Biowastes as a source of extracting chitin and chitosan for biomedical applications

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A B S T R A C T

Biomaterials are designed to interact with biological systems in aid to wound healing, regeneration of tissue, mechanical support, and drug delivery to eventually improve current therapeutic outcomes. The adoption of biomaterials is increasing constantly in health care practices by making it more biocompatible and non-toxic under physiological conditions. These adoptions have been associated with improvements in therapeutic outcomes across the population, however, the dosage of therapeutics needed to successfully treat a disease is generally different for each individual and relies a lot on experiences of consultant doctors. Many times, it leads to human errors in deciding on drug doses, un-fit implants and explants and eventually adverse effects or less positive effects. The personalized medicine and devices bring forth the idea that the medicine should be tailored for a patient based on various characteristics, such as gender, age, genetic makeup, and lifestyle. These personalized medicine approaches include type of drugs, activation methods, nano-assemblies, biomedical devices, etc. Among these approaches, personalized biomedical devices have become popular with the advent of 3D printing technologies, which can make customized implants for each patient with minimum price, limited time, and high accuracy. Personalized biomedicine also involves designing of drug to cater the need of an individual with minimum side effects. In this review an effort has been made to introduce different aspects of customized biomedical agents like therapeutic biomolecules, nanomedicine, implants, and explants. This comprehensive review of literature indicates that use of 3D printing technology in producing drug releasing, biodegradable personalized implants could be better therapeutic solution for a range of medical conditions.

1. Introduction

Biomaterials can be synthetic or obtained from natural sources for use in various applications including cure of various diseases like Alzheimer's disease (Hamedi et al., 2018), dental implants, prosthetic limbs (Williams et al., 1990), or for making scaffolds like collagen modified with lysine or hydroxy lysis (Sosnik and Sefton, 2005) or PEGylated fibrinogen (Ben-David et al., 2013). Thus, these biomaterials are very expensive because of the non-availability of the resources required for making the product or complex science and technology required to

develop (Bhat and Kumar, 2013). Their use in important biomedical interventions makes them vital for production on larger scale. Synthetic polymers were taken as source for biomaterial industry, but disclosures of their negative implications hard-pressed the researchers for course corrections. The problems of using synthetic polymers are non-biodegradability, associated toxicity, and non-biocompatibility. These concerns were behind the use of natural biopolymers as a very logical solution. Chitin and chitosan are two of the naturally available biopolymers which are known to overcome many of the reported shortcomings of synthetic polymers and can be extracted with higher benefits (Bakker et al., 1999). Additionally, chitin and chitosan are commercially viable because of their biocompatibility, biodegradability, chelation, non-toxicity and incredible adsorption power. All these attributes have allowed chitin and chitosan to find various uses in different kinds of industry like biotechnology, medical, food, and pharmaceutical to name a few. But they have established a place for themselves in biomedical chain of things. They have found their applications in wound healing, drug delivery, bio adhesives, etc. (Dhillon et al., 2013; Zhou et al., 2014; Wijesena et al., 2015)

Interestingly, after cellulose, chitin is the most inherent polymer in nature. Chitin and its derivatives have extravagant commercial value because of their diversified uses in biology and medical industry. However, some of its properties, like crystallinity and insolubility in water, hinder some of its applications. But, the use of its derivatives like chitosan, chito-oligosaccharides, etc. can be used to overcome those drawback (Kumar, 2000; Younes and Rinaudo, 2015; Ghormade et al., 2017; Yadav et al., 2019). The mentioned benefits of chitin and chitosan make them desirable polymeric materials for big scale industry productions and require better learning about their economical sources. This review article provides information regarding chitin and chitosan, that is, their extraction from the waste produced by different industries and advanced applications.

The chitinous waste are generally discarded by either flaring them or disposing it at a landfill. These methods are very hazardous to the environment (Xu et al., 2013). These wastes can be used to produce chitin and chitosan which are used further for different purposes like biomedical materials, fermentation, sewage treatment, etc. Chitosan is made up of both deacetylated and acetylated subunits of *D-glucosamine*. These two entities are linked by *β-(1,4) glycosidic* linkage. The linear polysaccharide is produced by de-acetylation where an acetate group and –NH₂ group is obtained by acetamide group's hydrolysis. *N-acetylglucosamine* ratio determines the degree of acetylation in chitosan (Fig. 1). This ratio is higher in chitin compared to chitosan (Ramírez et al., 2010; Viarsagh et al., 2010). Thus, chitin and chitosan although being extremely similar

structurally, can be used for different purposes based on required properties of final products. A large availability of these biowastes make them ideal candidate for extraction and use as industrial raw material benefitting the environment and providing economical starting material for various biomaterial industries.
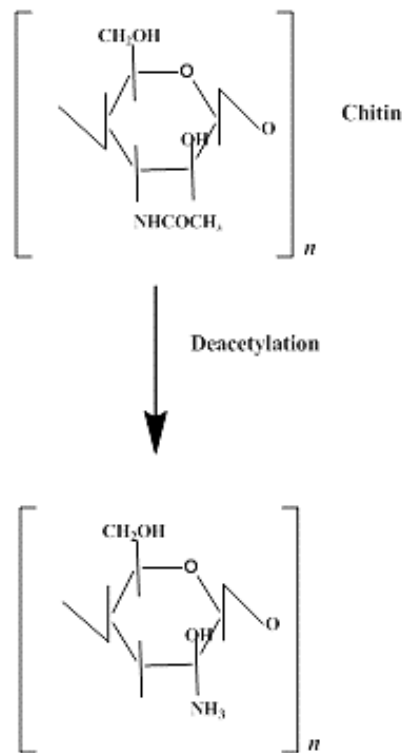

Figure 1. Conversion of chitin to chitosan by deacetylation. *N-acetamido* bonds in chitin monomer (*2-(acetylamino)-2-deoxy-d-glucose*) hydrolysed by chitin deacetylase to produce chitosan

2. Characteristics of Chitin

Chitin is present in the environment in three different forms which are crystalline in nature, namely *α, β,* and *γ.* These three different forms vary in the degree of de-acetylation (Aam et al., 2010). The *α-chitin* is generally present in yeast and fungal cell wall, insect cuticles, shrimp cells, etc. Polysaccharides are arranged in such a way that they are present in antiparallel fashion. This allows them to form maximum number of bonds. *α-chitin* is the main form of chitin present in the environment. Crystallinity index of these fibrils is approximately 80 % (João et al., 2015). *β -Chitin* exists in squid pens, some protozoa, and seaweeds. A diatom called *Thalassiosira fluviatilis* excretes a pure form of *β-chitin.* Polysaccharides are organized in a parallel manner. The crystalline index of this form of chitin is 70 %. Due to this arrangement, the distance between the chains increases, which make this structure more reactive and

easily soluble in solvents (João et al., 2015). *γ-Chitin* is made up of both *α* and *β* forms in a way that two parallel polymer units are organized alternatively with one anti parallel unit.

3. Generation of Chitinous wastes from bio-industries

As the population of the world is increasing, waste production has inevitably increased. The by-products of one industry can be used as a substrate to produce some other product. However, these by-products are treated as wastes and either dumped in sea or disposed at a landfill where they are not easily degraded. These wastes can be used for further isolation of chitin which can be further used for different purposes (Xu et al., 2013).

3.1. Seafood industry

Dumping of waste in oceans is one of the major factors leading to the environmental pollution where sea food industry is the major source of chitinous wastes. Chitin is generally present in all the aquatic crustaceans, shrimps and constitutes the major amount of dry weight in shrimps. Approximately 10^{12} - 10^{14} tons of chitin are manufactured annually by using the marine life forms. This amount of chitin should be enough to use as a raw material to produce different biomaterials or for other useful purposes. Chitin is generally extracted using chemical methods from such wastes, but biological methods are also used to some extent (Cauchie, 2002).

3.2. Silkworm industries

Silkworms are an unorthodox source of chitin and chitosan obtained from industries. The chrysalides of the silkworm (*Bombyx mori*) are the adult form of the larvae which form the silk threads and form cocoon. These chrysalides are a by-product of the silk industry. They are very cheap and easily available. Chitin constitutes around 20 % of the structure of silkworm. The yield with which chitosan is extracted from the chrysalides is very low but the purity of the chitin isolated is very high. (Paulino et al., 2006)

3.3. Honeybee industry

Obtaining chitin from honeybees is a very difficult process but large amount is generated for industrial purposes. Chitin is bound to different melanin and sclerotin-like proteins. They are a rich source of chitin and chitosan. In 2002, Russian Federation had 3.44 million bee colonies which constitute upto 3.5 - 4 kg of honeybees. This produces a lot of chitinous waste annually (Nemtsev et al., 2004).

3.4. From insect pests

Insects have grabbed a lot of attention as good source of chitin, and consequently chitosan. Cuticle present on the insect surface has less inorganic material than the crustaceans along with chitin. This makes the isolation of chitin much easier and convenient. For e.g. *Holotrichia parallela* is a beetle species which is a pest in China. Annually, this pest is caught in China to reduce the problem of pests. Chitin was isolated from this beetle quite effectively (Liu et al., 2012).

4. Methods of Chitinaceous material isolation from waste

The process of extraction of chitin and chitosan from bio-wastes can be done by chemical and biological procedures. During isolation of chitin from the natural sources, many different variables like molecular weight, degree of acetylation, purity, etc. are taken in consideration. Chemical methods are not eco-friendly and cause damage to the environment. Chemical interactions can also change the physical and chemical properties of chitin. Biological methods help in alleviating these problems. They are still in development phase and, therefore, not as popular as chemical methods (Abdou et al., 2008; Schmitz et al., 2019). Involvement of these processes in various industry based biowaste can be evaluated to understand the loss-benefit scenarios.

4.1. Seafood industry

Seafood industry is the primary source from which commercial chitin is being extracted. The chitin is drawn out from shells, crustaceans, and other sea life. Chitin is the major part of the waste produced by this industry. Chitin is extracted chemically as well as using biological methods (Fig. 2b).
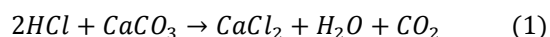
A. Chemical method

Shells which were obtained from different sources can dry after washing. They are then crushed into fine particles (Islam et al., 2004). Chemical withdrawal of chitin is done in three steps including (a) Deproteination (b) Demineralization, and (c) Decoloration.

(a) Deproteination

This step includes the removal of proteins from the chitin sample. These proteins are attached covalently to the biopolymer. This step helps in disrupting the chemical bonds that are present between these two components using chemical reagents like NaOH. This

step is important from biomedical point as the protein content is a major reason behind the allergic reaction that are caused in the organisms. Different types of chemicals in different concentrations were used for this step to increase the efficiency. It also leads to hydrolysis of biopolymer and deaceylation of chitin which decreases the molecular weight. It can also change the properties of the chitin or chitosan. (Yadav et al., 2019)

Figure 2. a) Extraction of chitin from chitonous waste; **b)** Extraction methods of chitin from biowastes using chemical methods like NaOH, H_2SO_4 etc. and biological method, eg: trypsin, alcalase etc; **c)** reaction mechanism of chitosan production from chitin using chemical and biological method

(b) Demineralization

Demineralizaton refers to the removal of minerals like $CaCO_3$. It is usually done by treating the polymer with

acids like HNO_3, HCl, CH_3COOH, etc. However, HCl is preferred for this process. In demineralization $CaCO_3$ is removed from the sample by converting it to CaO and CO_2. (Percot et al., 2003)

$$2HCl + CaCO_3 \rightarrow CaCl_2 + H_2O + CO_2 \qquad (1)$$

(c) Decoloration

This step is usually done only when colorless product is required. Generally, an organic solvent like acetone is used to separate pigments from the samples. (Mohammed et al., 2013)

B. Biological method

Biological method used for the chitin isolation revolves around the idea of 'Green chemistry'. The use of microbes and enzymes for chitin recovery is involved in biological method of chitin recovery. Generally, a chemical method is used for chitin isolation but the process is energy consuming (Dhillon et al., 2013) and cause negative effects on physico-chemical properties of the biopolymer. In comparison, biological methods are cleaner, more economical, and they allow the isolation of chitin with desired properties. (Khanafari et al., 2008) The two methods generally used to produce chitin and chitosan biologically are:

(a) Enzymatic deproteination

Proteases are enzymes that cleave the peptide bond present between two amino acids and thus can be used for the deduction of proteins during chitin extraction from the waste produced by seafood industry. Proteases like papain, alcalase, pancreatin, etc. can be implemented for the isolation of chitin. The use of proteases allows minimal de-acetylation and depolymerisation during the segregation of chitin. Protein accessibility to the proteases can be increased by doing demineralization step before the deproteination. Unrefined and purified enzymes both can be used for the purpose of deproteination. The use of crude enzymes from fish can bring the cost of extracting chitin down. The study with proteases on demineralised shrimp waste was done and it was found that the chitin extracted was satisfactorily pure (Rao and Sharma, 1997). Biological processes are environmentally and economically advantageous but efficiency of chemical deproteination is still higher. Around 5 - 10 % of protein remains bound to chitin even after treating with different enzymes. Therefore, additional NaOH treatment is needed for complete removal of protein attached to chitin.
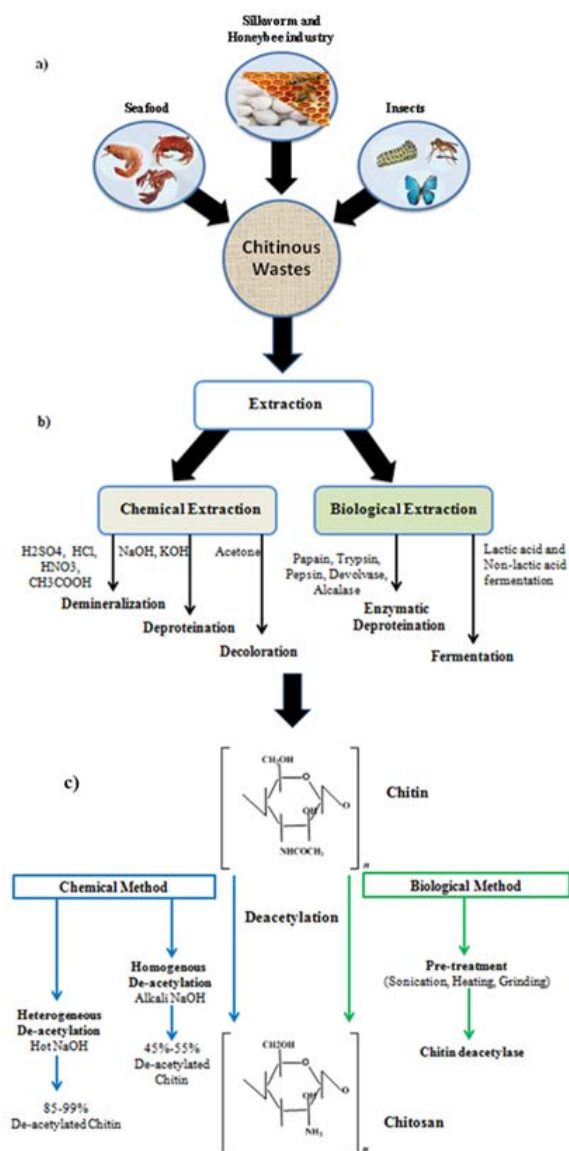
(b) Fermentation

The cost of enzymes can be significantly high, especially when purified enzymes are being used. This demands an alternative method by using microbes which can be more economical and efficient due to modification possibilities in microbial population. The selection of microbes is based on the fermentation which can be single or two-stage, successive fermentation or co-fermentation. (Arbia et al., 2013) Microbial fermentation for production of deproteination enzymes is divided into two broad types: lactic acid and non-lactic acid fermentation.

Lactic acid fermentation - Crustacean shells in presence of *Lactobacillus sp.* are generally used for this kind of fermentation. Lactic acid produced during this process by feeding on glucose further decreases the pH and does not allow any spoilage bacteria to grow. *Lactobacillus sp.* produces lactic acid along with a variety of proteases. The lactic acid fermentation's productivity relies on various components like microbial composition and quantity of inoculum, pH of the system during the process, temperature, time, carbon source, and its concentration, etc. (Prameela et al., 2010)

Non-lactic acid fermentation - Bacterial types generally used for this kind of fermentation are *Bacillus sp.*, *Aspergillus sp.*, etc. (Mahmoud et al., 2007; Sini et al., 2007) Different physico-chemical conditions are known to be affecting this fermentation and thereby deproteinization and demineralization proficiency. Ghorbel-Bellaaj et al. (2012) isolated proteases from *P. aeruginosa* and found that enzyme-substrate ratio and varying reaction time could influence the deproteinization efficacy of protease.

4.2. Silkworm industry

The chrysalides of the silkworm can be used for extraction chitinous materials two distinct methods post lyophilization. One of the methods is by using a closed reactor made of Teflon. The alternative method utilizes an open system of heating plate. HCl is added to dried chrysalides for eradication of catechols like Mg, Ca, etc. Filtration is done to isolate the residue which is then washed with deionized water to level the pH of the solution. These residues are then treated with NaOH to erase any trace of proteins. The treatment of the chrysalides is performed at higher temperature because of the presence of fat contents. If this reaction is performed at room temperature, it leads to saponification which makes the filtration process practically impossible. Reaction yield has been found out to be higher in an open reactor along with impurities, whereas chitin obtained from the closed reactor is reported to be almost free from any form of impurity, but with reduced overall yield (Paulino et al., 2006).

4.3. Honeybee industry

Dry dead bees were isolated and suspended in a specific amount of water w.r.t the bees. NaOH was put into the solution at high temperature to hydrolyze the protein present in the sample mixture. The solid component obtained is filtered followed by discoloration using H_2O_2. (Nemtsev et al., 2004)

4.4. Insect and pests

Various insects and pests can be used as source for isolation of chintinous materials. Isolation process involves starvation of insects/pests for 48 hours to remove any kind of food material present in the gut. Further they are cleaned with water and killed by freezing. These frozen corpses are then thawed and dried at 50 °C for next two days. These dried corpses are then crushed to a powder to store at 4 °C in airtight containers. This powder is then treated with HCl for demineralization, followed by rinsing with water to attain a neutral pH. The demineralized chitin is then treated with NaOH to eliminate the protein which is bound to chitin. Decolorisation is done by the use of potassium permanganate. (Chang et al., 2001; Liu et al., 2012)

5. Chitosan Production from Chitin

Chitin can be transformed into chitosan using either chemical or biological methods (Fig. 2c). Due to the requirement of mass production and procedures, chemical method is the one which is preferable, but high quality and environmental suitability makes biological method a better choice. (Tokuyasu et al., 2000; Philibert et al., 2017)

5.1. Chemical method

In chemical method of chitosan production from chitin, either acid or alkalis can be used for the process of de-acetylation but acid sensitivity of glycosidic bonds make alkalis more preferable (Hajji et al., 2014). De-acetylation of chitin can be generally classified into heterogeneous and homogenous de-acetylation processes. In heterogeneous de-acetylation, hot NaOH is added to the chitin for few hours and an insoluble substrate, chitosan, is obtained. It is generally obtained in the form of around 85 - 99 % of de-acetylated chitin. In the homogenous de-acetylation, chitin is treated with

alkali NaOH for three hours at 25 °C. After this, chitin is put in 0 °C which is achieved with the help of crushed ice. Chitosan obtained with the help of this process is 45 % - 55 % de-acetylated chitin. If this process is continued for around 580 hours, then it leads to formation with 90 % degree of de-acetylation. The acyl group present in chitosan are generally homogenously dispersed. (Aiba, 1991)

Under heterogeneous conditions, de-acetylation reactions lead to bumpy division of *D-glucosamine* and *N-acetyl glucosamine* in the polymer. Because of this, degree of aggregation, solubility differs in aqueous solution leading to change in the characteristics. Additionally, degree of acetylation, molecular weight, etc. can change due to changes during the chitosan preparation (Berger et al., 2005). Temperature and processing time are two of the most important factors that affect the degree of acetylation and molecular weight of produced chitosan (Rege and Block, 1999). Molecular weight and de-acetylation of chitosan are affected by concentration of NaOH too. (Tsaih and Chen, 2003)

5.2. Enzymatic method of converting chitin to chitosan

The enzymatic method for genesis of chitosan from chitin is done using enzyme chitin deacetylase. It hydrolyses the acetoamido present in *N-acetyl glucosamine* units of chitin and produces acetic acid and glucosamine units. It is a member of carbohydrate esterase. Different types of chitin deacetylase were isolated from different bacteria (*V. cholera*), fungi (*A. niger*, *M. racemosus*), insects (*D. melanogaster*, *Apismellifera*), and enzymatic deacetylation and then studied. Anyhow, chitin deacetylase is perceived to be not as successful on innate chitin which is insoluble and crystalline in environment. To increase the access of acetyl group to chitin deacetylase, pre-treatment is done which includes sonication, heating, grinding, etc. (Zhao et al., 2010)

6. Properties of Chitosan

The individual polysaccharides chains of chitosan (Table 1) have monomers which exhibit chiral properties with three crystal types including α, β, and γ, where *α-crystal* type is the most abundant in natural chitosan sources (Xia, 2003). The reactive functional groups present on monosaccharide unit are an amino/acetamido group, and hydroxyl groups (primary and secondary). The structural, physico-chemical, intra- and inter-molecular hydrogen bonds generation ability of chitosan have been correlated to these reactive functional groups. The amino group reacting with aldehyde or amide derivatives of acylating reagents allows imine formation

due to its nucleophilic properties. Cationic properties of polymer are demonstrated by its ability to generate salts and its correlation with chelation, flocculation, and biological functions. The dispersal of acetyl groups along the polysaccharide chain is responsible for solubility, as well as H-bonds inter-chain interaction and acetyl group hydrophobic nature of chitosan (Younes et al., 2014)

Table 1
Mechanical and thermal properties of chitosan (Martel-Estrada et al., 2015)

S.No	Property (Unit)	Value
1	Elastic Modules (KPa)	3,790
2	Comperession Strength (KPa)	589
3	Molecular Weight (Daltons)	3,800 and 20,000
4	Deformation at Maximum Strength (%)	22.9
5	Decomposition Range (℃)	232 to 326
6	Maximum degradation rate (Tmax) (℃)	271.35 ℃

Higher viscosity of chitosan in solution form is attributed to its high molecular weight (Martel-Estrada et al., 2015). Chitosan in its polymeric form is soluble in acidic conditions, but solubility decreases in solutions of pH values above 6.3. Solubility and low viscosity in oligomeric form of chitosan is observed at neutral pH (Hirano et al., 2002; Zhang et al., 2010). Chitosan in both polymeric and oligomeric form carry positive charges, which facilitates its association with negatively charged surfaces. Several biomedical applications are possible due to this chitosan-surface binding (Kurita, 1998). Different structures of chitosan exhibit different biological properties developed through chemical modification and enzymatic hydrolysis for various prospective applications.

6.1. Biological Properties of Chitosan

Chitosan is most frequently used natural polymers while molecular weight and degree of deacetylation acts as determining factor for its properties. Molecular weight and degree of acetylation are responsible for various properties of chitosan.

6.1.1. Biocompatibility of Chitosan

Chitosan is known to have low toxicity profile in comparison to other polysaccharides. The biocompatibility and toxicity of any material depend on its ability to be biologically degraded and resultant degradation products, respectively. Chitosan degradation leads to production of non-toxic products which are easily integrated in metabolic pathways or secreted with no body accumulation/retention issues.

6.1.2. Antimicrobial Agent

Chitosan and its derivatives have wide spectrum

antimicrobial properties which include activity against filamentous fungi, yeasts, and bacteria. The mode of action of chitosan involves interaction and subsequent disruption of cell membrane of microbe (Fradet et al., 1986; Lou et al., 2011; Mellegård et al., 2011; Costa et al., 2012; Lee and Je, 2013; Younes et al., 2014). Amine protonation of chitosan at certain pH leads to interaction of the positively charged ammonia (NH^{3+}) groups with cell wall causing hydrophilic and charge density changes in the cell surface. This cell permeability alteration causes the cytoplasmic constituent's leakage and ultimately death of the cell (Helander et al., 2001; Chung et al., 2003; Chung et al., 2004).

6.1.3. Hemostatic activity

Hemostasis is property of maintaining a balance of coagulation, complementary, and fibrinolytic pathways along various components of blood cells. The hemostatic activity of chitosan differ from normal mechanism observed in severe anti-coagulating conditions and abnormal activity of platelets through *in vitro* studies (Fradet et al., 1986; Subar and Klokkevold, 1992; Mankad and Odispoti, 2001). The cell membrane of blood cells (negative charge) and chitosan (positive charge) electrostatic interaction leads to agglutination of blood cells (Brandenberg et al., 1984; Rao and Sharma, 1997; Chou et al., 2003). Chitosan polymer chains are thought to be cross linked with these cells.

6.1.4. Antioxidant activity

Metabolic processes, exogenous factors and agents produce oxygen radical precursor of various degenerative diseases and aging process. These oxygen radicals react with biomolecules (proteins, carbohydrates, lipids, and DNA) leading to cell damage. The role of antioxidants is to eradicate free radicals and prevent oxidative stress in order to protect the biological system (Brasselet et al., 2019). The scavenging effect of chitosan-based compounds can be characteristic of their capacity to extract free radicals. Structural properties of chitosan, mainly amino and hydroxyl groups attached to the pyranose ring, can be attributed to radical scavenging property (Obara et al., 2003). The decline in intermolecular hydrogen bond leads to hydroxyl and amino group activation involved in radical scavenging process. Chitosan compounds work as radical locking along free alcohol and amino groups for free radical's entrapment and undergoing copulation reactions.

6.1.5. Anti-tumor activity

The antitumor property of chitosan and its derivatives is generally observed by low-molecular-weight hydrophilic chitosan, whereas oligo-chitosan is found to act as immunomodulator. Enhanced cytotoxic activities of chito-oligosaccharides against tumor are also associated with initiation of lymphocyte factor and increase in T-cell proliferation. High molecular weight of chitosan can induce death in cancer cells by neutralizing their strong charges and reducing viability in cancer cells (Rajasree and Rahate, 2013).

6.1.6. Anti-inflammatory activity

Treatment of inflammation by non-steroidal anti-inflammatory drugs acts through *cyclooxygenase-2* inhibition which leads to prostanoids production. The anti-inflammatory action mechanism of chitosan relies on ability to encourage inflammatory cells migration to healing site via *cyclooxygenase-2* inhibition pathway. As a result, huge collection of post inflammatory products and growth factors produced at healing site are reported (Zhang et al., 2010; Rajasree and Rahate, 2013).

6.1.7. Biodegradability of Chitosan

Chitosan is a polysaccharide with glycosidic bonds which are degraded by several proteases, lysozyme and chitinase enzymes (Kurita et al., 1998). Formation of non-toxic oligosaccharide of different length is noted as a result of chitosan biodegradation. (Kurita, 1998; Helander et al., 2001)

7. Modifications of Chitosan

Amine group along with primary and secondary hydroxyl represent reactive groups of chitosan where most of the modifications are carried out. Availability of these reactive groups is to mediate chemical properties and induce deviation in physical properties of chitin and chitosan as well. Modification, which could be introduction of a new group by replacing or adding to the reactive groups on chitosan backbone would generally not amend the basic structure of chitosan but improve or modify the exciting properties (Table 2). Modifications of chitosan can be categorized based on nature of functional groups introduced.

7.1. Chemical Modification of Chitosan

7.1.1. Quarternised Chitosan (QC)

The quarternisation of chitosan can be performed by introducing quaternary ammonium in chitosan chain utilizing various procedures including halo-alkylation (Rinaudo et al 1996; Rinaudo et al., 2005; Ortona et al., 2008; Luan et al., 2018). The positive charge on the chain and introduced alkyl moiety effect are responsible for improved properties of QC in comparison to the chitosan. The quaternary salts solubility is high in both acid and basic conditions rather than chitosan itself which has low solubility at physiological pH. The quaternary derivatives are mainly utilized due to their enhanced antibacterial potential where negatively charged cell

Table 2
List of modified chitosan with introduced group and improved properties (Rajasree and Rahate, 2013)

S. No	Modified chitosan	Introduced groups	Improved properties
1	Quarternised chitosan	Quaternary ammonium	Antimicrobial activity, solubility in water
2	Acyl chitosan	Carbonyl and keto groups	Hydrophobic properties
3	Thiolated chitosan	Thiol group	Muco-adhesive properties
4	Sulfated chitosan	Sulfate groups	Amphoteric properties
5	Sugar modified chitosan	Hydrophilic sugar moiety	Water solubility
6	Heterocyclic chitosan	Heterocyclic ring	Antibacterial activity
7	Cross-linked chitosan	Linking the chains together in 3D network	Improves strength and stability
8	Chitooligosaccharide (COS)	Reducing the molecular weight	Decreasing viscosity issues
9	Low molecular weight (LMW) chitosan	Reducing the molecular weight	Higher DPS
10	Phosphorylated chitosan	Phosphate	Osteo-conduction

surface acts as a favourable platform (Xia, 2003; Mohamed et al., 2013; Rajasree and Rahate, 2013).

7.1.2. *N-alkyl* chitosan

The *N-alkyl* derivative of chitosan are synthesized by reacting amino groups of chitosan with aldehydes and ketones which undergo Schiff reaction and subsequently reduce with reducing agents sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaBH_3CN$). Alkyl chain introduction on a modified chitosan (N-methylene phosphonic chitosan) enable both hydrophobic and hydrophilic branches to be present in its structure (Zhang and Hirano, 1995; Zong et al., 2000; Mourya and Inamdar, 2008). Introduction of alkyl group in *N-lauryl-N-methylene* phosphonic chitosan has been found to hinder hydrogen bond formation while increasing its hydrophobic properties. The amphiphilic properties increase along with surface activity which enhances its ability to act as surfactants, and it can find application in pharmaceutical and cosmetic field (Rinaudo, 2006; Zhang et al., 2010).

7.1.3. Carboxy Alkyl chitosan

Carboxy alkyl chitosan are synthesized by the reaction of chitosan with monohalocarboxylic acid in order to enhance chitosan's efficiency as absorption enhancer. This is achieved by overcoming the inadequate solubility. Absorption enhancement of carboxy alkylated derivatives at neutral pH values which are quite like those found in the intestinal tract and they can be utilized in pharmaceutical applications especially in drug-delivering systems, both controlled and sustained.

7.1.4. Acyl Chitosan

N-acyl chitosan is produced by the reaction of acyl halide or acid anhydride with chitosan in order to increase its hydrophobic character and to introduce some changes in structural features. Chitosan solubility is reported to be influenced by degree of acyl substitution and length of

the chain. The solubility in water decreases with rise in chain length and degree of acyl substitution (Chen et al., 2015). The modified chitosan will experience reduced hydration and increased hydrophobic interactions which also helps in network stabilization.

7.1.5. Thiolated chitosan

The thiolated chitosan are synthesized by the reaction of various reagents with chitosan; such as glutathione (GSH) and thioglycollic acid (TGA) for introduction of thiol group. Formation of disulphide bonds by oxidation process of the immobilized thiol groups result in thiolated chitosan. Excellent in situ gelling properties are demonstrated by thiolated chitosan. Chitosan with thiol group conjugation have been found to enhance the muco-adhesive and controlled drug releasing properties (Rajasree and Rahate, 2013).

7.1.6. Sulfated chitosan

Synthesis of sulphated chitosan occurs through site specific modification of hydroxyl and amino groups with sulfate group using sulfating agents. Sulfated chitosan have structural similarities with heparin, which is a known blood anticoagulant. It is also known to give rise to antiviral, anticoagulant, and anti-sclerotic activities to chitosan (Rajasree and Rahate, 2013; Al Ghamdi et al., 2017). This modified chitosan exhibits anti-obesity activities by inhibition of anti-adipogenesis and blocking of malignant melanoma cell adhesion in human. Sulfated chitosan with anticoagulant, antimicrobial, and osteogenic activities is reported as a water-soluble anionic material.

7.1.7. Phosphorylated chitosan

Phosphorylation of chitosan occurs through reaction between methane sulfonate and phosphorous pentoxide acid. Cation-exchange properties of phosphate functional group can be attributed for various orthopaedic applications. This ensures that calcium ions associated

with phosphate groups lead to initiation of calcium phosphate layer formation on polymeric implants. This can further facilitate the osteo-conduction of chitosan based implants (Qin et al., 2012; Rajasree and Rahate, 2013).

7.1.8. Cross-Linked Chitosan

Cross-linking of chitosan involves various chemical agents (glutaraldehyde, formaldehyde, tripolyphosphate, and polyaspartic acid sodium salt) for the purpose of linking the different chains together to create a multi-dimensional macromolecular chitosan. Resultant size and degradation rate of chitosan particles depend upon cross-linking preparation methods and chitosan employed in the process. Chitosan cross-linking is generally performed in two different ways, first by reacting with aldehyde compounds, such as glyoxal, formalin, or glutaraldehyde by formation of covalent bonds in acid or basic medium and secondly by the polyanions through formation of inter- and intramolecular cross-linkages (Mati-Baouche et al., 2014; Pellá et al., 2018). This could also be achieved by step wise utilization of ionic tail compounds covalently linked to enhance the mechanical properties in hydrogel formation (Rinaudo, 2006; Jătariu et al., 2013).

7.1.9. Chito-Oligosaccharide

High viscosity of chitosan due to its high molecular weight hinders its application at the commercial scale. This problem is solved by molecular weight reduction of chitosan through chemical or enzymatic hydrolysis procedures (Hsu et al., 2002; Cabrera and Van Cutsem, 2005; Delattre and Vijayalakshmi, 2009; Kasaai et al., 2013). The oligosaccharides are found to exhibit less viscous and more hydrophilic nature due to their reduced chain length. Chito-oligosaccharides with low degrees of polymerization (DPs) e.g. 20 and average molecular weight approximately 3,900 Da fall in category of less viscous and more hydrophilic chitosan. Many favourable biological effects such as infections protection, arthritis control, lowering blood cholesterol, antitumor properties, and calcium uptake improvement are among chito-oligosaccharide health benefits (Lodhi et al., 2014).

7.1.10. Low Molecular Weight (LMW) Chitosan

The Low Molecular Weight (LMW) Chitosan is also oligosaccharide with a degree of polymerization in the range between 11 up to 30. It is synthesized by depolymerization of chitosan through hydrolysis by acid and degradation by enzymes (Obaidat et al., 2010).

7.2. Enzymatic Modification of Chitosan

The environment issues surrounding reagents utilized in chemical modification initiated search for alternate

methods and reagents for chitosan modification. Enzymes offer an alternate way of chitosan modification without harming the environment and humans in the process.

7.2.1. Arginine functionalized chitosan

In Arginine-functionalized chitosan, arginine groups are substituted on chitosan backbone to different degrees. High solubility of functionalized chitosan in water is due to high pKa value of the side chain guanidinium of arginine (pKa = 12.48). Chitosan functionalization with arginine is carried out through reaction with *1-ethyl-3-(3-dimethylaminopropyl)* carbodiimide hydrochloride and its derivatives which generate different functionalized chitosan. Antibacterial activities of chitosan were restrained by reduced solubility above pH 6.5 but with improved solubility, arginine-functionalized chitosan improves antibacterial properties (Tang et al., 2010).

7.2.2. Chitosan thiosemicarbazones

Thiosemicarbazone chitosan compounds are synthesized through condensation reaction with aldehydes or ketones such as phenyl-aldehyde through different methods (Qin et al., 2012). Thiosemicarbazone chitosan derivatives are well known for its antiviral, antitumor, antibacterial, and antifungal activities. Thiosemicarbazone chitosan compounds are also reported for their antifungal activity against common crop pathogen like fungi. During structure-activity relationship analysis, it was observed that thiosemicarbazone, and not the produced Schiff base, is responsible for antifungal activity of the compounds. Aromatic ring substituent present in chitosan compounds has specific effect on its antifungal properties. (Qin et al., 2012)

8. Characterization of Extracted chitinous materials and their modified forms

The extracted chitinous materials can be characterized using range of methods (Flow chart 1).

8.1. Physical features

8.1.1. Solubility

Chitin is generally insoluble in water, while 50 % uniformly deacetylated chitin improves its water solubility and partial solubility in aqueous HCl is also possible as *β-chitin* is transformed into non-reactive α-chitin form. (Pillai et al., 2009) Linear chitin or chitosan is comparatively less soluble than the branched chitin or chitosan. Additionally, introduction of sugar derivatives in branched chain increases the solubility of the molecule. (Kurita, 1998) The solubility of chitosan can be obtained by mixing a weighed quantity of sample in
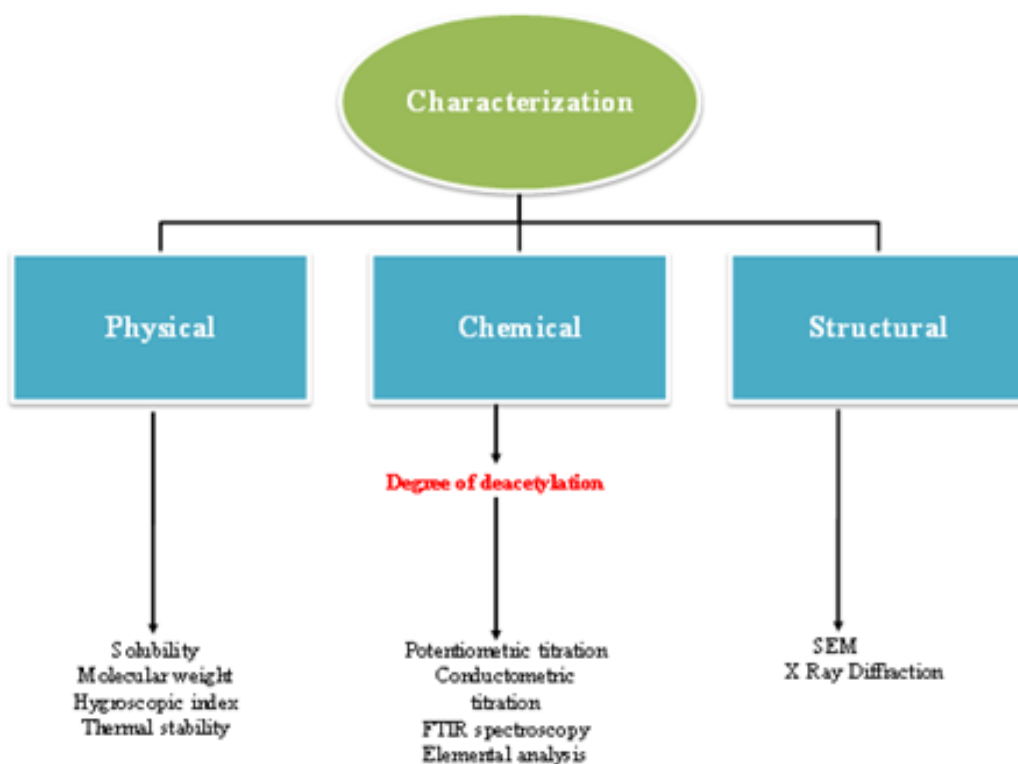
Figure 3. Various parameters included in characterization of chitin and chitosan. Different techniques involved in characterization of chitin and chitosan

measured volume of water. After a certain period, sample is filtered and undissolved part is collected, dried and weighed. From this the amount of sample solubilized could be measured. (Desbrières et al., 1996; de Queiroz Antonino et al., 2017)

8.1.2. High performance size exclusion chromatography

Size exclusion chromatography helps in determining molecular weight of samples and is extensively used for determining the mw of chitosan extracted from natural sources. The eluent that could be used is 0.2 M acetic acid / 0.1 M sodium acetate. Before running the sample in sephacryl columns, standard is plotted by using various molecular weight dextran samples. Later the samples are run in the column and eluents are analyzed using a RI detector (Kittur et al., 2002). In case of the absence of detector, the eluents could be analyzed using a colorimetric method. Here, the sample is treated with concentrated H_2SO_4 which breaks the glycosidic bond and converts pentose into furfural and hexose into hydroxymethyl furfural. When this product is treated with phenol, yellow-gold color is formed which could be estimated at 480 nm for pentose and 490 nm for hexose. From this absorbance, the concentration could be determined by plotting the values against the respective standard curves. (Nielsen, 2010)

8.1.3. Hygroscopic Index

Hygroscopic index of chitosan is the measure of its ability to absorb moisture and eventual trapping. This could be measured by keeping a weighed amount of dry sample in room conditions for 7 - 8 days after which the weight is again measured. Increase in weight shows that the sample has trapped moisture from the surrounding. Branched derivatives of chitin and chitosan tend to have a high hygroscopic index than the linear derivative of these molecules. (Kurita, 1998)

8.1.4. Thermal stability

Thermal stability of chitosan is generally analyzed by thermogravimetric approach. Here, the sample is heated at a certain temperature range and loss of weight is measured as a function of thermal stability. Pure chitin and chitosan show the initial peak at 90 - 99 °C which is due to the water molecules trapped in these samples. The second peak for chitosan appears at 303 °C, whereas for chitin it appears at 373 °C. Based on the data, it can be concluded that chitin is thermally more stable than chitosan. But few modifications of chitin or chitosan can alter their stability (Abdou et al., 2008).

8.1.5. Degree of Deacetylation

8.1.5.1. Potentiometric titration

As discussed in previous sections, deacetylation and its extent are important structural changes which govern many of the physico-chemical and biological properties of extracted chitosan, and it is important to evaluate degree of deacetylation after extraction and modification processes. Potentiometric titration is one of the important methods to evaluate degree of deacetylations. The experimental protocol involves dissolving of 0.5 g of sample in 25 ml of 0.1 M HCl whose ionic strength is maintained at 0.1 using KCl. During the next step, the sample is titrated against 0.05 M NaOH and a graph is plotted where Y-axis depicts pH and X-axis depict volume of NaOH. The graph will have two inflection corresponding to which the volume of NaOH is noted. With these values, DD % can be calculated using the formula (Abdou et al., 2008):

$$DD\% = \frac{1 - 161 \cdot Q}{1 + 42 \cdot Q} \ and \ Q = \frac{N \cdot \Delta V}{M} \qquad (2)$$

where: $DD\%$ is Degree of deacetylation, ΔV is Difference in NaOH volume between two points, N is NaOH Concentration, and M is dry weight of chitosan.

8.1.5.2. Conductometric titration

Conductometric titration is an indirect way of measuring the Degree of Deacetylation (DD) in chitosan. In this technique, the amine group of chitosan is saturated with excess of concentrated acid and the remaining unreacted acid is measured by titrating it against NaOH. The titration is monitored with the help of conductometer which senses the ion concentration as a function of conductivity. A 0.5 % amount of the sample is dissolved in 0.05 mol/L of HCl. After 18 hrs of mixing in shaker, 100 ml of water is added to the above mixture and titrated against NaOH (Dos Santos et al., 2009).

The DD can be calculated using the following formula:

$$x = 20{,}319.23 \cdot \frac{C \cdot \Delta V}{42.0367 \cdot C \cdot \Delta V + W \cdot m} \qquad (3)$$

where: x - Degree of Deacetylation; C - NaOH concentration; ΔV - Change in volume of NaOH, W - Solid mass fraction of Chitin.

8.1.5.3. Infrared Spectroscopy

FTIR is one of the sensitive techniques for the detection of DD. The most widely used procedure is pelleting out

1mg of sample with 100 mg of KBr such that the thickness is around 0.25 mm. Then the pellets are placed in the FTIR setup and scanned from 400 cm^{-1} to 4,000 cm^{-1} in absorbance mode. After scanning, DD % can be calculated using the formula as described in the paper (Brugnerotto et al., 2001):

$$DD\% = \frac{31.92 \cdot A_{1320}}{A_{1420}} - 12.20 \qquad (4)$$

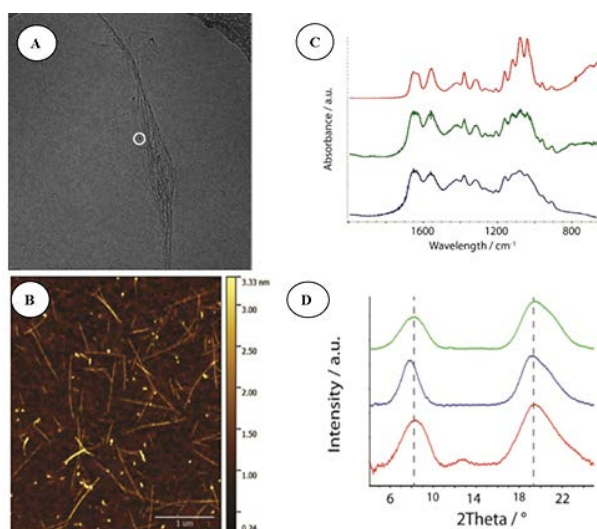where: A_{1320} - Absorbance at 1320 cm^{-1}; A_{1420} - Absorbance at 1420 cm^{-1}.

Figure 4. Characterisation of Chitin Nanofibril (CNF): (A) TEM image of β CNF; (B) AFM of β CNF; (C) FTIR spectra of β CNF; (D) XRD of β-Chitin (Blue) and β CNF (Red). (Montroni et al., 2019)

8.1.6. Elemental analysis

Using an elemental analyzer, the composition of individual atoms C, H, and N could be analyzed. Measurement of elemental composition pure chitosan with isolated chitosan determines whether the isolated sample has any other functional groups. (Yen et al., 2009) From elemental analysis data, DD % can also be calculated using the formula (Abdou et al., 2008):

$$DD\% = \frac{\left(6.857 - \frac{C}{N}\right)}{1.7143} \qquad (5)$$

where: C - elemental composition of Carbon, N - elemental composition of Nitrogen.

8.1.7. Structural and Topographical analysis

Even though chitin isolated from any source has the

same chemical composition, the topographical features may vary depending on the environment in which they were present. This makes it important to study their topographical features.

8.1.7.1. Scanning electron microscopy (SEM)

SEM is generally done to study the surface topography of the samples. For chitosan samples, SEM is performed
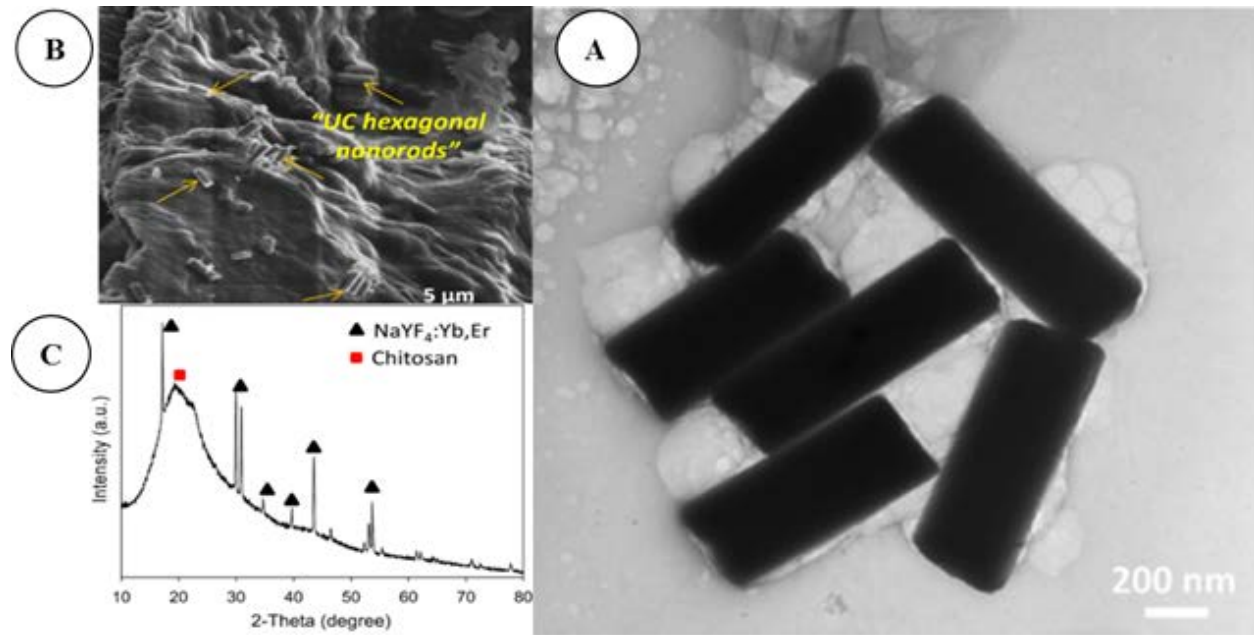

Figure 5. Characterisation of NaYF$_4$: Yb,Er /chitosan composit; (A) TEM image of aqueous dispersion; (B) SEM image of aerogel composite; (C) XRD patternof aerogel composite. (Duong et al., 2018)
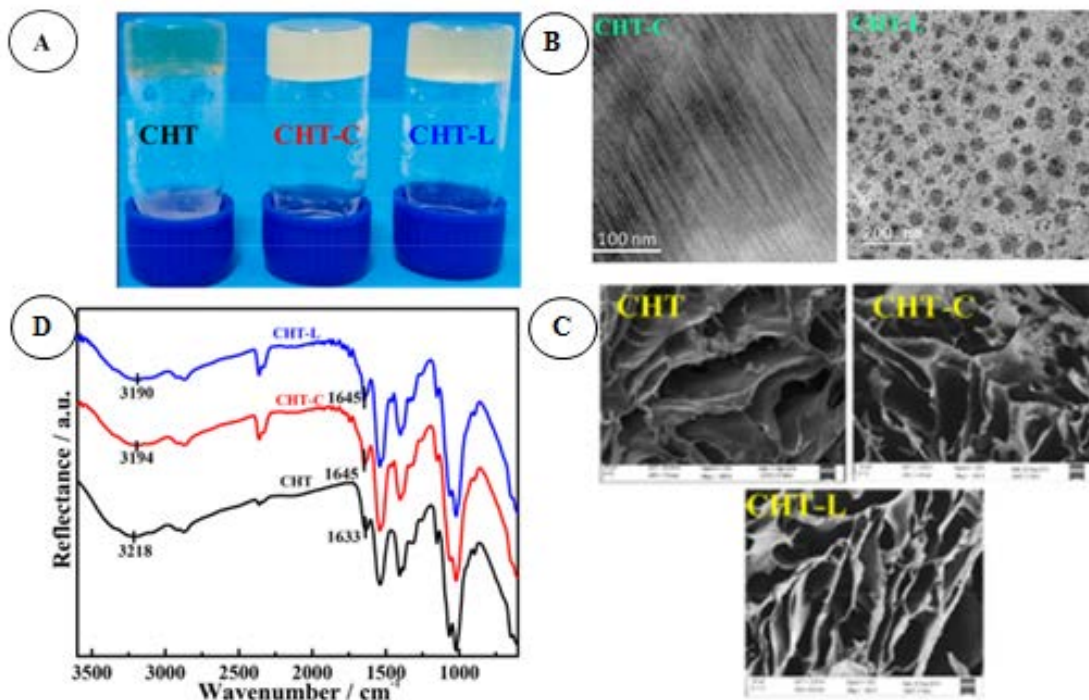

Figure 6. Characterisation of chitosan (CHT) nanohybrid in hydrogel and scaffold for bone regeneration. Nanohybrids developed with two nanofillers 30B nanoclay (CHT-C) and with layer double hydroxide (CHT-L); (A) Photographic image of CHT, CHT-C and CHT-L; (B) TEM image of CHT-C and CHT-L; (C) SEM image of CHT, CHT-C and CHT-L; (D) XRD pattern of CHT, CHT-C and CHT-L (Mahanta et al., 2019)

by making them electrically conductive. As carbon tapes are adhesive and conductive material, chitosan samples are coated with carbon tapes with additional gold sputtering, if required before performing SEM. These changes help in imaging the sample with good contrast.

8.1.7.2. Transmission Electron Microscopy (TEM)

TEM offers better spatial resolution than SEM. TEM analyzes inner structure and features, such as size, morphology on atomic scale, and surface properties. The chitin and chitosan samples used need to be thin (< 100 nm), in order to facilitate the transmission of electron beam.

8.1.7.3. Atomic Force Microscopy (AFM)

AFM offers statistical data, including size, surface area, and volume distributions. AFM helps in obtaining high resolution 3D image of chitosan. Through tip movement without any coating or damaging the sample, AFM measures the height of the chitin and chitosan samples. AFM can work effortlessly in room temperature without any vacuum environment.

8.1.7.4. X ray diffractometry

The atomic arrangements of any crystalline material including chitosan can be deduced using XRD method. By dividing the area of crystalline peaks with the total area under curve, the relative crystallinity of the sample could be analyzed. (Abdou et al., 2008) Studies show that pure chitin is more crystalline than chitosan and, hence, the stability of chitin is comparatively high. The X-ray diffractograms of the chitin and chitosan samples show two sharp diffraction peaks at 2θ of about 9 - 9.5 ° and 10 - 10.5 ° from (020) planes and 19 - 19.5 ° and 20 - 20.5 ° from (110) planes of crystalline unit cells. (Rout, 2001)

9. Biomedical Applications of Chitosan

9.1. Tissue engineering

Tissue engineering (Figure 7) is a modern advancement of biology which promises to replace, regain, or support biological functions of any damaged tissue. (Qazi et al., 2014; Ahmed et al., 2018) Different natural or artificial biomaterials have been explored for various tissue engineering applications in the form of hydrogels, composites, and bioceramics. Among naturally occurring biopolymers, chitosan is of great significance for tissue engineering applications owing to its sustainability, biocompatibility, and biodegradability. Another property of chitosan which makes it suitable for tissue

engineering is that, it is easily processable in various forms like nanofibres, hydrogels, membranes, nanoparticles, scaffolds etc. (Kim et al., 2008) These different forms have their different purposes in field of tissue engineering. Some of the major areas of tissue engineering include tissue engineering, liver tissue engineering, cardiac tissue engineering, and nerve tissue engineering, where chitosan based scaffolds are widely used (Artan et al., 2010; Park and Kim, 2010; Tan et al., 2012; Kulkarni et al., 2017).
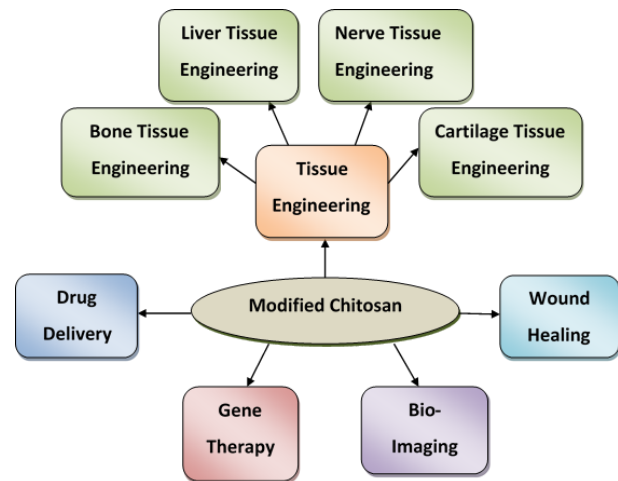

Figure 7. Block diagram showing various areas of chitosan utility. The modified chitosan with its improved properties fulfills the requirement of various biomedical applications

9.1.1. Bone tissue engineering

Bone tissue engineering is one of the most common and widely explored field of tissue engineering (Figure 8), which is concerned with creating artificial bone implants to support or replace the biological function of a damaged bone. In some clinical conditions like osteoporosis, arthritis, bone infections, or in some skeletal defects due to trauma, craniofacial surgeries etc. severe bone damages occur which cannot heal by themselves. In this condition artificial bone implants or bone tissue engineering strategies become important to regain the original functionality. The main objective of bone tissue engineering is to design a 3D scaffold that mimics ECM like environment and provide support to the newly growing bone tissue (Li et al., 2005; Saravanan et al., 2016). The scaffolds for bone tissue engineering are designed in such a manner that they promote adhesion, survival, and migration of osteogenic cells. The scaffold must also have all the physical, as well as biological factors i.e. growth factors, required for proliferation of osteoblasts. Simply put, a scaffold is devised with multiple techniques to enhance the growth of bone tissue. The modified chitosan is widely used as biomaterial for scaffolds fabrication. Some recent research studies suggest that chitosan nanofibre based 3D scaffolds are

very promising for bone tissue engineering applications (Logith-Kumar et al., 2016) because, fibrous structures provide larger surface area along with high porosity and they also have desired mechanical and physical properties (Amini et al., 2012). All these factors improve the regeneration capacity of the tissue. In some other studies combination of chitosan and bioactive glass nanoparticles are used to prepare novel bone regenerating membrane. These membranes are found to have high regenerative potential (Mota et al., 2012). Hu et al. (2017) fabricated hybrid scaffolds composed of natural polysaccharides in-situ strategy. Electrospining technique is generally used for the generation of nanofibers from chitosan, which can be used along with gel-based systems like gelatin to gain desired physical properties of the scaffolds.

9.1.2. Nerve tissue engineering

Various natural and artificial materials have been used in tissue engineering field to achieve regeneration of any body tissue or organ. Some clinical conditions like diabetes mellitus induced neuropathy, nervous system diseases, peripheral nerve injury, sensory disorders etc. have great impacts on patient's health. Approximately three decades back the only solution for damage nerve repair was autograft. But insufficient donor source, tissue mismatch, graft rejection, and unsatisfactory response limited this autograft strategy for human subjects. In recent years tissue engineering approaches have allowed the use of biomaterial derived scaffolds for nerve regeneration. (Yi et al., 2019) As mentioned earlier, the basic idea behind using biopolymeric scaffold is to improvecell attachment, cell growth, and

neovascularization for growth of new tissues by providing 3D porous structure. These scaffolds are designed in such a way that it should mimic the real environment of peripheral nerve matrix and should be biodegradable and non-toxic. Various in-vitro, as well as in-vivo studies, have proved that chitosan-based fibers and membranes support the survival and growth of hippocampal neurons and Schwann cells, suggesting their use in nerve tissue engineering. A recent in vivo study concluded that suturing a chitosan-based nerve scaffold into the damaged sciatic nerve of a rat model could induce a notable motor and sensory functional recovery. Chitosan based nerve implants are also proved to recover a long-distance peripheral nerve defect in diabetic rat (Mohammadi et al., 2013). Gonzalez-Perez et al. (2015) evaluated regenerative capability of chitosan tubes compared to silicone tube nerve autografts to bridge critical 15 mm nerve gap. In their study, they found that chitosan tubes had 57 % success rate, whereas regeneration failed with silicone tubes. A chitosan/polyglycolic acid nerve conduit was found to bridge and repair 30 mm long sciatic nerve gap in canine model. These chitosan/polyglycolic acid scaffolds can also repair delayed long-term peripheral nerve injury. In another study, the fabrication of an aligned cryomatrix-filled nerve guidance channel was reported. These nerve guidance channels were fabricated by 3D printing along with nerve growth factors for nerve regeneration (Singh et al., 2018). All these studies ultimately suggest that an engineered chitosan-based nerve scaffold is much better option for nerve damage treatment because it provides all the beneficial outcomes of an autograft and excludes autograft related problems.
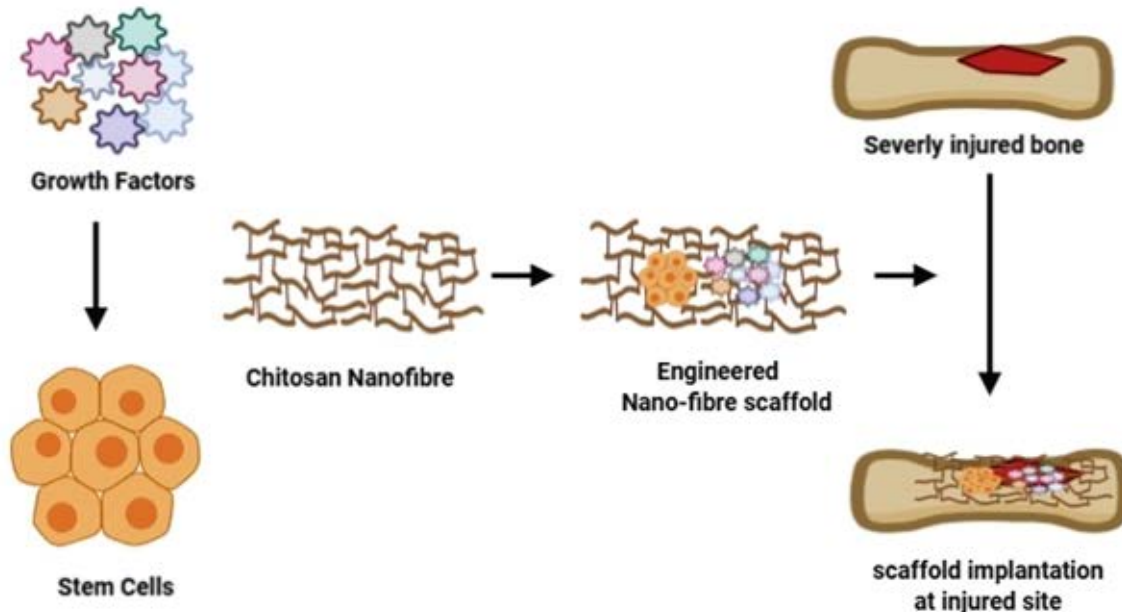

Figure 8. Schematic diagram showing bone tissue engineering strategy using chitosan nanofibre, growth factors and stem cell. The growth factors and stem cells are incorporated in the chitosan nanofibre scaffold to design nanofibre scaffold with combine benefits of better adhesion of scaffold as well as biological factors i.e. growth factors, required for proliferation of osteoblasts.

9.1.3. Cartilage tissue engineering

The treatment strategies for one of the most common age related disorder, osteoporosis, involves cartilage tissue engineering approaches. The basic idea behind this is quite similar to other branches of tissue engineering. The idea is to design a scaffold with a biomaterial incorporated with various factors that support adhesion and proliferation of cartilaginous cells at affected site and hence help the cartilage to regenerate. These scaffolds are designed in such a way that the cells adhere and increase their number, and these proliferating cells are in turn induced to show cartilaginous phenotype. The scaffold material should have some important properties like biocompatibility, elasticity, biodegradability, and non-immunogenic properties, i.e. degradation products of the material should not induce any immunogenic response in the body. By considering all these properties some naturally occurring biopolymers are suggested for this purpose that are proteinaceous in nature like collagen, keratin, fibroin, elastin, and polysaccharides such as chitosan, hyaluronan, and polyesters such as poly (hydroxybutyrate) (Alves da Silva et al., 2010). Among these materials, use of chitosan is preferred for scaffolds preparation since its structure is quite similar to many glycosaminoglycans which are found in articular cartilage (Kuo et al., 2015). In some of the recent studies, chitosan based hydrogels incorporated with TGF-β1 containing microsome have been found to be very efficient for cartilage regeneration therapy. Few years ago, infuseable chitosan hyaluronic acid hydrogel for cartilage tissue engineering was synthesized by Park et al. (2017). It was found that incorporation of Hyaluronic acid (HA) in hydrogels enhanced the proliferation and deposition of the cartilagenous extracellular matrix by the encapsulated chondrocytes. In another study, Tan et al. (2012), could synthesized infuseable biodegradable chitosan and HA based hydrogels by Schiff's base reaction of the amino groups of *N-succinyl* chitosan and the aldehyde groups of HA. The other properties of hydrogel like swelling capacity, porosity, gelation time, texture etc depended on *N-succinyl* chitosan and aldehyde HA ratio during the synthesis of the hydrogel. So, it should be considered during hydrogel preparation. In another study, tissue reconstruction efficiency of chitosan hydrogel scaffold for chondrocytes was tested in sheep models and it was revealed that chitosan hydrogels can repair cartilage defects in nearly 24 weeks (Hao et al, 2010).

9.1.4. Liver tissue engineering

In disease conditions like acute or chronic liver failure the only treatment option available is liver transplantation. But since availability of donors is very limited, the new tissue engineering based therapies are emerging. Liver tissue engineering approaches are used to regenerate damaged tissue and to replenish its original function. Structural resemblance of chitosan to glycosaminoglycans, makes it more suitable to use as a scaffold for hepatocyte culture. In some of the studies alginate-galactosylated chitosan scaffolds are proven to advance hepatocyte attachment. A basic need in liver tissue engineering is a perfect extracellular matrix for the hepatocytes to sustain high-level liver defined functions. Oxidized alginate covalently cross-linked with galactosylated chitosan is used for scaffolds preparation for liver tissue engineering. The swelling capacity and degradation rate depend on alginate concentration in chitosan scaffold. Hence alginate-chitosan ratio is very important during preparation. *In-vitro* biocompatibility research demonstrate that hepatocytes grow on scaffold with a distinctive spheroidal morphology which is further found very promising for liver tissue regeneration. It is also good for the improvement of liver defined functions and improvement of successful bioartificial liver devices through tissue engineering. There are two strategies, Exogenous and Endogeneous stem cell therapy, that can be followed for liver regeneration therapy An integrated hybrid poly(N-isopropylacrylamide)-chitosan cryogel based bioreactor was previously developed that showed better efficacy in terms of detoxification as well as synthetic functions than the conventional bioartificial liver device setup (Damania et al., 2017). Stem cell based therapies are also being used to achieve liver regeneration after injury. Two most common stem cell based therapies are endogenous stem cell therapy and exogenous stem cell therapy. (Yu et al., 2017)

9.2. Role of bio-extracted chitosan in wound healing

Wound healing is a complex process in which torn or injured site of skin and damaged tissue repair themselves. The overall process of wound healing involves a highly regulated pathway of biochemical reactions in order to repair the damage. In general, if wound follows the limited healing stages in a defined time period it is called **Acute wound** and if it takes too long to heal then it is considered as **Chronic wound**. Chronic wounds are difficult to heal and require treatment or wound dressing strategies in such a way that it provides a proper platform to accelerate the epithelialization, angiogenesis, and also reduce complications such as scar formation, discomfort, pain etc. Several studies have shown that moist local environment near the wound significantly enhances re-epithelialization.

9.2.1. Hydrogel based wound dressing material

Apart from various wound dressing materials available in the market the hydrogel-based systems are found more promising for wound management. Hydrogel systems can absorb high amount of water due to the presence of hydrophilic groups which results in gel formation. Since

hydrogels imbibe water and fluids, they eliminate the chances of wound desiccation. In some cases moist environment may delay the healing process, so the choice of dressing material depends on the type of wound.

Chitosan is found to be very promising for making hydrogel based wound dressing materials because of its superior properties like biocompatibility, biodegradability, antimicrobial activity, and the most important, optimum moisture absorption capacity. To achieve higher benefits, chitosan can be incorporated with polyvinyl alcohol (PVA), Sodium alginate, and Pluronics (polymers of polyethylene oxide). These are all hydrophilic, nontoxic, and FDA approved materials for biomedical applications. Several studies have proved that use of multiple polymers instead of a single polymer improves required mechanical and physical properties of hydrogel film. A recent study has revealed that hydrogel based wound healing materials prepared by combinations of different polymers show optimum swellability, flexibility, and crosslinking ability. These properties together accelerate wound healing by means of re-epithelialization (Park et al., 2009). In these properties, polymeric formulation of chitosan and pluronic are foundto be more suitable. The development of multifunctional collagen and chitosan hydrogel show enhanced wound healing and promote hemostatic ability. Different mass ratios are used to compare their antibacterial property apart from self-healing properties (Figure 9). (Ding et al., 2020)

9.2.2. Chitosan Nanofiber scaffold based wound dressing material

Nanofibrous wound dressing materials have many potential advantages over conventional dressings because of their higher surface area and microporous structure. These nanofibre based systems mimic a native extracellular matrix environment which attracts fibroblasts to the epidermal layer of wound sites. These fibroblasts secrete growth factors, cytokines, collagen, and ECM components which accelerate angiogenesis and re-epithelialization at wound site. This ECM like property occurs due to Chitosan, which has a structure similar to GAGs (glycosaminoglycans) of native ECM. Apart from these, microporous structures of chitosan materials absorb exudates from wound and allow proper ventilation at wound site for faster damage repairing. As discussed previously, some important properties of chitosan, such as biocompatibility, biodegradability, and the most important antimicrobial activity, make it more suitable for burn and wound healing. It is proven that nanofibers of chitosan accelerate the wound healing. Various hydrophilic polymers (PVA, PEO, PVP) are also used to prepare blend form of chitosan nanofibre scaffolds. The purpose of adding these hydrophilic polymers is to reduce viscosity and enhance electrospinability of the solution which is required to prepare nanofibers (Sweeney et al., 2014).
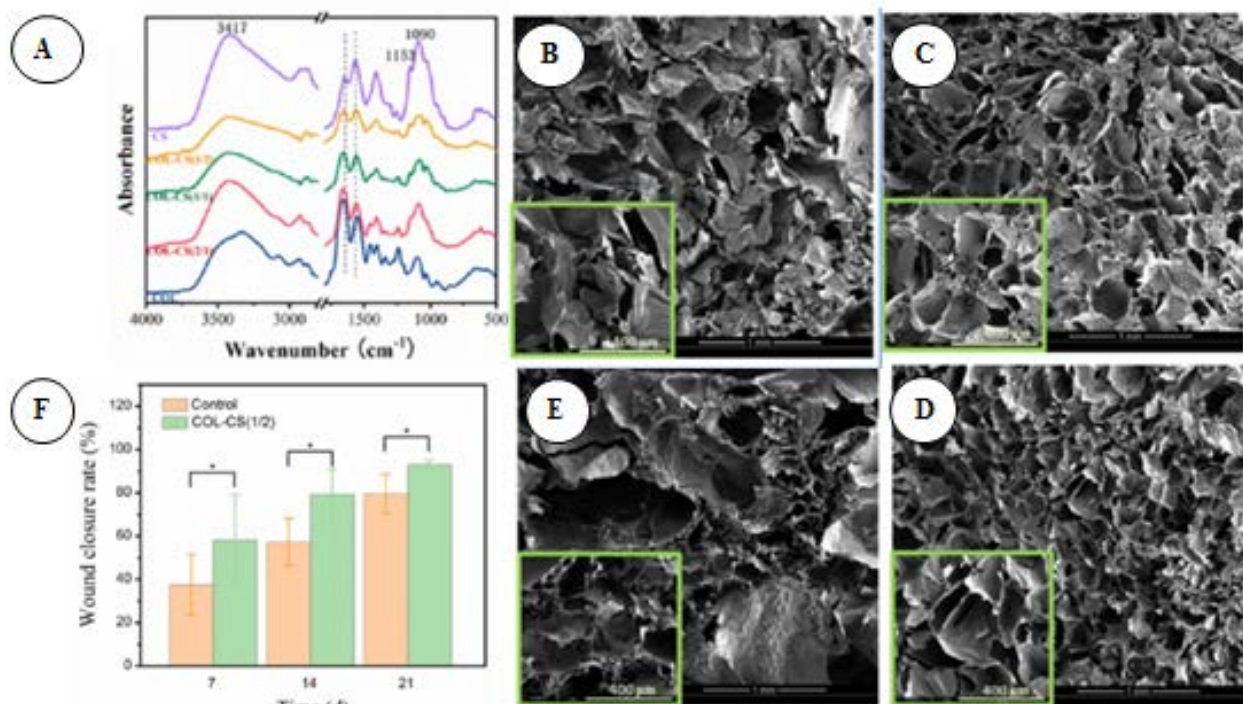

Figure 9. Collagen-Chitosan based self-healing hydrogel (COL-CS): (A) FTIR spectra of collagen, COL-CS (different mass ratio), Chitosan. SEM image of (B) Chitosan; (C) COL-CS (2/1); (D) COL-CS (1/1); (E) COL-CS (1/2); (F) Quantification of wound closing area. (Ding et al., 2020)

Oleoyl chitosan and gelatin in weight ratios (100:0, 90:10, 75:25) is utilized through electrospinning to develop nanofiber scaffolds (mat). The developed nanofibres have improved adhesion and proliferation in presence of human amniotic membrane derived stem cells (Figure 10). Additionally, some chemical crosslinkers are also used in system which improve optimum hydrophobicity of the material so that it can maintain its native structure in aqueous medium. Recent studies on canine model have proved that nanofibrous mat of chitosan along with Poly (caprolactone) and Poly (vinyl alcohol) in an optimum mass ratio of 1:2:1.5, enhances complete epithelialization with amount of new glands regenerations with complete regeneration of epidermal cell. (Datta et al., 2017)
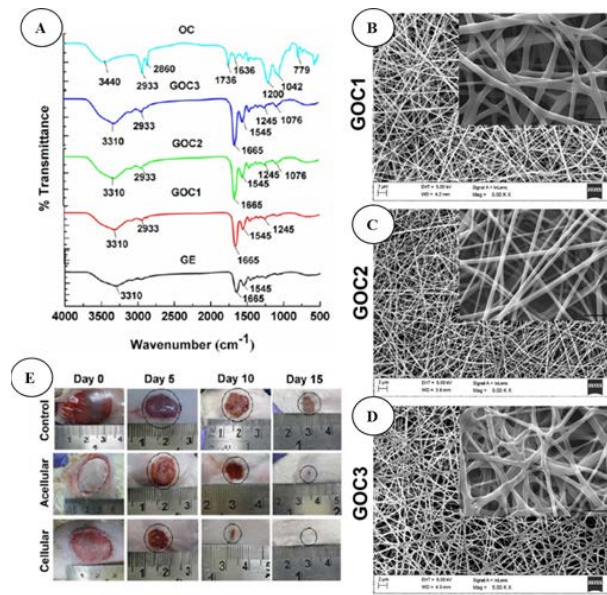

Figure 10. Nanofibre scaffold based on Oleoyl-Chitosan/ Gelatin (GOC): (A) FTIR spectra of OC, GOC (GOC1, GOC2, and GOC3), and Chitosan. SEM image of GOC: (B) GOC1; (C) GOC2; (D) GOC3; (E) Optical image of wound healing in control, acellular and cellular. (Datta et al., 2017)

9.3. Chitosan for drug delivery

Due to its favorable physiochemical properties such as biological degradability and biocompatibility, chitosan is preferentially used for targeted and localized drug delivery. Chitosan plays a role of most important polysaccharide for controlled drug delivery for targeted treatment of many diseases including cancers. The use of chitosan for cancer treatment overrules the side effects of non-targeted and non-specific therapies which are painful, low effective, and high cost. After oral administration nanopolymers possess easy absorption in gastrointestinal tract due to which these nanopolymers have proved as the potential candidates as drug carrier in cancer treatments. Chitosan nanopolymers prepared as a nano-carrier for delivering drug in chemotherapy by

mixing it with hydrophobic surface coating agents and making it stable nano-carrier till it reached target tumor site (Shanmuganathan et al., 2019). In 2018, Samrot et al. (2018) utilized crab shell derived chitosan for synthesis of polymeric nano particles, and these nano particles were further encapsulated with hydrophobic curcumin and analyzed for drug delivery *in vitro*. In 2019, host-guest chemistry-driven supramolecular chitosan nanogels were reported that were stimuli responsive thus allowing selective drug release in specific cancer cells or disease sites (Ding et al., 2019). In order to find the most suitable drug delivery system, the capacity of chitosan nanocomposite systems containing *N-doped* graphene or *P-doped* graphene nanoparticles for delivery of anticancer drug ifosfamide was examined by means of molecular dynamics simulations (Shariatinia and Mazloom-Jalali, 2019). Moreover, chitosan aerogels as drug delivery vehicles provided highly porous network, considerably large specific surface area, and polycationic feature and, thus, offered improved drug bioavailability and drug loading capacity (Wei et al., 2020). López-Iglesias et al. (2019) utilized vancomycin loaded chitosan aerogels which could devote to treat and prevent infections at the wound site (López-Iglesias et al., 2019).

Chitosan is proved to be versatile and so it could be used for application in controlled release of drug formulations for treating acute and chronic wounds. Chitosan hydrogels, being nontoxic, stable, biocompatible, and biodegradable, are mainly used for pharmaceutical and biomedical purposes such as drug delivery in wound healing (Hamedi et al., 2018).
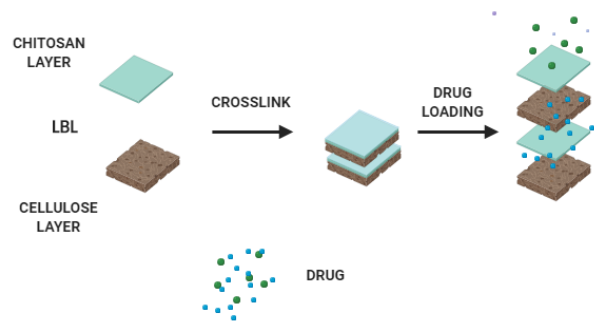

Figure 11. Multilayer nanofilm of Cellulose and Chitosan prepared by Layer by Layer Method (LBL) and chemically cross-linked to improve the inner structure of resultant multilayer. The inner structure of multilayer is responsible for drug loading and release

Chitosan as a promising matrix proved effective in treatment of chronic wounds like burns and diabetic ulcers too (Hamedi et al., 2018). Chitosan is being used for delivering local anesthetics which helps in controlled release of anesthetics to minimize side effect of uncontrolled and nonspecific anesthetics delivery like benzocaine, lidocaine, and tertacaine (Di Martino et al., 2019). Polyoxometalates - an antitumor agent inhibits the action of SOX_2 and it is effective in reducing the risk of metastasis - is found to be harmful for normal cells due

to its cytotoxicity. Development of chitosan nanogels loaded with covalently cross-linked Wells-Dawson type phosphomolybdate $[P_2Mo_{18}O_{62}]^{6-}$ was identified with great capability for targeted delivery of polyoxometalates in a noncytotoxic drug release manner at physiological pH (Pérez-Álvarez et al., 2019).

9.4. Chitosan in Gene Delivery

Chitosan is being widely used in gene therapies. The most critical step for gene therapy is release of gene sequence in intracellular space. Although viruses are proved to be the promising and useful gene delivery vectors, but limited to be used for gene delivery due to immunogenicity, toxicity, and sometimes uptake issues, a requirement for some non-viral vectors has risen. As nucleic acid and bio membrane are negatively charged, chitosan being natural cationic polysaccharide can interact with both and is one of the most versatile non-viral vectors.
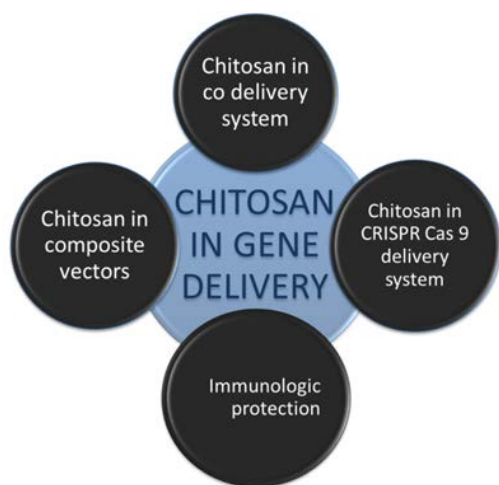

Figure 12. Various aspects of using chitosan in gene delivery

Native chitosan is modified to improve its ability as a vector (Fig. 12). One of these modifications includes improving solubility as chitosan is poorly soluble at neutral and alkaline pH conditions. Chitosan can also be modified with small molecules such as mannose and mannosylated chitosan is effective in gene therapies (Chuan et al., 2019). For escaping endosomal fusion chitosan is modified with PEI for improving buffering capability that leads to the proton sponge effect that depends on chitosan's buffering capacity (Chuan et al., 2019). Chitosan as successful non-viral vector has many applications in gene therapy systems including CRISPR/Cas9 delivery systems (Li et al., 2015). In basic research CRISPR/Cas9 is widely applied for therapeutic and genome regulation purpose but use of viral vectors have safety issues and have packaging limitations. Material science and nanotechnology improve by applying synthetic vectors in place of viral vectors which are capable of targeting cells and tissues with optimal

physiological conditions (Li et al., 2015). Chitosan encapsulated with red fluorescent protein proved to be successful vector for delivery of Cas9 ribonucleoproteins into the nucleus. Assembly of RFD-Chitosan Cas9 and ssDNA enters the cells through the endocytosis followed by entry to the nucleus to elicit homology directed repair (HDR). RFP-Chitosan can serve as the fluorescent probe to track the delivery and facilitate tacking of transfer efficiency (Qiao, 2019). Because of easy transport and adhesive properties in gut, oral delivery of chitosan-DNA construct has been demonstrated in several studies that is nearly impossible by using viral vectors. These oral administrations can be achieved when chitosan and DNA form a stable nanoparticle and enter gut. The endocytosis exhibits immunologic protection against several food allergies such as peanut allergy (Roy et al., 1999).

9.5. Chitosan in Bioimaging

In past several years, chitosan has been used for bioimaging. Chitosan derived nanostructures exhibit strong fluorescence upon excitation with ultraviolet. These polysaccharide-based nanostructures also proved to be stable in terms of photo-bleaching and some metal ions can have quenching effect on it. At a concentration of 10 mg/ml chitosan nanostructures show least cytotoxicity (Zu et al., 2016). In one study, immediately after the previous one, chitosan-based core shells proved to be used for near infrared imaging under NIR-I radiation. In this study, chitosan based nanospheres emitted bright NIR fluorescence that could be used in both in-vivo and in-vitro in tracing nanospheres (Tan et al., 2017). In one of the most recent study, the authors have developed $Fe_3O_4@SiO_2$ coated chitosan for bioimaging in Zebrafish model. GdOF: Ce^{3+}, Tb^{3+} were used as luminophore, which efficiently emitted green when excited at 280 nm. They have tried these nanomaterials in zebrafish and there was no acute cytotoxicity reported that proved biocompatibility (Khan et al., 2019).

10. Future Prospective

Chitin and chitosan, due to their diverse applications are estimated to make important advances in the field of biomedical. These materials can act as improvised prospects for health industry, financial growth, and development due to broad spectrum availability in biowaste, optimized methods of extraction and green methods of modifications. Extraction of chitin and chitosan from wastes produced by different industries is an economical and green process as no harmful chemicals are used. However, these processes are time-consuming and governed by various fermentation parameters like pH, temperature, growth span, etc. Microbial contamination is also one of the important factors that hinder the production of good quality of such materials. Additionally, one of the important drawbacks

of isolating chitin and chitosan from different wastes is that there is no single step process for the same which might reduce the overall yield of the product. These concerns are tuning future prospects of this area of interest as further optimization of all sustainable methods of interventions. It majorly includes extraction, modification, and application with high yield without losing gained properties of being better polymeric materials for various biomedical applications. Some of these can be achieved by minimizing harsh conditions of extraction from biowastes, less consumption, and high re-usability of enzymes used during chemical modifications and better and broad range tunability of physico-chemical properties for different applications.

Chitosan and their derivatives are one of the most promising biomaterials in the industry. These chitinous derivatives are used in various processes like wound healing, tissue engineering, drug delivery, etc. Since these biomaterials are produced by natural assets, these are more viable than synthetic materials produced chemically right now. Additionally, these biomaterials can find their way in the biomedical industry, as a scaffold for tissue engineering. This will reduce the cost of second surgery for removing the scaffold. Chitin and chitosan are highly biocompatible compared to the other synthetic polymers used. Therefore, they prevent any treatment for sensibility and rejection from the implants/materials formed from them. There are still some challenges associated to these materials regarding chemical and mechanical properties of these materials. The nanomaterials formed using these materials can have acute or chronic toxic effects on the body which is the primary challenge faced. Therefore, chitin and chitosan derived biomaterials are needed in tissue engineering implants which can simultaneously decrease the immune responses.

11. Conclusion

Chitin and its derivatives are the as important biopolymers present in the nature as cellulose. They can be isolated abundantly from seafood wastes like crustaceans, shrimps, etc. However, alternative methods for isolating chitin and its derivatives from industries like silkworm, honeybee industry, etc. have paved a way to produce chitin and chitosan from the industrial wastes. The isolation from these wastes can be done chemically or biologically. The chemical methods are used conventionally. However, the use of chemical methods can lead to change in the physical and chemical characteristics of the sample. It can change other properties like molecular size, charge, etc. Therefore, biological methods are making their way into production/isolation. They minimize the irregularities which are obtained during chemical isolation of chitin and chitosan. Isolation of these polymers from the waste materials is a way to maintain ecological balance as well economical preferences. Chitin and chitosan are very

versatile polymers which help in exploring diverse models of industrial function. Chitin and chitosan can also be used for improving the quality of existing polymers and implants. Thus, it is important to not only optimize the extraction of chitinaceous materials from natural sources but also their post extraction modifications, characterizations and plausible applications in various field. These understandings will motivate more industrial scale utilization of biowaste for production of chitinaceous materials and their use in developing economical products for human welfare in sustainable manner. Accordingly, this review has discussed various methods of extracting chitinaceous materials from biological sources, characterizations, modifications, and use in various industries. It also reveals the benefits associated with chitinaceous materials extracted from natural sources by biological methods and modified by bioenzymatic processes. They have also been correlated with areas of their application ranging from tissue engineering to drug and gene delivery. This review will surely be helpful to readers in providing overall information about sustainable availability and applicability of chitinaceous materials.

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Ekstrakcija hitina i hitozana iz biološkog otpada za primenu u biomedicini

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I Z V O D

Biomaterijali su dizajnirani tako da omoguće interakciju sa biološkim sistemima prilikom zarastanja rana, regeneracije tkiva, davanja lekova i kao mehanička potpora kako bi se poboljšali trenutni terapijski rezultati. Primena biomaterijala u zdravstvu se neprestano povećava u praksi i samim tim biomaterijal postaje biokompatibilniji i manje toksičan u fiziološkim uslovima. Primena ovih materijala je povezana sa poboljšanjem terapijskih ishoda kod ljudi, međutim, doziranje leka potrebnog za uspešno lečenje bolesti se obično razlikuje kod svake osobe i oslanja se na iskustvo lekara konsultanta. To može dovesti do ljudskih grešaka prilikom odlučivanja o dozi leka, neprilagođenih implanata ili uklanjanja istih što bi za posledicu imalo negativan ili manje pozitivan efekat. Personalizovana medicina i uređaji ukazuju na to da lek treba prilagoditi pacijentu na osnovu različitih karakteristika, kao što su pol, starost, genetika i način života. Ovakav personalizovani medicinski pristup podrazumeva različite vrste lekova, metode aktivacije, nano-sklopove, biomedicinske uređaje i slično. Među tim pristupima, personalizovani biomedicinski uređaji su postali popularni nakon pojave tehnologije 3D štampe, tehnike koja se koristi za pravljenje implantata koji se mogu prilagoditi svakom pacijentu uz minimalni trošak, za kraći vremenski period i sa velikom preciznošću. Personalizovana biomedicina takođe podrazumeva i dizajniranje leka koji bi udovoljio potrebi pojedinca sa minimalnim neželjenim efektima. U ovom radu je predstavljen pregled različitih aspekata prilagođavanja biomedicinskih sredstava poput terapijskih biomolekula, nanomedicine, implantata i uklonjenih implantata. Ovaj sveobuhvatni pregled literature ukazuje da bi upotreba tehnologije 3D štampe u proizvodnji personalizovanih biorazgradivih implantanata koji otpuštaju lekove mogla predstavljati dobro terapijsko rešenje za čitav niz medicinskih stanja.