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Anti-Idiotype scFv–Enzyme Fusion Proteins: A Clonable Analyte-Mimicking Probe for Standardized Immunoassays Targeting Small Biomarkers

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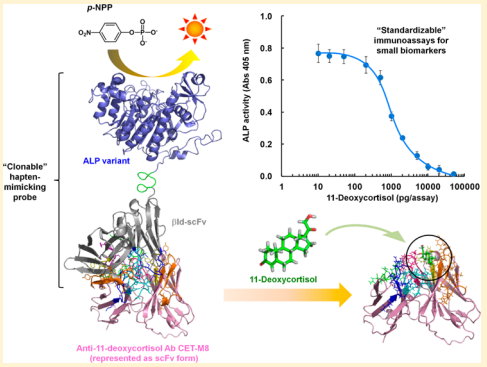
Supporting Information

ABSTRACT: Most immunoassays use probes that convert concentrations of analytes into signal intensity. To prepare the probes, analytes are usually linked to a reporter protein (e.g., enzymes) with the aid of chemical reagents. However, these conventional methods yield a mixture of heterogeneous products and consequently reduce assay performance. "Clonable" homogeneous probes, i.e., recombinant molecules in which a target protein is genetically fused to a reporter with a defined coupling ratio, are now available for analyzing protein biomarker concentrations. Here, we have expanded this strategy to measure small biomarkers (haptens) by genetically fusing proteinaceous molecules that mimic target haptens with enzymes. 11-Deoxycortisol (11-DC) was chosen as the model hapten, and the β-type anti-idiotype antibodies (βId-Abs) that recognize the paratope of anti-hapten antibodies were used as the target hapten mimic. The V_H and V_L genes of a βId-Ab, targeting a mouse anti-11-DC antibody (CET-M8), were assembled to encode a single-chain Fv fragment (βId-scFv), which was then fused with a gene encoding a variant of alkaline phosphatase. The product, βId-scFv-ALP′ protein, had satisfactory enzyme activity and bound to CET-M8 in a competitive manner with 11-DC. A colorimetric enzyme-linked immunosorbent assay (ELISA) for 11-DC, based on the competitive reaction between the analyte and βId-scFv-ALP′ against immobilized CET-M8, was found to be sensitive (limit of detection = 22 pg/assay) and specific (cross-reactivity with cortisol, 0.24%) for clinical use and could be used to determine serum 11-DC levels after a simple solvent extraction. The anti-idiotype scFv–enzyme fusion proteins proposed here can be prepared reproducibly as homogeneous products with a 1:1 coupling ratio and would facilitate standardization of immunoassays for small biomarkers.



Immunoassays are essential tools for trace characterization of physiologically active substances (biomarkers), because of their excellent specificity, feasibility, and higher sensitivity when compared to other methods.[1,2] Determination of small biomarker molecules, e.g., steroid/thyroid hormones, catecholamines, eicosanoids, and synthetic drugs/herbicides/industrial chemicals, which are called "haptens", has been based on the competitive format, in which unlabeled haptens and labeled haptens are reacted with a limited amount of anti-hapten antibodies. Another format, noncompetitive assays (immunometric assays), in which excess amounts of labeled-antibody are used to capture analyte, often provide higher sensitivity and precision, but are difficult to apply to the measurement of haptens.[3,4] Although the development of noncompetitive hapten assays has recently become a research trend,[3−9] the competitive formats are just as widely used as before, because of their simpler setup and well-established theoretical bases.

In modern nonisotopic competitive assays, enzymes or fluorescent/luminescent proteins (reporter proteins) are covalently linked to analytes or antibodies to prepare probes, which enable the conversion of analyte concentrations into signal intensities. For this purpose, analytes (or antibodies) and reporter molecules have been chemically linked using carbodiimides, glutaraldehyde, or bifunctional coupling reagents. However, these methods yield heterogeneous products that are composed of various molecules conjugated at different molar ratios. Therefore, it is almost impossible to prepare the probes as a single molecular species with a definite coupling molar ratio, which hampers the ability to standardize immunoassay systems. Moreover, unconjugated reactants (analyte/antibody and reporter molecules), which often remain in the products, reduce assay sensitivity.[3,4,7]

Recent advances in genetic engineering have led to the generation of molecules wherein proteinaceous analytes are

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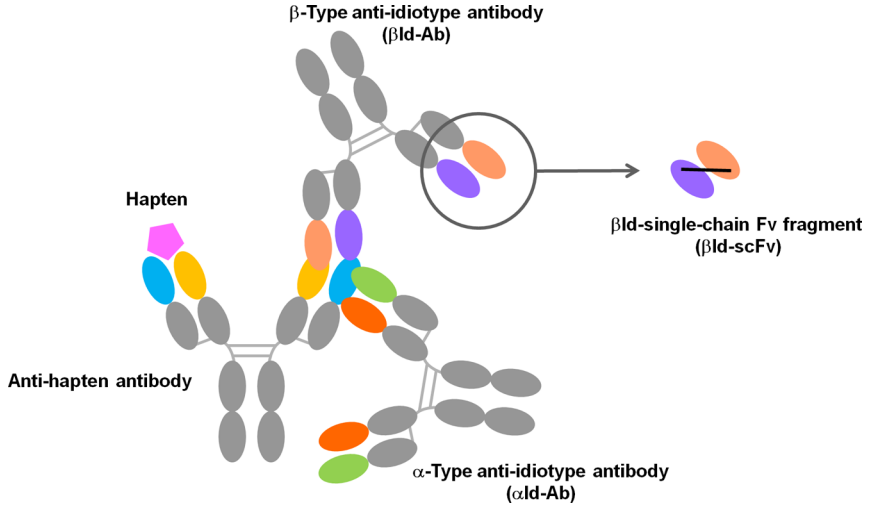

Figure 1. Schematic representation of the specificity of the α-type and β-type anti-idiotype antibodies (αId-Ab and βId-Ab) that bind to an anti-hapten antibody. The variable regions of βId-Abs (and, therefore, the scFvs prepared from βId-Abs) bind to the paratope of the anti-hapten antibody as they mimic the relevant hapten molecule.

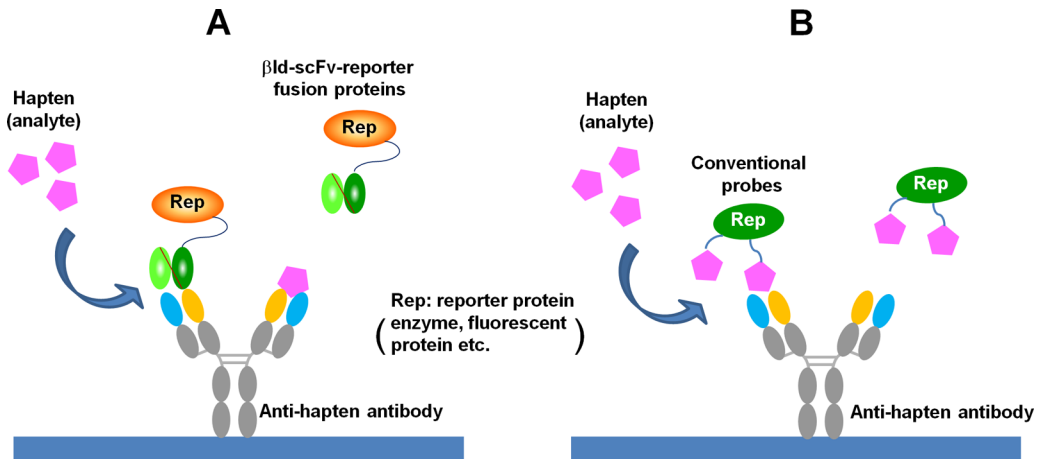

Figure 2. Principles of competitive hapten immunoassays using (A) βId-scFv-reporter fusion proteins (a novel clonable probe proposed in this study) and (B) conventional probes (chemically linked hapten-reporter conjugates).

fused to reporter proteins. Because of their "clonable" nature, which allows for reproducible preparability as a homogeneous molecule with a defined analyte/reporter molar ratio, these fusion molecules can work as ideal probes in immunoassays that measure protein levels. Here, we have expanded this strategy for the determination of small biomarkers, which are immunochemically categorized as haptens, by employing clonable proteinaceous molecules that are equivalently immunoreactive as the target hapten. The anti-idiotype antibodies (Id-Abs) that recognize the paratope of anti-hapten antibodies are the most promising molecules for this purpose.

Id-Abs are the second antibodies specific to idiotopes, which are the epitopes located on the variable regions of a particular primary antibody.[5,10−12] Id-Abs that bind to the paratope of the primary antibody, mimicking the original antigen, are called β-type Id-Abs (βId-Abs). βId-Abs compete with the original antigen when binding the primary antibody, while the α-type Id-Abs recognize the framework of the variable region, permitting the antigen to bind simultaneously (Figure 1). Thus, the variable regions of βId-Abs work as biomimics or surrogates of the original antigen and can be used as immunoassay probes after labeling with a suitable reporter molecule.

Single-chain Fv fragments (scFvs), in which the heavy-chain and light-chain variable domains (V_H and V_L, respectively) are genetically combined via flexible peptide linkers, are prepared by expressing a single gene.[13,14] Thus, scFvs of βId-Abs can be fused with reporter proteins, to produce fusion proteins that are excellent probes in competitive hapten immunoassay systems (see Figure 2).

In this study, a corticosteroid, 11-deoxycortisol (11-DC; M_r = 346.5), was used as a model analyte. 11-DC is the biosynthetic precursor of cortisol and a diagnostic index for pituitary-adrenal function.[15,16] We constructed an scFv by linking the V_H and V_L from a mouse βId-Ab that is specific to an anti-11-DC antibody (CET-M8) and fused it with a mutant of alkaline phosphatase (ALP). The resulting fusion protein worked successfully in a competitive enzyme-linked immunosorbent assay (ELISA) system for measuring human serum 11-DC levels.

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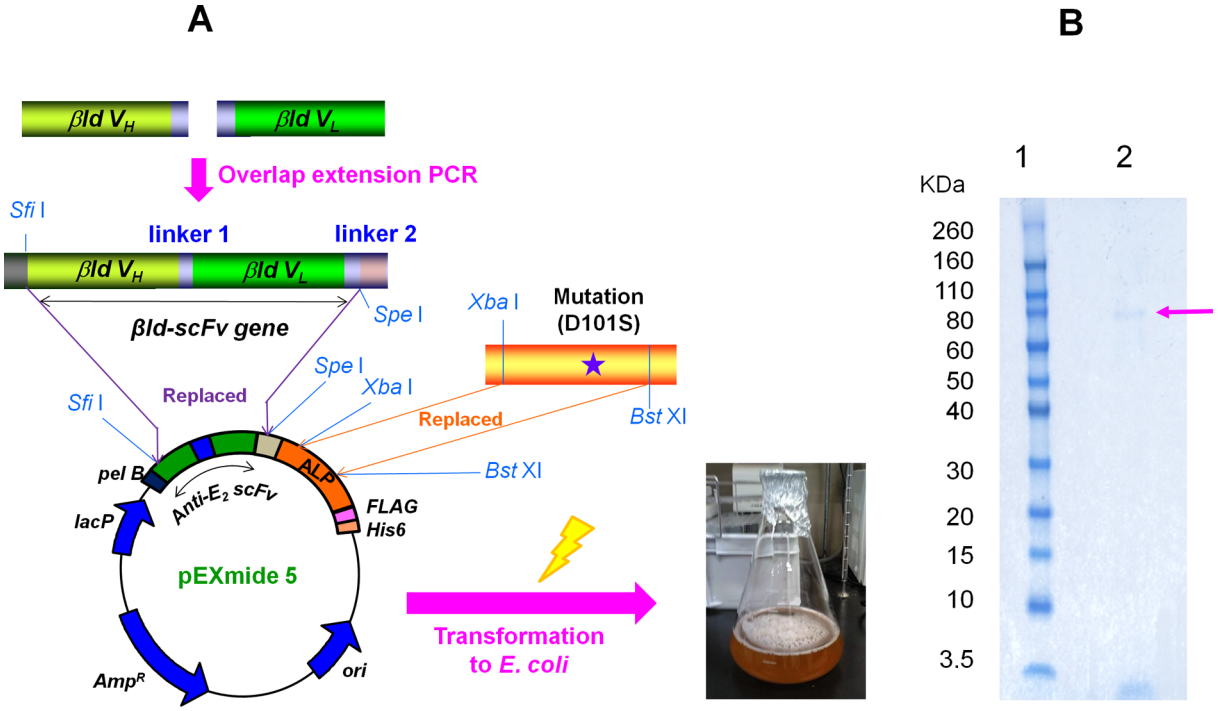

Figure 3. (A) Schematic diagram of construction and expression of the *βId-scFv-ALP′* fusion gene. (B) SDS-PAGE (Coomassie brilliant blue staining); the M_r marker resides in lane 1, and the affinity-purified βId-scFv-ALP′ fusion protein is indicated with a red arrow in lane 2.

MATERIALS AND METHODS

Buffers. The buffers used in this study were as follows: PB, 50 mM sodium phosphate buffer (pH 7.3); PBS, PB containing 9.0 g/L NaCl; G-PBS, PBS containing 1.0 g/L gelatin; M-PBS, PBS containing 20 g/L skim milk; T-PBS, PBS containing 0.050 (v/v)% Tween 20; and G-TBS, 50 mM Tris-HCl buffer (pH 7.4) containing 9.0 g/L NaCl and 1.0 g/L gelatin.

Hybridomas and Antibody. Hybridoma clones S.CET.M8.1.1[17] and BA1#38,[18] each of which secretes mouse anti-11-DC antibody CET-M8 (γ1, κ) and mouse βId-Ab BA1#38 (specific to the paratope of CET-M8) (γ1, κ), respectively, were previously established in our laboratory.

Preparation of scFv-ALP′ Fusion Protein. *Cloning of βId-Ab V Genes.* Total RNA was extracted from BA1#38 hybridoma cells (~1 × 10^7) and reverse-transcribed using a mγ1-GSP1 [C_H1(γ1)-specific] primer[19] to produce first-strand cDNA. The V_H gene fragment was cloned by rapid amplification of cDNA 5′-ends (5′RACE),[20] as described previously.[19] The V_L gene fragment was amplified by polymerase chain reaction (PCR) from the cDNA using V_L-IV/VI[21] and mκ-GSP ($C_κ$-specific) primers.[22] Both DNA fragments were gel-purified, ligated into pBluescript II (Toyobo), and transformed to *Escherichia coli* (*E. coli*) XLOLR cells (Stratagene).[19,23] Plasmid was extracted from the transformants and sequenced using standard methods.[23]

Preparation of the βId-scFv Gene. The V_H and V_L genes were separately reamplified to add a part of the linker sequence, FLAG tag, and restriction sites, using the first-strand cDNAs, as described previously.[19] The primers used were V_H-Rev and V_H-For for amplifying V_H and V_L-Rev and V_L-For for amplifying V_L, respectively. The nucleotide sequences of these primers are shown in Table S1 in the Supporting Information. The amplified products (modified V_H and V_L DNA fragments) were gel-purified and spliced by overlap extension PCR to generate the *scFv* gene (Figure 3A).[22] This procedure was performed using a 25-μL reaction mixture containing both DNA fragments (each 200 ng) and *Ex Taq* polymerase (0.65 U), with the following cycling profile: 10 cycles each of 95 °C (1 min), 55 °C (1 min), 72 °C (3 min), followed by a single incubation step at 72 °C (10 min). An aliquot (10 μL) of this solution was used for the next PCR, performed in a 100-μL reaction mixture containing the V_H-Rev and V_L-For-2 primers (see Table S1 in the Supporting Information) (each 100 pmol) with the same conditions as described above, except that 15 cycles were performed. In this *scFv* gene, the V_H and V_L genes were combined via a *linker1* sequence that encodes the common joint sequence $(GGGGS)_3$.[19,22] A *linker2* sequence encoding a peptidase-resistant peptide (GSTSGSGKSSEGKG),[24] was attached at the 3′-end.

Cloning of the βId-scFv-ALP′ Gene. We had previously cloned a fusion gene encoding an anti-estradiol-17β (E_2) scFv linked with a variant of ALP (EC 3.1.3.1) that has a D101S substitution (ALP′, which has been reported to increase enzyme activity)[25] in a pEXmide 5 expression plasmid[26] (Figure 3A: procedure is described in the Supporting Information). The *Sfi* I–*Spe* I fraction of this recombinant plasmid, which includes the entire *anti-E_2 scFv* gene and several 5′-nucleotides of *linker2*, was replaced with the *βId-scFv* DNA (described above) that had been digested with *Sfi* I and *Spe* I. Consequently, in the resulting plasmid, a *βId-scFv-ALP′* fusion gene (5′-*V_H-linker1-V_L-linker2-ALP′-FLAG tag-His6 tag*) was constructed (Figure 3A).

Expression and Purification of the βId-scFv-ALP′ Fusion Protein. The recombinant plasmid prepared above was transformed to *E. coli* XL1-Blue cells. A single colony of the transformants was grown and protein expression was induced.[19,23] Periplasmic extracts of the cells, in which the βId-scFv-ALP′ fusion protein was present, then were prepared[19,23] and used in the subsequent immunoassays. For gel electrophoresis and to determine enzyme activity, βId-scFv-

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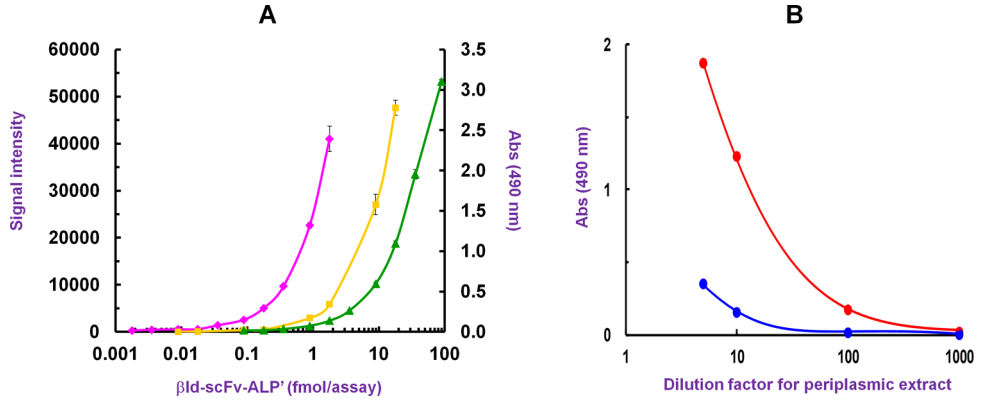

Figure 4. (A) Dose−response curves for enzyme activity of the affinity-purified βId-scFv-ALP′ fusion protein assessed by colorimetric (green triangle, −▲−), fluorometric (magenta diamond, −◆−), and chemiluminometric (yellow-orange square, ■) assays. The vertical bars indicate the SD (n = 4). Concentrations of purified βId-scFv-ALP′ solutions were determined based on absorption at 280 nm with the molar extinction coefficient estimated to be 88010.[55] (B) Binding of the βId-scFv-ALP′ fusion protein (periplasmic extracts) to the CET-M8 antibody immobilized on microplates in the presence (blue circle, ●) and absence (red circle, ●) of 100 ng of 11-DC. Bound enzyme activity was measured colorimetrically.

ALP′ was purified by affinity chromatography, using an anti-FLAG M2 affinity gel (Sigma).[23] Both the crude (periplasmic extracts) and purified βId-scFv-ALP′ protein were stored at −20 °C until use, and these were stable for over a year.

Measurement of the ALP′ Activity of the βId-scFv-ALP′ Fusion Protein. *Colorimetric Assay.*[27] For this assay, 0.10 M carbonate buffer (pH 10.0) containing 1.0 mM *p*-nitrophenyl phosphate and 0.10 g/L $MgCl_2 \cdot 6H_2O$ (100 μL) was added to microwells in 96-well microplates (Costar No. 3590) in which purified βId-scFv-ALP′ diluted in G-TBS (20 μL) had been placed, and was mixed; subsequently, the mixture was incubated at 37 °C for 1 h. After addition of 0.10 M NaOH (100 μL) and subsequent mixing, absorbance at 405 nm was measured using a Model 680 microplate reader (Bio-Rad).

Fluorometric Assay.[28] A substrate buffer containing 1.0 mM 4-methylumbelliferyl phosphate (100 μL) was added to microwells in 96-well black microplates (Costar No. 3925) in which purified βId-scFv-ALP′ diluted in G-TBS (20 μL) had been placed and was mixed; the mixture was incubated at 37 °C for 1 h. Then, 0.50 M K_2HPO_4−KOH buffer (pH 10.4) containing 10 mM EDTA (100 μL) was added and mixed, and fluorescence at 460 nm (excitation, 320 nm) was measured using a FLUOstar OPTIMA microplate reader (BMG Labtech).

Chemiluminometric Assay.[29] The BM chemiluminescence ELISA substrate (Roche Applied Science) that contains CSPD (disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate) (150 μL) was added to microwells in No. 3925 black microplates, in which purified βId-scFv-ALP′ diluted in G-TBS (10 μL) had been placed and was mixed; the mixture was incubated at room temperature for 10 min. Luminescence at 475 nm then was measured using the FLUOstar OPTIMA microplate reader.

ELISA Using the βId-scFv-ALP′. *Evaluation of Fundamental Assay Performance.* Microwells in No. 3590 microplates were coated with a solution of affinity-purified goat anti-mouse IgG+IgM antibody (Jackson ImmunoResearch) and then blocked with M-PBS.[30] The antibody CET-M8 (hybridoma supernatants) diluted with G-PBS (100 μL/well) was added and incubated at 37 °C for 1 h. After washing the wells three times with T-PBS, a diluted βId-scFv-ALP′ (periplasmic extract) (100 μL/well) and various concentrations of 11-DC standard (or its analogues) (50 μL/well), both diluted with G-PBS, were added and mixed; the mixture was incubated at 37 °C for 1 h. After washing three times with T-PBS, the enzyme activity in the wells was determined with the colorimetric, fluorometric, or chemiluminometric assays described above.

Pretreatment of Serum Specimens. Serum specimens (200 μL each) collected from 6 healthy male (age 20−52 years) and 18 female (age 18−24 years) individuals were extracted with methylene chloride (1.0 mL), as described previously.[7] The residue was dissolved in G-PBS (250 μL), and 50-μL aliquots (in duplicate) were used to determine 11-DC levels as described below.

Determination of Serum 11-DC Levels. Diluted βId-scFv-ALP′ (periplasmic extract) (100 μL/well) and the serum extract (or 11-DC standard) (50 μL/well), both diluted with G-PBS, were added to the microwells on which CET-M8 had been indirectly coated as described above. The solution was mixed and incubated at 37 °C for 1 h. After washing three times with T-PBS, the enzyme activity on the wells was determined with the colorimetric assay described above.

Conventional ELISA Using Enzyme-Labeled 11-DC. Preparation for the ALP-labeled and peroxidase (POD)-labeled 11-DC and ELISA procedure, in which these conventional probes were used, have been described in the Supporting Information.

■ RESULTS

Gene Cloning for the V_H and V_L Domains of βId-Ab. The V_H and V_L genes of the mouse monoclonal βId-Ab (BA1#38)[18] that recognizes the paratope of mouse anti-11-DC antibody CET-M8[17] were cloned and sequenced. The amino acid sequences of the V_H and V_L were deduced from these nucleotide sequences (see Figure S1 in the Supporting Information), and the complementarity-determining regions (CDRs) therein were determined according to the Kabat definition.[31]

Construction and Bacterial Expression of the βId-scFv-ALP′ Fusion Gene. A gene fragment that encodes scFv of βId-Ab (βId-scFv) was prepared by splicing the V_H and V_L genes, using overlap extension PCR in the orientation of 5′-V_H-*linker*-V_L-3′ (Figure 3A). The βId-scFv protein, which was expressed in *E. coli*, showed anti-idiotype activity similar to that

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of the parent βId-Ab (data not shown). A protein ribbon structures of this βId-scFv and the primary antibody CET-M8 (as scFv form) were constructed using the SWISS-MODEL protein modeling server[32] (see Figure S2 in the Supporting Information). Both of these model structures had the characteristic V-domain architecture, i.e., the CDR amino acids formed flexible loops that were raised above the surface of the rigid β-sheet scaffold. Conformation of the idiotype-anti-idiotype complex between the βId-scFv and the scFv form of CET-M8, and conformation of the CET-M8 scFv when docked with 11-DC, were also predicted with the ZDOCK[33] and SwissDock[34] docking servers, respectively (Figure S2 in the Supporting Information).

This *βId-scFv* gene was then fused with a gene encoding a variant of alkaline phosphatase (ALP′), that had the D101S substitution; this substitution has been reported to enhance enzyme activity[25] (Figure 3A). The amino acid sequence of ALP′ is shown in Figure S1 in the Supporting Information. Genes for His6 and FLAG tags were added to the 3′-terminus of the *βId-scFv-ALP′*. The pEXmide 5 phagemid[26] with this fusion gene was transformed to XL1-Blue cells, and the βId-scFv-ALP′ fusion protein was expressed in the periplasm of a cloned transformant. During sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), βId-scFv-ALP′ ran as a single band at the predicted molecular mass (calculated as 75783.9 for the 1:1 fusion of βId-scFv and ALP′) (Figure 3B, lane 2).

Enzyme Activity and Anti-Idiotype Reactivity of βId-scFv-ALP′. The enzyme activity of βId-scFv-ALP′ was assessed with colorimetric, fluorometric, and chemiluminometric assays (Figure 4A). As expected, the fluorometric and the chemiluminometric assays allowed for more sensitive detection than the colorimetric assay. The limit of detection (LOD) of these assays, defined as the amount (per assay) that provided signals two standard deviation (SD) greater than the average (n = 10) of the signal at zero concentration, was 62.5 amol for the colorimetric assay, 2.20 amol for the fluorometric assay, and 11.0 amol for the chemiluminometric assay, respectively.

βId-scFv-ALP′ strongly bound to the relevant primary antibody (CET-M8) in a dose-dependent manner, and this binding was inhibited by addition of 11-DC, which is the original antigen for CET-M8 (Figure 4B). Thus, βId-scFv-ALP′ retained binding activity to the CET-M8 paratope.

ELISA for 11-DC Using βId-scFv-ALP′ as the Probe. ELISA was performed according to the principle shown in Figure 2A. The analyte 11-DC was reacted to the antibody CET-M8 that had been coated on microplates in a competitive manner with a fixed amount (see below) of βId-scFv-ALP′. The bound βId-scFv-ALP′ was detected with a common colorimetric method as described above. The amount of βId-scFv-ALP′ used in these competitive reactions was adjusted to give enzyme activities at an absorbance of $B_0 \approx 1.0$ after a 30-min enzyme reaction. The blank absorbance (ELISA signals in the absence of βId-scFv-ALP′) was below 5% of the B_0 value. Dose−response curves of 11-DC were constructed in the range of 10−100000 pg per assay (Figure 5). The midpoint (the amount of 11-DC causing 50% inhibition)[23,35] was 800 pg, and the LOD [the amount of the analyte required to give bound enzyme activity that was two SDs below the average (n = 10) of the B_0 value] was calculated to be 22 pg (63 fmol).

For comparison, this ELISA format was also performed with fluorometric and chemiluminometric detection (Figure 5). An obvious improvement in assay sensitivity (but with slightly greater within-assay variances) was observed in those ELISAs, and this improvement was reasonably associated with the detection sensitivity of βId-scFv-ALP′ described above. The midpoint was 330 pg with the fluorometric assay, and 260 pg with the chemiluminometric assay. These βId-scFv-based ELISAs were as sensitive as the conventional competitive hapten ELISA systems (the principle is shown in Figure 2B), which we performed using ALP-labeled 11-DC or POD-labeled 11-DC as the probes (Figure 5).

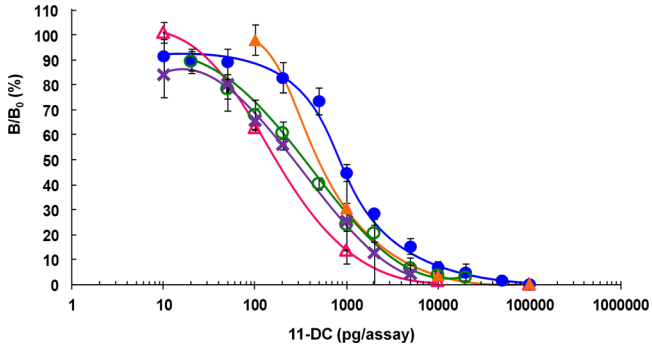

Figure 5. Dose−response curves of the ELISAs for 11-DC using βId-scFv-ALP′ fusion protein as the probe obtained with colorimetric (blue circle, ●; where the B_0/blank absorbance ratio was ∼20), fluorometric (green open circle, ○; where the B_0/blank fluorescence ratio was ∼60), or chemiluminometric (purple cross, ×; where the B_0/blank luminescence ratio was ∼35) detection and dose−response curves of the conventional ELISAs for 11-DC using ALP-labeled 11-DC (orange solid triangle, ▲) or POD-labeled 11-DC (magenta open triangle, △). The enzyme labeling of 11-DC and ELISA procedure are described in the Supporting Information. The vertical bars indicate the standard deviation, SD (n = 4).

The cross-reactivity with eight related endogenous steroids, determined based on the 50% displacement method[23,35] (Table 1), was compatible with, or even lower than, that of the ELISA using the conventional hapten-enzyme conjugate and radioimmunoassay (RIA)[17] using CET-M8. Very low cross-reactivity with cortisol (0.24%) is particularly favorable for clinical applications, because it usually circulates in the blood at much higher concentrations than 11-DC [serum (or plasma) reference values for adults: 11-DC, <10 ng/mL;[36,37] cortisol, 10−250 ng/mL[36−39]]. Although direct measurement was not

Table 1. Percent Cross-Reactivity[a] of the Present βId-scFv-Based ELISA and Related Immunoassays Using Anti-11-DC Antibody CET-M8

		conventional assay	
steroid	βId-scFv-based ELISA (present method)	ELISA[b]	RIA[c]
11-deoxycortisol	100	100	100
cortisol	0.24	0.50	0.2
cortisone	0.59	0.73	0.4
corticosterone	<0.05	0.05	0.02
11-deoxycorticosterone	2.5	9.5	7
17α-hydroxyprogesterone	10	13	7
progesterone	0.47		0.2
aldosterone	<0.05		
testosterone	<0.05		

[a]Determined using the 50% displacement method.[23,35] [b]Performed in this study using the POD-labeled 11-DC (see text). [c]Previously reported liquid-phase assay using a tritium-labeled 11-DC.[17]

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successful due to an unacceptable reduction in assay sensitivity (data not shown), simple methylene chloride extraction[7] (extraction rate >90%) provided reasonable assay values. The analytical recovery, when the 11-DC standard was added to charcoal-stripped serum at concentrations of 4, 20, and 100 ng/mL, was 5.1 (128%) ± 0.8, 19.4 (97.0%) ± 1.6, and 92.0 (92.0%) ± 19.8 ng/mL (mean ± SD; each n = 4), respectively. The serum 11-DC levels of healthy volunteers determined with the currently described ELISA, were as follows: 6.4 ± 1.8 (SD) ng/mL (18.5 ± 5.2 nmol/L) for healthy male individuals (n = 6) and 2.5 ± 1.5 (SD) ng/mL (7.2 ± 4.3 nmol/L) for healthy female individuals (n = 18), both of which were within the reference range (see above). It should be noted that a time-resolved fluoroimmunoassay of serum 11-DC that employed a chromatographic pretreatment afforded rather lower, and similar results both for male and female individuals (1.38 ± 1.12 and 1.60 ± 0.41 nmol/L, respectively).[40]

DISCUSSION

Over the past few decades, attention has been paid to the utility of βId-Abs that mimic small biomarker molecules (i.e., antibodies that bear an internal image of the cognate small antigens) in biomedical analytical fields. For instance, several murine monoclonal βId-Abs, for example, those specific to the primary antibody paratopes for alprenolol (a synthetic drug),[41] triamcinolone (a synthetic glucocorticoid),[42] aldosterone,[43] progesterone,[44] bradykinin,[45] and taxol[46] were produced by the standard immunization-based techniques and used to monitor the interaction of these haptens with their responsible receptors or cellular/tissue components. The βId-Abs mimicking cotinine,[47] estradiol,[48] and thyroxine[49] were used as the competitor in competitive enzyme immunoassay systems. In the cotinine immunoassay, the cotinine-mimicking βId-Ab was coated on microplates, and after the competitive reaction, bound anti-cotinine antibody was monitored by additional incubation with an enzyme-labeled protein A. In the latter two cases, estradiol-mimicking and thyroxine-mimicking βId-Abs were chemically linked to europium chelate and ALP, respectively. Subsequently, the use of βId-Abs, in combination with αId-Abs, paved the way for the measurement of haptens through the use of noncompetitive assay principles.[3,5] We have previously reported such an immunoenzymometric assay system for a steroid with attomole-range sensitivity.[4,7,50]

In the current study, we first described an advanced application, in which the variable regions of βId-Abs are used as the scFv formats that are fused genetically with a reporter protein (e.g., enzyme, as used in this study). The scFv-reporter fusion proteins are "clonable" homogeneous products expressed from a monocistronic transcript, which we can prepare reproducibly. Thus, by combining with cloned anti-analyte antibodies (i.e., monoclonal antibodies obtainable via hybridoma or genetic engineering techniques), we are able to construct completely standardized hapten immunoassay systems. The ideal 1:1 molar ratio between the βId-scFv and reporter provides practical sensitivity in the standard competitive assay systems, without developing the avidity issue that might be faced in ELISA systems using bivalent IgG-form antibodies with microplates on which multiple target antigens are immobilized.[30] Notably, the use of the βId-moiety as the competitor did not reduce the specificity of the assay, as seen with the results for cross-reactivity. Therefore, the present assay format would be an excellent choice, with respect to immunoassay developments.

The versatility of the present assay systems depends on the feasibility of establishing βId-Abs that mimic target analytes. Anti-idiotype antibodies are usually generated by immunization of animals with purified primary antibodies, which are often used after conjugation with immunogenic proteins (e.g., keyhole limpet hemocyanin).[51] This procedure requires a rather long period and laborious experiments. It has been reported that a wide variety of βId-scFvs can be selected from mutant antibody libraries.[52−54] The most recent instance reported an interesting application of βId-Ab to a ELISA system for measuring aflatoxins.[54] A camelid (alpaca) heavy-chain antibody, specific to an anti-aflatoxin antibody paratope, was isolated from a heavy-chain antibody library that had been constructed from RNAs extracted from an alpaca (immunized with the anti-aflatoxin antibody) blood, and its relevant V_HH domain proteins, which mimic aflatoxins, were, instead of conventional hapten-protein conjugates, used for coating microplates.[54] Rapid advances in antibody engineering will help accelerate the progress of trace characterization of a variety of bioactive molecules.[4]

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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