

# Phage Anti-Immune Complex Assay: General Strategy for Noncompetitive Immunodetection of Small Molecules

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Due to their size, small molecules cannot be simultaneously bound by two antibodies, precluding their detection by noncompetitive two-site immunoassays, which are superior to competitive ones in terms of sensitivity, kinetics, and working range. This has prompted the development of anti-immune complex antibodies, but these are difficult to produce, and often exhibit high cross-reactivity with the unliganded primary antibody. This work demonstrates that anti-immune complex antibodies can be substituted by phage particles isolated from phage display peptide libraries. Phages bearing specific small peptide loops allowed to focus the recognition to changes in the binding area of the immune complex. The concept was tested using environmental and drug analytes; with improved sensitivity and ready adaptation into on-site formats. Peptides specific for different immune complexes can be isolated from different peptide libraries in a simple and systematic fashion allowing the rapid development of noncompetitive assays for small molecules

Owing to their excellent sensitivity and specificity, immunodetection techniques, which use the fine specificity of antibodies to detect trace amounts of target compounds, are particularly suitable for a wide range of applications in biomedical and environmental analysis. Depending on the format, the assays fall into two main categories, noncompetitive and competitive immunoassays. Noncompetitive assays, mostly used for large molecules and proteins, are based on two different antibodies (or other binding molecules) able to bind simultaneously to two independent epitopes on the analyte (two-site assays). Analysis of low-molecular-mass analytes, such as drugs, hormones, toxins, pesticides, explosives, etc., not large enough to bind two antibodies simultaneously, requires a competitive immunoassay format. In this format, the analyte competes with a labeled (or immobilized) analyte analogue (hapten), and the measured signal is inversely proportional to the concentration of analyte in the sample. In this way, the formation of the analyte–antibody immune complex is

indirectly quantitated by measuring the empty binding sites of the unreacted antibody. Mathematical modeling of immunoassay performance shows that competitive assays are inferior to noncompetitive ones in terms of sensitivity, precision, kinetics, and working range.<sup>1</sup> In addition, noncompetitive immunoassays are more easily adapted into rapid “on-site” formats such as dipstick or immunochromatography, as well as to microfluidics and biosensor approaches. For this reason, several attempts have been made to implement small-molecule noncompetitive assays, but they have usually been limited to certain chemical structures or require analyte labeling.<sup>2,3</sup>

A method based on the blockage of the unreacted sites of the antibody with a polydentate ligand has been described<sup>4</sup> and successfully applied to the detection of cortisol, but a major drawback appears to be the difficulty in obtaining a stable blockage of the unoccupied antibody binding sites. An elegant alternative is the development of the so-called open-sandwich ELISA, an immunoassay based on antigen-dependent stabilization of antibody variable regions (V(H) and V(L) domains). In this format, the analyte-dependent association of the antibody domains is used to bring together a tracer enzymatic activity, such as by joining the N- and C-terminal domains of  $\beta$ -galactosidase.<sup>5</sup> The open-sandwich ELISA is quick and highly sensitive, but the technology is laborious and requires a stringent control of the background association of the recombinant V(H) and V(L) domains in the absence of analyte, which is again case specific.

The most common approach for the development of noncompetitive ELISAs for small molecules relies on the use of anti-immune complex antibodies;<sup>6–11</sup> however, these antibodies are

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- (1) Jackson, T. M.; Ekins, R. P. *J. Immunol. Methods* **1986**, *87*, 13–20.
- (2) Pradelles, P.; Grassi, J.; Creminon, C.; Boutten, B.; Mamas, S. *Anal. Chem.* **1994**, *66*, 16–22.
- (3) Piran, U.; Riordan, W. J.; Livshin, L. A. *Clin. Chem.* **1995**, *41*, 986–990.
- (4) Giraudi, G.; Anfossi, L.; Rosso, I.; Baggiani, C.; Giovannoli, C.; Tozzi, C. *Anal. Chem.* **1999**, *71*, 4697–4700.
- (5) Yokozeki, T.; Ueda, H.; Arai, R.; Mahoney, W.; Nagamune, T. *Anal. Chem.* **2002**, *74*, 2500–2504.
- (6) Towbin, H.; Motz, J.; Oroszlan, P.; Zingel, O. *J. Immunol. Methods* **1995**, *181*, 167–176.
- (7) Nagata, S.; Tsutsumi, T.; Yoshida, F.; Ueno, Y. *Nat. Toxins* **1999**, *7*, 49–55.
- (8) Kobayashi, N.; Shibusawa, K.; Kubota, K.; Hasegawa, N.; Sun, P.; Niwa, T.; Goto, J. *J. Immunol. Methods* **2003**, *274*, 63–75.
- (9) Kobayashi, N.; Kubota, K.; Oiwa, H.; Goto, J.; Niwa, T.; Kobayashi, K. *J. Immunol. Methods* **2003**, *272*, 1–10.

difficult to obtain. In general, when antibodies are elicited against the antigenic determinant of an antibody's combining site (idiotope), the interface of the idiotope–antiidiotope antibody complex buries a large surface that involves most of the complementarity determining regions of both antibodies.<sup>12</sup> When the idiotope under consideration corresponds to that of an anti-hapten antibody, it is important to consider that its modification upon reaction with the hapten will be modest and restricted to the binding pocket. In these antibodies, up to 85% of the accessible surface of the hapten can be buried after binding,<sup>13,14</sup> and therefore, the small portion of the hapten that remains exposed to the solvent has little contribution to the formation of the new idiotope. This imposes a serious limitation to the preparation of anti-immune complex antibodies for two-site assay development, because the residual affinity of the anti-immune complex antibody for the free site of the anti-hapten antibody can be significant and a major cause of high background noise in the assay.

In order to overcome this limitation, we sought “to focus” the recognition of the immune complex to the region of the idiotope where major changes are produced after binding of the hapten. For this, we substituted the large surface of the antiidiotope antibody binding site by short peptide loops that specifically react with the exposed region of the hapten and the conformational changes caused by its binding. For the selection of these peptides, we used phage display peptide libraries, reviewed by Smith and Petrenko,<sup>15</sup> which have shown to be an excellent source of peptide ligands for a large number of selector molecules. We demonstrate the potential of this approach using the herbicides molinate (MW = 187) and atrazine (MW = 215) as small model analytes and further test the concept with the drugs cyclosporin A (MW = 1203) and digoxin (MW = 780). Due to the control that can be exerted during the selection process, the isolation of these peptides is more systematic and much less laborious than the trial-and-error process of anti-immune complex antibody preparation.

## EXPERIMENTAL SECTION

**Antibodies, Herbicides, and Other Compounds.** Molinate and the thiocarbamate compounds used in the cross-reactivity assays were gifts from Stauffer Chemical Co. Thiobencarb was a gift from Chevron Chemical Co. Development of the monoclonal anti-molinate antibody (MoAb 14D7) has been described in detail previously.<sup>16</sup> Atrazine and related triazines were a gift from Dr. Shirley Gee, and the anti-atrazine antibody was a gift from Dr. T. Giersch.<sup>17</sup> Anti-cyclosporine (Catalog No. G86313M), anti-digoxin (Catalog No. G45330M) antibodies, and digoxin–BSA conjugate (Catalog No. Z6D53-3405) were purchased from BioDesign International. Cyclosporine and digoxin standards were purchased from Sigma.

- (10) Ullman, E. F.; Milburn, G.; Jelesko, J.; Radika, K.; Pirio, M.; Kempe, T.; Skold, C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1184–1189.
- (11) Self, C. H.; Dessi, J. L.; Winger, L. A. *Clin. Chem.* **1994**, *40*, 2035–2041.
- (12) Pan, Y.; Yuhasz, S. C.; Amzel, L. M. *FASEB J.* **1995**, *9*, 43–49.
- (13) Lamminmaki, U.; Kankare, J. A. *J. Biol. Chem.* **2001**, *276*, 36687–36694.
- (14) Monnet, C.; Bettsworth, F.; Stura, E. A.; Le, Du, M. H.; Menez, R.; Derrien, L.; Zinn-Justin, S.; Gilquin, B.; Sibai, G.; Battail-Poirot, N.; Jolivet, M.; Menez, A.; Arnaud, M.; Ducancel, F.; Charbonnier, J. B. *J. Mol. Biol.* **2002**, *315*, 699–712.
- (15) Smith, G. P.; Petrenko, V. A. *Chem. Rev.* **1997**, *97*, 391–410.
- (16) Rufo, C.; Hammock, B. D.; Gee, S. J.; Last, J. A.; Gonzalez-Sapienza, G. *J. Agric. Food Chem.* **2004**, *52*, 182–187.
- (17) Giersch, T. *Journal of Agriculture and Food Chemistry* **1993**, *41*, 1006–1011.

**Phage Display Peptide Libraries.** The phage display peptide library used for initial panning experiments was a kind gift from Dr. Peter Schatz (Affymax Research Institute, Palo Alto, CA). This is a constrained library constructed in the phagemid vector p8V2,<sup>18</sup> expressing peptides containing seven or eight random amino acids flanked by two cysteine residues and linked to the N-terminus of the major pVIII phage coat protein through a long glycine-rich spacer (GG-C(X)<sub>7/8</sub>C(GGGGS)<sub>3</sub>). The library size is  $2.4 \times 10^9$  independent clones. For mutagenesis library construction, 200  $\mu$ g of p8V2 was digested with the *Bst*XI restriction enzyme and the digested vector was purified by ultracentrifugation on a potassium acetate gradient at 48 000 rpm. The band visualized with ethidium bromide corresponding to linearized p8V2 vector was separated, the dye was removed with butanol, and the DNA was precipitated with ethanol. Double-stranded DNA coding for the randomized eight amino acid constrained peptides was constructed by annealing of 100 pmol of oligonucleotide mol mut 1 (5'-GGCCCA-GTGCTCACGCAGGAGGCTGTNNKNNKTGGGAHACNNKNN-KNNK TGTGGAGGCGGGGGTAGC-3') and 100 pmol of oligonucleotide ON-2 (5'-TAGGGCCACCTTGCTGGGATCGTCACT-TCCC CCACCGCCGCTACCCC CGCCTCC-3') in 40 mM Tris-HCl, pH 7.5, 20 mM MgCl<sub>2</sub>, 50 mM NaCl in a final volume of 45  $\mu$ L. After heating for 5 min at 70 °C and slowly cooling to room temperature to allow annealing, a fill-in reaction was performed by adding 80 units of sequenase V 2.0 (Stratagene) and 200  $\mu$ M dNTPs. After incubation at 37 °C for 1.5 h, excess dNTPs were removed by gel filtration using a Microspin S-200 HR column (Pharmacia). The purified double-stranded DNA was digested with *Bst*XI and *Bsi*HKAI by incubating ON at 55 °C, and the digested fragment was purified by gel filtration using Microspin S-300 HR columns (Pharmacia). Twenty micrograms of digested p8V2 was ligated with 1.25  $\mu$ g of the dsDNA insert using T4 DNA ligase (InVitrogen). The ligated DNA was precipitated with ethanol and dissolved in 20  $\mu$ L of water. ARI 236 cells (Affymax Research Institute, Palo Alto, CA) were electroporated, transferred to SOC (2% Bacto tryptone, 5% Bacto yeast extract, 0.05% NaCl, 2.5 mM KCl, pH 7.0 with NaOH) medium, and incubated without selection for 1 h at 37 °C. Dilutions of a small aliquot of cells were plated on Luria–Bertani medium (LB) agar petri dishes with ampicillin, allowing an estimation of library diversity at  $10^8$  independent clones. Cells were diluted in 500 mL of SOP medium (2% Bacto tryptone, 1% Bacto yeast extract, 100 mM NaCl, 15 mM K<sub>2</sub>HPO<sub>4</sub>, 5 mM MgSO<sub>4</sub>, pH 7.2 with NaOH) containing 2.5 mL of 20% glucose, and 1.5 mL of 50 mg/mL ampicillin, and grown at 37 °C with vigorous shaking. After the culture reached an OD<sub>600</sub> = 0.5,  $1 \times 10^{12}$  transducing units of M13KO7 helper phage (Pharmacia Biotech) were added and incubated without shaking for 30 min to allow phage infection. After that, 0.6 mL of 20 mg/mL kanamycin and 6 mL of 20% arabinose were added to the culture, and phages were cleared the next day as described below.

Panning experiments for isolation of peptides specific for digoxin and cyclosporin immune complexes were performed with a disulfide constrained 10-mer peptide library fused to the phage coat protein pVIII (a kind gift from Dr. Peter Schatz Affymax Research Institute). The general structure of this peptide library

- (18) Wrighton, N. C.; Farrell, F. X.; Chang, R.; Kashyap, A. K.; Barbone, F. P.; Mulcahy, L. S.; Johnson, D. L.; Barrett, R. W.; Jolliffe, L. K.; Dower, W. J. *Science* **1996**, *273*, 458–464.

was the same as that of the 8-mer library and has a size of  $5.8 \times 10^{10}$  independent clones.

**Biopanning.** For the selection procedure, Nunc-Immuno plates were coated with 100  $\mu\text{L}$  of MoAb 14D7 (2  $\mu\text{g}/\text{mL}$ ) in phosphate-buffered saline (PBS) overnight at 4 °C. After a blocking step of 1 h at 37 °C with 5% skimmed milk in PBS, 100  $\mu\text{L}$  of 1000 ng/mL molinate was added to form the molinate–MoAb 14D7 immune complex. After 20-min incubation, the wells were rinsed twice with cold PBS, then 100–1000 library equivalents were diluted in 600  $\mu\text{L}$  of 5% skimmed milk in PBS containing 100 ng/mL molinate, dispensed into six microtiter wells, and incubated for 2 h at 4 °C. Unbound phages were removed by 20 washes with cold PBS containing 0.05% Tween 20 (PBST). Bound phages were eluted by a 10-min incubation with 0.1 N glycine-HCl, pH 2.5, 0.1% BSA, and immediately neutralized with 2 M Tris base. The eluted phage was directly amplified as described below, or alternatively, a postselection–adsorption step was included in some panning experiments to deplete the eluted phage of clones with affinity for the uncombined antibody. For this step, the eluted phages were incubated for 30 min in ELISA wells coated with MoAb 14D7 in the absence of molinate. These phage preparations (600  $\mu\text{L}$ ) were added to 10 mL of log-phase *Escherichia coli* ARI 292 cells (Affymax Research Institute) and amplified in LB media containing 0.25%  $\text{K}_2\text{HPO}_4$ , 0.1%  $\text{MgSO}_4$ , 0.1% glucose, and 100  $\mu\text{g}/\text{mL}$  ampicillin to an  $\text{OD}_{600} = 0.4$ . M13KO7 helper phage at a multiplicity of infection 10:1 was added. After a period of 30 min at 37 °C without shaking, arabinose and kanamycin were added to a final concentration of 0.02% and 40  $\mu\text{g}/\text{mL}$ , respectively, and the cultures incubated overnight at 37 °C with vigorous shaking. Phage from liquid cultures were obtained by clearing the supernatants by centrifugation at 12 000  $\mu\text{g}$  for 15 min, precipitated with 0.2 volume of 20% poly(ethylene glycol) 8000–2.5 M NaCl, (PEG-NaCl) on ice during 1 h, and centrifuged as above. Phage pellets were resuspended in 2 mL of sterile PBS and titrated in ARI 292. For the next round of selection,  $10^{10}$  transducing units were used. The same procedure of panning was used for the isolation of phage clones specific for the atrazine immune complex. The panning experiments performed with digoxin and cyclosporin were essentially the same, except that in these cases a high concentration (5  $\mu\text{g}/\text{mL}$ ) of the analytes was added during the adsorption step on the immune complexes, and after elution and neutralization phages were postadsorbed on uncombined antibody.

After three to four rounds of panning, serial dilutions of individual amplified phage clones were tested for their ability to bind to the analyte–antibody immune complex by phage ELISA. Positive clones were further selected by checkerboard titration and submitted for DNA sequencing using the primer ON891 (TGAGGCTTG CAGGGGTC) (Division of Biological Sciences, Automated DNA Sequencing Facility, UC Davis).

**Phage ELISA.** Nunc-Immuno Maxi-Sorp plates were coated with the corresponding antibody or the analyte–antibody immune complex as described before. The wells were blocked with 5% skimmed milk PBS and washed three times with PBST. A total each of 100  $\mu\text{L}$ /well of an overnight culture of individual amplified phage clones were dispensed into the wells. The microtiter plates were then incubated for 1 h at room temperature with gentle rocking. After three washes with PBST, 100  $\mu\text{L}$  of a 1/5000 dilution of horseradish peroxidase (HRP)-labeled anti-M13 monoclonal

antibody (Pharmacia, Uppsala) in PBST was added to each well. One hour later, the plates were thoroughly washed and 100  $\mu\text{L}$  of peroxidase substrate (25 mL of 0.1 M citrate acetate buffer pH 5.5, 0.4 mL of 6 mg/mL DMSO solution of 3,3',5,5'-tetramethylbenzidine, and 0.1 mL of 1%  $\text{H}_2\text{O}_2$ ) was dispensed into each well. The enzymatic reaction was stopped after 15–20 min by the addition of 50  $\mu\text{L}$  of 2 M  $\text{H}_2\text{SO}_4$ , and the absorbance at 450 nm (corrected at 600 nm) was read in a microtiter plate reader (Multiskan MS, Labsystems). The supernatants showing high readings in wells coated with the immune complex and low response in antibody-coated wells were prepared as stabilized phage suspensions (see below) and used for further analysis. For checkerboard titration, 100  $\mu\text{L}$  of various concentrations of the antibody (0.3–2  $\mu\text{g}/\text{mL}$ ) was used for coating.

**Stabilization of Phage Suspensions.** Individual amplified phage clones were obtained as described above. After two steps of precipitation with PEG-NaCl, the phage particles were suspended in 1/50 volume of the original culture volume in PBS, which was supplemented with the Complete Protease Inhibitor Cocktail of Roche Diagnostics and sodium azide 0.05%. The preparations were filtered through a 0.22- $\mu\text{m}$  filter and stored in aliquots at 4 and –20 °C.

**Noncompetitive ELISA, the Phage Anti-Immune Complex Assay (PHAIA).** The 50  $\mu\text{L}$ /well of serial dilutions of analyte standard in PBST were dispensed into microtiter plates coated with the corresponding antibody, followed by addition of 50  $\mu\text{L}$ /well of the adequate dilution of the stabilized phage suspension. The antibody concentration used for coating and the proper dilution of the phage suspension had been previously optimized by checkerboard experiments. After 1 h of incubation at room temperature, the plates were washed and developed as previously described. Absorbance values were fitted to a four-parameter logistic equation using the Genesis Lite 3.03 (Life Sciences Ltd.) package software. The lower detection limit of the assays was estimated as the analyte concentration causing a 10% increase in signal when compared to the zero analyte signal.

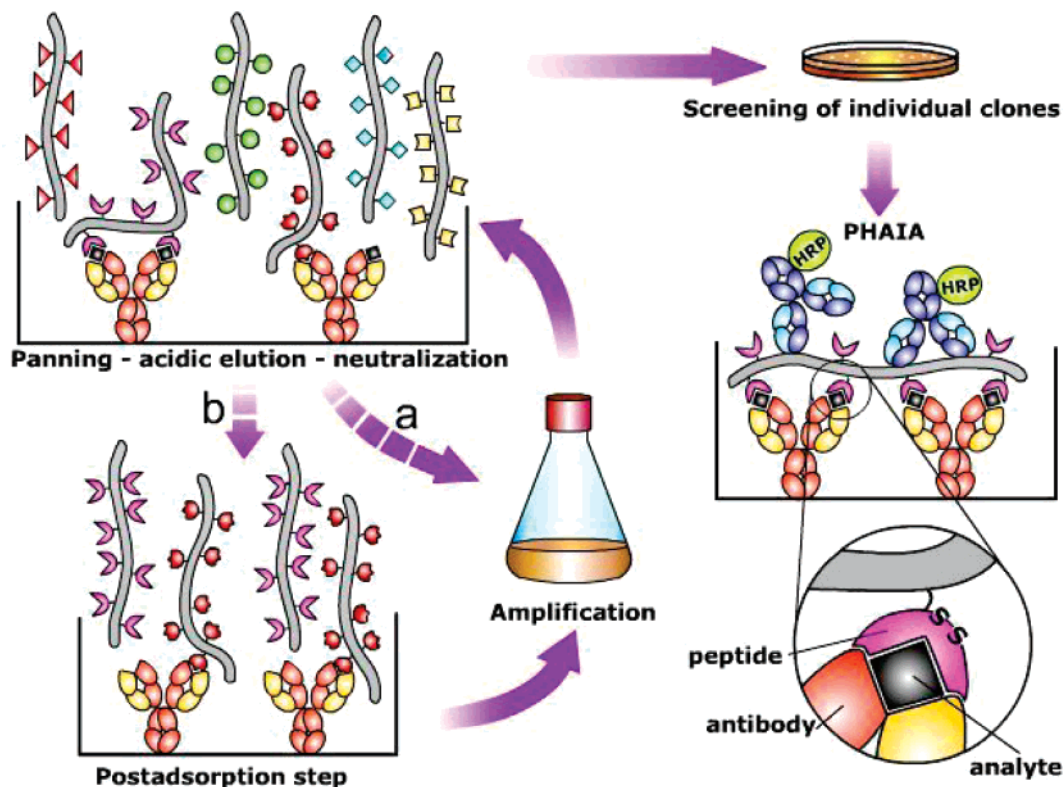
**Assay Cross-Reactivity.** The specificity of the noncompetitive assay setup with the phage-borne peptides was characterized by determining the cross-reactivity with related pesticides. Analyte concentrations in the 0–10 000 ng/mL range were used in the noncompetitive ELISAs. After the data were normalized, the molar compound concentration corresponding to the midpoint of the curve, which corresponds to the concentration of analyte producing 50% saturation of the signal ( $\text{SC}_{50}$ ), was used to express the cross-reactivity of the assay according to the equation

$$\% \text{ cross-reactivity} = 100[\text{SC}_{50}(\text{analyte}) / \text{SC}_{50}(\text{cross-reacting compound})]$$

**Matrix Effect Analysis.** For the analysis of matrix effect, runoff water samples from rural areas of Uruguay, with no register use of molinate or atrazine, were spiked with known amounts of analyte and assayed by PHAIA in the 0–100 ng/mL range, using 100  $\mu\text{L}$  of undiluted water samples plus 10  $\mu\text{L}$  of  $10 \times$  PBST.

**Dipstick Assay.** The 1.5- $\mu\text{L}$  aliquots of serial dilutions of MoAb 14D7 in PBS (7.5–30 ng/spot) were spotted onto nitrocellulose membranes (0.45  $\mu\text{m}$ ) (Biorad Laboratories Inc.). Immediately after drying, the membranes were quenched by soaking in 5%





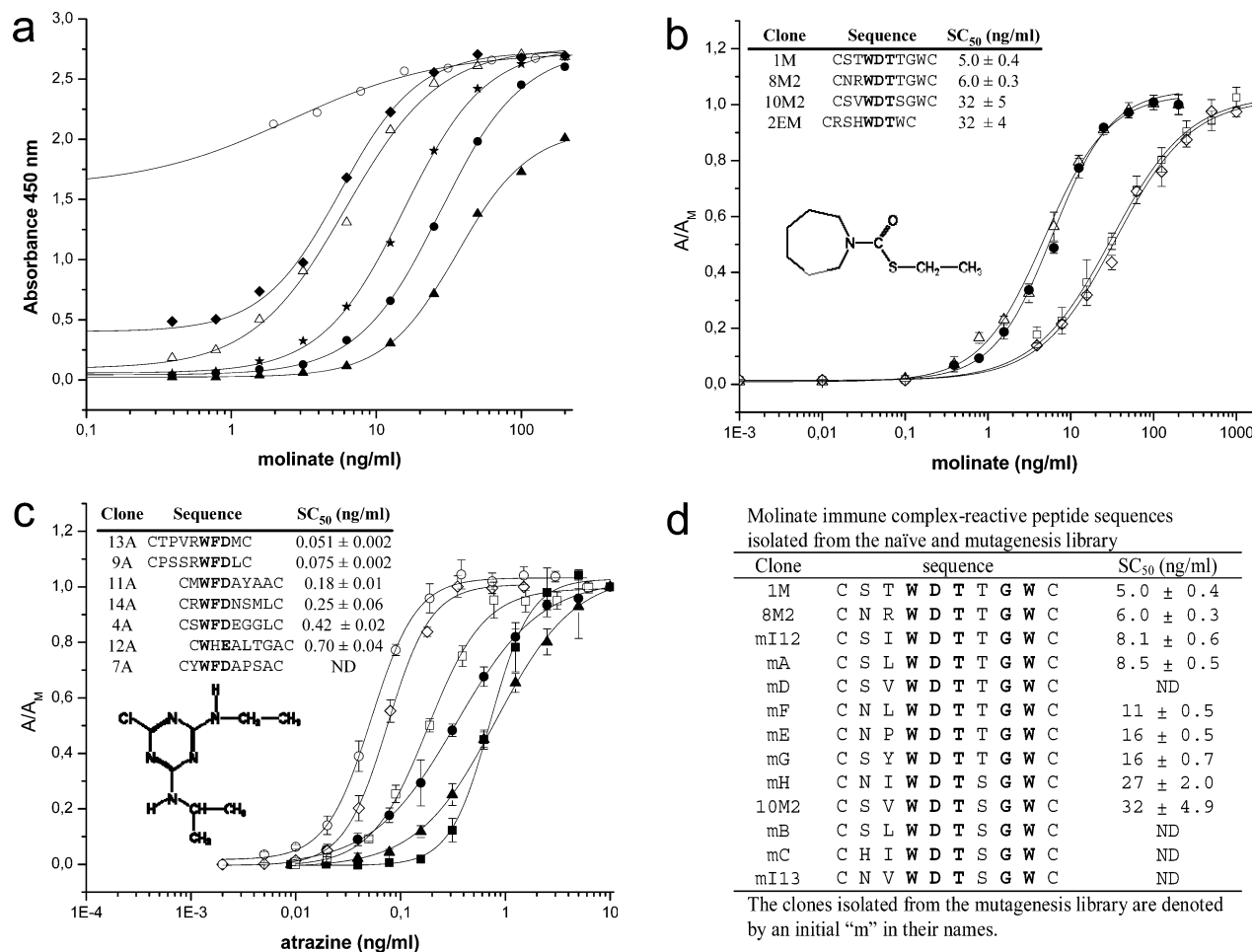
**Figure 1.** Scheme of the procedure for the selection of anti-immune complex phage and setup of phage anti-immune complex assay (PHAIA). An anti-analyte antibody adsorbed onto the solid phase of an ELISA plate is used to capture the analyte (black square). A phage display peptide library is then added, and after several washing steps, specific phages recognizing the immune complex are eluted by addition of low pH buffer. The supernatant is neutralized, and the selected subpopulation of phages can be used directly for *E. coli* infection and phage amplification (a) or submitted to an optional post adsorption step to eliminate phages with strong affinity for the uncombined antibody (b). After several rounds of selection (panning), individual clones are tested for differential binding to the immune complex and the free antibody by phage ELISA. Phage clones showing low reactivity with the empty antibody are selected for development of PHAIA. In this assay, the immune complex formation is detected by its reaction with the phage borne peptide, followed by an anti-pVIII phage coat protein antibody coupled to HRP). A possible scheme of the antibody-analyte-peptide trivalent interaction that allows development of PHAIA is shown in the lower right part of the figure. Note that the overall affinity of the peptide for the immune complex is postulated to be the result of their interaction with the analyte and the antibody. The relative role of these contributions is further discussed in the text.

skim milk in PBS for 30 min; after rinsing in PBST, the membranes were cut into strips, dried, and kept at 4 °C until used. The assay was performed by dipping the membrane strips for 15 min into PBST spiked with different amounts of molinate and containing the appropriate dilution of specific phage ( $\sim 10^{10}$  phage particles/mL), washed under tap water, incubated for 10 min in a 1/5000 dilution of HRP-labeled anti-M13 monoclonal antibody, and developed using diaminobenzidine with metal ion enhancement substrate (6 mg of 3,3'-diaminobenzidine in 9 mL of 50 mM Tris pH 7.6, 1 mL of 0.3% NiCl<sub>2</sub>, and 10  $\mu$ L of 30% H<sub>2</sub>O<sub>2</sub>). The reaction was stopped by washing the membrane in tap water.

## RESULTS

**Antibody-Analyte Immune Complex Specific Peptides Can Be Isolated from Phage Display Peptide Libraries.** A general scheme of the procedure for isolation of anti-immune complex peptides is shown in Figure 1. Our first attempts to select anti-immune complex peptides were done using the anti-molinate monoclonal antibody 14D7<sup>16</sup> and cyclic (disulfide constrained) peptide libraries consisting of 7- and 8-mer length peptides. This was performed on plates coated with the molinate-MoAb 14D7

complex in the presence of 100 ng/mL molinate. After three rounds of selection (panning), individual phage clones were grown and assayed by ELISA for differential reactivity with the immune complex and the unreacted antibody, using wild type phage as a control. Four different sequences were obtained, three of them from the 8-mer library (inset in Figure 2b). All clones had the consensus sequence **WDT**, shown in boldface type. To test whether the selection of anti-immune complex peptides could be extended to other analyte-antibody systems, we also explored its applicability to the competitive immunoassay for the herbicide atrazine described by Giersch.<sup>17</sup> This is a highly sensitive assay (IC<sub>50</sub> (analyte concentration producing 50% signal inhibition) = 0.5 ng/mL) that utilizes the MoAb K4E7. Using the selection strategy described above, several peptide sequences highly specific for the atrazine-MoAb K4E7 immune complex were isolated (inset in Figure 2c). All of these clones exhibited strong reactivity with the immune complex, showing that the isolation of peptides that bind to the molinate-14D7 antibody complex is not a specific phenomenon associated with this system. Again there was an evident consensus motif, **WFD** which occurred in position 2 or 5 of the random 8-mer peptide. The only exception



**Figure 2.** PHAIA development: noncompetitive ELISAs for molinate and atrazine. (a) Effect of phage concentration in assay performance. Different phage concentrations of clone 1M were used to detect binding of molinate on plates coated with 50 ng/well MoAb 14D7. Phage concentrations (phage particles/mL) were:  $10^{10}$  (white circles),  $5 \times 10^9$  (diamonds),  $2.5 \times 10^9$  (white triangles),  $1.25 \times 10^9$  (stars),  $6.3 \times 10^8$  (black circles), and  $3.1 \times 10^8$  (black triangles). (b) Noncompetitive ELISA for molinate (structure shown in the inset) performed with different phage-borne peptides. Clone sequences and their corresponding midpoint values ( $SC_{50}$ ) are shown in the inset. (c) Noncompetitive ELISA for atrazine (structure shown in the inset) performed with different phage-borne peptides. Clone sequences and their corresponding midpoint values ( $SC_{50}$ ) are shown in the inset. (d) Peptides isolated from the mutagenesis library with the molinate–MoAb 14D7 immune complex.

was clone 12A, which had a three-residue stretch that resembles the WFD consensus sequence, with His substituted for Phe, and Glu, another acidic residue, substituting for the aspartic acid residue of the consensus.

**Phage Bearing Peptides Can Be Used To Set Up the Noncompetitive Phage Anti-Immune Complex Assay.** Checkboard titration analysis was used to optimize the amount of coating antibody in the presence of a fixed amount of analyte, in order to set up conditions for noncompetitive PHAIA. Once the amount of antibody was fixed, it was possible to develop a noncompetitive format to measure the analyte-bound sites using the setup shown in Figure 1. As expected, the sensitivity of PHAIA increased with the concentration of phage particles used in the assay; however, after a certain point, the additional increase of phage concentration resulted in a high background signal due to direct binding of the peptide to the uncombined antibody (Figure 2a). The molinate assay performance was greatly influenced by the sequences flanking the conserved WDT motif of the phage-borne peptides used for immune complex detection (Figure 2b). The most sensitive test for molinate was attained with clone 1M. With this phage-borne peptide, the detection limit was 0.73 ng/

mL, and the concentration of analyte producing 50% of signal saturation ( $SC_{50}$ ) was  $5.0 \pm 0.4$  ng/mL. This sensitivity was  $\sim 30$ -fold better than that of the competitive ELISA set up with the same antibody<sup>16</sup> (detection limit = 22 ng/mL and  $IC_{50} = 69.2 \pm 1.4$  ng/mL). In a similar way, peptides isolated with the atrazine immune complex also allowed the setup of sensitive noncompetitive assays, Figure 2c. As found for the molinate system, there was a marked influence of the residues flanking the WFD motif in the sensitivity of the assay. Clone 13A showed the best performance with a detection limit of 0.016 ng/mL and  $SC_{50} = 0.051 \pm 0.002$  ng/mL. Once more, this represented a significant improvement with regard to the competitive assay setup with the same antibody,<sup>17</sup> which exhibited a detection limit of 0.18 ng/mL and  $IC_{50} = 0.64 \pm 0.06$  ng/mL.

The role of the flanking residues was further explored for the molinate–MoAb 14D7 system by building the mutagenesis library CXXwdtXXXC, where X indicates a fully random position and lowercase letters indicate 50% probability of finding this residue in that position. Different strategies were used to pan this library, including extensive washing and postadsorption steps on fresh uncombined antibody; in addition, the screening of individual

**Table 1. Cross-Reactivity (%) of Molinate and Atrazine PHAIA<sup>a</sup>**

Compound	Structure	Chemical hapten <sup>19</sup>	Clone 1M CSTWDTTGWC	Clone 2EM CRSHWDTWC
molinate		100	100	100
thiobencarb		1	0	0
butylate		1	0	0
EPTC		1	5	15
cycloate		1	9	0
pebulate		4	7	13
vernolate		4	4	5

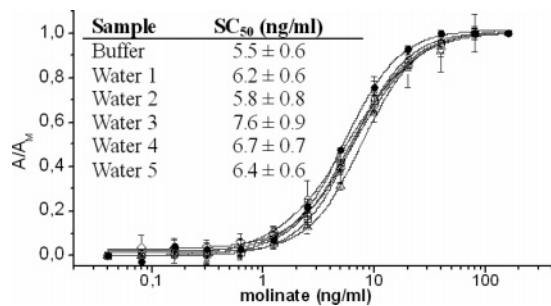
Compound	Structure	Chemical hapten <sup>17</sup>	Clone 13A CTPVRWDFMC
	R1      R2      R3		
atrazine	Cl      C <sub>2</sub> H <sub>5</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	100	100
simazine	Cl      C <sub>2</sub> H <sub>5</sub> C <sub>2</sub> H <sub>5</sub>	48	0
propazine	Cl      CH(CH <sub>3</sub> ) <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	116	144
cyanazine	Cl      C <sub>2</sub> H <sub>5</sub> C <sub>2</sub> N(CH <sub>3</sub> ) <sub>2</sub>	91	0
ametryn	SCH <sub>3</sub> C <sub>2</sub> H <sub>5</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0	0
simetryn	SCH <sub>3</sub> C <sub>2</sub> H <sub>5</sub> C <sub>2</sub> H <sub>5</sub>	0	0
prometryn	SCH <sub>3</sub> CH(CH <sub>3</sub> ) <sub>2</sub> CH(CH <sub>3</sub> ) <sub>2</sub>	0	0
terbutryn	SCH <sub>3</sub> C <sub>2</sub> H <sub>5</sub> C(CH <sub>3</sub> ) <sub>3</sub>	0	0

<sup>a</sup> All data are the mean of two independent experiments. A value of 0 means that there was no observable cross-reactivity with the highest concentration tested 10<sup>4</sup> ng/mL

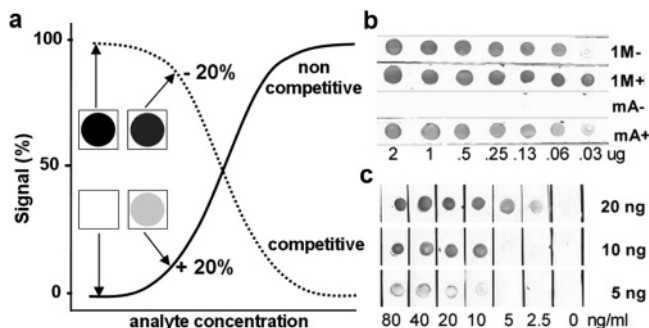
clones was biased toward phage exhibiting low binding affinity for the unreacted antibody. Ten new peptides were isolated using this strategy (Figure 2d), with the consensus sequence (S/N)-XWDT(T/S)GW, where in addition to the WDT motif, Gly and Trp were also essential in positions 7 and 8, respectively. While Thr in position 6 appeared to improve the assay sensitivity, the role of Ser and Asn at position 1 was less clear. As expected due to our biased selection, most clones showed negligible cross-reactivity with the free antibody, particularly clone mA (see below in Figure 4b). However, this intuitive advantage of clone mA was not directly translated into a more sensitive assay; clone mA had a detection limit of 2 ng/mL and SC<sub>50</sub> = 8.5 ± 0.5 ng/mL versus a detection limit of 0.73 ng/mL and SC<sub>50</sub> = 5.0 ± 0.4 ng/mL for

clone 1M. This highlights the relevance of the overall affinity of the peptide for the immune complex as a critical feature that determines the sensitivity of the assay.

**PHAIA Cross-Reactivities and Matrix Effect.** The simultaneous recognition of the analyte by the antibody and the peptide has a great potential for discriminating haptens, improving the sensitivity of the assay. This was observed, particularly for atrazine, when the cross-reactivity of PHAIA and the competitive assays set up with the same antibodies were examined using a panel of molinate- and atrazine-related herbicides (Table 1). PHAIA was further validated by performing molinate standard curves in runoff water obtained from representative agricultural areas of Uruguay. No relevant matrix effect was observed in the molinate PHAIA



**Figure 3.** Matrix effect of PHAIA. Molinate standard curves performed in assay buffer (black squares) and similar curves performed with undiluted water from agricultural runoffs.



**Figure 4.** Adaptation of the molinate PHAIA into dipstick format. Dipstick assay for molinate using phage-borne peptides. (a) Theoretical representation of competitive (dotted line) and noncompetitive (solid line) immunoassays and their adaptation into dipstick format. Notice that even in the case that both assays have equal sensitivity, the change in signal obtained in the noncompetitive format is much more convenient to detect trace amounts of analyte by naked eye visualization. (b) Capture antibody titration. Different amounts of MoAb 14D7 (ranging from 2 to 0.03  $\mu\text{g}$ ) spotted onto nitrocellulose strips were incubated with  $10^{10}$  phage particles/mL bearing the peptides 1M or mA in the presence (+) or absence (-) of 50 ng/mL molinate. (c) Dipstick assay setup with 20, 10, or 5 ng of MoAb 14D7 and clone 1M ( $10^{10}$  phage particles/mL) in the range of 0–80 ng/mL molinate.

(Figure 3) when undiluted samples were analyzed. Similar results were obtained for the atrazine PHAIA (data not shown).

**PHAIA Can Be Easily Adapted into Dipstick Formats.** The most attractive advantage of noncompetitive assays is the possibility of measuring near-zero signals at low analyte concentrations with much more ease than a competitive format. This advantageous property makes them the method of choice when rapid on-site test formats are needed, particularly when the presence of the analyte needs to be visualized in a yes or no fashion (Figure 4a). To illustrate this concept, we adapted the molinate assay into a dipstick format where the antibody immobilized onto a nitrocellulose strip is dipped into a molinate solution and the formation of the immune complex is revealed by its reaction with the phage-borne peptide. Two clones were evaluated for this purpose, 1M and mA. The latter was chosen on the basis of its high specificity for the immune complex, and as shown in Figure 4b, clone mA did not react with the empty antibody even at the highest coating density. Conversely, clone 1M showed intense cross-reactivity with the uncombined antibody over a wide range of coating densities. However, once the proper conditions were set, clone 1M performed with higher sensitivity in the dipstick assay, allowing the detection by visual examination of up to 2.5 ng/mL molinate (Figure 4c). This is an interesting

result considering that the WHO guideline value for the concentration of this compound in drinking water is 6 ng/mL,<sup>19</sup> and 20 ng/mL in the State of California.<sup>20</sup>

**Isolation of Anti-Immune Complex Peptides Is of General Applicability.** To further demonstrate the concept, we selected two additional target analytes, cyclosporin and digoxin. These are larger molecules that need careful monitoring during medication. We performed our panning experiments using two commercial MoAbs, cyclosporin ( $K_d 1 \times 10^{-8}$  M) and digoxin ( $K_d 1.4 \times 10^{-12}$  M). The selection process was done as previously, but using a 10-mer library and including postadsorption steps. In the case of both analytes, after three rounds of panning, most clones showed strong binding to the immune complex with negligible reactivity with the uncombined antibody. Out of 15 clones sequenced, three different sequences were obtained for cyclosporin and four for digoxin (insets in Figure S-1, Supporting Information). As observed in the case of molinate and atrazine, a three-amino acid consensus sequence, **FPD**, was present in the family of peptides isolated for cyclosporin, but no consensus sequence was found in the case of digoxin. Sensitive PHAIAs could be set up with selected phage-borne peptides (Figure S-1). Cyclosporin PHAIA using the peptide CELFPDLIHVWC attained a detection limit of 0.62 ng/mL and  $SC_{50} = 3.4 \pm 0.2$  ng/mL, while the digoxin PHAIA set with the peptide CMVLPESLPTCTC performed with a detection limit of 0.070 ng/mL and  $SC_{50} = 0.22 \pm 0.01$  ng/mL. This represented a 10-fold improvement when compared with the competitive assay performed with the same antibody (detection limit 0.72 ng/mL,  $SC_{50} = 2.1 \pm 0.3$  ng/mL). Because of the lack of commercially available coating antigens for cyclosporin, the comparison between the direct and competitive formats using the same antibody could not be done.

## DISCUSSION

Despite the many advantages of two-site immunoassays, to date, no systematic and simple methods exist for the development of these assays when the analyte is a low molecular weight molecule. The small size of these analytes is the critical obstacle that precludes the simultaneous binding of the capture and detecting antibody. The closest approach to overcome this situation has consisted in the use of antiidiotype antibodies that show differential reactivity with the antibody binding site upon binding of the analyte. However, as discussed before, due to the large surface buried in the antiidiotype immune complex interface, these antibodies constitute a rare event in the polyclonal immune response against immune complex antigens and therefore are difficult to produce. Here we demonstrated that it is possible to “focus” the recognition of the immune complex using short peptide loops isolated from phage display libraries, allowing the trivalent detection of small analytes in a noncompetitive format.

In the different models examined, the transition from detection of unreacted antibody binding sites (competitive assay) toward detection of the analyte–antibody reaction boosted the sensitivity of the tests. The formation of the trivalent complex appears to be the result of the combined interactions of the peptide with the exposed regions of the analyte plus additional bonding to residues

(19) Hamilton, D.; AMbrus, A.; Dieterle, R.; Felsot, S.; Harris, C.; Holland, P.; Katamaya, A.; Kurihara, N.; Linders, J.; Unsworth, J.; Wong, S.-S. *Pure Appl. Chem.* **2003**, *75*, 1123–1155.

(20) OEHA. [http://www.oehha.ca.gov/public\\_info/public/phg4.html](http://www.oehha.ca.gov/public_info/public/phg4.html), 1988.



in the antibody. While the latter is an undesirable source of cross-reactivity with the free antibody, it may also work as a key contribution to the detection of trace amounts of immune complex in the PHAIA system. Thus, the proper balance of this characteristic appears to be a critical feature of the peptide, and care should be taken in the design of the panning and screening experiments in order to select convenient candidate phage-borne peptides for PHAIA. In addition, the shift to a positive or noncompetitive readout facilitates the detection of near-zero signals and, therefore, makes easier the adaptation of the PHAIA into rapid yet sensitive dipstick formats for "point of need" testing, as shown here for the molinate assay.

Another important aspect of PHAIA is its general applicability and simplicity. The availability of commercial phage display peptide libraries makes this technology accessible to nonspecialized laboratories and, therefore, prone to be applied in biomedical, agricultural, and environmental sciences. In addition to the four examples presented here, the principle has also worked for other analytes such as phenoxibenzoic acid, a pyrethroid metabolite. In this case the peptides were isolated utilizing affinity purified polyclonal antibodies and the immunoassay performed with the total IgG fraction (manuscript in preparation). Moreover, we have found a great flexibility with regard to the type of library that can be used. Anti-immune complex peptides have been isolated from phage libraries expressing different lengths of random peptides and also from libraries expressing the peptide on the phage minor coat protein pIII. Conversely to the challenging and laborious preparation of detection antibodies, the selection of phage-borne peptides is systematic and can be accomplished in a few days. Furthermore, the final product of this selection, namely, the phage particle bearing the specific peptide sequence, can be directly used

(21) Cardozo, S.; Gonzalez-Techera, A.; Last, J. A.; Hammock, B. D.; Kramer, K.; Gonzalez-Sapienza, G. G. *Environ. Sci. Technol.* **2005**, *39*, 4234–4241.

as a convenient immunoassay reagent.<sup>21</sup> It is worth noting that the robustness and filamentous polymeric structure of the phage particle opens many possibilities for its chemical modification using dyes, fluorescent compounds, acridinium esters, enzymes, etc., which could enable the automation of the noncompetitive PHAIA technology in microfluidic and biosensor platforms.

## CONCLUSIONS

The principle described here represents a new concept for the development of noncompetitive immunoassays that is simple and of general applicability. In addition to providing a positive readout, in all cases examined, PHAIA resulted in an increased sensitivity (~10-fold). The trivalent detection principle possesses the potential to increase the specificity of the assay and is amenable to be adapted into rapid on-site dipstick formats.

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## SUPPORTING INFORMATION AVAILABLE

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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