# Chitosan Biotinylation and Electrodeposition for Selective Protein Assembly

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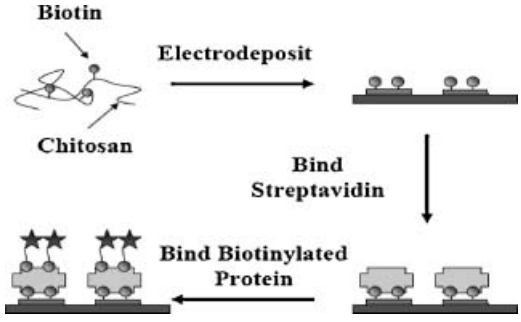
An alternative route to protein assembly at surfaces based on using the unique capabilities of biological materials for the spatially selective assembly of proteins is described. Specifically, the stimuli-responsive properties of aminopolysaccharide chitosan are combined with the molecular-recognition capabilities of biotin-streptavidin binding. Biotinylated chitosan retains its stimuli-responsive properties and is capable of electrodepositing at specific electrode addresses. Once deposited, it is capable of binding streptavidin, which can mediate the subsequent assembly of biotinylated proteins. Spatially selective protein assembly using biotinylated Protein A and fluorescently-labeled antibodies is demonstrated.

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## Introduction

The interfacing of proteins to devices is important for applications that range from point-of-care diagnostics to high-throughput drug screening. For many applications, protein assembly must be spatially controllable so individual proteins in an array can be assembled at a specific device address. Spatial control of protein assembly is often achieved using printing or photolithographic approaches.

An emerging alternative approach for spatially-controlled assembly is to use the pH-responsive aminopolysaccharide chitosan to guide protein assembly.[1–3] Scheme 1a illustrates that chitosan can be induced to undergo a localized sol-gel transition in response to the localized pH gradient created by electrochemical proton consumption at a cathode surface.[4–9] Once electrodeposited, the chitosan film is stable and adheres to the electrode surface without the need for an applied voltage, provided



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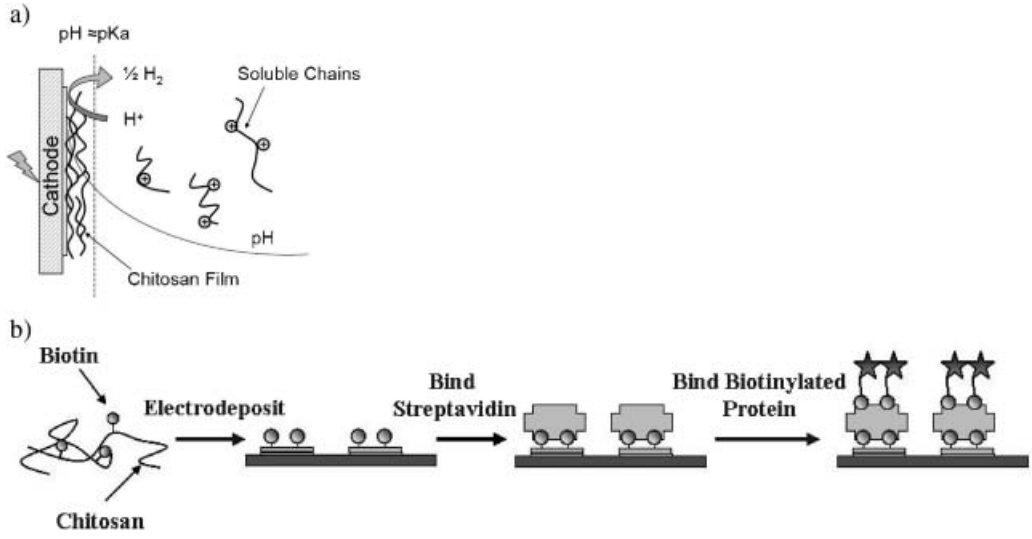


*Scheme 1.* (a) Chitosan can be induced to undergo a localized sol-gel transition in response to the localized pH gradient created by electrochemical proton consumption at a cathode surface; (b) a method for streptavidin-mediated protein assembly.

the film's exposure to acidic conditions is minimized – chitosan's sol-gel transition is reversible and chitosan can re-dissolve when the pH is reduced near or below its pKa (≈6.3).[10–15] Chitosan's electrodeposition is spatially selective in the lateral dimensions,[16] and the film thickness can be controlled by the deposition conditions.[4,6,7]

Previous research has shown chitosan electrodeposition provides a versatile means of assembling proteins. One assembly method is to blend the protein with chitosan, and then codeposit the protein-chitosan solution so the protein becomes entrapped within the electrodeposited gel network. This "codeposition" method has been used to assemble catalytically-active glucose oxidase[17–20] and peroxidase[21,22] enzymes. A second method for protein assembly is to first conjugate protein to chitosan's backbone, to confer pH-responsive and film-forming properties, and then to electrodeposit the protein-chitosan conjugate. This "conjugate-then-deposit" method has been used to assemble the green fluorescent protein (GFP)[23] and gelatin at separate electrode addresses.[24] A third method reported for protein assembly is to first electrodeposit chitosan and then to conjugate protein to the deposited film. This "deposit-then-conjugate" method has been demonstrated to assemble GFP through covalent bonds.[25] This method has also been extended for the non-covalent assembly of the enzyme acetylcholinesterase[26] and even cells[27] to previously-deposited chitosan films.

The above discussion indicates that chitosan's electrodeposition provides a unique opportunity for protein assembly. Here, we extend the previous work and report a streptavidin-mediated protein assembly method as illustrated in Scheme 1b. For this method, we first conjugate biotin to chitosan[28–33] to generate a stimuli-responsive biotinylated chitosan. The (strept)avidin-biotin system is widely used for high affinity and selective binding[34] and there has been considerable interest in conferring stimuli-responsiveness to this binding system.[35–41] Next, Scheme 1b shows that the biotinylated chitosan is electrodeposited in response to localized cathodic signals imposed from patterned electrodes. Several groups have recognized the convenience of electrical signals and pursued a range of electrochemical approaches for the assembly of biotin at surfaces.[42–45] The last two steps in Scheme 1b show streptavidin binding to the electrodeposited biotinylated chitosan, and finally the assembly of a biotinylated protein to streptavidin. Here, we report streptavidin-mediated protein assembly and demonstrate that it is complementary to other protein-assembly methods.

## Experimental Part

### Materials

The following materials were purchased from Sigma-Aldrich: poly(oxyethylenesorbitan) monooleate (TWEEN 20), chitosan from crab shells (85% deacetylation), tyrosinase from mushrooms (2 130 $U \cdot mg^{-1}$), streptavidin from *Streptomyces avidinii*, ATTO-565-labeled biotin, human immunoglobin G (IgG) labeled with fluorescein, and ATTO-590-labeled rabbit anti-chicken IgG. The following materials were purchased from Pierce Biotechnology: sulfosuccinimidyl-6-[biotin-amido]hexanoate (sulfo-NHS-LC-biotin) and biotinylated Protein A (99% biotinylation). The following were purchased from Quality Biologicals: phosphate-buffered saline (PBS; 10× concentrate, molecular biology grade, pH = 7.4) and Tris-buffered saline (TBS; 10× concentrate, molecular biology grade, pH = 7.4). Streptavidin conjugated with HiLyte Fluor™ 488 was purchased from AnaSpec. Nonfat dry milk was purchased from BioRad.


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Macromolecular Journals

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The green fluorescent protein (GFP) was engineered and expressed in *E. coli* as previously described.[46] Specifically, this GFP has an *N*-terminal hexahistidine tag (to facilitate immobilized-metal-affinity separations) and a *C*-terminal pentatyrosine tag. The $(Tyr)_5$ tag can be selectively activated by the enzyme tyrosinase to initiate GFP grafting to chitosan.

## Preparation of the Chips

Patterned chips were prepared as previously described.[16] Briefly, we used standard photolithographic methods to generate a pattern on a silicon wafer with a 1 μm thick thermal oxide film and deposited films of chromium (150 Å) and gold (2 000 Å). Three chip patterns were prepared for this work. Figure 1a shows a pattern of 20 μm parallel gold lines separated by 500 μm spaces. Figure 1b shows a grid pattern with 200 μm × 360 μm rectangles. Figure 1c shows a chip with two electrically-independent gold rectangular patterns (8 mm × 1 mm) separated by a 1 mm space.

## Preparation of Biotinylated Chitosan

Chitosan was biotinylated using sulfo-NHS-LC-biotin. Initially, a chitosan solution (1.6% w/v) was prepared by adding chitosan to 1% HCl, mixing overnight, and filtering using a vacuum filter to remove undissolved particles. A chitosan film was prepared by pouring the chitosan solution (5 mL) into a Petri dish, drying overnight at 50 °C, neutralizing the dried chitosan films with 1 N NaOH (10 mL for 30 min), and extensively washing the film with distilled water and then PBS buffer. The neutralized film was then immersed into 10 mL of PBS (pH = 7.8), and biotinylation was initiated by adding 10 mg of sulfo-NHS-LC-biotin (equivalent to a degree of chitosan substitution of 3.6%). The biotinylation reaction was allowed to proceed overnight at room temperature. After reaction, the biotinylated film was washed with PBS buffer and then dilute HCl was added drop-wise to dissolve the biotinylated chitosan. The pH of the biotinylated chitosan solution was adjusted to 5.6 using NaOH and the final polymer concentration was 0.8% w/v.

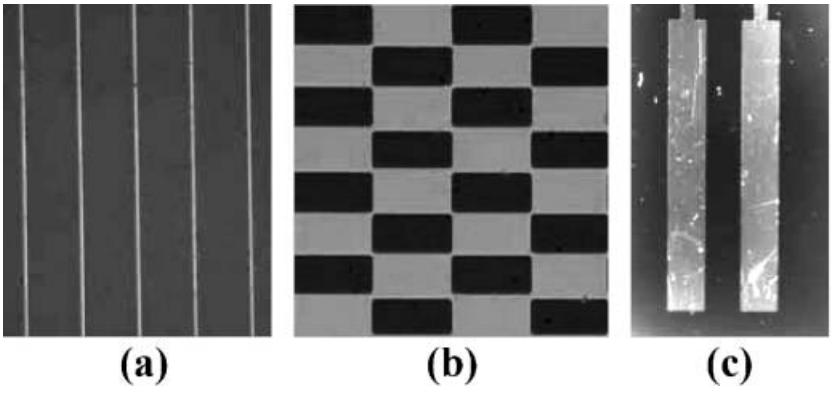


*Figure 1.* Photomicrographs of patterned chips used in this study: (a) parallel gold lines (20 μm wide lines separated by 500 μm spaces) that are connected to a single lead; (b) grid of interconnected gold rectangles (200 μm × 360 μm) that are connected to a single lead; (c) chip with two gold rectangular electrodes (8 mm × 1 mm) that are connected to separate leads (i.e., the electrodes are electrically independent).

## Electrodeposition

Electrodeposition was performed by partially-immersing the chip in a solution of chitosan (or biotinylated chitosan) to ensure the patterned region of the chip was submerged while the lead was above the liquid level. An alligator clip was used to connect the chip to a DC power supply (2400 Sourcemeter, Keithley) and the chip was biased to serve as the cathode (negative electrode) while a platinum wire served as the anode. Electrodeposition was performed at a constant current density of 3 $A \cdot m^{-2}$ for 1 min (typical voltages of 2–3 V). After electrodeposition, the chip was immediately removed from the deposition solution, briefly rinsed with water, and stored in PBS buffer. The thickness of the electrodeposited chitosan film was measured by profilometry (after drying) to be 0.5 μm, but the thickness could be altered by the deposition conditions.[4]

## Solutions and Methods for Assembly

We assembled various components onto patterned chips by immersing the chips in a series of solutions (2 mL) that varied with the experiment, as detailed in the Section Results and Discussion. For these experiments, the following solutions were used: (i) chitosan or biotinylated chitosan dissolved to 0.8% w/v at pH = 5.6; (ii) TWEEN-containing buffers prepared with either PBS (0.1% TWEEN) or TBS (0.05% TWEEN); (iii) streptavidin labeled with HiLyte Fluor™ 488 (0.01 $mg \cdot mL^{-1}$) dissolved in TWEEN-containing PBS; (iv) streptavidin (0.01 $mg \cdot mL^{-1}$) dissolved in TWEEN-containing PBS; (v) biotin labeled with ATTO 565 (0.01 $mg \cdot mL^{-1}$) dissolved in PBS buffer; (vi) biotinylated Protein A (0.01 $mg \cdot mL^{-1}$) dissolved in TWEEN-containing PBS; (vii) fluorescein-labeled human IgG (0.01 $mg \cdot mL^{-1}$) dissolved in TWEEN-containing TBS with 1% milk added; (viii) blocking solution containing 5% milk dissolved in PBS; and (ix) rabbit anti-chicken IgG labeled with ATTO 590 dissolved in TWEEN-containing TBS with 1% milk added.

After each assembly step, the chip was washed multiple times (5 min for each wash) with various buffers to remove any physically-bonded reagent. Specific washing procedures were: after electrodeposition the chip was washed twice with PBS; after binding with streptavidin, the chip was washed twice with TWEEN-containing PBS; after incubation with biotinylated Protein A, the chip was washed twice with TWEEN-containing PBS; after blocking in 5% milk, the chip was washed 3 times with TWEEN-containing TBS; after incubating with antibody, the chip was washed 3 times with TWEEN-containing TBS; and before microscopic examination, the chip was washed twice with PBS.

## Fluorescence Analysis

The chips were examined using a Leica fluorescence microscope (MZFL III). To observe green fluorescence, a GFPplus filter with excitation filter at 480/40 nm and emission

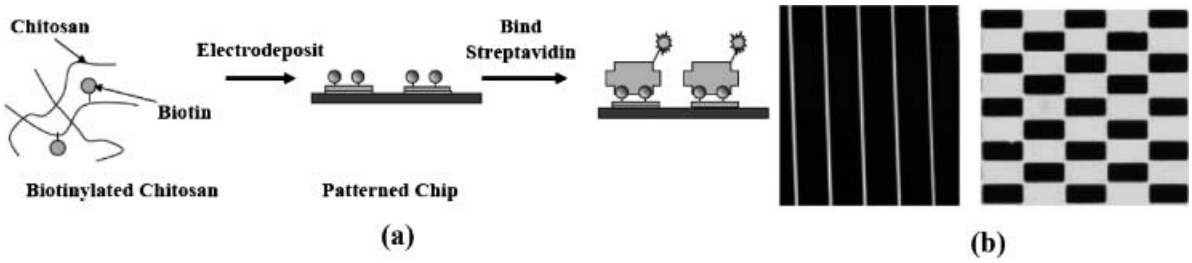


*Figure 2.* Spatially-selective assembly of fluorescently-labeled streptavidin onto patterned chips: (a) schematic of experiment; (b) fluorescence photomicrograph showing fluorescently-labeled streptavidin is assembled onto the patterned electrodes used to electrodeposit biotinylated chitosan.

filter at 510 nm was used. To observe red fluorescence, we used a Leica 41004 TXRD filter with excitation filter at 560/55 nm and an emission barrier at 645/75 nm. Fluorescence micrographs were obtained using a digital camera (spot 32, Diagnostic Instrument) connected to the fluorescence microscope. The fluorescence profiles of the micrographs were analyzed using Image J software (http://rsb.info.nih.gov/ij/).

## Results and Discussion

In our initial experiment, we examined the spatial selectivity for streptavidin-mediated assembly onto electrodeposited biotinylated chitosan. In this experiment, we used the patterned chips shown in Figure 1a and 1b, and performed the experiment as outlined in Figure 2a. Specifically, we electrodeposited biotinylated chitosan from a dilute acidic solution (0.8% polymer, pH = 5.6) onto the patterned chips (3 $A \cdot m^{-2}$ for 1 min). After washing the chip with PBS buffer, the labeled streptavidin was bound by contacting the chip for 1 h with a solution of fluorescently-labeled streptavidin. After streptavidin binding, the chip was washed with TWEEN-containing PBS buffer and examined using fluorescence microscopy. The fluorescence photomicrographs in Figure 2b show that the labeled streptavidin is assembled onto the patterned lines or grid with high fidelity. These results demonstrate that electrodeposition of biotinylated chitosan enables the spatially-selective assembly of streptavidin.

Next, we examined the selectivity for streptavidin-mediated assembly onto biotinylated chitosan using the patterned chip of Figure 1c and the experimental approach illustrated in Figure 3a. Using the procedures described above, we sequentially electrodeposited biotinylated chitosan on the left electrode and then electrodeposited unconjugated chitosan on the right electrode. After electrodeposition and washing, streptavidin was bound by incubating the chip for 30 min in the streptavidin solution. After washing, the chip was incubated for 1 h with the fluorescently-labeled biotin solution. The fluorescence photomicrograph and associated image analysis, shown in Figure 3b, show strong fluorescence on the left electrode with little, if any, fluorescence on the right electrode. This result indicates that the labeled biotin can bind (via streptavidin) to the biotinylated chitosan, and that there is little non-specific binding of either streptavidin or labeled biotin to the unconjugated chitosan. Thus, streptavidin-mediated assembly is selective for the biotinylated chitosan.

We next examined the streptavidin-mediated assembly between the biotinylated chitosan and biotinylated Protein A as illustrated in Figure 4a. *Staphylococcal* Protein A is a bacterial cell wall protein that has a broad affinity for binding immunoglobulins from many species.[47] We detected binding to Protein A using fluorescently-labeled human IgG. Using the methods described above we sequentially electrodeposited unconjugated chitosan on

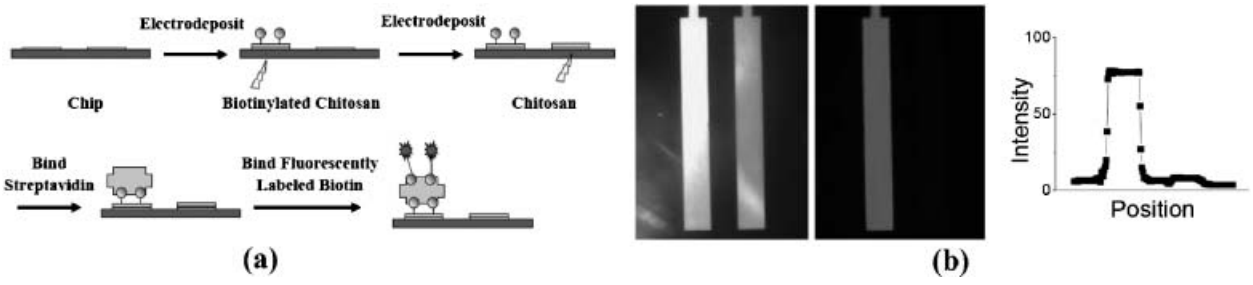


*Figure 3.* Streptavidin-mediated binding for selective assembly: (a) schematic of experiment to test the selectivity of streptavidin-mediated assembly for the biotinylated chitosan; (b) bright-field photomicrograph and fluorescence photomicrograph with corresponding fluorescence intensity profile showing fluorescently labeled biotin is assembled onto the left electrode (with biotinylated chitosan) and minimal non-specific binding to either the substrate or the control chitosan electrodeposited on the right electrode.


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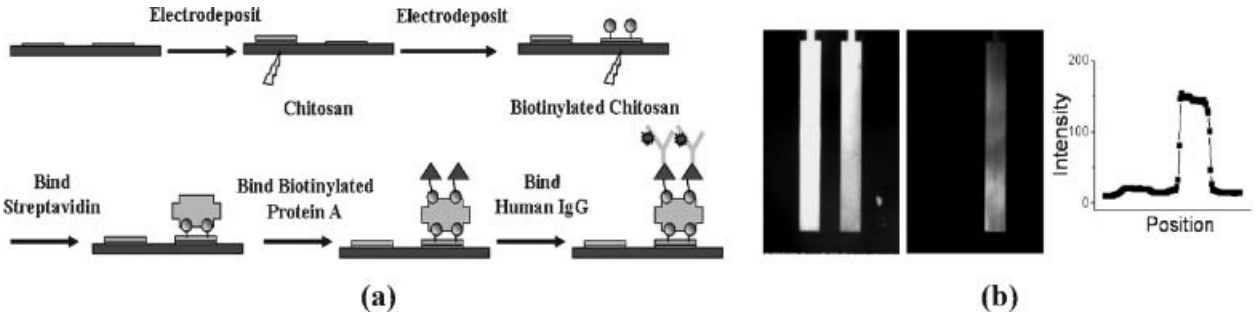


*Figure 4.* Streptavidin-mediated protein assembly: (a) schematic of experiment; (b) bright-field photomicrograph and fluorescence photomicrograph with fluorescence intensity profile showing fluorescently labeled IgG is assembled onto the right electrode (with biotinylated chitosan) with little non-specific assembly on the left electrode (with chitosan).

the left electrode and biotinylated chitosan on the right electrode, and then we bound streptavidin to the biotinylated chitosan. Next, we bound biotinylated Protein A to the streptavidin by incubating the chip for 2 h in the biotinylated Protein A solution. After Protein A binding, we washed the chip with TWEEN-containing PBS and then performed blocking by incubating the chip for 2 h with 5% milk in PBS. Antibody binding to Protein A was achieved by incubating the chip for 1 h with the labeled human IgG solution. The chip was then washed with TWEEN-TBS and then PBS. The fluorescence photomicrograph and image analysis of Figure 4b show that fluorescence is predominantly confined to the right electrode where the biotinylated chitosan was deposited. This observation indicates that: (1) streptavidin-mediated Protein A assembly is selective for the biotinylated chitosan; (2) IgG assembly is selective for the Protein A; and (3) non-specific binding to chitosan of streptavidin, Protein A and IgG can be controlled. Importantly, this result also demonstrates the assembly of an antibody at a specific electrode address.

Finally, we demonstrated that streptavidin-mediated assembly can complement other methods for the sequential assembly of multiple proteins at different electrode addresses. Specifically, we used streptavidin-mediated assembly to complement a deposit-then-conjugate method based on tyrosinase-mediated conjugation. Tyrosinase selectively activates proteins by oxidizing accessible tyrosine residues into reactive *o*-quinones that can graft onto chitosan to generate protein-chitosan conjugates.[48–53] In previous studies, a tyrosine-rich fusion tag composed of 5 tyrosine residues was created to facilitate tyrosinase-mediated protein-chitosan conjugation.[23,46]

We used the experimental approach illustrated in Figure 5a to demonstrate sequential protein assembly through complementary methods. First, we electrodeposited chitosan on the left electrode. After washing, the chip was immersed in a buffered solution containing tyrosinase (100 $U \cdot mL^{-1}$) and tyrosine-tagged green fluorescent protein (GFP; 5 $\mu g \cdot mL^{-1}$), and this solution was incubated overnight at room temperature. The fluorescence photomicrograph of Figure 5b indicates that GFP was assembled on this left electrode. After washing the chip, the biotinylated chitosan was electrodeposited on the right electrode. We should note that this second electrodeposition step must be performed with care since exposure of the chip to low pH conditions for extended periods

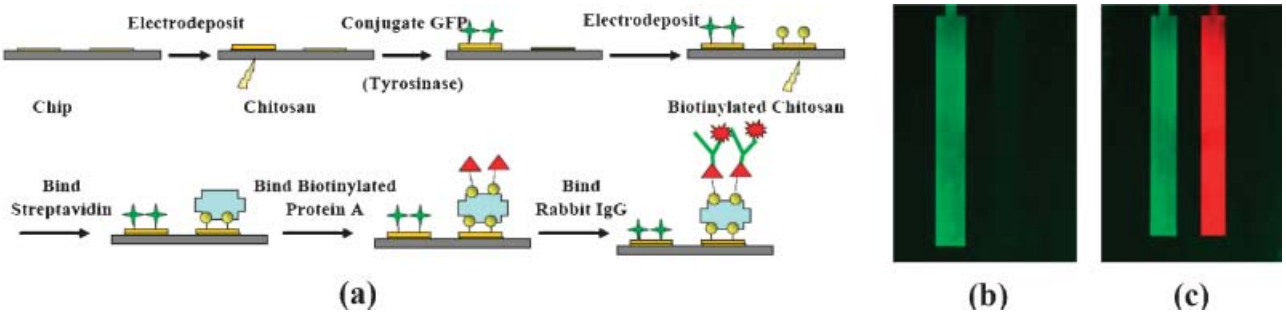


*Figure 5.* Sequential protein assembly at separate electrode addresses using complementary assembly methods: (a) schematic of experiment in which GFP is assembled on the left electrode by a "deposit-then-conjugate" method, and the labeled antibody is assembled on the right electrode by streptavidin-mediated assembly; (b) fluorescence photomicrographs after the first assembly step; (c) fluorescence photomicrograph after second assembly step demonstrates sequential protein assembly (note: fluorescence images using green and red filters are superimposed in this photomicrograph).

promotes resolubilization of the chitosan initially deposited on the left electrode (the left electrode is not biased during this second electrodeposition step). To minimize resolubilization of the previously-deposited chitosan, we performed electrodeposition from a buffered solution of pH = 5.6 which is close to chitosan's p$K_a$ (≈6.3);[10–15] we electrodeposited for a short time (1 min), and immediately removed the chip from the deposition solution after electrodeposition. After the second electrodeposition and washing, we contacted the chip with solutions in the following sequence: streptavidin, biotinylated Protein A, milk blocking solution and a fluorescently-labeled rabbit anti-chicken IgG. The fluorescence photomicrograph of Figure 5c shows green fluorescence (due to GFP) on the left electrode and the red fluorescence (due to the labeled IgG) on the right electrode. This result demonstrates that streptavidin-mediated assembly can be used to complement existing assembly methods for the sequential assembly of separate proteins at different electrode addresses.

## Conclusion

We report the coupling of streptavidin-mediated binding with chitosan electrodeposition for the spatially-selective assembly of proteins. Compared to other chitosan-based protein assembly methods, there are several potential advantages of streptavidin-mediated assembly: the protein and chitosan do not need to be dissolved together; a pH-responsive protein-chitosan conjugate is not required; and the protein is not exposed to high pH during electrodeposition. A disadvantage of streptavidin-mediated assembly is that biotinylation (of both chitosan and the protein), and the use of streptavidin, add complexity and cost. Nevertheless, avidin-biotin binding has become a routine and generic method for a range of medical and biotechnological applications. On balance, we believe the value of streptavidin-mediated assembly is that it complements existing chitosan-based methods to allow for sequential protein assembly at separate electrode addresses (for example as in Figure 5). In a broader perspective, we believe chitosan's ability to "recognize" localized electrical stimuli and respond by electrodepositing as stable films provides unique opportunities for lab-on-a-chip applications.[54–56]

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