aromatic derivative [72].

Synthesis of Aromatic chitosan derivatives:

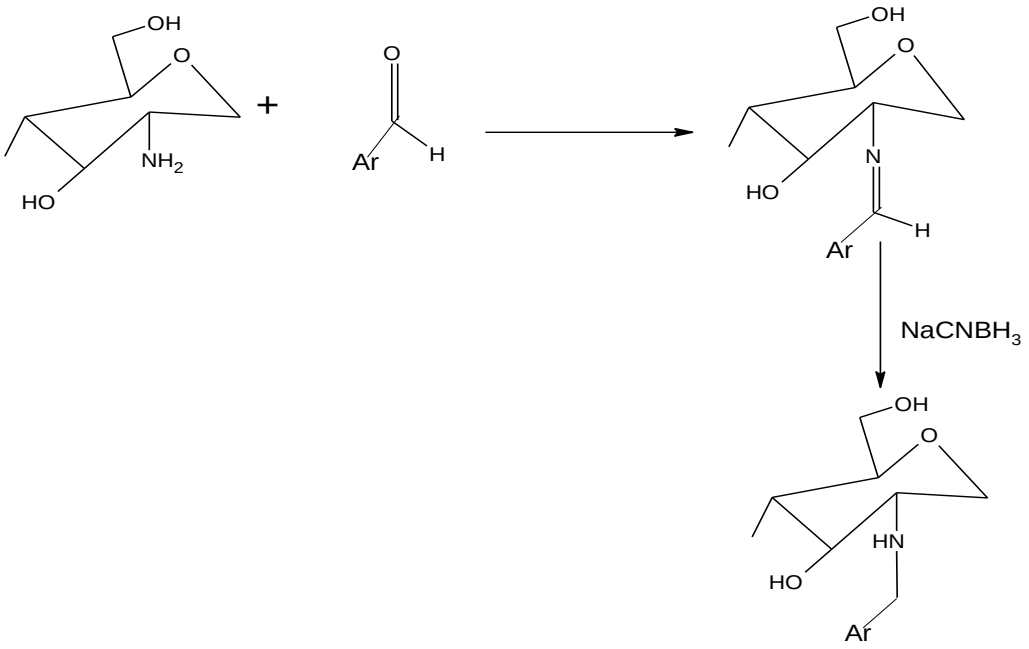


FIGURE 16: SYNTHESIS OF AROMATIC CHITOSAN DERIVATIVES

The piperazine derivatives of chitosan as antibacterial agents are also proposed. As shown in **fig. 17,** the amino group was protected by phthaloylation (A) and the hydroxyl group by trityl group (B). After the deprotection of amino group by treatment with hydrazine hydrate (C), N-chloroacylation was carried out by chloroacetyl chloride (D) to form the intermediate N-chloroacyl 6-O-triphenylmethyl chitosan. The piperazine derivative (F) was then prepared after deprotection of hydroxyl group (E) [73].

Synthesis of N heterocyclic derivatives of chitosan:

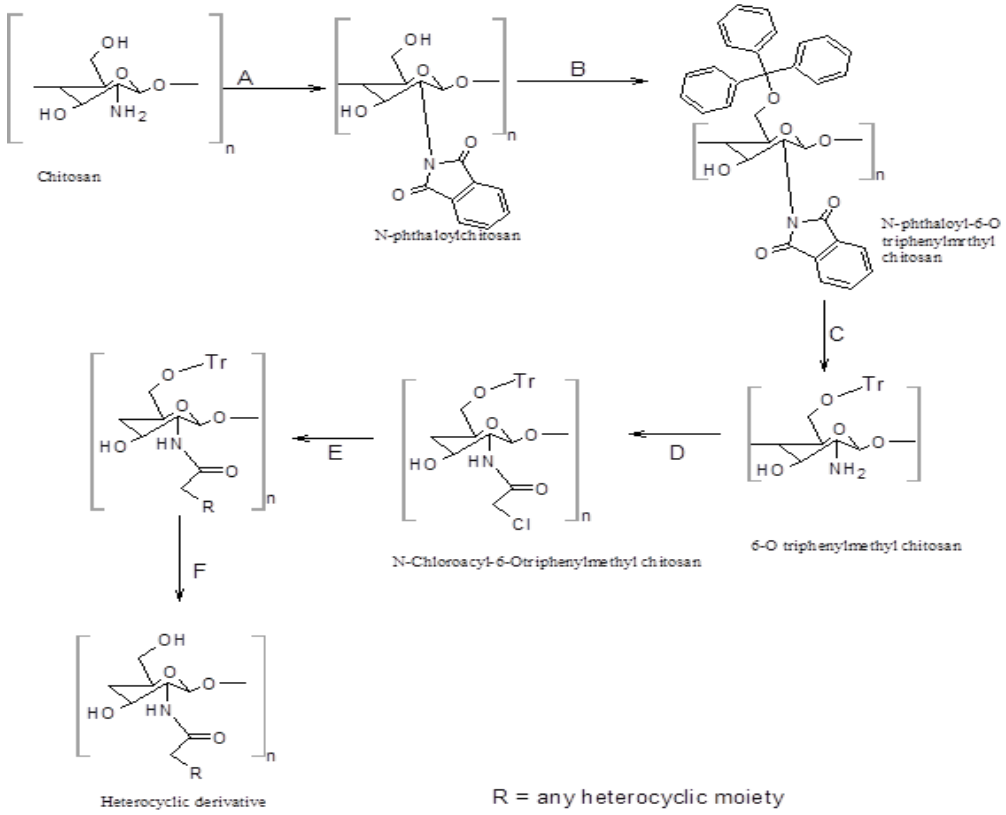


FIGURE 17: SYNTHESIS OF N HETEROCYCLIC DERIVATIVES OF CHITOSAN

Applications of Chitosan and its derivatives: Chitosan, which is a versatile natural polysaccharide, is having wide applications.

1. Biological applications
2. Pharmaceutical applications

1. **Biological applications:** The biological applications of chitosan include antimicrobial effects, immunity-enhancing, Hypocholesterol emic, antitumor and anticancer activity, acceleration of calcium and iron absorption, anti-inflammatory and antioxidant activities.

a. **Antimicrobial activities:** Microorganisms are found in air, water and soil. Some human friendly organisms are useful in several fields, but on the other hand, the pathogenic organisms cause life intimidating infectious diseases. It is reported that yeasts and moulds are the most susceptible group, followed by Gram-positive bacteria and finally Gram-negative bacteria. In order to promote the general health and to control the infection of these harmful microorganisms potent or specific antimicrobial systems are introduced. Due to the proficient intrinsic properties chitosan is strongly employed in the field of health as antimicrobial agent.

The antibacterial property of chitosan can be enhanced by altering the hydrophobic as well as hydrophilic nature of the polysaccharide backbone. Activity varies considerable with the type of chitosan, the target organism and the environment in which it is applied. The antibacterial activity of chitosan is also applied in the field of agriculture for the activity against plant pathogenic bacteria and fungi. They persuade decay on a large number of economically important agricultural crops during the growing season and during postharvest storage. Control of these infections is predominantly important and can be achieved by synthetic pesticides.

However, there is growing environmental difficulty caused by bactericides and fungicides, especially by synthetic products [74].

As a result there has been mounting interest in finding alternatives to chemical bactericides and fungicides considered as safe and with minor risk to human health and environment. Recently chitosan, a biopolymer of common interest have been anticipated because of its unique physiochemical characteristics and biological activities [75].

Along with various bioactive properties of chitosan, its antimicrobial activity has received considerable interest due to problems connected with fungicidal agents [76]. The fungicidal activity of chitosan has been well recognized both in *in vitro* and *in situ* studies. It is reported that the level of inhibition of fungi is highly correlated with chitosan concentration, indicating that chitosan performance is related to its application at suitable rate. It is believed that the polycationic nature of this compound is the key to its antifungal properties and that the length of the polymer chain enhances its antifungal activity [77].

Recently, research has been focused on the opportunity of developing chitosan as a natural disinfectant. It can also be applied to extend the storage life of fresh fruits and some foods. Much of the interest in the antimicrobial properties of chitosan has focused on the possibility of plant protection [78].

Bactericidal effectiveness of chitosan has also been well reported. It arises from various factors, and according to the roles playing, these factors can be classified into four categories as follows:

(1) Microbial factors, related to microorganism species and cell age;

(2) Intrinsic factors of chitosan, including positive charge density, molecular weight, concentration, hydrophilic/hydrophobic characteristic and chelating capacity;

(3) Physical state, namely water-soluble and solid state of chitosan;

(4) Environmental factors, involving ionic strength in medium, pH, temperature and reactive time [79].

Chitosan is an adaptable material with proved antimicrobial activity. Three antibacterial mechanisms have been proposed:

i. The ionic surface interaction resulting in cell wall leakage;

ii. Inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms and

iii. Formation of an external barrier, chelating metals and provoking the suppression of essential nutrients to microbial growth.

It is likely that all events occur at the same time but at different intensities. The molecular weight (MW) and the degree of acetylation (DA) are also important factors in determining such activity.

In general, the lower the MW and the DA, the higher will be the effectiveness on reducing microorganism growth and multiplication. From the literature it has not lead to any conclusive data as to whether the chitosan has higher activity on gram-positive or on gram-negative bacteria. On both species chitosan seems to act differently, though in both cases satisfactorily [80-82]. Anyway, research on modification of chitosan scaffold is going on which leads to better antimicrobials in future.

b) **Antioxidant activity:** Synthetic works are going on for producing more potent and naturally derived antioxidant compounds. Chitosan and several of its derivatives belong to this class, which being safe and non-toxic offer protection from free radicals, thus retarding the progress of numerous chronic diseases [83]. It is known that the antioxidant effect of chitosan varies with its molecular weight and viscosity [84, 85]. It is attributed to differences in the availability of net cationic amino groups in the molecule, which impart intermolecular electrostatic repulsive forces leading to increase in the hydrodynamic volume of the extended chain conformation. The highly unsaturated fatty acids commonly found in seafood are particularly sensitive to oxidative change during storage. Treatment of herring fish samples with chitosan, however, showed lower peroxide values and total volatile aldehydes than the untreated samples. The low viscosity chitosan showed the strongest anti-oxidative effect [86].

c) **Antitumour activity:** Recently conducted research on antitumour effect of chitosan showed that the low-molecular-weight water-soluble chitosans and oligochitosans might be useful in preventing tumor growth, partly through enhancing cytotoxic activity against tumors as an immunomodulator [87].

Most anti-cancer chemotherapeutic agents have harmful side effects that are not favourable to prolonging the lives of cancer patients. Therefore, it is important to find new anti-cancer agents that are non-toxic and biocompatible.

The antitumor mechanism of chito-oligosaccharides was probably related to their initiation of lymphocyte factor, increasing T-cell proliferation to produce the tumor inhibitory effects. Through analysis of the splenic cell changes in cancerous mice, it is also proved that the antitumor mechanism of chitooligo-saccharides is to enhance acquired immunity by accelerating T-cell differentiation to increase cytotoxicity and maintain T-cell activity [88].

It has been reported that low-molecular-weight chitosans were useful in preventing tumor growth in sarcoma 180-bearing mice through the activation of intestinal immune functions [89]. The charge properties of chitosan are also very important for the anticancer activity also the quarternised chito-oligosaccharides and sulphated chito-oligosaccahrides could reduce viability of cancer cells mainly by interacting via their electronic charge.

It is reported that, high molecular weight of chito-oligosaccharides can be overcome by their strong negative or positive charge to induce death of cancer cells [90, 91].

d) **Anti-inflammatory activity:** A large number of studies have broadly investigated the effects of chitin, chitosan and their derivatives, few investigating anti-inflammatory activity have recently been published. Inflammation is a physiological body immune response against pathogens, toxic chemicals or physical injury. Acute inflammation is a short-term normal response that usually causes tissue repair by recruitment of leukocytes to the damaged region, chronic inflammation is a long-term pathological response involving induction of own tissue damage by matrix metallo-proteinases [92, 93].

The treatment of inflammation relies mainly on the selective inhibition of cyclooxygenase-2 (COX-2) activity responsible for producing prostanoids. The most widely prescribed drug for treatment of many inflammatory diseases is the non-steroidal anti-inflammatory drugs (NSAIDs). However, they display a high occurrence of gastric, renal and hepatic side effects. In recent years, it has been reported that chronic inflammation is associated with an increased risk of malignant transformation [94].

This is mainly because phagocytic leukocytes in chronic inflammatory processes produce large amount of reactive metabolites of oxygen and nitrogen that induce oxidative stress and lead to oxidation of fatty acids and proteins in cell membrane, thus impairing their normal function. Although the anti-inflammatory effects of chitin and its derivatives have been rarely reported, in recent years data has been accumulating [95].

It is reported that chitosan promotes the migration of the inflammatory cells which are capable of the production and secretion of a large collection of pro-inflammatory products and growth factors at a very early phase of healing. Chitosan also activates immuncytes and inflammatory cells such as PMN, macropromotion of tumor growth and invasion, phage, fibroblasts and angio-endothelial cells. So the research in anti-inflammatory activity of chitosan is emerging now days [96].

2. **Pharmaceutical applications:** The main pharmaceutical application of chitosan is in drug delivery. It involves the oral, parenteral, nasal, ocular and gene delivery [97]. Chitosan is also used as diluents in direct compression of tablets, binder in wet granulation, slow-release of drugs from tablets and granules, drug carrier in micro particle systems, films controlling drug release, preparation of hydrogels, agent for increasing viscosity in solutions, wetting agent and improvement of dissolution of poorly soluble drug substances, disintegrant, bioadhesive polymer, absorption enhancer [98].

CONCLUSION: Chitin is the second richest polysaccharide of animal origin found in nature mainly obtained from the shell waste of shrimps, lobsters, and crabs. Chitin is a derivative of cellulose whereas chitosan is a derivative of chitin. Chitosan is a biopolymer of high molecular weight. Due to better solubility and unique biological properties, chitosan molecule has been extensively studied by scientists. Chitosan is manufactured by shells deprotination, separation of calcium and then deacetylation of chitin. Chitin and chitosan are highly basic polysaccharides. Their unique properties include polyoxy salt formation, ability to form films, chelate metal ions and optical structural characteristics.

Chitosan is mainly characterised by its molecular weight and degree of deacetylation owing to presence of highly reactive amino group. It was observed that chitosan with a low DD tended to be degraded more rapidly. Chemical modification of chitosan provides a new way for developing new derivatives with promising biological activities and physiochemical properties. Variety of derivatives of chitosan have been synthesised to advance its solubility in physiological media, to improve its biological properties and to widen its applications.

The quaternary derivatives of chitosan with additional alkyl moiety produce synergistic antimicrobial effect. The carboxy alkylated derivatives exhibited special physicochemical and biophysical properties and generated interest in controlled or sustained drug-delivering systems. The pharmaceutical applications include wound healing, tissue engineering in drug delivery of anti-inflammatory, anti-cancer, proteins peptides and vaccines, in gene therapy, bio imaging and in green

chemistry. The *N*-acylated chitosans are characterised by increased hydrophobic character but when acylated with cyclic acid anhydrides the derivatives exhibited good solubility in water. The thiolated chitosan derivatives prolonged the stability and the adhesion of different dosage forms on various mucosal tissues and thus these derivatives represent a promising type of new mucoadhesive polymers**.**

Sulfated chitosan derivatives were obtained by using chlorosulphonic acjd in DMF as the sulphating complex. The water-soluble anionic chitosan derivative exhibited antiviral, anticoagulant, antimicrobial and osteogenic activity. Studies have shown that this derivative also blocks human malignant melanoma cell adhesion and showed anti-obesity effect by the promotion of anti-adipogenesis inhibition. The phosphorylated chitosan derivatives prepared by treating chitosan in methane sulphonic acid with phosphorous pentaoxide became an interesting molecule in orthopaedic applications. Phosphate groups formed a calcium phosphate layer known to endorse the osteo-conduction of polymer-based implants.

The heterocyclic derivatives of chitosan with substitution at the free amino group at C-2 position reported to exhibit greater pharmacological activity with wider applications due improved bioavailability.

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