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Stimuli-Responsive Array of Microbeads Conjugated with Elastin-like Polypeptide

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Abstract

It describes a platform of microarray for immunoassay using polystyrene microbeads (μbeads) conjugated with elastin-like polypeptides (ELP) as a responsive molecule to external stimuli. Prior to the highly resolved localization of μbeads for an array, photoresist micropatterns were fabricated onto the spun-cast adhesive thin layer. A UV-curable adhesive was applied to enhance the adhesion of μbeads to the substrate by controlling UV irradiation time. Spatial placement of μbeads on the photoresist micropatterns was easily performed with a flow of colloidal suspension through a device. The μbead array conjugated with ELP molecules was used for the immunoassay of prostate specific antigen (PSA), a cancer marker. The capture of PSA from a protein mixture was successful with high sensitivity and the release of PSA from the array was complete and reversible.

Keywords: Microarray, Microbead, ELP, Immunoassay, PSA

Introduction

In the paper, we describe a simple microfluidic bead -based immunoassay for detection of prostate-specific antigen (PSA). PSA is a 33 kDa serine protease that is produced by epithelial cells of the prostate gland[1]. PSA has been used as a tumor marker for the monitoring of patients with prostate cancer. In serum, PSA exists in two different isoforms, free PSA (f-PSA) and complex PSA bound to α-1-antichymotrypsin (PSA-ACT)[2,3]. Currently employed methods for detection of PSA usually measure the total PSA level in serum and PSA value of 4.0 ng/mL is generally regarded as a cut-off value[4]. Besides the traditional immunoassay methods, several strategies have been developed for detection of PSA, including recombinant antibody[5], binding peptide[6], quantum dot[7], carbon nanotube[8], surface-enhanced Raman scattering (SERS)[9], immuno PCR[10], micro cantilever[11], microbead[12], and surface plasmon resonance (SPR)[13]. In addition to the fabrication methods, recent biochips have been requested to be functional for more rapid and accurate detection and analysis[14]. Among the materials to introduce a smart functionality to the biochips, elastin-like polypeptide (ELP) can be a potential candidate due to its effective and rapid separation and purification by simple phase transition. ELP containing repeats of the pentapeptide sequence Val-Pro-Gly-Xaa-Gly (VPGXG, where Xaa is any amino acid except Pro) has been previously identified as a smart biopolymer, as it undergoes a reversible phase transition in response to changes in temperature, pH, light, and ionic strength at its lower critical solution temperature (LCST)[15-17]. Characteristic hydrophobic-hydrophilic phase transition of ELP molecules by external stimuli enables the controllable and effective binding or separation of analytes from the surface[18,19]. In the paper, the thermally controlled capture and release of PSA will be described using bead-based biochips functionalized with ELP.

Results and Discussion

By PCR and recursive directional ligation (RDL) method[20], an ELP molecule with repeating amino acid sequence of $\{[(VGPVG)_5]_8+[(VGVPG)_{14}(KGVPG)]_8\}$ was synthesized and successfully purified by repetitive phase transition. The transition temperature (T_t) of ELP was determined to be 37˚C by UV spectrometry, which meant that the ELP solution was transparent at temperatures below 37˚C, but became turbid above 37˚C due to the formation of ELP aggregates.

Sulfo-SANPAH is a heterobifunctional photoactivatable crosslinker containing amine-reactive NHS ester and photoactive nitrophenyl azide. Conjugation of ELP to polystyrene (PS) μbeads is achieved by reacting the amine groups of ELP with NHS groups on the PS μbead surface previously photocrossliked

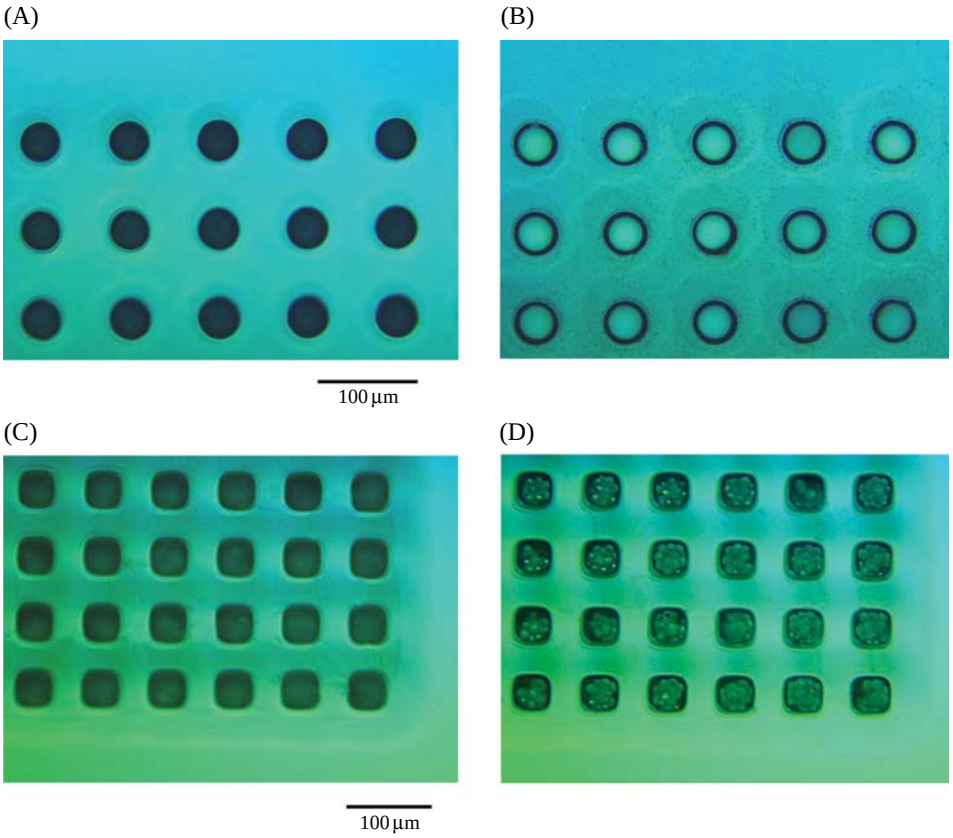

Figure 1. Optical images of ELP-PS μbead microarrays. (A) 40 μm circle features of photoresist micropatterns. (B) 30 μm ELP-PS μbead array in (A). (C) 40 μm square features of photoresist micropatterns. (D) 10 μm ELP-PS μbead array in (C).

with Sulfo-SANPAH. The conjugation of ELP on the surface was confirmed by fluorescence detection using Alexa Fluor 488 conjugated ELP (data not shown). It is important to retain the responsiveness of ELP molecules attached on the surface for the reliable function in the system. Therefore, the stability of ELP-PS ubead against the changes of temperature, and ionic strength was evaluated. First, the stable attachment of ELP molecules on the ELP-PS μbeads was evaluated by changing its surrounding temperature up to 60°C. Second, the beads were immersed in various concentration of NaCl solution to observe the effect of salt concentration on the stability of ELP molecules bound to the bead surface. The fluorescence intensity was not decreased at high temperature and high salt concentration and confirmed the robust attachment on the surface (data not shown).

Figure 1A and 1C present the photoresist-micropatterned glass surface with exposed adhesive layer. UV-curable acrylate adhesive used in the experiment consisted of prepolymer, reactive monomer and photoinitiator and is generally applicable to paper, film, plastic, steel, wood, and glass surfaces. The UV curing reaction is induced by the absorption of UV light by the photoinitiator and subsequent free radical cross-linking of the resins. A UV-curable adhesive was spun-cast on the glass surface at 3,000 rpm for 30 sec and ~50 μm thick layer was obtained by profilometer. The adhesive layer well covered the glass surface without any visible defect in the region by reflection microscopy and was cured by UV irradiation for micropatterning a photoresist on the surface. The UV dose of 40-140 mJ/cm^2 provided the good adhesion of the layer to the ELP-PS μbeads array even under ultrasonication with 50 : 50 (v/v) H_2O/ethanol mixture for 2 min. Moreover, the adhesion performance of the adhesive layer was maintained during photolithographic process.

Single- and multi-bead arrays of ELP-PS μbead were spatially placed and immobilized onto the photoresist-micropatterned glass surface by a simple microfluidic flow of μbead suspension. ELP-PS μbead with various sizes could be successfully arrayed to the micropatterns by controlling the flow rate and evaporation process (Figure 1B, D). The adhesive layer on the bottom of micropatterns was important for the stable maintenance of arrayed μbead-structures against the fast flow rate and evaporate rate.

PSA immunoassay was performed with the microfluidic ELP-PS μbeads array chip. At first, an ELP-

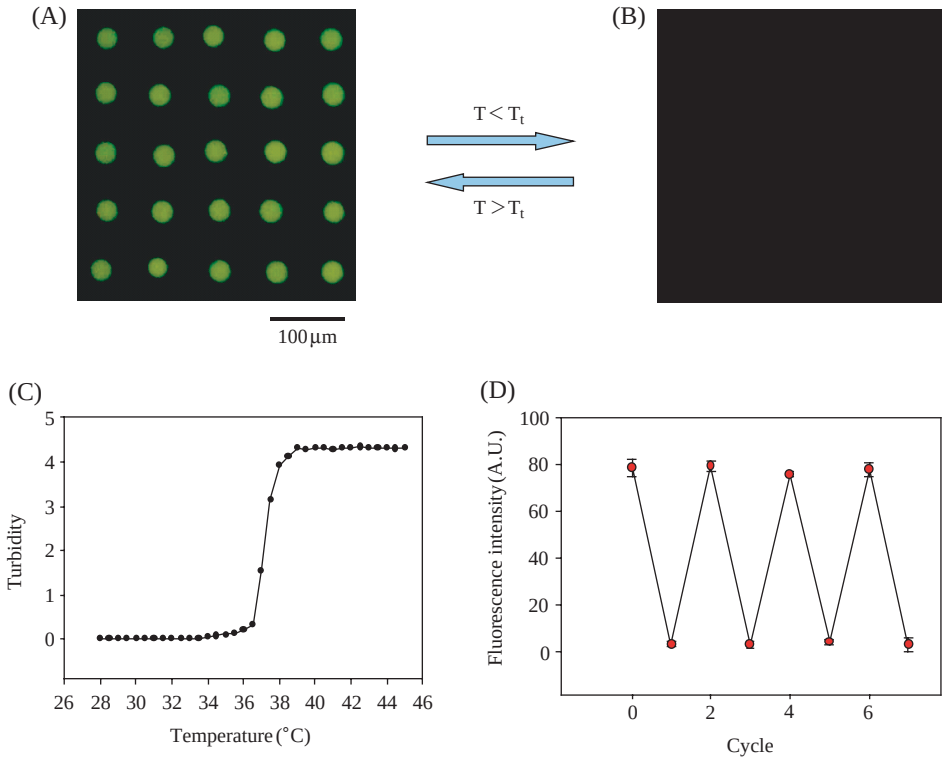

Figure 2. Reversible responsiveness of μbead arrays to thermal stimuli in immunoassay of PSA. Fluorescence images (A) after binding of Alexa Flour 488 conjugated secondary antiPSA to the ELP-PS μbeads array above LCST and (B) after removal of antigen-antibody complex below LCST. (C) Phase transition of ELP molecules according to temperature of solution. (D) Cyclic experiments in which the temperature was changed from 45°C to 10°C.

PS μbeads array was washed with fresh PBS buffer (pH 7.4) to remove any possible impurities on the surface. Then, the antiPSA-ELP conjugates were added to the ELP-PS μbeads array at the temperature above LCST of ELP to induce the specific binding of anti-PSA-ELP conjugate to the ELP-PS μbeads through the hydrophobic interactions between ELP molecules. Subsequently, unreacted antiPSA-ELP conjugates were removed with fresh 1M NaCl solution and the protein mixture composed of PSA, IgG, and BSA was added to the array. Unbound proteins were easily removed by washing the array twice with 1M NaCl solution. Fluorescence assay was performed by labeling PSA bound to the array with antibody-Alexa Fluor 488 conjugates in PBS buffer (pH 7.4) (Figure 2A).

ELP-PS μbeads can be recovered by cooling the array to the temperature below the LCST of ELP. At the temperature below LCST, the hydrophobic interactions between ELP molecules disappeared and consequently, we could remove antigen-antibody complex from the ELP-PS μbeads array. The removal of antigen-antibody complex from the array was simply observed by fluorescence microscopy as shown in Figure 2B. The LCST of ELP used in this study was designed to close to the human body temperature (Figure 2C). The reversible phase transition of ELP molecules enabled the repetitive binding and removal of analytes with the arrays. 4 cyclic experiments showed reliable smart property in specific binding of ELP-PS μbeads array (Figure 2D).

By utilizing the reversible phase transition of ELP, various concentrations of PSA molecules were successfully characterized and separated from the protein mixture. The dose response of ELP-PS μbeads array for the PSA immunoassay was shown in Figure 3. It showed linear relationship between the PSA concentration injected to the ELP-PS μbeads array and the fluorescence intensity measured from the ELP-PS μbeads array after PSA-antiPSA binding showing a minimum detection limit of 0.05 ng/mL. These results demonstrate the ELP-based assay systems as a reliable and promising detection tool for biomolecules.

Conclusions

It described a spatial localization of smart PS μbeads in the photoresist micropatterns and the highly selective detection of PSA by stimuli-responsiveness. The negative micropatterns of photoresist were fabricated on the curable adhesive layer and PS μbeads functionalized with ELP were placed with high resolution. The spatial localization of ELP-PS μbeads was successfully carried out by a simple flow of colloidal suspension. The ELP-PS μbeads in photoresist micropatterns were stable at harsh conditions of high tem-

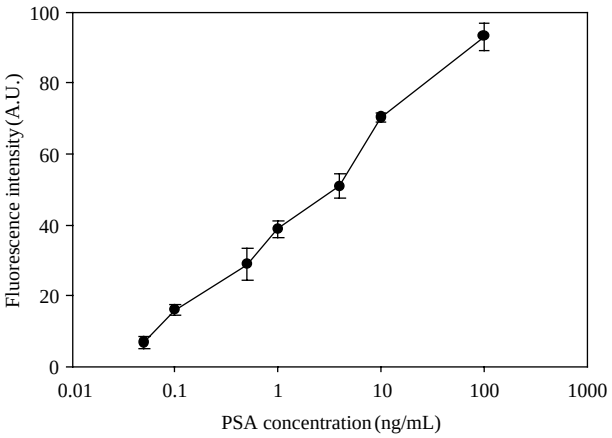

Figure 3. Dose response curve for PSA detection.

perature and rapid flow rate of solutions. The controllable phase transition of ELP molecules enabled the reversible capture and release of PSA from the protein mixture inferring its reusability of devices as well as continuous analysis of multi-analytes with one biochip.

Materials and Methods

Construction and Characterization of ELP

Polymerase chain reaction (PCR) was performed to create a synthetic gene coding for $(VGVPG)_5$ (KGVPG) peptide sequence. ELP with various repeating peptide units have been constructed by employing RDL method[20]. ELP expression was achieved by *E. coli* strain

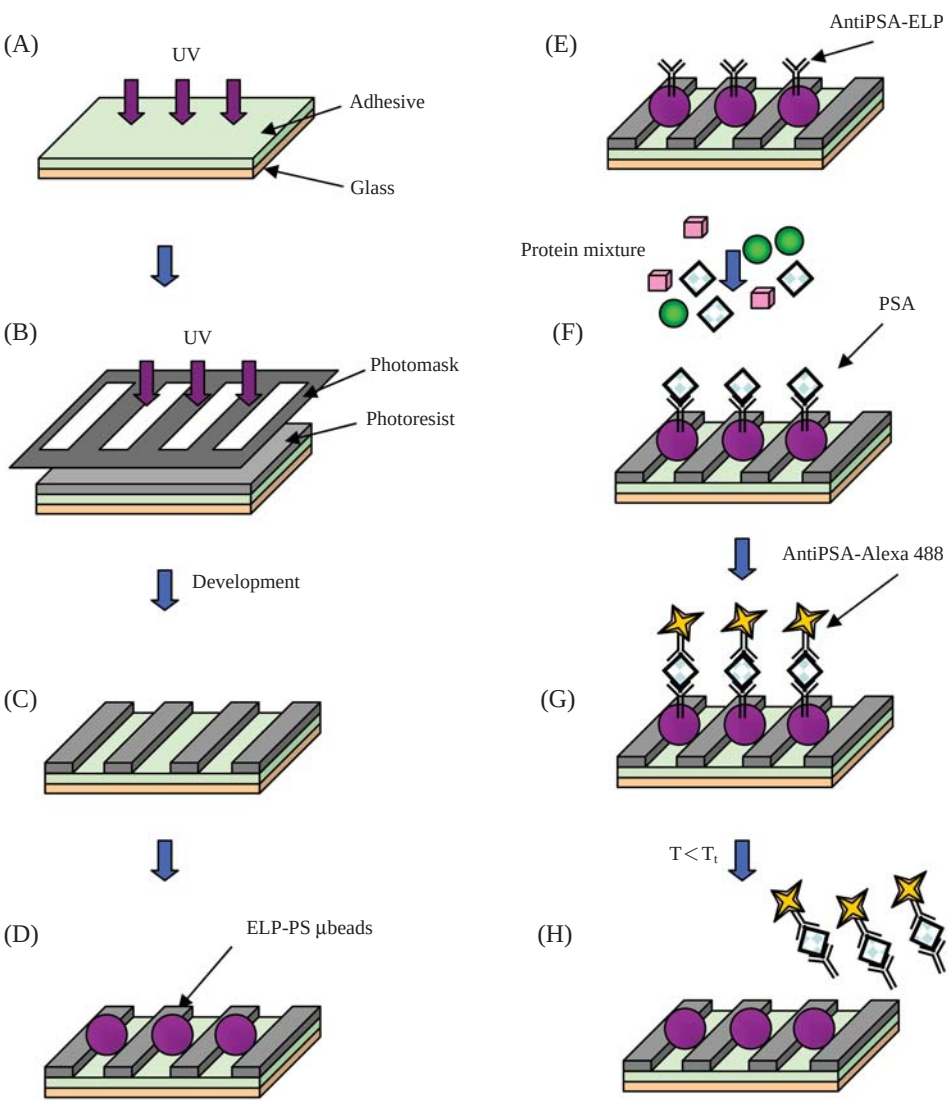

Figure 4. Schematic procedure for the fabrication of ELP-PS μbeads array and the smart immunoassay of PSA. (A) UV irradiation onto a spun-cast thin adhesive layer. (B) UV irradiation onto a spun-cast photoresist SU-8 2050 layer. (C) Removal of unexposed photoresist with SU-8 Developer. (D) ELP-PS μbeads array. (E) Binding of antiPSA-ELP conjugates to ELP-PS μbeads at the temperature above LCST. (F) Specific binding of PSA from protein mixture. (G) Binding of Alexa Flour 488 conjugated secondary antiPSA. (H) Release of antigen-antibody complex from ELP-PS μbeads by cooling to the temperature below LCST.

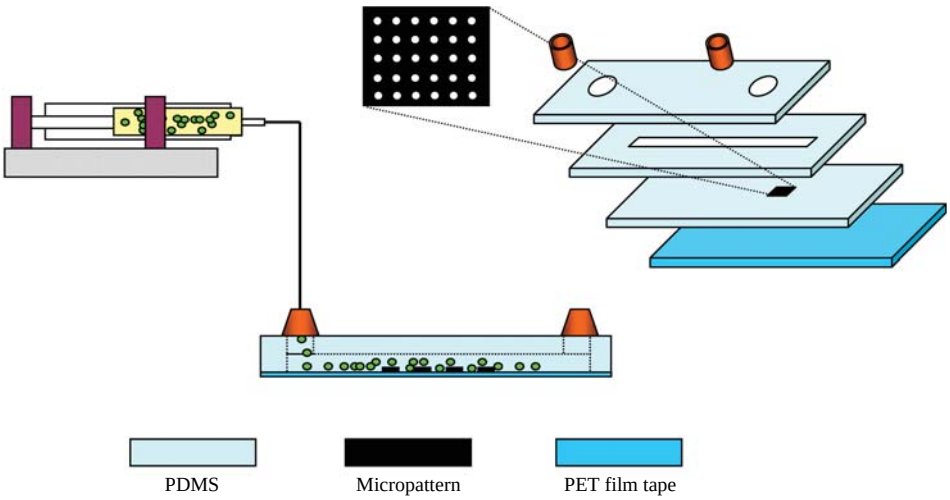

Figure 5. Schemetic representation of a microfluidic bead array chip.

BLR(DE3) (Novagen) using the modified pET-32b(+) vector (Novagen). All cultivation was carried out at 37°C in Circlegrow media (Q. Bio gene) supplemented with 100 μg/mL ampicillin. After 20 h of cultivation, cells were harvested and resuspended in PBS buffer (pH 7.4). The resuspended cells were lysed by sonication and cell debris was removed by centrifugation. Further purification of ELP was achieved by repetitive phase transition followed by ultracentrifugation as described previously[20]. The T_t of purified ELP in PBS buffer (pH 7.4) was characterized by monitoring its absorbance at 350 nm using a UV-spectrophotometer, and was determined as the solution temperature at the half maximum of the turbidity gradient.

Preparation of ELP Conjugated PS μbead (ELP-PS μbead)

Suspensions of polystyrene microbead (PS μbead) with a diameter of 10 and 30 μm (Polysiences, Inc. USA) were mixed with 0.5 mM N-sulfosuccinimidyl-6-[4′-azido-2′-nitrophenylamino] hexanoate (sulfo-SANPAH) (Pierce Chemical Co.) in 4 mL PBS buffer (pH 7.4) containing 0.5 mL dimethyl sulfoxide (DMSO). Photoactivation was performed by exposing the PS μbead suspension at 350 nm for 8 minutes. After the removal of unreacted reagents by centrifugation at 12,000 rpm for 15 min, 1 mL of 1 mM ELP solution in PBS buffer (pH 7.4) was added to the suspension. A crosslinking reaction between the SANPAH bound PS μbeads and ELP was performed for 2 h. The stability of ELP crosslinked to PS μbeads was investigated by changing incubation temperature and salt concentration using ELP labeled with the Alexa Fluor 488 conjugate (Invitrogen).

Fabrication of Photoresist Micropatterns on Adhesive Thin Layer

The UV-curable acrylate adhesive was coated onto a cleaned glass plate (15 × 15 × 1 mm) using a spin-coater at 3,000 rpm for 30 sec to make a uniform thin adhesive layer. The surfaces were then dried at room temperature for 2 to 5 min, and cured using a custom-built UV curing equipment (~350 nm) by controlling the irradiation time and intensity (Figure 4A). The UV dose was measured with an IL 390C Light Bug UV radiometer (International Light, USA). The SU-8 2050 (MicroChem Corp.) photoresist was used to make a defined micropattern above the adhesive thin layer. Photoresist was poured onto the adhesive coated glass plate and spin coated at 2,000 rpm for 30 sec. The glass plate was then soft baked at 65°C for 1 min on the hot plate and 95°C for 5 min in the oven. The soft baked glass plate was then slowly cooled down to room temperature and was exposed to UV radiation through the photomask (Figure 4B). The micropatterned glass plate was then hard baked at 95 °C for 8 min in the oven and followed by immersed in SU-8 Developer (MicroChem Corp.) (Figure 4C). After the development process, the stickiness of the exposed adhesive layer was characterized with a probe tag test using the TAXT2i texture analyzer (Stable Micro Systems, UK) and a cylindrical probe. The test was performed with a contact force of 100 g/cm^2, a contact time of 1 sec, and a 5 mm/sec debonding rate; the stickiness was determined as the debonding force.

Fabrication of a Bead Array Chip

The μbead array chip consisted of photoresist-micropatterned glass surface, poly(dimethylsiloxane) PDMS layer, and polyethylene terephthalate (PET)

film tape (Figure 5). We have employed simple microfluidic method to make a spatially localized array of ELP-PS μbead onto the micropatterned glass surface. The suspension of ELP-PS μbead in PBS buffer (pH 7.4) was introduced into the device using microsyring pump connected to the inlet tubing. Spatial arrangement of ELP-PS μbead was controlled by flow rate, concentration of bead suspension, and evaporation rate (Figure 4D).

Smart Immunoassay of PSA

A 50 fold-dilution of antiPSA (Invitrogen) in PBS buffer (pH 7.4) was activated with 5 mM N-hydroxysuccinimide (NHS) and 2 mM 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and subsequently mixed with 10 μM ELP solution in PBS buffer (pH 7.4) for molecular conjugation (antiPSA-ELP). AntiPSA-ELP conjugates were then added to the ELP-PS μbead and the temperature was raised above the T_t of the conjugated ELPs (Figure 4E). Anti-PSA-ELP that had not bound to the ELP-PS μbead array was removed by washing the array with a 1 M NaCl solution. Subsequently, the protein mixture composed of PSA, IgG, and BSA was then introduced to the the ELP-PS μbead array and washed with 1 M NaCl (Figure 4F). Finally, Alexa Fluor 488 conjugated secondary antibodies were added and the bead array was visualized using fluorescence microscopy (BX21, Olympus, Japan) (Figure 4G). The release of analytes from the array surface was performed by washing the array with ice-cold PBS buffer (pH 7.4) (Figure 4H).

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