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Review

Chitosan: An undisputed bio-fabrication material for tissue engineering and bio-sensing applications

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ABSTRACT

Biopolymers have been serving the mankind in various ways since long. Over the last few years, these polymers have found great demand in various domains which includes bio medicine, tissue engineering, bio sensor fabrications *etc*. because of their excellent bio compatibility. In this context, chitosan has found global attention due to its environmentally benign nature, biocompatibility, biodegradability, and ease of availability. In last one decade or so, extensive research in active biomaterials, like chitosan has led to the development of novel delivery systems for drugs, genes, and biomolecules; and regenerative medicine. Additionally, chitosan has also witnessed its usage in functionalization of biocompatible materials, nanoparticle (NP) synthesis, and immobilization of various bio-recognition elements (BREs) to form active bio-surfaces with great ease. Keeping these aspects in mind, we have written a comprehensive review which aims to acquaint its readers with the exceptional properties of chitosan and its usage in the domain of biomedicine, tissue engineering, and biosensor fabrication. Herein, we have briefly explained various aspects of direct utilization of chitosan and then presented vivid strategies towards formulation of chitosan based nanocomposites for biomedicine, tissue engineering, and biosensing applications.

Contents

1. Introduction

Biopolymers are proven structural materials in a number of biosystems, for example, cellulose supports the structure of higher plants, chitin forms the exoskeleton of several molluscs, keratin provides thermo insulation to hair, collagen acts as a mechanical support in connective tissues, and silk imparts strength to spider webs [1–3]. We can thus, conjecture that polymers may play an

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essential role in the preparation of artificial organs owing to their tremendous biomedical properties. Tissue engineering is an emerging field in the area of regenerative medicine which combines the knowledge of biology and engineering in an attempt to develop substitutes for damaged tissues [4]. It involves generation of scaffolds from biocompatible polymers to enhance cell adherence, proliferation, and differentiation that eventually leads to regeneration of a new tissue. Marine biomaterials have been found to be excellent resources towards tissue engineering [2,3,5–9]. Drug delivery and biomedicine are other prospects for marine biomaterials to be utilized in medical domain. Drug delivery systems with NPs have become alternative method of targeted drug delivery, as they increase drugs' availability to target cells and leave normal cells unaffected [10]. Different types of NPs have been extensively studied for cancer drug, peptide, and nucleic acid delivery. Over the last five decades, organic NPs like, liposome-, polymer-, dendrimer-, and protein-based NPs and inorganic NPs like, metal and metal oxide NPs have been utilized as drug carriers to treat cancer [11]. New products that help in making human tissue and organ regeneration more effective are in high demand and include materials, structures and substrates that drive cell-to-tissue transformations, orchestrate anatomical assembly and tissue integration with biology [12]. Natural derived polysaccharides, such as chitin, chitosan, alginate, fucoidan, carrageenan, and chondroitin sulfate are widely used versatile biomaterials for biomedical applications. These polysaccharides can be divided into two groups depending on their charge, for example, chitosan belongs to the group of positively charged (cationic) polysaccharide, whereas alginate, carrageenan, and fucoidan belong to the group of negatively charged (anionic) polysaccharides. Generally, these polysaccharides are considered safe, biocompatible, stable, hydrophilic, biodegradable, and they can be modified into different forms, such as hydrogels, scaffolds, fibers and NPs. NPs have many advantages for drug delivery purposes compared with larger (micro-sized) particles because they can easily penetrate into targeted areas [13–15]. In addition, chemical and physical modifications of these polysaccharides can bring in new properties toward further suitable biomedical applications [16].

Among various polysaccharides, chitin is a naturally occurring muco polysaccharide present in the exoskeleton of crustaceans, insects and fungal cell walls. It is a derivative of cellulose with the hydroxyl groups replaced by amine groups thereby making it polycationic. The major derivative of chitin is chitosan, obtained usually by the alkaline deacetylation of chitin [17]. Owing to its negligible toxicity and biodegradability, chitosan has found considerable application in the pharmaceutical industry. More recently it has been utilized in the pharmaceutical industry as a matrix molecule for drug delivery and tissue engineering applications [18]. In this review, our aim has been to discuss the importance of chitosan as bio-fabrication material in biomedicine, tissue engineering, and biosensing applications. Over past several years, myriad of reports has been published in this regard, however, our focus has been solely towards the reports published in last one decade. In addition, we have presented a comprehensive report on the current status of chitosan in these fields.

2. Chitosan in biomedicine and tissue engineering

2.1. Chitosan as a delivery vehicle in biomedicine

The molecular weight and degree of acetylation are the two major property of chitosan which affect its use as a matrix molecule for drug delivery. An important property of chitosan for its implications in drug delivery is its muco-adhesive nature and its ability to transiently open epithelial tight junctions. This particular ability of chitosan has been exploited in delivery of drugs across various well-organized epithelia, such as nasal [19,20], intestinal [21–24], ocular [25], buccal [26], and pulmonary [27]. Another property of chitosan that enables its utilization as a drug carrier and coating molecule is the availability of functional groups which facilitates chemical modifications [28–30]. Apart from the aforementioned properties, the biodegradability of chitosan also adds to its usage as a drug delivery vehicle. Chito-oligosaccharide (COS) has been used to functionalize carbon nanotubes (CNTs) for effective delivery of gliotoxin against cancer [31]. Researchers have prepared chitosan-CNT hydrogels by the freeze-lyophilization method and examined their antimicrobial activity against *Staphylococcus aureus, Escherichia coli,* and *Candida tropicalis*. The composite Chitosan-CNT hydrogel showed greater antimicrobial activity with increasing CNT concentration, suggesting a promising role of Chitosan-CNT hydrogels in biomedical applications [32].

In a recent report, a drug delivery system was fabricated using chitosan NPs loaded with Silibinin (SCN), a plant derived flavonolignan known for its spectacular biological properties and complex hydrophobic nature that limits its bio availability. The SCN vehicles were then incorporated into scaffolds containing alginate and gelatin for the sustained and prolonged release of SCN. It was found that the addition of SCN did not affect the porosity of the scaffolds, yet increased the protein adsorption, degradation rates, and bio-mineralization. These scaffolds were found to be biocompatible with mouse mesenchymal stem cells and promoted osteoblast differentiation [33].

Nucleic acid delivery through synthetic polymers has been comparatively less successful when compared to viral vectors and therefore, in order to mitigate this issue chitosan-based particles have been used. The positive charge on chitosan enables its interaction with the negatively charged nucleic acid molecules forming a polyelectrolyte complex. Such, complex formation has been shown to protect the nucleic acids from nuclease mediated degradation [34]. Moreover, the positive charge on chitosan also enables its interaction with the negatively charged cellular membranes leading to an increase in cellular interaction and uptake efficiency [35]. The most popular methods used for the preparation of these NPs are ionic gelation [36–38] and complex coacervation [38–41]. Similar strategies of chitosan NP preparation have been employed for the delivery of siRNA, to carry out efficient knock down of various therapeutically relevant genes, such as TGFβ1, Bcl-2, *etc.* (Fig. 1)[42–44].

Chitosan-based matrices and gels also offer several advantages over traditional materials employed in the wound healing process [32]. Although, chitosan is known to confer broad spectrum antimicrobial properties, treatment of several easily infected wounds still remains a serious problem. For this reason, gentamicin loaded chitosan bars were evaluated for their efficacy post-implantation in rabbit tibia. The implant led to a low gentamicin concentration in the blood, however the local bone concentration of gentamicin was found to be high [45]. Ishihara et al. demonstrated the use of a photo-crosslinked chitosan hydrogel in wound healing. It was shown that the application of chitosan hydrogels induced wound contraction and accelerated wound healing in mouse models. Histological studies suggested that the gel induced epithelialization and tissue formation in the mouse models [46]. Cho et al. demonstrated the application of water soluble chitosan (WSC) on wound healing in full-thickness skin incision in rats [47]. The application of the WSC accelerated the wound healing process and showed a high tensile strength as compared to controls. Histologically, the collagen fibers in the wound showed arrangement similar to normal skin. The use of chitosan based wound dressings has also been shown for the treatment of open-skin wound infections [48], burn infections [49,50], surgical-site infections [51], oral mucositis [52], hemorrhagic cystitis, *etc* [53,54].

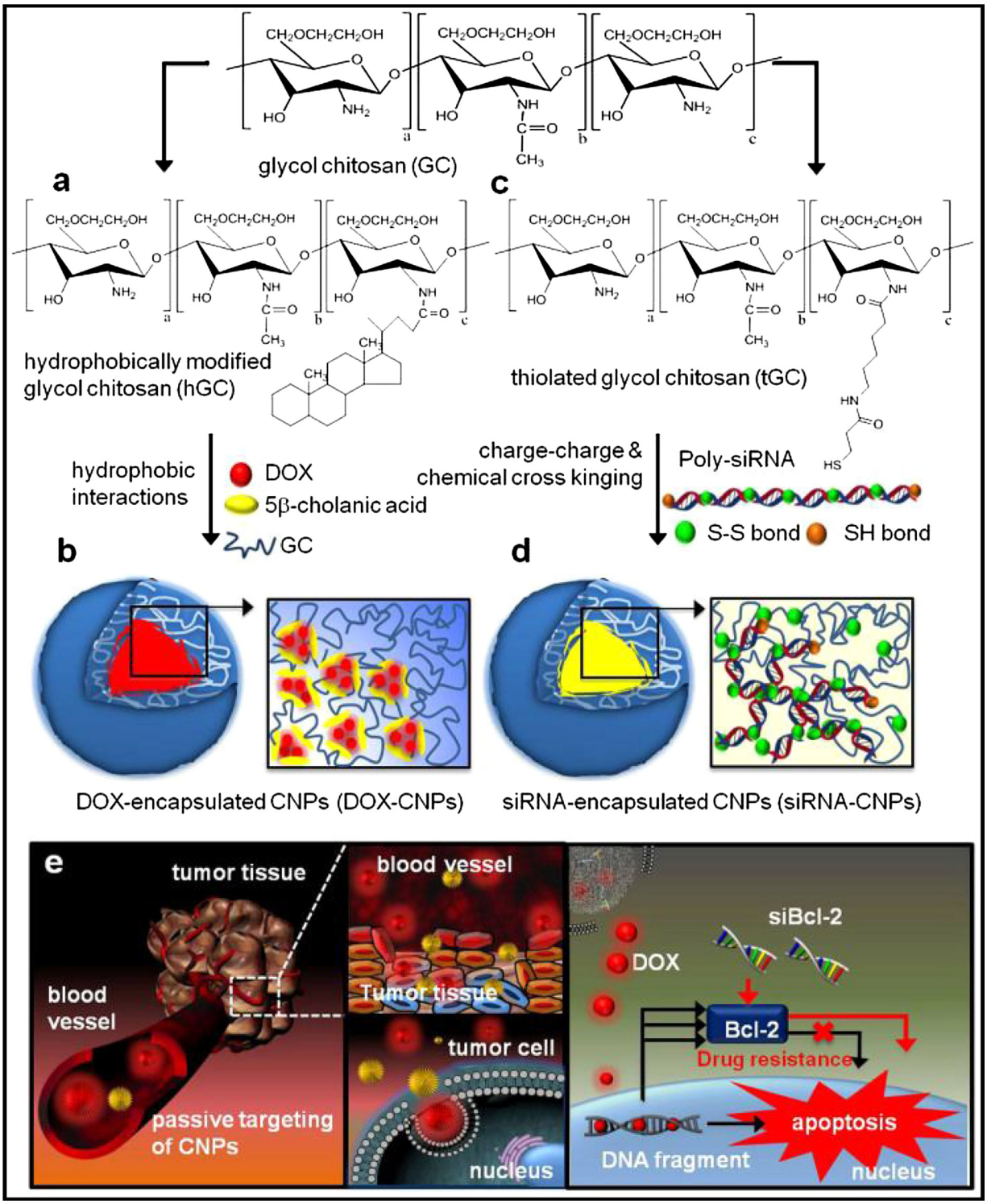

Fig. 1. (a) Synthetic scheme of hydrophobic 5β-cholanic acid modified GC, (b) Schematic illustration of effective self-assembly of DOX-CNPs by hydrophobic interactions between DOX and cholanic acid moieties of GC polymers in mild conditions, (c) Synthetic scheme of thiolated glycol chitosan (tGC) by chemical conjugation of Sulfo-LC-SPDP to the amine group of GC polymers, (d) Schematic illustration of the formulation process of small and compact Poly-siRNA/tGC complexes, (e) Schematic representation of the combinational delivery process by GC-based nano-platforms. Reproduced from [43] (licensed under a Creative Commons Attribution 4.0 International License). Copyright © 2014, Macmillan Publishers Limited.

2.2. Chitosan as a matrix molecule in tissue engineering

Tissue engineering involves designing of polymer based scaffolds for the modulation of cellular growth and differentiation [55]. The prerequisite considerations for choosing a biomaterial for scaffold regeneration in tissue engineering are: (a) presence of interconnecting pores of appropriate dimensions to promote tissue integration and vascularization, (b) controlled biodegradability and bioresorbability to match tissue replacement, (c) favorable surface chemistries for modifications, (d) mechanical properties similar to the intended site of implantation, (e) negligible toxicity, and (f) easy fabrication procedures to achieve desirable shapes and sizes [56].

Table 1
Tabular representation of methods commonly used for chitosan scaffold fabrication. Adopted from [82] Copyright © Mary Ann Liebert, Inc. 2011.

Scaffold structure	Processing method	Cell type (source)
Chitosan scaffolds	Freeze-drying	ROS17/2.8 cells MG63 human cell line MC3T3-E1cell line
Chitosan-TCP sponges	Freeze-drying	Fetal rat calvaria cells
Chitosan-gelatin scaffolds	Freeze-drying	–
Chitosan-TCP sponges	Freeze-drying	MG63 human cell line
Chitosan-HA scaffolds	RP and freeze-drying	–
Chitosan-silk scaffold	Freeze-drying	–
Chitosan-calcium phosphate scaffolds	Freeze-drying	MG63 human cell line
Chitosan sponges	Freeze-drying	Rat calvaria cells
Chitosan scaffolds with HA formation	Freeze-drying	Human SAOS-2 cell line
Chitosan-nano-HA scaffolds	Freeze-drying	MC3T3-E1cell line
Chitosan-coralline scaffolds	Freeze-drying	CRL-12424 cell line
HA-chitosan scaffold	Freeze-drying	Goat bone marrow cells
Chitosan-gelatin scaffolds	Freeze-drying	HUVECs
PLGA-chitosan scaffolds	Freeze-drying	Human BMSCs
Chitosan sponges	Freeze-drying	Chicken embryo chondrocytes
BCP-chitosan scaffolds	Freeze-drying	MC3T3-E1cell line
Chitosan-collagen sponges	Freeze-drying	Rat BMSCs
Chitosan sponges	Freeze-drying	MG63 human cell line
Chitosan scaffolds	Freezegelation	
Chitosan scaffolds	RP	Porcine BMSCs
Chitosan scaffolds	Wet spinning	Mouse osteoblast 7F2 cell line
Chitosan fibre mesh scaffolds	Wet spinning	Human SAOS-2 cell line
Chitosan scaffolds	Electrospinning	–
Chitosan-gelatin scaffolds	Freeze gelation	Human BMSCs
Chitosan scaffolds	Freeze gelation	–
Chitosan and chitosan-starch scaffolds	Freeze gelation	Human SAOS-2 cell line
Chitosan and chitosan-starch + lysozyme scaffolds	Freeze gelation	Rat BMSCs
Chitosan-PLAGA scaffolds	Particle aggregation	MC3T3-E1cell line
Chitosan-PBS/PBTA/PCL	Compression molding/salt leaching	Mouse BMC-9 cell line
Chitosan-PBS scaffolds	Melt spinning/fibre bonding	Human BMSCs
Chitosan-PBS/PCL/PBTA/PBSA	Compression molding/salt leaching	–
PCL-chitosan	Solvent casting/Salt leaching	Rat osteoblasts
Chitosan-PCL scaffolds	Electrospinning	MC3T3-E1cell line
Chitosan scaffolds	Precipitation/Particle aggregation	ADAS
CPC-Chitosan scaffold	Cement/Particle leaching	MG63 human cell line
Chitosan-starch scaffolds	Solvent-exchange phase separation	–

Chitosan based structures, due to their structural similarity with glycosaminoglycans in the extracellular matrix and the ability to mold them into scaffolds with similar morphological and mechanical properties as that of collagen along with controllable pore size, provide excellent alternatives for tissue engineering applications [57,58]. Moreover, properties such as tunable degradation rates, biocompatibity, ease of preparation, encapsulation and sustained release of cytokines/growth modulators, *etc.* adds to the utilization of chitosan as a matrix molecule for tissue engineering applications. Chitosan based scaffolds thus find extensive applications in the engineering of a wide variety of tissues (Table 1) [55]. A number of fabrication methods have been suggested for chitosan based scaffold generation which are shown in Fig. 2 [59].

Composites of chitosan with collagen and gelatin have been extensively reported [60–65]. These blended scaffolds have been shown to have enhanced physicochemical properties, such as tensile strength, swelling behavior and biodegradability, which makes it extremely suitable for skin tissue engineering. Polyelectrolyte complexes (PEC) of chitosan with alginate and anionic glycosaminoglycans, such as hyaluronic acid, chondroitin sulphate, and heparin have also been explored for their efficacy as dressing materials [66–69]. The chitosan-alginate PEC membranes have been reported to be biodegradable and therefore, can acts as effective controlled-release membranes. Not only this, polyelectrolyte gelatin-chitosan hydrogel has also been reported for three-dimensional (3D) bioprinting applications [70].

Bone tissue engineering strategies utilizing synthetic grafts offer promising alternatives to autograft and allo graft bone regeneration [59,71]. Role of chitosan in bone tissue engineering has been extensively studied owing to its osteoconductivity, porosity, biodegradability, and most importantly biomechanical compatibility; to support the ingrowth of bone at the implantation site along with maintaining structural integrity during *in vivo* tissue remodeling [71,72]. Chitosan and hydroxyapatite (HA) are two biocompatible and biodegradable materials with well-established osteoconductivity [73–75]. They have thus been used either alone or in combination for bone tissue engineering applications [76]. Biodegradable chitosan-nanohydroxyapatite (nHA) scaffolds have been reported by Thein-Han et al. [77] and Kim et al. [78], independently. These scaffolds were demonstrated to have superior mechanical, physicochemical, and biological properties as compared to chitosan scaffolds. Another piece of work reported from Korea demonstrated the fabrication of tri-component scaffolds of chitosan/natural hydroxyapatite with chondroitin sulfate (chitosan–CS/HAp) and amylopectin (chitosan–AP/HAp) towards bone tissue engineering. Cell proliferation, alkaline phosphatase activity, and type-1 collagen production was evaluated *in vitro* using MG-63 cell line, which was observed to be higher in the composite scaffolds and they depicted excellent interconnected porosity, controlled biodegradation, and enhanced cell proliferation [79]. Chitosan based nanofibers have also been well described in literature as potential biomaterials towards bone tissue engineering since electro spun nanofibers possess unique properties, such as the high surface area to volume ratio, high porosity, and enhanced stability [80]. Chitosan complexes with natural polymers, such as alginate, collagen, gelatin, *etc.* have also been reported for bone tissue engineering applications. It has been reported that biodegradable porous scaffolds of chitosan and alginate have sig-

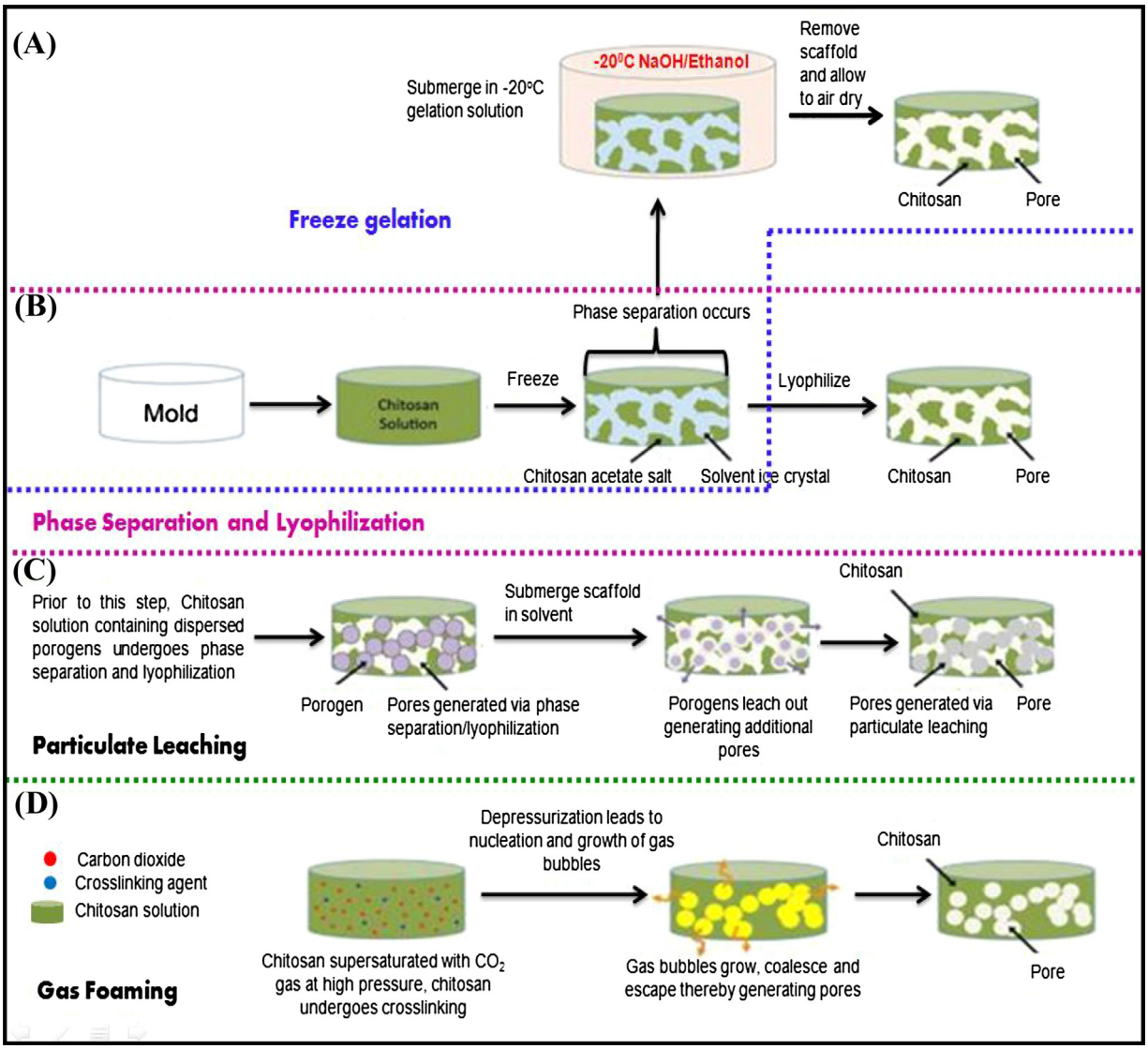

Fig. 2. Schematic representation of four commonly-used chitosan scaffold fabrication methods. (A) Freeze gelation technique, which initially involves phase separation due to freezing, (B) Phase separation and lyophilization technique, (C) Particulate leaching technique, and (D) Gas foaming technique. Redrawn from [59]. Copyright © 2014, Royal Society of Chemistry.

nificantly improved mechanical and biological properties [81] as compared to chitosan alone. More exploratory work with chitosan in the field of tissue engineering is required to utilize the medical potential of this polysaccharide to the fullest.

3. Chitosan in biosensing

In addition to its application in tissue engineering and regenerative medicine, chitosan has also been acknowledged as a promising material in biosensing technologies of various genres, targeting a number of biological and chemical molecular species. Owing to their unique properties and undisputed applications in numerous fields, biosensors have gained humungous success as an efficient analytical device consisting of BREs (such as, DNA, RNA, proteins, antibodies, enzymes, cell organelles, or whole live cells) coupled with a transducer surface that enables translation of a signal into detectable and quantifiable electrical signal [83–87]. These signals could be either in the form of intensity or color depending on the type of analyte and BRE. The fundamental benefits offered by a biosensor includes miniaturized system, high sensitivity and selectivity, portability, adaptability, ease of handling, and quick response in most cases [6,88–90]. However, certain challenges like development of inexpensive, durable, multi-purpose, and point-of-care testing volumes are faced even today when scientific community tries to achieve ASSURED standards for globally acclaimed biosensors [7,84,91]. Therefore, in order to meet the requirements and overcome these challenges, scientists have focused their attention towards polymer and (nano-) composite based biosensing strategies.

Chitosan, an environmentally benign, natural polymer has proven to be a suitable modifier of transducer surface by enabling mild yet stable immobilization of BREs. However, it is not the only biological polymer that can act as surface modifier (natural), other modifiers include amino acids in polymeric form (such as, poly-lysine, poly-glutamate, and poly-arginine), alginic acid, and hyaluronic acid [84]. Though, these modifiers have potential to be used in sensor development but, chitosan seems to supersede them by offering attractive benefits like non-toxicity, low cost, and being soluble in aqueous solution at mild acidic pH (6.3) which ensures immobilization of BREs without actually causing their denaturation. Additionally, properties like remarkable biocompatibility, prominent ability to form films, ease of availability

in different commercial forms, and choice of controlled electrodeposition as thin but tough film on negatively charged electrode (cathodes) make chitosan a great immobilization matrix for fabrication of wide range of biosensors [84,88]. Moreover, the chitosan based nanocomposites also play a very crucial role in developing biosensors by integrating BREs with varied types of transducers, like potentiometric, amperometric, thermometric, optical, piezoelectric, and conductimetric biosensors [92]. This section presents a comprehensive discussion on the usage of chitosan in biosensor fabrication incorporating first two types of transducers exclusively.

3.1. Chitosan modified electrochemical (EC) DNA biosensor

Biosensing of target single-stranded (ss) cDNA and short oligonucleotide chain (SOC) ladders in biological samples that have been pre-declared as biomarkers for inception or manifestation of clinical conditions by using conventional DNA microchip or microarray techniques have long been known [93–97]. Although, these techniques are of gold standard and highly effective but, they have certain issues against which better analytical techniques are being developed. In last few decades, EC biosensors have attracted huge researchers' attention as they can allow quick, yet selective analyte detection in a facile experimental setting without requiring exhaustive procedure and trained personnel [15,83,98,99]. Prerequisites of diagnosing biological samples by using these biosensors include synthesis of nucleotide probes, their accurate immobilization onto the surface of transducer, and ultimately detection of signal generated by analyte hybridization with the biosensing probe. The DNA based biosensing probes have made use of both carbon and gold as electrode material, however, use of gold is more common because immobilization of DNA probes onto the gold surface utilizes strong binding affinity amid gold and sulphur atoms, that in turn results in quick self-assembly of covalently attached 2-D monolayers when exposed to thiol (—SH) group containing organic molecules [100]. As, thiol based probe fixation is restricted to gold substrates, different procedures like, immobilization of biotinylated DNA probes through formation of a stable complex or covalent binding *via.* O_2 onto pre-activated carbon electrodes are required for anchoring DNA probes on the transducer surface. So far, several studies have shown that chitosan has potency to immobilize DNA probes and even mediate hybridization detection [101]. Not only this, it can also be used as a coating system when unmodified and as a nanocomposite material when in combination with CNTs or metal/Metal oxide NPs. In addition, chitosan mediated DNA probe immobilization facilitate both covalent as well as electrostatic interaction where, latter is formed due to interaction between chitosan's positively charged amino groups (protonated) and negatively charged phosphate groups in DNA backbone.

Different approaches have been applied for the immobilization of DNA that utilize protonated amino groups of chitosan to ensure either covalent or electrostatic bond formation. For instance, Zang et al. developed a DNA modified nanocomposite of reduced graphene oxide (rGO) and chitosan particles by a novel single-step technique for the EC detection of Hg^{+2} in drinking water. They proposed that this EC sensing technique exhibited excellent stability, high selectivity, and repeatability [102]. A recent study published by Chen et al. also reported development of chitosan based DNA biosensor, where, probe fabrication was done using nitrogen-doped graphene and Fe_3O_4NPs (as shown in Fig. 3(A)) [103]. In another work, a biosensor was developed by immobilizing ds-DNA on PAMAM (dendrimer)-encapsulated Au-Pd bimetallic nanoparticles (BNP) with chitosan composite as an immobilization layer, for successful detection of DNA damage and sericin's antioxidant activity(as shown in Fig. 3(B)) [104]. Similarly, other research groups also developed a DNA biosensing techniques by exploiting ds-DNA from fish sperm and immobilizing it on graphite [105] and chitosan-CNT modified screen printed carbon electrodes (SPCE) [106,107] in order to allow detection of deep DNA damage. Table 2(A) shows various other examples of EC based DNA biosensors where, chitosan has been used for the detection of pathogens like, viruses (*Yersinia enterocolitica* and *Cauliflower Mosaic virus*), bacteria (*Salmonella typhi* and *E. coli*), cancer, pesticides, *etc.* Though chitosan based DNA biosensors are extremely promising, however, an inherent limitation of non-specific interaction with functionalized interface coatings can compromise the analytical performance of the sensing probe. To address this limitation, two strategies have been employed first, reduction of amino groups within chitosan surface during or post attachment of probe strand and second, treatment of chitosan modified electrode surface with urea and Ca^{+2} or Mg^{+2} salt solutions after completion of hybridization step. Therefore, proper washing of modified biosensor surface with a buffer containing $CaCl_2$ and/or urea should serve the purpose of preventing non-specific binding and hence, improve the quality of analytical performance.

3.2. Chitosan modified EC enzyme biosensors

A large number of reports are available on biosensors which employ chitosan-enzyme complex when compared with reports on chitosan-DNA complex systems. Therefore, it is almost impossible for us to discuss all these reports in this review; however, we have included some representative works which will present chitosan's potential in enzymatic biosensors. Many of the recently proposed architectures for such biosensors are complex-multi-component systems, which integrate unmodified or pre-modified chitosan variants with an enzyme specific for the analyte molecule and an additional functional (nano)-material like metal/metal oxide nanoparticles, CNTs, graphene sheets, clays, ionic liquids, *etc.* Till today, a wide range of enzymes like oxidase, laccase, peroxidase, dehydrogenase, tyrosinase, urease, alliinase, phosphatase, reductase, hydrolase, and acetylcholinesterase have been exploited as BRE for various analyte detection [92]. The aforementioned functional materials have been used independently or in combination in order to generate redox cycle which link encapsulated or entrapped enzymes' active sites with the electrode interfaces electrically and increase the conductivity of immobilization matrix.

Since, the properties of an immobilized enzyme are greatly influenced by both support material and enzyme itself [84,92,108,109], it becomes really important to choose the material very judiciously. Though, it is very obvious that no material can act as a universal support for every enzyme, however, some characteristics, such as high binding affinity towards protein, accessibility of active functional groups, firmness, low cost, mechanical stability, hydrophilicity and reusability, and ease of fabricationare highly desirable for any and every support material. In addition to this, material should also be non-toxic and biocompatible if it is intended to be used for medical, pharmaceutical, agricultural, or food applications.

Of the several organic or inorganic, natural or synthetic support/carrier materials that have been exploited for immobilizing enzymes, chitosan qualifies for most of the desired qualities for enzyme immobilization permitting unhindered mobility of cofactor and substrate for their incessant accessibility to enzyme. So far, two feasible means of enzyme immobilization have been employed *viz.* physical entrapment and covalent binding of enzymes with poly-cationic chains of chitosan and most of the published approaches of binding enzyme-chitosan have made use of common cross-linkers, such as; glyoxal, carbodiimides, epichlorohydrin, glutaraldehyde, or N-hydroxysuccinimide, however, some specific cross-linking reagents (like cyanuric chloride) have also been successfully used [110]. A work published in 2010 studied electrical properties of thin chitosan films produced *via.* different cross-

Table 2
Various chitosan based EC biosensors have been summarized depending on (A) DNA as recognition element (B) Enzyme as recognition element, and (C) antibody as recognition element.

(A) Chitosan modified EC DNA biosensors

Sensing Probe Fabrication	Target Molecule	Dynamic Range	LOD	Real Sample	References
ssDNA/Chit-V_2O_5-MWCNTs/CILE	Loop-mediated isothermal amplification product of *Yersinia enterocolitica* gene sequence	1.0×10^{-11}–1.0×10^{-6} mol L^{-1}	1.76×10^{-12} mol L^{-1}	NR	[134]
ssDNA/Chit/PB/GP	Complementary DNA target sequence	2.12×10^{-11}–2.12×10^{-8} mol dm^{-3}	1.58×10^{-11} mol dm^{-3}	NR	[135]
ssDNA/GTD/Chit-MWNTs/GC	Short DNA species of gene fragment of transgenic plants (*Cauliflower Mosaic virus* 35S promoter)	1.0×10^{-13}–5×10^{-10} M	8.5×10^{-14} M	NR	[136]
ssDNA/Chit-Co_3O_4-GR/CILE	*Staphylococcus aureus* nuc gene sequence	1.0×10^{-12}–1.0×10^{-6} M	4.3×10^{-13} M	NR	[137]
ssDNA/GO-Chit/ITO	Complementary DNA strand for *Salmonella typhi*	10 fM–50 nM	10 fM and 100 fM	Serum	[138]
ssDNA/GO/Chit/GC	Detection of *Escherichia coli* O157:H7	1×10^{-14}–1×10^{-8} M	3.584×10^{-15} M	NR	[139]
ssDNA-coated Chit/CP	Mitoxantrone	0.03–120 mg L^{-1}	0.0013 L^{-1}	Human blood serum	[140]
ssDNA/Chit-NVTO/ITO	Breast cancer susceptible gene	1.0×10^{-15}–1.0×10^{-6} M	1.09×10^{-15} M	NR	[141]
CB/rGO-Fe_3O_4-Chit/GC	H_2O_2	0.1–1.1 μM and 17–38 μM	42 nM	NR	[142]
ssDNA/3DN-G/Fe_3O_4/GC	Complementary DNA target sequence	1.0×10^{-14}–1.0×10^{-6} M	3.63×10^{-15} M	NR	[103]
ssDNA/MnTPP/rGO	Complementary DNA target sequence	100 aM–10 pM	6×10^{-14} M	NR	[143]

(B) Chitosan modified EC enzyme biosensors

Sensing Probe Fabrication	Target Molecule	Dynamic range	LOD	Real Sample	References
Chit/GOD/Pt	Glucose	50–3000 μM	30 μM	NR	[144]
AChE/Chit/GC	Acetylthiocholine	0.005–0.039 mM and 0.064–0.258 mM	1.58×10^{-10} M.	NR	[145]
Chit/GOD@AgTNPs/Pt	Glucose	9–3000 μM	1 μM	NR	[144]
ChOx/Chit/GC	Choline	1.0×10^{-7}–5×10^{-4} molL^{-1}	1×10^{-8} molL^{-1}.	Milk choline hydrolysate samples	[146]
ChOx/nan-NiO-Chit/ITO	Cholesterol	10–400 mg dL^{-1}	43.4 mgdL^{-1}	Serum	[147]
Au/CeO_2–Chit	H_2O_2	50–2500 μM	7 μM	NR	[148]
GOD/ZrO_2/Chit/ER-GO	Glucose	0.2–1.6 mM	45.6 mM	NR	[149]
GluOx/cMWCNT/AuNP/ChitAu	Glutamate	5–500 μM	1.6 μM	Blood serum	[150]
GlOx/Chit-CNT-AuNW/GC	Glutamate	5–20 mM	1.2 μM	Food items	[151]
Tyrs-rGO/Mn_3O_4/ITO	Bisphenol A	0.05–100 mM	10 nM	NR	[114]
GOD/Chit/Pd@Pt NC	Glucose	1–6 mM	0.2 μM	NR	[152]
(Chit/GLM)8-GC	Hg^{2+}	0.5–5 μM	76 nM	NR	[153]
AChE/Chit@TiO_2-Chit/rGO/GC	Organophosphate pesticides	0.036–22.6 μM	29 nM	NR	[154]

(C) Chitosan modified EC immuno-sensors

Sensing Probe Fabrication	Target Molecule	Dynamic range	LOD	Real Sample	References
BSA/Ab-Vc/Chit–NiO/ITO	*Vibrio cholerae*	20–700 ng mL^{-1}	0.108 ng mL^{-1}	NR	[155]
HRP/HA-Ab/PB-Chit-AuNP	Histamine	0.01–100 μg mL^{-1}	1.25 pg mL^{-1}	NR	[156]
Ag-NC/Chit	Mouse IgG	0.60–40.00 μg mL^{-1}	–	NR	[157]
thGP/thCTS/AuNPs	Carcinoembryonic antigen (CEA)	0.3–8.0 ng mL^{-1} and 8.0–100 ng mL^{-1}	0.03 ng mL^{-1}	Human serum	[158]
12HC-G/SP	Fibrinogen	938–44542 μg dL^{-1}	500 μg dL^{-1}	Human serum and unprocessed blood	[132]
anti-PSA/AuNPs–PAMAM-dendrimer/MWCNTs/IL/Chit/GCE	PSA	0.05–80 ng mL^{-1}	0.001 ng mL^{-1}	Human serum	[159]
SWA/Chit-Glut/SP	*Schistosoma Mansoni* Antibodies	0.038–20 ng mL^{-1}	–	NR	[160]
HRP-anti-AFP-ISNGs	α-fetoprotein	0.02–4.0 ng mL^{-1}	10 pg mL^{-1}	Human serum	[161]
Antibody/AuNPs-Chit-GC	*Bacillus cereus*	5.0×10^1–5.0×10^4 cfu mL^{-1}	10 cfu mL^{-1}	NR	[162]
Chit/AuNPs/anti-CEA	CEA	0.1–2 and 2–200 ng mL^{-1}	0.04 ng mL^{-1}	Human serum	[163]
BSA/anti-CEA/Chit-CNTs-AuNPs	CEA	0.01k80 ng mL^{-1}	0.0034 ng mL^{-1}	Human serum	[164]
Anti-humanIgG/coral-shaped AuNPs-Chit	Human IgG	0.05 to 50 ng mL^{-1}	0.005 ng mL^{-1}	NR	[165]
anti-CEA/AuNPs-Nafion/Fc-Chit/GC	CEA	0.01–150 ng mL^{-1}	0.003 ng mL^{-1}	NR	[166]
BSA/anti-hCG/AuNPs-TiO_2/Thi/GTD/MWCNTs-Chit/GC	Human Chorionic gonadotrophin	0.2–300 ng mL^{-1}	0.08 ng mL^{-1}	NR	[167]
HRP-anti-*S. flexneri*/MWCNT/Chit	*Shigella flexneri*	10^4–10^{11} cfu mL^{-1}	3.1×10^3 cfu mL^{-1}	NR	[168]

Table 2 (*Continued*)

(A) Chitosan modified EC DNA biosensors

Sensing Probe Fabrication	Target Molecule	Dynamic Range	LOD	Real Sample	References
anti/PSAAu-hydroxyapatite nanocomposite/Chit	PSA	3.5–30 ng mL^{-1}	2.6 ng mL^{-1}	Serum	[169]
BSA/anti-AFP/AuNPs/Thi/Chit-AuNPs	α-1-fetoprotein	0.4–200 ng mL^{-1}	0.24 ng mL^{-1}	Human serum	[170]
anti-CEA/AuNPs/CNT/Chit	CEA	0.3–2.5 and 2.5–20 ng mL^{-1}	0.01 n gmL^{-1}	Human serum	[171]
QDs/CNTs-PDDA/AuNPs-Chit	Human IgG	0.006–150 ng mL^{-1}	0.001 ng mL^{-1}	NR	[172]
anti-humanIgG/Chit-AuNPs/CP	Human IgG	0.3–120 ng mL^{-1}	0.1 ng mL^{-1}	Serum	[173]

*Single stranded (ss), Multi-walled Carbon Nanotubes (MWCNTs), Carbon Ionic Liquid Electrode (CILE), Chitosan (Chit), Prussian blue (PB), Graphene Paste (GP), Glutaraldehyde (GTD), Glassy Carbon (GC), Graphene (GR), Graphene oxide (GO), Carbon Paste (CP), NbV co-doped TiO_2(NVTO), Celestine Blue (CB), Reduced graphene oxide (rGO), 3-Dimensional N doped Graphene (3DN-G), Manganese (III) tetraphenylporphyrin (MnTPP), Glucose oxidase (GOD), Acetylthiocholine esterase (AChE), Triangular Silver nanoparticles (AgTNPs), Nanostructured NiO (nan-NiO), Electrochemically reduced graphene oxide (ER-GO), Glutamate oxidase (GluOx), Carboxylated Multi-walled Carbon Nanotubes (cMWCNT), Gold Nanowires (AuNW), Tyrosinase Reduced graphene oxide (Tyrs-rGO), Nanocomposite (NC), GOD liposome microreactors (GLM), Bovine serum albumin (BSA), Antibody *Vibrio cholerae* (Ab-Vc), Indium Tin Oxide coated (ITO), Histamine antibody (HA-Ab), Horseradish Peroxidase (HRP), Gold nanoparticles (AuNPs), Silver nanocomposites (Ag-NC), Thiol Graphene (thGP), Thiol Chitosan (thCTS), Screen printed (SP), Prostate specific antigen (PSA), Antibody specific to Prostate specific antigen (anti-PSA), Polyamidoamine (PAMAM), Interleukin (IL), *Schistosoma* antigen (SWA), α-fetoprotein (AFP), Irregular-shaped gold nanoparticles (ISGNG), Carcinoembryonic antigen (CEA), Antibody CEA (anti-CEA), Chitosan-ferrocene (Fc-Chit), Thionine (Thi), Antibody *Shigella flexneri* (anti-*S. flexneri*), Carbon Nanotubes (CNT), Quantum Dots (QDs), Polydiallyldimethylammonium chloride (PDDA)

linking chemistries and proposed that the type of cross-linking reagent may influence the sensor performance [111]. Therefore, an approach which avoids such enzyme modification strategies can be a better method of choice. Physical entrapment is one such method which does not require chemical modification and entraps the enzyme within chitosan network by electrostatic attraction amid protonated amino groups in chitosan and multi-valent anion, tri-polyphosphate [112], for instance.

In efforts to fabricate an enzyme based EC biosensor with better analytical performance, chitosan composites modified by redox mediator have been developed. For instance,Wang and coworkersdesigned a tyrosinase biosensor by exploiting iron oxide NPs and chitosan nanocomposites, for the determination of phenolic compounds. The designed biosensor offered better analytical performance because iron oxide NPs ensured huge surface area that allowed effective loading of tyrosinase enzyme in large amount and chitosan provided high porosity which ensured enzyme entrapment by retaining its biological activity [113]. Similarly, chitosan based enzymatic biosensors has been reported by the Reza et al. for the determination of plasticizer bisphenol A (BPA), where the probe was fabricated using rGO/Mn_3O_4 based modification on an ITO electrode (as shown in Fig. 4(A)) [114]. In another work, Dalkiranand coworkers fabricated a novel biosensor that made use of Co_3O_4NPs, chitosan, and MWCNTs in order to modify glassy carbon electrode. This system investigated immobilization and co-immobilization of two different enzymes, mono-enzyme xanthine oxidase and bi-enzyme xanthine oxidase (XO)/horseradish peroxidase in respective order. Development of this complex system allowed efficient detection of xanthine (shown in Fig. 4(B)) by electro-oxidation and electro-reduction of hydrogen peroxide that was produced as a result of enzymatic reaction [115]. In a different study published by Zhao et al. a novel nanocomposite was developed by direct electrodeposition of rGO, gold NPs, β-cyclodextrin and Prussian blue-chitosan (PBC) on a glassy carbon electrode. This PBC modified electrode not only allowed efficient immobilization of acetylcholinesterase enzyme, but also catalyzed oxidation of thiocholine and reduced its oxidation potential to 0.2 V from 0.68 V thus, ensuring successful fabrication of an organophosphorus pesticide biosensor [116]. Several other examples of chitosan composite modified EC biosensor which allowed detection of analytes like carbohydrate, neurotransmitters, heavy metals, *etc*., are mentioned in Table 2(B).

3.3. Chitosan modified EC immuno-sensors

The ability of EC immuno-sensors to allow analyses with detection limit in the range of attomolar [117,118] and sub-attomolar [119] has led to the development of various types of immunosensors that have found their applications in various domains like food analyses [120,121], clinical diagnoses [122–127], and environmental monitoring [128–130]. An immuno-sensor whether EC based or not, employs antibodies (Abs), which interact only with

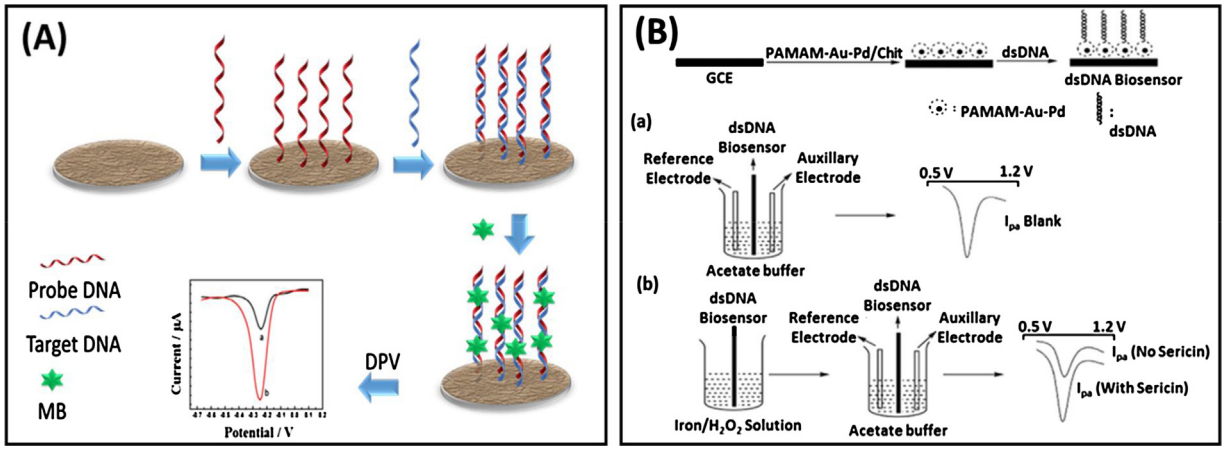

Fig. 3. Schematic representation of chitosan modified EC DNA biosensors for detection of (A) target DNA sequence, adapted with the permission from [103],© 2016 Published by Elsevier B.V. and (B) DNA damage and sericin's antioxidant activity, reprinted with the permission from [104], © 2017 Springer International Publishing AG. Part of Springer Nature.

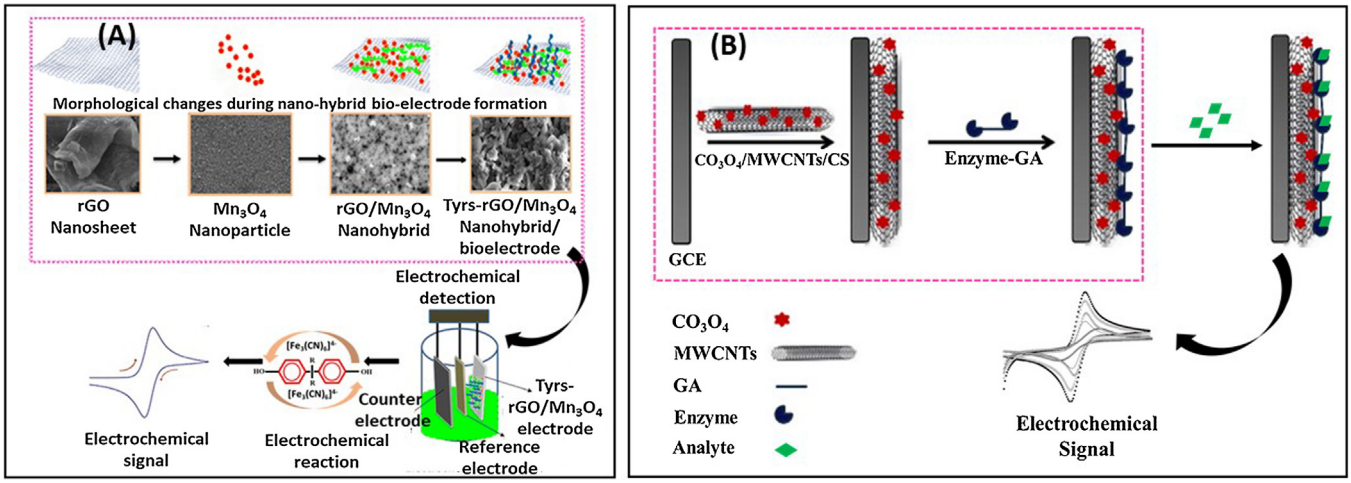

Fig. 4. Schematic representation of Chitosan modified EC enzyme biosensors for determination of (A) plasticizer bisphenol A (BPA), adapted with permission from [114],© 2017 Springer International Publishing AG. Part of Springer Nature, and (B) xanthine, adapted with permission from [115], Copyright © 2014 Elsevier B.V.

their complementary analytes, antigens (Ags) specifically and eventually allows their detection. A very important step in designing of EC immuno-sensors is, firm yet gentle immobilization of Ab or Ag onto the electrode interface, for which several fabrication strategies have been proposed. As mentioned in several published works both research and review articles, one condition that is always sought in sensing techniques is achievement of high sensitivity. This condition can be easily realized by loading large amount of analyte capturing immuno-chemical moieties (ICMs) onto the working electrode surface as, higher the density of ICM on electrode, higher will be the magnitude of electrical signal generated on binding of analyte molecule. In the view of this, approaches for stable but facile, effective, and reproducible loading of Ab or Ag in large amount on electrode surface have been highly sought. In this regard, the advantage of using different forms of chitosan films is that the number of binding sites available for covalently attaching protein molecules on chitosan material is highly flexible and easily attainable. Another advantage of using chitosan as immobilization material is that it allows possibility of automated, voltage-driven EC deposition which is not seen in other traditional materials [84].

Over last one decade, several reports on chitosan-supported EC immuno-sensors for detection of different biomarkers *viz.* aflatoxin B1, carcinoembryonic Ag, ferritin, prostrate-specific-Ag, ochratoxin (food contaminant), fibrinogen, *etc.* have been published. A report published by Yang et al. showed development of an EC immuno-sensor by using single wall carbon nanotubes (SWCNTs) and chitosan modified electrode for analysis of fumonisin B_1 (FB_1) in corn (Fig. 5(A)). The detection mechanism exploited by this immuno-sensor was indirect competitive binding of free FB_1 and BSA modified FB_1 to a particular amount of immobilized anti-FB_1 on SWNTs-chitosan modified glassy carbon electrode. The anti-IgG Ab from rabbit (secondary Ab) labelled with alkaline phosphatase was then bound *via.* α-naphthyl phosphate to electrode interface, which ultimately generated EC signal [131]. Similarly, Saleem et al. reported chitosan based biosensor for the detection of fibrinogen, where the sensing assembly was mounted over the screen printed electrode, the detailed scheme has been shown in Fig. 5(B) [132]. In another study, performed by Xia and coworkers allowed fabrication of highly sensitive EC immuno-assay for the detection of serum amyloid A (SAA), a serum inflammation biomarker. This sensing strategy made use of carboxy-endcappedpolypyrrole (CE-PP), multi-walled carbon nanotubes (MWCNTs), ionic liquid, and chitosan as support interface for modification of a glassy carbon electrode. Use of nanocomposite as modifier material, enhanced the sensitivity and stability of immunoassay. Anti-SAA was fixed on single layered surface comprising of CE-PP that allowed specific binding of SAA and thus, generated EC signal to quantify the presence of SAA [133]. Table 2(C) shows other representative works on EC immuno-sensors. Many of the EC immuno-sensors which exploit chitosan matrices for immobilization, undergo an issue of non-specific binding, which is necessary to avoid in order to ensure high analytical performance of the immuno-sensor. Usage of a protein, bovine serum albumin (BSA) for instance, can easily prevent this issue and successfully retain the analyte sensitivity. Other blocking agents used for the purpose may include pre-immune serum, ovalbumin, skimmed milk proteins, and surfactants/detergents like Tween 20 [84].

4. Conclusions

Myriad reports including original and review articles have been published on chitosan and its nano material based composites in last few decades. The incessant work on chitosan and its composites' application in numerous fields reveal the importance of chitosan as a functional material. Chitosan is a natural polymer which possesses unique properties like biodegradability, biocompatibility, hydrophilicity, *etc.* and can be used in biomedical and biosensing domain, if combined with non-toxic matrices. In the present review, research advancements focusing on application of chitosan and its composites in biomedicine, regenerative medicine, and biosensing applications have been explicitly discussed. In recent years, tissue engineering has evolved as a novel interdisciplinary field to repair or replace injured/damaged parts and thus, restore their function. We seriously hope that the tissue engineering approaches with novel materials, chitosan, for instance will provide methods for developing a better understanding of body parts and associated pathologies. The ability of chitosan to exhibit spectacular muco-adhesive, permeation enhancing, *in situ* gelling, and efflux pump inhibitory properties along with ionic interaction has made it suitable to develop systems for targeted drug delivery and controlled drug release. Owing to its additional properties, such as low cost, high biocompatibility, ability to solubilize in aqueous solution, ease of availability in varied commercial forms, and ability to form thin films, chitosan has become a great immobilization matrix for fabrication of wide range of biosensors. These properties altogether impart significant versatility to chitosan and imply it to have exceptional future in aforementioned fields.

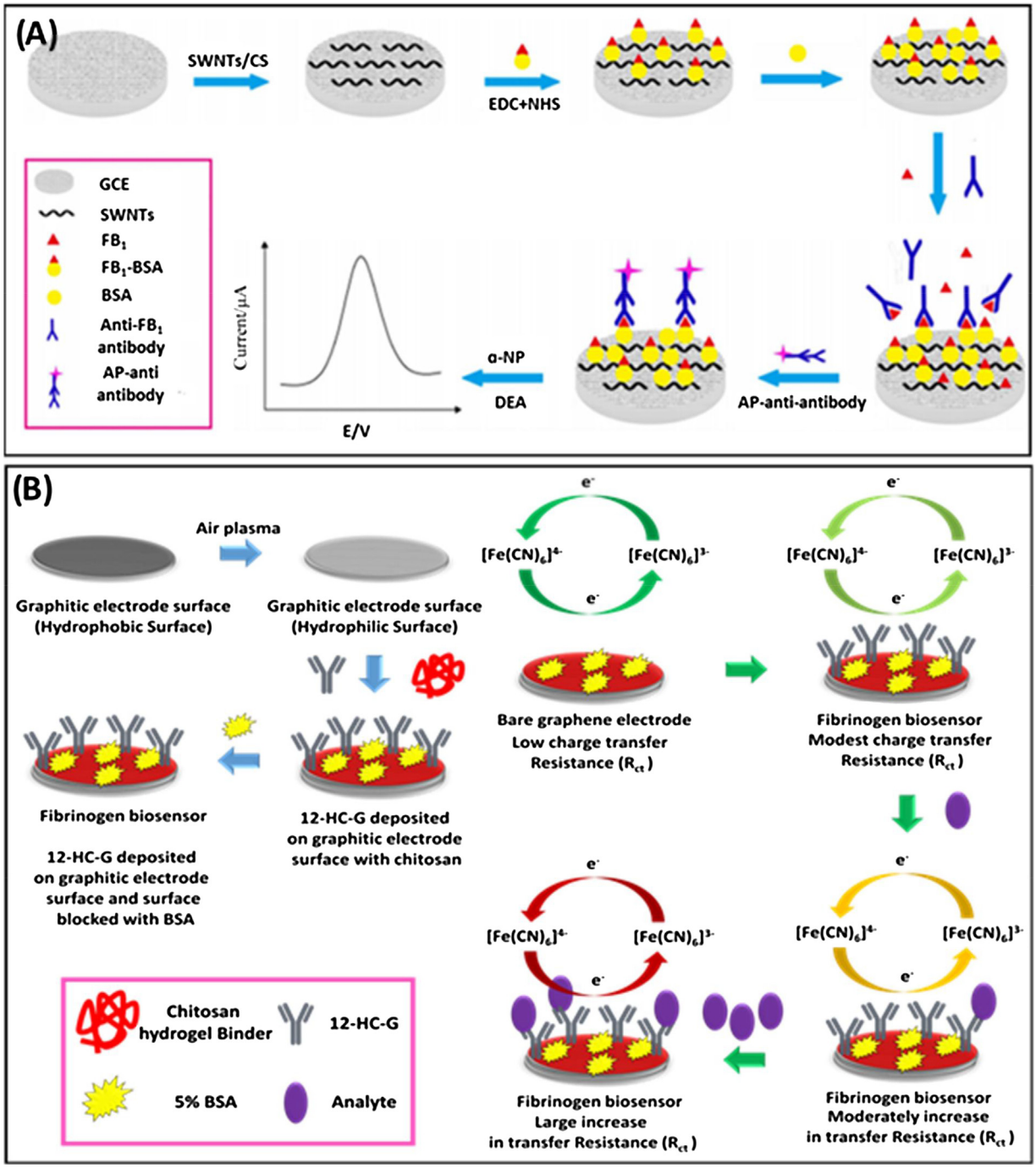

Fig. 5. Schematic representation of Chitosan modified EC immuno-sensors for determination of (A) corn Fumonisin B_1, reprinted with permission from [131] Copyright © 1999–2017 John Wiley & Sons, Inc.; and (B) fibrinogen, an indicator of trauma induced coagulopathy, adapted with permission from [132] © 2016 Elsevier B.V. Abbreviations: chitosan (CS), 1-ethyl-3-(3-dimethyllaminopripyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), alpha-naphthyl phosphate (NP), Diethanolamine (DEA).

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