Anal. Chem. **2005,** *77,* 2318−2322

Genetically Engineered Elastin-Protein A Fusion as a Universal Platform for Homogeneous, Phase-separation Immunoassay

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A simple and universal platform for competitive phase-separation immunoassay is reported based on a fusion protein composed of a temperature-responsive elastin-like polypeptide (ELP) and the antibody-binding staphylococcal protein A (SpA). The basic principle is to take advantage of the ability of SpA to bind a variety of antibodies with high affinity, allowing simple separation of antigen−antibody complex by thermal precipitation. The resulting ELP−SpA fusion was shown to preserve the ability to reversibly precipitate as well as its high affinity toward different IgGs and IgMs. As a model system, a competitive phase-separation immunoassay based on the ELP−SpA format was established for paclitaxel (taxol) with IC_{50} (20.18 nM) and the lower detection limit (2.94 nM) very similar to those reported for the ELISA format. Unlike the heterogeneous interaction in ELISA, which decreases the antibody-binding activity, the reported homogeneous immunoassay not only alleviates this problem but also enables the potential for high-throughput automation. We believe that the reported ELP−SpA fusion will find applications not only as a powerful diagnostic tool for diverse analytes but also a potential useful tool for purification and immobilization of antibody.

Immunoassays allow rapid and inexpensive analysis of analytes with high affinity and specificity. Enzyme-linked immunosorbent assay (ELISA), which is based on immobilized antibodies on various solid surfaces, has been widely used in clinical and environmental monitoring. However, ELISA requires extensive sample handling, a considerable amount of costly plastic trays, and a long analysis time. More importantly, immobilization of antibodies can greatly affect their binding affinity due to steric hindrance by random antibody orientations and environment-induced denaturation.[1] This heterogeneous interaction between antibodies and antigens results in nonuniform kinetic and thermodynamic properties that significantly limit the sensitivity and reproducibility of the ELISA assay.[2]

Phase-separation immunoassay[3,4] is a newly designed immunomethod developed in recent years that allows antigens and antibodies to interact under homogeneous conditions, as opposed to heterogeneous conditions in ELISA. The immunocomplex formed can be separated from the solution by a simple thermal precipitation. The homogeneous nature of phase-separation immunoassay is preferred due to the ease of automation and the potential for higher throughput analysis.[5] Typically, thermally reversible polymers such as poly(N-isopropylacrylamide) (PNIPAM) are chemically conjugated to the antibodies to provide the thermally tunable property.[6] The polydispersity, use of environmentally unfriendly synthetic chemistry for polymer synthesis, polymer−antibody conjugation, and random/uncontrolled conjugation of antibody to polymer are some of the limitations of PNIPAM-based phase-separation immunoassay. To circumvent these problems, we have recently reported a novel phase-separation immunoassay based on thermally tunable immunosorbents composed of an elastin-like polypeptide (ELP) and a single-chain antibody (scAb) for the detection of atrazine from contaminated water.[4] ELP, consisting of the repeating pentapeptide VPGVG, is structurally similar to the mammalian protein elastin that undergoes a reversible phase transition from water-soluble forms into aggregates as the temperature increases.[7] The unique phase transition property of ELP has been exploited for thermally triggered protein purification,[7] and the same property enables the immunosorbent−atrazine complex to be separated from the reagents by a simple thermal precipitation. Although tunable immunosorbents can be generated by creating ELP fusions for each individual antibody of interest, this is a tedious and expensive strategy. A universal method, in which any available antibody can be easily and readily conjugated to the ELP biopolymer, will provide a cost-effective and efficient means for generating the antibody-based immunosorbents.

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10.1021/ac0484326 CCC: $30.25
Published on Web 03/02/2005

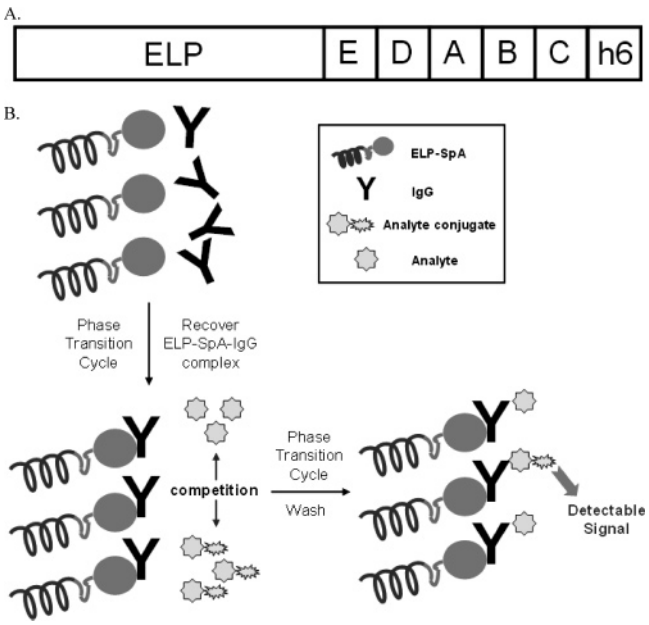

Figure 1. (A) Synthetic gene encoding for the ELP−SpA fusion. Abbreviations: ELP, elastin-like polypeptide; E, D, A, B, and C, IgG-binding region of protein A; h6, six-histidine amino acid tail. (B) A schematic representation of the competitive phase-separation immunoassay by the ELP−SpA fusion.

Staphylococcal protein A (SpA), a cell wall component of *Staphylococcus aureus*, binds immunoglobulin G (IgG) from several mammalian species.[8] It has been extensively applied in immunoassays, owing to its high specific avidity for the Fc portion of IgG without interrupting its antigen-binding ability.[9,10] A series of protein fusions with SpA have been generated while maintaining the IgG-binding affinity.[11,12] Similarly, ELP can be easily fused to SpA, enabling rapid binding to any IgG molecules of interest. The resulting ELP−SpA−IgG complex, providing both antigen-binding and reversible phase-transition properties, is highly desirable for a wide range of applications because the antigen−antibody complex can be easily separated by inducing aggregation of the ELP domain (Figure 1).

In this paper, we describe the construction and characterization of a genetically engineered ELP−SpA fusion. The utility of the ELP−SpA fusion in an immunoassay format is demonstrated for the sensitive detection of paclitaxel (taxol), an important anticancer agent that must be monitored for therapeutic applications. Our results demonstrate that this ELP−SpA fusion has great potential as a universal platform for phase-separation immunoassay for a variety of antibodies.

EXPERIMENTAL SECTION

Materials. Taxol was purchased from ICN Biomedicals Inc. (Aurora, OH). A mouse anti-paclitaxel monoclonal antibody type IgG2a specific to paclitaxel was purchased from Hawaii Biotechnology (Aiea, HI). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Dimethyl formamide (DMF), *N*-hydroxysuccinimide (NHS), dimethyl pimelimidate, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCA), human plasma, horseradish peroxidase (HRP), donkey IgG-HRP, and goat IgG−HRP conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). 7-Succinyltaxol (7-SucTax) was a gift from Hawaii Biotechnology.

Synthesis of HRP-Labeled Taxol Derivative. The synthesis of HRP-labeled taxol was performed as described previously.[13] Briefly, to 700 μL of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7) a 500 μL solution of 7-SucTax (0.6 mg, 0.63 μmol) in anhydrous DMF was added, followed by 300 μL of a 1.0 M EDCA solution (191.4 mg in 1 mL of water) and 500 μL of 0.92 μmol of NHS (0.2 mg, 0.92 μmol) dissolved in PBS. The reaction mixture was stirred at room temperature for 10 min before a solution of HRP (2.4 mg, 60 nmol) in 1 mL of PBS was slowly added. The mixture was stirred for 24 h at room temperature followed by dialysis overnight at 4 °C in PBS. The final product was stored in the freezer with 50% glycerol until use.

Molecular Biology, Bacterial Strains, and Plasmids. DNA manipulations were performed according to standard procedures unless specified otherwise.[14] PCR was performed using the *Pfu* DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer's instruction. *Escherichia coli* strains JM109 (*rec*A1 *sup*E44 *end*A1 *hsd*R17 *gyr*A96 *rel*A1 *thi* Δ(*lac-pro*AB) F′ [*tra*D36 *pro*AB$^+$ *lac*I^q *lac*Z ΔM15]) and BLR(DE3) (*recA*$^-$ *hsdS gal* (λ*c*I*ts*857 *ind*1 Sam7 *nin*5 *lac*UV5-T7 gene *1*)) (Novagen, Madison, WI) were grown on LB agar for solid culture and in terrific broth for liquid culture. All media contained 0.1 mg/mL ampicillin for selection. Plasmid pET-Ela78h6[15] carrying the 78 ELP (ELP78) repeat used as the source of the ELP gene and plasmid pRIT12[16] carrying the protein A gene was used as a template for PCR amplification.

Construction of pELP−SpA. A DNA fragment coding for SpA from pRIT12 was amplified as a 926-bp PCR fragment using primers (Loma Linda University, CA) SpA1 (5′-atacccggga ggaggaggag gagcgcaaca cgatgaagct-3′) and SpA2 (5′-tatggtgacc aggcttgtta ttgtcttc-3′). The PCR product was digested with *Xma*I and *BstE*II and inserted into a similarly digested pET-Ela78h6, resulting in pELP−SpA.

Expression and Purification of ELP−SpA. *E. coli* strain BLR(DE3) containing plasmid pELP−SpA was inoculated from a single colony and grown in 3 L of terrific broth medium in a BIOFLO 3000 fermenter (New Brunswick Scientific, Edison, NJ) at 37 °C and pH 7.0 until $OD_{600} = 1$. Expression was induced by the addition of IPTG to a final concentration of 1 mM. After 6 h, the culture was harvested, washed, and resuspended in PBS (pH 7). Cells were then lysed with a French press at 20,000 psi (SLM Instruments, Inc.), and cell debris was removed by centrifugation for 15 min at 30000*g*.

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Purification of ELP−SpA was achieved by repeated temperature transitions. After adding NaCl to the cell free extract to a final concentration of 1 M, the sample was heated to 37 °C and centrifuged at 30,000 *g* in 37 °C for 15 min. The pellet containing ELP−SpA was dissolved in ice-cold PBS and centrifuged at 30,000 *g* at 4 °C for 15 min to remove undissolved proteins. This temperature transition cycle was repeated once more, and the pellet containing ELP−SpA was finally dissolved in ice-cold PBS. The purity of the protein preparation was determined by silver staining (Bio-Rad, Hercules, CA) after SDS−PAGE electrophoresis. To confirm the expression of the fusion protein, western blot was performed using a goat IgG-alkaline phosphatase (AP) conjugate and the AP color reagent (Bio-Rad).

Characterization of ELP−SpA

The inverse-phase transition of the ELP−SpA was determined spectrophotometrically in a 96-well microplate reader (POLARstar Optima, BMG Labtechnologies, Inc.). Either 0.1 mM ELP78 or ELP−SpA in 0.1 mL of PBS containing 0.5 M NaCl was added to the wells. The well temperature was increased from 24 to 40 °C, and the absorbance at 620 nm was measured.

To demonstrate the antibody-binding property of the ELP−SpA fusion during phase transition, 0.1 mg of ELP and ELP−SpA was mixed with 1:2500 dilutions of HRP, human IgM−HRP, goat IgG−HRP, and donkey IgG−HRP (1 mg/mL each) in 100 μL of PBS. After 30-min incubation at room temperature, precipitates were recovered by the addition of 0.5 M NaCl and centrifugation at 15000*g* while keeping temperature at 37 °C. All precipitates were washed with 100 μL of PBS, resolubilized in 50 μL of ice-cold PBS, and transferred into microplate wells. Recovered HRP activity was signalized with 100 μL of substrate (20 mg of *o*-phenylenediamine in 10 mL of 0.1 M pH 4.6 citrate−phosphate buffer, containing 4 μL of 30% H_2O_2) for 10 min, and the absorbance was measured at 490 nm using a microplate reader (model 3550-UV, BioRad). The stability of the ELP−SpA−IgG complex was investigated by repeating the precipitation and solubilization cycle two more times as described above.

Phase-Separation Immunoassay for Paclitaxel. A stock solution of paclitaxel was prepared in DMSO to a final concentration of 10 mg/mL. To prepare the ELP−SpA−IgG complex, 10 μg of ELP−SpA and 1 mg of ELP in 500 μL of PBS were mixed with 0.1 μg of anti-paclitaxel IgG. The complex was recovered by precipitation and solubilized in 500 μL of cold PBS. For the paclitaxel assay, serial dilutions (100 μL) of paclitaxel were prepared in PBST with 0.1% DMSO and mixed with 2.5 ng of taxol−HRP. After mixing the samples with 10 μL of ELP−SpA−IgG complex at room temperature for 30 min, 10 μL of 5 M NaCl was added and the mixture was heated to 37 °C to precipitate the ELP−SpA−IgG-antigen complex. The precipitate was pelletized by centrifugation at 15000*g* while maintaining the temperature, washed with 100 μL of PBS for 5 min, and resolubilized in 100 μL of ice-cold PBS. The amount of bound taxol−HPR was quantified with 100 μL of HRP substrate, and the absorbance of each well was measured at 490 nm.

RESULTS AND DISCUSSION

Production and Purification of the ELP−SpA Fusion Proteins. To generate a fusion protein composed of a temperature-responsive ELP domain and an antibody-binding SpA domain, the gene fragment coding for the IgG-binding domain of SpA was PCR amplified and fused to the 3′ end of a gene coding for 78 ELP repeats (Figure 1). A linker consisting of five glycine residues was inserted between ELP and SpA to provide flexibility and to minimize interference between the domains.

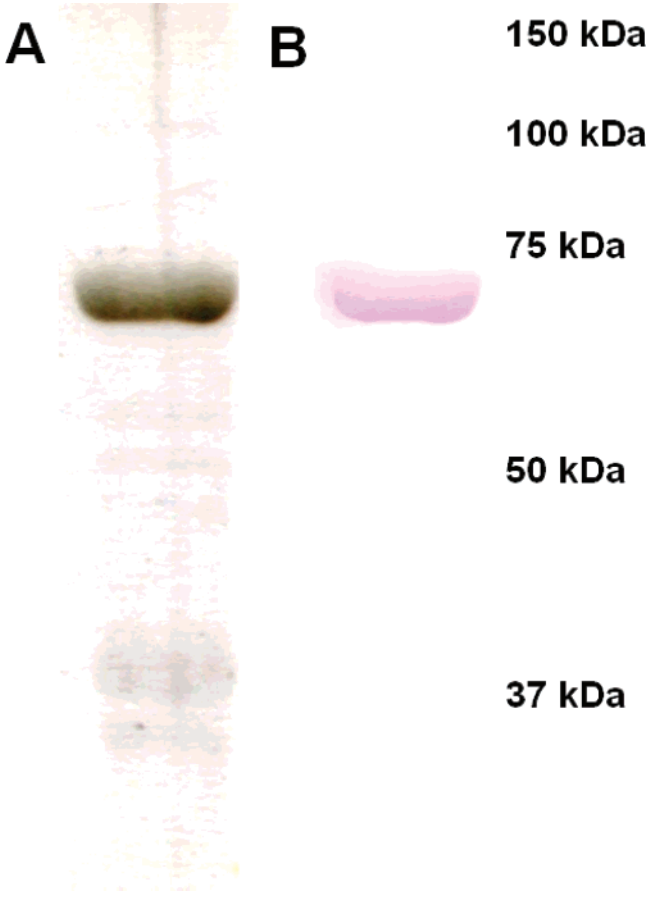

Figure 2. Purification of ELP−SpA fusions by two cycles of inverse phase transition. The purity of the fusion proteins was analyzed by (A) 10% SDS−PAGE gel and (B) Western blot analysis with goat IgG−alkaline phosphatase conjugates.

The ELP−SpA fusion protein was produced in *E. coli* BLR(DE3) and easily purified by taking advantage of the reversible phase-transition property of the ELP domain. Phase transition was induced by adding 1 M NaCl to the cell lysate, and aggregation was immediately visible at room temperature. A pellet containing primarily the ELP−SpA fusions was obtained after centrifugation. After solubilization at 4 °C, the supernatant was subject to an additional round of inverse temperature cycles resulting in highly purified ELP−SpA fusions. The purity of the protein was determined by silver staining of SDS−PAGE gel (Figure 2A), and only a single protein band corresponding to the expected size of the fusion (67 kDa) was detected. The presence of the SpA domain was confirmed by Western blot analysis using a goat IgG−AP conjugate (Figure 2B), which interacts specifically with the SpA domain.[17] Up to 68 mg/L purified ELP−SpA fusion was obtained using the inverse transition cycle. This yield is in the range of ELP[15] and is significantly higher than that obtained previously with ELP−scAb (1 mg/L).[4] This is a major benefit of the reported ELP−SpA fusion technology for immunoassay because of the increased protein production.

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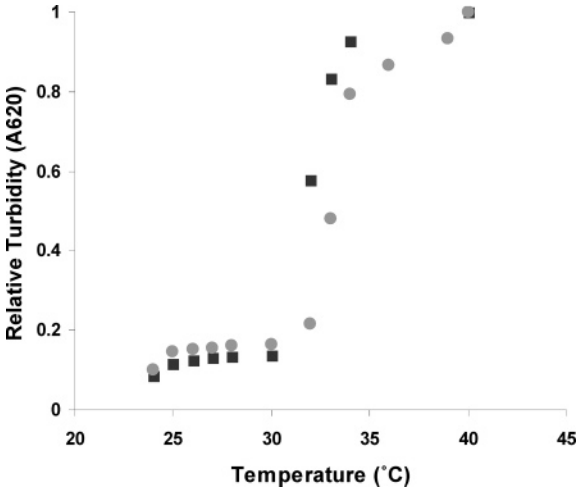

Figure 3. Turbidity profiles of (■) ELP and (●) ELP−SpA undergoing inverse temperature transition.

Table 1. Binding of Antibodies to ELP−SpA after Thermal Precipitation as Indicated by the Conjugated HRP Activity

Δ*A*490	ELP−SpA	ELP
donkey IgG-HRP	1.455	0.038
goat IgG-HRP	1.070	0.030
human IgM-HRP	1.843	0.098
HRP[a]	0.153	0.061

[a] The same concentration of HRP instead of antibody−HRP conjugate was used.

Characterization of ELP−SpA Fusion. The functionality of the ELP domain was demonstrated by its ability to obtain highly purified fusion protein with the inverse temperature cycling. The transition profiles of purified ELP−SpA fusions were further characterized (Figure 3). Turbidity measurements were used to determine the onset of folding and aggregation. The value of T_t, defined as the temperature at which 50% turbidity occurred, was used to indicate the phase-transition properties.[18] The resulting T_t (33 °C) of the ELP−SpA fusion was very similar to ELP (32 °C), indicating that the transition property was not affected by fusion to the SpA domain.[19] The relatively low transition temperature of the fusion also allows simple phase separation to occur at mild conditions compatible with those required for preserving antibody-binding activity.

To investigate whether the binding capability of the SpA domain is preserved during the reversible phase-transition, binding experiments with different types of antibodies (human IgM, goat IgG, donkey IgG) were performed by mixing them individually with ELP−SpA fusions. After binding for 30 min, the complex was recovered by precipitation and the amount of IgG or IgM bound was quantified by the conjugated HRP activity. As shown in Table 1, ELP−SpA bound all three antibodies and produced appreciable signals, clearly demonstrating that the interaction between SpA and antibodies is not affected by the phase transition.

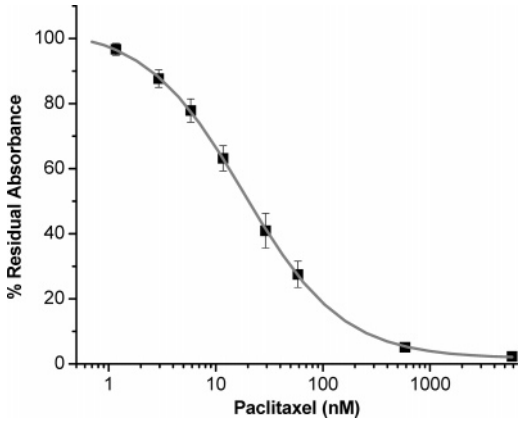

Figure 4. Calibration plot for paclitaxel using the phase-separation immunoassay. The results are the average of four independent measurements, with error bars showing ± standard deviation.

Conversely, only background levels of binding were detected when either ELP or HRP was used as a control. This property is extremely desirable because any antibody of interest could be easily complexed with ELP−SpA and recovered by the thermally triggered precipitation. The amount of bound IgG remained relatively constant even after three repeating transition cycles (data not shown), suggesting very strong interaction between the SpA domain and IgG.

Phase-Separation Immunoassay for Paclitaxel. As a model system to demonstrate the utility of the ELP−SpA fusion in phase-separation immunoassay, paclitaxel, which has antineoplastic activity against several tumors but a very narrow therapeutic range before showing toxicity in clinical studies, was chosen as the detection target.[20] A competitive phase-separation immunoassay was performed based on thermally triggered precipitation of the ELP−SpA−IgG−paclitaxel complex in the presence of HRP-labeled taxol derivatives. After separation of the immunocomplex from the reagents, the amount of HRP-labeled taxol bound to the antibody can be easily quantified. Figure 4 shows a calibration curve for paclitaxel generated by the phase-separation competitive immunoassay. The IC_{50} of 20.18 nM and the lower detection limit of 2.94 nM (10% inhibition) are comparable to results reported with the same antibody using a fluoroimmunoassay.[21] In addition, the lower detection limit was on a par with that (4.10 nM) reported by the manufacturer based on ELISA, suggesting that binding to the SpA domain had no adverse effect on the paclitaxel-binding affinity of the antibody. The assay also showed a broad dynamic range, displaying high linearity between 3 and 60 nM ($y = -20.764\ \mathrm{Ln}(x) + 112.39$, $R^2 = 0.9936$) and good reproducibility as demonstrated by the low residual standard deviation of less than 10% for four replicates.

CONCLUSIONS

In this study, we exploit the reversible phase-transition property of ELP to thermally precipitate proteins and their corresponding ligands[7,15,22,23] in engineering an ELP−SpA fusion that is useful

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as a universal platform for phase-separation immunoassay. The resulting ELP−SpA fusion retained the ability to reversibly aggregate and to bind IgG or IgM with high affinity. Utility of the ELP−SpA fusion in a competitive phase-separation immunoassay format was successfully demonstrated using a paclitaxel-specific IgG with a detection limit similar to that reported in the literature, validating that the sensitivity of the IgG is not compromised by the thermal precipitation. To further extend our capability to target a broader class of immunoglobulins, Streptococcal protein G and peptostreptococcal protein L, which have binding preferences different from those of SpA, could be similarly employed to provide a cocktail of ELP fusions useful for any target analytes. In addition, the facile recovery of the ELP−SpA−IgG complex by a simple thermal precipitation may also be exploited for the economical and highly efficient purification of immunoglobulins directly from cell cultures, providing a simple approach to generate ELP−SpA−IgG complexes for analysis. These strategies are currently under investigation.

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ACKNOWLEDGMENT

This work was supported by a grant from the University of California Water Resources Center and NSF (EIA0330451). We thank Dr. George Georgiou for providing the plasmid coding for SpA.

Received for review October 22, 2004. Accepted January 23, 2005.

AC0484326