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ARTICLE *in* NANO LETTERS · JULY 2007

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NANO LETTERS

2007
Vol. 7, No. 6
1575−1579

SP1 as a Novel Scaffold Building Block for Self-Assembly Nanofabrication of Submicron Enzymatic Structures

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Received February 25, 2007; Revised Manuscript Received May 1, 2007

ABSTRACT

In this study, SP1, a ring-shaped highly stable homododecamer protein complex was utilized for the self-assembly of multiple domains in a predefined manner. Glucose oxidase (GOx) was fused in-frame to SP1 and expressed in *Escherichia coli*. Complexes where GOx encircled SP1 dodecamer were observed, and moreover, the enzymatic monomers self-assembled into active multienzyme nanotube particles containing hundreds of GOx molecules per tube. This work demonstrates the value of SP1 as a self-assembly scaffold.

In this so-called Age of Designed Materials, new knowledge is required to build advanced materials. Moreover, recent progress in molecular biology in general and protein engineering in particular has enabled us to enter the molecular realm, designing nanoscale structures and engineering novel materials.[1,2] Molecular self-assembly systems represent a significant advance in the molecular engineering of simple molecular building blocks useful for a wide range of applications. Biomolecules in general are capable of self-assembling into a wide variety of structures with nanoscale architecture. Proteins in particular can form intricate structures that can be readily manipulated and functionalized because their synthesis is genetically directed. By combining nature's molecular tools with synthetic nanoscale constructs or pre-engineered biological structures, one can obtain the self-assembly of a desired nanostructure/material.[3]

Realization of the full potential of nanotechnological systems, however, has so far been limited by difficulties in their synthesis and subsequent assembly into useful functional structures and devices.[4] The use of biological entities, such as proteins, for that matter, sounds both tempting and promising but introduces another challenge in terms of system instability to even mild conditions. A protein building block or a molecular scaffold has to be able to withstand extreme environmental conditions before it can be exploited for that purpose. Today, despite all the promise of science and technology at the nanoscale, the control of nanostructures and ordered assemblies of materials in two and three dimensions still remains a challenge.[5,6]

Here, we present a novel molecular scaffold based on the boiling-stable protein SP1. This recently discovered and characterized protein[7−10] has a fascinating ringlike structure that enables it to be a pivotal scaffold for self-assembled nanostructures. SP1 is a homo-oligomeric 148.8 kDa protein complex, first isolated from aspen (*Populus tremula*) plants.[7] The complex is composed of 12 subunits of 12.4 kDa that are tightly bound to each other, thus forming a homo-dodecamer. Electron microscopy studies have indicated that SP1 rings tend to stack into nanotubes.[10] SP1 is resistant to extreme conditions such as diverse pHs, high temperatures (T_m of 107° C),[9,10] organic solvents, and various proteases.

To demonstrate SP1's ability to assemble large complexes, and display catalytic modules, we initially designed the basic scaffold unit in silico, taking into account the size of the counterpart and the estimated linker length that would enable full operational flexibility for both the scaffold and the displayed partner. Glucose oxidase (GOx), the assembly counterpart, is a relatively large homodimeric glycoprotein (monomer molecular mass is 80 kDa) that catalyzes the oxidation of β-D-glucose by molecular oxygen to δ-gluconolactone and hydrogen peroxide. GOx is of considerable commercial importance[11] and is extensively applied in food processing, in the production of gluconic acid,[12] and in the

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10.1021/nl070450q CCC: $37.00
Published on Web 05/26/2007

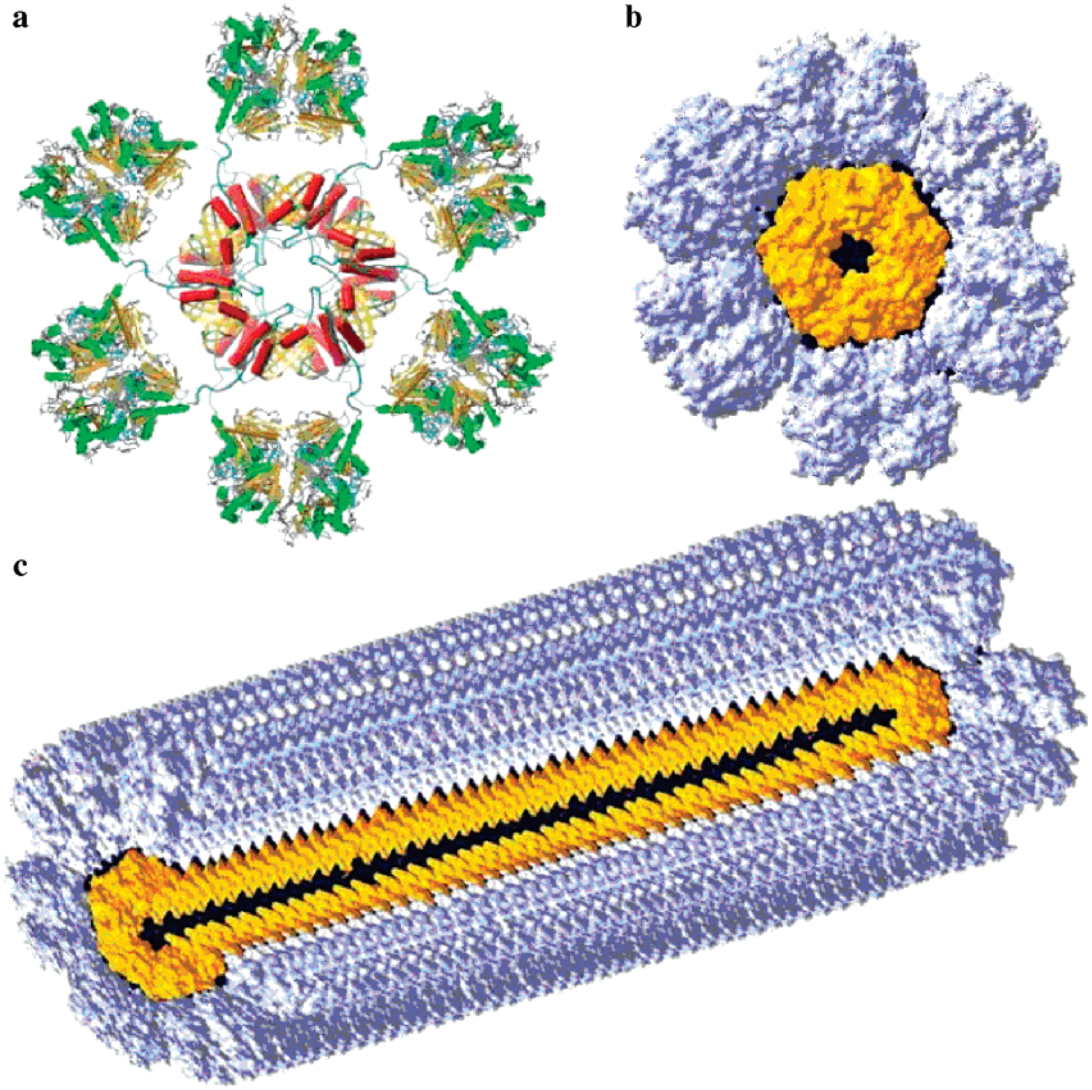

Figure 1. An illustration of GOx-L-SP1 complex. (a,b) SP1 dodecamer in the center and six GOx dimers encircling it. (c) Dodecamers clinging together to form an enzyme nanotube particle. (the SP1 and the GOx files can be found in the PDB database 1TR0 and 1GPE respectively).

quantitative determination of D-glucose in fermentation processes and medical diagnostics.[13,14] However, perhaps the most interesting application for GOx is its utilization as a pivotal enzyme in biofuel cells.[15−17]

We used a genetic-engineering approach and fused *Aspergillus niger* GOx along with a 23-amino-acid native peptide linker taken from *A. niger* glucoamylase to SP1. The linker was chosen to minimize steric hindrance of the two linked proteins. Because both proteins' 3D structures are known,[8,9,18] we were able to model the assembly of the new fusion protein, GOx-L-SP1 (Figure 1a).

GOx-L-SP1 was expressed in *E. coli* as a nonsoluble protein and accumulated in inclusion bodies. The mass of the fused protein was approximately 80 kDa, which correlates with the calculated MW of the fusion protein (Figure 2). The recombinant GOx (rGOx) appeared as expected as a 60 kDa nonsoluble protein, and both GOx-L-SP1 and rGOx were refolded in order to gain GOx activity.

Gel-filtration FPLC analysis of refolded GOx-L-SP1 indicated that large protein complexes (over 669 kDa) were

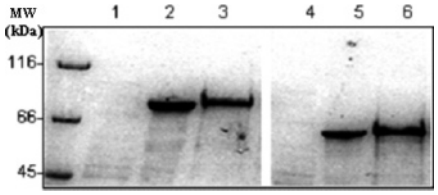

Figure 2. SDS-PAGE analysis of GOx-L-SP1 and GOx protein expression in *E. coli*. Lane 1, GOx-L-SP1 bacterial soluble fraction; lane 2, GOx-L-SP1 bacterial insoluble fraction; lane 3, GOx-L-SP1 refolded protein; lane 4, GOx bacterial soluble fraction; lane 5, GOx bacterial insoluble fraction; lane 6, GOx refolded protein.

eluted in the void volume of the column (Figure 3). The calculated molecular mass of the GOx-L-SP1 complex is about 1 mega Dalton, and it was therefore expected that only monomers or dimers would be resolved in the column. Fractions of protein samples were collected at different elution intervals, and specific activity of GOx was determined (Table 1). Clearly the high-MW complex of GOx-L-SP1 possessed GOx activity, although its specific activity was lower than that of rGOx. The half-life ($T_{1/2}$ at 65 °C) of GOx-

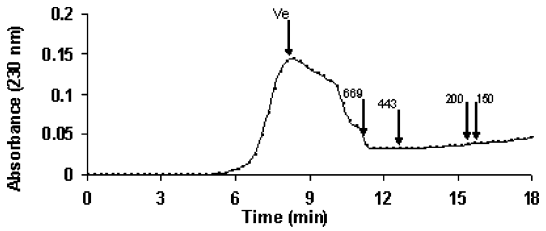

Figure 3. Gel-filtration chromatography of GOx-L-SP1. Ve: GOx-L-SP1 elution time (8.24 min), also determined by GOx activity and SDS-PAGE analysis. Molecular weight markers, indicated by arrows, are in kDa.

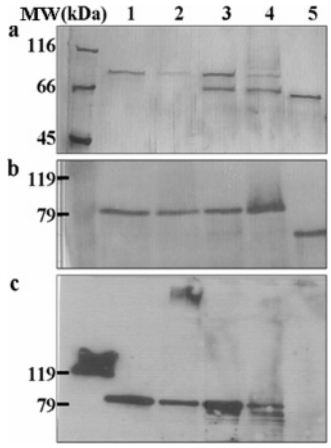

Figure 4. SDS-PAGE and Western Blot analyses of GOx-L-SP1 and GOx gel-filtration fractions. (a) Silver staining of: lanes 1−2, GOx-L-SP1 multimer (boiled and nonboiled, respectively); lanes 3−4, GOx-L-SP1 dimer (boiled and nonboiled, respectively); lane 5, GOx dimer. (b) Western Blot analysis of gel in (a) with anti-GOx antibodies. (c) Western Blot analysis of gel in (a) with anti-SP1 antibodies.

Table 1. Specific Activity of Glucose Oxidase (GOx)

sample	activity (u/mg protein)	$T_{1/2}$[a]
A. niger native GOx	278	0.9 ± 0.1
rGOx[b] (refolded before gel filtration)	27	3.8 ± 0.45
rGOx dimmer	110	
rGOx-L-SP1 (refolded before gel filtration)	13	
rGOx-L-SP1 dimer	60	
rGOx-L-SP1 multimer	9	8 ± 0.55

[a] $T_{1/2}$: half-life (min) at 65 °C. [b] rGOx: recombinant glucose oxidase.

L-SP1 was 2 and 10 times higher than that of rGOx and native GOx, respectively. SDS-PAGE analysis of the gel filtration eluted fractions (Figure 4a), indicated that the high-MW complex was indeed the GOx-L-SP1 fusion protein appearing at approximately 80 kDa as expected. When not boiled, only a small fraction of those complexes disassembled and entered the gel. Fractions collected at a volume corresponding to a dimmer MW revealed the protein dimer accompanied with some degradation products. Western Blot analyses using anti-GOx or anti-SP1 antibodies were performed on the gel-filtration FPLC fractions. The two antibodies detected the same GOx-L-SP1 protein, at the expected MW of 80 kDa, both as a high-MW complex

Table 2. Dynamic Light Scattering (DLS) Measurements of SP1 and GOx-L-SP1

sample	particle diameter (nm) [% int[a]]
SP1	10 ± 1.7
GOx-L-SP1	200 ± 20 [76]
	50 [24]
GOx-L-SP1 with 0.1 M Gu-HCl	65 ± 12
GOx-L-SP1 with 1 M Gu-HCl	65 ± 12
GOx-L-SP1 with 4 M Gu-HCl	60 ± 6

[a] Intensity. Gu: guanidine.

(eluted in the void volume) and as a dimer. When samples were not boiled prior to electrophoresis, anti-SP1 antibody detected a high-MW complex accumulated in the stacking gel, indicating that some complexes were still stable under high SDS concentrations (Figure 4b,c). The dimmer degradation products mentioned above are recognized only by the anti SP1 antibodies, meaning that this minor proteolysis occurs near the GOx enzyme N-terminal. This correlates with the position of the enzyme as it was fused through its C-terminal and should be presented on the SP1 scaffold (see Figure 1). It is also consistent with SP1's known protease resistance.

Dynamic light scattering (DLS) analysis of the GOx-L-SP1 multimers revealed two distinct particle types. The first type, approximately 24% of the particle population, had a 50 nm diameter. These particles represented the GOx-L-SP1 dodecamer complexes in which one SP1 dodecamer holds together 12 monomers of GOx (Figure 1a,b). The second type of particle was much larger, with a diameter of 200 nm (Table 2), representing multimers assembled from the former group and creating a multienzyme nanotube particle (Figure 1c). Moreover, incubation of the high-MW complex with guanidine-HCl resulted in disassembly of the nanotube enzyme particle into smaller complexes (Table 2).

Transmission electron microscopy (TEM) further substantiated our observations. As expected, TEM images demonstrated the assembly of GOx molecules around the circumference of the SP1 dodecamer scaffold, creating the pre-engineered structure displayed in Figure 1a,b (Figure 5). One SP1 dodecamer holds 12 GOx monomers (or six dimers, Figure 5a,b), and the large multimer assemblies confirmed that GOx-L-SP1 disclike complexes stack to form multienzyme nanotube particles (Figure 5c,d,e). Deviations observed at the edges of the nanotube particle were suspected to occur due to GOx intermolecular interaction.

Displays of exogenous biologically active molecules on external surfaces of large biological complex structures have been previously demonstrated. Most commonly, viruses are engineered for the presentation of peptide epitopes, proteins, and antibodies as part of their coat protein.[19−24] The use of protein scaffolds usually results in 2D assemblies.[3,25] Here we demonstrate a single protein that has unique stability properties as a 3D molecular scaffold for multimer assemblies. This approach is a simplified bottom-up approach for the self-assembly nanofabrication of complex biological structures displaying catalytic modules.

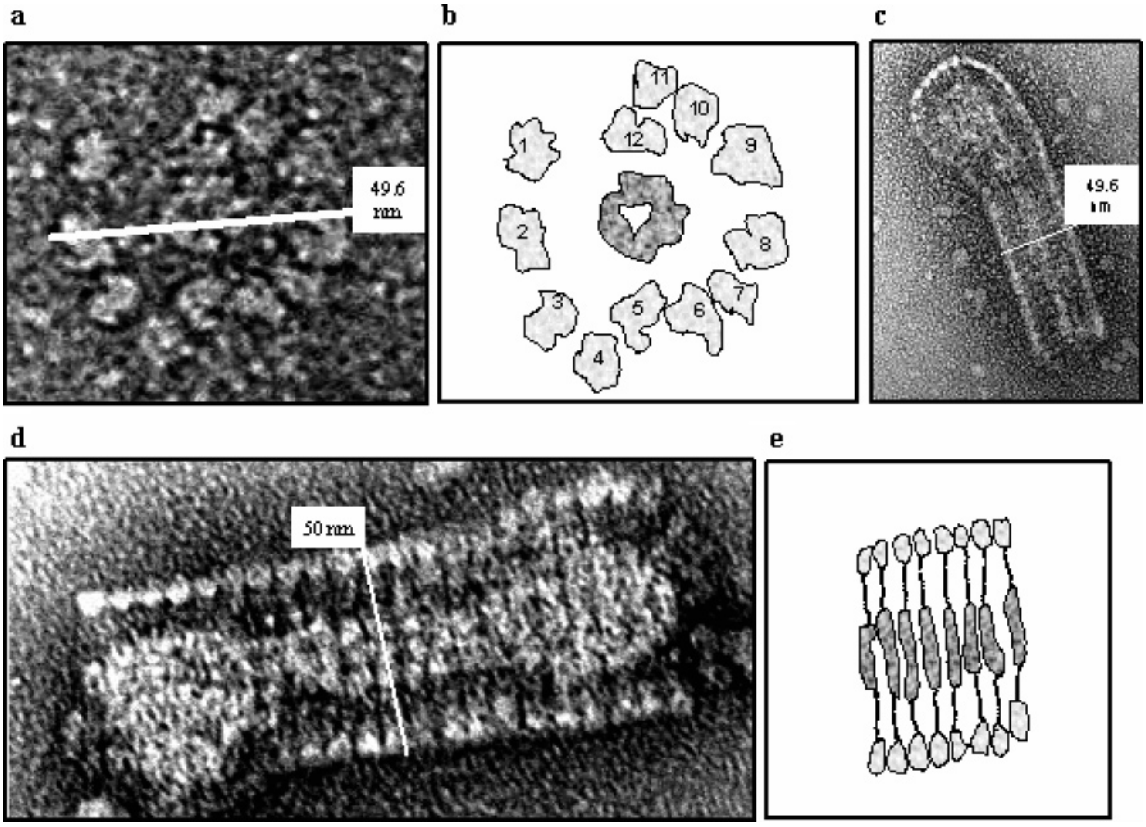

Figure 5. Transmission electron microscopy imaging of GOx-L-SP1. (a) GOx-L-SP1 complex, 12 GOx monomers around the SP1 dodecamer (49 nm diameter). (b) Graphical representation of the complex. (c,d) Multimers: dozens of dodecamers clinging together to form an enzyme nanotube particle. (e) Graphical representation of the enzyme nanotube particle.

The advantage of using SP1 as a molecular scaffold is twofold: its modular design and its inherent stability to varied conditions. Here, the added virtue of the new complex is a 2-fold higher inactivation time at high temperature (65 °C) and a remarkable 10-fold higher $T_{1/2}$ relative to native GOx. This can be attributed to the inherent stabilizing nature of SP1 combined with the close proximity of the two fused proteins. As reported, the stability of immobilized enzymes is dictated by factors such as the number of bonds between the enzyme and the carrier, the nature of the bond (covalent, noncovalent, etc.), and degree of confinement.[26] A similar approach for enzyme stabilization involves cross-linked enzyme crystals (CLEC). These are produced by stepwise crystallization and molecular cross-linking to preserve their crystalline structure. Uniform-size crystals can be obtained in the range of 1−100 μm; CLEC are extremely stable, not only with respect to temperature but also in the presence of other inactivating agents such as organic solvents. Stabilization is a consequence of intense polar and hydrophobic interactions, and molecular rigidity is responsible for thermal stability.[27] A similar approach uses chemical cross-linkers to produce cross-linked enzyme aggregates (CLEA).[28]

Of additional importance is the controlled stoichiometric ratio of enzymes or any other nanoparticles assembled through SP1. We demonstrate a 1:6 scaffold-to-enzyme stoichiometric ratio (1:12 if the enzyme is a monomer), and overall, 90% (w/w) of the total particle consists of active enzyme. In ordinary enzyme immobilization, less than 10% of the total mass is immobilized enzyme and the rest is attributed to the matrix; therefore, the enzyme is a dense monolayer at best. Immobilized enzymes with a higher protein-to-matrix ratio are clearly advantageous. A typical GOx-L-SP1 nanotube contains hundreds of enzymes per tube, and its nanometer dimensions retain the advantages of single molecular enzymes that remain in solution and favor substrate and product mass-transfer. Furthermore, the submicron enzymatic complexes are large enough to be removed by standard filtration. Considering all of its unique characteristics, the use of this approach is appealing, especially in view of its potential application in reactor-type production of biomolecules.[26]

We conclude that SP1, a novel protein with unique characteristics, can be exploited as a pivotal protein in molecular self-assembly. By exploiting the protein's inherent virtues, we can place and spatially orient specific nanoscale molecules and particles. These self-assembled objects could be metal particles, peptides, protein domains, or even whole enzymes creating a nanoplatform for chemical reactions. Future directions involve handling and controlling the nanoparticles assembly and disassembly, thus generating a bottom-up nanoconstruction protocol. Furthermore, we intend to create heterocomplexes with the SP1 scaffold by using other fusion candidates and domain exchange within the SP1 dodecamer.[8] We believe that SP1 will enable us to bridge and combine the "soft" organic biological sciences with the durable inorganic materials sciences.

Acknowledgment. We thank Prof. Ehud Gazit from Tel Aviv University for assistance with the dynamic light scattering analysis.

Supporting Information Available: Materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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NL070450Q