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An Immunoassay Utilizing DNA-Coated Cage Protein As a Signal Generator

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Enzyme-linked immunosorbent assay (ELISA) is a conventional immunoassay technique based on a specific interaction between antigens and antibodies.[1,2] This method has been regarded as a gold standard in detection of disease-related biomarkers in clinical area and has been used for the detection and quantitation of various biomolecules such as peptides, proteins, antibodies, and hormones.[3-6] Despite these wide applications, ELISA is often limited because of its relatively low detection sensitivity. Recently, we have developed a microwell plate-based immunoassay, called oligonucleotide-linked immunosorbent assay (OLISA).[7] OLISA utilizes DNA oligonucleotides conjugated on detection antibodies (dAbs) that mediates the cleavage of fluorogenic RNA probes by RNase H for generation of the fluorescent detection signal (Fig. 1, left). Considering that the DNA strand is the template for the signal generation, the increased number of DNA strands per dAb in the DNA-dAb conjugate would improve the detection sensitivity. In that regard, nanomaterials having multiple reactive sites in a restricted surface area are potentially useful cross-linkers for connecting multiple DNA strands to a dAb, leading to improved detection sensitivity in immunoassays.[8]

Cage proteins are self-assembled and nanostructured spheres that are biological in origin and 10-100 nm in diameter.[9] Since the proteins display nano-sized, monodisperse structures, they have recently been utilized as functional nanomaterials for various aims, including inorganic nanoparticle synthesis,[10] medical imaging,[11-13] and drug delivery.[14] In addition to their utilities as a building block in nanoconstructs, the multimeric self-assembled proteins have the potential to be used for signal amplifier in immunoassays. As an effort to utilize cage proteins in development of an immunoassay, multiple DNA strands were loaded on a cage protein, heat shock protein 16.5 (HSP16.5) by utilizing multiple functional groups on the surface of the protein in this study (Fig. 1, right). This DNA-coated protein was further conjugated with a dAb and used in the OLISA assay for detection of a liver cancer marker, α-feto-protein (AFP). The detection sensitivity of our cage protein-based OLISA (CP-OLISA) was examined carefully in comparison with OLISA and ELISA. Further, the accuracy and reproducibility of the assay were also evaluated by detecting the target antigen in human sera.

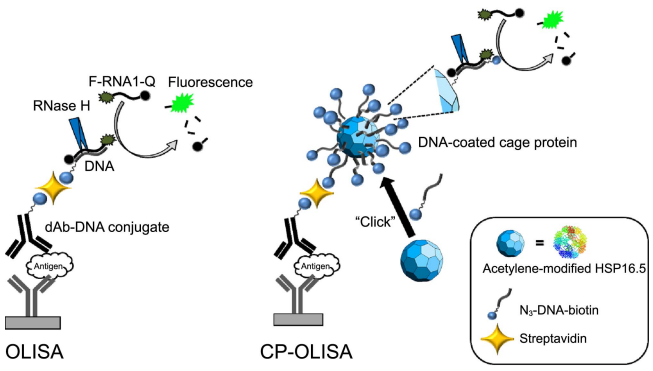

Figure 1. Schematic presentation of OLISA (left) and CP-OLISA (right).

HSP16.5 from *Methanococcus jannaschii* is known to construct a 12 nm-diameter cage with octahedral symmetry by self-assembly of 24 monomers.[15] For chemical modifications, a cysteine residue was added at the C-terminal of the monomer protein, which would be displayed at the outer surface after assembly of the nanocage construct.[16] The recombinant HSP16.5 was expressed in *Escherichia coli* (*E. coli*) and purified by fast protein liquid chromatography (FPLC) as previously described.[15] The cysteine residues on the surface of the cage protein were conjugated with biotinylated DNA using the click chemistry (Fig. 1). For preparation of azide-functionalized DNA, the DNA containing iodo group at the 5'-end and biotin at the 3'-end, respectively, was synthesized by following the standard protocol for oligonucleotide synthesis and was subject to reaction with sodium azide to transform the iodo group into azide group. The acetylene moiety was introduced on the surface of the cage protein by reacting the thiol group of cysteine with a commercially available, maleimide-modified acetylene. The DNA-coated multimeric protein could be obtained from

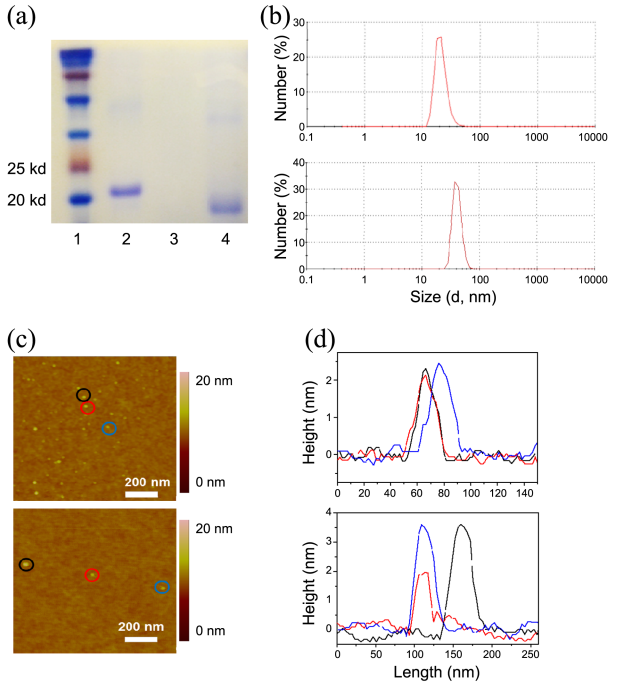

Figure 2. Characterization of DNA-coated cage protein. (a) SDS-PAGE analysis of the conjugated product. Lane 1: size markers, lane 2: DNA-coated cage protein, lane 3: DNA alone, and lane 4: cage protein alone. (b) DLS data of DNA-coated protein (bottom) and protein alone (top). (c) The AFM image of DNA-coated protein (bottom) and protein alone (top). (d) Heights of particles indicated by circles in Figure 2(c).

click reaction of the acetylene-modified cage protein with an excess of N_3-DNA-biotin in the presence of Cu^{2+}/THPTA (tris(3-hydroxypropyltriazolylmethyl)amine). Unreacted DNA strands were removed by using a size exclusion filter with 10 kDa of MWCO (molecular weight cut-off). The conjugation product was analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Figure 2(a), the DNA-protein conjugate (lane 2) was moving more slowly than the unreacted protein monomer (lane 4), suggesting that the conjugate was successfully prepared.

After preparation of the protein-DNA conjugate, the hydrodynamic size of the DNA-coated cage protein was estimated as *ca.* 30 nm in dynamic light scattering (DLS) (Fig. 2(b), bottom). The increase in the hydrodynamic size of the construct compared with that of the protein itself (*ca.* 10 nm) is likely from the DNA conjugation on the protein (Fig. 2(b), top). The conjugate was further characterized on atomic force microscopy (AFM) by which uniformly constructed protein assemblies could be observed (Fig. 2(c), bottom). The enlarged widths (50-60 nm) and shortened heights (3.5 nm) of conjugate particles determined in the AFM images compared to the size measured in DLS might be due to the dried state of the particles during AFM experiments (Fig. 2(d) bottom). The conjugates, however, were slightly larger than the unmodified protein particles, demonstrating the contribution of DNA to the size of the DNA-coated protein (Fig. 2(c) and (d), top).

The cage protein interacts with the detection antibody in

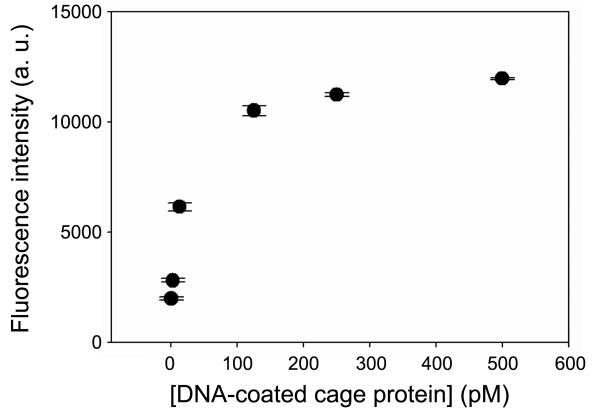

Figure 3. Optimization of the DNA-coated cage protein concentration for immunoassays.

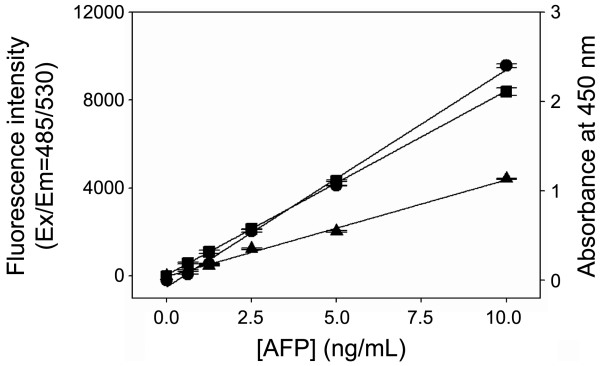

Figure 4. Immunoassays of AFP using ELISA (●), OLISA (▲), and CP-OLISA (■). The detection signals were absorbance for ELISA or fluorescence for OLISA and CP-OLISA, respectively.

immunoassays through the streptavidin which connect between the biotin moieties on the dAb and on the surface of the protein-DNA conjugate. The DNA would play as a signal generator on which the RNA probe of OLISA hybridizes and subsequently is cleaved by RNase H to produce fluorescence signal. According to the previously reported procedure for OLISA,[7] immunoassays were performed on a 96-well plate pre-coated with capture antibody (cAb) for AFP. After construction of the cAb-AFP-dAb sandwich, streptavidin was allowed to bind to the biotin tethered from dAb. The DNA-coated cage protein was added and the RNase H-mediated signal generation was conducted by the oligonucleotides on the protein. To determine the optimal concentration of the DNA-coated cage protein for the assays, CP-OLISA assays were carried out to detect AFP (10 ng/mL) with several concentrations of the DNA-coated cage protein. Figure 3 shows that immunoassay signal increased with the concentration of the DNA-coated protein up to 125 pM. At the concentration higher than 125 pM, additional signal increase was not observed, and thus 125 pM was chosen as the concentration of the DNA-coated protein for immunoassays.

After determining the optimal concentration of the cage protein for the signal generation, we performed CP-OLISA at varying concentrations of AFP. As show in in Figure 4,

the fluorescence intensities linearly increased with elevated concentration of AFP (squares in Fig. 4). To evaluate the performance of CP-OLISA in comparison to OLISA, we also carried out OLISA assays in parallel in which biotinylated oligonucleotides was used instead of the DNA-coated cage protein to detect the bound streptavidin-conjugated dAbs for the marker (triangles in Fig. 4). The increase of fluorescence intensity observed in CP-OLISA was about 4-fold higher than that in OLISA. The enhanced signal amplification was due to the transducer based on the multimeric protein assembly. We additionally performed conventional ELISA assays as the control immunoassay in which streptavidin-HRP conjugate was employed as the signal generator to detect the bound biotinylated dAbs for the marker. The absorbance signal increased linearly depending on the concentration of the marker as reliable as those in OLISA and CP-OLISA (circles in Fig. 4).

To assess detection sensitivity of CP-OLISA, we determined the limit of detection (LOD) for the marker by interpolating the curve with the average value of the blank plus 3 times the standard deviation of the blank. The same protocol was used to determine LODs of OLISA and ELISA and the LOD values calculated for all the assays are summarized in Table 1. As expected from the previous study, OLISA and ELISA showed similar LOD values, 0.83 ng/mL and 0.83 ng/mL for the marker, respectively.[7] In contrast, the LOD value of CP-OLISA was estimated about 0.21 ng/mL for the marker, showing approximately four-fold higher sensitivity than OLISA or ELISA. It seems that the increased amplification power in CP-OLISA also contributed to improve the detection sensitivity. The level of improvement in the sensitivity was, however, not as high as expected from the increased number of DNA strands per dAb (24 strands) in CP-OLISA compared to that in OLISA (up to 3 strands). We believe that some of the DNA strands immobilized on the protein surface might not be active for the signal generation reaction because of hindrance by other neighboring DNA strands and the DNA strands stretching to the solid phase of the microwell which are not fully available for the enzymatic reaction.

To further test the potential of the CP-OLISA in clinical applications, the precision (%CV) and the accuracy (% recovery) values of the method were determined for the biomarker in human sera. Eighteen healthy human serum samples containing AFP (20, 40 or 80 ng/mL) were analyzed by using CP-OLISA. As presented in Table 2, the %CV values were less than 5% at all concentrations examined, demonstrating that CP-OLISA is a reliable method for clinical uses. In addition, the %recovery expressing the accuracy of the assay ranged from 94.9 to 98.8%, indicating that the method could be used potentially for practical samples.[17]

In summary, we have prepared the DNA-coated cage protein and employed it in an immunoassay system, called CP-OLISA utilizing the cage protein for conjugating dAb with multiple signal generating DNA strands. Compared with the previous methods such as ELISA and OLISA, the CP-OLISA showed improved sensitivity in quantitation of AFP in buffer solution. In addition, the CP-OLISA showed acceptable levels of accuracy and reproducibility that are required for practical applications. We therefore expect that the protein-OLISA presented in this study can be useful for clinical diagnosis as well as research applications.

Table 1. The limit of detection (LOD) of ELISA, OLISA and CP-OLISA in AFP detection

Assay	LOD (ng/mL)
ELISA	0.80
OLISA	0.83
CP-OLISA	0.21

Table 2. Precision (%CV) and accuracy (%recovery) of CP-OLISA

AFP (ng/mL)	20	40	80
%CV	4.5	1.9	3.7
%Recovery	94.9	95.1	98.8

Experimental

To perform CP-OLISA, black 96-well microplates (Nunc, Denmark) were coated with cAb in PBS (5 μg/mL, 100 μL/well) by incubation for 1 h at room temperature, followed by washes with PBS (3 × 300 μL). The wells were then treated with Blocking Buffer (200 μL, PBS containing 3% (w/v) BSA and 0.1% (v/v) Tween 20) and incubated for 2 h at room temperature. After rinses with PBST (PBS containing 0.05% (v/v) Tween 20), AFP solutions (0, 0.0625, 1.25, 2.5, 5.0, 10.0 ng/mL) in Assay Buffer (100 μL, PBS containing 1% (w/v) BSA and 0.05% (v/v) Tween 20) were added to the wells, followed by incubation for 1 h at room temperature. The plate was washed with PBST before addition of biotin-dAb conjugates (2 μg/mL, 100 μL) in Assay Buffer and incubated for 1 h at room temperature. After rinsing with PBST (3 × 300 μL), the solution of streptavidin (2 μg/mL, 100 μL) was added to the wells and incubated for 1 h at room temperature. After rising with PBST (3 × 300 μL), solutions of DNA-coated protein (125 pM, 100 μL, DNA sequence: AACCACAGTG) were added to the wells, followed by incubating for 1 h at room temperature, and the plate was rinsed with PBST (3 × 300 μL). Finally, the RNase H (100 μL) solution containing F-RNA-Q probe (200 nM, FAM-5′-CACUGUGGUU-3′-BHQ1), PRI (0.4 U, Protector RNase Inhibitor, Roche, Germany) and 20 U of RNase H in RNase H buffer (40 mM Tris-HCl, 4 mM $MgCl_2$, 1 mM DTT, 0.003% BSA, pH 7.7) was added and incubated for 1 h at 37 ºC. The fluorescence intensities were measured by Apliskan (Thermo scientific, Waltham, MA, USA) with the excitation/emission filter sets of 485/535nm.

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Supporting Information. Experimental procedures for preparation of DNA-coated cage protein, ELISA, and OLISA.

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