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Recent Insights on Chitosan's Applications

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CHITOSAN (CS) is a biopolymer procured from the second most bountiful natural polysaccharide, chitin. CS has been investigated for a wide assortment of applications in the industrial, environmental, and biomedical fields owing to its bountiful beneficial traits. CS is a cost effective, safe, biodegradable, and biocompatible biopolymer. It exhibits anti-microbial, anti-oxidant, haemostatic and wound-healing traits. Thus, it was investigated as a wound dressing material and in tissue engineering applications. CS's structure with its abundant hydroxyl and amine moieties conferred it adsorptive capabilities. Thus, it was utilized to adsorb and eliminate pollutants and also to adsorb and purify important enzymes. Moreover, the hydroxyl and amine moieties of CS permitted its derivatization so as to be capable of immobilizing enzymes via the sturdy covalent bonds. CS also served as an immobilization matrix for medications in order to provide targeted sustained medications' delivery. Owing to the importance of CS, we attempted to discuss its bountiful applications while shedding the light on its beneficial traits.

Keywords: Chitosan; Wound dressing; Tissue engineering; Food packaging; Immobilization

Introduction

Chitin is a natural polysaccharide that is found in the exoskeleton of crustaceans and insects, and also in fungal cell-walls. Chitin is bountiful. Actually, it is the second most bountiful natural polysaccharide after cellulose. The main derivative of this bountiful chitin is chitosan (CS) (Figure 1) [1]. CS offers numerous beneficial traits which make it a candidate for a wide array of applications in the industrial, environmental, and biomedical fields. CS is a safe, biodegradable, and biocompatible biopolymer. It also exhibits anti-microbial, haemostatic and wound-healing traits. Thus, it has been considered as a wound dressing material [1,2]. CS's construction mimics the glycosaminoglycans which are primary constituents of the natural extracellular matrix. Accordingly, CS has been investigated for tissue engineering applications [3-5]. Another valuable trait of CS is its film forming capability which, together with the abovementioned advantages, caused CS to be investigated as an edible packaging for foods which could protect such foods and lengthen their shelf-life [6-8]. Moreover, CS's structure offers plentiful amino and hydroxyl groups (Figure 1). These groups imparted adsorptive capabilities to CS; thus CS could be utilized to adsorb and eliminate pollutants [9, 10]. Such amino and hydroxyl groups also enabled CS's derivatization so as

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to be capable of immobilizing enzymes via the sturdy covalent bonds. Immobilization of enzymes is one of the approaches to augment the enzymes' stability. Moreover, immobilizing enzymes onto solid supports would enable their partitioning from the reaction solution and their reuse [11, 12]. Enzymes were also immobilized by CS via adsorption and entrapment [13, 14]. Other moieties that were entrapped within CS included cells and medications. The cells could be microbial cells that would be guarded within the CS networks against harsh environmental circumstances. This is particularly useful for the microbial cells which are intended to function in an open uncontrolled environment [15]. Medications were also entrapped within CS so as to allow for the controlled and targeted release [16]

Chitosan structure

CS and chitin are both polysaccharides of linear orientation. They are constituted from N-acetyl-2-amino-2-deoxy-D-glucose moieties together with 2-amino-2-deoxy-D-glucose moieties which are appended together via β(1→4) connections [17]. The proportions of these two moieties in either CS or chitin differ appreciably. Actually, these proportions are what set CS and chitin apart. The proportion of N-acetyl-2-amino-2-deoxy-D-glucose moieties is denominated as degree-of-acetylation (DA) whereas that of 2-amino-2-deoxy-D-glucose moieties is the degree-of-deacetylation (DDA). Chitin's DA is commonly ~0.9 whereas CS should possess DA less the 0.35. The majority of the CS's moieties are the deacetylated moieties [18]. Thus, CS evinces ionizable amine moieties whose protonation in acidic vehicles solubilizes it. On the other hand, chitin is eminently insoluble which lays restrictions on its usance although it is nature's second most copious polysaccharide. Chitin is eminently spread in nature in crustaceans' exoskeletons, cell walls of fungi, and also in the cuticles of insects whereas CS natural occurrence has been reported in some fungi. CS is chiefly procured from chitin via deacetylation (Figure 1). This deacetylation is mediated either by a baleful alkaline chemical approach or a green enzymatic based approach via chitin deacetylase. Unfortunately, the enzymatic approach is exorbitant and mainly restricted to lab-sale [17]. The deacetylation of chitin into CS is generally accompanied by a dwindle in its molecular weight (M.W.) (depolymerization) [18].

The commercial CS preparations evince variable MW, DDA, and purity depending on the chitin's origin and its procuring technique and also on the adopted deacetylation approach. These variables appreciably affect critical CS traits, and thus, they should be attentively considered so as to select the CS type that would fit for the intended application. The CS should be of escalated purity and devoid of contaminants as the low purity CS would be accompanied with contaminates which might trigger solubility difficulties and hamper its utilization. The purity scale of CS also affects its biodegradability and immunogenicity [19].The biodegradability is an indispensable trait for any polymer to be involved in tissue regeneration and medication's delivery. Biodegradable CS packagings would also constitute greener surrogates to the plastic ones. The CS's low immunogenicity has promoted its application in wound healing, tissue regeneration, and medication's delivery. As regards to the CS's MW and DDA, they affect its solubility, biodegradability, and anti-microbial effectiveness [19, 20]. The CS's anti-microbial effectiveness has encouraged its application in wound healing, tissue regeneration, and medication's delivery. The anti-microbial traits would also serve to prolong the shelf life of CS packaged films. These anti-microbial traits would be boosted upon escalating the CS's DDA. The DDA reflects the abundance of amino groups in a CS specimen. These amino groups are the cationic moieties which were postulated to mediate the CS's anti-microbial traits. Such cationic moieties would interact with the anionic residues on microbial surfaces. These interactions would induce variations in the cells' permeability. The permeability could be escalated, and the cell membrane could be lysed. The CS could also constitute some sort of CS-membrane polymer which could prohibit the nutrients' entry

and could also act as an oxygen barrier prohibiting the growth of aerobic microbes. Moreover, anionic substrates in micro-organisms, such as proteins could be adsorbed by the cationic CS. This would disturb the physiological activities of these micro-organisms and eventually trigger their death. Considering the effect of the CS's MW on its microbial effectiveness, it could be considered that the CS's M.W. dictates the location of its anti-microbial action. The CS of escalated MW can't permeate the microbial membrane, and accordingly, it piles on the microbial surface. On the other hand, CS of low MW could permeate into the cell's nucleus where it could get attached to DNA and prohibit mRNA construction [20, 21].

Chitosan applications

Wound dressing

Wound healing is a natural process that takes place through four main stages. These are the haemostatis stage, the inflammation stage, the proliferation stage and finally the tissue remodeling stage which includes scar formation [22]. CS could be employed as a wound dressing material as it has the ability to assist in different stages of the wound healing process. CS was reported to exhibit a haemostatic effect. It was also shown that this effect would be boosted upon increasing the DDA of the employed CS as this would allow the CS to bind to more platelets [2, 23, 24]. CS was also reported to activate macrophages [23, 25]. Macrophages are essential to the wound healing process as was confirmed via different studies. In one study, the wounded tissues were deprived of macrophages during either the early inflammation stage or the early proliferation stage. Such macrophage deprivations significantly impaired the wound healing process in both cases [26]. Moreover, CS was shown to exhibit analgesic effect which would be beneficial to combat the pain of the inflammation. In an attempt to fathom the mechanism of the CS's induced analgesia, CS was administered to combat the pain of intra-peritoneally administered acetic acid. The CS's amino groups took up protons from the inflammatory site and this induced analgesia [23]. CS also exhibits scar repairing capacity. Other CS traits that favor its application in wound dressings include its biocompatibility, antioxidant, antitumor properties, and its superior film forming capability. CS also adheres well to injured tissues, sealing off the wounds [2, 23].

Cutaneous wounds expose their underlying tissues, and these tissues are prone to infections as they provide a nutritious, warm, and moist environment for micro-organisms. Hence, wound dressing materials with anti-microbial activity are highly desirable. CS was proven to posses anti-microbial traits against both gram-positive and negative bacteria and also against fungi and yeast [23, 24]. Moreover, CS was blended with other materials to augment its anti-microbial traits; such as cell-penetrating peptides and chlorhexidine [24, 25]. The cell-penetrating peptides are small peptides that have the ability to transfer different macromolecules through biological membranes. They also exhibit antibacterial traits. On one occasion, CS was amalgameted with the fibrous protein, collagen, and the amalgamate was combined with a cell-penetrating peptide known as oligoarginine. The produced gel was then tested as a wound dressing material. Blending oligoarginine with CS-collagen augmented the gel's anti-microbial activity against *Staphylococcus aureus*. This could be regarded to the fact that both CS and oligoarginine exhibit a cationic nature. Hence, both could interact with the anionic peptidoglycans in bacterial cell wall, disturb the cell wall's structure, and prohibit cell growth. Oligoarginine might have also augmented the gel's anti-microbial activity through another mechanism. CS's escalated molecular weight (100 000 Da) would impede its ability to cross the bacterial outer-membrane. Oligoarginine might help transfer CS through the bacterial outer-membrane and augment its anti-microbial activity. It is worth mentioning that blending oligoarginine with CS-collagen also improved the wound healing rate. The oligoarginine might have augmented the gel's adherence to the cells causing such an improvement [24].

As regards to chlorhexidine, it's a synthetic bisbiguanide derivative that exhibits anti-microbial traits. Chlorhexidine offers the advantage of not being absorbed into the body. On the other hand, it is rapidly absorbed by bacteria. Upon its absorption, it disturbs the osmotic function of the bacterial cell membrane and degrades it. This leaves the bacteria, more specifically the bacterial cytoplasm, open for the action of both CS and chlorhexidine. Chlorhexidine, having the ability to precipitate proteins, works on the precipitation of the bacterial cytoplasm. Blending chlorhexidine into CS gel at a concentration of 2% inhibited the growth of *S. aureus* to an extent similar to that attained by 4% chlorhexidine without CS. The CS-2% chlorhexidine gel also offered a bigger and a faster decline in wound size as compared to the pure CS gel. This superior performance was accompanied with excessive angiogenesis and production of fibroblasts [25].

Eggshell membrane (ESM) was another material that augmented the anti-microbial activity of CS films. ESM is derived from the membranes attached to the waste eggshells. It consists of an assortment of proteins and glycoproteins. It is biocompatible and safe. ESM has long been employed in the Chinese traditional medicine for healing burns. Recently, ESM was blended with CS and the sugar alcohol, glycerol, in order to formulate wound dressing films. The anti-microbial activity of these films was assessed against *Escherichia coli* (gram-negative) and *S. aureus* (gram-positive). In both cases, the films formulated with 1%CS-2% glycerol-0.01g/ml ESM offered superior anti-microbial activity to that offered by the pure CS films. This superior anti-microbial activity was regarded to the lysozyme and ovotransferrin residuals present in ESM. Another benefit from blending ESM into CS films was the acquisition of an enhanced ability to take up bovine serum albumin (BSA). BSA was a model protein employed to assess the protein uptake capacity of the CS films. A film with a high protein uptake capacity would be capable of retaining enough nutrients for the proliferation of the cells; thereby, this film would facilitate wound healing. The CS films blended with 0.01g/ml ESM were capable of up taking 1.31 folds higher amount of BSA relative to the pure CS films [2].

It is worth mentioning that during the investigation of wound dressing materials CS has been frequently blended with collagen. Collagen is a biocompatible, low antigenic compound as it is natural constituent of skin. It also has cell adhesion traits and is involved in the tissue healing process. Nevertheless, collagen biodegrades rapidly and exhibits impaired mechanical strength. Thus, it could not be employed alone as a wound dressing material. Blending collagen with CS would be beneficial as it would create cross-linked networks that would augment the biostability of the produced wound dressing [24, 27].

Tissue engineering

CS based scaffolds were investigated for the regeneration of an assortment of soft tissues including skin, blood vessels, neural tissues, and tendons [3-5]. Bone-tissue regeneration has also been attempted via CS based scaffolds [28, 29]. These scaffolds are expected to supply a foundation where cells could proliferate till they construct new regenerated tissues [3].

Bone tissue regeneration (BTR)

Certain traits should be available in a synthetic scaffold in order to be considered a candidate for BTR. The scaffold should promote bone construction on its surface. It should be porous in order to permit the diffusion of nutrients' and wastes' and also to provide the space necessary for the newly formed vasculature and bones. The mechanical stability of the scaffold should be adequate to its intended implantation site so as to retain structural integrity and to provide the necessary support for bone in-growth. The scaffold should also be biodegradable but in a controlled rate [30] so as to provide its supportive function during bone regeneration without the need for eliminating its residuals via any post-surgical interventions. Furthermore, scaffolds with anti-microbial traits would help guard against orthopedic infections [31]. Scaffolds could also be seeded with growth factors or cells

to help promote the bone regeneration [30].

Considering the ability of CS to meet the aforementioned criteria, CS exhibits a hydrophilic surface which permits the anchoring and the proliferation of osteoblasts, the bone-forming cells [30]. CS's content of amino and carbonyl groups could also facilitate the consecutive sedimentation of calcium and phosphate ions on its surface. This will eventually form an apatite layer onto the CS's surface (biomineralization). This apatite layer represents an anchoring layer for osteoblasts which will eventually formulate the bones' extracellular matrix. Such a biomineralization property permits the binding to host bones [31, 32]. It is worth pointing out that CS has been coupled to other materials, especially hydroxyapatite (HA), so as to augment the cell proliferation traits. HA is an inorganic compound whose structure comprises calcium and phosphorus ions. Hence, HA resembles the inorganic constituent of bones. HA could also be naturally derived [30, 33]. Further converting the binary CS-HA system into ternary systems has also been reported to further enhance the cell proliferation traits. On one occasion, CS-HA was amalgamated with two different polysaccharides, amylopectin or chondroitin sulfate, and CS-HA-amylopectin and the CS-HA-chondroitin sulfate scaffolds were prepared. These scaffolds showed superior preliminary cell attachment, proliferation, and spreading as compared to the CS-HA scaffolds [33]. Another superior ternary system was prepared via combining a galactomannan polymer known as *Trigonella foenum graecum* seed polysaccharide (TSFP) with CS- nano hydroxyapatite (CS-n-HA) and forming a new nano-composite [31]. It is worth mentioning that the n-HA offers the advantage of mimicking the natural bone apatite with respect to size. Nano-sized particles also offer amplified surface area to volume ratios, hence, augment their interactions with their surrounding polymeric matrices [30]. The CS-n-HA-TSFP nano-composite permitted a significantly superior propagation of MG-63 cells, which mimic osteoblasts, than did the CS-n-HA nano-composite. However, this was noticed up to a nano-composite concentration of 64μg/ml. At a nano-composite concentration of 256μg/ml, the propagation of the MG-63 was superior in case of the CS-n-HA rather the CS-n-HA-TSFP. This was regarded to toxicity of TSFP. TSFP is derived from fenugreek seeds, and it was shown that the dietary assimilation of low amounts of fenugreek seeds exerted a positive effect on rats' skeletal system whereas the assimilation of high amounts caused skeletal damage. Hence, in-vivo studies should be performed to decide on the safe amount of the CS-n-HA-TSFP nano-composite. The CS-n-HA-TSFP nano-composite was also superior in terms of biominaeralization where a dense, regular carbonated HA layer was deposited on their surface after their storage in a simulated-body-fluid for 4 weeks as opposed to a non-uniform apatite deposition on the surface of the CS-n-HA nanopcomposite. Other advantages were also offered by the CS-n-HA-TSFP nano-composite. TSFP exhibits anti-microbial traits which were combined with the CS's anti-microbial traits and produced a nano-composite with augmented anti-microbial traits against both gram positive and negative bacteria. Moreover, the CS-n-HA-TSFP exhibited superior water absorption and protein adsorption properties [31].

Porous CS could be simply obtained via lyophilization. Moreover, such a porous structure could be tuned via the incorporation of different materials with CS. The incorporation of HA into CS scaffolds decreased their porosity and this was argued to be secondary to the interactions occurring among the CS's amino groups and the HA's hydroxyl groups. Furthermore, adding amylopectin to the CS-HA scaffolds further lessened their porosity. Amylopectin is a branched polysaccharide and such a branched nature could have caused the lessening of the scaffold's porosity [33]. The incorporation of TSFP into CS-n-HA nano-composites also caused the porosity of these nano-composite to drop from 60.3% to 52.0% (both still in range of the porosity off the human cancellous-bone which is 50-90%). Again this was regarded to the interactions among the amino and hydroxyl groups of CS and

n-HA, respectively and the hydroxyl and C-O-C moieties in TSFP [31]. On the contrary, the porosity of CS scaffolds was amplified after the incorporation of alginate or chondroitin sulfate. This was also regarded to the interactions between CS and alginate or chondroitin sulfate. However, these were strong ionic interactions among the cationic polysaccharide, CS, and the anionic polysaccharides, alginate or chondroitin sulfate, which were postulated to trigger precipitation among these polymers, and produce a more open network porous structure [29].

Regarding the mechanical strength of pristine CS scaffolds, it is insufficient for BTR applications. Thus, different materials should be incorporated with CS to produce scaffolds of appropriate mechanical strength [30]. For instance, the amalgamation of TFSP significantly boosted the mechanical stability of CS-n-HA [31].

CS is a biodegradable polymer that can be hydrolyzed via the action of lysozymes. Moreover, its biodegradation products, mainly amino sugars, could be exploited in metabolic pathways [32]. The rate of CS biodegradation relies on both its molecular weight and DDA. Less biodegradation will be observed in scaffolds made from highly deacetylated CS. The increased CS's molecular weight also acts against its degradation. Hence, the choice of CS type would help tune the biodegradation of the scaffold to meet the requirements of its implantation site so that that the regenerated bone constantly takes over the place of the degraded scaffold [30]. Other approaches were also adopted to tune the biodegradation rate of CS scaffolds. For instance, CS was blended with hydroxyapatite (HA) which was proven to offer very limited biodegradation. The produced CS-HA scaffold exhibited much lower degradation rate relative to the CS scaffold, after both scaffolds were oscillated for 21 days in PBS/lysozyme solution. The addition of biodegradable polysaccharides (chondroitin sulfate or amylopectin) to the CS-HA scaffolds altered their biodegradation rate and accelerated it beyond that of the CS-HA scaffold owing to the hydrolysable nature of these polysaccharides. Nevertheless, such rate was still much lower than that of the CS scaffolds [33]. Somewhat similar findings were observed when TSFP was incorporated into CS-n-HA nano-composite. Both the CS-n-HA and the CS-n-HA-TSFP offered similar biodegradation results after 14 days storage in PBS/lysozyme. Afterwards, the CS-n-HA-TSFP degraded more rapidly indicating that such a nano-composite would be cleared off from the body somewhat rapidly after bone regeneration [31]. A different approach to tune the CS's biodegradation rate comprised blending CS, the cationic polymer, with the anionic protein gelatin and forming a polyelectrolyte complex that was less prone to degradation [28].

The incorporation of growth factors into bone regeneration scaffolds, and their controlled release from these scaffolds would help deliver the therapeutic concentrations of these growth factors for an extended period. Hence, these growth factors would function throughout the bone regeneration process. On one ocassion, CS-tricalcium phosphate (CS-TCP) scaffold was seeded with a polypeptide growth factor known for its powerful mitogenic and chemotactic effects on osteoblasts, the platelet derived growth factor (PDGF). The PDGF loaded CS-TCP scaffolds were evaluated in terms of the in-vitro release of PDGF and for their effect in repairing rats' calvarial defects. The CS-TCP scaffold provided a controlled release of the PDGF in vitro. Despite the gradual decline in the release rate, a therapeutic dose was still released even after 21days. The PDGF loaded CS-TCP scaffold also ameliorated the bone regeneration in vivo [34]. It is worth mentioning that the TCP, which was combined with CS, is similar to HA where both are calcium phosphates that bear structural similarity to the bones' inorganic components [30].

Tendon regeneration

Tendons are critical to the musculoskeletal system as they are the fibrous tissues that transfer stress among muscles and bones. Degeneration in tendons commonly occurs to athletes and elderly people. Tissue engineering has lately become a prevailing technique for tendon repair, and

CS has been exploited in this application. CS is biocompatible; biodegradable, and offers anti-microbial traits. The structure of CS also offers resemblance to the glycosaminoglycans of the tendons' extracellular matrix. The ideal tissue engineering implant should resemble the native tendon. The tendons are covered with a sheath. This sheath is constituted of an external fibrotic layer and an internal synovial layer which encloses a cavity having synovial fluid. The synovial fluid allows the gliding of the tendons. The tendons themselves are constituted of fascicles. Each of these fascicles is in turn constituted of bundles of collagen fibers [5, 35]. In an attempt to mimic the tendon structure, four heat-treated micrfibrous poly(L-lactic acid) (PLLA) membranes (5cm*1cm each) were twisted together. Such a bundle of twisted fibers was then rolled within a 3cm*3cm heat-treated micrfibrous PLLA membrane whose inner surface was coated with CS hydrogel. The produced scaffolds were then lyophilized so as to confer a porous structure to the CS inner layer. Such a porous structure served to promote cells infiltration into the scaffolds to assure their homogeneity [5]. It is worth mentioning that the base material of these scaffolds, PLLA, is biocompatible aliphatic polyester that is procured from inexhaustible sources, such as sugar cane and potato. Moreover, PLLA is biodegradable, and its degradation process was shown to last from six months to one year. [5, 36]

It is worth mentioning that the one of the main complications observed during tendon repair is the peritendinous adhesion where adhesion occurs between the tendon and its enclosing synovial sheath. This impedes the gliding of the tendons and can produce disabilities. CS may act against such adhesions via a sirtuin-1-signaling pathway. Moreover, a CS asymmetric scaffold was specially designed in order to impede such adhesions and at the same time promote tendon repair. The asymmetric scaffold was constituted of a compact CS membrane and a porous CS sponge. Both the compact CS membrane and the porous CS sponge were prepared similarly via the addition of NaOH. However, the compact membrane was allowed to dry at 60°C whereas the porous sponge was lyophilized. The membrane and the sponge were then attached to each other and were spun with the compact membrane on the outer side. This outer compact membrane served as a smooth barrier that could impede the peritendinous adhesions. It also aimed at amplifying the mechanical stability of the scaffold. On the other hand, the porous inner sponge layer was postulated to enclose cells and allow for nutrients infiltration, thereby promoting cell proliferation. This inner porous layer was also seeded with tendon stem-progenitor cells (TSPC). Both the CS and the CS-TSPC asymmetric scaffolds enhanced the tendon healing and diminished the peritendinous adhesion as was proven via the in vivo investigations. However, the tendon healing was slightly superior in case of the CS-TSPC scaffolds. It should be noted that the outer compact CS membrane actually did boost the mechanical stability of the asymmetric CS scaffold to the extent that the elastic modulus of the dry scaffold was higher than that of the native tendon. Nevertheless, this elastic modulus dropped considerably reaching only 5.6% of the value of the native tendon's elastic modulus, in case of the wet scaffolds [35]. Hence, the mechanical properties of these asymmetric CS scaffolds should be further amplified.

Neural tissue regeneration

Peripheral nerves are flimsy and unguarded tissues; hence, they could be damaged at the exposure to physical injuries. Damaged nerves could regenerate themselves at a rate of 1-3mm/day. Nevertheless, such intrinsic regenerations are highly limited in case of grievous nerve injuries that are accompanied with big nerve gaps, and surgical interventions are required. Tissue engineered neural scaffolds are highly recommended for such surgical interventions. Neural scaffolds are required to guide the regenerating nerve fibers to their target tissues. They should also provide the essential physical support, and should function as carriers for the cells and the bio-moieties that would be seeded into them in order to boost the neural regeneration process. CS was shown to

support the in-vitro growth of two types of neural cells (Schwann cells and hippocampal neurons). It was also reported that chitooligosaccharides (COS), which are produced upon the degradation of CS, could act against neurotoxicity and could guard against the apoptosis of hippocampal neurons. COS were also shown to augment axon's myelination, and increment the discharge of neurotrophic factors, such as nerve growth factor. Thus, it was favored to construct CS based neural scaffolds. On one occasion, a CS neural scaffold was sutured into 10 or 15 mm long sciatic nerve gaps in rats, and prominent recoveries of the motor and sensory functions were observed. CS was also blended with synthetic polymers in order to construct neural scaffolds. For instance, a neural conduit was constructed with CS as the external microporous tube. The interior of this microporous tube contained filaments of the poly(α-hydroxy acid), polyglycolic acid. This neural conduit was employed to bind sciatic nerve across a 30mm gap in dogs, and it was shown that the nerve's trunk was regenerated with the re-establishment of nerve continuity. [37-38]

Blood vessels' regeneration

Cardiovascular diseases are among the leading reasons of world-wide morbidity and mortality. Surgical interventions are sometimes necessary in order to fix or substitute damaged blood vessels, and restore normal blood flow [39]. Tissue engineered scaffolds could be utilized in such surgeries. One of the techniques to construct ideal blood vessels scaffolds, which wouldn't be accompanied with vascularization problems, is a computer based 3D printing technique termed the fused deposition modeling technique. In this technique, the material is heated (fused) until it melts then it is deposited layer by layer till the desired construct is fabricated. Accordingly, this technique requires highly thermo-stable matrices. The polyester, poly (ε-caprolactone), is known for its superb thermo-stability; hence, it was employed. Nevertheless, poly (ε-caprolactone) is hydrophobic, and this would hinder cell adhesion. Thus, hydrophilic CS together with another hydrophilic hydrogel mixture were blended with poly (ε-caprolactone), and blood vessel constructs were fabricated [40, 41].

Another form of vascular scaffolds is the vascular patches. Vascular patches could be employed in endarterectomy. Endarterectomy is a kind of surgery in which an artery is longitudinally opened in order to remove atherosclerotic plaques. Afterwards, the artery is closed. Employing vascular patches as arteries' closure aids could diminish the incidence of post-operative complications, which might result in the reocclusion of the treated artery. CS was blended with two polysaccharides, pectin (P) or alginate (A), and CS-P and CS-Alg vascular patches were constructed. However, the CS-P vascular patch was superior to the CS-Alg patch. The CS-P patches exhibited a longer stability against degradation in presence of lysozyme. They were also stiffer and exhibited superior hemocompatibility. Moreover, the adherence and proliferation of primary-human umbilical-artery smooth-muscle cells was superior in case of the CS-P vascular patches than in case of the CS-Alg patches[39]

Food wrapping and packaging

CS has been extensively employed to construct bio-based packaging and wrapping materials. CS is biodegradable; hence, it would offer a greener substitute to the non-biodegradable polluting plastic packages. CS is also known for its anti-microbial traits which would allow it to guard packaged food against microbial spoilage [6-8]. Another type of food spoilage that CS could help guard against is lipid oxidation. Lipid oxidation triggers alterations in foods' flavor and odor that would revolt consumers and cause them to reject these foods. CS would hinder lipid oxidation owing to its fine oxygen barrier traits which would impede the commencement of the free-radical chain reactions of lipid oxidation. Moreover, CS exhibits antioxidant traits which are related to its amino content, and consequently, its molecular weight and its DDA. The CS's amino moieties would scavenge free radicals and establish stable

moieties. Additionally, CS exhibits metal chelation traits which could impede the commencement of lipid peroxidation [8, 42-44].

On the other hand, the CS's mechanical stability is not sufficient to withstand the stresses encountered throughout the packaging, convection, and handling of foods. Thus, it should be subsumed with other materials to escalate its mechanical strength and offer it sufficient plasticity. Glycerol is the most commonly utilized additive to escalate the plasticity of CS membranes [6, 8, 42, 43].

Different bio-active materials were incorporated into CS films so as to boost the bio-activity of the produced films [7, 42-45]. Some of these bio-active materials also imparted pH sensing traits to the produced films [44]. For instance, a bio-active CS film was synthesized upon the subsuming of fish-purified antioxidant peptide. Animal acquired antioxidant peptides constitute substitutes to synthetic antioxidants. Nevertheless, they are heat and oxygen sensitive. Thus, they should be efficiently encapsulated in order to conserve their bioactivity. Electrospun nano-fibers could serve as an entrapment matrix for these antioxidant peptides owing to the simplicity and cost-effectiveness of the electrospinning process. Moreover, the nano-size and the highly porous construction of the electrospun nano-fibers would help control the release of the entrapped peptides out of the packaging film. However, the CS's ionic repulsions make it very hard to prepare electrospun CS nano-fibers. Hence, CS was blended with polyvinyl alcohol, which is a synthetic, safe and simply electrospinnable polymer, and CS-PVA electrospun nano-fibrous sheets were prepared. The fish-purified antioxidant peptide was then entrapped within the nano-fibers of these sheets. Elevated entrapment efficiencies (>94%) were attained upon blending different peptide concentrations. These entrapment efficiencies were superior to those attained upon entrapping a peptide within CS nano-particles (74.7% entrapment efficiency), suggesting that the nano-fibers are superior entrapment matrices. The CS-PVA nano-fibers also regulated and extended the release of the fish-purified peptides. The fish-purified peptides entrapped within the CS-PVA nano-fibers still offered antioxidant activity but inferior to that of the free peptides. The entrapped peptides required time to be released from the entrapment matrix and exert their antioxidant effect. Furthermore, the decline in the antioxidant activity could be triggered by the interactions of these peptides with the CS-PVA [7]. Another approach to subsume CS into electro-spun nano-fibers comprised formulating CS nano-particles and then entrapping them within the safe, biodegradable gelatin nano-fibers. This double matrix approach was adopted during the construction of moringa oil enriched bioactive packaging. Moringa oil exhibits superior anti-microbial traits. Accordingly, the CS-moringa oil-gelatin nanofibers capably hampered the propagation of both *Listeria monocytogenes* and *Staphylococcus aureus* in cheese samples that were initially inoculated with these pathogenic micro-organisms. Another positive trait that was acquired by the CS-moringa oil-gelatin nanofibers relative to the gelatin nano-fibers was their diminished water vapor permeability (WVP). The WVP of the nano-fibers diminished progressively with escalating their content of the CS-moringa oil nano-particles from 0 to 15 mg/ml of the spinning solution. Thus, these fibers would better guard against moisture convection among foods and their contiguous environment [45]. The subsuming of the oil loaded nano-particles into the gelatin nano-fibers could have decreased the nano-fibers hydrophilicty. This in turn decreased their WVP [8].

Anthocyanins are phenolic moieties which were verified to exhibit potent antioxidant traits. Moreover, their structure, and consequently, their color are altered upon altering their surrounding pH. This trait is a key to attaining smart packaging materials that would point out food spoilage as pH changes occur during numerous food spoilage mechanisms. Hence, an anthocyanin affluent extract from purple fleshed sweet potato was subsumed into CS films in order to establish smart bioactive packaging films. The CS-anthocyanin

extract films possessed much superior antioxidant traits relative to the CS films, and such superior antioxidant traits were further amplified upon increasing the concentration of the subsumed extract from 5 to 15% (relative to CS). Furthermore, the color of the CS-anthocyanin extract films was altered, after their immersion in diverse buffer solutions (pH 3.0-10.0). They acquired pink-red, purple-brown, and greenish-green colors at pHs 3.0-6.0, 7.0-8.0, and 9.0-10.0, respectively. An additional positive trait of the CS-anthocyanin extract films was their diminished light transmittance. This trait was further boosted upon raising the concentration of the subsumed extract from 5 to 15% (in relation to CS) indicating that the CS-anthocyanin extract films could be utilized to wrap up light-sensitive food. Another concentration dependent amendment in the CS-anthocyanin extract films was the amendment in their water-vapor permeability (WVP). At a 5% extract concentration, no significant amendment occurred in the WVP relative to CS films. On the other hand, subsuming 15% extract significantly amplified the films' WVP which was disadvantageous. The dispersion of this high extract concentration was impaired, and this could have disturbed the compact construction of the films [44]. An anthocyanin affluent extract from the black-soybean seed-coat was also subsumed into CS films. Unlike the sweet-potato anthocyanin extract, the soybean anthocyanin extract diminished the films' WVP. This diminishing became more pronounced upon escalating the extract's concentration. This extract was argued to confer a denser and more compact construction to the CS's films owing to the interactions among CS and the phenolic anthocyanins. The soybean anthocyanin extract-CS films also exhibited beneficial alterations similar to those offered by the sweet-potato anthocyanin extract-CS films. The soybean anthocyanin extract-CS films offered boosted antioxidant traits, diminished light transmittance, and their color was visually altered in response to pH alterations. All of these beneficial alterations were boosted upon escalating the extract concentration from 5 to 15% (in relation to CS) [46].

On another occasion, a carotenoproteins extract was subsumed into CS films. Carotenoproteins are stable conjugates, in which a high-density lipoprotein is attached to carotenoids. Carotenoids are highly unsaturated tetraterpenoids which display anti-microbial and antioxidant traits. Besides, they are widely utilized as colorants in food, cosmetics, and also in pharamaceuticals. Hence, the CS-carotenoprotein extract films displayed superior anti-microbial, antioxidant, and light barrier traits relevant to CS films. The amplifications in the films' anti-microbial and antioxidants traits were in concert with the increase in carotenoproteins extract concentration, and the upmost anti-microbial and antioxidant traits were displayed upon utilizing the highest carotenoproteins extract concentration (50% w/w relevant to CS's dry weight). The superior anti-microbial traits and antioxidant traits of the CS-carotenoproteins extract films were referred to the bioactivities of both CS and carotenoprotein extract. Moreover, the CS's hydrophilic nature allowed the swelling of the matrix. Hence, the release of carotenoprotein extract, which was only physically bonded to CS, was enhanced [42].

Immobilization

CS has been extensively utilized in the immobilization of versatile moieties ranging from metal ions to whole cells. Versatile immobilization techniques were also adopted including the adsorption, the entrapment, and the covalent. The CS's content of amino and hydroxyl moieties promoted its adsorption capacities [10]. Moreover, CS moieties were chemically modified via various techniques to enable its exploitation in covalent immobilization [11, 47]. The CS chemical modifications were also adopted to escalate its performance as an entrapment matrix for medications to allow for their targeted and controlled discharge [16]. Various modifications were also adopted to escalate the CS's impaired mechanical stability [11, 48] as a mechanically impaired immobilization matrix could break at the exposure to any stress, and waste all the efforts and costs that were exerted to construct and load

such a matrix.

Entrapment

Enzymes' entrapment

Enzymes' entrapment is among the immobilization approaches that serve to create a solid enzyme preparation which could be simply partitioned from the reaction solution and could be then rehashed for numerous catalytic cycles. In this approach, the enzymes are embedded within a polymeric matrix. In doing so, the enzymes become protected from their contiguous environment which might contain baleful materials. The entrapped enzymes are restricted within the network of the polymeric matrix. This network should be tight enough to impede the leaching of the enzymes. Meanwhile, it should allow for the distribution of the substrates throughout the polymeric matrix so as to reach all the entrapped enzyme moieties [49]. Regarding CS, its network configuration was significantly influenced by the concentration and the pH of the ionotropic gelation inducer, tripolyphosphate (TPP), which is an inorganic anionic moiety. Alsarra et al. [13] entrapped lipase within a sea-cure-242 CS. The most efficient CS network, which provided the upmost entrapment efficiency (69.3%) and the slightest enzyme leaching, was acquired with a 0.5% TPP solution (pH 7). Declining the TPP's pH to 6 or its concentration to 0.4% diminished the entrapment efficiency to 17.6% and 45.3%, respectively, and also escalated the enzyme's leaching. The decline in the TPP pH diminished the TPP's anionic charge density which impeded its ionic cross-linking with the cationic CS. This might have caused the construction of a looser CS network which permitted the enzyme's leaching. Moreover, declining the concentration of the cross-linker (TPP) would also impair the CS network construction. It is worth mentioning that alterations in the solution pH could also induce variations the CS's cationic charge density which would in-turn affect the CS-TPP cross-linking [13]

The CS's entrapped enzymes were also adopted for the construction of biosensors. For example, glucose oxidase was introduced into a solution of a polyelectrolyte complex established among the cationic CS and the anionic carrageenan. This mixture was then deposited onto a gold electrode. The biosensor fabricated thereof offered a linearity range of 5μM till 7mM glucose concentration, and a 5 μM glucose detection limit [50].

Cells' entrapment

Cells have also been entrapped within biopolymeric matrices. These biopolymeric matrices could be the scaffolds intended for tissue regeneration. For instance, mesenchymal stem cells could be subsumed within bone scaffolds to boost the regeneration process [30]. Microbial cells could also be entrapped within biopolymeric matrices. In doing so the microbial cells would be protected against harsh environmental circumstances, such as the existence of baleful chemicals. This is particularly useful for the microbial cells which are intended to function in an open uncontrolled environment, such as the petroleum oil degrading micro-organisms which are expected to degrade petroleum oil and eliminate them from their contaminated sites. Of these oil degrading organisms, *Serratia* sp. was entrapped within CS-activated-carbon (CS-AC) beads and its oil degrading capacity was investigated [15]. The activated carbon is an organic material whose porous configuration caused it to manifest large surface area (≥1500m2g-1). Hence, activated carbon displays colossal adsorptive capacity [51]. As regards to CS, it has biosorption capacity towards diesel oil, and hence, it participated in the oil elimination process. The immobilized *Serratia* cells were efficacious in oil elimination, and a 73% oil elimination percent was recorded after incubating them for 14 days within an oil containing medium, which was regularly (every 2 days) inoculated with additional fresh oil amounts (200 mg/L). The stored immobilized cells (30days, 30°C) were also as efficient as their freshly prepared analogues indicating the potential of the immobilization matrix for protecting its entrapped cells against environmental stresses. The immobilized cells were also lucratively reused for 12 degradation cycles [15].

It is worth pointing out that a concern about the viability of the CS entrapped microbial cells

might arise owing to the eminent anti-microbial activity of CS. Chanratana et al. [52] evaluated the viability of *Methylobacterium oryzae* among CS prior to its entrapment.. The CS, up to a 1.5% concentration, didn't elicit any determinal effects on the *M. oryzae* viability. Hence, a CS concentration range up to 1.5% was utlilized to entrap *M. oryzae,* and an entrapment efficiency of 80% was achieved with 1.5%CS. *M. oryzae* is a plant-growth-promoting micro-organism with biocontrol traits. The entrapping of *M. oryzae* within CS would protect it and would permit its slow and prolonged discharge into the soil. Such a slow and prolonged discharge was deduced from the controlled CS biodegradability in soil where the *M. oryzae* entrapping CS beads were not completely degraded even after 30 days residence in soil. Moreover, CS itself could also assist in plant growth owing to its ability to adsorb nutrients which would facilitate the assimilation of these nutrients by the plants. Upon evaluating their effect on the development of tomato seeds, the CS entrapped *M. oryzae* significantly escalated the seeds' development in terms of shoot and root length and also dry weight. The CS beads alone also boosted the seeds' development with respect to the aforementioned criteria.

The entrapped microbial cells could also act as surrogates to immobilized enzymes in industry. This is particularly handy in case of intracellular enzymes. There will be no need to spend effort and money extracting and purifying such intracellular enzymes to utilize them in industry. Instead, the whole cells together with their intracellular enzymes will be immobilized and will be employed to catalyze the required reactions in a handy reusable manner. For instance, the whole cells of *Pseudomonas diminuta* were entrapped within CS and this biocatalyst was investigated as a surrogate to cephalosporin-C-acylase for synthesizing 7-aminocephalosporanic acid (7-ACA). 7-ACA is a intermediate adopted for manufacturing cepalosprin antibiotics. The entrapped *P. diminuta* lucratively synthesized 7-ACA and retained 67% of its preliminary activity along the sixth production cycle [53].

Medications' entrapment

Diminutive plasma half-life of medications necessitates their administration in recurrent and rather big doses so as to constantly deliver them at their therapeutic doses. The big doses could trigger toxic side effects [16], and the recurrent administration of medications is not favored by patients and could even be rather annoying. Entrapping medications into biopolymeric formulations could constitute a solution to this issue. The biopolymeric formulations would entrap and store the medications among their networks and would allow the controlled discharge of these medications over an extended period, thereby reducing the urge for their recurrent administration. CS based formulations have been extensively investigated for the delivery of a wide variety medications. CS is available, cost effective, biocompatible, safe, and biodegradable. CS degrades via the actions of lysozyme and chitinase. Different mucosal surfaces secrete lysozyme whereas chitinase could be produced by intestinal flora. Moreover, CS could degrade owing to its dissolution in the acidic pH of the stomach [1]. Another advantageous trait of CS that could be exploited upon utilizing it in medications' delivery is its muco-adhesiveness. Electrostatic attractions could be constructed among the cationic CS and the anionic mucus membranes [16, 54, 55]. Neverthess, the major challenge that impedes CS biomedical application is its mechanical instability [54, 55].

It is worth pointing out that upon utilizing pristine CS networks as medications' entrapment matrices, a burst discharge of the medications is often observed [55]. Such burst discharge could cause over-dosage of the medications. Thus, it should be minimized and a slow continuous drug discharge should be promoted so as to provide the therapeutic doses of medications over an extended period. Subsuming different materials with CS could modulate the discharge of the entrapped medications.

On one occasion, clotrimazole was entrapped within various CS beads. Clotrimazole is an imidazole derivative that is utilized as an antifungal

medication. The gelling technique of these beads was manipulated in order to diminish the burst discharge of clotrimazole. These beads were constructed from either CS or polyethylene glycol (PEG) conjugated CS (CS-g-PEG). The conjugation among CS and the polyether compound, PEG, would diminish the polymer's ionic interactions with bio-moieties, such as proteins, and this would assist medication delivery. The polysaccharide, starch (S), was also added to the CS-g-PEG with the aim of escalating the beads' hydrophilic traits and palatability. Clotrimazole was subsumed with CS, CS-g-PEG, or CS-g-PEG-starch (CS-g-PEG-S) dispersions and, the utilized mixture was extruded into a NaOH solution. More or less analogous initial burst and cumulative discharges of clotrimazole were attained from both the CS and CS-g-PEG beads after 120 min in phosphate-buffer solution (PBS) of pH 7.4. The amalgamation of starch into the beads amplified both the initial burst and the cumulative discharge of clotrimazole. Starch escalated the hydrophilic traits of the beads. This facilitated the penetration of the aqueous PBS (pH 7.4) solution into the beads and triggered their swelling and the consequent discharge of the entrapped clotrimazole. In order to diminish the initial burst and the cumulative discharge from the CS-g-PEG-S, the clotrimazole loaded CS-g-PEG-S dispersions were subjected to a two stepped gelling protocol. This protocol comprised the initial dripping of a tripolyphosphate (TPP) solution onto the loaded CS-g-PEG-S dispersion until the dispersion was opalescent, and then the dispersion was extruded into a NaOH solution. The additional TPP cross-linking step triggered a considerable decline in both the initial burst and the cumulative discharge of clotrimazole. In the TPP untreated beads, the cationic CS's amino groups mediated repulsions among the CS chains. The anionic TPP then cross-linked such cationic groups. Thus, repulsions among the CS chains were diminished, and CS acquired a more compact construction which impeded the clotrimazole's release [54].

Subsuming nano-particles, such as rectorite (a synthetic silicate-nanoclay) into CS has been shown to diminish or even avoid the burst discharge of the entrapped biomoieties. Nano-hydroxyapatite (n-HA) was also subsumed into CS with the aim of diminishing the burst discharge of the fluoroquinolone antibiotic, ciprofloxacin. The n-HA was not just subsumed with CS, it was also in-situ synthesized within the CS solution. CS macro-molecular chains inhibited the aggregation of n-HA, and retained it in the nano-scale. The sulfated polysaccharide, κ-Carrageenan (Car) was also added to the CS-n-HA dispersion. Car was intended to cross-link CS. Hence, the pH of the CS-n-HA-Car dispersion was set at 5.7 so as to permit the ionic cross-linking among the cationic CS and the anionic Car. n-HA devoid CS-Car hydrogels were also prepared and ciprofloxacin was entrapped within both hydrogels. The utilized 5.7 cross-linking pH permitted the ionic cross-linking among the cationic ciprofloxacin and the anionic Car, and this augmented the ciprofloxacin's entrapment efficiency (79.5%) in CS-Car. Subsuming n-HA into CS-Car diminished the ciprofloxacin entrapment efficiency (50.85%) owing to the n-HA induced interactions. As regards to the ciprofloxacin's discharge, a burst discharge was still observed in both the n-HA supplemented and deprived samples. Nevertheless, the subsuming of n-HA diminished this burst discharge, during their initial 7.5 h residence in PBS (pH 7.4), from 69.6% to 34.2%. The cumulative discharge of ciprofloxacin after 120h was also diminished from 98% to 52%., upon the subsuming of n-HA. H-bonds were established among n-HA and both CS and Car. This established a nano-composite with a compact construction which impeded the diffusion and the discharge of ciprofloxacin [55].

Another approach to prolong the discharge of drugs from CS matrices involved subsuming the hydrophilic CS with the hydrophobic polyester, polycaprolactone. Nano-fibers were prepared from these two dissimilar polymers, and these nano-fibers were utilized to entrap the fluoropyrimidine anticancer medication, 5-fluorouracil. The hydrophilic CS with its ability to interact with the hydrophilic 5-fluorouracil caused the 5-fluorouracil entrapment efficiency to escalate

from 69.69% to 90.53% upon escalating its polymer percent from 7 to 23%. Further escalating the CS's polymer percent didn't trigger significant alterations in the 5-fluorouracil entrapment efficiency. On the other hand, the hydrophobic polycaprolactone diminished the nano-fibers' degradation and also the 5-fluorouracil's release upon escalating its polymer percent from 69 to 93%. Hydrophobic polymers limit the diffusion of the hydrophilic medications' to the exterior of the nano-fiber, and their subsequent release [41, 56].

Targeted medication delivery was also attempted while utilizing CS based entrapment matrices. Delivering medications at their desired action site would limit their baleful side-effects on other tissues. Moreover, the medications wouldn't be wasted away from their desired action site, and this would escalate their therapeutic effectiveness [16, 41]. For instance, upon the oral administration of capecitabine, a fluoropyrimidine carbonate intended to treat colon cancer, part of capecitabine could function and destroy healthy cells in the upper gastrointestinal tract before reaching its target site in the colon. Consequently, attempts have been conducted to establish colon targeted delivery for capecitabine. CS succinate (Figure 2), a compound chemically procured from CS, was shown to be effective in colon targeted medication delivery. The CS succinate (CS-SU) was subsumed with the polysaccharide, alginate (Alg), and CS-SU-Alg beads were formulated via $CaCl_2$ mediated ionotropic gelation. These beads were investigated for the colon targeted delivery of capecitabine. The beads formulated with a 1:1 ratio of CS-SU:Alg didn't discharge any capecitabine after their 2h residence in pH 1.2, which mimicked the stomach pH. These beads didn't swell in pH 1.2, and consequently, no capecitabine was discharged. The 1:1 CS-SU:Alg beads provided a dense compact inner lattice of dipole interactions, such as H-bonds. This dense compact inner lattice hindered the diffusion of the acid solution into the beads, and impeded their swelling. Moreover, at pH 1.2 the carboxylic groups of CS-SU were unionized. Hence, they couldn't bring about charge repulsions among the polymers' chains, and the porosity of the polymers wasn't escalated and the beads didn't swell. On the other hand, at pH 7.4 the carboxylic groups of CS-SU were ioinized and hydrophilic. Thus, they triggered charge repulsions among the polymers' chains and caused the beads to swell. The swollen beads discharged capecitabine but in a controlled manner over 22h. This showed the adequacy of the CS-SU-Alg beads for the sustained discharge of colon targeted medications [16, 57]. Nano CS was also investigated as a vehicle for targeted medications' delivery. CS's solubility is escalated among acidic media, and the extracellular microenvironment contiguous to cancer cells is acidic. Hence, CS nano-formulations could provide targeted medications' delivery to cancer cells, and diminish the systemic side-effects of these medications [41].

Adsorption

Adsorption is the most straightforward immobilization approach. The carrier and the target adsorbate are just blended together under appropriate conditions (e.g. the solution's ionic strength and pH). Reversible physical bonds are then established linking the adsorbate to the carrier. Nevertheless, such physical bonds are feeble and could be simply disrupted by; for instance, a change in pH or an escalated ionic strength. This would lead to the desorption of the adsorbate [49]. However, such desorption could be advantageous as the carrier would be regenerated in the process. The unloaded carrier could then be reutilized to uptake fresh amounts of the adsorbate. This is particularly valuable in environmental applications where the carrier is employed to adsorb a certain pollutant or undesired moiety. Afterwards, the loaded carrier is collected, the adsorbate is desorbed, and the unloaded carrier is utilized again to adsorb further amounts of the adsorbate. Ciprofloxacin, a fluoroquinolone antibiotic, is among the pollutants that are commonly found in waste-water, and CS-biochar beads were concocted in order to adsorb it in a successive batch manner. Biochar is an organic porous, carbon-rich material which is known for

its ability to adsorb organic moieties. Moreover, it could be simply concocted. On one occasion, it was concocted from the peels of grapefruit simply via placing them in a furnace and escalating the temperature to 450°C. Nevertheless, Biochar is utilized as a powder whose partitioning from waste-water is difficult and pricey. Thus, it was entrapped within CS to facilitate its partitioning. Furthermore, the CS-biochar beads were much superior adsorbants for ciprofloxacin relative to the biochar powder where sorption capacities of 36.72mg/g and 3.31mg/g were attained, respectively. The CS could have participated in the adsorption process where it was shown that such adsorption involved hydrogen bonds among amine moieties in the CS-biochar beads and the ciprofloxacin's carboxyl moieties. The CS-biochar beads were regenerated via 1 N NaOH, and were utilized for six successive adsorption cycles with >64% of their initial sorption capacity retained [9, 58].

CS was reported to be efficacious in the adsorption of mercury, copper, and also chromium ions. Hence, CS, together with nano-zero-valent iron (NZVI), were formulated as beads, and these beads were intended for treating chromium contaminated waste-water. These beads didn't just target the adsorption and the removal of chromium, but they also aimed at converting the chromium to its less baleful form. Chromium (Cr) exists in the environment as hexavalent(VI) and trivalent(III) ions. The Cr(VI) is more baleful than Cr (III); hence, the CS-NZVI beads aimed at reducing Cr(VI) to Cr(III). The CS served initially to protect NZVI particles, entrapped within the beads, against agglomeration and oxidation so as to be capable of efficaciously reducing Cr(VI). Furthermore, upon the introduction of the CS-NZVI beads into the Cr(VI) solution, the CS adsorbed the Cr(VI) via the action of its available amino and hydroxyl moieties. This caused enriched amounts of Cr(VI) to be in contact with the beads' NZVI . The NZVI then reduced the Cr(VI) to Cr(III). This led to the removal of 82% of the Cr(VI) within 30 min at ambient circumstances [10].

The adsorption-desorption model could also be adopted to purify enzymes. The carrier could be introduced into the crude solution containing the target enzyme. This carrier would then adsorb this target enzyme. After the removal of the carrier from the solution, the target enzyme would be desorbed and collected. Nevertheless, in order for the carrier to specifically adsorb the target enzyme, this carrier should possess a ligand moiety that specifically interacts with the target enzyme (affinity sorption). The ligand for enzymes could be a substrate or an inhibitor. A tyrosinase inhibitor, p-amino benzoic acid (PABA), was loaded onto an organic-inorganic hybrid macro-porous film comprising both CS and silica, and the concocted affinity film was attempted to purify tyrosinase. This CS based affinity film was efficacious. It selectively adsorbed tyrosinase from its crude solution as was evident by the single band offered by the eluate during electrophoresis [59].

It is worth mentioning that adsorbed enzymes have been investigated for different applications. Adsorbed enzymes were investigated for their efficiency in the versatile lipase mediated esterification applications, the hydrolysis of various moieties, including polysaccharides (cellulose) and proteins, and also in the synthesis of bioactive moieties such as galacto-oligosaccharides. Adsorbed enzymes have also been investigated for the establishment of biosensors [60]. On one occasion, the cholesterol-oxidase (CO) and hydrogen-peroxidase (HP) were adsorbed via CS-polyvinyl alcohol (CS-PVA) nano-fibers, and were explored as the potential bio-element in a biosensor. This biosensor aimed at providing a simple visual semi-quantitative estimation of cholesterol. The blood cholesterol concentration should be monitored as the incidence of widespread cardiovascular diseases, such as hypertension, inflammatory and also coronary heart diseases might be triggered by the escalated blood cholesterol concentrations. Hence, both CO and HP were adsorbed onto CS-PVA nano-fibrous films. The hydrophilic CS-PVA nano-fibrous films swelled upon their introduction into the enzymes'

solution. This escalated the pore size of the nano-fibers and permitted the diffusion of the enzymes' into such pores. Hence, hefty enzymes' amounts were adsorbed on and also within the nano-fibrous films. Afterwards, cholesterol was introduced. CO oxidized cholesterol yielding hydrogen peroxide as a byproduct. The HP reduced this hydrogen peroxide and electrons were discharged. A chromogen, 3,3',5,5'-.tetramethylbenzidine hydrochloride (TMB HCl), was then added. The TMB was reduced via the action of the electrons discharged from the HP reaction, and it attained blue-green color whose intensity is reliant on the cholesterol concentration (Figure 3). It is worth mentioning that the adsorption of CO and HP and their catalytic reactions took place at pH 7.4. The introduced chromogen ratio was also kept to a minimum to impede pH alterations [14].

The CS's chemical structure permits its derivatization. Such derivatizations could amend the CS's adsorptive traits. Carboxymethyl CS (CMCS) is a CS derivative that was regarded by Wang et al. [61] as an anionic adsorbent. The carboxymethyl groups could be introduced onto the CS's NH_2 moieties (Figure 4), thus diminishing the CS's cationic traits. Wang et al. [61] further escalated the anionic traits of CMCS beads and subsumed ($-SO_3^-$) moieties into their construction. In doing so CMCS was made to mimic heparin where now both possessed sugar, hydroxyl, carboxyl, and sulfo moieties. Heparin is known to adsorb the low-density-lipoprotein-cholesterol (LDL-c), which is the deleterious lipoprotein as it may escalate the incidence of cardiovascular diseases. The refined CMCS was intended to fulfill that same goal. The refined CMCS beads selectively adsorbed the LDL-c rather than the protective high-density-lipoprotein-cholesterol (HDL-c) with a ratio of 24.8/7.7 mg/g whereas the crude CMCS beads offered a ratio of 2.3/1.5 mg/g. Moreover, the refined CMCS beads didn't elicit obvious cytotoxicity. Hence, they could be regarded as an innocuous adsorbent that can be adopted in blood purification.

Covalent

Covalent immobilization is usually applied to enzymes where it provides the sturdiest linkages among enzymes and solid supports. This impedes enzymes' leakage; hence, it helps retain the activity of the bio-catalyst which could then be rehashed for numerous catalytic cycles. Moreover, the products' contamination via the leaked proteinaceous enzymes will be circumvented. Nevertheless, CS lacks the functional moieties necessary to mediate its covalent interactions with enzymes. Accordingly, CS was refined via glutaraldehyde (GA), genipin, and the carbodiimide chemistry in order to acquire covalently active moieties.

GA could be regarded as the traditional cross-linker utilized to confer covalently active moieties to a variety of biopolymeric formulations [11, 47]. GA is a dialdehyde compound ($C_5H_8O_2$). Thus, upon introducing GA to CS, a fraction of the GA reactive moieties would interact with the CS's amine moieties whereas the remaining GA reactive moieties would covalently link the target enzyme. Among the attempts to escalate the immobilization ability of the GA refined CS, an attempt comprised the subsuming of Na_2CO_3 into the CS's initial gelling solution. Thus, the CS's gelling solution comprised both the ionotropic gelation inducer, tripolyphosphate (TPP), and Na_2CO_3. The Na_2CO_3 reacted with both the cationic CS's amine moieties and the acetic acid, in which CS was dissolved (Equations 1&2). These reactions triggered the evolution of CO_2, which in turn triggered pore concoction in the CS beads. These pores escalated the beads' surface which could host CS's interactions with both TPP and GA. Accordingly; the amounts of CS's physical cross-links with TPP and chemical cross-links with GA were amplified. This boosted the beads mechanical stability. Moreover, the amount of immobilized enzyme also escalated as a consequence of the escalated amount of bound GA. Actually, a 1.91 escalation in the observed activity of the immobilized enzyme was acquired just by subsuming 0.35M Na_2CO_3 [11]. Another attempt comprised the refining of the $NaHCO_3$-gelled CS rather than the TPP-gelled CS. The $NaHCO_3$-gelled CS is known to be of escalated mechanical stability. Moreover, the amine

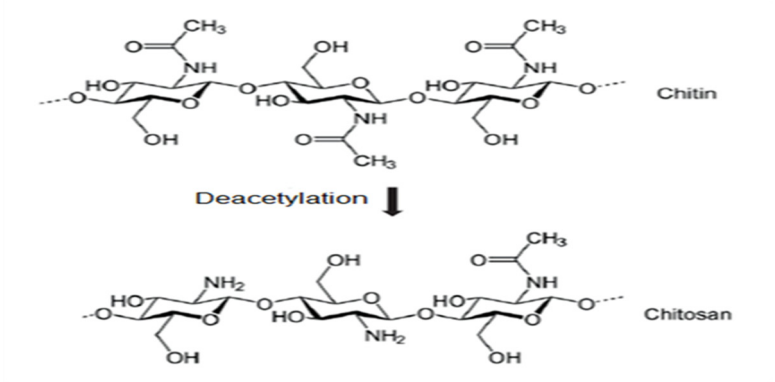


Figure 1: The structure of chitin and chitosan [18].

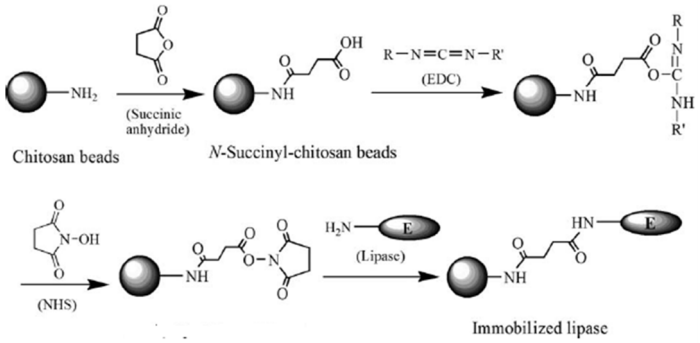
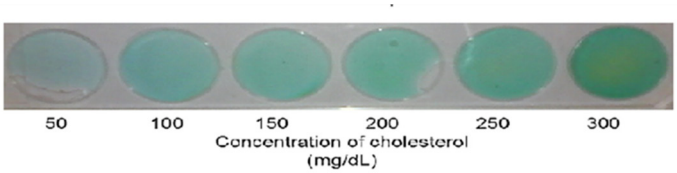


Figure 2: EDC-NHS activation of N-succinyl-CS beads [65].



Concentration	Color scale	Remark
Below 200 mg/dL		Desirable
200-239 mg/dL		Borderline
Above 240 mg/dL		High
300 mg/dL		Very high

Figure 3: TMB color variations relative to cholesterol concentration [14].

moieties of $NaHCO_3$-gelled CS are in their free nucleophilic form (NH_2) which is the from that is reactive towards GA. The abundance of these nucleophilic amine moieties escalated the gel's interaction with GA. Consequently, the amount of bound GA was escalated together with the amount of immobilized enzyme. These beads acquired a 2.45 escalation in the observed activity of the immobilized enzyme relative to their analogous TPP-gelled CS beads [48]. In other covalent immobilization attempts, the GA refined CS represented an encrusting layer deposited onto the surface on the solid immobilization carrier. Carriers concocted from anionic polysaccharides, such as pectin and gellan gum beads, were allowed to interact with the cationic CS. This caused the deposition of a CS layer onto the beads surface. Afterwards, this CS layer was refined with GA to enable the covalent immobilization of enzymes [62-63]. It is worth mentioning that GA could be considered toxic as, under physiological conditions, it might cross-link DNA and various body proteins [47]. Thus, it should be confirmed that the GA present in the immobilization matrices will not leach out during the industrial utilization of the biocatalyst. However, such studies are deficient.

$Na_2CO_3 + 2\ CH_3COOH \rightarrow 2\ CH_3COONa + H_2O + CO_2$ (Equation 1)

$CO_3^{2-} + 2\ CS\text{-}NH_3^+ \rightarrow 2\ CS\text{-}NH_2 + H_2O + CO_2$ (Equation 2)

Genipin is an aglycone procured from the glycoside, geniposide. Geniposide occurs naturally in the fruits of *Genipa Americana* and *Gardenia jasminoides Ellis.* Hence, genipin is safe and could be incorporated in food industries [47]. Genipin could covalently link proteins to CS as it could react with nucleophilic amino groups via two dissimilar mechanisms which eventually lead to the binding of the attacking nitrogen at two dissimilar locations (Figure 5). Moreover, genipin polymerization was also reported to occur after its cross-linking with amino containing moieties [64]. Klein et al.[47] immobilized β-gal by adsorption onto CS beads, and then covalently linked it via both genipin and GA. Immobilization efficiencies of 36% and 66% were recorded for the GA and the genipin treated CS, respectively. The β-gals immobilized onto both beads' types exhibited the same pH and temperature optima. Nevertheless, the genipin refined CS caused the immobilized β-gal to acquire an inferior thermal stability relative to that acquired by the GA refined CS. This was referred to the different covalent linking mechanisms adopted by both samples [47].

For CS to be refined via the carbodiimide chemistry, carboxylic groups were first subsumed into the CS's structure. For instance, Cui et al. [65] synthesized N-succinyl-CS beads via the reaction of the CS beads with the cyclic dicarboxylic anhydride, succinic anhydride (Figure 2). The newly acquired free carboxylic groups in the N-succinyl-CS beads were permitted to react with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), and an unstable ester was created. This ester was transformed to another more stable intermediate ester via the action of N-hydroxysuccinimide (NHS). The ester activated N-succinyl-CS beads were then introduced into a lipase solution. Finally, the Lipase's amino groups established direct amide linkages with the succinic carboxylic groups in the N-succinyl-CS beads, and neither EDC nor NHS was retained in the final loaded beads. This is advantageous as there is no need to worry about the leakage of such chemicals during the consumption of the final biocatalyst. This immobilization approach acquired a 74.56% lipase activity recovery percent. Furthermore, the activity offered by immobilized lipase during its fifth 15 min olive oil hydrolysis cycle amounted to 60%. [65].

On another occasion, carboxylic groups were subsumed into CS via cross-linking with the cost-effective, non-toxic tricarboxylic acid. citric acid. CS encrusted alginate (Alg) beads were formulated. These beads were introduced into a citric acid solution. The mixture was then placed in an oven (50°C) to be dehydrated. Afterwards, the temperature was escalated to 100-120°C and was set as such for 2h. These consecutive thermal treatments allowed for the dehydration of citric acid (Figure 6) and for the ester formation

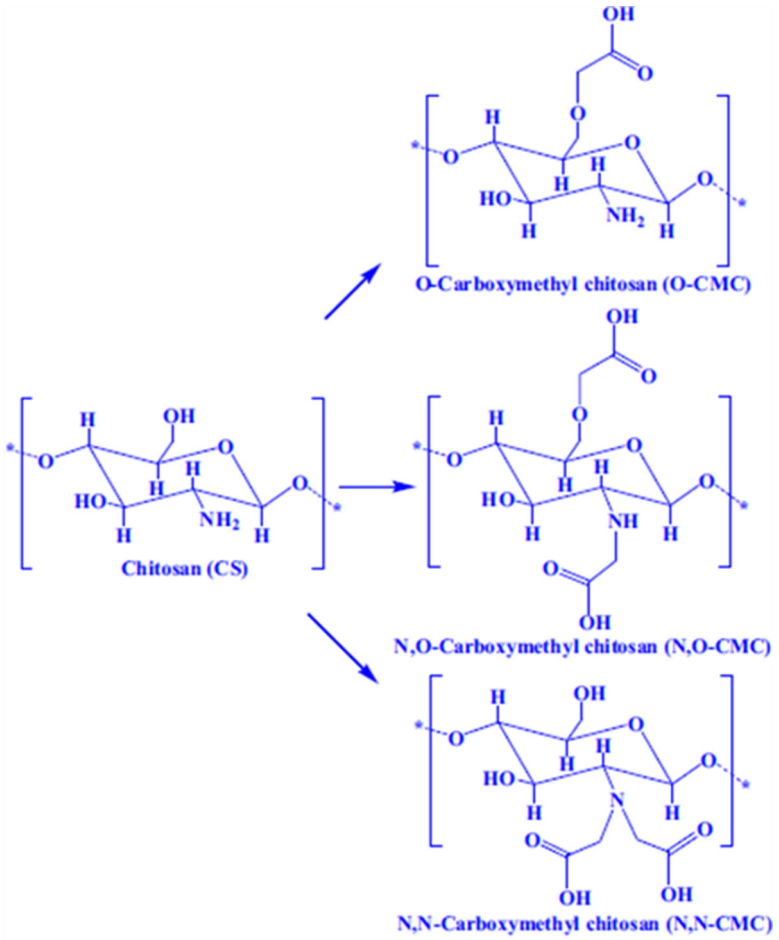


Figure 4: CS and its derivative CMCS [72].

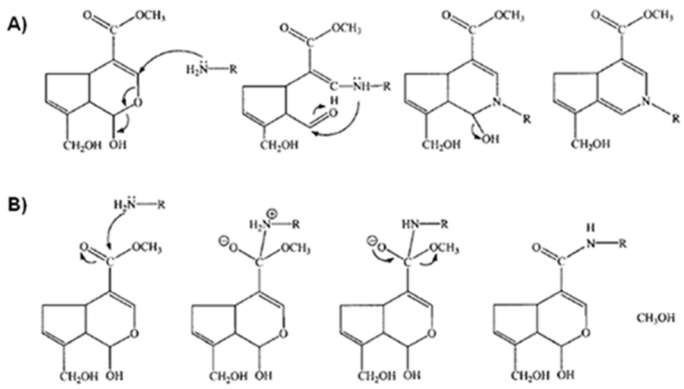


Figure 5: Reaction of genipin with amino containing compounds [64].

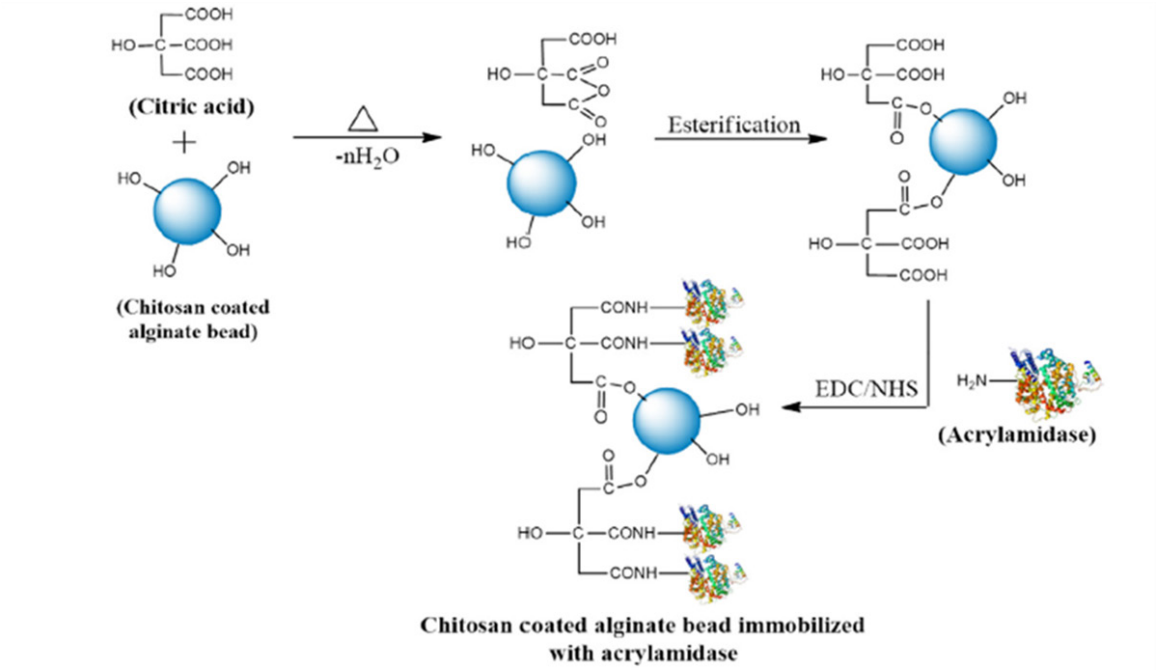


Figure 6: EDC-NHS activation of citric cross-linked CS encrusted Alg beads [12].

among citric acid and the CS's hydroxyl moieties. The EDC and NHS were then employed, and the enzyme, acrylamidase, was immobilized. Direct amide linkages were constructed among the acrylamidase's amino moieties and the citric acid carboxylic moieties. This approach was also triumphant with an acrylamidase's activity recovery percent of 75%. Moreover, the activity exhibited by the immobilized acrylamidase during its fourth and seventh 1h catalytic cycles amounted to 80% and 40%, respectively. Such cutback in the immobilized acrylamidase activity was regarded to the products' accumulation and the subsequent active sites blockage [12].

CS magnetic nano-particles (CMNP) could function as competent covalent immobilization matrices. Their nano-size would provide an escalated surface-area-to-volume ratio. This would permit for the immobilization of escalated enzymes' amounts and would promote mass transfer. On the other hand, despite their nano-size, they could be simply partitioned from the products' containing solution via the action of a magnetic field. Divergent methods have been adopted to concoct CMNP. In one method, CS nano-particles were first concocted via the cross-linkings established among the carboxylic moieties of citric acid and the CS's hydroxyl and amine moieties. Magnetic (Fe_3O_4) nano-particles were then introduced. Citric acid, which was already present in solution, was postulated to cross-link these magnetic particles and cause their association with the nano CS. Finally, NaOH was added and the CMNP were concocted. These CMNP were then activated via the subsuming of GA, which interacted with the CS's amine moieties. The activated CMNP were utilized to immobilize lipase. The immobilized lipase acquired superior storage stability relative to its free compeer where >95% and ~31% of their starting activities were recorded after 30 days residence at room temperature, respectively. The CMNP immobilized lipase was also lucratively reused for five consecutive cycles [66]. On another occasion, CS nano-particles were concocted via the action of tripolyphosphate. Fe^{2+} ions were then introduced. It was postulated that these Fe^{2+} ions were adsorbed by the nano-CS. Afterwards, NaOH was added which concocted $Fe(OH)_2$ and further precipitated CS. The $Fe(OH)_2$ was then oxidized into the magnetic Fe_3O_4 via 0.05% v/v of dissolved oxygen. These magnetic Fe_3O_4 (10-20 nm) were well covered with CS in the 50-100 nm CMNP. The CMNP were then activated via the subsuming of GA, and were also utilized to immobilize lipase. This CMNP immobilized lipase was lucratively reused for five consecutive cycles with only 12% activity loss [67]. Another CMNP concoction method involved dispersing the Fe_3O_4 nano-particles into a CS emulsion. Thereafter, NaOH was added and the CMNP were concocted and were later activated via GA [68].

Some alterations occur in enzymes after their covalent immobilization. The covalent immobilization usually triggers an escalation in the enzymes' optimum temperature and amplification in their thermal stability. The covalent linking rigidifies the enzymes' construction, and thus, escalates their ability to resist distortion and deactivation under harsh conditions, such as escalated temperatures [11, 12, 48, 65].

The enzymes' pH optima usually shift as a consequence to the immobilization process. Some debate that the nature of these shifts, whether they are to a more alkaline or a more acidic value, is related to the carrier's charge. Anionic carriers would induce alkaline shifts whereas cationic carriers would trigger acidic shifts. Solutions contain hydroxyl anions. These anions would be attracted towards cationic carriers. Hence, their concentration in the vicinity of the carrier would be escalated relative to their concentration in the main solution. This kind of unequal hydroxyl anion distribution was debated to trigger the acidic shift in enzymes' pH optima and vice versa [47, 69, 70]. CS (pKa~6.3) is considered a cationic carrier at pHs <6. Thus, enzymes with pH optima <6 would experience an acidic shift after their covalent immobilization onto CS. This was actually the case with β-gal (free enzyme's optimum pH 4.6-5.1) enzymes covalently immobilized via GA or genipin refined CS [11, 47]. Furthermore,

pectinase (free enzyme's optimum pH 5) also experienced an acidic shift in its optimum pH following its immobilization onto chitin beads encrusted within a GA refined CS coat [71]. Nevertheless, alkaline shifts were also observed with CS carriers. For instance, the optimum pH of the N-succinyl-CS immobilized lipase was more alkaline relative to its free analogue [65]. The acrylamidase immobilized onto the citric cross-linked CS encrusted Alg beads also offered a more alkaline pH optimum relative to its free analogue [12]. In the two aforementioned cases, carboxylic moieties were subsumed into CS. These carboxylic moieties could have imparted anionic charges to the CS carriers which caused them to trigger alkaline shifts in the enzymes' pH optima.

Conclusion

CS is suited for a wide variety of applications that could serve the biomedical and industrial sectors. This is regarded to the CS's beneficial traits and also to the possibility of tuning the CS's traits or even repairing its defects via a wide array of approaches. CS can be prepared in many forms, such as beads, membranes, and even scaffolds. Moreover, its morphology could be altered from porous to compact via simply altering its preparation technique. CS's chemical structure could also be simply modified, and this enabled it to function as a targeted medications' delivery vehicle, as a covalent immobilization support, and as an affinity matrix for the purification of different moieties. Some of the CS's traits, such as its anti-microbial and antioxidants traits could be boosted by subsuming other bioactive moieties into CS's structure, and this is advantageous in biomedical applications, such as wound healing and tissue regeneration. CS's adsorptive capabilities could also be boosted upon subsuming other adsorptive materials with CS. Other traits, such as CS's porosity and its biodegradation rate could be tailored, increased or decreased, so as to fit its intended applications. On the other hand, CS's defects and limitations with respect to any of its intended applications could be surmounted via subsuming other moieties into the CS structure whether through physical or chemical approaches. For instance, the CS's impaired mechanical stability could be overcome, and its plasticity could be boosted after adding glycerol to the food packaging CS membranes.

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