Direct Labeling of Protein Nanoparticles with Fluorescent Compounds for Immunoassay Applications

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Abstract

A fusion protein, designated ELP-D-C, comprised of a hydrophobic elastin-like polypeptide unit, a hydrophilic aspartic acid-rich peptide unit, and an antibody-binding domain as a functional unit, was constructed. Upon heat induction, ELP-D-C forms micellar nanoparticles displaying antibody-binding domains on their surfaces. The protein nanoparticles were able to incorporate hydrophobic fluorescent compounds and subsequently detect target molecules via antibody binding by the resulting fluorescence intensity, which was proportional to the log of the concentration of the target molecule.

Keywords: Direct labeling, elastin-like polypeptide (ELP), protein nanoparticles, antibody-binding domain, fluorescence immunoassay
Introduction

Immunoassays allow for the presence and concentration of a target molecule (antigen) in solution to be measured using a detection antibody, with high specificity and sensitivity. In solid phase immunoassay, the target molecule is bound to a solid surface, such as a plastic microplate, directly or via capture polyclonal immunoglobulin (IgG), and then measured using detecting polyclonal IgG that has been conjugated with, e.g., enzymes, fluorescent compounds, chemiluminescent compounds, radioactive isotopes, and DNA reporters. The concentration of the target molecule in solution is determined from a calibration curve.¹ In fluorescence immunoassays (FIAs), IgG is typically labeled with fluorescent compounds, such as fluorescein isothiocyanate (FITC), cyanine, rhodamine, Oregon Green®, Texas Red®, and Alexa Fluor® series. These detecting probes are typically either covalently linked or conjugated to primary amino groups or a thiol group of IgG via chemical reaction.² These methodologies are well established; however, the IgG coupling site is limited and labeling via chemical reaction may result in deterioration of antigen-binding activity.³⁴ To overcome such limitations, detection probes conjugated with antibody-binding proteins (e.g., Protein A, G or L) have been developed, such as Protein A-enzymes (e.g., horseradish peroxidase, alkaline phosphatase, luciferase), and Protein A-fluorescent compounds (e.g., FITC, Alexa Fluor®), to label the desired IgG without chemical modification.⁵

Nanoparticles are generally defined as small size-controlled particles ranging from 1 to 100 nm in diameter, and can be made of metals, silica, polymers, carbon, lipids, peptides and proteins.⁶ As a source of peptide-based nanoparticles, elastin-like polypeptides (ELPs) comprised of the repeating amino acid sequence (Val-Pro-Gly-Xaa-Gly)n have been widely used.⁷ Smits and Fujita proposed that amphiphilic ELP-based peptide block copolymers comprised of highly hydrophobic and hydrophilic units self-assemble to form spherical micelles upon stimulation with heat and/or
salt, with the constituent hydrophobic units constituting the core of the nanoparticle. Due to their highly concentrated hydrophobic core, ELP-based nanoparticles have been proposed as drug delivery carriers for hydrophobic pharmaceutical compounds.

Reports suggest that nanoparticles displaying antibodies on their surfaces are useful in immunoassays. Liu et al. and Yuan et al. produced metal- and polystyrene-based nanoparticles, respectively, that directly displayed antibodies, for application in a homogeneous turbidity immunoassay. Iijima et al. developed bionanocapsules displaying the Z domain of Staphylococcus aureus Protein A for binding to enzyme-antibody conjugates. In addition, nanoparticles can be used as signal enhancers in immunoassays. Ikeda et al. designed self-assembling ELP-based nanoparticles displaying luciferase as a signal enhancer and biotin as an accepter peptide to bind biotinylated secondary antibodies.

In this study, we focus on the highly concentrated hydrophobic core of ELP-based nanoparticles for efficient incorporation of hydrophobic fluorescent compounds via hydrophobic interactions. Herein, we designed protein-based nanoparticles displaying an antibody-binding domain, designated “ELP-D-C/micelles,” in accordance with a previous study. ELP-D-C is comprised of 42 repeats of the sequence of the elastin-like pentapeptide PAVGV as a hydrophobic unit (ELPs unit), 4 repeats of the sequence of a highly hydrophilic aspartic acid-rich polypeptide (D11L) as a hydrophilic unit (D unit), and 3 repeats of the IgG binding domain sequence derived from Protein G (streptococcal bacteria) as the functional unit (C unit). Upon heat induction, ELP-D-C generates micellar nanoparticles (ELP-D-C/micelles) displaying antibody-binding domains on their surfaces. The highly hydrophobic core of ELP-D-C/micelles enables efficient incorporation of hydrophobic compounds via hydrophobic interactions. The concept of this study is schematically illustrated in Fig. 1. This study is model examinations and the ELP-D-C/micelles may have a potential for fluorescence immunoassay.
Experimental

Materials and apparatus

BLR(DE3) competent *E. coli* cells were purchased from Merck KGaA (Germany). Hi-TrapTM chelating column was purchased from GE Healthcare UK Ltd (UK). Bicinchoninic acid (BCA) protein assay kit was purchased from ThermoFisher Scientific Inc. (USA). Protein G was purchased from Abcam plc. (UK). Mouse IgM was purchased from ReliaTech GmbH (Germany). Anti-mouse IgM rabbit IgG was purchased from RayBiotech Inc. (USA). Anti β-galactosidase rabbit IgG was purchased from Rockland Immunochemicals Inc. (USA). The FIA was performed using a microplate reader (EnspireTM; PerkinElmer Inc.; USA).

Preparation of ELP-D-C/micelles

ELP-D-C was expressed in *E. coli* BLR(DE3) cells in accordance with a previous study\(^\text{17}\), using the transfected plasmid pET28b-PAVG42-D44-C3-CHis, which encodes ELP-D-C. After fermentation, *E. coli* cells were collected by centrifugation, washed with phosphate-buffered saline (PBS) and then disrupted with an ultrasonic homogenizer. After removing debris by centrifugation, the supernatant was applied to a Hi-TrapTM chelating column to purify ELP-D-C via a poly-histidine tag in immobilized metal ion affinity chromatographic mode. The purified ELP-D-C was dialyzed with 20 mM phosphate buffer (pH 7.0) and the concentration was adjusted to 0.2 mg/mL using a BCA protein assay kit. ELP-D-C/micelles were generated upon heat incubation for 10 min at 70°C. Likewise, ELP-D, comprised of 42 repeats of PAVGV and 2 repeats of D11L, was expressed in *E. coli* BLR (DE3) cells using the transfected plasmid pET28b-PAVG42-D22, and similarly purified.
Fluorescence immunoassay

ELP-D-C/micelles, ELP-D-C, ELP-D or Protein G solution was adjusted to a concentration of 0.2 mg/mL, and subsequently mixed with an identical volume of a 1 mg/mL solution of FITC. Twenty-five microliters of mouse IgM or bovine serum albumin, at various concentrations, was applied to the 96-well half area microplate and then incubated for 2 hours at room temperature. After washing the wells 3 times with 180 μL of PBS-T (PBS with 0.05% Tween20), 180 μL of blocking solution (PBS with 1% casein) was applied to each well. After incubating for 1 hour at room temperature, the wells were washed 3 times with 180 μL of PBS-T. Twenty five microliters of 2 μg/mL anti-mouse IgM rabbit IgG or anti-β-galactosidase rabbit IgG was added to each well at room temperature and then incubated for 1 hour at room temperature. After washing the wells 3 times with 180 μL of PBS-T, 40 μL of the premixed protein solution with FITC was added to the wells and incubated for 1 hour at room temperature. After washing the wells 3 times with 180 μL of PBS-T, 40 μL of 20 mM phosphate buffer (pH 7.0) was added and the fluorescence intensity was measured at 525 nm (excitation wavelength: 490 nm). The difference of fluorescence intensity between assay solution and base line (without mouse IgM) was calculated, respectively.

Results and Discussion

Nanoparticles displaying antibody on their surfaces have a potential tool for immunoassay. As described in a previous study, ELP-D-C/micelle displaying IgG generated the large complexes upon interactions between antibody and target molecules in proportion to the target molecule concentration, which could be detected by measuring the turbidity of the assay solution. This turbidity immunoassay system enabled to detect the concentration of target molecule directly without any difficult operation. But it was low sensitivity and subject to the condition of assay.
solution. By taking these advantage/disadvantage into consideration, this report proposed another application of ELP-D-C/micelle for fluorescence immunoassay.

ELP-D-C exists as a monomer at low temperatures, but self-assembles to form spherical micelles with diameters of ~40 nm above the phase transition temperature (>40°C) via intermolecular hydrophobic interactions of the ELP units. Further, ELP-D-C/micelles demonstrate the promising ability to display antibody-binding domains on their surfaces, allowing for immobilization of IgG molecules. The core of ELP-D-C/micelles was originally assumed to be highly hydrophobic, and it was suggested that hydrophobic fluorescent compounds, such as FITC, should be easily incorporated into the micelle core via hydrophobic interactions.

Based on this assumption, a composite of ELP-D-C/micelles and FITC (ELP-D-C/micelles+FITC) was prepared and subsequently evaluated for application in a fluorescence immunoassay system. The target molecule, namely mouse IgM, was first immobilized onto a microplate surface and anti-mouse IgM rabbit IgG was subsequently added to bind to surface-immobilized mouse IgM. As shown in Fig. 2, the ELP-D-C/micelles+FITC composite was able to detect mouse IgM via rabbit IgG (anti-mouse IgM), resulting in high fluorescence intensity. In contrast, there seemed to be detected approximately one-third difference of fluorescence intensity in ELP-D-C (which did not form nanoparticles) with FITC, ELP-D (which lacks the antibody-binding domain) with FITC, or Protein G (in which the nanoparticle-forming unit is absent) with FITC, respectively. These results suggest that ELP-D-C/micelles were able to efficiently incorporate fluorescent compounds in the micelle core via hydrophobic interactions. Changing the target molecule from mouse IgM to bovine serum albumin, or replacing the detecting antibody (anti-mouse IgM rabbit IgG) to anti-β-galactosidase rabbit IgG, resulted in a little observed fluorescence from the ELP-D-C/micelles+FITC composite (Fig. 2).
When various concentrations of mouse IgM were coated onto the microplate, the deference
of fluorescence intensity in ELP-D-C/micelles+FITC was found to increase linearly with an
increase in the log of the concentration of mouse IgM within a range of 0.1 to 10 μg/mL (Fig. 3).
These results indicated that ELP-D-C/micelles is successfully incorporating fluorescent
compounds not only FITC but other hydrophobic fluorescent compounds as well and there
seemed to be a potential for use as fluorescence carriers in applications such as fluorescence
immunoassays. Although it is a little low detectability compared with conventional fluorescence
immunoassay, it has a high flexibility to select fluorescence compounds without any preparation
for chemically-modified detecting antibody. Furthermore, it may be possible to improve the
detection limit by optimizing the micelle size and/or the number of antibody binding sites per
micelle, and to detect multiple targets at the same time by labeling the various fluorescent
compounds.

Although this work is at a proof concept stage, obtained results suggest that ELP-D-C/micelles incorporating fluorescent compounds may have a potential as fluorescence probes. In
future study, it is necessary to examine applicability of ELP-D-C/micelles in various immunoassay
systems.
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Figure Captions

Fig. 1 Schematic illustration of ELP-D-C/micelles fluorescence immunoassay.

Fig. 2 Difference of fluorescence intensity of each immunoassay combination. Error bars represent standard error (n=6).

Fig. 3 ELP-D-C/micelles fluorescence immunoassay for detecting specific target molecules. FITC labeled ELP-D-C/micelles detected the mouse IgM via rabbit IgG (anti mouse IgM) as deference of fluorescence intensity in proportion to the concentration of mouse IgM. Error bars represent standard error (n=6).
Fig. 1

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### Fig. 2

| Target molecule | Mouse IgM | Mouse IgM | Mouse IgM | Mouse IgM | Mouse IgM | Mouse IgM | BSA |
|-----------------|-----------|-----------|-----------|-----------|-----------|-----------|-----|
| Antibody        | Rabbit IgG #1 | Rabbit IgG #1 | Rabbit IgG #1 | Rabbit IgG #1 | Rabbit IgG #1 | Rabbit IgG #2 | Rabbit IgG #1 |
| Protein         | NA        | Protein G  | ELP-D     | ELP-D-C   | ELP-D-C /micelle | ELP-D-C /micelle | ELP-D-C /micelle |

*a: Rabbit IgG #1 is reacting mouse IgM and rabbit IgG #2 is β-galactosidase, respectively.
*b: Protein is composed with FITC*
Fig. 3

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