Nature-Inspired Creation of Protein−Polysaccharide Conjugate and Its Subsequent Assembly onto a Patterned Surface

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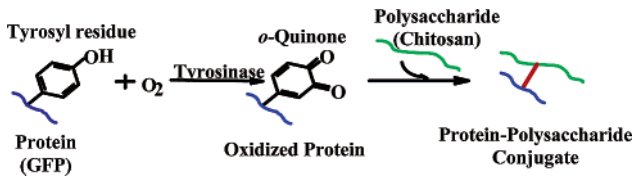
A protein's functional properties can be adjusted by conjugating it to other polymers. We used a nature-inspired route to create a protein−polysaccharide conjugate and examined the properties of this conjugate. Specifically, the enzyme tyrosinase was used to oxidize accessible tyrosine residues of the model protein green fluorescent protein (GFP). Oxidation yields quinone residues that are "activated" for the covalent conjugation of GFP to nucleophilic groups of the aminopolysaccharide chitosan. Conjugation to chitosan conferred distinct properties to GFP. The GFP−chitosan conjugate was observed to have pH-responsive, "smart" properties, and GFP could be conjugated onto a gel matrix. Additionally, the GFP−chitosan conjugate can be selectively deposited onto a micropatterned surface in response to an applied voltage. This nature-inspired method provides a simple and safe method to conjugate proteins to chitosan, and these conjugates can be readily assembled onto patterned surfaces.

Introduction

Polymers are commonly grafted to proteins to adjust the protein's properties (e.g., solubility), to confer stimuli-responsive "smart" properties, or to entrap the protein within a matrix. In nature, proteins are commonly conjugated with sugars. We are adapting a nature-inspired route for creating protein−polysaccharide conjugates.

In nature, tyrosinase-initiated reactions are responsible for the setting of mussel glue. The mussel uses an open chain adhesive protein that is rich in tyrosine or dihydroxyphenylalanine (DOPA) residues.[1−3] Tyrosinase oxidizes some of these residues and the oxidized *o*-quinone residues undergo nonenzymatic cross-linkling reactions with amino acid residues on other protein chains. This cross-linking leads to the formation of a three-dimensional gel network that is believed to confer cohesive strength to the mussel glue.[4−7] As illustrated in Scheme 1, we are extending tyrosinase-based biochemistry to create protein−polysaccharide conjugates. Specifically, we use tyrosinase to react with, and "activate", accessible tyrosine residues of proteins. These "activated" quinone residues can then react with nucleophilic substituents—in our case with nucleophilic substituents of the polysaccharide chitosan.

Scheme 1



Chitosan is an amino-polysaccharide with a pK_a of about 6.5—the exact pK_a value varies depending on the degree of acetylation and degree of ionization.[8−11] At low pH, the amine groups are protonated making chitosan a water-

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soluble, cationic polyelectrolyte. At higher pH (above about 6.3), when a significant number of amine groups are deprotonated, chitosan becomes insoluble and can either form a precipitate or a hydrogel depending on conditions.[12] Chitosan's amine groups are also nucleophilic and can undergo reactions with a variety of electrophiles, including tyrosinase-generated quinones.[13−17] Currently, the quinone−chitosan conjugation chemistry is incompletely understood because quinones can undergo a complex set of reactions.[18−23] Recent studies indicate that tyrosinase-generated quinones are conjugated to glucosamine's (or chitosan's) amines through Schiff base and/or Michael-type adduct linkages.[14,17]

Previous studies showed that tyrosinase can oxidize accessible tyrosyl residues of the open chain protein gelatin, and that the "activated" gelatin chains can be conjugated to chitosan.[24−26] It is not clear, however, how readily the tyrosinase-initiated conjugation approach can be extended from proteins with open-chain conformations (e.g., structural proteins) to proteins with compact, globular structures (e.g., catalytic and recognition proteins). Specifically, the tyrosyl residues of compact proteins may not be readily accessible for tyrosinase-catalyzed activation.[27]

Here, we examined green fluorescent protein (GFP) as a model of a protein with a compact structure. Figure 1 shows the structure of GFP and indicates that four of its nine tyrosine residues may be present on the protein's outer surface and therefore may be accessible for tyrosinase-catalyzed oxidation. We specifically studied GFP fusion proteins containing a hexahistidine tail at the N terminus ($(His)_6$GFP).[28,29] The His-tail facilitates purification of the fusion proteins. Additionally, we created a fusion of the $(His)_6$GFP that contains 5 tyrosyl residues at the C terminus ($(His)_6$GFP$(Tyr)_5$). The Tyr-tail was designed to increase the number of accessible residues to facilitate tyrosinase-catalyzed activation. The goals of this study were to show that tyrosinase could be used to create a GFP−chitosan conjugate and to demonstrate useful properties of this graft copolymer.

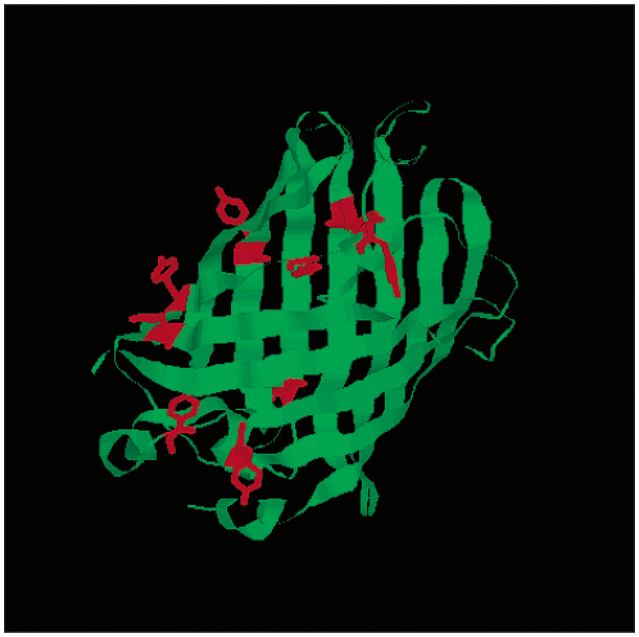

Figure 1. Structure of GFP showing tyrosine residues in red.

Materials and Methods

Chitosan was purchased from Sigma and reported by the manufacturer to have a molecular weight of approximately 200 000 and a degree of acetylation of about 15%. Chitosan solutions were prepared by dissolving chitosan in HCl solutions as described elsewhere.[25] Chitosan hydrogels were prepared by pouring a 2% chitosan solution into a small Petri dish and then immersing the dish in a caustic solution (1 M NaOH). After gel formation, the gels were washed extensively with water and PBS buffer. Specific conditions used for preparing GFP−chitosan conjugates are described in the text and legends to each figure. GFP fluorescence was quantified using a PerkinElmer LS55 luminescence spectrometer with an excitation and emission wavelengths of 395 and 509 nm, respectively.

Dr. H. J. Cha (Postech University, Seoul, South Korea) provided the pHis-GFP plasmid containing a *gfp* gene inserted into a pTrcHisB (Invitrogen) expression vector and transformed into *Escherichia coli* BL21 (Invitrogen).[28,29] The pHis-GFP plasmid contained a hexahistidine tail on the N-terminus of *gfp*, and contained the gene for ampicillin resistance. For this work, a pentatyrosine tail was inserted at the C-terminus of *gfp* (resulting in pHis-GFP-Tyr) and transformed into *E. coli* BL21 (Invitrogen). Cells were grown to an OD_{600} of 0.6−1.0, induced with 1 M isopropylthiogalactoside (IPTG), grown for an additional 5 h, and then harvested by centrifugation (30 min at 12000*g*, Sorval). Pellets were resuspended in 1:50 ratio (by volume) of 1× phosphate buffered saline (PBS) pH 7.4. Aliquots of cell suspension were lysed using sonication. The cell lysate was centrifuged (10 min at 12000*g*) to remove insoluble cellular debris and then filtered through a 0.2 μm filter. The cell lysate was then purified using Immobilized Metal Affinity Chromatography (IMAC; AP Biotech Hi-Trap) using the manufacturer's protocol. Fractions with high GFP content were eluted (100 and 300 mM imidazole) and dialyzed (#2 Spectra/Por dialysis tubing) in 1X PBS pH 7.4.

Gold was patterned onto silicon wafers using standard microfabrication techniques as previously described.[30,31] For deposition of the GFP−chitosan conjugate, the patterned surfaces were immersed in a conjugate-containing solution and the patterned gold surfaces were polarized to serve as negative electrodes (i.e., cathodes). The anode in these experiments was an unpatterned gold film on a silicon surface. The two electrodes were connected to a DC power supply (model 6614C, Agilent

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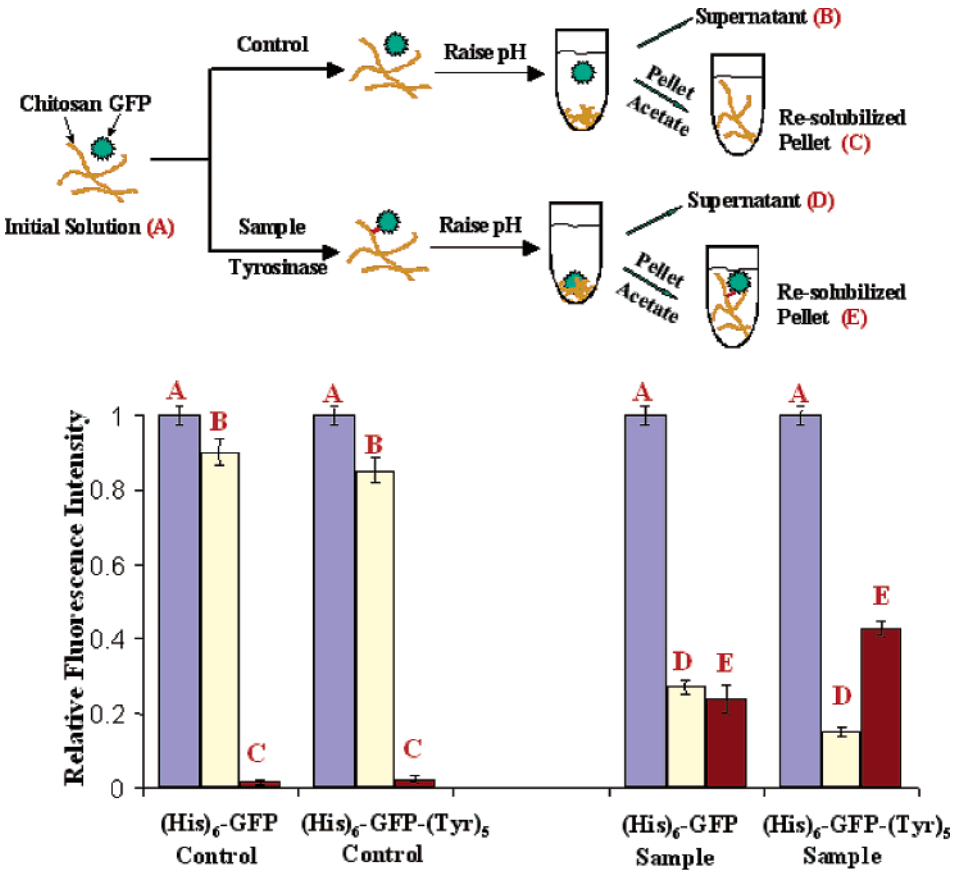

Figure 2. Tyrosinase-initiated conjugation of GFP-fusions with chitosan. Solutions (pH ≈ 6) were prepared with GFP-fusion (0.17 μg/mL) and chitosan (0.7% w/v), in either the absence ("Controls") or presence ("Samples") of tyrosinase (60 U/mL). After overnight incubation at room temperature, freshly prepared $NaBH_4$ (6.6 mM final concentration) was added for 1−2 h. Chitosan and conjugates were precipitated by raising the pH with phosphate buffer (pH = 9). After centrifugation, the fluorescence of the supernatant was measured. The pellet was washed twice with PBS buffer to remove physically bound protein and then resolubilized in aqueous acetic acid solution (0.5 v/v %, pH = 4).

Technologies) using alligator clips and a fixed current density was maintained for a specified time. The patterned surfaces were examined using a fluorescence stereomicroscope (MZFLIII, Leica) with an excitation filter at 425 nm (bandwidth of 60 nm) and an emission barrier filter at 480 nm.

Results and Discussion

Conjugate Formation. Conjugation was studied by blending individual fusion proteins with chitosan under slightly acidic conditions (pH = 6) and, in some cases, adding tyrosinase to the solutions. The upper scheme in Figure 2 indicates that the fusion protein was blended with chitosan (but not tyrosinase) for the "Controls". After blending, the pH was increased to precipitate the chitosan. Figure 2 shows that most of the initial fluorescence in the "Controls" remained in the supernatant (bars "A" and "B" in the chart). The pellet was washed to remove physically bound protein and then resolubilized in aqueous acetic acid (bars "C" in the chart). Figure 2 shows that little fluorescence appeared in the resolubilized pellet for these "Controls". These results indicate that neither the $(His)_6$GFP nor the $(His)_6$GFP$(Tyr)_5$ fusion proteins strongly associates with chitosan. Further, the observation that neither fusion protein is conjugated to chitosan in these "Controls" rules out the possibility that auto-oxidation of tyrosine residues leads to conjugation.

The lower scheme in Figure 2 indicates that the "Samples" were prepared by including tyrosinase in the solutions of the fusion protein and chitosan. A slight browning of these solutions was visually observed—consistent with an enzymatic oxidation of accessible tyrosyl residues. After overnight incubation at room temperature, the reducing agent sodium borohydride was added, and the "Samples" were incubated for an additional 2 h. (A similar borohydride treatment was performed with the "Controls" of Figure 2.) After borohydride treatment, the pH of the solutions was raised to precipitate chitosan and the GFP−chitosan conjugates. Figure 2 shows that less than half the initial GFP fluorescence remained in the supernatant from these tyrosinase-treated "Samples" (bars "D" in the chart). The pellet was recovered, washed and resolubilized in an aqueous acetic acid solution (bars "E" in the chart). In contrast to the "Controls" in Figure 2, substantial fluorescence is observed in the resolubilized pellets for the "Samples". This observation indicates that tyrosinase activates both the $(His)_6$GFP and the $(His)_6$GFP-$(Tyr)_5$ fusion proteins for conjugation to chitosan. Comparison of the resolubilized pellets for these two "Samples" (bars "E") indicates that the $(His)_6$GFP$(Tyr)_5$ fusion is more effectively conjugated to chitosan than the $(His)_6$GFP fusion (43 and 24% of the initial fluorescence was observed in the pellets for the $(His)_6$GFP$(Tyr)_5$ and the $(His)_6$GFP fusions, respectively). This comparison indicates that the Tyr-tail enhances, but is not required for, conjugation. [It should be noted that the sum of the fluorescence of the supernatant plus that of resolubilized pellet is not equal to the initial fluorescence because some fluorescence is lost in washing, and because the intrinsic fluorescence of GFP is reduced at the low pH of the resolubilized pellet.]

Quantitatively, we performed the conjugation by reacting 0.02 mg of GFP per gram of chitosan. This is far less

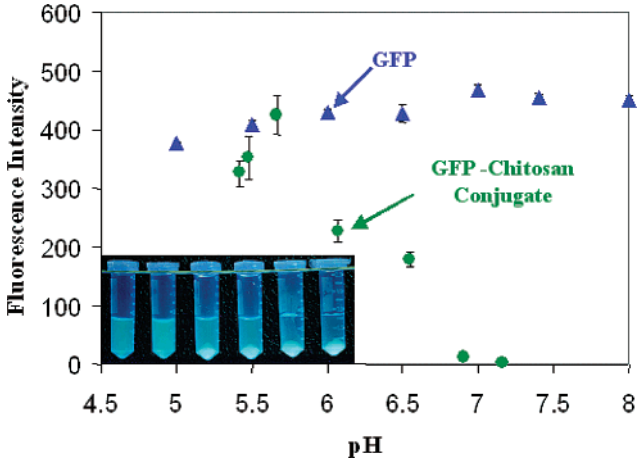

Figure 3. pH-responsive properties of GFP−chitosan conjugate generated using $(His)_6GFP(Tyr)_5$. Conditions for reaction, precipitation, washing, and resolubilization are the same as described in the legend to Figure 2. Aliquots from these resolubilized pellets were mixed with phosphate buffer (30 mM final concentration) of varying pH. The final pH was measured, the samples were centrifuged, and the fluorescence of the supernatant was determined. For the "Control", the $(His)_6GFP(Tyr)_5$ was added directly to phosphate buffers of differing pH. The insert shows photographs using UV illumination to demonstrate precipitation of the fluorescent GFP−chitosan conjugate when the pH is increased from 5.5 to 7.2 (left to right in the photograph).

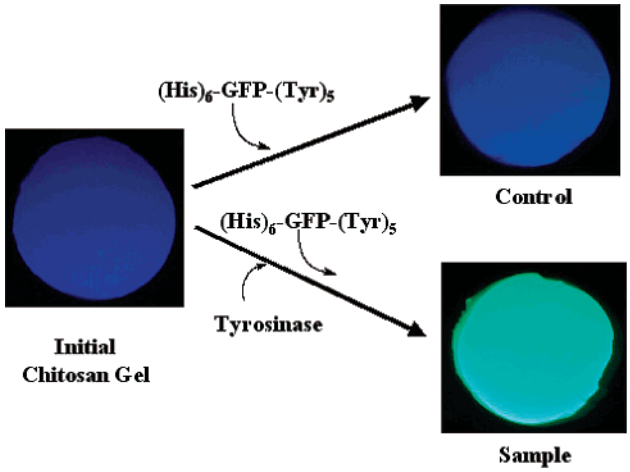

Figure 4. Conjugation of GFP to chitosan hydrogel. Tyrosinase-containing solution (100 U) was spread onto the surface of a chitosan hydrogel (1.2 cm diameter), the solution was allowed absorb into the gel for 10 min, and the gel was then immersed into a solution containing $(His)_6GFP(Tyr)_5$ (2 mL at 0.32 μg/mL). After incubating at room-temperature overnight, $NaBH_4$ (6.6 mM final concentration) was added and the gel was incubated for an additional 1−2 h after which the gel was washed extensively with PBS buffer. The "Control" was prepared in the same manner except tyrosinase was not added.

than the amount of GFP that would be required to conjugate a single GFP molecule onto each chitosan chain (approximately 100 mg/g would be required for one GFP protein to be added to each chitosan chain). The fluorescence results (bars "E" in Figure 2) suggest that the partially purified conjugate contained approximately 0.005 or 0.01 mg of GFP per gram of chitosan, for the $(His)_6GFP$ and $(His)_6GFP(Tyr)_5$ fusion proteins, respectively.

The first conclusion from Figure 2 is that tyrosinase is required for conjugation of the GFP fusion proteins to chitosan (no conjugation occurs in the "Controls"). Thus, tyrosinase "activates" the fusion protein to initiate covalent conjugation. The second conclusion is that the tyrosine-rich fusion tail enhances, but is not required for, GFP conjugation. Presumably the Tyr-tail provides additional accessible tyrosyl residues for tyrosinase-catalyzed activation.

Properties of GFP—Chitosan Conjugates. Conjugation to chitosan confers three important properties to GFP. First, the conjugate offers pH responsive (i.e., "smart") properties characteristic of chitosan.[32] This property is illustrated in Figure 3 that shows the GFP−chitosan conjugate is soluble under acidic conditions but precipitates when the pH is raised above about 6. The insert in Figure 3 shows photographs of the GFP−chitosan conjugates taken with UV illumination. Fluorescence is observed to precipitate as the pH is raised from 5.5 to 7.2 (left to right in the photograph). Interestingly, the GFP−chitosan conjugate appears to become insoluble at lower pHs than chitosan (i.e., the solubility of the conjugate is reduced even at a pH of 6). Presumably, differences in solubility between chitosan and the GFP−chitosan conjugate are due to interactions between the chitosan chain and the conjugated GFP. It seems likely that these solubility differences would depend on the number of GFP chains grafted to chitosan.

The second property conferred by chitosan to the GFP conjugate is the ability to be covalently immobilized as a hydrogel. This property is illustrated by an experiment in which tyrosinase was coated onto the chitosan hydrogel and the gel was then immersed in a solution containing $(His)_6GFP(Tyr)_5$. After immersion, the gel was washed extensively with buffer to remove noncovalently bound GFP. Figure 4 shows this "Sample" retains substantial fluorescence under UV illumination. For the "Control" in Figure 4, a chitosan hydrogel without tyrosinase treatment was immersed in a similar $(His)_6GFP(Tyr)_5$−containing solution. After washing, Figure 4 shows that this "Control" gel had little GFP fluorescence.

The third property conferred to GFP by chitosan is the ability to be assembled onto a patterned surface in response to an applied voltage. Previous studies have shown that chitosan can be deposited onto patterned gold surfaces that have been polarized to serve as the negative electrodes (i.e., as cathodes).[30,31,33] Initial studies to demonstrate deposition of the conjugate were performed using the patterned surface illustrated in Figure 5a. The 1 mm wide gold patterns on this silicon oxide surface were prepared using standard microfabrication techniques.[31] A photomicrograph of the bottom section of the two gold patterns is shown in Figure 5b. If this surface were viewed using a fluorescence microscope, no image would be visible (not shown). For deposition, the surface was immersed in a conjugate-containing solution (the concentration of chitosan plus the GFP−chitosan conjugate was 0.6 w/w %, and the pH was 4.9) and one of the gold patterns was polarized to serve as a cathode. After contacting the solution for 40 s at a current density of 1.2 A/m^2, the surface was rinsed with distilled water and viewed using a fluorescence microscope. The fluorescence micrograph in Figure 5c shows that the right-most gold pattern that had been polarized to serve as the cathode is fluorescent, while the control electrode on the left, which was not polarized, is not fluorescent. These results are consistent with those for the selective deposition of chitosan[31] and demonstrate

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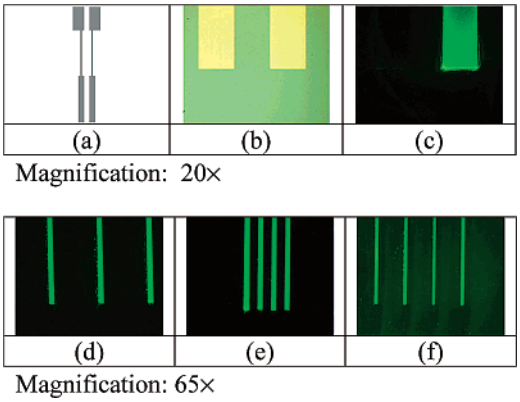

Figure 5. Spatially selective deposition of GFP−chitosan conjugate onto patterned gold surfaces in response to an applied voltage. (a) Schematic showing two, 1 mm wide gold patterns on a silicon oxide surface. The upper regions of the gold patterns are the leads that can be connected to a power source. (b) Photograph from an optical microscope that shows the surface with the two gold patterns before being contacted with the conjugate-containing surface. (c) Photomicrograph from a fluorescence microscope after the surface was contacted with a solution containing the GFP−chitosan conjugate. The right-most gold pattern was polarized to serve as a cathode during deposition and the left-most gold surface was not polarized during deposition. The resolution for deposition was tested with a separate surface containing gold patterns of varying widths. The widths of gold pattern and spacing are as follows: (d) 50 and 500 μm, (e) 50 and 100 μm, and (f) 20 and 300 μm, respectively.

that the GFP−chitosan conjugate deposits onto this patterned surface only in response to an applied voltage (no fluorescence is observed on the unpolarized electrode or on the substrate). A separate control (not shown) was a blend of chitosan and $(His)_6GFP(Tyr)_5$. No GFP fluorescence was observed to deposit on the cathode in this control − thus conjugation was necessary for GFP deposition.

To examine the resolution for deposition of the GFP−chitosan conjugate we immersed a silicon wafer that had gold lines of varying widths and varying spacings[31] into the same conjugate-containing solution and maintained a current density of 2.2 A/m^2. Parts d and e of Figure 5 show that the GFP-conjugate can be deposited onto patterned gold lines that are separated by distances as small as 100 μm. Figure 5f shows that the conjugate can be deposited onto gold patterns as narrow as 20 μm (this is the resolution limit for fabricating the gold patterns using our optical mask). The results in Figure 5 demonstrate that the GFP−chitosan conjugate can be controllably deposited onto a patterned surface. Spatial control of deposition is based on where the gold is patterned on the surface while temporal control of deposition is achieved according to when the voltage is applied. We have not investigated the limits of resolution for deposition but have observed well-resolved lines and spaces that are tens and hundreds of microns wide.

Conclusions

Tyrosinase provides a simple and safe means to conjugate proteins to the polysaccharide chitosan. By oxidizing accessible tyrosyl residues, tyrosinase "activates" proteins for conjugation. The ability to "fuse" tyrosine residues via a fusion tail suggests that tyrosinase-catalyzed conjugation may be possible even for wild-type proteins that lack accessible tyrosine residues. One potential limitation to the tyrosinase-catalyzed conjugation method is selectivity. Although tyrosinase selectively activates only tyrosine residues, it may be necessary to use protecting groups if activation must be limited to a single or specific tyrosine residue(s). More importantly, the reactivity of the quinone may allow reactions to occur between the activated protein and other proteins, and not simply to occur between the activated protein and chitosan. This selectivity concern does not appear to be as serious as might be suspected because there is a preference for the quinones to react with chitosan because of chitosan's relatively low pK_a[8−11] and the abundance of its nucleophilic amines. A second potential limitation to the tyrosinase-initiated conjugation of proteins to chitosan is that some proteins precipitate upon mixing with chitosan. If precipitation does occur, it may be possible to overcome this limitation by performing conjugation reactions under heterogeneous conditions where the protein is conjugated to the surface of a chitosan film or hydrogel (as illustrated in Figure 4).

The polysaccharide chitosan confers three important properties to the GFP−chitosan conjugates. First, the chitosan conjugate has pH-responsive (i.e., smart) properties. Responsive polymers are gaining increasing attention for medical applications since they allow materials to be injected as solutions but become insoluble at the site of delivery.[34−38] Second, tyrosinase-initiated conjugation enables proteins to be covalently tethered to a three-dimensional chitosan gel network. This covalent coupling should permit the easy fabrication of biocatalytically active hydrogel-based membranes, films, and coating for a range of applications.[39,40] Finally, the electrostatic nature of chitosan enables the GFP−chitosan conjugate to be selectively deposited onto micropatterned surfaces in response to an applied voltage. Current methods to assemble proteins onto patterned surfaces include variations of photolithography[41,42] and microcontact printing.[43−49] The combination of biochemical conjugation with selective deposition provides an alternative approach for the assembly of proteins onto patterned surfaces and should be useful for biosensor, microarray, and MEMS applications.

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