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Signal amplification by a self-assembled biosensor system designed on the principle of dockerin–cohesin interactions in a cellulosome complex

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To construct a self-assembled biosensor with signal amplification, a cellulosome system, comprising type I and type II dockerin–cohesin interactions with different specificities, from the anaerobic *Clostridia* bacterium was applied. The self-assembled biosensor was highly sensitive and achieved 128.1-fold increase in detection levels compared to the control.

Genomic and proteomic researchers have proposed various types of new biosensors to diagnose and monitor cancers and other medical conditions with greatly improved sensitivity and selectivity.[1] To detect multiple specific molecules in a complex mixture, such as serum, a rapid and sensitive detection method is necessary. An important goal in biosensor development is the production of simple and reliable nanoscale sensors for the continuous monitoring of target species in a complex mixture.[2] Thus, much attention has been focused on signal amplification for sensitive detection. Protein-based recognition, such as antigen–antibody or protein domain interactions, is one of the most promising strategies for signal simplification.[3] Immunoassays using antigen–antibody interactions typically use reporters, such as radioactive tracers or luminescent probes, to transduce immune interactions into a quantifiable change.[4] Among these methods, fluorescence signals can typically be several orders of magnitude more sensitive than colorimetric signals.[5] We developed a self-assembled fluorescence biosensor for signal amplification through the deposition of fluorophores. Our methods should provide a simple, chip-based detector with high sensitivity.

The principle of the self-assembled biosensor with signal amplification is illustrated in Fig. 1. The dockerin–cohesin interaction of the cellulosome was used to construct the designed self-assembled sensor system. Anaerobic bacteria, such as *Clostridium cellulovorans* and *Clostridium thermocellum*, produce cellulosomes as complexed enzyme systems that involve the nonenzymatic scaffolding protein CbpA binding of a variety of cellulolytic subunits *via* dockerin–cohesin interactions.[6] The dockerin–cohesin interaction between enzymes with duplicated sequences (dockerin modules) and a non-catalytic scaffolding protein with repeated sequences (cohesin modules) is a high-affinity protein–protein interaction.[7] Also, the dockerin module recognizes the cohesin module with the K_D range from 1.9×10^{-9} to 2.4×10^{-10}. This property is high-affinity interaction ($>10^9$ M^{-1}) in comparison with other protein interactions. Cohesin and dockerin modules have several types based on sequence homology, and interactions between these modules have been classified into type I and type II.[8] Type I involves an interaction between the cohesin module of the scaffolding protein and the dockerin module of a cellulosomal catalytic component and type II dockerin–cohesin interactions are the counterparts of type I dockerin–cohesin interactions.[9] Type I–type II cross-interactions have been not reported, meaning that the dockerin–cohesin interaction is specific to the type. Additionally, dockerin–cohesin interactions from different *Clostridia* strains have interspecies specificity.[10] Although this classification is based on sequence homology, recognition among the same types of modules with similar homology is not necessarily implied, which means that recognition for interactions is affected by subtle differences in the structures of cohesins and dockerins, despite having highly conserved sequences with significant homology.[11] Thus, the cellulosome system comprising type I and type II dockerin–cohesin interactions with different specificities from the *Clostridia* anaerobic bacterium was applied to construct a self-assembled biosensor with high sensitivity.[12] For biosensor applications, a scaffolding protein and dockerin–cohesin interaction based signal amplification molecular unit was developed.

To design the dockerin–cohesin-interaction based self-assembled biosensor system, we constructed a self-assembled unit (SA unit) containing a type I cohesin module from *C. cellulovorans* that was fused to a type II dockerin module from *C. thermocellum* at the N-terminus and two type II cohesin modules from *C. thermocellum* at the C-terminus. Additionally,

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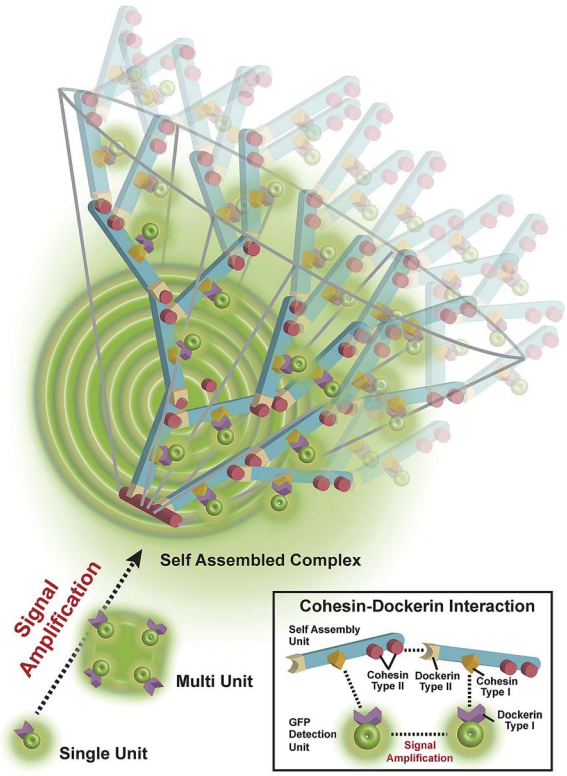

Fig. 1 Illustration of the self-assembled fluorescence biosensor based on a dockerin–cohesin interaction of the cellulosome. The self-assembled unit (SA unit) contained a type I cohesin module fused to a type II dockerin module and type II cohesin module at the N-terminus and C-terminus, respectively. The GFP detection unit (GFP Doc unit) was generated based on the type I dockerin module with a fluorescence signal. SA units were designed to connect the GFP Doc detection unit and SA unit *via* a type I dockerin–cohesin interaction derived from *C. cellulovorans* and a type II dockerin–cohesin interaction based on the *C. thermocellum* system, respectively. This self-assembled biosensor system was assembled into a complex and enhanced the fluorescence intensity due to a signal amplification effect.

to develop optical biosensors, a green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was used as an output signal. The GFP detection unit (GFP Doc unit) was generated based on the type I dockerin module from *C. cellulovorans* to construct a fluorescence biosensor based on the cellulosome system. SA units were designed to connect the GFP Doc detection unit *via* a type I dockerin–cohesin interaction derived from the *C. cellulovorans* system and SA units *via* type II dockerin–cohesin interactions based on the *C. thermocellum* system. The genetic fusion of each domain in the SA unit to the GFP Doc unit was performed using a multistep PCR strategy using overlapping primers.[13] To fuse the different fragments, each of the overlapping primers possessed a 10-nucleotide-long 5′ extension that was complementary to the end of the adjacent fragment. The final PCR products of the SA and GFP Doc units were separated and ligated into the pET22b (+) *Escherichia coli* vector (Novagen, San Diego, CA, USA), resulting in the pET22b (+) SA unit and pET22b (+) GFP Doc unit plasmids, respectively. After construction of the plasmids, DH5α and BL21 (DE3) were used as hosts for recombinant DNA manipulation and gene expression, respectively.

The expression of the induced SA unit protein in the recombinant *E. coli* strain (SA unit) harboring the pET22b (+) SA unit plasmid was confirmed by SDS-PAGE and western blotting (Fig. 2A). The calculated molecular mass of the SA unit protein was 61.1 kDa (29.4 kDa dockerin I + 32.2 kDa residues for the type II dockerin, type II cohesin and His-tag). A single, 61 kDa band was observed on the SDS-PAGE gels that corresponded with the molecular size of the SA unit (61.1 kDa) predicted by the nucleotide sequence. The nucleotide sequence of the GFP Doc unit coding region is 966 bp, and this encodes a polypeptide of 328 amino acids. The pET22b (+) GFP Doc unit plasmid containing the recombinant gene encoding the GFP-Doc unit with a C-terminal His-tag was transformed into *E. coli* BL21 (DE3), and the resulting recombinant strain was named *E. coli* (pET22b (+) GFP-Doc unit). After the expression and purification of the GFP Doc unit protein from *E. coli* (pET22b (+) GFP-Doc unit) as mentioned above, a homogeneous band was observed by electrophoretic analysis (Fig. 2B). The purified GFP Doc protein showed fluorescence intensity at 488 nm and had an apparent molecular mass of 38 kDa, which was in good

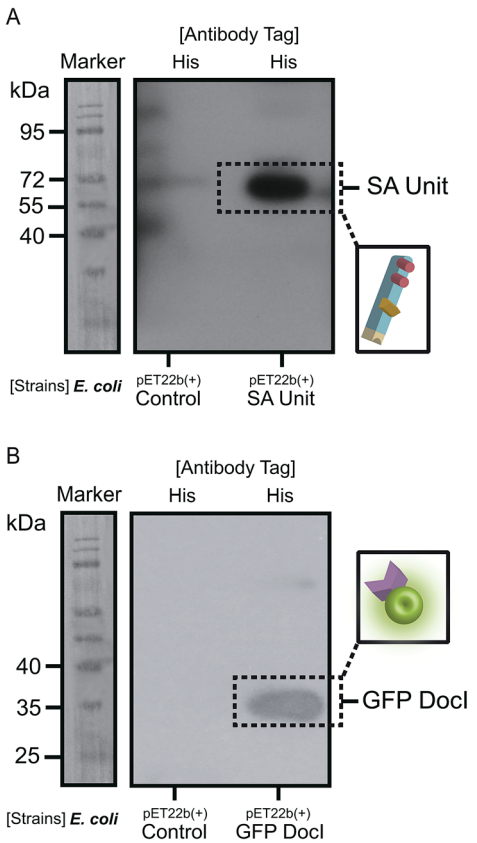

Fig. 2 Electrophoretic analysis of the purified, self-assembled unit (SA unit) and GFP detection unit (GFP-Doc unit) by SDS-PAGE and western blotting. Analysis of (A) the self-assembled unit in *E. coli* (pET22b (+) SA unit) and (B) GFP detection unit in *E. coli* (pET22b (+) GFP-Doc unit) visualized by western blotting with luminal reagents and a rabbit anti-6x-His antibody and goat anti-rabbit IgG–HRP antibody as primary and secondary antibodies, respectively.

agreement with the calculated molecular mass of 34.3 kDa (26.8 kDa GFP + 7.5 kDa residues for the linker peptide, type I dockerin and His-tag). These results indicated that each unit of the self-assembled biosensor was expressed in the non-degraded, active form and that the fusion of the different domains at the C- or the N-terminus did not interfere with the correct folding of the proteins.

After expressing the individual subunits to construct the biosensor and signal amplification, the formation of the self-assembled biosensor was confirmed by native PAGE and western blotting, as previously described.[14] To confirm the consistent construction of the biosensor system, various amounts of SA units were assembled and then analyzed. A new band appeared after the formation of the self-assembled complexes in all samples (Fig. 3A). However, the assembled samples comprising over 20 ng of SA unit showed the remained non-complexed SA unit band under the self-assembled complex band. These results provided more concrete evidence of complex formation, and the new band confirmed that the self-assembled unit was correctly assembled into a complex system. Also, complex formation was limited over the maximum capability. Additionally, when compared to the individual GFP Doc protein as a single detection unit, the self-assembled biosensor caused a 128.1-fold increase in fluorescence intensity at the maximum capability (Fig. 3B). Over the maximum capability, the signal amplification effect was not shown. These results indicated that the SA and GFP Doc units were assembled into a complex form, and that the dockerin-fused GFP in the designed, self-assembled complex had enhanced fluorescence intensity due to a signal amplification effect. The signal amplification clearly suggested that the SA and GFP Doc units were all correctly folded and that their high-affinity interactions were sufficient to direct the assembly of the self-assembled biosensor. Experimental results by other researchers showed that a maximum, single-molecule fluorescence enhancement results in a 40-fold increase.[15] Here, we report large enhancements of fluorescence by introducing a self-assembled biosensor system without any additional equipment.

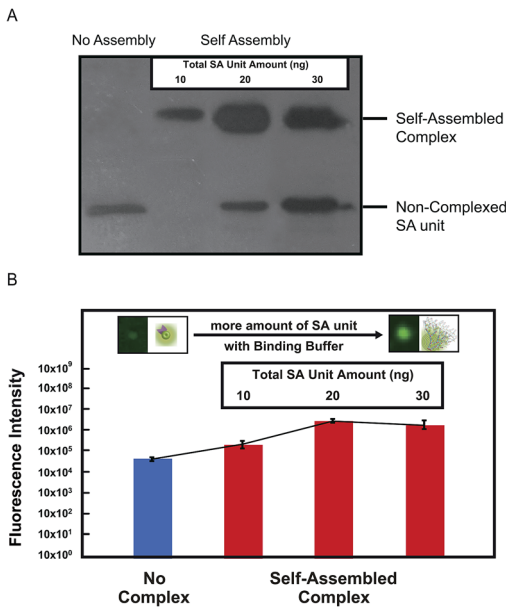

Fig. 3 The construction of the self-assembled biosensor is confirmed by two methods: native-PAGE and fluorescence signal analysis. (A) Native PAGE and western blotting of the assembled complexes show a shifted band, indicating the formation of the self-assembled biosensor. (B) The fluorescence intensity of the non-complexed GFP detection unit and self-assembled complexes is compared with the same detection spot. The differences between the non-complexed unit and self-assembled complex reflect the signal amplification through an integrated detection system.

Conclusions

In conclusion, we present a protein complex based on dockerin–cohesin interactions as a signal-amplifiable reporter with high sensitivity and selectivity. The designed, self-assembled biosensor system successfully increases the upload of signal tags by amplifying the signal readout with high sensitivity. These results suggest that the designed, fluorescence-signal-amplification system may be a promising strategy for utilizing an enzyme and a whole-cell based biosensor to analyze small amounts of materials. For example, because this biosensor system was constructed by protein-based composition, simple fusion between small molecular binding modules such as glucose binding module or calcium ion binding module with one of the SA units will lead to the application for quantitative analysis of glucose or calcium ions. This amplification was accomplished by designing sensor components to amplify output signals through an integrated detection system. The use of protein complexes for fluorescence signal amplification is advantageous because they are easily self-assembled by only interactions between protein modules, without any additional equipment. Due to the self-assembly of the protein complex, a cascaded signal amplification method has been developed, indicating the wide applicability of the new paradigm for protein assembly and useful biosensor development.

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