Biosensors

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Integration of a Fluorescent Molecular Biosensor into Self-Assembled Protein Nanowires: A Large Sensitivity Enhancement**

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Protein or peptide nanowires can occur either naturally in, for example, amyloid proteins, or be genetically fabricated by gene fusion or phage display technology.[1,2] One of the major advantages of protein nanowires is that they can self-assemble into predetermined patterns with functionalized surfaces. Because of their unique properties, nanowires have great potential in the development of next-generation circuits,[3] new functional materials,[4] nanostructures,[5] ordered liquid crystalline systems,[6] high powered batteries,[7–9] and diagnostic tools.[10] The self-assembly of biological structures has been shown to be advantageous in the study of biosynthesis and nanobiotechnology.

One of the aims of the current study is to construct a fluorescent molecular biosensor. The combination of molecular recognition with a fluorophore, which acts as a signal transducer, enables a fluorescent molecular biosensor to be constructed based on a pH-sensitive fluorescence probe.[11,12] In the search of more effective fluorescent pH indicators, much attention has recently been dedicated to protein-based fluorescent pH indicators.[13–16] The recently developed F64L/S65T/T203Y/L231H green fluorescent protein mutant (E^2GFP) was reported as an effective ratiometric pH indicator for intracellular studies.[15,17] The excitation and emission spectra of E^2GFP show two distinct spectral forms that are interconvertible upon pH change, with a pK value close to 7.0. Excitation of the protein at 458 and 488 nm represents the best choice in terms of signal dynamic range and ratiometric deviation from the thermodynamic pK value.[15] These properties render E^2GFP ideally suited for use as a signaling element in molecular biosensors.

Methyl parathion hydrolase (MPH) is an enzyme that can catalyze the hydrolysis of the pesticide methyl parathion (MP) to *p*-nitrophenol and dimethylthiophosphoric acid[18] (Figure 1a). A pH-stat titration experiment demonstrated

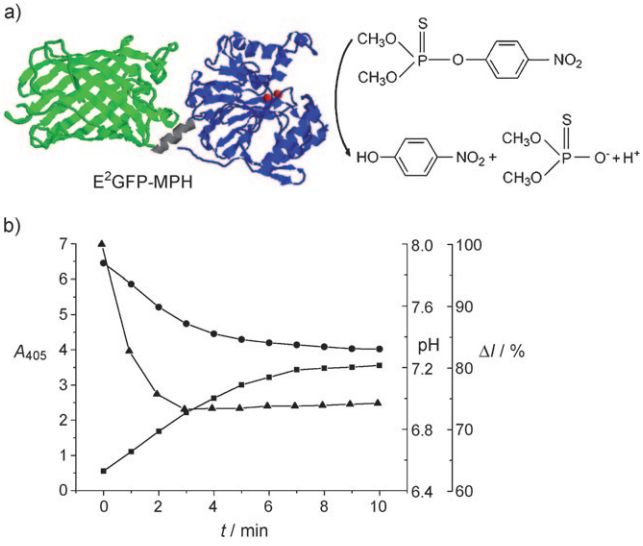

Figure 1. a) Structure of the protein molecular biosensor E^2GFP-MPH (left; green: E^2GFP, blue: methyl parathion hydrolase with two Zn-centers in red, gray: linker peptide), and hydrolysis of methyl parathion (MP) by methyl parathion hydrolase (MPH) into *p*-nitrophenol (*p*NP) and dimethylthiophosphoric acid (right). b) Correlations between the pH change caused by MP hydrolysis and the pH dependency of the fluorescence change of E^2GFP-MPH. The curves represent the production of *p*NP (absorption at 405 nm) (■), bulk pH change (●), and fluorescence intensity change over time (▲) in the same solution, respectively.

that the MP hydrolysis was dependent on the pH change (Figure S2 in the Supporting Information). We have previously demonstrated that MPH is a monomer that has its C and N terminals exposed at its surface.[19,20] These features enable MPH to be genetically fused to another species for building a molecular biosensor without impairing its activity.[10,20]

By linking E^2GFP to MPH, a fluorescent biosensor could be built that allows the detection of MP by sensing the H^+ ions released during the enzymatic reaction (Figure 1a). To verify this concept, the genes coding for E^2GFP and MPH were fused and cloned in *E. coli*, and the resulting fusion protein E^2GFP-MPH was successfully expressed (details about the plasmid construction, protein expression and purification are

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given in the Supporting Information). When using this fusion protein to detect MP, we found that when the amount of enzymatic reaction products increased, both the pH and the residual fluorescence intensity decreased (Figure 1b), thus indicating that the concentration of H^+ ions surrounding E^2GFP could increase because of MP hydrolysis by MPH, and the fusion protein could be used as a biosensor.

The major aim of this study is to integrate E^2GFP-MPH into the protein nanowires to yield a highly sensitive assay through gene fusion and self-assembly of prion proteins. Sup35p, from *Saccharomyces cerevisiae*,[21,22] is one such amyloid protein. The amyloid fibrils formed by the self-assembly of Sup35p provide a convenient model to study both amyloid formation and conformational transformation.[22] Many studies have shown that amyloid fibrils are non-native protein aggregates that contain glutamine/asparagine-rich (Q/N-rich) amino-terminal (N) domains, numerous β sheets, and a highly ordered cross-β core structure.[23,24] It has been observed that Sup35p and its amyloid-forming fragment $Sup35^{1\text{-}61}$ can fuse to exogenous functional molecules without significantly impairing their own respective self-assembly capabilities.[10] Furthermore, Sup35p fibrils have high chemical stability.[25]

Therefore, to improve the sensitivity of the molecular biosensors in this study, the E^2GFP-MPH fusion protein was further attached to $Sup35^{1\text{-}61}$ by gene fusion. A protein nanowire could thus be created by self-assembly through aggregation of the $Sup35^{1\text{-}61}$ subunit. After immersing the purified fusion protein in the assembly buffer (50 mM sodium phosphate at pH 8.0 and 4 °C) for 24 hours, a large number of $Sup35^{1\text{-}61}$-E^2GFP-MPH filaments in the solution were observed by transmission electron microscopy (TEM), as shown in Figure 2b (details about the protein expression and purification are given in the Supporting Information). The diameter of the filaments was approximately (21.5 ± 1.8) nm, and the length was up to 800 nm. These results indicate that the fusion of E^2GFP-MPH molecules to $Sup35^{1\text{-}61}$ did not impair the formation of the nanowires.

The MPH catalytic activity of the nanowires was measured. The enzymatic activity of the $Sup35^{1\text{-}61}$-E^2GFP-MPH nanowires was found to be about three times higher than that of the E^2GFP-MPH sensors, and about 10.4% higher than that of the native free MPH , as shown in Figure 2c. These observations were consistent with those of previous reports,[10,26,27] and our expectations. Investigation of the effect of pH on the fluorescence of the nanowires showed that the nanowires have a similar pH profile to free E^2GFP. The fluorescence intensity of the nanowires also decreased with decreasing pH within the pH range 6.0–8.0 (Figure 2d). These results demonstrate that the constructed nanowires retain the activities of MPH and E^2GFP.

The fluorescence measurement results obtained by using the nanowires are shown in Figure 3c. Surprisingly, it was found that the nanowire biosensor could detect a concentration as low as 1 pmol mL^{-1} (0.26 ng mL^{-1}) of MP, which was about 10000 times lower than the concentration detected by the fluorescent molecular sensor E^2GFP-MPH (Figure 3b). It is apparent that such a dramatic enhancement could not be explained by the higher activity of MPH in the nanowires

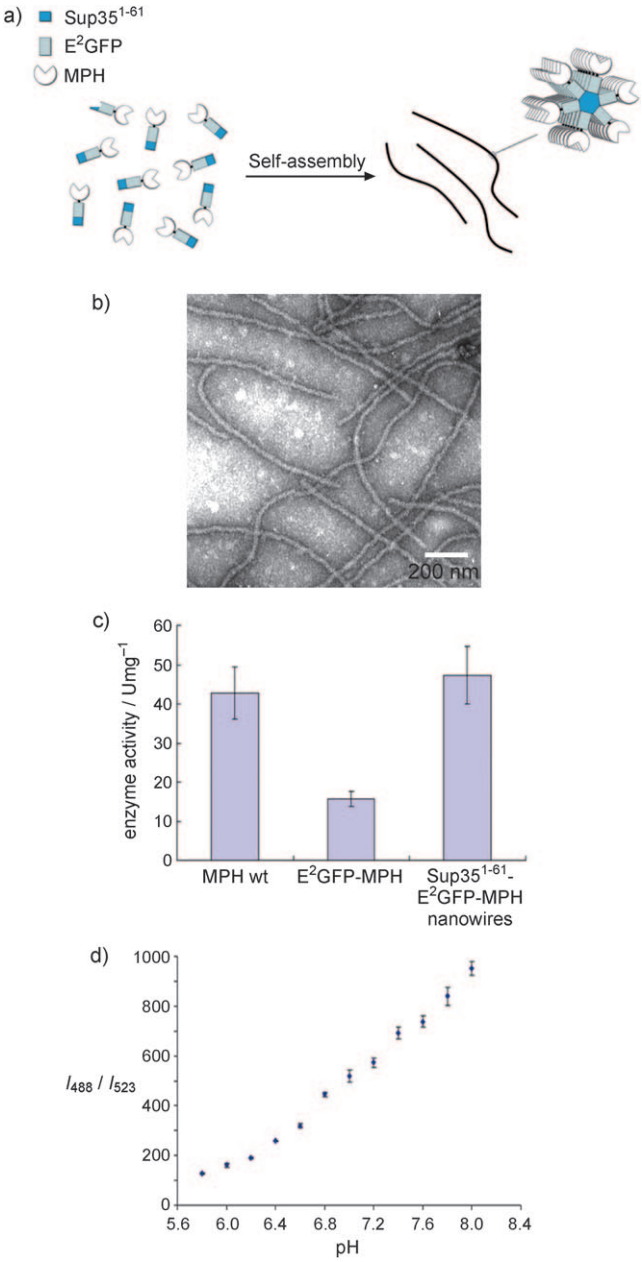

Figure 2. a) Self-assembly of the nanowire fluorescent biosensor $Sup35^{1\text{-}61}$-E^2GFP-MPH. b) TEM micrograph of $Sup35^{1\text{-}61}$-E^2GFP-MPH nanowires. c) Specific enzyme activities of $Sup35^{1\text{-}61}$-E^2GFP-L-MPH nanowires, E^2GFP-MPH and free MPH. d) Dependence of the nanowire fluorescence ratio (I_{488}/I_{523}) on the pH value.

alone. For comparison, the presence of MP was detected by using the solution prepared by simply mixing E^2GFP and MPH molecules (molar ratio 1:1). The quenching of the fluorescence by H^+ ions was largely reduced, thus resulting in a detection sensitivity that was about 100 and 10^7 times less than the fusion fluorescent sensor E^2GFP-MPH and the nanowire sensor, respectively (Figure 3a). Since free MPH showed a higher catalytic activity than the fusion sensor E^2GFP-MPH, the lower sensitivity obtained by using free MPH demonstrates that the molecular assembly, especially

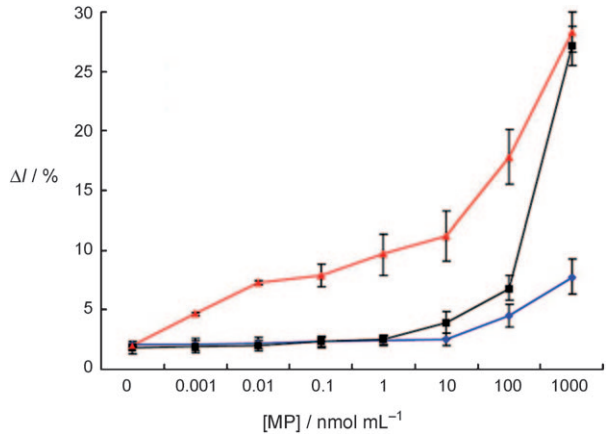

Figure 3. Comparison of the sensitivity of the mixtures of protein E^2GFP and MPH (blue diamonds), E^2GFP-MPH (black squares), and $Sup35^{1-61}$-E^2GFP-MPH nanowires (red triangles) for MP detection. ΔI represents the relative reduction in fluorescence intensity.

the one obtained by $Sup35^{1-61}$-guided self-assembly of the molecular sensor, plays a more important role in enhancing the molecular biosensor sensitivity than the enzyme activity.

The schematic structure of the nanowire fluorescent biosensor shows that $Sup35^{1-61}$ molecules form the backbone core, which is surrounded by E^2GFP-MPH so that the outer layer is formed from MPH (Figure 2a). Theoretically, this supramolecular structure may have two effects. Firstly, the fusion and aggregation of $Sup35^{1-61}$-E^2GFP-MPH creates a rather hydrophobic inner interface between the fusion partners, which is favorable for MP, a hydrophobic substance, to access and accumulate, and thus ensure the high rate enzymatic reaction (Figure 2c). Secondly, the packed nanostructures may limit the diffusion of the acidic product of the enzyme reaction. Thus, the accumulation of H^+ ions around the E^2GFP molecules in the nanowire should be much greater than that around the fusion sensor E^2GFP-MPH without the nanowire structure. As shown in the dynamic fluorescence intensity changes (Figure S3 in the Supporting Information), the time for the nanowire biosensor to reach the minimum of the fluorescence intensity was about 4 minutes, which was 1 minute or so longer than that for the fusion sensor, thus implicating a slowed diffusion of H^+ ions in the nanowires and enhancement of the binding of H^+ ions to E^2GFP. The microenvironment of the highly packed protein nanostructure would therefore cause the equilibriums of both the MPH catalysis and the H^+ ion binding reaction to shift towards the forward direction, which would promote H^+ ions to combine with the E^2GFP in the nanowires, thus resulting in a greatly enhanced biosensing activity. Conformational changes of the fusion partners might be another reason for such an enhancement, but this hypothesis needs to be validated.

In conclusion, a new type of fluorescent molecular biosensor for the detection of the pesticide MP was developed by construction of the E^2GFP-MPH fusion protein, in which the fluorescence intensity of E^2GFP is regulated by its genetic fusion partner MPH through pH change caused by an enzyme-catalyzed reaction. The response sensitivity of the molecular biosensor could be enhanced over 10000 fold by the integration of the E^2GFP-MPH fusion protein biosensor structure into protein nanowires through self-assembly of $Sup35^{1-61}$. Because there are various kinds of amyloid proteins that retain the aggregation function after being fused to different functional proteins,[10,28–30] the proposed method may serve as a model to develop versatile sensitive molecular biosensor systems.

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