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Chitosan to electroaddress biological components in lab-on-a-chip devices

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ABSTRACT

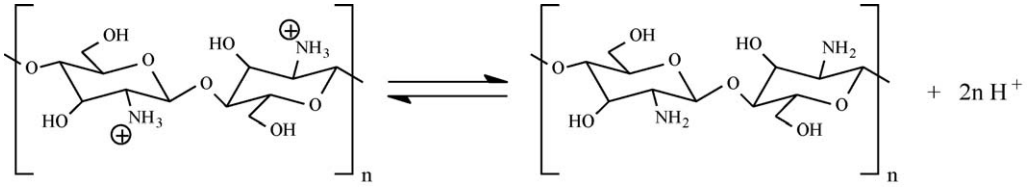
Chitosan offers a unique set of properties that suggest its potential for interfacing biological components into electronic devices for lab-on-a-chip (LOC) applications. Specifically, chitosan's pH-responsive film-forming properties allow it to be electrodeposited as a stable thin film in response to localized cathodic signals. In addition, the electrodeposited chitosan film can be electrochemically activated for protein conjugation by applying an anodic potential to the chitosan-coated electrode while it is immersed in an aqueous solution containing NaCl. Together electrodeposition and electrochemical protein conjugation allow proteins to be electroaddressed to electrode surfaces quickly and without the need for reactive reagents. On-going efforts aim to extend protein electroaddressing from patterned chips to microfluidic LOC devices. Additional efforts are beginning to show the potential for integrating chitosan with sensor technology to transduce chemical and biological events (e.g., molecular recognition) into device-compatible optical, electrical and mechanical signals. Highlights of the progress in using chitosan as the bio-device interface are reviewed.

1. Introduction

There is great interest in developing lab-on-a-chip (LOC) devices that can perform high throughput analysis with small sample volumes. For instance, LOC devices are being pursued to enable biological fluids (e.g., blood or saliva) to be rapidly analyzed for metabolites (e.g., glucose) or protein biomarkers. We believe chitosan offers a unique combination of properties for LOC applications and as illustrated in Fig. 1 we are examining how chitosan can be employed to mediate the assembly of proteins (e.g., antibodies) at individual sensor addresses within a microfluidic channel.

2. Electrodeposition

The primary amines in chitosan's repeating glucosamine residues confer *pH-responsive* properties as indicated by the following reaction.



Soluble Insoluble

At low pH, the amines are protonated and chitosan is a water-soluble cationic polyelectrolyte. At high pH, the amines are deprotonated, chitosan looses its charge and becomes insoluble. Importantly, chitosan's pKa is near neutrality (Anthonsen & Smidsrod, 1995; Rinaudo, Pavlov, & Desbrieres, 1999; Sorlier, Denuziere, Viton, & Domard, 2001; Strand, Tommeraas, Varum, & Ostgaard, 2001; Varum, Ottoy, & Smidsrod, 1994) and the soluble-insoluble transition occurs near a pH of 6.3. Also important, is that

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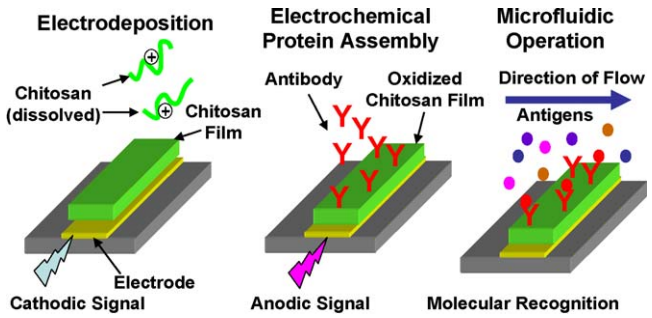

Fig. 1. Schematic illustrating chitosan's potential to interface biological components (e.g., antibodies) to sensor addresses of microfluidic LOCs.

chitosan does not simply precipitate at high pH, but rather chitosan is *film-forming* and can undergo a sol–gel transition to form a robust hydrogel network upon an increase in pH (Montembault, Viton, & Domard, 2005).

Chitosan's pH-responsive film-forming properties enable this polymer to electrodeposit as a stable film in response to cathodic signals. Fig. 2 illustrates the electrodeposition mechanism. Cathodic reactions lead to the net consumption of H^+ and the generation of a localized pH gradient. Chitosan chains from solution "recognize" the localized region of high pH adjacent to the cathode and respond by electrodepositing as a thin film (Fernandes et al., 2003; Pang & Zhitomirsky, 2005; Wu et al., 2002). Importantly, electrodeposited chitosan forms a 3D hydrogel network that is stable in the absence of an applied voltage (although this network re-dissolves at pH < 6). For instance, the photograph in Fig. 2 shows a thick electrodeposited chitosan film that has been peeled from the cathode surface. This photograph illustrates that the electrodeposited films are stable—provided the pH is retained above about 6.3. Previous studies demonstrated that the thickness of the electrodeposited chitosan film can be controlled by the deposition conditions (Wu et al., 2002) and that deposition is spatially selective in the lateral dimensions (Wu et al., 2003).

Chitosan's diverse properties also enable various mechanisms to be used to assemble proteins with the electrodeposited film. The Chen group in Nanjing University demonstrated that proteins could be co-deposited with chitosan and entrapped within the chitosan gel network. These co-deposited proteins were observed to retain their biological activity (e.g., enzymatic activity) (Luo, Xu, Du, & Chen, 2004). Proteins can also be physically (i.e., reversibly) linked to chitosan by exploiting chitosan's cationic or metal-binding properties (Shi, Wu, et al., 2008). Further, the nucleophilic properties of chitosan allow various chemical (Vazquez-Duhalt, Tinoco,

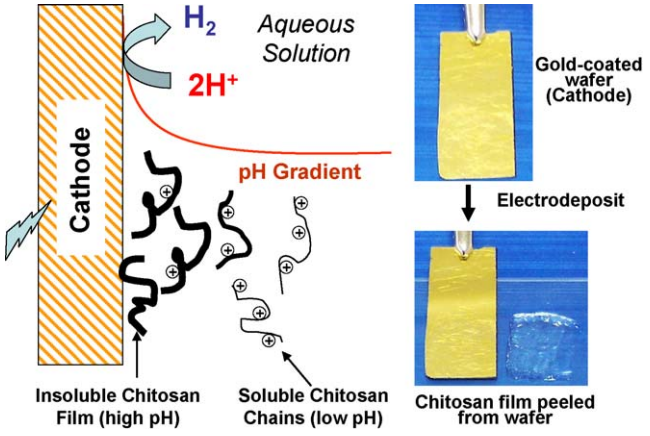

Fig. 2. Chitosan's electrodeposition. Cathodic reactions generate a locally high pH that induces chitosan to undergo a sol–gel transition to form a hydrogel film.

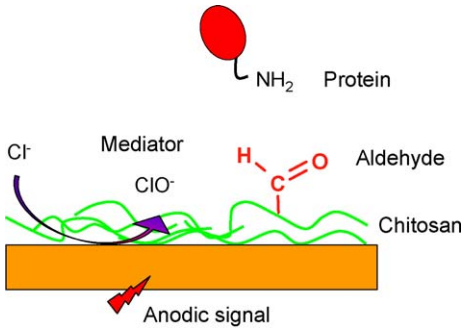

Fig. 3. Proposed mechanism for the anodic activation of chitosan and subsequent protein conjugation.

D'Antonio, Topoleski, & Payne, 2001) and biochemical (Chen et al., 2003) approaches to be employed to covalently attach proteins to chitosan. Finally, standard biotinylation chemistries can be employed with chitosan to permit selective protein assembly through (strept)avidin–biotin linkages (Shi, Liu, et al., 2008). In comparison, co-deposition is simple and rapid although the possibility exists that co-deposited proteins can diffuse through and leak from the chitosan films. Protein assembly methods that employ coupling chemistries can yield stable protein–chitosan linkages but these methods generally require more preparation steps, incur greater costs (for the coupling reagents) and may require greater care due to the hazard of some reagents. Biological assembly methods based on metal binding or enzyamtic conjugation may require the protein to be engineered to have added amino acid sequences (i.e., fusion tags) that permit assembly and potentially even orient the protein. Thus, the appropriate choice of assembly methods depends on the specific details of the application.

3. Electrochemical protein assembly

Recently, an electrochemical protein conjugation approach was developed in which anodic signals are used to activate chitosan for protein assembly (Shi et al., 2009). The proposed mechanism for chitosan activation and protein conjugation is illustrated in Fig. 3. Known anodic oxidation reactions of NaCl are hypothesized to generate a reactive mediator (e.g., hypochlorite; ClO^-) (Bechtold, Turcanu, Campese, Maier, & Schrott, 2006; Cheng & Kelsall, 2007). The mediator diffuses from the electrode surface into the film and oxidizes chitosan. Polysaccharides are known to be oxidized by various oxidants (e.g., NaOCl) and oxidized substituents (e.g., aldehydes) are capable of reacting with proteins (Bragd, van Bekkum, & Besemer, 2004; Ehrenfreund-Kleinman, Domb, & Golenser, 2003; Fang, Takahashi, & Nishinari, 2005; Lee & Frasch, 2001; Sierakowski, Freitas, Fujimoto, & Petri, 2002). Consistent with this hypothesis are results from X-ray photoelectron spectroscopy (XPS) which show an increase in carbonyl (presumably aldehyde) content for anodically oxidized chitosan films (Shi et al., 2009). Importantly, anodic activation is *reagentless* (except for NaCl) and can be spatially controlled due to the localized generation of mediator (Shi et al., 2009).

Two proof-of-concept experiments were performed to demonstrate protein electroaddressing by combining chitosan electrodeposition and chitosan electrochemical activation. For these experiments, we fabricated the chip in Fig. 4a to possess 6 electrically independent gold electrodes (250 μm wide gold lines spaced 250 μm apart). For each assembly step, the "electrode" portion of the chip was immersed in solution while the "lead" portion of the chip was connected to the power supply. In these experiments, chitosan was first electrodeposited onto all 6 electrode addresses using chitosan solutions (0.9%, pH 5.6) and applying a cathodic voltage to achieve a constant current density (4 A/m^2 for

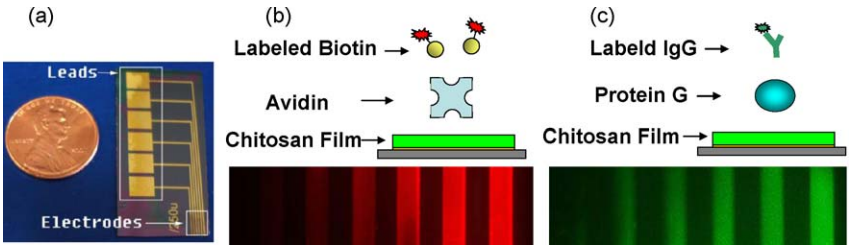

Fig. 4. Assembly of avidin and protein G at electrode addresses. Film activation progressively increased from left to right. Visualization was achieved using fluorescence labels.
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15 s, typical voltages of 2–3 V). After deposition, the chips were rinsed.

The first demonstration experiment aimed to show controllable protein assembly (i.e., electroaddressing) at the individual electrode addresses of the chip in Fig. 4a. A chip with electrodeposited chitosan films on the electrodes was immersed in a solution containing NaCl (0.1 M) and each film was individually activated by biasing the underlying electrode (0.9 V vs. Ag/AgCl) for a specific time. It is important to note that the entire chip remained immersed in the activation solution during the sequence of film-activation steps, and the process of activating all 6 electrode addresses required approximately 2 min. In the first set of studies, we assembled a single protein (either avidin or Protein G) at the individual electrode addresses. Proteins were assembled by immersing the chips with the activated chitosan films into solutions (4 ml) containing either avidin or Protein G (a streptococcal Fc-binding protein). Protein assembly was visualized by incubation with either fluorescently labeled biotin (Fig. 4b) or fluorescently labeled human IgG (Fig. 4c). In both cases, a progressive increase in fluorescence intensity is observed with increase in film activation (left to right in the images).

The second demonstration experiment is illustrated in Fig. 5a which shows two different proteins (Green Fluorescent Protein, GFP and Red Fluorescent Protein, RFP) are assembled at the electrode addresses of the chip in Fig. 4a. Chitosan was first electrodeposited onto all 6 electrodes. Next, the chitosan films on the 3 left-most electrodes were anodically activated and the chip was contacted with a GFP-containing solution. After assembling GFP onto the left 3 electrodes, the chip was washed and then blocked with bovine serum albumin (BSA, 5%) to consume any residual oxidized substituents on the chitosan film. For RFP assembly, the 3 right-most electrodes were electrochemically activated, and RFP was assembled. Fig. 5b shows a fluorescence photomicrograph of this chip which indicates the selective assembly of the two proteins at the appropriate electrode addresses. The quantitative control of protein assembly is illustrated in Fig. 5c which shows a plot of the fluorescence intensity (a measure of the assembled protein amount) vs. charge transfer ($Q = \int i dt$, where i is current density for anodic film activation).

In summary, the above results demonstrate that electrode-imposed signals can be enlisted to spatially and quantitatively control protein assembly. This on-demand electroaddressing is performed from aqueous solution using mild conditions and preserves the proteins' native structure and biological function. Further, since no reactive reagents are required, and the oxidative mediator is generated electrochemically from NaCl, this assembly approach is simple, safe, and inexpensive. Finally, this assembly approach appears to be generic for the sequential assembly of various proteins at individual electrode addresses.

4. Microfluidic operation: assembly

A common requirement for performing biological operations in a LOC format is that the biological components (e.g., the antibody in Fig. 1) must be assembled within a microfuidic channel. In many cases, it is desirable to assemble multiple biological components, each at a separate location within the LOC in order to perform multiple operations. For instance, the analysis of multiple biomarkers from a blood sample might require the assembly of multiple antibodies at separate addresses within the microfluidic channel. In these cases, methods must be available to controllably assemble the individual antibody proteins with spatial selectivity.

Two general approaches are commonly considered for the spatially controlled assembly of proteins. First, proteins could be printed onto a surface using some variation of microcontact or ink-jet printing. These printing methods require either direct contact of a stamp or indirect contact so the jetting fluid can strike a surface. Thus, printing must be performed before the LOC device is closed in order to allow access to an internal surface. A second approach for the spatially controlled assembly of proteins is based on photolithographic patterning. Photolithographic methods require a

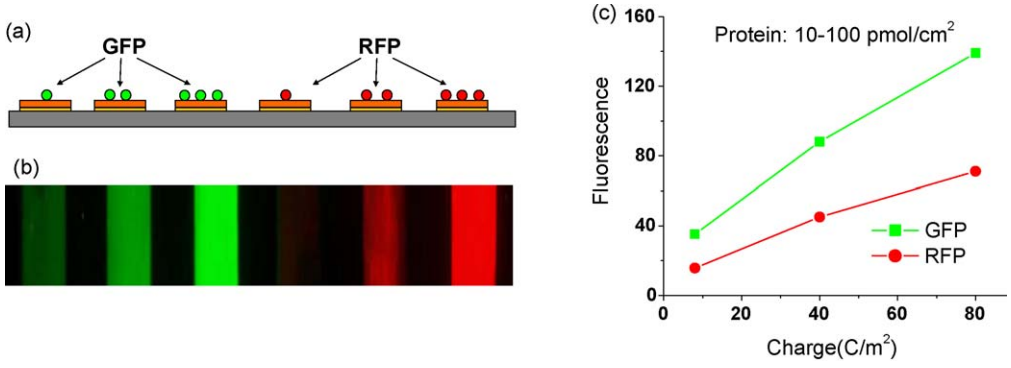

Fig. 5. Assembly of two model proteins (GFP and RFP) at individual electrode addresses. (a) Schematic. (b) Fluorescence image. (c) Quantification.
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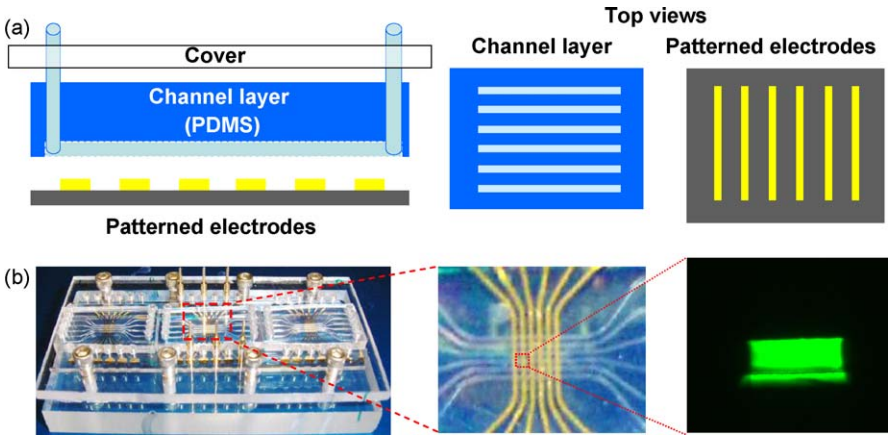

Fig. 6. Microfluidic device. (a) Schematic illustrating components of a typical device. (b) Photographs and fluorescence photomicrograph of microfluidic device at various magnifications.

"line-of-sight" to permit photoinitiation of the spatially controlled reaction. While both printing and photolithographic approaches have advantages, we believe chitosan's electroaddressing provides interesting opportunities for the spatially controlled assembly of proteins within LOC devices.

To employ chitosan's capabilities for spatially controlled protein assembly we are fabricating microfluidic devices such as the one illustrated in Fig. 6. These devices have multiple gold electrodes patterned onto a solid substrate (e.g., glass). Each electrode can serve as the site for chitosan's electrodeposition and subsequent protein assembly. The microfluidic channels are molded from polydimethylsiloxane (PDMS). In the device shown in Fig. 6, the channels are 200 μm wide, 150 μm high and 1 cm long. The cover for the device is also a solid substrate and could serve multiple functions; a transparent cover allows optical imaging of the electrode addresses while a conducting cover could serve as a counter-electrode if it directly contacts the liquid. Fluid is pumped through the channels using tubes attached to the cover.

The left-most photograph in Fig. 6b shows a system with 3 sets of microfluidic devices. One device is shown in the photograph at the center and this image shows that the microfluidic channels are aligned perpendicular to the electrodes so a sample that is pumped through the channel can access all 6 electrodes. The fluorescence photomicrograph at the right shows fluorescently labeled chitosan that was electrodeposited from a microfluidic channel onto one of the electrode addresses.

Previously published results demonstrated that chitosan can be electrodeposited from solutions within a covered microfluidic channel (Park et al., 2006). In addition, a biosynthetic enzyme was assembled at an electrode address and its activity monitored under differing conditions (Luo et al., 2008). These results demonstrate that chitosan enables the spatially controlled assembly of proteins from solution within the microfluidic channel after the LOC device has been covered. The ability to fully fabricate and assemble the LOC device prior to biofunctionalization allows labile biological components to be rapidly assembled immediately before use. Further, the fact that chitosan films can be removed from the electrodes after use indicates that the LOC device can be created to be reusable. Reusability is potentially important because it allows LOC devices to be fabricated that can access the power of electronics (e.g., for data processing and wireless transmission) while meeting the constraint of low cost analysis per sample. In contrast, single-use disposable analytical systems (e.g., dipsticks) that allow low cost detection often have limited capabilities for quantitative and multiplexed analysis.

5. Microfluidic operation: detection

In addition to the need for assembling biological components into an LOC device, it is desirable for the LOC device to offer the capabilities of converting biological events (e.g., antigen–antibody binding) into device-compatible signals. In many of the previous studies, activities occurring within the microfluidic channels were observed optically using fluorescence microscopy (e.g., Fig. 6b) or quantified off-line by HPLC (Luo et al., 2008). Obviously, the need for extensive off-chip instrumentation (microscope or HPLC) limits the potential for a stand-alone LOC device to perform analysis at remote sites (e.g., in the physician's office). Thus, there has been a growing effort to integrate the chitosan platform with existing sensor technology to detect and quantify biological events.

The fact that the chitosan film is deposited atop an electrode suggests electrochemical methods for detection and quantification. Initial studies indicated that electrochemically active small molecules (e.g., phenols and H_2O_2) can readily diffuse through the chitosan film to access the underlying electrode for electrochemical detection (Liu, Gaskell, Cheng, Yu, & Payne, 2008; Meyer et al., 2009). The deposited chitosan film is also optically transparent and devices have been designed and fabricated with integrated optical waveguides to probe the deposited chitosan at an electrode address within a microfluidic channel (Dykstra et al., 2009; Powers et al., 2005). Finally, the mechanical properties of the chitosan film are sensitive to the environment and this has enabled the coupling of chitosan with micromechanical sensors (e.g., cantilevers) (Koev et al., 2007). These initial studies illustrate the potential of chitosan to be intergrated with conventional sensor technology to convert biological recognition events into device-compatible optical, electrical and mechanical signals.

6. Conclusions

Chitosan offers a combination of properties that we believe enable it to serve as the bio-device interface for LOC applications. Chitosan's pH-responsive film-forming properties allow this aminopolysaccharide to "recognize" localized electrical stimuli and respond by electrodepositing as a stable thin film. In addition, chitosan's cationic, nucleophilic and metal-binding properties, along with its susceptibility to oxidation, enable proteins to be "connected" to chitosan through various mechanisms. Finally, chitosan films are optically transparent, permeable to small molecules and responsive to their environment which facilitates the transduction of biological events (e.g., antigen-antibody binding) into device-compatible optical, electrical and mechanical signals. Thus,

we believe chitosan has an exciting future as an "interconnect" between biology and electronics

Acknowledgements

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