Signal-Directed Sequential Assembly of Biomolecules on Patterned Surfaces

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The signal-guided and sequential assembly of biomolecules onto patterned surfaces is demonstrated. Readily transmittable electric signals are used to guide spatially selective deposition of the pH-responsive polysaccharide, chitosan, and functionalized chitosan conjugates, by generating localized pH gradients. The nucleophilic primary amine groups of chitosan enable facile conjugation of proteins and nucleic acids by two approaches, one an enzymatic approach and the other a standard chemical modification, thus providing flexibility when sequentially assembling biomolecules in a spatially selective manner. Moreover, we developed an agarose gel "biomask" for the sequential assembly of single-stranded DNA and confirmed its functionality through nucleic acid hybridization assays.

Introduction

We report the spatially selective, sequential, and signal-guided assembly of nucleic acids and proteins onto prefabricated patterned surfaces. The patterning of biomolecules onto substrates is of great interest, and parallel deposition processes are now widespread (e.g., spotting techniques for DNA microarrays[1,2] and soft lithographic stamping[3] for BioMEMS). Formats that are sequential or that enable postfabrication biofunctionalization in aqueous environments are lacking, however. Especially useful would be techniques where transmittable signals direct the in situ assembly of biomolecules (e.g., minimal mechanized equipment). We employ standard methods to microfabricate devices designed to transmit signals which, in turn, exploit the signal-responsive properties of biopolymers to direct assembly. Specifically, silicon-based chips with micropatterned gold surfaces are polarized to create localized regions of high pH.[4] In response to this imposed signal, the pH-responsive aminopolysaccharide chitosan becomes insoluble and deposits onto the polarized surface with high spatial selectivity.[5] Chitosan's primary amines are deprotonated at neutral or high pH ($pK_a \cong 6.5$) and are therefore nucleophilic, allowing facile conjugation of biomolecules.[6−8]

Materials and Methods

Reagents. Chitosan (deacetylated chitin, 85% minimum) from crab shells, gelatin (type A from porcine skin, 175 Bloom), tyrosinase (3400 U/mg), agarose, glutaraldehyde (grade I 50% aqueous solution), urea (SigmaUltra grade), PerfectHyb Plus hybridization buffer, phosphate-buffered saline (PBS) buffer tablets, saline sodium citrate (SSC) buffer (20× concentrate, molecular biology grade), and Tris-EDTA (TE) buffer (100× concentrate) were all purchased from Sigma (St. Louis, MO). Sodium hydroxide pellets, glacial acetic acid, anhydrous DMSO, $MgCl_2 \cdot 6H_2O$ (enzyme grade), and sodium borohydride powder ($NaBH_4$) were purchased from Fisher Chemical (Fair Lawn, NJ). Fluorescein and Texas Red derivatives 5-(and 6-)carboxyfluorescein, succinimidyl ester (NHS-fluorescein) and Texas Red-X, succinimidyl ester (NHS-Texas Red) were purchased from Molecular Probes (Eugene, OR). Distilled and deionized water (ddH_2O, 18 MΩ·cm, Milli-Q) was autoclaved before use. Unless otherwise noted, 1× SSC buffer with 0.1 M $MgCl_2$ was used for equilibration and rinsing throughout this study. To prepare this SSC buffer, we first diluted the SSC buffer 20× concentrate with ddH_2O. After autoclaving, we added autoclaved 4 M $MgCl_2$ solution and adjusted the pH to 7.1. All single-stranded DNA (ssDNA) molecules were purchased from Gene Probe Technologies, Inc. (Rockville, MD). These DNAs are HPLC-purified and were used without further purification. DsRed protein was a generous gift from Chesapeake PERL, Inc. (College Park, MD).

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Preparation of the Chips. The chip for chitosan and protein assembly consists of two gold rectangular patterns (8 mm × 1 mm) separated by a 1 mm gap. Each pattern is linked to a 3 mm × 4 mm rectangular region for alligator clip connection by an 8 mm long gold connecting line. The chip for ssDNA assembly consists of two gold square patterns (3.5 mm × 3.5 mm) vertically separated by a 4 mm gap. The patterned surfaces were fabricated by depositing 50 Å thick chromium and then 2000 Å thick gold films on 4 in. diameter silicon wafers, which had previously been coated with 1 micron thick silicon oxide film. Patterning was achieved using photolithography in which a primer and then photoresist were spin-coated onto the gold surface. After soft-baking the coated wafer at 100 °C for 1 min, a specially designed mask was placed over the surface and the wafer was exposed to UV light (total dosage, ~190 mJ/cm^2). After 30 s of development, the wafer was then hard-baked at 120 °C for 10 min. The exposed areas were then etched away by gold and chromium etchants, and the photoresist was removed using acetone. After the patterning was complete, the chips were manually cut to fit in a standard 2 mL microcentrifuge tube. Bright view images of these chips (Supporting Information 1) are available free of charge via the Internet at http://pubs.acs.org.

Preparation of Fluorescently Labeled Chitosan. NHS-fluorescein and NHS-Texas Red were stored at −20 °C with desiccation in a dark container until use. These fluorescent derivatives were dissolved in a 1:4 mixture of DMSO and absolute ethanol at 5 mg/mL for labeling. For fluorescent-labeling of chitosan, 5 mL of 1% (w/v) chitosan solution was poured into each well of a 6-well plate and dried overnight at 45 °C. After drying, 5 mL of 1 N NaOH solution was added to each well to neutralize the chitosan. After 30 min of incubation at room temperature with 75 rpm gyratory shaking, NaOH solution was removed and the chitosan was extensively rinsed with 5 mL portions of distilled water and then SSC buffer until the pH reached 7. A total of 20 μL of 5 mg/mL NHS-fluorescein or NHS-Texas Red was added dropwise to each well containing 5 mL of SSC buffer. After 2 h of incubation at room temperature with 75 rpm gyratory shaking, fluorescently labeled chitosan was extensively rinsed with distilled water and dilute HCl was then added into the well dropwise while shaking, to dissolve the fluorescently labeled chitosan. The concentration and pH of the recovered chitosan with fluorescent label were then adjusted to 0.5% and 6 using distilled water and NaOH solution, respectively.

Preparation of Protein−Chitosan Conjugates. To prepare gelatin−chitosan conjugate, gelatin was first labeled with NHS-Texas Red. Gelatin solution was first prepared by adding 2.5 g of gelatin in 50 mL of distilled water and then heating at 50 °C. After the pH was adjusted to 7.2 using 1 N NaOH, 200 μL of 5 mg/mL NHS-Texas Red-X solution was added dropwise to the gelatin solution while shaking at 32 °C. After 4 h of reaction (at which point, all NHS-Texas Red is either hydrolyzed or reacted with gelatin), the pH was adjusted to 5.6 and 15 mL of 1% chitosan solution (pH = 3.8) was added to this gelatin solution. Enzymatic conjugation of gelatin to chitosan was initiated by adding 200 μL of tyrosinase (~5000 U/mL) into the gelatin−chitosan mixture. After 1 h of incubation at 32 °C while stirring, the reaction was stopped by heating to 60 °C for 20 min. The gelatin−chitosan conjugate was then precipitated by adding a small amount of HCl. Precipitated conjugate was then filtered out using a Whatman filter paper (no. 5, particle retention > 2.5 μm) under vacuum. The flow-through, containing the unconjugated gelatin and Texas Red dye, was discarded. The precipitate was then resolubilized by adding distilled water and a small amount of dilute HCl while stirring on a hot plate. This precipitation/resolubilization was repeated three times to remove the unconjugated gelatin and the unreacted Texas Red dye.

Green fluorescent protein (GFP)−chitosan conjugate was prepared as previously described.[7] Briefly, GFP with an N-terminal hexahistidine tag and C-terminal pentatyrosine tag was purified from an *Escherichia coli* culture using immobilized metal affinity chromatography. This GFP (0.17 μg/mL) and tyrosinase (60 U/mL final) were added to a chitosan solution (0.7% w/v) and incubated overnight at room temperature. After 1 h of incubation of the reaction mixture with 6.6 mM $NaBH_4$, the conjugate was precipitated by raising the pH with phosphate buffer (pH = 9). The conjugate was then centrifuged, rinsed twice with phosphate buffer to remove physically bound protein, and resolubilized in aqueous acetic acid solution (0.5% v/v, pH = 4).

Deposition of Fluorescently Labeled Chitosan and Protein−Chitosan Conjugates. Chitosan solution was prepared by adding chitosan flakes to water and incrementally adding small amounts of HCl to the solution to maintain the pH near 3. After mixing overnight, the chitosan solutions were filtered to remove undissolved material, and the pH was adjusted to 3.8 using 1 N NaOH. For deposition, the patterned chip was immersed in a chitosan solution (fluorescently labeled or protein-conjugated) and one of the patterned gold surfaces was polarized to serve as a negative electrode (i.e., the cathode). The positive electrode in these experiments was an unpatterned gold-coated silicon wafer. These two electrodes were connected to a DC power supply (Keithley 2400 SourceMeter) using alligator clips. Deposition was performed by applying 4 A/m^2 current for 1 min. After deposition, the chip was removed from the chitosan solution, disconnected from the power supply, rinsed briefly with distilled water, and immersed in 5 mL of 1×SSC buffer.

Assembly of DsRed. Deposited chitosan was neutralized (dilute NaOH) and equilibrated (in SSC buffer) before activation. Activation was done by immersing the chip in 1 mL of a solution of 0.05% (w/v) glutaraldehyde and incubating for 30 min at room temperature with 75 rpm gyratory shaking. After activation, the chip was rinsed three times with distilled water and immersed in a DsRed protein solution (~1.3 mg/mL) overnight at 4 °C.

Assembly of Single-Stranded DNA. The two probe ssDNAs used in this study were amine-modified at the 5′ end. Probe 1 (5′-CTTTCGCGTTGTTTGCAGAA)[8] is complementary to a region in *E.coli dnaK* mRNA. Probe 2 (5′-ATGATGATGATGATGATG) is complementary to a hexahistidine tag-encoding region of a plasmid pTrcHis B from Invitrogen. These two probes were selected to monitor nucleic acid sequences of heterologous genes (histidine tag) and native *E. coli dnaK* mRNA. Target 1 (TTCTGCAAACAA-CGCGAAAG) is complementary to probe 1 and is fluorescein-labeled at the 5′ end. Target 2 (TTCTGCAAA-CAACGCGAAAG) is complementary to probe 2 and is Texas Red labeled at the 5′ end.

Assembly of the ssDNA probe and hybridization/denaturation were done as previously reported.[8] Briefly, unlabeled chitosan was deposited onto one of the two square patterns by applying voltage (4 A/m^2 current density) for 1 min. After extensive rinse with distilled water and SSC buffer, the patterned chip was immersed in 1 mL of 0.05% (w/v) glutaraldehyde solution for activation. After this 30 min activation reaction at room temperature with 75 rpm gyratory shaking, the activated chip was rinsed with 5 mL portions of SSC buffer twice and immersed in the probe DNA solution (20 μg/mL) overnight at 4 °C for coupling. After this coupling reaction, the chip was rinsed extensively with SSC buffer and immersed in a 5 mM $NaBH_4$ solution for 5 min.

Hybridization/Denaturation. The DNA solution for both hybridizations described in Figure 4 contained both target 1 and target 2 at 1 μM concentration in Sigma PerfectHyb buffer. Hybridization was done by immersing the DNA probe chip in 1 mL of target DNA solution for 30 min at room temperature. After briefly rinsing with SSC buffer, the hybridization was examined using a Leica MZ FLIII fluorescence microscope. Denaturation of the hybridized target DNA was done by immersing the chip in 1 mL of a solution of 4 M urea at 70 °C for 30 min. We performed several hybridization/denaturation cycles afterward, and the resulting fluorescence from each event was consistent (result not shown), consistent with our previous results.[8]

Masking/Demasking with Agarose Gel. The agarose gel biomasking was done by pouring 40 μL of hot 2% agarose solution onto the probe 1 region and cooling to room temperature for the gel to solidify. Demasking was done by putting the chip in a standard 2 mL microcentrifuge tube containing 1.5 mL of SSC buffer and heating the microcentrifuge tube at 97 °C for 10 min using a heat block. Upon this demasking, the gel was detached from the chip but not fully melted.

Analysis. A fluorescence microscope (Leica, MZFLIII) was used to observe fluorescence throughout this study. Green fluorescence was observed using the GFPplus filter set from Leica (an excitation filter at 480/40 nm and an emission barrier filter

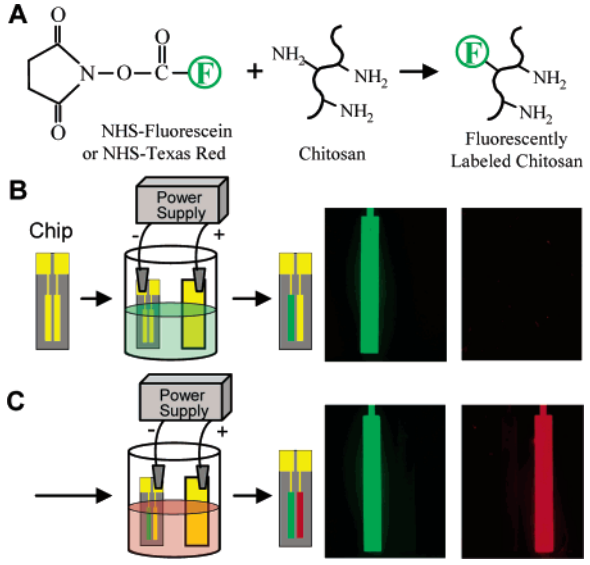

Figure 1. Sequential deposition of chitosan onto patterned surfaces. (A) Fluorescent labeling of chitosan with NHS-fluorescein or NHS-Texas Red. (B) Deposition of fluorescein-labeled chitosan. (C) Deposition of Texas Red labeled chitosan.

at 510 nm). Red fluorescence was observed using the Texas Red filter set from Leica (14004TXRD, excitation filter at 560/55 nm and an emission barrier filter at 645/75 nm). Photomicrographs were prepared from the fluorescent microscope using a digital camera (Spot 32, Diagnostic Instruments). The fluorescence micrographs with two colors shown in Figure 2 and Figure 3 were taken by double exposure, with red light exposure first and green next, using the Texas Red filter set and then the GFPplus filter set, respectively. Our assembly technique allows chitosan to be deposited up to several micrometer thickness ranges,[4] which will prevent fluorescence quenching in the close proximity (several nanometers) of gold surfaces. The fluorescence profile plot in Supporting Information 2 was obtained from ImageJ, free image analysis software available from the NIH Web site (http://rsb.info.nih.gov/ij/).

Results and Discussion

Sequential Deposition of Fluorescently Labeled Chitosan. In Figure 1, we demonstrate the feasibility of a sequential and in situ approach by depositing labeled chitosans onto micropatterned chips. First, separate batches of chitosan were labeled with succinimidyl ester derivatives of fluorescein and Texas Red (Figure 1A). Then, chips designed to elicit voltage-dependent pH gradients were dipped into the fluorescein-labeled chitosan solution and a negative voltage was applied to the left pattern (Figure 1B). After rinsing the chip with water, fluorescence micrographs were taken with green and red light filters, respectively. These images show that the fluorescein-labeled chitosan was selectively deposited only onto the left pattern.[5] We then performed the second deposition by dipping the chip in a solution of Texas Red labeled chitosan and applying voltage to the right pattern. The fluorescence micrographs of Figure 1C show that the Texas Red labeled chitosan was selectively deposited only onto the right pattern. Importantly, Figure 1C indicates that (i) the fluorescein-labeled chitosan deposited in the first step was retained on the left pattern without loss of fluorescence, while (ii) no Texas Red labeled chitosan was deposited onto this left pattern. This result shows the signal-directed, spatially selective sequential assembly of different chitosans from an aqueous environment.

Sequential Assembly of Proteins via Biochemical Conjugation. Our next goal was to demonstrate signal-directed and sequential assembly of proteins. For this, we

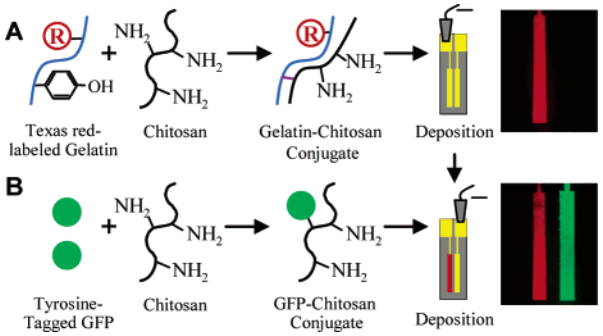

Figure 2. Sequential assembly of proteins onto patterned surfaces. (A) Tyrosinase-catalyzed conjugation of Texas Red labeled gelatin with chitosan and the subsequent deposition of the conjugate. (B) Tyrosinase-catalyzed conjugation of GFP with chitosan and the subsequent deposition of the conjugate.

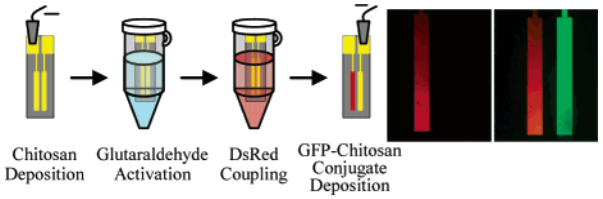

Figure 3. Assembly of DsRed by deposition−chemical conjugation and the subsequent deposition of a GFP−chitosan conjugate.

generated two fluorescent protein−chitosan conjugates using an enzyme-catalyzed conjugation reaction. Conjugation to chitosan confers proteins with the pH-dependent solubility that enables them to be deposited in response to an applied voltage. As shown in Figure 2, conjugation is based on the tyrosinase-catalyzed conversion of tyrosine residues of proteins into activated *o*-quinones that undergo uncatalyzed reaction with chitosan's amines.[6] The first protein−chitosan conjugate was prepared with a Texas Red labeled gelatin, while the second conjugate was prepared with a green fluorescent protein. Figure 2A depicts the gelatin−chitosan conjugate preparation, its subsequent deposition onto the left pattern, and the resulting fluorescence micrograph.

The image of Figure 2A shows spatially selective deposition of the gelatin−chitosan conjugate onto the left pattern. The micrograph of Figure 2B shows that the GFP−chitosan conjugate was deposited only onto the right pattern and that the gelatin−chitosan conjugate on the left pattern was left intact. Important to note is that the GFP−chitosan conjugate retained its fluorescence (and presumably GFP's β-barrel structure), indicating that the conjugation and deposition procedures are sufficiently mild to prevent denaturation. This result demonstrates the sequential assembly of protein−chitosan conjugates from aqueous solution onto patterned surfaces in response to applied signals.

Flexible Sequential Assembly of Proteins via Chemical/Biochemical Conjugation. In Figure 2, we assembled the proteins by first conjugating them to chitosan using an enzyme-mediated biochemical reaction and then depositing the resultant conjugate onto the gold surface. However, the nature of the chitosan scaffold provides additional flexibility: conjugation may be performed with already-deposited chitosan. That is, in Figure 3, chitosan was deposited onto the left pattern by the process in Figure 1B. The deposited chitosan was then "activated" by glutaraldehyde, a homobifunctional chemical coupling agent that reacts only with the primary amine groups of chitosan. This activated chip was then dipped

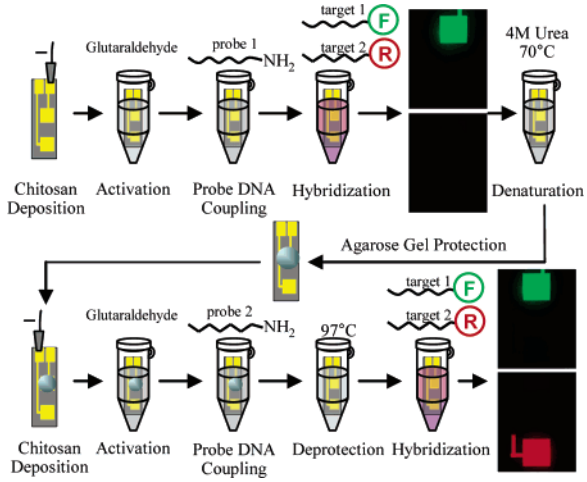

Figure 4. Sequential assembly of functional ssDNAs as shown by postassembly hybridization. The target DNA solution contains two target DNAs (fluorescein-labeled target 1 and Texas Red labeled target 2).

into a solution containing a red-fluorescent protein, DsRed, completing the first assembly. The second protein, GFP, was then assembled onto the right pattern by the same sequence as in Figure 2 (conjugation prior to deposition). The fluorescence micrographs of Figure 3 show good resolution of the chemically coupled DsRed on the left pattern and the deposited GFP−chitosan conjugate on the right pattern. This result demonstrates that chitosan's pH-dependent solubility and nucleophilicity enable this polysaccharide scaffold to be flexibly exploited for sequential protein assembly.

Sequential Assembly of ssDNA via Biomasking. Our final goal was to sequentially assemble single-stranded DNAs with different sequences onto a patterned substrate and test these probes for hybridization with their complementary targets. Specifically, we assembled ssDNA probes complementary to *E. coli dnaK* mRNA (probe 1) and an artificial hexahistidine-encoding region (probe 2). The top row of Figure 4 shows that we first assembled the amine-terminated probe 1 by depositing chitosan onto the upper pattern, followed by glutaraldehyde activation and coupling. This assembly was confirmed by hybridization with the target DNA solution containing *both* fluorescein-labeled *dnaK* target DNA (target 1, complementary to probe 1) and Texas Red labeled histidine tag-encoding sequence DNA (target 2, complementary to probe 2). The fluorescence micrographs in the top row of Figure 4 show that target 1 is hybridized to the upper pattern while target 2 does not hybridize anywhere, as expected. After denaturing this hybridized target 1 in urea solution (verified by a reduction in fluorescence, not shown), we assembled probe 2 onto the lower pattern. As illustrated in Figure 4, we developed a removable "biomask" using agarose gel to protect probe 1 throughout this probe 2 assembly procedure. Absence of this biomask results in deactivation of probe 1 during probe 2 assembly (Supporting Information 2). Agarose is inert to aldehyde−amine coupling chemistry, and this polysaccharide is thermoresponsive (mp 95 °C), enabling it to be easily applied and removed from the chip by heat. This biomasking was done by pouring hot 2% (w/v) agarose solution onto the probe 1 region and cooling to room temperature for the agarose to solidify. The bottom row of Figure 4 shows assembly of the amine-terminated probe 2 by depositing chitosan onto the lower pattern, followed by activation and coupling. After removing the agarose mask by heating the chip at 97 °C, a second hybridization was performed by immersing the chip in the target DNA solution containing both targets. The final micrographs show that target 2 was hybridized to the lower pattern only, confirming the spatially selective assembly of probe 2. Importantly, probe 1 on the upper pattern retained its hybridization capacity throughout the second assembly procedure. This result demonstrates that our prefabricated chip enabled signal-directed sequential assembly of nucleic acids onto readily addressable sites. Hence, this technique of sequentially assembling biomolecules may provide simple ways to improve a biosensor's sensitivity by avoiding probe species at unwanted (e.g., proximal) locations.

Conclusion

In conclusion, we demonstrated that a readily transmitted signal, in our case voltage, can guide spatially resolved and sequential assembly of biomolecules in aqueous environments by exploiting the stimulus-responsive biopolymer, chitosan. We envision this simple technique might be advantageous in many sensor and device assembly applications.

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Supporting Information Available: Bright view images of the chips (1) and fluorescence micrographs of partially protected sequential ssDNA assembly (2). This material is available free of charge via the Internet at http://pubs.acs.org.

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