Recent development in chitosan nanocomposites for surface-based biosensor applications

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Abbreviations:

2-D	Two dimensions
AChE	Acetylcholinesterase
AFP	α-fetoprotein
APTES	(3-Aminopropyl) triethoxysilane
AuNP	Gold nanoparticle
BRE	Biological Recognition Element
BSA	Bovine serum albumin
CA	Carbohydrate antigen
CEA	Carcinoembryonic antigen
CFU	Colony-forming unit

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CHI, CS	Chitosan
CNT	Carbon nanotube
CV	Cyclic voltammetry
DNA	Deoxyribonucleic Acid
DPV	Differential pulse voltammetry
E. coli	*Escherichia coli*
EDC	(N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride)
Fc	Fragment crystallizable region
FTIR	Fourier-transform infrared spectroscopy
GA	Glutaraldehyde
g-C3N4	Graphitic carbon nitride
GCE	Glassy carbon electrode
GQD	Graphene quantum dot
HER2	Human epidermal growth factor receptor 2
ITO	Indium tin oxide
kDa	Kilodalton
LOD	Limits of detection
M	Mole
MEMS	Microelectromechanical systems
miRNA	Micro ribonucleic acid
MWCNT	Multi-walled nanotube
NHS	N-Hydroxysuccinimide
NVTO	Niobium and vanadium codoped titanium dioxide
QD	Quantum dot
RAC	Ractopamine
RSD	Relative standard deviation

S/N	Signal-to-noise ratio
SAM	Self-assembled monolayer
SEM	Scanning electron microscope
SPR	Surface plasmon resonance spectroscopy
ssDNA	Single-stranded DNA
SWCNT	Single-walled nanotube
TMD	Transition metal dichalcogenide

Abstract

Recent years have witnessed ever expanding use of biosensors in the fields of environmental monitoring, homeland security, pharmaceutical, food and bioprocessing, and agricultural industries. To produce effective and reliable biosensors, good quality immobilization of biological recognition elements is critical. Chitosan and its nanocomposites emerge as an excellent immobilization matrix on biosensor surface. As a natural polysaccharide, chitosan has many useful characteristics, such as high permeability and mechanical strength, biocompatibility and non-toxicity, availability and low cost. Due to the presence of amino and hydroxyl groups on chitosan, chitosan can easily crosslink with a variety of nanomaterials. This investigation of chitosan nanocomposite-based biosensors presents recent development and innovations in the preparation of chitosan nanocomposites in coordination with biosensors for various bio-detection applications, including chitosan nanocomposites formed with carbon nanomaterials, various inorganic and biological complexes. These chitosan nanocomposite based biosensors have demonstrated good sensitivity selectivity and stability for the detection of different types of targets ranging from glucose, proteins, DNAs, small biomolecules to bacteria. It is our hope that this review will offer guidance for the development of novel biosensors and open up opportunities in the field of biosensor research.

Color online: See article online to view Figs. 1, 3–8 in color.

1. Introduction

Over the past few decades, biosensors have expanded their applications in various fields such as the food industry [1-5], medical diagnostics [6-10] and treatment, national security [11, 12], and environmental monitoring [13-19], owing to their simplified operat ion, low consumption of reagents and energy, cost-effectiveness, and small form factor. Much research effort has been devoted to the design and development of sensitive and specific biosensors [20-22]. In many biosensors, detection occurs when a soluble analyte target binds with a complementary ligand, known as biological recognition element (BRE), immobilized on the sensor surface, which is known as a surface-based biosensor. It has been widely recognized that the performance of biosensors is closely related to the intrinsic characteristics of immobilized BREs, which includes their biological activities, size, and binding capability for target analytes [23, 24]. In fact, the characteristics of immobilized BREs are determined not only by the intrinsic properties of BREs, but also by the substrate material that the

BREs are immobilized on, because the surface interaction can lend BREs additional kinetic and physic-chemical properties [25]. Improving the surface modification and immobilization of BREs on sensors can greatly enhance the specificity and sensitivity of surface-based biosensors, which is crucial to the sensor performance. Thus, a judicious choice of substrate materials can greatly enhance the immobilized system.

A wide variety of nanomaterials, such as carbon nanotubes, graphene, and polymers, have been extensively studied as an intermediate substrate material between BREs and electrode surfaces [26-29], in an effort to realize highly sensitive and selective surface-based biosensors. While there has been progress in the sensitivity and detection limit of biosensors when using these inorganic nanomaterials, there continues to be challenges in obtaining uniform dispersion and high-electrodes surface coverage of BREs due to the hydrophobic nature of inorganic nanomaterials [30]. As a result, production of nanomaterial-based biosensors faces difficulty associated with the lack of stability and repeatability. Natural polymer materials such as cellulose and chitosan are low-cost and environment-friendly with strong gel-forming ability, thus most of them are suitable for use in biosensor preparation [31]. Among common natural polymers, chitosan possesses physical advantages of good mechanic strength, hydrophilicity, easy-processability and chemical advantages of biocompatibility, stability in bio-environment, capability of chemical modification [32]. There have been reports over the past decade using chitosan as a substrate material instead of conventional inorganic nanomaterial for the preparation of electrochemical biosensors [32, 33]. In these reports, the electrochemical sensors have shown improved stability and repeatability even after weeks or months storage, in addition to reduced cost. Recently, to take advantage of its ability to interlink between the hydrophobic nanomaterial and metal/carbon/resin substrate, research has start combining chitosan with conventional nanomaterials to form commixture nanomaterial [27, 28].

In this report, most recent development (2013-2018) of chitosan modification methods for surface-based biosensors will be reviewed, aiming to offer guidance for the development of nanomaterial-based biosensors and open up opportunities in the field of biosensor research. The basic concepts and operation principles are reviewed first. Then, related characteristics of chitosan, and surface modification methods as well as the BREs immobilization approaches on chitosan nanocomposites are discussed. Finally, some advanced examples of surface-based sensors, such as immunosensors, enzyme sensors, and DNA based sensors are presented.

2. Surface-based biosensor principles

Surface-based biosensor has been a hot research area in recent years. A typical biosensor system can be generally defined as an analytical transducer that can convert a biological input response to a processable and quantifiable electronical output signal [34]. As schematically shown in Figure 1 [35], a surface-based biosensor usually has following components: a) a bio-probe that specifically bind to or react with the target analyte; b) an interfacial structure which supports the reaction sites and gives rise to an electrical signal that can be captured by c) the transducer architecture for signal recovering, amplifying and enhancement; and d) and e) digital processing units for data analysis and human-interface display. The transducer can be an optical device based on surface plasmon resonance or absorbance or induced fluorescence, a mass sensitive sensor such as a quartz microbalance or a microcantilever, or an electrical or electrochemical biosensor.

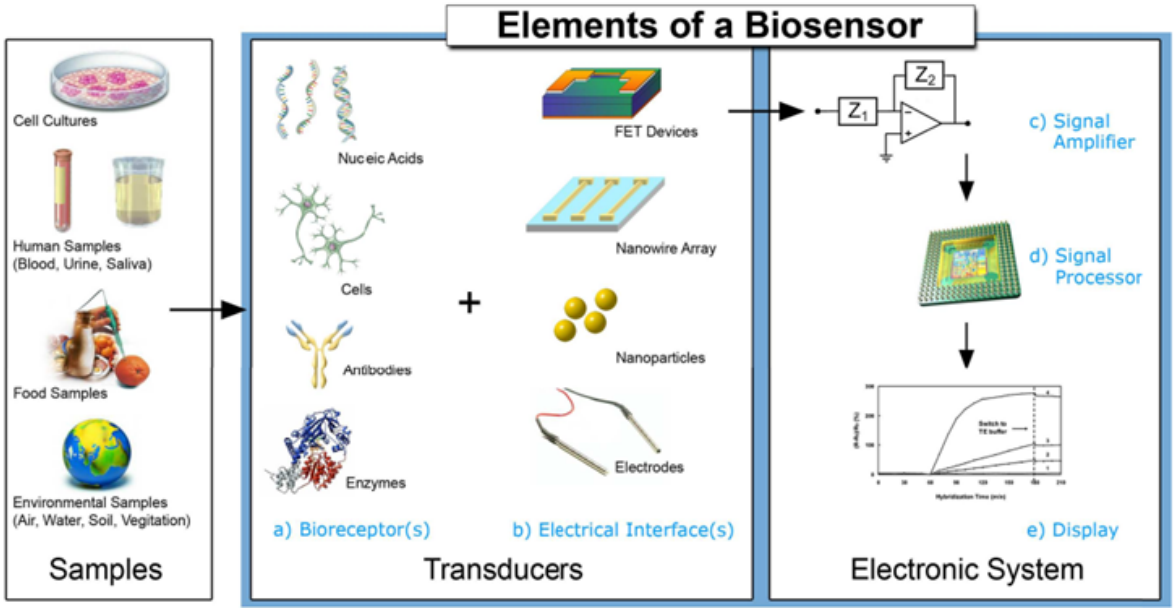

Figure 1 Elemental components of a typical biosensing system [35].

Antibodies [35], single-stranded (ss) DNA [36] are the most commonly used BREs that offer good selectivity and strong affinity toward specific targets. Hybridization or conjugation reaction between BREs and target analytes will induce surface morphology changes or localized disturbance [37, 38], which can be converted into an electrical signal by the transducers. Enzyme is another type of widely used BREs. Instead of affinity reaction between targets and probes, enzyme biosensor is based on the specific catalytic reaction between substrate and immobilized enzyme [21]. Once the target reagents or biomolecules are applied, reduction-oxidation reaction will be activated, augmented or inhibited, which is often monitored by an electrochemical sensor such as cyclic voltammetry (CV) or differential pulse voltammetry (DPV).

As the name implies, surface-based biosensors have their BREs immobilized on the sensor surfaces. Commonly used supportive sensor material includes quartz/Silicon/silicon dioxide, noble metal/metal oxide, carbon, and polymer film [39]. In addition to some general requirements e.g., non-toxicity, chemical stability, insolubility in testing solution, there are extra requirements for sensor materials, depending on the detection mechanism and the BREs characteristics. For examples, enzyme biosensors usually prefer the sensor materials to have good conductivity and high charge carrier mobility to obtain high sensitivity [21].

Nondestructive immobilization of BREs onto the transducing surfaces (also known as "functionalization") is a dominant issue to achieve reliable measurement. A successful surface immobilization should be able to attach BREs to the sensor surface firmly and maintain their biological activity in the meanwhile.

Most of the widely reported immobilization methods can be classified as a) physical adsorption and b) covalent chemical bonding [40]. The physical absorption method is simple and low-cost, but has the shortcomings of poor uniformity and low probe coverage, while the covalent bonding can provide a more reliable attachment quality but need sophisticated handling [40]. Some typical examples of covalent bonding include self-assembled monolayer (SAM) of thiolated- ssDNA on bare gold surface

[40], and (3-Aminopropyl) triethoxysilane (APTES) as a linker to connect the carboxyl group of protein to hydrophilic electrode surface, with the aid of N-Hydroxysuccinimide (NHS)/(N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) (EDC) [41].

The last step in sensor preparation is usually blocking. To minimize non-specific binding and improve the selectivity, a blocking agent, such as Tween-20, 6-Mercapto-1-hexanol and bovine serum albumin (BSA), is subsequently applied to cover any non-occupied spaces on the sensors [42]. This blocking process needs to be compatible with the immobilized BREs, i.e., the blocking molecules should not replace the BREs.

To achieve lower limits of detection (LOD), much research work has been done to increase the effective immobilization density. Some study [43] found that the detection limit and specificity of surface-based sensors often depend more strongly on the effective BRE concentration, i.e., the density the BREs are loaded on the sensor surface, than on the true affinity of the BREs. Different types of surface-based biosensors have different additional considerations. Immunosensors often prefer capture of antibody in a specific orientation (e.g., Fc) and control the interlinking position, to ensure that antibody's binding site is more assessible for binding [44]. DNA based biosensors, on the other hand, demand an optimized immobilization density since dense ssDNA surface immobilization will result in repulsion effect and dramatically reduce the sensitivity [45]. So the density of ssDNA probe needs to be optimized. In addition to the requirement of controlled immobilization density of BREs, the durability and preservation of activity of sensing film on sensor surfaces are also important considerations, especially for point of care and on-site testing with complex and unpredictable matrices. Biosensors with chitosan-based substrate have demonstrated long storage time [32, 33].

To further increase BRE load to achieve more sensitive biosensors, it becomes increasingly challenging for traditional 2-D immobilization strategies. Two dimensional structures of planar sensor surfaces can only support a limited capacity of immobilization density owing to their low area-to-volume ratio. While analytical performance of biosensors can be improved by optimizing architectures and geometric parameters of biosensor designs, deploying nanostructured interfacial materials between BREs and sensor material is demonstrated to be an effective approach that has been extensively explored in recent years [46]. The investigation has included nanomaterials made from metals, semiconductors, and organic compounds.

3. Basic concepts of Chitosan

Chitosan is a natural polycationic linear polysaccharide, and is one of the most important derivation of chitin. The main sources of chitin can be found from many types of living creatures in terrestrial, marine, and freshwater ecosystems, including the exoskeletons of arthropods including crustaceans, arachnids, and insects, e.g., crustacean shells, shellfish such as crabs, shrimp, prawns, and beaks of cephalopods. Chitosan also exists in some microorganisms, for example the cell walls of fungi to provide structure supporting strength and maintain the cell's shape. It is the second most abundant natural polysaccharide just after cellulose in the ecosphere. Usually, chitosan is marketed and stored in the physical forms of white powders, flakes, and small beads. Chitin and chitosan exist mainly in the form of highly basic polysaccharides. While the majority of naturally occurring polysaccharides, e.g., pectin, dextrin, agarose and carragenas are usually either neutral or negatively charged in an acidic environment, chitosan exhibits cationic characteristic, i.e., being positively charged [47]. This

characteristic allows chitosan to readily form composites or multilayer structures with other synthetic or natural polymers owing to electrostatic attraction.

Chitosan can be derived from chitin with N-deacetylated method. It belongs to linear polysaccharide, and contains copolymers of D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units) linked by b (1,4) glycosidic bonds [48] as shown in Figure 2 A. Based on the degree of deacetylation, chitosan possesses different characteristics and molecule weights. By different degrees of deacetylation, chitosan can be classified three categories as high (>∼500 kDa), medium (∼50−500 kDa), and low (<∼50 kDa) molecular weights [49]. Chitosan exhibits several special physical properties such as viscosity, mucoadhesivity and possible solubility in various media.

The solubility of chitosan in aqueous solution can be adjusted according to the needs of biosensors, by modifying the pH value, (usually below pH 6.5) ionic strength of aqueous medium, and the degree of deacetylation. Besides tunable solubility, chitosan possesses reactive amino and hydroxyl groups in its linear polyglucosamine chains, which makes chitosan amenable to chemical and biological adjustability. For example, the active primary amino groups in chitosan backbone can provide special chemical-active platform for side group attachment under mild reaction conditions, which expands the applications of chitosan in bio-fabrication [50]. As a result, the biological and physical characteristics of chitosan are flexible and can be readily modified to achieve specific functions [51].

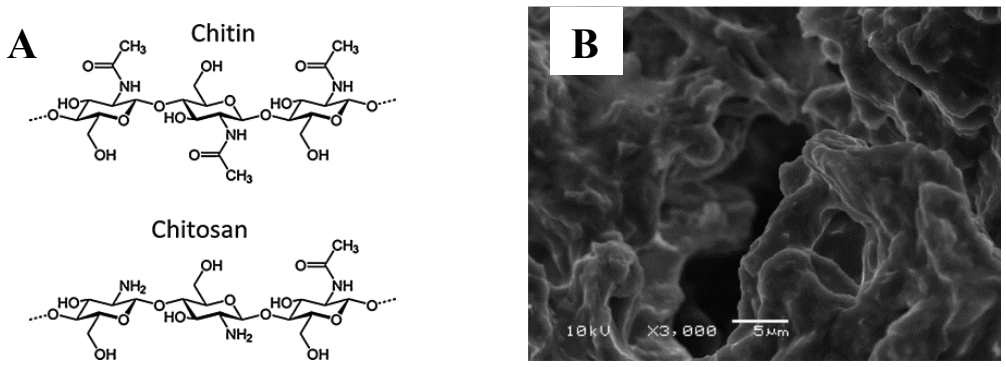

Figure 2 (A) Structures and different function groups of chitin and chitosan and (B) SEM images of porous chitosan membranes (B). Reproduced from [52]

As discussed in the earlier section, proper choice of materials can increase the efficiency of BREs immobilization. Although it is recognized that it is impossible to find a universal substrate to support all kinds of BREs and sensors, chitosan ranks close to the top as a suitable substrate material for biosensor design, owing to its abundant lateral functional groups for biological/chemical binding, high mechanical rigidity and area-to-volume ratio. As shown in Fig. 2 B, a chitosan membrane has very porous surface topography, with pore size on the order of micrometer [52]. The porosity of chitosan can dramatically expand the surface area of traditional planar sensors and increase the loading capacity of BREs in unit surface area, thus amplifying the response of output signals. Additionally, when detecting analytes smaller than bacteria, porous structures of chitosan films have negligible effect on analyte diffusion towards BREs. Besides the diversity and flexibility of structures and functional groups of chitosan, other important benefits of chitosan are that it is non-toxic, safe for humans, and also eco-friendly in all stages, since the raw materials are biodegradable and renewable. Being nontoxic is important to biosensor materials, because it leads to low immunogenicity, which

ensures no or little false responses incurred from incompatibility between samples and sensor materials.

4. Preparation of surface-based biosensors using chitosan

Because the solubility of chitosan in aqueous systems can be simply modified by pH value, chitosan polymer film can be attached to most sensor surface with controlled thickness and desired modification. The most basic method of making chitosan membrane on a solid surface is solution casting [53]. Chitosan powder is originally dissolved in acetic acid, then sensors are coated with the chitosan solution. As solvent evaporates out the chitosan solution, the pH value will gradually climb and chitosan will become insoluble. Finally chitosan film forms on the surface after several hours. The thickness of chitosan during casting can be roughly determined by the raw solution concentration. When denser and smoother chitosan films are desired, spin coating is usually adopted during the accretion of chitosan solution [54]. Primary limitation of casting and spin coating methods arises from the requirement of flat surface [53]. Sensors with uneven surface or complicated topography will cause localized chitosan film accumulation.

Another interesting method is layer by layer assembly, an extension of solution casting [55]. By alternately exposing the substrate to the cationic chitosan solution and anionic solution several times, a multi-level film can be produced by electrostatic attraction.

Chitosan can also be integrated onto sensor surfaces by electrodeposition [56]. In fact, chitosan films are not good conductors and the term “electrodeposition” is not exactly what used in other fields. There is no direct charge exchange between chitosan and sensor materials. As shown in Figure 3 A, when a pair of anode and cathode with a typical voltage are applied to the chitosan solution, the electrochemical reaction will result in a locally higher pH region near the cathode surface and cause a shift in the solubility, which is shown in Figure 3 B. Because chitosan’s solubility is pH value dependent, if the local pH is above a threshold value (usually 6.5), chitosan becomes deprotonated and forms a thin film over the cathode surface. The deposition rate of chitosan is controlled by molecule weight, solution pH value, structures of anode and cathode as well as the applied voltage. What is superior to solution casting and spin coating methods is that electrodeposition can be applied to device with various geometries, e.g., complicated micro nanoscale sensors and MEMS devices, which is crucial to novel sensor design [56].

Chitosan film preparation can be used to immobilize BREs directly, which is known as the entrapment method for BRE immobilization. The porous morphology of chitosan film makes it possible to physically trap large BREs as surface probes. It is reported that moderate mixture of biological molecules with chitosan solution will not alter its property of pH dependent solubility [53]. Thus, by simply adding BREs into chitosan raw solution and implementing solution casting or electrodeposition procedure, copolymer of chitosan doped with BREs will be coated over the sensing area. Since biomolecules are comparatively large, they will be trapped in the chitosan matrix even without chemical bond or electrostatic attraction. No additional preparation or pre-labeled of BREs is needed during BREs immobilization, except that the BREs should be in weak acidic environment to keep their stability. One shortcoming of physical entrapment method is its poor stability, because small BREs can leak out during test or storage due to their week interaction with chitosan.

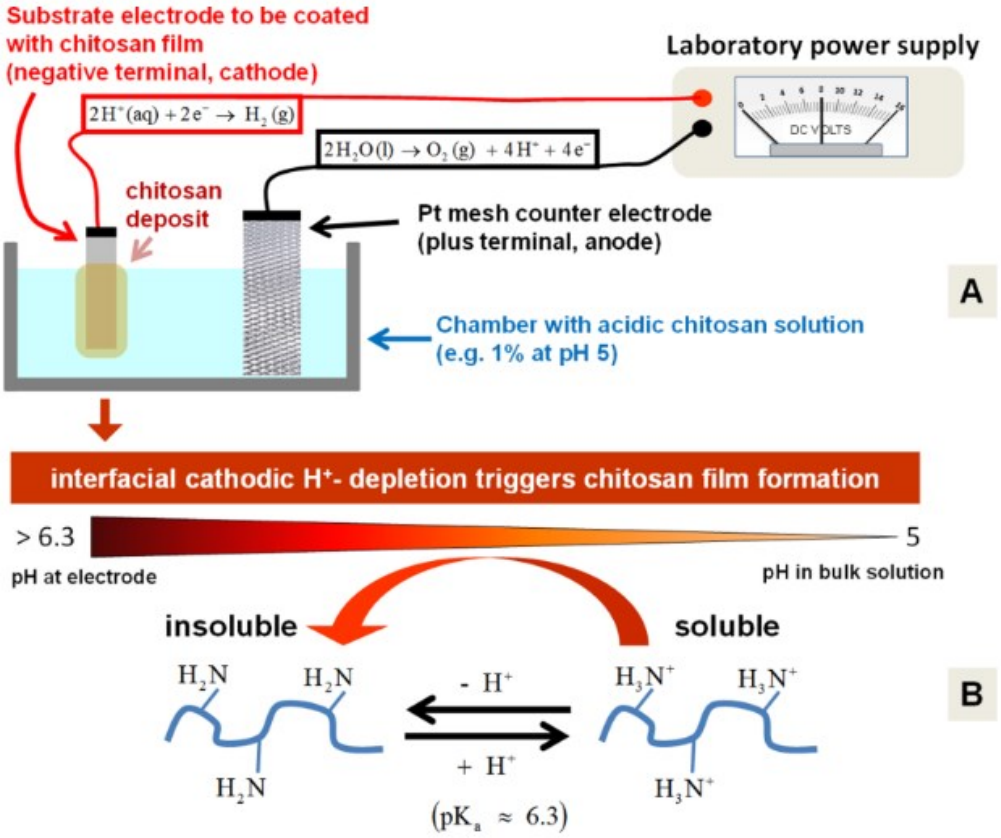

Figure 3 Scheme of chitosan electrodeposition. (A) Instrumental setup for electrodeposition of thin chitosan films on substrate electrodes. (B) Mechanism of chitosan precipitation onto cathode surface. Electrolysis of water can induce local pH change near the electrode surface. When the localized pH is higher than 6.5, deprotonation will happen, then chitosan will become insoluble and deposit on the electrode surface. Reproduced from [57]

For most biosensors, BRE immobilization is usually implemented by adding BRE solutions after chitosan film formation on sensor. Amines groups on the chitosan surface provides good reaction sites for covalent binding, which were shown in the Figure 4. BREs such as proteins also have amines groups, and they can be attached to chitosan surfaces with the aid of glutaraldehyde. Glutaraldehyde has aldehydes [57] on both sides and can be used as strong linkers between the amine groups in chitosan and BREs. For nuclei acid strands that end with phosphate groups, EDC/NHS, i.e., (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide) / N-hydroxysuccinimide, can be applied to form covalent crosslinks between primary amines in chitosan and phosphate groups. In such cases, EDC acts as an activator to increase the reaction efficiency and create stable intermediates. EDC/NHC can also be used to couple amines to carboxyl group. In addition to chemical linkers, avidin/biotin pair can also be used as a biochemical linker. Biotins can be modified onto chitosan surface and the 5' end of DNA molecules separately, then avidins link them together.

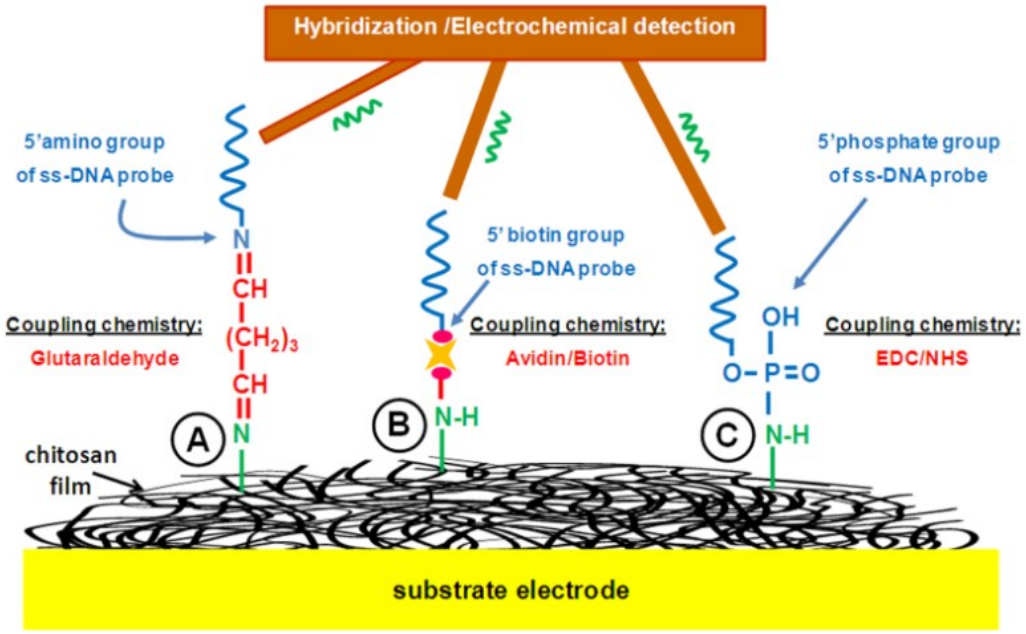

Fig. 4 some mostly used immobilization strategies of chitosan films. (A) Utilization of glutaraldehyde-based coupling chemistry. (B) Exploitation of biotin-modified chitosan and DNA and use of avidin as a high-affinity bridging molecule. (C) Coupling the phosphate groups of the DNA to the amino groups of chitosan with EDC/NHS. Reproduced from Ref. [57].

In addition to homogenous chitosan film, some recent work adopts chitosan nanocomposites as substrate material for BRE immobilization. In these reports, nanomaterials such as CNTs [28], metal nanoparticles, graphene nanosheets [58] and quantum dots [29] have been adopted as doping elements to offer additional carboxyl groups for BREs immobilization. When mixing with carbon nanotubes or graphene nanosheets, the rich protonated amino groups on chitosan backbone can form stable chemical bonds with carboxyl or hydroxyl on nanoparticles [59]. The benefit is to provide inorganic nanoparticles that are mostly hydrophobic with water solubility for uniform dispersion, which as a result improves the biosensor stability. In such cases, chitosan films are used more as a dispersion material rather than an immobilization linker, and BREs are linked to those nanoparticles. Compared with homogeneous chitosan materials, chitosan mixture incorporating the aforementioned nanoparticles/sheets usually has good conductivity [60] and surface flatness [61], thus such heterogeneous materials are more suitable for high-performance enzyme [62] and impedance biosensors.

5. Recent developments

5.1 Immunosensors

Immunosensor, which utilizes antibodies as BREs, is a well-developed sensing technology that take advantage of the specificity of antibody-antigen binding. With suitable sensor designs and optimized test protocols, detection limit as low as atto molar and even sub-atto molar of protein is possible according to some of the reported immunosensors [63]. One major approach to achieve higher sensitivity for immunosensors is to enlarge the active area of sensor for denser BRE loading, since extensive research has shown that the magnitude of sensor response correlates well with the surface concentration of BREs [57]. In such context, the advantage of chitosan copolymer is that it can

support expanded binding sites and surface area for antibody immobilization. Additionally, chitosan is widely used as a dispersion matrix to offer water solubility, thus greatly simplifying the surface coating or electrodeposition processing due to its excellent biocompatibility, non-toxicity and pH dependent solubility. The below are some examples.

Using chitosan nanocomposites with carbon nanotubes, Zhang et al. demonstrated a SWCNTs/chitosan composite-based biosensor for aflatoxin B1 detection in corn [64]. In their work, SWCNT is added to enhance the antibody binding, while chitosan is applied to entrap and attach the SWCNTs to the glass carbon electrode. After solution coating of chitosan composite, antibody probe is immobilized onto the sensors by linking the antibody with the carboxylic group of SWCNTs by NHS/EDC. A detection limit of 3.5 $\mathrm{pg/mL}$ is achieved by differential pulse voltammetry (DPV) measurement. The detection schematic is shown in the figure 5 (A). Recently, Ahmet Güner et al. [65] reported a hybrid sensing platform that utilizes chitosan, MWCNTs and gold nanoparticles to detect O157:H7 Escherichia coli (E. coli). The chitosan nanocomposite in their work is modified by polypyrrole to form pyrrole branched chitosan structure for improved antibody immobilization. After mixing polypyrrole modified chitosan with MWCNTs, the solution coating method is adopted to form chitosan-MWCNTs-AuNPs film. Then amine groups of the composite is activated by glutaraldehyde (GA) to form covalent bonds to immobilize antibody. The detection limit is found to be 30 $\mathrm{cfu/mL}$ with a linear range from 3×10^1 $\mathrm{cfu/mL}$ to 3×10^7 $\mathrm{cfu/mL}$. The detection schematic is shown in the figure 5 (B). Instead of using CNTs, Jia et al. reports an immunosensor based on gold nanoparticles modified chitosan for α -fetoprotein (AFP) detection [66]. In their work, gold nanoparticles are synthesized in chitosan thin film for physical absorption of antibody. Figure 6A illustrates the electrodes fabrication schematics. The LOD is determined to be 1.6 pg/mL using DPV, which is shown in Figure 6B and 6C. In another work, Liang et al. use chitosan as dispersion material, which is used to bind graphene quantum dots and peptide onto glassy carbon electrode [67]. The electrochemiluminescence resonance energy transfer is used for detection of protein kinase activity and inhibitor. The LOD is reported to be 0.023 U/mL (S/N = 3).

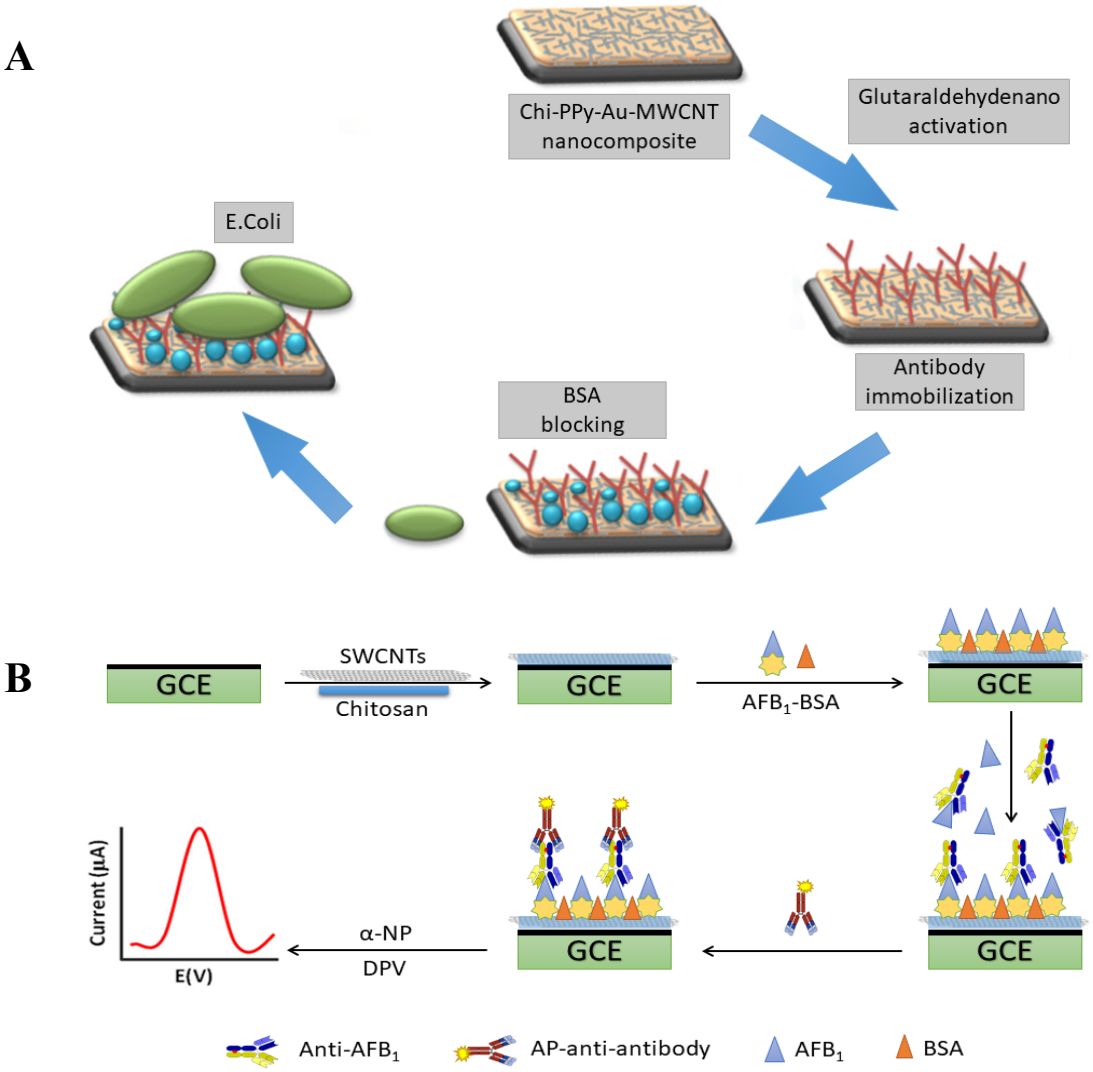

Figure 5 flow diagram of immunosensor detection. (A) Schematic diagram of the experimental set up of an immunosensor fabrication for E. coli detection [65]. (B) Schematic illustration of the strategy for AFB1 assay using GCE modified with a single-walled carbon nanotubes/chitosan film. After coating, the AFB_1 is first immobilized, then the electrode will be blocked using BSA. The competing reaction will occur between AFB_1 and anti-AFB_1 [64].

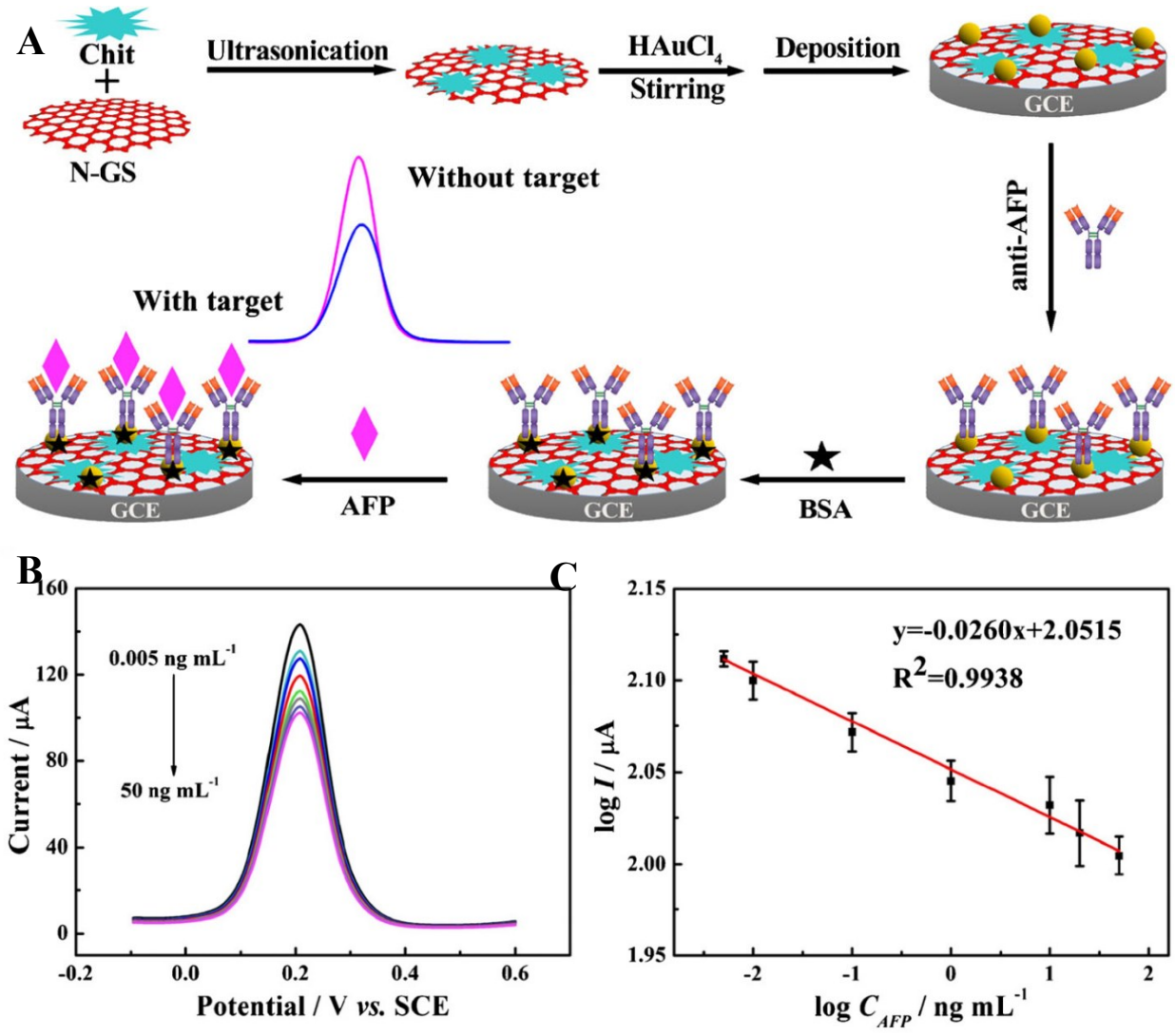

Figure 6 (A) Schematic diagram of the fabrication and detection of the AFP immunosensor. (B) DPV curves of the immunosensor after being incubated with different concentrations of AFP. (0, 0.005, 0.01, 0.1, 1, 10, 20, 50 ng/mL). (C) Plots of the logarithm of peak current versus the logarithm of AFP in a potential of −0.1-0.6 V. Error bars represent standard deviation (n = 5). Adapted from [66]

Chemically modified chitosan thin film has also been developed to offer reliable binding sites for antibody immobilization. Rong et al. designed a one-pot method to synthesize chitosan–poly(acrylic acid)-metal ions nanospheres and use them to produce electrochemical signals. Four different metal ions, copper ions, cadmium ions, lead ions and zinc ions, possess independent peak overpotentials, so they can provide a possibility for simultaneous detection of more than one tumor markers. The metal ion doped chitosan-poly (acrylic acid) nanospheres then react with glutaraldehyde (GA) to immobilize different labeled antibodies [68]. Chitosan and metal ions are polymerized by acrylic acid to form nanospheres structure, combining the excellent biocompatibility of chitosan and independent peak potentials of metal ions. By immobilization four different antibodies on chitosan surface, this nanosphere prototype is further developed to build a sandwich-type immunosensor that could detect four tumor markers (CEA, CA199, CA125 and CA242) of pancreatic cancer. Their work establishes a good linear relationship in range from 0.1 to 100 ng for CEA, 1 to 150 U for CA199, CA125 and CA242, and corresponding detection limits of 0.02 ng, 0.4 U, 0.3 U and 0.4 U.

5.2 DNA based sensors

In contrast to antibodies, DNA probes have smaller size (3-60kDa), as typically they are around 20 nucleotides [36], and flexible structure, which allows them to detect tiny analyte and reduces the restraints from screening effects of electrical double-layer for electrical affinity sensors [46]. The amino groups on chitosan film can provide bonding sites for direct DNA immobilization if the DNA probes are terminated with amino groups [69], in which case the glutaraldehyde is usually used as chemical linker. When incorporating nanoparticles etc. to form nanocomposites, chitosan can either act as a substrate material that will contribute the bonding sites or just function as dispersion material that makes the component material more stable and the surface coating process easier [70]. Nanomaterial such as carbon nanotubes and graphene nanosheets are usually added into chitosan as enhancement materials [71]. It is reported that the graphene nanosheets can improve the uniformity and evenness of chitosan thin film [58] and promote DNA hybridization reaction.

Recently, Tian et al. have reported a miRNA sensor, in which two-dimension transition metal dichalcogenide (TMD) nanosheets instead of graphene nanosheets are used for repeatability and sensitivity enhancement. [72]. In their work, the chitosan substrate is processed to contain carboxy group by N-carboxymethyl for better immobilization of DNA probe. Their developed miRNA-21 biosensor demonstrates a linear range of 1.0 fM to 1.0 nM with a detection limit of 0.34 fM and shows good selectivity, reproducibility, and stability with direct human serum samples tests. Singh et al. also have reported a graphene oxide-chitosan nanocomposite based electrochemical DNA biosensor for detection of typhoid [73]. In their work, *Salmonella typhi* specific ssDNA is tagged with amine for immobilization on graphene oxide-chitosan decorated ITO electrodes via glutaraldehyde covalent binding. Amino groups in chitosan skeletal chains provide binding sites for ssDNA probes. The hybridization between target DNAs and ssDNA probes is detected using DPV. The LOD for analytic samples is reported to be 10 fM. For complementary targets present in serum samples, the LOD is 100 fM.

As an alternative to amine-terminated DNA probe, self-assembly of thiolated DNA probes on gold surface has become a popular DNA immobilization technique due to the strong affinity between thiol functional groups and gold atoms [36]. Using Au-S bond, a high-quality single molecule layer of DNA probes can be formed without any special manual intervention. Furthermore, the density of DNA probes can be tuned by mixing DNA probe and spacer, e.g., 6-Mercapto-1-hexanol at a given ratio [44]. Similarly, Au-S bonding method can be applied for chitosan-based sensors simply by doping chitosan with gold nanoparticles.

Conventional method, which usually use chitosan as dispersion/film substrate material, takes the advantages of gel-forming ability of chitosan and used the chitosan film as supportive layer to carry the gold nanoparticles. Bhatnagar et al. developed a new method to use a single or few chitosan molecular layers as a stabilizing medium between gold nanoparticles and other linkers to detect invasive aspergillosis DNA sequence. [74]. The sensor achieves a dynamic range between 1×10^{-14} and 1×10^{-2} M with a detection limit of $0.32 \pm 0.01 \times 10^{-14}$ M (RSD < 5.2%). Chitosan composites with other noble metal nanoparticles or metal oxide particles have also been frequently investigated. In 2015, Tiwari et al. reported a graphene oxide–chitosan composite substrate for DNA immobilization to detect specific DNA sequence of pathogenic Escherichia coli (O157:H7 E. coli), in which nickel ferrite is first reported to be adopted as enhancement material in biosensor application. Chitosan is used as disperse nanomaterials to load spinel ferrites and graphene oxide. After synthesis of mixed solution, electrophoretic deposition is used to electroplate chitosan on to ITO electrodes. Finally, DNA probes are assembled by glutaraldehyde via amine-amine interaction. In their work, a

detection limit of 1×10^{-16} M/L with a liner response range of $10^{-6} - 10^{-16}$ M/L is achieved, based on CV and DPV measurements [75]. Wang et al. fabricated chitosan-novel (Nb, V) codoped TiO_2 (NVTO) nanocomposite electrodes to detect the breast cancer susceptible gene and acquired the detection limit of 1.09×10^{-16} M/L when signal to noise ration equal to 3 [76].

The capability of coordinating with metal ions is another unique property of chitosan. Zhang et al. reported a novel DNA-modified nanocomposite of 3D reduced graphene oxide and chitosan particles to detect mercury ion in drinking water [77]. The 3D graphene is induced to increase surface areas while chitosan film is adopted to provide the DNA bonding sites and help to gather metal irons. A low detection limit of 0.016 nM and a dynamic range from 0.1 nM to 10 nM is reached in their work.

5.3 Aptamer sensors

Aptasensor uses a special kind of short single-stranded oligonucleotide or peptide, known as aptamer, as its specific probe. Aptamer recognizes its target via its ability of folding upon binding with special target molecules, as opposed to the hybridization reaction between conjugated nucleotide sequences in a nuclei acid sensor [78]. Compared with natural BREs based biosensors such as immunosensors and enzyme sensors, aptasensor posseses advantages in following aspects: (1) high specificity and affinity toward almost any kinds of target. (2) better stability. (3) ease of synthesis/better reproducibility.

The abundant amino groups in chitosan surface can provide immobilization sites for aptamers, which is very similar to DNA based biosensors. Arezoo Tabasi et al. reported an ultrasensitive electrochemical aptamer-based assay for detection of human epidermal growth factor receptor 2 protein (HER2) cancer biomarker [79]. Chitosan together with reduced graphene oxide is combined to provide amino groups for immobilization, improve the homogeneity ofsensing surface and increase the sensor surface area, respectively. Using methylene blue as electrochemical indicator, a very low detection limit of 0.21 ng/ml and two linear concentration ranges of 0.5–2 ng/ml and 2–75 ng/ml is obtained using DPV.

Strong physical interactions, e.g., electrostatic interaction, $\pi - \pi^*$ interaction also widely exist in most of nanomaterials and have been applied to immobilize aptamer in some recent work. $\pi - \pi^*$ interaction is noncovalent attraction between aromatic rings that contain pi bonds. Duan et al. have designed a biosensing platform, based on a two-dimensional (2D) nanocomposite of graphitic carbon nitride (g-C3N4) nanosheets, MoS2 quantum dots (MoS2 QDs), and chitosan-stabilized Au nanoparticles (CS-AuNPs) for prostate specific antigen detection [80]. Due to localized surface plasmon resonance of CS-AuNPs and excellent electrochemical activity of MoS2QDs, this work has constructed a surface plasmon resonance (SPR) spectroscopy sensor and an electrochemical aptasensor simultaneously. Subsequently, the two sensors' performances are compared, and it is found that the LOD of the electrochemical aptasensor is three orders of magnitude lower than that of the SPR sensor, with 0.71 pg/mL versus 0.77 ng/mL. Hills et al. [81] report a multi-walled carbon nanotubes-chitosan nanocomposite based aptasensor for hepatitis C virus core antigen detection. In this work, the multi-walled carbon nanotubes-chitosan base offers an immobilization interface with better conductivity and larger surface areas. As a result, a linear range from 5.0 fg/mL to 1.0 pg/mL with a detection limit of 1.67 fg/mL is reached.

Very recently, a chitosan-based nanobrush-structured aptamer sensor has been developed for the detection of *Listeria* monocytogenes in food samples [82]. Unlike conventional substrate configuration, in this work, chitosan is polymerized to produce brush-like nanostructures, which can

be actuated by a change in pH values to stimulate bacteria capture by protrusions. As shown in the figure 7, by combining the electrostatic interactions from chitosan nanobrush and receptor–cell binding from aptamer immobilized on the structure surface, the detection range can be from 9 to 10^7 CFU/ml.

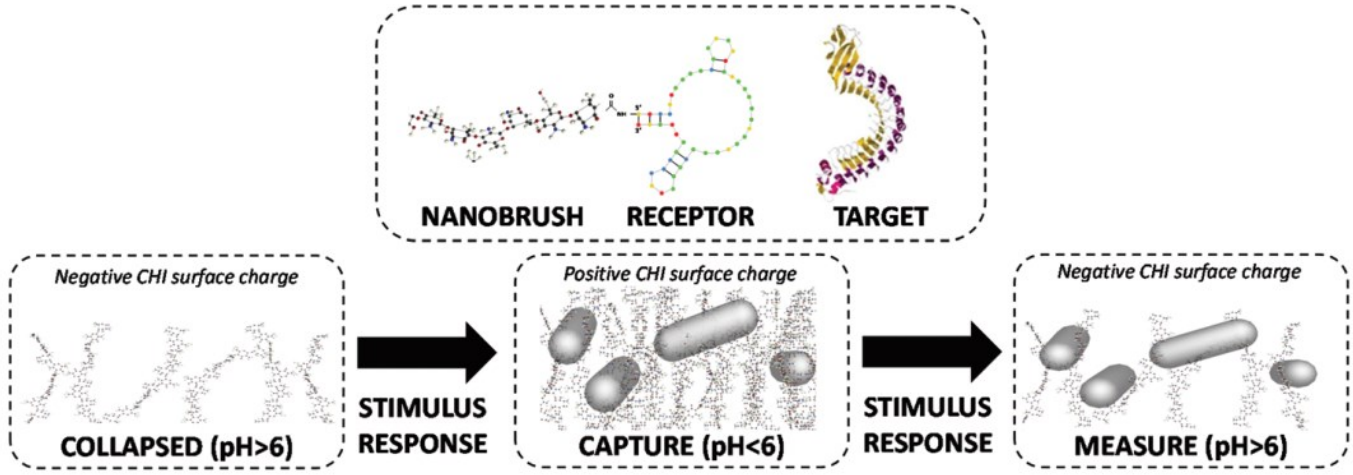

Figure 7 Nanobrush sensing strategy for selective bacteria capture. Top: Stimulus-responsive chitosan (CHI) nanobrushes are decorated with receptors that bind a cell surface target (a DNA 47-mer targeting Internalin A on Listeria monocytogenes is shown as an example). Bottom: Nanobrush is actuated from collapsed to extended states based on pH changes. The nanobrush is first extended (pH < 6), facilitating cell capture due to a combination of electrostatic interactions and receptor-target binding, and then measurement (sensing) is conducted in the collapsed nanobrush state (pH > 6). Reproduced from [82]

5.4 Enzyme sensors

Chitosan is a biocompatible polymer that adheres tightly to other materials [34]. It is often used as an intermediate material between enzymes and sensors to provide reliable immobilization of enzyme to sensor surface.

Particularly advantageous to enzyme sensors, chitosan matrix poses little restriction to substrate and cofactor's access to enzyme due to its porosity. Physical entrapment, covalent bonding and electrostatic absorption/interaction have been demonstrated to be feasible methods of enzyme immobilization with controllable leakage [57]. Due to relative large size of enzyme and relaxed requirement for enzyme orientation, it is a suitable choice to use chitosan material as a substrate or dispersion material in enzyme-based biosensors. There are also reports thatphysical properties of chitosan membrane can be enhanced by nanoparticle doping or mixing. In [61], the controlled mingling of Graphene nanosheets or carbon nanotubes enhanced the electrical conductivity or carrier mobility of chitosan film, which can further improve the sensitivity of enzyme sensors.

One representative application of chitosan-based enzyme sensor is pesticide/organophosphate detection by entrapping an enzyme, acetylcholinesterase (AChE), into chitosan polymer [81] and measuring the redox current/potential. When the targets are applied, the activity of AChE will be inhibited. The target concentration can be quantified by the decrease in peak current at a certain

overpotential when using CV or DPV measurement. Chitosan' biocompatibility helps to preserve enzyme activity even at high immobilization concentration [83].

Previous research has demonstrated that comparing with chemical linkers such as glutaraldehyde, chitosan physical entrapment is advantageous in preserving enzyme activities, which is crucial to enzyme biosensors [83]. The non-toxicity of chitosan film also bestows chitosan-based biosensors the capability of long-time storage and better reproducibility. According to some recent reports [84], the performance of enzyme biosensors did not exhibit obvious decrease in performance after several weeks even several months storage.

To further exploit the potential of chitosan as a support material of nanocomposites, some recent work investigated in great detail the morphologies of chitosan nanocomposites, in an effort to obtain more advantageous nanostructures and functions. El-Moghazy et al. tried to combine the chitosan with electrospun technology and reported a nanofiber structured chitosan composite for pirimiphos-methyl detection in olive oil [85]. The new processing technology lead to more porous 3D structures with far more specific surface area than traditional plane configuration. As a result, it can load high density enzyme to further lower the limit of detection. Delezuk et al. studied the chitosan layer by layer deposition for optimized enzyme immobilization [86]. In their work, stacked chitosan films with different molecule weights as well as silk fibroin is fabricated and tested for phytic acid detection. They found that chitosan molecule weights can affect the conformation of silk fibroin and result in different enzyme loading efficiency. A large change in conformation from random coils to β-sheets for silk fibroin is observed when high molecular weight chitosan is adopted. A LOD down to 10^{-9}M is reached when high molecule weight chitosan is used. Zhao et al. explored the possibility of using chitosan as structure-directing agent for zinc oxide mesocrystal growth. Figure 8 [87] shows different structures of zinc oxide mesocrystal. In their work, the dimension, growing direction and shape of zinc oxide mesocrystal can be controlled and guided through Coulomb forces and adjustment of surface energy. They concluded that the starfish-like CS-ZnO mesocrystal with a grain size of 20−30 nm shows a better sensing performance compared with other mesocrystal shapes and sizes for glucose detection. The response range is reported to be 0.05 - 0.3 mM.

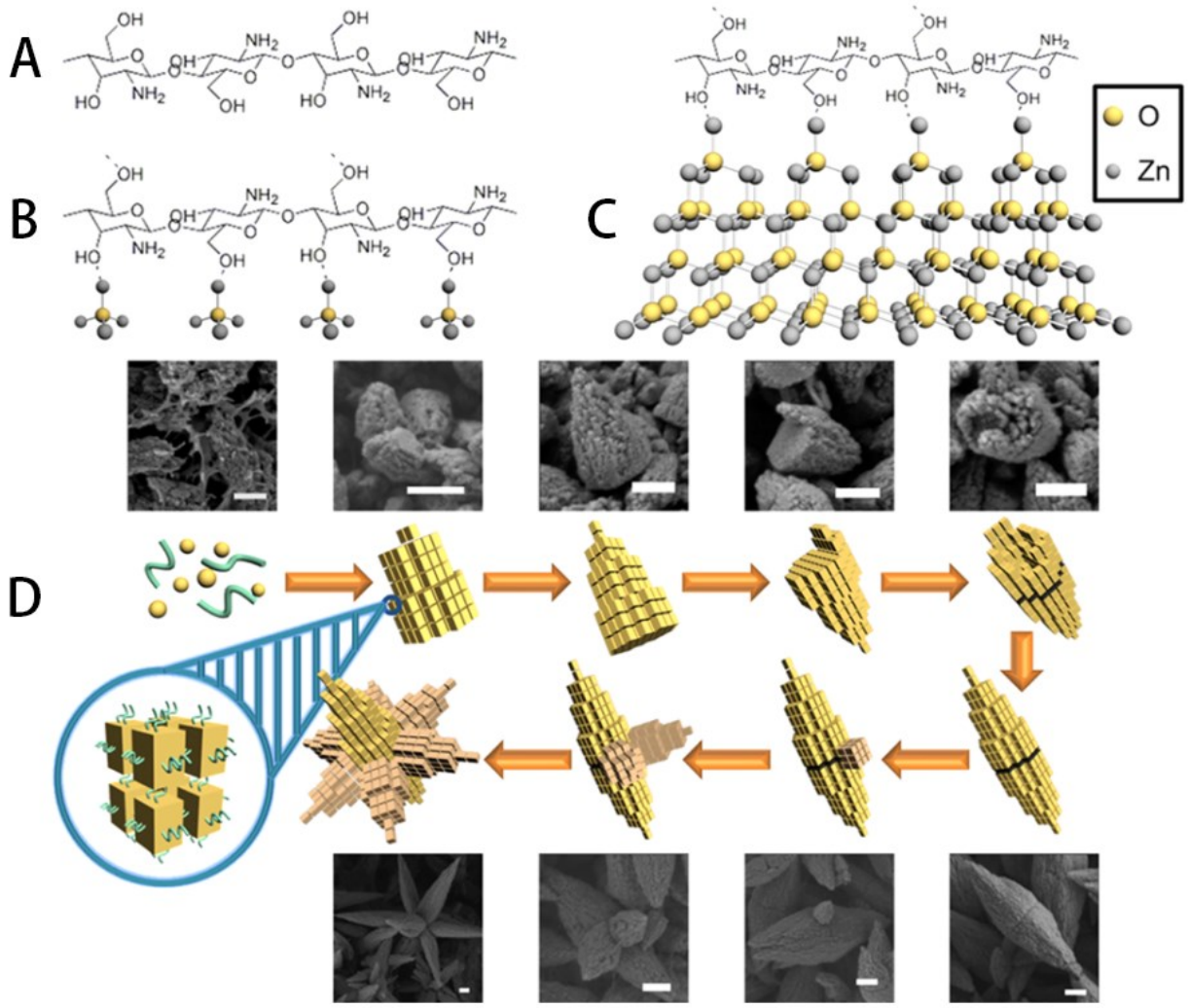

Figure 8 Schematic illustration of the CS-ZnO structures self-assembly process. (A) Schematic graph of chitosan chains, (B) C−O groups of chitosan chains binding with zinc ions of ZnO nuclei via Coulomb forces, (C) the change of surface energy guiding the preferential formation of zinc oxide, (D) morphological evolution of the CS-ZnO structures. (Each scale bar in FESEM images is 200 nm) [87].

5.5 Other advanced biosensors

The porous morphology of chitosan membrane and its nontoxicity have been used to produce cell-based biosensors. It is usually an easier and low-cost alternative BRE immobilization approach in comparison to chemical linkers. Fang et al. developed a reagentless electrochemical microbial biosensor by physically entrapped the thionine decorated E.coli onto chitosan surface [88]. The E.coli is used as an indicator of wastewater toxicity level. The biosensor is used to evaluate the toxicity of four heavy metal ions (Cd^{2+}, Cu^{2+}, Pb^{2+}, Zn^{2+}). The LOD of four metal ions are 36.2 mg/L, 20.2 mg/L, 34.3 mg/L and 53.2 mg/L, respectively.

Taking advantage of chitosan's gel-forming capability, Xia et al. explored molecular imprint sensor using chitosan material [89]. In their study, a chitosan/ionic liquid-graphene modified glassy carbon electrode is fabricated for bovine serum albumin (BSA) detection. The chitosan is added for better

biocompatibility and more stable, non-destructive contact between BSA and substrate. The concentration of chitosan, graphene sheets and polypyrrole are optimized. The detection limit was finally reported to be 2×10^{-11} g/L and the liner response range is from 1×10^{-10} to 1×10^{-4} g/L (R = 0.996).

There also exist reports that chitosan films incorporating nanomaterial can possibly exhibit specificity in electrochemical biosensing without use of BREs. Such sensors are known as enzyme-free sensors. Mane et al. reported an atropine biosensor based on single walled carbon nanotube (SWCNT)-chitosan film on glassy carbon electrode, in which chitosan was used as a dispersion matrix and stabilizer [90]. Uniform dispersion of SWCNTs in chitosan is realized through covalent attachment, which is verified by Fourier transform infrared (FTIR) and Raman spectra. Their work also demonstrates long time-span stability over 7 days for the atropine sensors, which can be attributed to chitosan's excellent gel-forming ability and nontoxicity. Similar research on chitosan-nanocomposite based electrochemical sensors also demonstrated that chitosan nanocomposite materials can acquire addition chemo-physical properties from different nanomaterials such as specificity to atropine from SWCN-chitosan [90] and to dopamine from graphite-chitosan composite [91]. Together with nanomaterials, conductive polymer such as polypyrrole can also be added into chitosan nanocomposite for improved conductivity in such enzyme-free sensors. AL-Mokaram et al. reported a chitosan- polypyrrole-iron oxide composite material based biosensors to detect glucose [92]. Chitosan is used as dispersion material. The inclusion of iron oxide nanoparticles is to realize the goal of non-enzyme detection. A detection limit of (234 μM) at a signal-to-noise ratio (S/N)=3.0 is reached.

6 Conclusion

As a natural polysaccharide, chitosan has the merits of intrinsic biocompatibility, wide variety of production sources, low-cost and bio-degradability. Extended advantages of chitosan in the application of medical and biological detection includes its adhesive film-forming properties and abundant nitrogen/oxygen residues which can be used to optimize the material's characters in accordance to particular applications. The major drawback of chitosan arising from its inherent chemical reversibility of hydrogel film formation. When being exposed to aqueous system with low enough pH to cause reprotonation of chitosan polymer amine groups, adverse internal matrix conversion will be trigged, sharp decline of surface adhesion even exfoliation will be caused [48].

In this review, the emphasis is on the use of chitosan in surface-based sensor design. The reviewed publications of chitosan-based nucleic acid, enzyme, and antibody-based biosensors are based on the role that these natural biopolymers play as thin-film surface modifiers for electrodes of all sorts. A majority of recent works still focus on analytic samples or spiked/simulated samples. However, when testing real samples, e.g., blood samples and natural water samples, unknown interferences may cause false positive prediction or inhibit true positive response. Biosensors are typically used in a setting outside central laboratory facilities, which lacks sophisticated sample pretreatment capabilities, which makes it more demanding for biosensors to reach desired sensitive, specificity and repeatability. Such constraints limit the applications of biosensors. Hence, future developments should focus on easy-to-reproduce materials/designs. Therefore, a major goal in the next stage of development is to further improve the robustness and specificity of chitosan-based biosensors, in an effort to build competitive commercial products that can be used for wearable personal health care devices, point-of-care clinical diagnostics, and environmental real-time monitoring.

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