Chitosan: an integrative biomaterial for lab-on-a-chip devices

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Chitosan is a naturally derived polymer with applications in a variety of industrial and biomedical fields. Recently, it has emerged as a promising material for biological functionalization of microelectromechanical systems (bioMEMS). Due to its unique chemical properties and film forming ability, chitosan serves as a matrix for the assembly of biomolecules, cells, nanoparticles, and other substances. The addition of these components to bioMEMS devices enables them to perform functions such as specific biorecognition, enzymatic catalysis, and controlled drug release. The chitosan film can be integrated in the device by several methods compatible with standard microfabrication technology, including solution casting, spin casting, electrodeposition, and nanoimprinting. This article surveys the usage of chitosan in bioMEMS to date. We discuss the common methods for fabrication, modification, and characterization of chitosan films, and we review a number of demonstrated chitosan-based microdevices. We also highlight the advantages of chitosan over some other functionalization materials for micro-scale devices.

1 Introduction

One of the main challenges in the development of miniaturized sensors and systems for life science applications continues to be the integration of biological components. These types of microdevices typically need to be functionalized with biomolecules such as DNA, enzymes, or antibodies to operate with sufficient specificity and sensitivity. However, the harsh fabrication techniques and materials involved in traditional MEMS fabrication are incompatible with the labile biological components. Specialized materials and processes are needed to allow for seamless integration of biology into microdevices. Several approaches toward this goal have been demonstrated based on the use of self-assembled monolayers or surface-immobilized polymers.[1,2] The polymer chitosan is one of the most promising candidates for interfacing biology and microdevices, and it is the subject of this review paper.

Chitosan is a polysaccharide derived from naturally occurring chitin. Its unique properties make it attractive for many industrial and biomedical applications. Due to its pH dependent solubility, it forms stable films on various surfaces under neutral and basic pH conditions. Its amine groups serve for covalent

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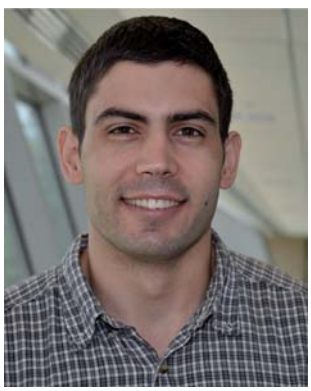

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attachment of biomolecules, and it can be co-deposited with other polymers or nanoparticles. Several review papers survey the diverse applications of chitosan,[3–11] including controlled drug release, wound healing, nutrition supplements, water purification, removal of toxins, scaffolds for tissue engineering, and semipermeable membranes.

Recent advances in chitosan fabrication have allowed this material to be integrated into lab-on-a-chip devices.[6,12–14] The primary role of chitosan in the microdevices is to immobilize biomolecules, cells, or nanoparticles. This enables the devices to perform advanced functions such as specific recognition of analytes and enzymatic conversions. The present article starts with a brief review of chitosan's properties. Next, we describe the common methods for microfabricating chitosan films, for modifying them with other substances, and for characterizing them. This is followed by a survey of reported microdevices that utilize chitosan. We conclude by summarizing the advantages of chitosan mediated assembly and by discussing possible future developments in the field.

2 Properties of chitosan

Chitosan is obtained by the deacetylation of chitin. Chitin is the structural material of the shells of crustaceans and insects, and it is the second most widespread natural polymer on earth after cellulose. Therefore, chitosan is abundant, biocompatible and biodegradable. Its degradation products are harmless natural metabolites, and it is non-antigenic (it does not cause an immune response in an organism).

The structural formulas of chitin and chitosan are shown in Fig. 1 Many of chitosan's properties are explained by the presence of its primary amine groups with pK_a ~6.5.[6] At pH lower than 6.5, the amines are positively charged and chitosan is soluble. At higher pH, the amines are increasingly deprotonated, and it becomes insoluble. This pH dependent solubility allows stable chitosan films to be deposited on device surfaces through several methods that will be reviewed in section 3. Importantly, the film can be dissolved and removed by a mildly acidic wash, and the device can be re-used multiple times. The structure of the film depends on the deposition conditions, the chitosan molecular weight, and its degree of deacetylation.

Chitosan films immersed in liquid experience strong pH dependent swelling.[15,16] The pH of the solution determines the

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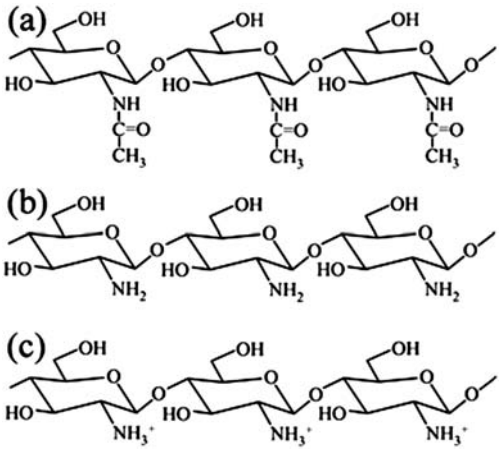

Fig. 1 Structural formulas of chitin and chitosan showing the three possible states of the amine group: (a) chitin, amine group acetylated; (b) chitosan, amine group free; (c) chitosan, amine group protonated.

amount of positive charge on the chitosan chains, which in turn affects the electrostatic repulsion between the chains and the volume of the polymer. This swelling behavior can be utilized for actuation[17] or for controlled drug release.[18]

Perhaps the most significant property of chitosan is its ability to be modified with other substances. The amine groups can be used as sites for covalent attachment of various biomolecules, including enzymes, DNA, and antibodies. The modification can also be performed by physical interaction (*e.g.* surface absorbance, entrapment) instead of chemical bonding, especially in the case of negatively charged substances. The types of chitosan modifications that have been demonstrated are discussed in section 4. The chitosan chains can also be covalently crosslinked with each other instead of with biomolecules. As explained in section 3.5, this crosslinking improves the strength and chemical resistance of the film.

Unmodified chitosan films are transparent and therefore suitable for use in optical sensors.[19–21] Crosslinking of the chitosan somewhat increases its optical absorbance in the UV region, but it still remains low in the visible range.[22] The electrochemical properties of chitosan are also favorable. Wet chitosan films are porous and highly permeable to ions.[23] This enables the construction of electrochemical sensors with chitosan coated-electrodes.[24–26]

The mechanical properties of chitosan vary widely with the preparation method. For example, its Young's modulus has been reported to be 9 MPa[22] and 3.50 GPa,[14] both for unmodified films. Note that the mechanical properties of chitosan do not significantly affect the design of bioMEMS devices since it is normally used as a coating rather than a structural material. The thickness of the chitosan is typically smaller than that of the structural material, and its stiffness is much lower (for example, the Young's moduli of Si, Si_3N_4 and SiO_2 are on the order of 100 GPa). The reported residual stress of dried electrodeposited chitosan films is approximately 60 MPa[27] (in comparison, the residual stresses of Si_3N_4 and SiO_2 are normally hundreds of MPa). Therefore, the addition of chitosan is likely to have a minimal impact on the mechanical characteristics of the microdevice.

3 Methods for chitosan microfabrication

Chitosan is normally purchased in the form of dried flakes. These flakes are dissolved in an acidic solution, typically dilute hydrochloric or acetic acid, which is then filtered to remove particulates. The preparation of chitosan solutions is presented in detail in many articles.[12–14,23,28] Several different methods have been developed to deposit and pattern chitosan films that are compatible with bioMEMS fabrication.

3.1 Solution casting

The simplest fabrication method consists of coating the surface with chitosan solution and evaporating the solvent. This leaves behind a solid chitosan film with a thickness dependent on the solution concentration. The coating procedure can be performed with a pipette or with a spin coater (if a highly uniform film is needed). Elevated temperatures can be applied to the substrate to speed up the solvent evaporation.

Solution casting is the predominant method for applying chitosan to macroscale devices. However, it does not inherently provide the spatial control necessary for use in bioMEMS. For this reason, several approaches have been demonstrated to pattern the cast film. These include a containment method in which chitosan droplets are laterally constrained[24] and a thermolithography method in which areas of the chitosan film are masked with a heat sensitive thermoresist.[29] More recently, spin-cast chitosan films were patterned by conventional photolithography by masking them with photoresist and etching them with oxygen plasma[14] (Fig. 2). In another work, chitosan was mixed with hydroxyethyl methacrylate (HEMA) and a photoinitiator, making it UV-curable.30 This suggests that photopatternable formulations of chitosan may be developed in the future, which will make the solution casting method more applicable to bioMEMS integration.

Unpatterned solution-cast films are quite useful for fabricating chitosan test samples and simple glass slide based optical sensors.

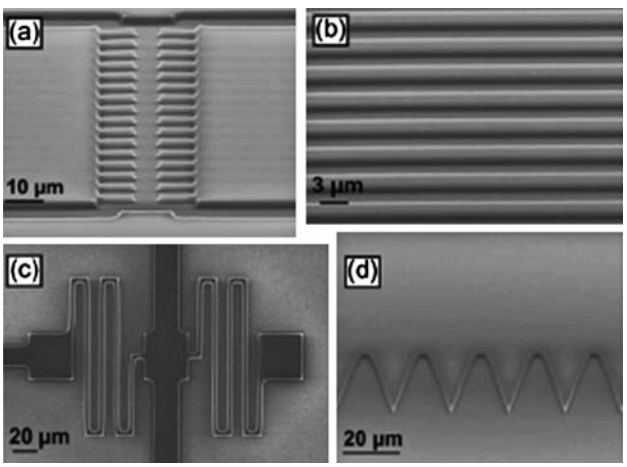

Fig. 2 Photolithographically patterned chitosan features. (a) SEM of matched comb structure at a 50r tilt. (b) SEM of 2-μm-wide lines with 4-μm pitch. (c) SEM of serpentine spring structures. (d) SEM of a sawtooth structure. Note: All patterns are in a 2-μm-thick chitosan layer. Reprinted with permission from ref. 14. © 2008 IEEE.

The film is deposited onto a transparent substrate, and its absorbance is measured with a spectrophotometer.[19,31–33] This approach can be used either to determine the optical properties of the chitosan or detect an analyte bound to it. Films of sufficient thickness (typically greater than 10 μm) can be completely peeled off the substrate after drying and subjected to further optical and mechanical testing.[22,34] Common chitosan characterization methods are discussed in section 5.

An interesting extension of the solution casting method is the layer-by-layer assembly (LBL) technique. The substrate is exposed to alternating washes in the cationic chitosan solution and a solution of some anionic species. Due to electrostatic attraction, a multi-level composite film is produced. This method has been used for the entrapment of enzymes,[35–38] nanoparticles,[39–41] DNA[42] and cells.[43] The modifications of chitosan with other substances are described further in section 4.

3.2 Printing

Both nanoimprinting and microcontact printing have been successfully demonstrated for patterning chitosan films with nanoscale resolution. These methods make use of a patterned mold or stamp, which is typically a flexible polymer such as PDMS. For nanoimprinting, the PDMS mold is placed over a substrate coated with chitosan solution while applying heat and pressure. After cooling, the PDMS is peeled off, and molded structures of chitosan remain patterned on the substrate. Park *et al.* demonstrated the use of nanoimprint lithography to create nanowire and nanodot chitosan structures.[13] Tan *et al.* added plasticizers to the chitosan to allow for imprinting at reduced temperature and pressure.[44] For microcontact printing, the chitosan solution is applied to the PDMS stamp surface, which is then contacted with the substrate. The pattern formed on the substrate corresponds to the geometry of the raised features on the stamp. Feng *et al.* demonstrated co-patterning of chitosan and bovine serum albumin on the same substrate using microcontact printing.[45]

Printing methods can easily create arrays of chitosan structures for spatially resolved functionalization. However, both nanoimprint lithography and microcontact printing are inherently planar processes and cannot be used to pattern chitosan films on non-planar surfaces (such as microchannels or sidewalls). Furthermore, alignment of the mold or stamp to preexisting features on the substrate can be difficult.

3.3 Electrodeposition

A very promising method for chitosan integration in microdevices is electrodeposition.[12,28,46–48] It allows for both spatial and temporal control of the chitosan film location and thickness, and it can be used with a variety of device geometries. Electrodeposition takes advantage of chitosan's pH dependent solubility. At pH lower than 6.5, chitosan is soluble and cationic due to its protonated amino groups. At higher pH values, chitosan becomes deprotonated and is no longer soluble. When an anode and cathode are immersed in a chitosan solution and a voltage is applied, electrochemical reactions lead to a locally high pH adjacent to the cathode surface. While Fig. 3 illustrates these cathodic reactions as a net consumption of hydrogen ions, we should note that the mechanistic details are more complex and likely involve the reduction of oxygen ions as well.[23] Chitosan forms a thin film over the cathode surface as a result of the high pH there. The rate of the chitosan deposition is influenced by many factors: the molecular weight of the chitosan, the pH of the solution, the sizes and separation of the anode and cathode, and the applied voltage. Buckhout-White *et al.* has extensively characterized the spatial resolution of electrodeposited chitosan films.[49] Recent *in situ* visualization and characterization of electrodeposited chitosan done by Cheng *et al.* further proved that the gelation and immobilization of chitosan onto the cathode are due to the electrochemically generated pH change by OH^- ions and the density distribution of deposited chitosan hydrogel is electric field-dependent.[50] Further dependence of electrodeposition rates on applied stimuli has been investigated in several articles.[6,23,28,47,51]

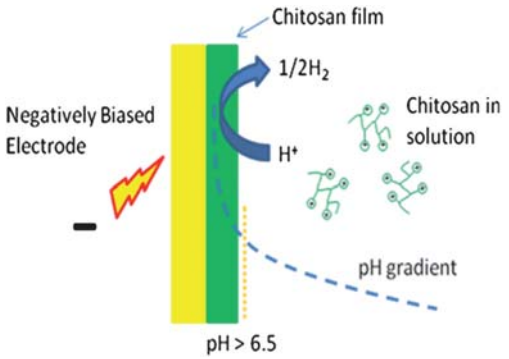

Fig. 3 Schematic demonstrating chitosan film electrodeposition. Adapted with permission from ref. 6. Copyright 2005 American Chemical Society.

The electrodeposition method inherently provides a patterning capability. The resulting chitosan film pattern is identical to the electrode geometry, which can be easily defined by standard microfabrication techniques. Unlike other chitosan deposition methods such as solution casting or printing, electrodeposition does not require an exposed, planar surface. This allows for the deposition of chitosan films on a variety of micro-scale geometries such as released structures,[27,52] out-of-plane surfaces,[20,53–55] and sealed microchannels.[56–58] Fig. 4 shows electrodeposited chitosan on the side of a thick polymer film (SU-8), highlighting the ability of this method to coat non-planar surfaces.

Importantly, chitosan can retain its pH responsive and electrodeposition properties when combined with other substances. The electrical co-deposition of chitosan with several proteins[46,59–61] and nanoparticles has been successfully demonstrated.[62,63] This facilitates the fabrication of modified chitosan films.

3.4 Other methods

Several other methods for chitosan fabrication have been reported that could be viable for use in bioMEMS devices. Very thin chitosan films can be formed by starting with a self-assembled monolayer of cysteamine on a gold surface.[64] Chitosan chains are then anchored to the cysteamine with a glutaraldehyde cross-linker, forming a chitosan monolayer. Chitosan microstructures other than thin films have also been developed. For

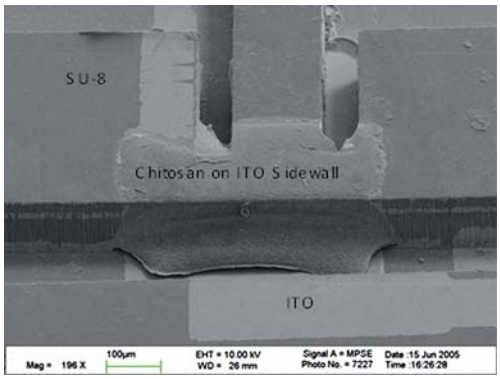

Fig. 4 SEM of chitosan film electrodeposited on the sidewall of a microchannel. A thin film of patterned indium tin oxide acted as the cathode surface for the deposition. Reprinted with permission from ref. 162.

example, chitosan fibers with an average diameter of 100 μm were used as pH sensors.[17] These fibers are created by a wet spinning method, in which the chitosan solution is ejected through a small nozzle into a coagulation solvent of NaOH. The fibers can be further cross-linked using glutaraldehyde and conjugated with polyaniline or carbon nanotubes to improve their strength and conductivity.[65,66]

Porous 3D chitosan scaffolds have been developed to aid the attachment and proliferation of cells for tissue engineering. Tangsadthakun *et al.* fabricated such structures by freeze drying a collagen/chitosan blend and crosslinking it.[67] Ang *et al.* constructed a micropatterned scaffold of chitosan-hydroxyapatite using a robotic dispensing system.[43] Slavik *et al.* used a PDMS mold to form chitosan fibers, which were coagulated to create a scaffold.[68]

Luo *et al.* recently utilized chitosan's pH responsive solubility to form freestanding chitosan membranes in a microfluidic channel.[69] A pH gradient is formed at the interface between two converging flows of slightly acidic chitosan and slightly basic buffer solution. Due to laminar flow conditions, the two solutions do not mix. A chitosan membrane 30–60 μm thick forms at the solution interface with pore size in the range of a few nanometers.

In one particularly interesting demonstration, chitin membranes were obtained directly from the carapaces of blue crabs and used to construct a glucose biosensor.[70] Although this approach requires extensive manual manipulation and is not applicable to microdevices, it highlights the versatility of chitosan fabrication methods.

3.5 Chemical compatibility and adhesion of chitosan

Once deposited, the chitosan films must be kept at a pH above 6.5 to prevent them from dissolving. They are typically washed with a basic solution after deposition in order to neutralize any remaining acid and reduce the positive charge on the amine groups. The films can be crosslinked with reagents such as phenols,[22,34] DTBP,[71] or glutaraldehyde[5,6] to make them more chemically resistant and physically harder. However, the crosslinking consumes chitosan's amine groups and may limit the ability to modify the film with other substances.

Chitosan films are fully compatible with the near-neutral physiological conditions encountered during their use. However, they can be damaged by any strong acids, solvents, plasmas, or extreme temperatures commonly used in MEMS or bioMEMS fabrication. Therefore, it is a better approach to deposit chitosan in the device after the fabrication is completed.[57]

The adhesion of the chitosan to the substrate can vary greatly with the deposition conditions and surface properties. Films with high water content (hydrogels) have very poor adhesion and may be removed even by rinsing the substrate.[47] More compact films have excellent adhesion and withstand the high shear forces in microfluidic channels.[57,58] It is believed that electrodeposition produces films with better adhesion than the other deposition methods.[12,28] The attraction of the positively charged chitosan chains to the electrode can lead to tightly packed films with low water content. The density of the film can be varied by adjusting the applied voltage to produce either compact films or hydrogels. Although the mechanism of chitosan adhesion to substrates is not entirely clear, problems with the adhesion have generally not been encountered.

4 Strategies for chitosan modification

Chitosan films can be readily functionalized with other substances such as polymers, nanostructures, biomolecules, or dyes. The purpose of these modifications is typically to improve the film's mechanical properties, to make it selectively responsive to certain stimuli, or to immobilize biomolecules. Table 1 lists common modifications of chitosan that have been reported in literature and their applications. Many of these examples come from work on macroscale devices. However, the same modifications should be readily applicable to microdevices as well. The table aims to show the versatility of chitosan and is by no means exhaustive.

Multiple different methods have been demonstrated for chitosan modification. The modifying substance can be dissolved in the chitosan solution and co-deposited with the chitosan to form a composite film. Alternatively, a pure chitosan film can be deposited first, followed by attachment of the modifying substance. The chitosan deposition can be performed by solution casting, printing, or electrically. Therefore, a large variety of chitosan films can be formed by choosing a different modifying substance, modification method, and deposition method.

4.1 Assembly of biomolecules

Due to its porous structure and dense amine groups, chitosan is well-suited for attachment of biomolecules. Chitosan films have been modified with proteins, enzymes, antibodies, and DNA (Table 1). The typical application of chitosan films with biomolecules is selective coatings for biosensors.[27,62,64,79,82,103] When a matching element (enzyme substrate, antigen, or complementary DNA) binds to the coating, a measurable physical signal is generated that is transduced by the sensor. Chitosan films modified with enzymes can also be used for catalysis in microfluidic reactors.[58] The modification of chitosan films with certain structural proteins such as gelatin or hemoglobin improves their mechanical properties, making them useful as a cell scaffold[67] or pH responsive material.[72,73]

Table 1 Survey of chitosan modifications reported in literature

Modifying substance	Purpose of modification	References
Proteins		
Collagen	Scaffolds for cell assembly	67
Gelatin	Used as a model protein	61
Gelatin	pH responsive hydrogels	72,73
Green fluorescent protein (GFP)	Used as a model protein	29,46,57,59,61
Hemoglobin	Direct electrochemistry of proteins	74,75
Myoglobin	Direct electrochemistry of proteins	74
Protein A	Attachment of antibodies	76
Protein G	Attachment of antibodies	77
Enzymes		
Acetylcholinesterase	Pesticides detection	25,62
Alcohol oxidase	Ethanol detection	78
Catalase	Hydrogen peroxide detection	74,79
Creatinine iminohydrolase	Creatinine detection	80
Glucose dehydrogenase	Glucose detection	81
Glucose oxidase	Glucose detection	35,36,38,63,82–89
Glutamate oxidase	Glutamate detection	90
Horseradish peroxidase	Hydrogen peroxide detection	26,74,91–95
Laccase	Catechol detection	19
Lactate oxidase	Lactate detection	96
Lipase	Processing of lipids	97,98
Polyphenol oxidase	Phenol detection	99
S-adenosylhomocysteine nucleosidase (Pfs)	Studies of bacterial signaling	58,60
Sulfite oxidase	Sulfite detection	30
Tyrosinase	Phenol detection	31,33,100
Urease	Urea detection	101
Uricase	Uric acid detection	102
Antibodies		
Alginate-factor B	Detection of factor B in blood plasma	64
Anti-CEA	Detection of carcinoembryonic antigen	103
Carbohydrate antigen 19-9	Detection of carbohydrate antigen	104
Immunoglobulin G	Used as model antibody	76
DNA		
dsDNA	Effect of drugs on dsDNA	42
ssDNA	Sequence specific detection of DNA (hybridization)	27,37,61,105,106
Nanostructures		
Carbon nanotubes	pH responsive hydrogels	17,66
Carbon nanotubes	Glucose detection	63,88,107
Carbon nanotubes	Fabrication of composite films	108
Carbon nanotubes	Insulin detection	109
Fe3O4 nanoparticles	Phenol detection	94
Gold nanoparticles	Pesticides detection	62
Gold nanoparticles	Glucose detection	35
Gold nanoparticles	Hydrogen peroxide detection	92
Metallophthalocyanines	Dopamine detection	41
Tobacco mosaic virus	Assembly of nanoscale materials	110
Vesicles (liposomes)	Drug delivery, chemical signaling	111
Polymers		
Cellulose	Electroactive polymers for actuation	112,113
Poly(acryl amide)	pH responsive hydrogels, drug delivery	18,73,114
Poly(diallyldimethylammonium chloride)	Temperature and pH responsive hydrogels	16
Poly(o-ethoxyaniline) and poly(methacrylic acid)	Impedance spectroscopy for copper ion detection	39
Polyaniline	Electroactive polymers for actuation	17,65
Polyvinyl alcohol (PVA)	pH responsive hydrogels	15
Dyes		
Acetyl yellow 9	Permeability control	86
Azure C	NADH detection	115
Lucifer yellow VS	Permeability control	116
4-(2-Pyridylazo)resorcinol	Detection of Cobalt	117
Procion brown, procion green	Affinity ligand for lysozyme purification	118–120
Prussian blue		121,122

Table 1 (*Contd.*)

Modifying substance	Purpose of modification	References
	Detection of glucose, glutamate, galactose	
Redox salts	Glucose detection	123
Thionine	Optical pH sensor	124
Toluidine blue O	NADH detection	125
Tungsten oxide (WO3)	Electrochromic films	40

The simplest method for attaching biomolecules to chitosan is by surface adsorption. The deposited chitosan film is exposed to a solution of biomolecules, and some of them bind to the surface.[25,33,37,38,42,62,64,80] Since the chitosan chains are positively charged, this approach works particularly well for negatively charged molecules due to the electrostatic interaction.[38,42,64] The biomolecules can also be co-deposited with the chitosan by mixing them in the chitosan solution prior to deposition.[19,31,74] Since biomolecules are normally large, they are trapped in the chitosan matrix even if there is no chemical bond or electrostatic attraction. The content of biomolecules in the film can be varied by their concentration in the initial solution. Both the surface adsorption and physical entrapment methods are straightforward, but they might not provide stable immobilization. The biomolecules can leak out over time since the interaction with the chitosan is weak.

An improved method for attaching biomolecules to chitosan is covalent bonding. For this purpose, the biomolecule of interest must have a functional group that can be linked to chitosan's amines. For example, proteins[56,57] and DNA[27,61,105,106,126] tagged with amine groups can be attached to the chitosan's amines by glutaraldehyde. The glutaraldehyde molecule has aldehydes on both sides that covalently bind to amines, therefore establishing a strong link between the chitosan and the biomolecules. Biomolecules tagged with tyrosine can be bound to the chitosan by first activating them with tyrosinase. This enzyme converts the tyrosine residue into a reactive o-quinone, which binds to the amine groups of chitosan.[29,58,59,61] The covalent binding of biomolecules to chitosan can be performed after a plain chitosan film is deposited,[56,57,105,106] resulting in biomolecule attachment to the film surface only. It can also be performed prior to the deposition by conjugating the biomolecules to chitosan chains in solution.[46,58,61] This produces a composite chitosan film with biomolecules dispersed throughout its volume. We should note that since the chitosan deposition solution is mildly acidic (pH less than about 6) and deposition takes a few minutes, extremely acid-labile biomolecules may not be good candidates for co-deposition. For such labile biomolecules, the first approach would be more appropriate. Once a chitosan film is deposited, it is neutral and the biomolecules are not exposed to harsh conditions.

The covalent bonding approach results in more stable attachment of biomolecules than the surface absorption and physical entrapment methods. It also provides some control over the spatial orientation of the biomolecule, which is attached to the chitosan matrix at a known site. However, the covalent bonding is more complicated, and it requires the biomolecule to have accessible functional groups. Ultimately, the choice of coupling approach depends on the biomolecule in use and on the application of the sensor.

4.2 Modification with nanostructures

Chitosan films have been modified with various nanostructures such as carbon nanotubes, metallic nanoparticles, vesicles, and even viruses (Table 1). The modifications with carbon nanotubes are aimed at increasing the mechanical strength of chitosan-based pH responsive hydrogels and to improve the film's conductivity, resulting in better electrocatalytic activity of chitosan-coated electrodes for amperometric sensors.[17,66,88,107,126,127] Similarly, chitosan films modified with gold nanoparticles have been used in enzyme-based amperometric biosensors.[35,62,92] In addition to improving the electron transfer between the electrode and the solution, the nanoparticles have been shown to enhance the specificity of the sensor due to their interaction with the analyte. Nanoparticles also increase the chitosan surface area available for enzyme immobilization and thus improve the overall catalytic activity.[94]

Chitosan films have been successfully modified with vesicles, including both synthetic surfactant vesicles and liposomes.[111] The vesicles can be filled with other substances such as drugs or signaling chemicals, and they can be released from the chitosan by dissolving it in a low pH solution. Therefore, chitosan films with vesicles can serve for storage and controlled release of substances.

The modification of chitosan with nanostructures can be performed either by co-deposition[88,107,108,111] or layer-by-layer deposition (LBL).[35,39,41] The success of the co-deposition approach depends on how the nanoparticles impact the film-forming properties of chitosan. Sonic agitation can be used to disperse the nanotubes or nanoparticles in the chitosan solution if they aggregate.[66] The LBL approach relies on ionic interactions between a deposited chitosan film exposed to a solution of nanoparticles, and it works mainly for particles with negative charges (since chitosan is cationic).

Improved control over the assembly of the nanostructures in chitosan can be achieved if they contain a specific recognition element. For example, Yi *et al.* demonstrated assembly of viruses on chitosan functionalized with DNA.[110] Exposed RNA at one end of the virus hybridized with the chitosan-bound DNA, thereby anchoring the virus on the chitosan film. The viruses attach only to chitosan with the correct DNA sequence, allowing for spatial selectivity of the assembly.

4.3 Codeposition with other polymers

Chitosan can be co-deposited with other polymers to alter its mechanical properties (Table 1). Chitosan hydrogels with large water content are very sensitive to pH and are useful for

controlled drug release or actuation; however, they have low mechanical strength. It has been shown that combining the chitosan with polymers such as polyvinyl alcohol (PVA)[15] or poly(acryl amide)[18] considerably increases the fracture strength and the elastic modulus of the films. The combination of chitosan with other polymers also alters its micropore structure and allows for tuning its swelling behavior.[114,128]

Chitosan modified with cellulose has been used to make actuator films, also called electroactive paper.[112] Cellulose exhibits a piezoelectricity effect, which is conferred to the chitosan-cellulose composite. Chitosan modified with polyaniline has also been demonstrated as an actuator.[17] In this type of composite material, two modes of actuation are possible. Changing the solution pH causes large strains due to chitosan's swelling, while redox reactions of the polyaniline cause small-scale strain adjustments.

4.4 Modification with dyes

Chitosan films have been successfully functionalized with several different dyes (Table 1). Dyes that act as chromogenic agents undergo an optical absorbance change due to certain chemical or physical conditions and are useful for optical sensing. For example, chitosan modified with 2-(4-Pyridylazo)resorcinol is capable of detecting cobalt ions.[117] Chitosan modified with thionine undergoes a color change with pH variation. Chitosan modified with tungsten oxide changes its color due to redox reactions.[40] Other types of dyes are added to chitosan to exploit their chemical properties and not necessarily their color. For example Prussian blue has catalytic capabilities for hydrogen peroxide, and it has been used with chitosan in amperometric glucose sensors.[121,122] Procion brown and procion green dyes are ligands for certain proteins and have been combined with chitosan to make affinity membranes for lysozyme purification.[118–120]

5 Chitosan characterization methods

A variety of methods have been employed to determine the physical and chemical properties of pure and modified chitosan. Some of these methods are not applicable to chitosan deposited in lab-on-a-chip devices, and specialized samples need to be fabricated (*e.g.* freestanding films or films on transparent substrates). Advanced characterization procedures, such as atomic force microscopy (AFM), Fourier Transform Infrared Spectrometry (FT-IR), and X-Ray Diffraction (XRD) are performed to investigate subtle structural or compositional changes in the chitosan film following fabrication or modification with other substances. The results from relatively simple characterization techniques such as absorbance measurements, tensile testing, or cyclic voltammetry can also be quite valuable, especially for the development of sensors. As discussed in section 6.2, the operation of most chitosan-based sensors is based on a change in optical absorbance, mechanical strain, or electrical conductivity.

5.1 Fluorescence microscopy

Fluorescence microscopy is commonly used to confirm the localized patterning of chitosan films.[12,47] Unmodified chitosan is transparent and is difficult to image with a standard optical microscope. However, it can be readily conjugated with fluorescent dyes and imaged with a fluorescent microscope. Fig. 5 is a micrograph of electrodeposited chitosan labeled with fluorescein, demonstrating the selective pattering of the film.

Fluorescence microscopy is also used to verify the presence of fluorescently active biomolecules in a deposited chitosan film.[61] For example, green fluorescent protein (GFP) has been used to demonstrate the capability of chitosan to bind proteins.[57] Fluorescent intensity images of chitosan films were used to confirm the entrapment of vesicles filled with fluorescent substances.[111] The fluorescence microscopy method is compatible with nearly all types of bioMEMS devices, and it does not require any specialized sample fabrication.

5.2 Fourier transform infrared (FT-IR) and UV-VIS spectroscopy

FT-IR spectroscopy identifies the molecular structures that are present in a substance based on their respective absorption bands in the infrared spectrum. Chitosan exhibits characteristic absorption bands at 3400, 2886, 1643 and 1593 cm^{-1}. These features correspond to the stretching or bending vibrations of chitosan's hydroxyl groups (–OH), protonated amine groups ($–NH_3$), carbonyl groups (–NH–C=O), and amine groups ($–NH_2$), respectively.

Cheng *et al.* and Ma *et al.* performed FT-IR measurements to verify that their respective fabrication techniques with chitosan did not alter its chemical structure.[14,129] Similarly, Kam *et al.* used FT-IR to characterize any change to chitosan that may occur due to storage conditions.[130] The results of chemical reactions, including covalent bonding of molecules to chitosan's amine groups or enzyme-catalyzed hydrolysis, can also be observed by FT-IR.[22,83] Therefore, this method is commonly used to confirm that certain proteins or nanoparticles are

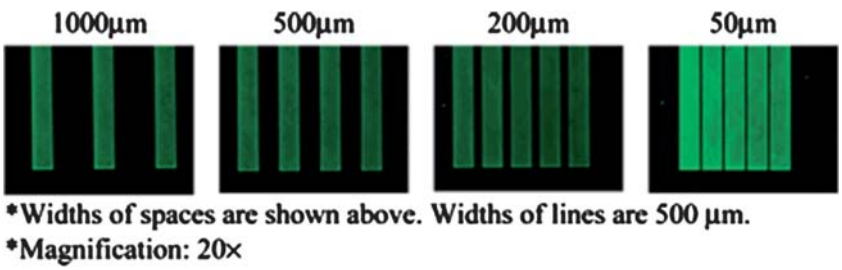

Fig. 5 Resolution of spaces between 500 μm wide gold lines after chitosan deposition and subsequent reaction with NHS fluorescein. The widths of spaces were 1000, 500, 200, and 50 μm. Reprinted with permission from ref. 12. Copyright 2003 American Chemical Society.

immobilized within the chitosan and that they have retained their structure following deposition.[25,30,41,62,86,104,131–133] The co-deposition of chitosan with other polymers can also be studied with FTIR.[15,67,134,135] For example, Cai *et al.* added cellulose to chitosan to decrease the blend's sensitivity to humidity and used FT-IR to characterize the effect of blending different ratios together.[113]

UV-VIS spectroscopy measures the light absorbance of the sample in the UV and visible ranges. Traditionally, it has been used along with the Beer–Lambert law to measure the concentration of an absorbing species in liquid, but it is also suitable for transparent solids. Many research groups have used UV-VIS measurements to study changes that occur in chitosan upon conjugation with other substances such as enzymes or metallic nanoparticles.[41,62,86,92,103] For example, Wu *et al.* noted a large absorption increase around 420 nm when chitosan is subjected to oxidized catechol molecules.[22] This effect has been used to make absorbance-based optical sensors for catechol detection,[54] which are discussed in section 6.2.

5.3 X-Ray diffraction

X-Ray diffraction (XRD) can yield further information regarding the crystalline structure of the solid. Chitosan exhibits a fairly broad peak due to its amorphous structure at 2θ of 20°. Studies have suggested that neutralization of chitosan by NaOH induces crystalline formations as evidenced by the creation of peaks at 2θ of 10° and 15°.[130] Ma *et al.* performed XRD measurements to characterize structural changes in the chitosan resulting from different degrees of chitin deacetylation.[129] Ogawa *et al.* used XRD data to directly compare the molecular and crystallographic differences between chitin and chitosan.[136]

Characterizing the changes in chitosan's crystalline structure may be useful for determining the density of exposed surface amine groups. These amine groups are crucial for the conjugation of biomolecules to the chitosan and are what make it a functionalization layer in sensors.

5.4 Atomic force microscopy (AFM)

Nanometer changes in the morphology of the chitosan film can be determined using atomic force microscopy (AFM). A micro-scale cantilever with a sharp probe tip is scanned over the chitosan surface. The minute deflections of the cantilever are measured to create a surface topography map. Cai *et al.* used AFM to study the morphology of adsorbed chitosan films on glass slides.[137] AFM measurements are also often used to confirm cross-linking reactions at the chitosan surface and to verify successful conjugation of chitosan with other materials such as metallic nanoparticles. Lin *et al.* observed an increase in thickness and surface regularity for crosslinked chitosan *vs.* non-crosslinked chitosan.[104] Du *et al.* used AFM imaging to ensure that gold nanoparticles were embedded in the chitosan film following the chemical reaction between tetrachloroauric (III) acid, acetic acid and chitosan.[62] Similar studies have been performed on chitosan films containing metallic nanoparticles.[94] The morphology of chitosan films that have been patterned using either nanoimprint lithography or microcontact printing is also commonly examined using AFM.[13,44,45] Example images of chitosan patterned using nanoimprint lithography can be seen in Fig. 6

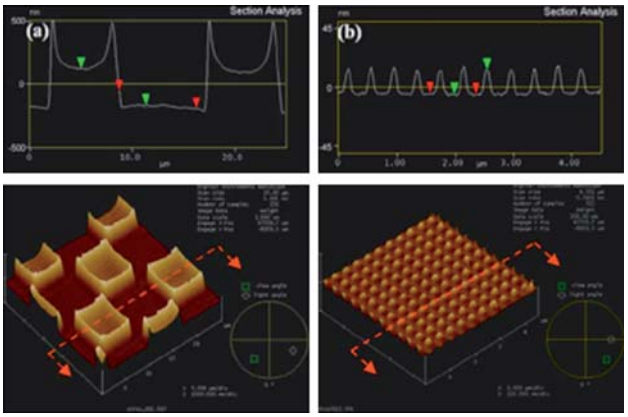

Fig. 6 Examples of AFM images taken of (a) printed chitosan squares 6.7 μm across and (b) chitosan nanodots with 150 nm width and 400 nm pitch. Reprinted with permission from ref. 13. Copyright 2007, American Institute of Physics.

5.5 Cyclic voltammetry

Cyclic voltammetry (CV) is an electrochemical method for quantifying redox reactions of target species within the chitosan films. The redox activity of compounds such as thionine, DNA, dopamine and other proteins conjugated with chitosan have been reported.[37,74,124] Siqueira *et al.* utilized cyclic voltammetry measurements to confirm the number of bilayers and the types of metallic nanoparticles embedded in layer-by-layer chitosan films.[41] Since the electrodeposition of chitosan involves a reduction reaction at the cathode, cyclic voltammetry has been utilized to characterize deposition conditions and the permeability of chitosan films to various redox species.[23] By detecting the onset of hydrogen gas evolution at the cathode due to increasing potential, the electrodeposition conditions of chitosan can be more accurately controlled with regard to film thickness and uniformity.

Although most of these studies involve large electrodes placed in a beaker of solution, the CV method can be implemented on the microscale through standard fabrication techniques. Many groups have already demonstrated CV measurements for film characterization or analyte detection with patterned micro-scale electrodes.[138–140]

5.6 Tensile testing

Understanding the mechanical properties of chitosan and their dependence on environmental conditions aids in the fabrication of stronger or more flexible films. The stiffness and strength of chitosan can be determined by tensile testing. This requires preparation of a freestanding film or fiber that is significantly longer in one direction compared to the other two. The sample is stretched with increasing tensile force until it breaks. The Young's modulus can be extracted from the resulting stress-strain curve; it has been measured for several different fabrication methods and for blends of chitosan with other materials.[14,22,66,113] Changes in chitosan's mechanical properties

can be used to characterize structural variations in the chitosan film as well as fluctuations in environmental conditions. For example, Cai *et al.* observed a decrease in Young's modulus for chitosan/cellulose blends with increasing chitosan content and attributed this to a decrease in crystallinity combined with an increase in moisture uptake caused by the chitosan.[113] Kim *et al.* also used a chitosan/cellulose blend to investigate the effect that free ions such as Cl^- and NO_3^- have on the mechanical behavior of the film.[112] Chitosan fibers have also been shown to undergo strain variations with the pH of the solution.[17,65]

As expected, crosslinking the chitosan chains yields an increase in the mechanical stiffness.[22,34,71] Adekogbe *et al.* observed this effect after crosslinking chitosan with DTBP and also found that tensile strength increases for higher degrees of chitosan deacetylation.[71]

6 Microscale devices using chitosan

The majority of chitosan-based devices reported in literature to date are macroscale. However, significant progress has been made in the area of microfabrication with chitosan as discussed in section 2, and the number of chitosan-based microscale devices is rapidly increasing. The unique properties of chitosan and its multiple possible modifications make it particularly useful for bioMEMS applications. Here, we review recent demonstrations of microdevices functionalized with chitosan. These examples fall under two broad categories: platforms for assembly of other substances and sensors.

6.1 Immobilization platforms

Chitosan can serve as a matrix for immobilizing other substances such as enzymes, drugs, and even living cells. This capability is essential in microdevices used for biocatalysis, drug delivery, and cell studies.

Xie *et al.* reported a microneedle array for transdermal transport of drug compounds dispersed in chitosan films.[141] The model drugs calcein and bovine serum albumin (BSA) are dissolved in a chitosan solution. A thin chitosan film is then deposited on a silicon microneedle array by solution casting. When pressing the array on rat skin, the chitosan-coated microneedles penetrate the outer protective layer of the skin and increase its permeability to the drugs. The permeation rate of the drugs can be regulated by the chitosan film thickness and by the concentration of drugs in the chitosan. This device provides a way for controlled transdermal delivery of therapeutic agents as an alternative to oral administration.

Co *et al.* used chitosan to micropattern two different cell types on the same substrate sequentially.[142] First, the chitosan-coated substrate is patterned with a cell-resistant polyelectrolyte by microcontact printing. The first set of cells adhere only to the exposed chitosan. Then, the cell-resistant polyelectrolyte is covered with fresh chitosan, making these regions adhesive to cells again. The substrate is exposed to a second type of cells, which adhere to the new chitosan but not to the regions already occupied by cells. This approach can be used for incorporating different cell types into microdevices with sub-cell patterning resolution.

Kastantin *et al.* constructed a polymeric microfluidic device capable of patterning proteins by means of chitosan electrodeposition.[56] It consists of SU-8 channels with a PDMS capping layer and polypyrrole electrodes. Chitosan is deposited on the electrodes inside the fluidic channel, and the model protein GFP (green fluorescent protein) is assembled in the device by glutaraldehyde covalent bonding. The GFP retains its fluorescence, indicating that the structure of the protein is preserved. The chitosan deposition and GFP attachment are addressable, and they occur only at the electrodes on which a voltage is applied. This demonstrates for the first time the feasibility of chitosan-mediated protein assembly inside microfluidic devices with spatial selectivity and electrical control.

Park *et al.* developed another microfluidic device for biomolecule assembly using electrodeposited chitosan.[57] This implementation consists of SU-8 channels, gold electrodes, a PDMS gasket, and a Plexiglass compression package for reversible sealing. The package can be disassembled after use, allowing the chitosan film thickness inside the device to be measured by contact profilometry. As seen in Fig. 7, the model protein GFP is assembled only on electrodes where a voltage is applied. Traditional protein patterning methods such as microcontact printing require physical access to the device surface and must be performed before packaging. However, the chitosan electrodeposition method enables the biomolecule patterning to be performed after packaging and therefore decouples the manufacturing of the device from its use. The microfluidic device can be completely fabricated, packaged and stored without any perishable biomolecules. The biomolecules can be later added by chitosan mediated assembly when the device is ready for use.

The microfluidic platform demonstrated by Park *et al.* was further developed by Luo *et al.* and adapted to enzyme assembly.[58] The device in this work is aimed at studying a bacterial metabolic pathway. The enzyme S-adenosylhomocysteine nucleosidase (Pfs), which participates in the synthesis of bacterial signaling molecules, is tagged with a tyrosine residue and is conjugated with chitosan in solution. The chitosan-Pfs conjugate is electrodeposited on electrodes inside the microfluidic channel after complete packaging of the device as shown in Fig. 8 It was shown that the chitosan-immobilized Pfs retains its catalytic activity and even that it has better long-term stability than the free enzyme in solution. The chitosan can be dissolved with a mildly acidic wash, allowing the device to be reused multiple times.

Fernandes *et al.*, in collaboration with Luo, used a similar microfluidic device to conjugate biological nanofactories to an immobilized chitosan film.[143] These nanofactories shown schematically in Fig. 9 consist of linked enzymes for substrate conversion as well as antibodies for cell entrapment. In this study, *Escherichia coli* was captured by the nanofactories while their response to an autoinducer signaling molecule (AI-2) was observed *via* the production of GFP by the bacteria. The chitosan-provided spatial control of the biological nanofactories allows for the response of the captured bacteria to be observed as a function of outwardly controlled parameters such as pH, temperature or flow rate.

Luo *et al.* continued the work involving enzyme assembly with chitosan by improving the microfluidic packaging in order to optimize the signal-to-noise ratio of the enzymatic conversion.[144] The device is improved by using an alignment scheme for the

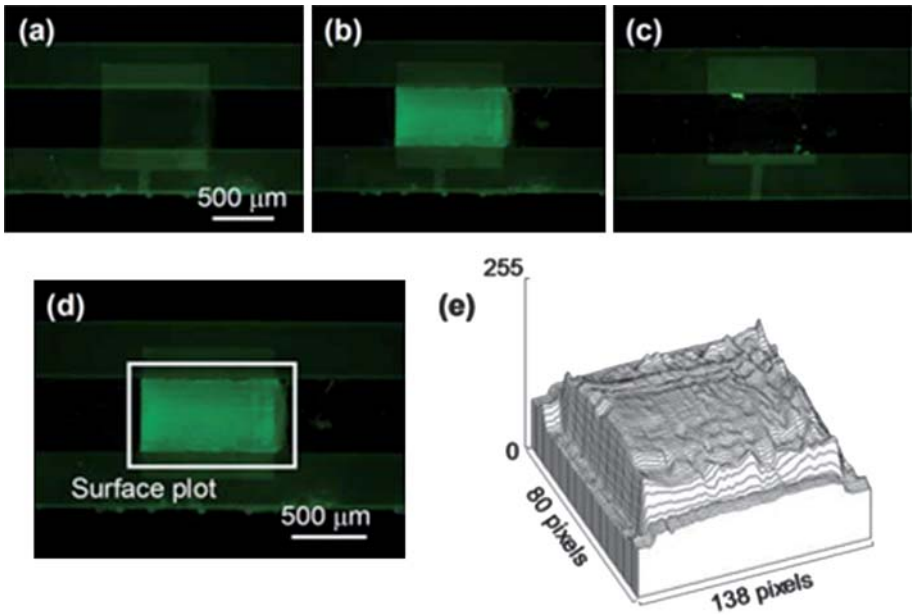

Fig. 7 Chitosan mediated assembly of GFP in micro fluidic device. (a) Fluorescence image of the assembly site after glutaraldehyde reaction; (b) after GFP reaction; (c) negative control (no bias); (d) GFP conjugated chitosan film; (e) Image J fluorescence surface plot.[57]—Reproduced by permission of the Royal Society of Chemistry.

fluidic interconnects which reduces the dead volume created by reservoirs at the inputs and outputs. In addition, a cross channel design over the sensor site allows the user to separate the path of the enzyme flow from that of the substrate. This separation reduces parasitic enzymatic conversion that may result from non-specifically bound enzymes on the channel walls. By implementing both improvements into the design of the device, the signal-to-noise ratio of the enzymatic conversion was improved from 0.72 to 2.43.

6.2 Sensors

The other category of microdevices that employ chitosan are sensors. Typically, in this case the chitosan is modified with probe biomolecules that serve as specific recognition elements. When a target biomolecule in the sample binds to the probe, a physical signal is produced such as a change of mass, strain, optical absorbance, or electrical conductivity. This signal is then detected by mechanical, optical, or electrochemical means.

Zhu *et al.* reported micromachined amperometric sensors for glucose, glutamate, and galactose using chitosan membranes.[24] The device has a pyramidal chamber array etched in silicon anisotropically (Fig. 10). Each chamber contains a platinum electrode at the bottom covered with a solution-cast chitosan membrane. The membranes are modified with the enzymes glucose oxidase or galactose oxidase by means of glutaraldehyde crosslinking. The enzymes perform highly specific conversion of the samples, and the products of the conversion are detected by

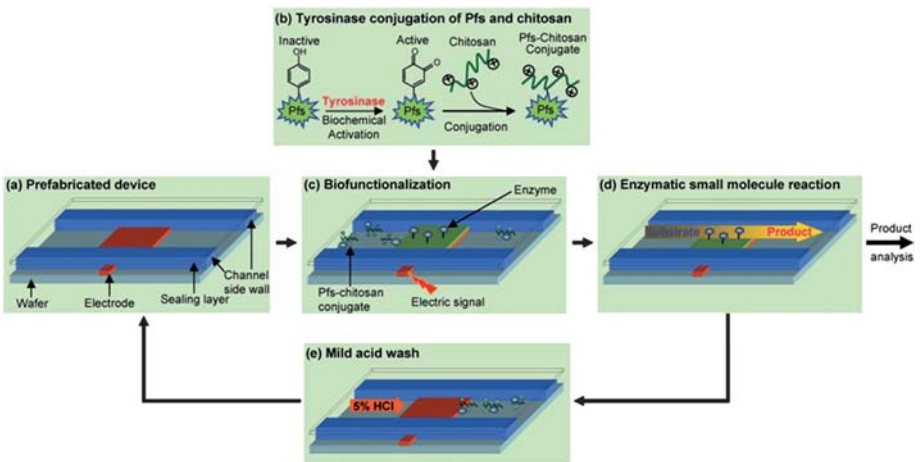

Fig. 8 Schematic of chitosan conjugation and electrodeposition in a microfluidic channel. (a) Prefabricated device, (b) conjugation between chitosan and the enzyme Pfs, (c) electrodeposition of Pfs-chitosan conjugate, (d) enzymatic small molecule detection, (e) mild acid wash removes chitosan and refreshes electrode surface.[58]—Reproduced by permission of the Royal Society of Chemistry.

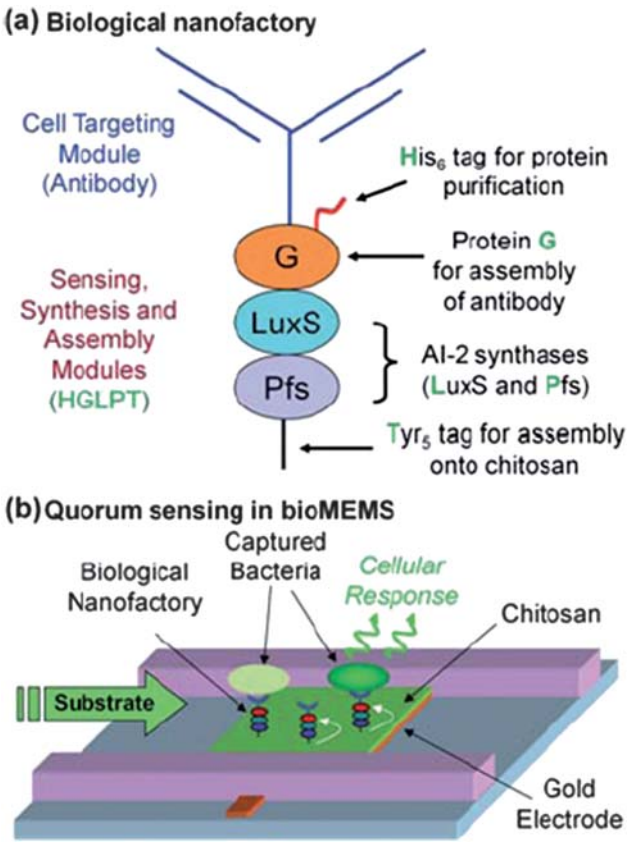

Fig. 9 Assembly and manipulation of quorum sensing (QS) bacteria in a bioMEMS device *via* biological nanofactories. (a) Components of a biological nanofactory: cell targeting module (antibody); sensing, synthesis and assembly modules (HGLPT nano-construct). (b) The nanofactories are spatially assembled onto chitosan electrodeposited within the device; they capture targeted bacteria and manipulate their QS response.[143]—Reproduced by permission of the Royal Society of Chemistry.

cyclic voltammetry. In addition to enzyme immobilization, the chitosan membrane also has a second role. Due to its selective permeability, it reduces the penetration of interferents such as uric acid or ascorbic acid to the electrode and improves the specificity of the sensors.

Odaci *et al.* demonstrated the use of chitosan for the immobilization of microbial cells and further sensing of their respiratory activity.[145] Chitosan solutions are mixed with carbon nanotubes for enhanced electrical conductivity and deposited over an electrode by solution casting. The bacteria *Pseudomonas fluorescens* and *Pseudomonas putida* are then allowed to adhere to the chitosan film over a period of 30 min. The reduction of the dissolved oxygen produced by bacterial respiration in the presence of glucose is electrochemically detected. The biosensor is used to test the effect of varying pH, temperature, cell density, and sugar type to the respiratory activity of the bacteria. This device demonstrates how the biocompatible nature of chitosan can be used to help analyze biomolecules *in vitro*.

Powers *et al.* developed a multipurpose optical detection platform with an electrodeposited chitosan film.[20] The device consists of an SU-8 microfluidic channel and an optical waveguide defined on a Pyrex substrate as shown in Fig. 11. One of the facets of the optical waveguide is located at the sidewall of the fluidic channel and is covered with a transparent ITO electrode for chitosan deposition. This allows a chitosan film to be formed in the path of light passing through the waveguide. Molecules attached to the chitosan film are detected by the change in the output light spectrum, which is analyzed with an optical fiber coupled spectrometer. The concentration of analytes attached to the chitosan can be much higher than their concentration in the solution. Therefore, the chitosan film acts as a pre-concentrator and enhances the optical detection signal. Furthermore, the ability to deposit the chitosan film out-of-plane (*i.e.* perpendicular to the substrate surface) and the use of in-plane optics allows for dense integration of the sensors. This demonstrated chitosan

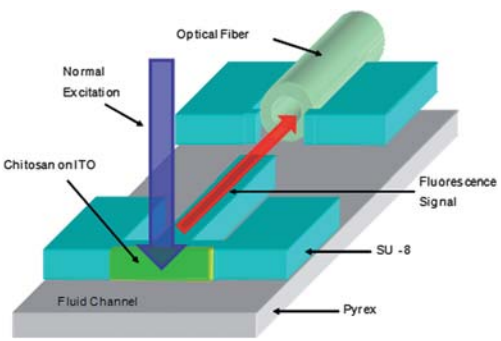

Fig. 11 Perspective view of optical waveguide sensor with chitosan film on sidewall. The waveguide is shortened to show fiber coupling. The blue arrow represents the optical excitation signal while the red arrow represents the collected fluorescence signal.[20]–Reproduced by permission of the Royal Society of Chemistry.

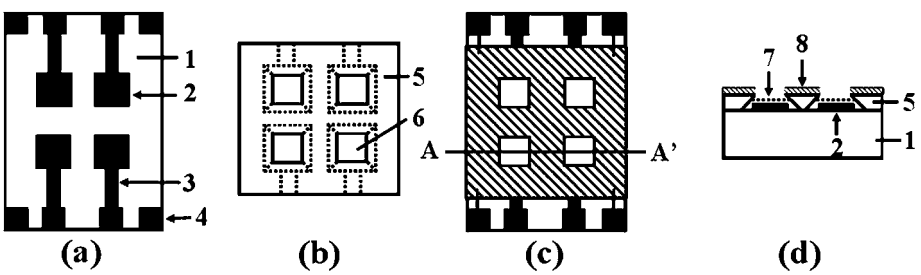

Fig. 10 Schematic of micromachined amperometric sensor. 1. Pyrex glass down-substrate, 2. Pt WE, 3. Conduction line, 4. Pads, 5. Up-substrate made of silicon, 6. Quadratic hole, 7. Chitosan membrane with cross-linked enzyme, 8. Ag/AgCl RE. (a) Down-substrate, (b) up-substrate, (c) bonding between up- and down-substrates, (d) cross section along A-A′. Redrawn with permission from ref. 24.

sidewall patterning technique is crucial for the design of future optical, in-plane, lab-on-a-chip sensors.

The same optical platform was applied to detection of DNA hybridization.[21] In this work, the chitosan film is functionalized with amine-tagged probe DNA by glutaraldehyde crosslinking. It is then exposed to fluorescently labeled target DNA with two different sequences. The spectrum changes of the output light confirm that sequence specific binding of the target to the probe occurs. The device is also used for detection of phenols after some modifications to the microfluidic design.[54] The microfluidic channel is enclosed using a capping layer of PDMS while metal capillaries and plastic tubing allow for continuous fluid flow through the device as shown in Fig. 12. It is shown that the products of phenol electrochemical oxidation readily bind to the amine groups of the chitosan film and cause a measurable change in its optical absorbance. Various phenol concentrations are detected and a dramatic increase in the absorbance signal is observed for devices that include the chitosan film *vs.* those that do not. Common interferents such as ascorbic acid do not react with the chitosan, which results in good selectivity of the phenol sensor.

Some other demonstrations of optical sensors using chitosan have also been reported. Lin *et al.* constructed a chemiluminescent immunosensor for Carbohydrate Antigen 19-9 (CA19-9) containing a chitosan membrane.[104] The membrane is functionalized with CA19-9 and placed inside a fluidic channel monitored by a photomultiplier tube. For detection, the CA19-9 sample is mixed with enzymatically labeled CA19-9 antibodies that produce a chemiluminescent signal when injected into the channel. The concentration of CA19-9 in the sample is determined indirectly from the power of that signal. Yusof *et al.* reported an optical sensor for measuring cobalt ion concentration in solution.[117] A chitosan membrane doped with the chromogenic reagent 4-(2-Pyridylazo) resorcinol (PAR) is prepared and placed inside a flow cell. The PAR undergoes a color change when exposed to cobalt. Optical fibers are used to shine light on the membrane, collect the reflected light, and guide it to a spectrometer. The cobalt concentration can be reliably determined from the measured membrane reflectance.

Resonant sensors using chitosan have also been demonstrated. Deng *et al.* reported a piezoelectric immunosensor for the detection of the enzyme factor B in human serum.[64] A piezoelectric quartz crystal (PQC) with gold electrodes is first coated with a self assembled monolayer of cysteamine and then with chitosan by glutaraldehyde crosslinking. Factor B antibodies conjugated with a negatively charged polymer, alginate, are adsorbed on the chitosan. When the device is exposed to samples containing factor B, its resonant frequency decreases in response to the mass loading caused by the immunorecognition. Experiments without chitosan were also performed, in which the antibodies were attached to the cysteamine monolayer directly by glutaraldehyde crosslinking. It was discovered that the use of chitosan significantly improves the response of the sensor, presumably by better preserving the biological activity of the antibodies. The chitosan mediated assembly also allows the sensor to be more easily regenerated by washing off the alginate-antibody complex with a saline solution.

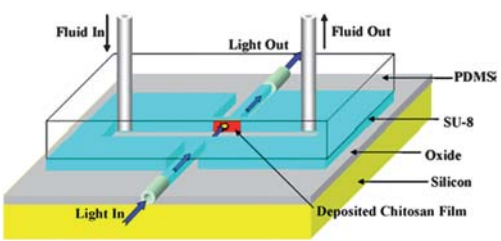

Fig. 12 Schematic of the microfluidic sensor for phenol detection. Both waveguides patterned perpendicular to the channel allow for in-plane light interrogation. Reprinted from ref. 54 with permission from Elsevier.

Another chitosan-based PQC sensor was developed by Zhao *et al.* for determination of fish freshness.[146] The device surface is coated with a chitosan film containing pimelic acid. The sensor exhibits a specific response to trimethylamine (TMA) vapor, which is a byproduct of fish decomposition and can be used as an indicator of its freshness. TMA concentrations down to 50 ppm are reliably detected, and the response to interfering gases such as water vapor is shown to be negligible.

A different type of chitosan-coated resonator was constructed by Gao *et al.* for the detection of glucose in urine.[83] A 28 μm thick magnetoelastic element is covered with a pH responsive polymer and with chitosan conjugated to glucose oxidase. Any glucose in the sample is converted by the enzyme into gluconic acid and reduces the pH of the solution, increasing the mass of the pH responsive polymer. The magnetoelastic element is excited by a time-varying magnetic field to vibrate at its resonant frequency, which depends on the mass loading of its surface. As the element vibrates, it generates a secondary magnetic filed that is measured by a pickup coil and used to determine the resonant frequency. Therefore, the concentration of glucose can be measured wirelessly by the resonant frequency shift.

Koev *et al.* has demonstrated microcantilever sensors functionalized with electrodeposited chitosan.[27] These devices are micromachined beams (Fig. 13) that can be used for detection either in dynamic (resonant) or static mode. In the dynamic mode, the cantilever is actuated electrostatically, and its resonant

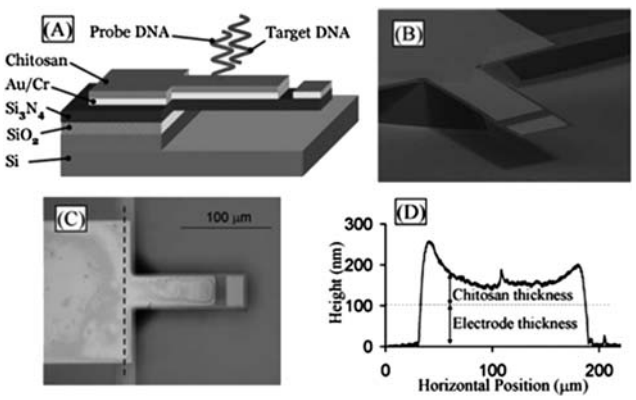

Fig. 13 (a) Schematic of microcantilever with chitosan used for detection of DNA hybridization and for detection of dopamine electrochemical oxidation. (b) SEM of fabricated cantilever. (c) Optical micrograph of cantilever after chitosan electrodeposition. The chitosan is deposited everywhere except at the electrically isolated tip. (d) Contact profiler scan of chitosan film along dashed line in C.[27]–Reproduced by permission of the Royal Society of Chemistry.

frequency is measured. Any change of mass on the surface causes a resonant frequency shift. In the static mode, the position of the cantilever tip is measured. Substances that bind to the surface create a stress and deflect the beam. The cantilevers are functionalized with a chitosan-based coating to make them specific, and are used for the detection of DNA hybridization or phenols. For the DNA hybridization experiments, the coating consists of probe DNA immobilized on chitosan by glutaraldehyde crosslinking. Sequence-specific detection of target DNA was demonstrated by both the dynamic and static methods. The measured sensor response was significantly higher than values reported in literature for similar sensors without chitosan.

For the phenol detection experiments, the cantilever coating is an unmodified chitosan film.[27,52] The phenol samples are electrochemically oxidized; the products of the oxidation are known to crosslink chitosan and change its structure. The crosslinking causes a stress in the film and bends the cantilever, allowing for static-mode detection. This chitosan mediated detection was shown to be specific to phenols. For example, the oxidation of ascorbic acid did not cause chitosan crosslinking and cantilever bending.

Mao *et al.* used microcantilever sensors to characterize the swelling behavior of chitosan/gelatin hydrogels.[72] Due to protonation and deprotonation of the amine groups in the chitosan, the hydrogels undergo volume changes when the pH is varied and can be useful for controlled release devices. Cantilevers are coated with mixtures of chitosan and gelatin, and their deflections under different pH conditions are measured. It was shown that the swelling depends on the ratio of chitosan to gelatin and the content of the crosslinking agent. The device demonstrated in this work can potentially be used for biosensors by incorporating enzymes that cause pH changes.

The examples of devices presented so far used chitosan as a coating layer but not as a structural material. Cheng *et al.* reported a cantilever made entirely of chitosan.[14] The device is fabricated by spin casting of chitosan, conventional photolithography with photoresist, etching of the chitosan with oxygen plasma, and etching the Si substrate under the chitosan with XeF_2. An SEM of the released cantilever is shown in Fig. 14. The ability to construct devices entirely of chitosan can potentially simplify the fabrication process and lower the cost.

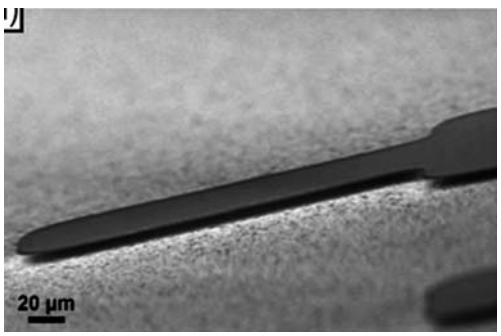

Fig. 14 Partially released chitosan cantilever on silicon with dimensions 240 × 25 × 2 μm. Chitosan serves as a sufficient structural material to support itself. Reprinted with permission from ref. 14. © 2008 IEEE.

7 Conclusions

The polysaccharide chitosan provides a unique interface between microdevices and biology. It offers the ability to realize lab-on-a-chip devices with spatiotemporal control of biomolecules and other organic compounds. In reviewing the properties of chitosan, it is instructive to discuss briefly other biofunctional materials used in MEMS. These alternative approaches can be broadly divided into self assembled monolayers and surface immobilized polymers.

Self-assembled monolayers (SAMs) are organized layers of molecules with a head group and a tail.[147–150] The head group, typically silane or thiol, has a binding affinity for the substrate; the tail has a functional group, to which biomolecules can be anchored. A variety of SAM systems have been demonstrated. They provide very strong biomolecule attachment to the surface, and they are used extensively in the area of DNA microarrays.[151] Although SAMs have many excellent properties, chitosan has some potential advantages. It has been shown that chitosan provides a stronger response[27,64] and better long-term stability[58,100] of sensors with immobilized biomolecules than the ones bound directly to the surface by SAMs. This may be due to the large water content in the chitosan film, which provides a favorable microenvironment for the biomolecules and preserves their three-dimensional structure. In addition, the high density of amine groups on the chitosan network and its large surface area allows for high loading of the film with biomolecules. These effects are still not completely understood and need further study.

Another potential advantage of chitosan over SAMs is its robustness. SAMs require atomically clean surfaces and fail to form even if there is minor contamination.[148,149] They require rigorous cleaning procedures with piranha solution or oxygen plasma, which are typically not feasible for already-packaged devices and for microfluidic channels. In contrast, chitosan is much more tolerant to the properties of the device surface because it does not form a chemical bond with it. A mildly acidic wash is sufficient for cleaning the surface and even removing previous chitosan to allow for reusing the device multiple times[58]

The other approach for functionalization of bioMEMS is based on surface immobilized polymers. A wide variety of polymers in addition to chitosan have been used in microdevices, including alginate,[152,153] cellulose,[154] collagen,[155] gelatin,[156] polyethylene glycol,[157] polypropylene glycol,[152] polylysine,[158,159] PLGA,[160] and even spider silk.[161] The functions performed by these polymers are similar to the ones performed by chitosan: drug release,[152,160] cell culture adhesion,[155,156,158,159] structural support and actuation,[154,161] scaffolding for tissue engineering,[153] and biomolecule entrapment.[157]

There is insufficient data at present to compare the properties of chitosan comprehensively with the other polymers. Depending on the application, some of the polymers may offer better performance than chitosan. For instance polylysine is more popular for cell culture adhesion,[158,159] and the mechanical properties of spider silk are superior to those of chitosan.[161] However, in general, chitosan has two advantages over other biofunctional polymers in MEMS: the availability of dense amine groups for biomolecule attachment and fabrication through electrodeposition. Most other polymers can entrap

biomolecules only by physical absorption, which is weak and may allow them to leak out over time. Chitosan's amines enable for strong covalent attachment as described in Section 4.1 and improved long-term stability.

Most of the fabrication techniques used for chitosan have also been demonstrated for other biopolymers as well: solution casting,[152,159] spin casting,[161] printing,[154] molding,[160] and photolithography.[156,157] However, the electrodeposition method is unique to chitosan, and it allows improved pattern control and more fabrication flexibility. The other patterning methods (printing and lithography) require physically accessible planar surfaces. In contrast, chitosan can be electrodeposited on any surface geometry (including vertical sidewalls[20] and cantilever beams[27]) and inside enclosed fluidic devices.[57] Therefore, the functionalization can be performed after complete packaging of the device and just prior to its use.[57] In addition, chitosan electrodeposition is (i) reagentless (no requirement for complex conjugation chemistries or extensively-cleaned surfaces) and (ii) reversible with a change in conditions (potentially, reversibility allows recovery of the biomolecule and enables post-fabrication biofunctionalization).

While the reviewed demonstrations of chitosan functionalization have been successful, many opportunities for future improvement exist. Only a small portion of the possible chitosan modifications have been employed in bioMEMS. We expect that more of the knowledge generated by macroscale applications of chitosan will be used for microdevices. In addition, new modification methods will be developed to further enhance the functionality of chitosan based devices. We also believe that chitosan biofabrication methods will be improved, possibly by transitioning toward 3D shaped structures instead of 2D thin films. This will provide more area for surface immobilization of biomolecules, cells and other substances.

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