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Protein Assembly Onto Patterned Microfabricated Devices Through Enzymatic Activation of Fusion Pro-Tag

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ABSTRACT: We report a versatile approach for covalent surface-assembly of proteins onto selected electrode patterns of pre-fabricated devices. Our approach is based on electro-assembly of the aminopolysaccharide chitosan scaffold as a stable thin film onto patterned conductive surfaces of the device, which is followed by covalent assembly of the target protein onto the scaffold surface upon enzymatic activation of the protein's "pro-tag." For our demonstration, the model target protein is green fluorescent protein (GFP) genetically fused with a pentatyrosine pro-tag at its C-terminus, which assembles onto both two-dimensional chips and within fully packaged microfluidic devices in situ and under flow. Our surface-assembly approach enables spatial selectivity and orientational control under mild experimental conditions. We believe that our integrated approach harnessing genetic manipulation, in situ enzymatic activation, and electro-assembly makes it advantageous for a wide variety of bioMEMS and biosensing applications that require facile "biofunctionalization" of microfabricated devices.

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KEYWORDS: pro-tag; protein assembly; bioMEMS; biofabrication

Introduction

Integrating proteins with microfabricated devices has gained significant attention for various applications such as biosensors (Chen et al., 2003b; Luo et al., 2004; Miao and Tan, 2001; Wang et al., 1999, 2003; Yang et al., 2004), medical diagnostics (Kartalov et al., 2006; Lee et al., 2005; Sebra et al., 2006), microarrays (Caelen et al., 2002), bioMEMS (biological microelectromechanical systems) (Grayson et al., 2004; Kartalov et al., 2006; Kim et al., 2001), and metabolic engineering (Jung and Stephanopoulos, 2004). Current methods for protein assembly include physical entrapment within sol–gel films (Chen et al., 2003b; Miao and Tan, 2001; Wang et al., 2003), affinity capture via ligands (Choi et al., 2002; Li and Lee, 2004; Zhen et al., 2004), mechanical methods such as microstamping onto polymer surfaces (Hyun et al., 2001), spotting (Caelen et al., 2002; Lee et al., 2005), and soft lithography (Kane et al., 1999; Suh et al., 2004), optical methods such as photolithography (Fodor et al., 1991; Sebra et al., 2006), and chemical methods such as attachment to self-assembled monolayers (Tender et al., 1996; Veiseh et al., 2002) and to chemically modified surfaces (Williams and Blanch, 1994). Chemical methods also include cross-linking proteins

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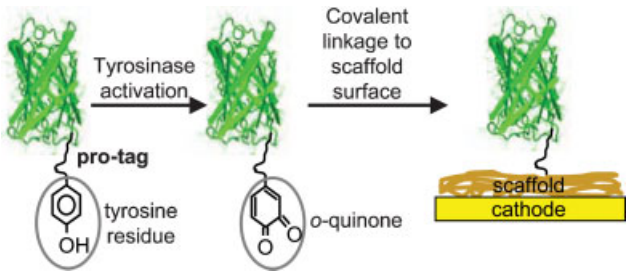
through their amines to surfaces or polymer films activated by glutaraldehyde (Diao et al., 2005; Lee et al., 2003; Park et al., 2006; Yang et al., 2004; Yi et al., 2005a). Despite these advances, protein assembly onto devices remains challenging due to the need for special facilities and instrumentation, arduous multi-step procedures, and dry environments, and other experimental conditions not ideal for maintaining biological activity.

We report an approach based on two strategies: scaffold electro-assembly onto a patterned conductive surface and covalent assembly of the target protein onto the patterned scaffold surface upon enzymatic activation of a genetically fused pro-tag. First, the scaffold is the pH-responsive aminopolysaccharide chitosan that electrodeposits as a stable thin film onto a conductive surface due to its pH-responsive properties that are conferred by its abundant primary amine groups. At low pH (<6.3), the amine groups are protonated, and chitosan is a water-soluble cationic polyelectrolyte. At neutral to high pH, these amine groups become deprotonated, and chitosan forms an insoluble hydrogel network. Due to its pH-responsive solubility transition, chitosan electrodeposits onto a negatively biased electrode surface due to localized high pH at the cathode surface (Luo et al., 2004, 2005b; Pang and Zhitomirsky, 2005; Wu and Payne, 2004; Wu et al., 2002, 2003; Yi et al., 2005b). Once deposited, the chitosan film is stable and adheres to the cathode surface in the absence of an applied voltage. Second, tyrosinase enzyme activates the C-terminal pentatyrosine pro-tag that is genetically fused to the target protein. As illustrated in Scheme 1, tyrosinase converts accessible tyrosine residues of the pro-tag into reactive *o*-quinones that form covalent linkage to the nucleophilic amine groups of the chitosan scaffold (Chen et al., 2002, 2003a; Freddi et al., 2006; Sampaio et al., 2005; Yi et al., 2005b). The target model protein is green fluorescent protein (GFP). GFP preferentially grafts to the chitosan scaffold through its C-terminal pro-tag versus its native tyrosines by a ratio of over 4:1 (Lewandowski et al., 2006); that is, over 80% of GFP molecules are grafted through the C-terminal pro-tag. Thus the GFP molecules are specifically oriented through the pro-tag with respect to the scaffold surface and are subsequently accessible in aqueous solution.



Scheme 1. Enzymatic activation and surface-assembly of specifically oriented target protein. The tyrosine residues of the C-terminal pentatyrosine "pro-tag" of the target protein GFP are enzymatically activated by tyrosinase into reactive *o*-quinones, which then form covalent linkages to the electro-assembled scaffold surface.

We report assembly of GFP fused with a pro-tag onto pre-fabricated devices using our two-step approach: electro-assembly of the scaffold onto selected electrode patterns followed by covalent assembly of GFP onto the scaffold surface upon enzymatic activation of the pro-tag. We performed assembly onto both two-dimensional chips and within fully packaged microfluidic devices in situ and under flow. We also demonstrate on-site activation of the pro-tag through assembly of the activating enzyme, which allows for sequential assembly of multiple target proteins onto a single device. Our strategy covalently assembles specifically oriented proteins in a spatially selective manner onto device surfaces under mild experimental conditions, ideal for maintaining protein biological activity. We envision many potential bioMEMS and biosensing applications that require facile in situ "biofunctionalization" of microfabricated devices.

Materials and Methods

Materials

Chitosan (minimum 85% deacetylated chitin; molecular weight 200,000 g/mol) from crab shells, phosphate buffered saline (PBS) (2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.5), and tyrosinase from mushroom were purchased from Sigma (St. Louis, MO). Tyrosinase was reported by the manufacturer to have an activity of 1,530 Units/mg solid. Sodium hydroxide was purchased from J.T. Baker (Phillipsburg, NJ). Acetone, hydrochloric acid, sulfuric acid, and glycerol were purchased from Fisher Chemical (Fair Lawn, NJ). Bleach was purchased from James Austin Co. (Mars, PA). De-ionized water (ddH_2O, 18 MΩ cm, Milli-Q) and PBS (dissolved in de-ionized water) were autoclaved before use.

Chitosan Preparation

Chitosan solution was prepared by adding chitosan flakes in de-ionized water, with HCl added dropwise to maintain pH ~ 2, and mixing overnight. The pH was then adjusted to 3.5 by adding 1 M NaOH dropwise, and the chitosan solution was then filtered and stored at 4°C.

Chip Fabrication

The microfabrication process for the chips was reported previously (Yi et al., 2004). Briefly, 4″ diameter silicon wafers were coated with 1 μm silicon nitride film, followed by deposition of 50 Å chromium film, and finally, deposition of 2,000 Å gold film. The patterns were created by photolithography, and the photoresist removed using acetone. The chips contain two upper gold rectangular

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patterns (6 mm long × 3 mm wide). The left upper pattern was where the alligator clip was attached. The upper patterns are each linked by 8 mm gold lines to two lower gold rectangular patterns (8 mm long × 1 mm wide). The left lower pattern was where assembly was performed. Chips were cleaned by incubation in ~1.4 M HCl for 30 min (to remove deposited chitosan), followed by incubation in concentrated bleach for 20 min (to sanitize and to ensure that organic molecules are removed), with thorough rinsing with de-ionized water after each cleaning step.

Assembly Onto Chips

First, chitosan was deposited onto the left gold electrode by dipping the chip into chitosan solution (0.5% (w/w), pH 3.7) and applying negative bias to the electrode (2 min at 16 A/m^2). This was done by connecting the cathode and anode (nickel chromium wire) using alligator clips to a DC power supply (2400 SourceMeter, Keithley Instruments, Cleveland, OH). After deposition, the chip was rinsed thoroughly with de-ionized water and rinsed with PBS. The chip was then incubated in both tyrosinase (0.1 mg/mL or 166 Units/mL) and $(His)_6$-GFP-EK-$(Tyr)_5$ (0.2 mg/mL) in PBS for 16 h at 4°C. The production of $(His)_6$-GFP-EK-$(Tyr)_5$ was previously described (Lewandowski et al., 2006). Briefly, the GFP was expressed in *E. coli* BL21, and IMAC-purified using a 5 mL HiTrap Chelating HP column charged with Ni^{2+} ions (GE Healthcare Bio-Sciences, Uppsala, Sweden). Controls for this experiment were done by incubating a chip with deposited chitosan in GFP alone or in tyrosinase alone. For the method with two chips, the activator chip was created by incubating a chip with deposited chitosan in tyrosinase solution for 16 h at 4°C. After rinsing thoroughly with de-ionized water and washing 3 × 5 min in PBS with gentle shaking, the activator chip was then incubated face-to-face with the assembly chip (2nd chip with deposited chitosan) in GFP solution for 2 h at 30°C such that the chitosan patterns were directly opposite one another and less than 1 mm apart. This was accomplished by holding the chips in place with an alligator clip. An additional experiment was performed where the two chips were incubated in GFP back-to-back with the chitosan patterns ≥5 mm apart. A control was done where the activator chip did not contain deposited chitosan. All chips were rinsed thoroughly with de-ionized water, and washed in PBS 3 × 5 min with gentle shaking before viewing under the fluorescence microscope (5 s exposure). ImageJ software (National Institutes of Health) was used to analyze the fluorescence intensity of the fluorescence micrographs.

Microfluidic Device Fabrication and Packaging

The fabrication process of our microfluidic device with packaging was reported previously (Park et al., 2006). Briefly, our microfluidic device features six identical microchannels evenly distributed on a 4″ pyrex wafer with two rectangular gold electrodes underneath each microchannel. A Cr adhesion layer (90 Å) and then a gold layer (2000 Å) were deposited onto a 4″ Pyrex wafer, and rectangular gold electrode patterns (1 mm × 0.5 mm) were created by photolithography. SU8-50 (MicroChem, Newton, MA) was patterned on the top of substrate and electrode surface to form structures which serve a dual function, namely (1) sidewalls for a microfluidic channel, and (2) sharp "knife-edge" structures for reliable leak-tight sealing to a PDMS layer above. The wafer was leak-tightly sealed by a 300-μm-thick top sealing PDMS layer spun on a sealing Plexiglas plate, and the SU8-50/PDMS junction was compressed by two packaging Plexiglas plates with six pressure-adjustable compression bolts (1/4″-28) hexagonally spaced on the ring and six force tunable socket screws (4–40) between every two microchannels. The microchannels thus formed were 500 μm wide by 150 μm high. Fluidic connectors (Nanoport™ Technologies, Portland, OR) and electric Pogo pins (Interconnect Devices, Kansas City, KS) were assembled through punched holes on the sealing PDMS and drilled-holes through the top sealing and packaging Plexiglas plates, and then connected to external pressure-driven aqueous transport, and electrical signal, correspondingly.

Assembly in Microfluidic Device

To avoid cross contamination among different solutions, a LabView based microfluidic control system enabling selection from different solutions with separate tubing was developed to enhance control over different solutions and processes. First, the experimental microchannel and all connecting tubing (0.02″ ID, Tygon®) were rinsed with de-ionized water at 50 μL/min for 30 min using a micropump (Masterflex® pump drive, Cole-Parmer, Vernon Hills, IL). Chitosan (0.375% (w/w), pH 5) was pumped into the microfluidic system at 5 μL/min. After the microchannel was completely filled with chitosan solution, the pump was stopped. The DC power supply was then used to maintain negative bias voltage on the gold (working) electrode under constant current conditions of 3 A/m^2 for 240 s, while a second gold electrode served as the anode. The chitosan solution was then drained from the system, and the deposited chitosan was washed with PBS (30 min at 5 μL/min). After draining the PBS buffer, a PBS solution with $(His)_6$-GFP-EK-$(Tyr)_5$ (0.2 mg/mL) and tyrosinase (0.1 mg/mL or 166 Units/mL) was pumped at 5 μL/min over the deposited chitosan. As a control, a PBS solution with GFP but without tyrosinase was pumped at 5 μL/min over the deposited chitosan. (Between experiments the system was cleaned by rinsing with ~1.4 M HCl and then concentrated bleach at 5 μL/min for 10 min each, followed by thorough rinsing with de-ionized water at 50 μL/min for 30 min.) For real-time in situ fluorescence and observation, the microfluidic device was placed under a microscope (model 310, Carl Zeiss, Thornwood, NY) and a UV source (Zeiss HBO 100). Fluorescence micrographs were acquired every minute

from the microscope using a digital camera (Carl Ziess AxioCam MRc5). Finally, the system was washed with PBS (30 min at 5 μL/min). ImageJ software (National Institutes of Health) was used to analyze the fluorescence intensity of the final fluorescence micrograph.

Results and Discussion

Enzymatic Activation and Assembly of Target Protein Onto a Patterned Chip

We demonstrate enzymatic activation of GFP for its assembly onto the patterned electro-assembled scaffold surface of a two-dimensional chip, shown schematically in Figure 1a. For this, we first electro-assembled chitosan scaffold onto a selected electrode pattern by dipping the chip in chitosan solution and applying negative bias to the left gold pattern (Fig. 1b). This chip with assembled scaffold was then incubated at 4°C in a PBS solution containing the pro-tagged GFP (in excess of available scaffold) and the activating enzyme tyrosinase, which activates the pro-tag of GFP for its assembly onto the scaffold. Finally, the chip was thoroughly washed with PBS buffer. The fluorescence micrograph in Figure 1c illustrates that (1) the target protein GFP assembled only onto the scaffold pattern with high spatial selectivity and uniformity, (2) the assembled GFP remained fluorescent, indicating the retention of its three-dimensional structure, and finally, (3) non-specific binding of GFP to the other surfaces of the chip was minimal, as there is negligible fluorescence of the right gold electrode pattern or the silicon oxide. Importantly, incubating the chip with assembled scaffold in GFP alone (Fig. 1d) or tyrosinase alone (Fig. 1e) yielded significantly less fluorescence (<3% of the fluorescence of Fig. 1c, based on ImageJ software analysis), indicating minimal non-specific binding of GFP to the scaffold, and that activation of the pro-tag by tyrosinase is required for GFP assembly. As GFP is covalently assembling onto the scaffold, the chip could be repeatedly washed with negligible loss in fluorescence (not shown). Additionally, the chips were reused by simply cleaning with dilute hydrochloric acid followed by concentrated bleach. Combined, these results demonstrate patterned and covalent assembly of biologically functional protein onto a two-dimensional chip through enzymatic activation of the C-terminal pro-tag.

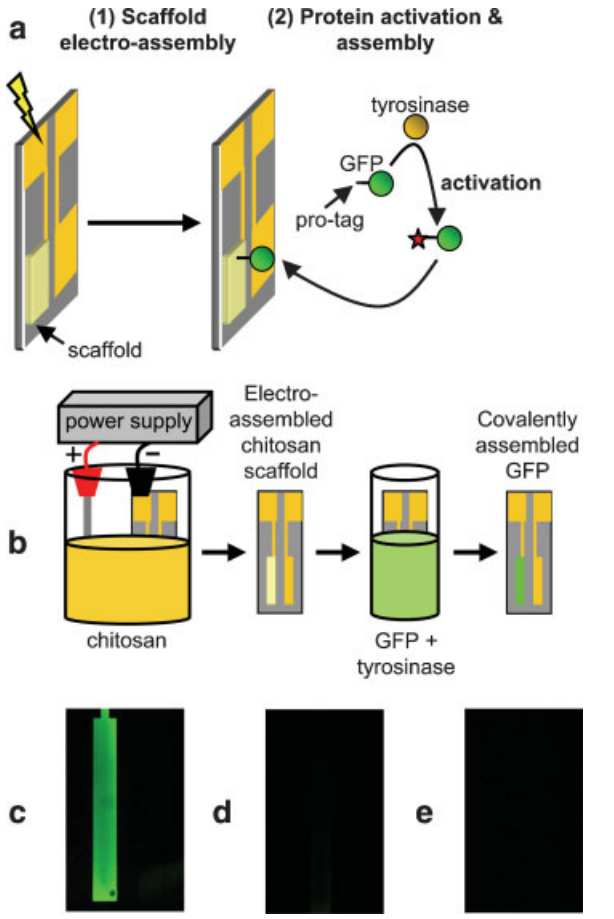

Figure 1. Enzymatic activation and assembly of target protein onto a patterned chip. **a**: Electro-assembly of the scaffold onto a patterned electrode is followed by enzymatic activation and assembly of GFP onto the patterned scaffold surface. **b**: The scaffold is assembled by dipping the chip into chitosan solution and applying negative bias to the selected electrode pattern. Subsequent incubation in GFP and tyrosinase solution activates and assembles GFP onto the patterned scaffold surface. **c**: Fluorescence micrograph: chip with assembled scaffold incubated in GFP and tyrosinase. **d**: Fluorescence micrograph: chip with assembled scaffold incubated in GFP only (control). **e**: Fluorescence micrograph: chip with assembled scaffold incubated in tyrosinase only (control).

We have demonstrated a high degree of covalent grafting and a low degree of non-specific binding of the target protein GFP to electrodeposited chitosan scaffold. This agrees with previously reported results (Chen et al., 2003a; Lewandowski et al., 2006) which examined GFP binding to chitosan in solution. Electrodeposited chitosan may have structural and chemical properties that are distinctly different than soluble chitosan, particularly in that the chitosan experiences a high pH for a short time during the electrodeposition. In the present work, the chitosan scaffold is electrodeposited prior to target protein assembly, and thus, there is no pH-dependence for the protein assembly. Indeed, the protein assembly process takes place under mild experimental conditions ideal for maintaining protein activity and three-dimensional structure: at low temperature, through enzymatic activation, in aqueous solution, and at neutral pH. Thus, our current assembly technique is particularly advantageous for pH- or temperature-sensitive proteins.

Due to the distinct differences between electrodeposited chitosan and soluble chitosan, the current assembly procedure was investigated as an alternative to that of previous work where the procedural order was reversed: the protein was first conjugated to chitosan in solution, and then this protein–chitosan conjugate was electrodeposited (Chen et al., 2003a; Yi et al., 2005a). Conjugation occurred at pH ~ 6 so that chitosan remained soluble, that is below its pK_a of 6.3, and this may destabilize low pH-sensitive

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proteins and additionally proteins with isoelectric points at or near pH 6. Next, electrodeposition of the protein–chitosan conjugate solution occurred at pH 11–12, which may destabilize high pH-sensitive proteins. Additionally, the protein was embedded within the electrodeposited chitosan film and in no particular orientation. In the current work, in contrast, the protein presumably assembles onto the scaffold surface as diffusion of proteins into the chitosan film is low. Indeed, this low diffusion allows proteins to be physically entrapped within chitosan films (Luo et al., 2005a).

Finally, we expect the vast majority of GFP molecules to be specifically oriented through the C-terminal pro-tags as they assemble onto the scaffold surface, as we previously demonstrated that GFP preferentially grafts to chitosan through the pro-tag versus native tyrosines by a ratio of over 4:1; that is, over 80% of binding is through the pro-tag (Lewandowski et al., 2006). This enables orientational control and contrasts considerably with an additional previous approach, where the protein was covalently linked through its native amines to a glutaraldehyde-activated chitosan film surface and thus in no particular orientation (Yi et al., 2005a). These aspects of our strategy (surface-assembly under orientational control) are especially advantageous for biosensor applications involving enzymes, which require reproducible orientation and active site accessibility.

In summary, Figure 1 demonstrates covalent assembly of the target protein GFP onto selected patterned scaffold surfaces of two-dimensional microfabricated chips under mild experimental conditions. Assembly occurs through the C-terminal pro-tag upon selective enzymatic activation. This assembly strategy is advantageous for pH-sensitive proteins and biosensor applications involving assembled enzymes.

On-Site Activation and Assembly of Target Protein Onto Multiple Patterned Chips

Next, we demonstrate assembly of GFP onto the patterned scaffolds of two-dimensional chips through on-site enzymatic activation by assembled tyrosinase, shown schematically in Figure 2a. For this, we first assembled the activating enzyme tyrosinase (by incubation in tyrosinase solution) onto electro-assembled chitosan scaffold on one chip serving as the activator chip (Fig. 2b). After thorough rinsing, the activator chip was incubated in GFP solution directly opposite and <1 mm from the assembly chip, which contained only electro-assembled chitosan scaffold. The fluorescence micrographs in Figure 2c illustrate that (1) similar quantities of the target protein GFP assembled onto both activation and

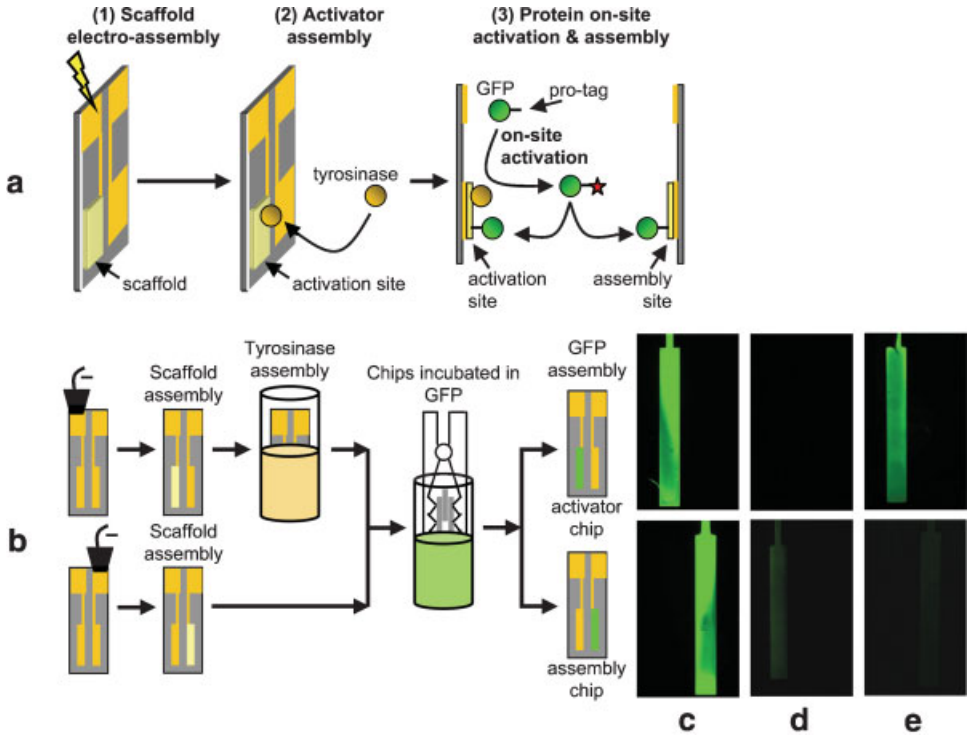

Figure 2. On-site activation and assembly of target protein onto multiple patterned chips. **a**: Tyrosinase activator is assembled onto the patterned scaffold surface to create an activation site. GFP is activated on-site by the assembled tyrosinase for its local assembly at both the activation site and the proximal assembly site. **b**: The activator chip is created by electro-assembly of the scaffold followed by tyrosinase incubation. The assembly chip is created by electro-assembly of the scaffold. Both chips are then incubated in GFP solution. **c**: Fluorescence micrographs: activator and assembly chips incubated in GFP solution directly opposite each other and <1 mm apart. **d**: Fluorescence micrographs: minimal tyrosinase assembled onto the activator chip (control with no electro-assembled scaffold). **e**: Fluorescence micrographs: activator and assembly chips incubated in GFP solution back-to-back with the scaffolds ≥ 5 mm apart.

assembly sites, and (2) GFP assembled only onto the patterned chitosan scaffolds with high spatial selectivity and uniformity.

These results demonstrate that (1) the tyrosinase assembled onto the activator chip, (2) the activator chip served as a heterogeneous catalyst for GFP activation, (3) the assembled tyrosinase activated GFP on-site for its assembly onto the activator chip, and finally, (4) the assembled tyrosinase activated GFP for assembly onto a second proximal chip (assembly chip). Importantly, a chip without assembled scaffold bound a minimal quantity of tyrosinase activator, and could not be used to activate, and assemble GFP, as illustrated by the minimal fluorescence of both chips in Figure 2d.

Finally, we demonstrate that the distance between activation and assembly sites is critical for target protein assembly. For this, the activator and assembly chips were incubated in GFP solution back-to-back with the scaffolds $\geq$5 mm apart. Here, GFP assembled only onto the activation site, as illustrated in Figure 2e. This indicates that a minimal amount, if any, of tyrosinase was released from the scaffold and into the GFP solution, as any released tyrosinase would have activated GFP for its assembly onto the assembly site. We propose that the reactive *o*-quinones created by activation of the pro-tag each have a short half-life and can only diffuse a short distance before losing activity. The distance between activation of the target protein and available scaffold must be significantly small to result in target protein assembly onto the scaffold. In a sense, this makes our approach (pro-tag activation and assembly) a self-passivating system. This short half-life, however, also indicates that formation of multimer forms of the target protein is possible (i.e., attachment of multiple activated proteins together), if activated protein molecules encounter each other before encountering the scaffold surface; this possibility was previously noted (Chen et al., 2003a). Nonetheless, we view the short half-life of activated protein as a strength of our assembly strategy for the biofunctionalizaton of microfabricated devices, as it demonstrates the flexibility of our strategy, and we envision many potential applications. For example, this method can be used for the sequential assembly of multiple proteins onto separate patterned scaffold surfaces of the same device. For chip assembly, each protein would be directed to a specific scaffold pattern by placement of the scaffold proximal to the activation site during target protein incubation. This could additionally be accomplished with microfluidic devices under flow of the target protein, where each assembly site would be directly downstream of each activation site.

In summary, Figure 2 demonstrates that tyrosinase enzyme can be assembled to form an activation site for on-site activation of the target protein, which then assembles locally onto both the activation site and onto a proximal assembly site. Local assembly of the target protein is presumably due to the short half-life of activated protein. These results demonstrate an additional flexibility to our assembly strategy, and we envision many potential applications involving sequential assembly of multiple target proteins.

In Situ Activation and Assembly of Target Protein Within a Completely Packaged Microfluidic Device

Finally, we demonstrate in situ activation and assembly of GFP onto a selected electrode pattern within a completely packaged microfluidic device, shown in Figure 3. For this, we fabricated a microfluidic device that features six identical microchannels evenly distributed on a 4″ pyrex wafer (Park et al., 2006); one microchannel is illustrated in Figure 3a. The microfluidic device employs a microfabricated SU8 layer on a transparent Pyrex wafer that defines the microchannel structure, two patterned gold electrodes at the bottom of each microchannel, a top PDMS gasket that seals the microchannel by compression bolts, and fluidic, and electrical input/output ports that connect to an external micropump, and electrical signals, respectively. The solution selection and pumping is controlled by LabView.

We electro-assembled chitosan scaffold onto a selected gold electrode pattern within a microchannel by transporting chitosan solution into the channel, and then stopping the pump and applying negative bias to the electrode pattern (Fig. 3b). Next, we continually pumped through the channel a PBS solution containing the target protein GFP and the activating enzyme tyrosinase (Fig. 3c) to activate and assemble GFP onto the electro-assembled chitosan scaffold. We observed the fluorescence profile of the 1 mm $\times$ 0.5 mm assembly site (scaffold) in real time through an on-site fluorescence microscope. As illustrated in Figure 3d, the fluorescence intensity of the assembly site gradually increased with time until reaching a maximum constant level. Importantly, the fluorescence remained high even after thorough PBS buffer rinsing, indicating that GFP was covalently bound to the chitosan scaffold. Next, we analyzed the fluorescence intensity of the final micrograph of the assembly site using ImageJ software, which illustrates that GFP assembled relatively uniformly onto the scaffold pattern. These results demonstrate that (1) the target protein GFP assembled only onto the chitosan scaffold with high spatial selectivity, and uniformity, (2) the assembled GFP remained fluorescent after thorough rinsing, and (3) non-specific binding of GFP to other channel surfaces was minimal, as there was no significant fluorescence of the microchannel floor, ceiling, or walls.

Next, we performed control experiments to examine non-specific binding of unactivated GFP to the electro-assembled scaffold. For this, we reused the same microchannel by removing the scaffold from the previous experiment with dilute hydrochloric acid. We then reassembled the chitosan scaffold onto the electrode pattern, and then continually pumped through the channel a PBS solution containing only the target protein GFP (without the activating enzyme tyrosinase). As illustrated in Figure 3e, the fluorescence intensity of the assembly site gradually increased with time

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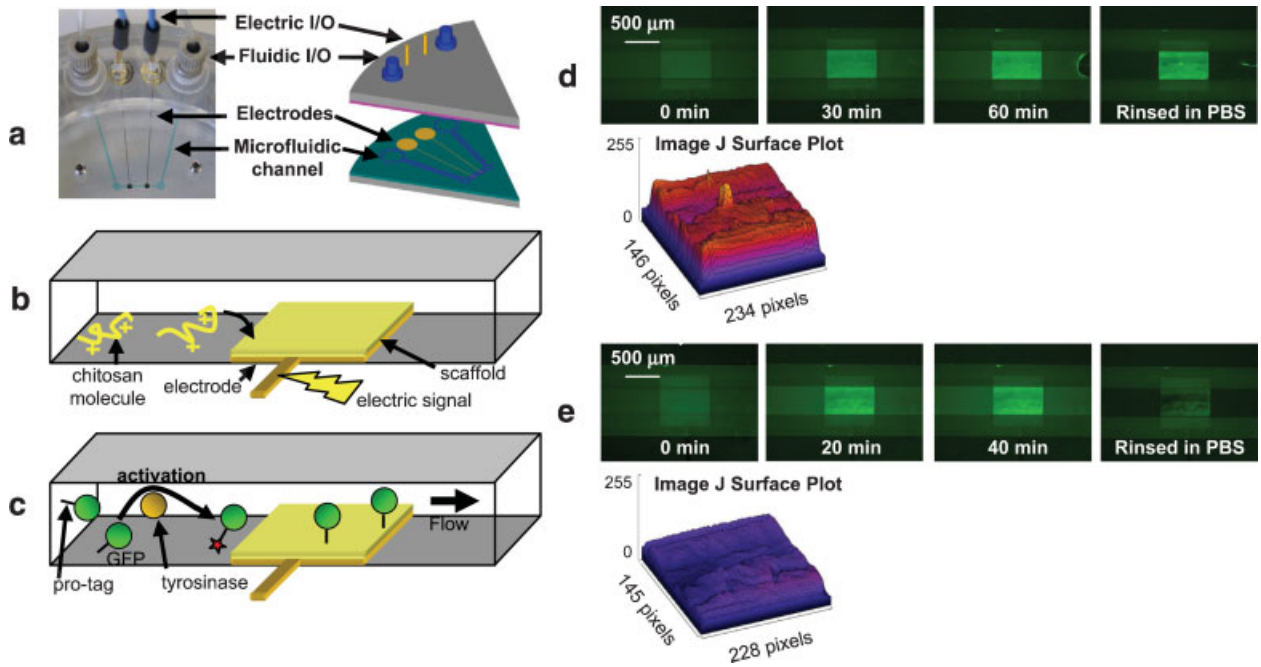

Figure 3. In situ activation and assembly of target protein within a completely packaged microfluidic device. **a**: One of the six microfluidic channels of the completely packaged microfluidic device. **b**: Chitosan scaffold is electro-assembled onto a gold electrode pattern within a microchannel (static state). **c**: In situ tyrosinase activation and assembly of GFP onto the patterned scaffold surface within a microchannel under flow. **d**: Fluorescence micrographs of the assembly site during in situ GFP activation, and assembly, and after rinsing the assembly site with PBS buffer, with a surface plot (ImageJ) of the rinsed assembly site. **e**: Fluorescence micrographs of the assembly site during GFP non-covalent assembly (control with no tyrosinase activation), and after rinsing the assembly site with PBS buffer, with a surface plot (ImageJ) of the rinsed assembly site.

until reaching a constant level. However, the fluorescence decreased significantly upon PBS buffer rinsing, as illustrated by ImageJ software analysis of the final fluorescence micrograph (the surface plot represents the pixel intensity over the deposition area). Further ImageJ analysis of the micrographs in Figure 3d and e, suggest that the loss of GFP upon rinsing after tyrosinase treatment (Fig. 3d) was about 8% (e.g., loss from the 60 min time point) and that the final level in Figure 3e (no tyrosinase) was ~25% of that in Figure 3d. These results indicate that the GFP without tyrosinase was loosely bound to the chitosan scaffold, and easily rinsed off, and confirm that activation of the pro-tag by tyrosinase is required for GFP assembly.

We have demonstrated here, for the first time, in situ enzymatic activation, and assembly of a target protein onto a patterned scaffold within a microfluidic channel under flow. The target protein GFP assembled covalently, and uniformly in a spatially selective manner, and was not released upon further flow, and buffer rinsing. This is significant, as we are unaware of any reports demonstrating the spatially resolved enzymatically activated covalent assembly of proteins in microchannels under flow. Additionally, as discussed earlier, GFP is assembling predominately through its C-terminal pro-tag, that is in a specific orientation onto the patterned scaffold surface. In contrast, in our previous work with microfluidic channels, the protein was covalently linked through its native amines to a glutaraldehyde-activated chitosan film surface and in no particular orientation (Park et al., 2006). Finally, assembly occurs in a completely fabricated and packaged device for reusability, and as mentioned before, occurs under mild experimental conditions ideal for maintaining protein biological function: in aqueous solution, through enzymatic activation, and at neutral pH. These unique advantages of our assembly strategy combined with the well-known advantages of microfluidic devices (i.e., rapid response time and small volumes of expensive reagents) make this particularly appealing for applications that necessitate microfluidic systems.

In summary, Figure 3 demonstrates in situ enzymatic activation and assembly of the target protein GFP within a pre-fabricated and fully packaged microfluidic device under flow. Assembly is covalent and robust, spatially selective (only onto selected patterned scaffold surfaces), and occurs under mild experimental conditions. Importantly, our assembly approach is readily applicable to microfluidic systems.

Conclusions

We report a versatile approach for covalent protein assembly onto the surfaces of selected patterned scaffolds of pre-fabricated devices. Our results demonstrate assembly of the model protein GFP genetically fused with a C-terminal

pentatyrosine pro-tag onto both two-dimensional chips and within fully packaged microfluidic devices.

We believe that our assembly approach holds several unique advantages. First, the entire assembly process occurs under mild experimental conditions: in aqueous solution, through enzymatic activation, and at neutral pH, making it ideal for maintaining protein three-dimensional structure and biological function. Second, the assembled protein is readily accessible, and specifically oriented as it assembles though the C-terminal pro-tag onto the scaffold surface. This makes our approach particularly appealing for assembling enzymes, where reproducible orientation, and active site accessibility are necessary for maintaining catalytic activity. Third, the activating enzyme tyrosinase can be assembled to create an activation site for on-site activation and local assembly of the target protein. This strategy can be exploited for local assembly of multiple proteins onto individual patterned scaffolds of the same device. Finally, the protein is enzymatically activated and assembled in situ within a microfluidic channel under flow, and thus, our approach is readily applicable to microfluidic systems, which have additional well-known advantages over batch systems. Combined, these advantages make our assembly approach appealing for a wide variety of bioMEMS and biosensing applications that require device biofunctionalization.

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