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Feasibility of asymmetrical flow field-flow fractionation as a method for detecting protective antigen by direct recognition of size-increased target-captured nanoprobes

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ABSTRACT

Asymmetrical flow field-flow fractionation (AF4) was evaluated as a potential analytical method for detection of a protective antigen (PA), an *Anthrax* biomarker. The scheme was based on the recognition of altered AF4 retention through the generation of the size-increased Au nanoparticle probes as a result of PA binding, in which a PA-selective peptide was conjugated on the probe surface. In the visible absorption-based AF4 fractograms, the band position shifted to a longer retention time as the PA concentration increased due to the presence of probe bound with PAs. The shift was insignificant when the concentration was relatively low at 84.3 pM. To improve sensitivity, two separate probes conjugated with two different peptides able to bind on different PA epitopes were used together. The band shift then became distinguishable even at 84.3 pM of PA sample. The formation of larger PA-probe inter-connected species using the dual-probe system was responsible for the enhanced band shift. In parallel, the feasibility of surface-enhanced Raman scattering (SERS) as a potential AF4 detection method was also evaluated. In the off-line SERS fractogram constructed using fractions collected during AF4 separation, a band shift was also observed for the 84.3 pM PA sample, and the band intensity was higher when using the dual-probe system. The combination of AF4 and SERS is promising for the detection of PA and will become a potential tool if the reproducibility of SERS measurement is improved.

1. Introduction

Studies on disease diagnosis through the detection of a relevant protein biomarker, especially using diverse nanostructures based on noble metals and quantum dots made of semiconductor materials, have been extensively reported [1–9]. There are several advantages of employing nanostructures in the analyses, such as sensitivity enhancement and multiplexed detection [3–9]. Probes prepared by conjugating target-specific receptors such as antibodies or peptides on the surface of nanoparticles or quantum dots are then used for selective detection of relevant biomarkers. When a nanostructure-based probe captures a target protein biomarker, its overall size is expected to increase. Therefore, a method capable of recognizing the increased size of the target-captured probe, such as field-flow fractionation (FFF) [10–14], is potentially useful since it could make the analysis reliable by directly observing the analyte-induced physical change in the probe and by eliminating sample-handling steps, such as washing of target-uncaptured probes, which is commonly needed in a normal plate-based immunoassay [15]. In addition, the immune-reaction is kinetically favored when nano- or micrometer-sized beads are used as an assay substrate compared to the conventional microtiter plate assays [15–19].

FFF is a family of separation techniques useful for size characterization of particles in the range from a few nm to approximately 100 μm [20–22], and its separation is performed by applying an external field to the sample flowing in a hollow channel without a solid stationary phase, relieving problems of non-specific protein adsorption or structural deformation on surface. In the case of FFF-based immunoassay for protein detection, a few studies utilizing micrometer-sized beads coated with capture antibody selective to target analytes have been reported [15–19], such as the detection of horseradish peroxidase (HRP) using chemiluminescence [15–18] and the measurement of protein A by combining flow cytometry

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[19]. In these studies, micron-sized polystyrene (PS) beads were used since they are readily separable in a FFF channel, chemically stable, and facile for use in surface functionalization.

Another way of utilizing FFF for protein detection is the direct recognition of the size-increased target-bound particle and use of an altered fractogram to measure target concentration. This method is simple since a mixture of target and probe particle is injected into an FFF channel for measurement without further sample treatments. Moreover, size increase of the particles can be easily recognized via migration time shift if the particle is directly detectable. To realize this strategy, several important issues should be considered. First, the size of the probe capturing a target protein needs to be properly selected in consideration of the relative size difference between bare probe and target-captured probe. As known size of a protein is usually a few nanometers [23], the use of a nano-sized probe would be advantageous because the relative size change is larger than conventionally adopted micron-size particles. Also, the probe needs to be quickly detected upon FFF elution. Considering these criteria, the above-mentioned noble metal nanoparticles and quantum dots seem to be good FFF probe candidates since their sizes are usually in the range of nanometers, and they have spectroscopic properties, such as visible absorption (color) and fluorescence [5–9], allowing fast optical detection.

In this study, we explored the feasibility of asymmetrical flow FFF (AsFlFFF, AF4) as an analytical method for the detection of a protective antigen (PA), an *Anthrax* biomarker [24–26], by recognizing the altered feature of the AF4 fractogram induced by the size increase of PA-captured Au nanoparticles (AuNPs). In AF4, the hydrodynamic diameter, d_H, of a particle can be determined directly from its measured retention time, t_r, according to

$$d_H = \frac{2kTV^o}{\pi\eta w^2 F_c t^o} t_r \quad (1)$$

where k is the Boltzmann constant, T is the absolute temperature, V^o is the channel void volume, η is the viscosity of the carrier liquid, w is the channel thickness, F_c is the volumetric crossflow rate, and t^o is the channel void time [27–30]. Eq. (1) shows that d_H is proportional to t_r, so that an AF4 fractogram is a direct representation of the size distribution of the sample that can be readily transformed to a size distribution. Therefore, increased retention time is indicative of the presence of a species with larger hydrodynamic diameter, such as PA-captured AuNPs.

Initially, ~60 nm AuNPs were synthesized, and a peptide that can selectively bind to PA was conjugated onto the AuNP surface using a linker molecule (5,5′-dithiobis(succinimidyl-2-nitrobenzoate), DSNB), as shown in Fig. 1. The peptide-conjugated AuNP probes were added to PA samples at various concentrations, and the mixture solutions were injected into an AF4 system equipped with a UV/Visible detector. Then the changes in AF4 fractograms with PA concentration were examined. When two probes conjugated with different peptides capable of binding on different PA epitopes were used, larger species such as PA bound with both probes and inter-connected multiple probes were formed. These complexes were more perceptible in an FFF system, thus further improving the sensitivity of PA detection. Dual probes separately conjugated with two different PA-selective peptides (Fig. 1) were used to measure the same PA samples, and the potential sensitivity improvement was examined.

AuNP is a widely adopted SERS material and thus SERS-based detection of FFF effluent is feasible since the linker molecule (DSNB) on the Au surface simultaneously operates as an effective Raman reporter [31]. SERS has been widely employed for analysis of diverse samples due to its ultra-sensitivity [32,33]. Therefore, synergetic combination of both methods, the capability of FFF for recognizing probe size increase and the high sensitivity of SERS detection, is worthwhile to explore. For the feasibility study, AF4 fractions were collected during an FFF elution, and Raman spectra of each fraction were acquired to construct off-line SERS-based fractograms. The positive potential and challenges of SERS-based detection are subsequently discussed.

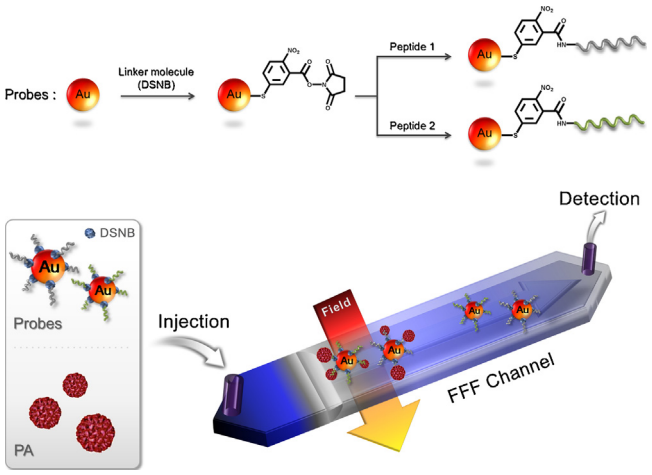

Fig. 1. Schematic descriptions of the preparation of peptide-conjugated AuNP probes (top) and the overall PA detection scheme using FFF (bottom).

2. Experimental

2.1. Preparation of AuNP probes

All reagents used in the study were of the highest available grade, purchased from Sigma–Aldrich, except FL-70, and used as received. FL-70, a dispersing agent, was obtained from Fisher Scientific (Fair Lawn, NJ, USA). First, 15 nm AuNPs were synthesized through a well-known citrate reduction method [34]. The average size of the synthesized AuNPs was 14.6 ± 1.4 nm, with a maximum absorption at 520 nm (concentration: 9.27×10^{12} particles/mL). Then, the 15 nm AuNPs were grown to a diameter of 30 or 60 nm through the surface-catalyzed reduction of Au^{3+} by NH_2OH, the so-called seed mediated growth method [35,36]. The average sizes of two synthesized AuNPs were 32.0 ± 3.3 (1.51×10^{11} particles/mL) and 59.6 ± 6.3 nm (2.29×10^{10} particles/mL), with maximum absorption observed at 525 and 530 nm, respectively. The size distribution of AuNPs was determined by the measured sizes of dispersed particles in SEM images (obtained by Hitachi S-4800) using an image analysis program (Image J, National Institutes of Health, USA).

To prepare the AuNP probes, 25 mL of the 60 nm AuNP solution was mixed with 25 mL of water, and then the pH of the solution was adjusted to 8.5 using 1 M NaOH or HCl solution. Then, 2 mL of 50 mM borate buffer was added to the pH-adjusted solution. To conjugate a linker (5,5′-dithiobis(succinimidyl-2-nitrobenzoate), DSNB) to the AuNP surface, 125 μL of 1 mM DSNB solution was added to the AuNP solution. This procedure generated a thiolate monolayer of DSNB on the AuNP surface, and the succinimidyl terminus in the DSNB-derived coating formed an amide linkage with a primary amine of the peptide (Fig. 1), as previously reported [31]. Two different AuNP probes were prepared by coupling two peptides with different amino acid sequences (peptide 1 sequence: HKHAHNYRLPASGGKK (K_d = 28.0 ± 3.6 nM), peptide 2 sequence: NAYKHHHPPVFYGKGK (K_d = 4050.8 ± 18.6 nM)). The peptides (synthesized by Peptron, Daejeon, Korea) bind to different epitopes on PA, as previously reported [37–39]. Either single or dual probes were mixed with a PA sample solution over three hours at room temperature before injection into the AF4 channel.

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