

Nanopeptamers for the Development of Small-Analyte Lateral Flow Tests with a Positive Readout

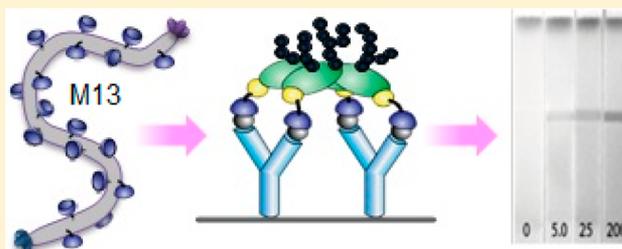
Lucía Vanrell,[†] Andrés Gonzalez-Techera,[†] Bruce D. Hammock,[‡] and Gualberto Gonzalez-Sapienza^{*,†}

[†]Cátedra de Inmunología, Facultad de Química, Instituto de Higiene, Universidad de la República, Montevideo 11600, Uruguay

[‡]Department of Entomology and UCD Cancer Center, University of California, Davis, California 95616, United States

S Supporting Information

ABSTRACT: There is a great demand for rapid tests that can be used on-site for the detection of small analytes, such as pesticides, persistent organic pollutants, explosives, toxins, medicinal and abused drugs, hormones, etc. Dipsticks and lateral flow devices, which are simple and provide a visual readout, may be the answer, but the available technology for these compounds requires a competitive format that loses sensitivity and produces readings inversely proportional to the analyte concentration, which is counterintuitive and may lead to potential misinterpretation of the result. In this work, protein-multipetide constructs composed of anti-immunocomplex peptides selected from phage libraries and streptavidin/avidin as core protein were used for direct detection of small compounds in a noncompetitive two-site immunoassay format that performs with increased sensitivity and positive readout. These constructs that we termed “nanopeptamers” allow the development of rapid point-of-use tests with a positive visual end point of easy interpretation. As proof of concept, lateral flow assays for the herbicides molinate and clomazone were developed and their performance was characterized with field samples.



Immunoassays are analytical tests that exploit the high capacity of antibodies for recognizing target analytes with high affinity and specificity. Because of their simplicity, sensitivity, cost-effectiveness, and matrix tolerance, immunoassays have been used in countless applications in pharmacology, food safety, environment, homeland security, narcotics, etc.^{1–3} In these methods, the antigen–antibody reaction is generally coupled to a signal generating molecule (tracer) which translates the interaction into a quantitative readout. If the analyte is a macromolecule, typically the antigen is first captured by a primary antibody and then detected with a secondary antibody coupled to the tracer. This two-site format allows the use of excess concentrations of reacting antibodies, promoting the formation of the trivalent immunocomplex, even in the presence of trace amounts of the analyte (high assay sensitivity). Unfortunately, this is not possible for the important group of small analytes (drugs, metabolites, toxins, additives, pesticides, explosives, etc.), because they cannot bind two antibodies simultaneously. The measurement of such small molecules (immunochemically classified as haptens) is typically accomplished by competitive reactions between the analyte and a labeled variant of the analyte in the presence of limiting amounts of the antihapten antibody. This competitive format performs with inferior sensitivity, precision, kinetics, and working range than the two-site noncompetitive format⁴ and produces readouts inversely proportional to the analyte concentration, making more difficult its adaptation into microarrays or microfluidic devices or into rapid “on-site” visual assays such as lateral flow tests.

Several attempts have been made to implement small-molecule noncompetitive assays, but most of them are limited to particular chemical structures or require analyte labeling.^{5–7} By focusing the recognition of the analyte–antibody immunocomplex (IC) into changes of the hapten binding pocket upon analyte binding, we were able to isolate short-peptide loops from phage display libraries that specifically react with the analyte-capture antibody IC.⁸ Using M13 phage particles displaying these peptides, we developed two-site phage anti-IC assays (PHAIA) (Figure 1A) for the herbicides molinate, atrazine, and clomazone,^{8,9} the drugs digoxin and cyclosporine,⁸ the flame-retardant brominated diphenyl ether,¹⁰ and the pyrethroid metabolite phenoxybenzoic acid.¹¹ Recently, other groups have also isolated anti-IC peptides for gibberellins¹² and a 1,3-diketone hapten derivative¹³ further showing the general applicability of the concept. The PHAIA method provides higher sensitivity than the conventional format (one or more orders of magnitude), and the two-site recognition also accounts for a higher specificity.⁹ The phage particles proved to be robust and versatile assay components,¹⁴ but they are “unconventional” reagents for the immunoassay industry and their biological nature (an *Escherichia coli* infecting phage) could be a matter of concern in some laboratories. For this reason we sought to develop PHAIA into a phage-free detection method by using synthetic peptides designed from

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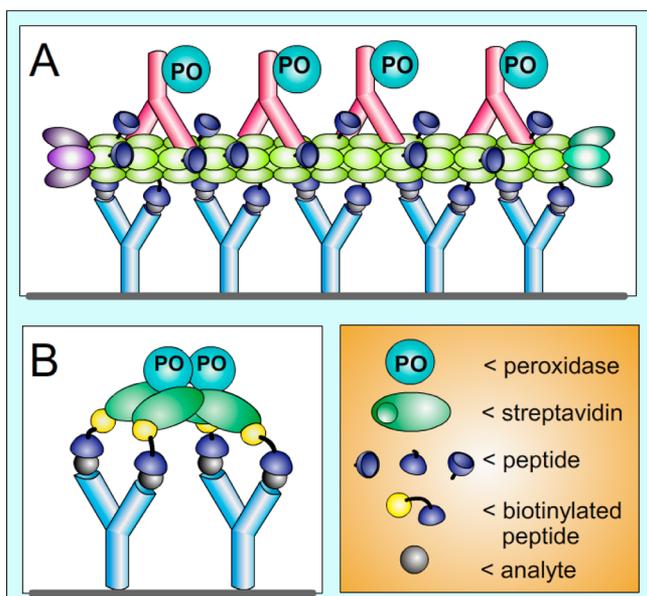


Figure 1. Scheme of the phage anti-immunocomplex assay (PHAIA) and the nanopeptamer noncompetitive assays. Panel A shows the scheme of PHAIA that typically uses filamentous M13 phage (light green) expressing disulfide constrained peptides (~100–200 copies along the ~2700 copies of the coat protein pVIII). Binding of the phage to the analyte–antibody (light blue) immunocomplex is detected with an anti-M13 antibody (pink) coupled to horse radish peroxidase (PO). Panel B schematizes the substitution of the phage particle by a nanopeptamer (a streptavidin conjugate, combined with four molecules of the biotinylated peptide).

the anti-immunocomplex peptide sequences isolated from phage display libraries attached to a protein scaffold. These constructs that we termed “nanopeptamers” allow sensitive detection of ICs and could be developed into lateral flow tests, a feat that turned up to be particularly difficult with phage particles due to their filamentous nature.

EXPERIMENTAL SECTION

Materials. Molinate and the thiocarbamate standards were gifts from Stauffer Chemical Co. Thiobencarb was a gift from Chevron Chemical Co. Development of the monoclonal antimolinate antibody (MoAb 14D7) and anticlomazone (MoAb 5.6) has been described in detail previously.^{1,2} Clomazone was purchased from Riedel-de Haen (Seelze, Germany), high-sensitivity streptavidin-peroxidase (SPO) from Pierce (Rockford, IL), and bovine serum albumin (BSA), Tween 20, 3,3',5,5'-tetramethylbenzidine (TMB), and avidin from Sigma (St. Louis, MO). Enzyme-linked immunosorbent assay (ELISA) and dilution microtiter polystyrene plates were purchased from Greiner (Solingen, Germany). Hi-Flow Plus 120 nitrocellulose membrane cards and cellulose absorbent pads were purchased from Millipore (Bedford, MA). Carbon black nanoparticles were from Degussa AG (Frankfurt, Germany).

Formation of Antimolinate and Anticlomazone Nanopeptamers. Two anti-IC peptide sequences previously isolated for molinate 3 and one for clomazone 4 were produced by a commercial manufacturer (Peptron Co., Daejeon, Korea). These peptides were synthesized to 80% of purity by high-pressure liquid chromatography (HPLC), with intramolecular disulfide bonds between cysteines, an N-terminal biotin

molecule and amidated C-terminus. The synthesized peptides were p1M, biotin-SGSGCSTWDTTGWC; pA, biotin-SGSGCSLWDTTGWC; and pX11, biotin-SGSGCLEAP-NIEGC for molinate, molinate, and clomazone, respectively (the anti-IC sequence is in black and the SGSG was used as a spacer). In total 250 pmol of SPO or avidin in 100 μ L of phosphate-buffered saline (PBS), 1% BSA were incubated with 50, 6.5, 1.5, and 0.6-fold molar excess of biotinylated peptides for 15 min on ice. The Nanopeptamers were initially separated from the excess of biotinylated peptides by gel filtration, but this step proved to be unnecessary.

Nanopeptamer ELISA. Microtiter plates were coated with 100 μ L of the antiherbicide monoclonal antibody in PBS at different concentrations (10–2.5 μ g/mL). After incubation for 1 h at 37 $^{\circ}$ C and blocking with PBS BSA 1% 1 h at 37 $^{\circ}$ C, the microtiter plates were washed three times with PBS 0.05% Tween 20 (PBST). Nanopeptamer (prepared with SPO and the corresponding anti-IC biotinylated peptide) were diluted with PBST to the appropriate dilution, and 50 μ L were mixed with the 50 μ L of the herbicide standard or water sample in nontreated polystyrene plates (low binding capacity), transferred to the reaction microtiter plate and incubated for 1 h at room temperature. After thoroughly washing, 100 μ L of the peroxidase substrate (0.4 mL of a 6 mg/mL DMSO solution of 3,3',5,5'-tetramethylbenzidine, 0.1 mL of 1% H₂O₂ in water in a total of 25 mL of 0.1 M citrate acetate buffer, pH 5.5) was dispensed into each well. The enzyme reaction was stopped after 20 min by addition of 50 μ L of 2 N H₂SO₄, and the absorbance was read at 450/650 nm in a microtiter plate reader (Multiskan MS, Thermo Labsystems, Waltham, MA). Absolute or normalized values were fitted to a four-parameter logistic equation using Genesis Lite 3.03 (Life Sciences, London) package software.

Molinate Nanopeptamer Assay Cross-Reactivity. The specificity of the assay was characterized with related S-thiocarbamate pesticides. Cross-reactant concentrations in the 0–10 000 ng/mL range were used in the noncompetitive ELISAs. Standard curves were normalized by expressing experimental absorbance values (B), as $(B/B_0) \times 100$, where B_0 is the absorbance value at zero analyte concentration, and the molar concentration corresponding to the midpoint of the curve, (SC_{50}), was used to express the cross-reactivity of the assay according to the equation

$$\% \text{ cross-reactivity} = 100 \times \left[\frac{SC_{50}(\text{analyte})}{SC_{50}(\text{cross-reacting compound})} \right]$$

Carbon Black Avidin Labeling. A 5% solution of carbon black in Milli-Q water was homogenized in a sonication water bath and was diluted 25-fold with 5 mM boric buffer, pH 8.8. A volume of 100 μ L of avidin (4 mg/mL) in 5 mM boric buffer, pH 8.8 were combined with 900 μ L of 0.2% carbon black and incubated for 3 h at room temperature. The suspension was then centrifuged a 14 000 rpm for 15 min, and the pellet was resuspended in 1 mL of 100 mM boric buffer, pH 8.8, 0.02% Tween 20 by sonication. After repeating this step three more times, the carbon-labeled avidin was resuspended in 1 mL of 100 mM boric buffer, pH 8.8, 0.02% Tween 20 and used to prepare the nanopeptamers as described below.

Lateral Flow Immunochromatography. MoAb 14D7 or MoAb 5.6 lines were printed on Hi-Flow Plus 120 nitrocellulose membrane cards at 0.92 μ g/cm using a BioDot AD 1500 liquid dispenser. Avidin was labeled with carbon black

nanoparticles as described. Approximately 40 μg (100 μL) of carbon black labeled avidin was preincubated with 15 μg of pA or pX11 (6 μL , 2.5 mg/mL in DMSO) in a final volume of PBS containing 0.025% Tween-20. After incubation for 15 min on ice, 2.5 μL of this carbon black labeled nanopeptamers mixture were transferred to microtiter plate wells containing 100 μL of PBS, 0.025% Tween previously spiked with known amounts of molinate and clomazone standards. Hi-Flow Plus 120 nitrocellulose membrane cards (printed with MoAbs 14D7 or 5.6) assembled with an absorbent cellulose pad were dipped into the wells and let stand for 10 min. After that, the strips were read for the formation of a visible reaction line by the naked eye by four independent observers in three different repetitions of the tests. Molinate assays were validated by performing spiking in runoff water samples of agricultural areas of Uruguay (95 μL of water sample + 5 μL of PBS \times 10, 0.025% Tween 20) and measuring them with strips as described above.

RESULTS AND DISCUSSION

Anti-IC Peptides Can React Specifically with ICs When Displayed in a Multivalent Manner. As a starting model system, we used the thiocarbamate herbicide molinate, the antimolinate monoclonal antibody 14D7,¹⁵ and the anti-IC synthetic peptides p1M (biotin-SGSGCSTWDTTGWC) and pA (biotin-SGSGCSLWDTTGWC). Both peptides were previously isolated from phage libraries panned against the molinate-14D7 IC. Peptide pA, which differs only by having Leu in the seventh position, was isolated from a mutagenesis library derived from the p1M sequence and, while performing with similar sensitivity in PHAIA tests, showed less residual cross-reactivity with the unliganded antibody.⁸ Initial ELISA experiments aimed at detecting binding of the biotinylated peptides to the molinate-MoAb14D7 IC failed. This was assumed to be caused by the loss of the avidity effect inherent to the display of the peptide on pVIII. This prompted us to express the peptides as N-terminal fusion with the *E. coli* alkaline phosphatase that forms dimers and therefore allows bivalent display of the peptides, but only marginal reactivity with the IC was observed (not shown). In order to further increase the avidity of the interaction, we used the tetrameric structure of streptavidin as scaffold for tetravalent display of the biotinylated synthetic peptides. These streptavidin-peptide complexes, that we termed “nanopeptamers”, have the additional advantage that several ready-to-use streptavidin commercial conjugates exist, which can be used as versatile signal-generation elements (Figure 1).

Preliminary experiments with different commercial streptavidin-peroxidase conjugates showed the best results when streptavidin conjugated to a polymeric form of horse radish peroxidase conjugated to streptavidin (high-sensitivity streptavidin-peroxidase, SPO, Pierce (Rockford, IL)) was used. The nanopeptamers were initially formed by using an estimated 50-fold molar excess of biotinylated peptide and their reactivity was assayed in microtiter plates coated with MoAb 14D7, in the presence or absence of molinate. Similarly to what we observed with anti-IC phage, the nanopeptamers reacted specifically with the IC but showed some residual reactivity with the uncombined antibody. This cross-reactivity was more evident at high concentrations of nanopeptamers and a high density of coating antibody (Figure S-1, Supporting Information). Since these conditions (excess of reagents) are expected to provide the best assay sensitivity, a trade-off selection of these parameters needs to be done by checkerboard experiments.

This is exemplified in Figure S-2 (Supporting Information) where increasing concentrations of the nanopeptamer (SPO-pA) improve the assay sensitivity, but it also shows that the background signal at zero concentration of analyte deteriorates after a certain point due to the residual cross-reactivity with the uncombined antibody. The influence of the peptide to SPO ratio on assay performance was also studied. The formation of the streptavidin–biotin complex is fast, and due to the exceptionally high affinity of the interaction,¹⁶ it is expected that rapidly after mixing all the biotinylated peptide is complexed. However, since the exact stoichiometry of the SPO commercial polymeric conjugate is unknown, the amount of saturating biotinylated peptide was determined empirically (Figure S-3, Supporting Information).

PHAIA and Nanopeptamer ELISAs Perform in a Similar Way. After optimization of these parameters, both nanopeptamers (SPO-pA and SPO-p1M) were used to develop noncompetitive assays for molinate, Figure 2. The dose

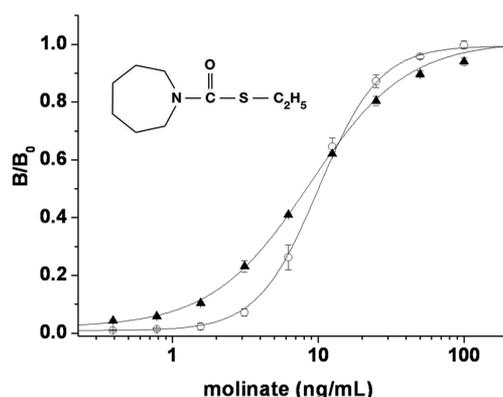


Figure 2. Noncompetitive nanopeptamer ELISA for molinate using peptides pA and p1M. MoAb 14D7 (10 $\mu\text{g}/\text{mL}$) was used for coating and SPO (0.75 $\mu\text{g}/\text{mL}$) complexed with a 50-fold molar excess of pA (\blacktriangle) or p1M (\circ) was used for detection.

response curves had a typical sigmoid shape with signal saturation at a high concentration of molinate. The midpoint corresponding to the concentration of analyte giving 50% of signal saturation (SC_{50}) was 8.3 ± 0.2 and 10.0 ± 0.3 ng/mL, and the limit of detection (LOD = analyte concentration giving a 10% increase over the zero signal) were 1.2 and 3.2 ng/mL for nanopeptamers pA and p1M, respectively. The LOD attained with the nanopeptamers were up to 18-fold better than that of the competitive ELISA set up with the same antibody (LOD 22 ng/mL, IC_{50} 69 ± 0.5 ng/mL).¹⁵

The SC_{50} obtained with nanopeptamer p1M was not as good as the SC_{50} obtained in the PHAIA format ($SC_{50} = 5.0 \pm 0.4$ ng/mL), but this was not the case for the pA complex that performed with a SC_{50} value equal to that obtained with the phage borne peptide (8.5 ± 0.5 ng/mL).⁸ On the basis of these results, nanopeptamer pA was then used to characterize the cross-reactivity, matrix interference and for adaptation of the nanopeptamer assay to other formats. Cross-reactivity was tested using a panel composed of common agrochemicals utilized in rice culture (quinclorac, glyphosate, molinate, bispiribac, propanil, and atrazine) as well as other S-thiocarbamate pesticides, Table 1. Only minor cross-reactivity with closely related thiocarbamate compounds was observed, which was similar to that obtained with the PHAIA assay. Likewise, no matrix effect was observed when standard

Table 1. Cross Reactivity (%) of Nanopeptamer pA and PHAIA Assays with Related Thiocarbamate Pesticides^a

Compound	Structure	SPO-pA	PHAIA
Molinate		100	100
Thiobencarb		0	0
Butylate		1	0
EPTC		2	5
Cycloate		10	9
Pebulate		6	7
Vernolate		5	4

^aAll data are the mean of two independent experiments. A value of 0 means that there was no observable cross-reactivity at the highest concentration tested, 10⁴ ng/mL.

molinate curves were performed with undiluted agricultural runoff-water samples from different areas of Uruguay (Figure S-4, Supporting Information).

Nanopeptamers Allow the Development of Non-competitive Lateral-Flow Tests. A major advantage of noncompetitive assays is that they can be developed into simple formats with a positive visual end point. Our initial attempts to adapt PHAIA into lateral-flow assays were not successful, most probably due to the filamentous nature of the phage that promoted the formation of aggregates with the colloidal labels. Fortunately, this limitation was overcome using nanopeptamers. Figure 3A shows the results of a lateral-flow assay set up using MoAb 14D7 as the capture reagent immobilized on

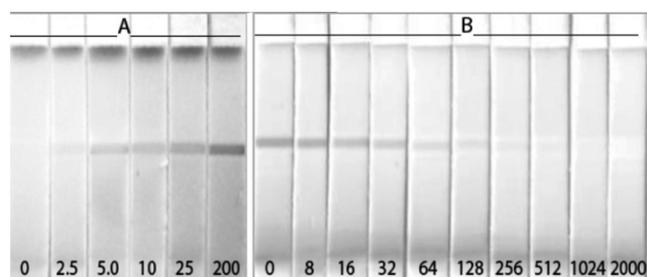


Figure 3. Noncompetitive nanopeptamer pA-based (A) and competitive (B) lateral-flow assays for molinate. The nitrocellulose strips were tested with buffer containing various concentrations of molinate (ng/mL) as denoted in the figure.

polyester-backed nitrocellulose membranes and colloidal-carbon labeled avidin complexed to pA for detection (see the Supporting Information). For the sake of comparison, we also developed the molinate assay in a lateral-flow competitive format using MoAb 14D7 as capture antibody and the molinate derivative 7b (S-2-(*p*-aminophenyl)-ethyl-hexahydroazepine-1-carbothioate) coupled to conalbumin, which was labeled with colloidal carbon for detection, Figure 3B. A molinate concentration of 2.5 ng/mL caused a visible test line in the noncompetitive assay, while 32 ng/mL produce a weaker test line than the negative control in the competitive assay, as agreed upon by four independent observers in three different repetitions of the test. This is in agreement with the densitometry analysis of the strips, Supporting Table S-1A (Supporting Information). In addition to the positive reading, the noncompetitive test performed with a 10-fold improved sensitivity. The assay was then tested for matrix interference using 10 runoff water samples from agricultural areas of Uruguay spiked with 0, 1.0, 1.5, 2.5, 5, and 20 ng/mL of molinate. The strips were read by four independent observers, all of whom detected a visible reaction line with concentrations of 2.5 ng/mL or higher, Figure S-5 (Supporting Information).

Nanopeptamer Based ELISA and Lateral-Flow Test for Clomazone. To explore the general utility of the method, we also developed nanopeptamer-based assays for the herbicide clomazone. To this end we used the anticlomazone MoAb 5.6¹⁷ and the anti-IC synthetic peptide X11 (biotin-SGSGCLEAP-NIEGC) complexed with SPO or avidin. This peptide has been

previously isolated from phage libraries panned against the clomazone-MoAb 5.6 IC.⁹ For ELISA, the assay conditions were optimized essentially as described for the molinate test, which allowed one to obtain a LOD = 1.2 ng/mL and SC50 = 3.4 ± 0.2 ng/mL using SPO-pX11 (Figure 4A). This represents

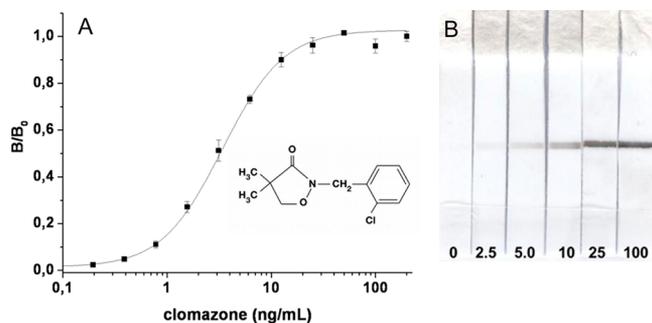


Figure 4. Noncompetitive nanopeptamer ELISA and lateral-flow test for clomazone using peptide pX11. (A) MoAb 5.6 (10 $\mu\text{g/mL}$) was used for coating and SPO (0.75 $\mu\text{g/mL}$) complexed with a 50-fold molar peptide excess for detection. (B) Nitrocellulose strips were tested with buffer containing various concentrations of clomazone (ng/mL) as denoted in the lower part of the figure.

an improvement of 3.3 and 8.3-fold regarding the assay setup with the same antibody in a competitive format (LOD = 4 ng/mL and IC50 = 28 ± 1.1 ng/mL, respectively).¹⁷ The assay was also adapted into a lateral-flow format using avidin-pX11 labeled with carbon black, which allowed one to detect up to 2.5 ng/mL of clomazone (Figure 4B); the densitometry data is shown in Supporting Table S-1B in the Supporting Information.

CONCLUSIONS

Our results show that in spite of big structural differences, the streptavidin/avidin-based nanopeptamer properly reproduces the binding characteristics of the phage particles. Indeed, the dihedral D2 molecular symmetry of the streptavidin homotetramer positions two pairs of biotin binding sites on opposite faces of the protein, and thus the distance between the biotin carboxylated oxygens that anchor the peptides are ~ 2 nm on the same face and ~ 3 nm on opposite faces of the complex.¹⁸ On the other hand, M13 has a ~ 6 nm \times 1000 nm rodlike structure, covered by ~ 2700 copies of the minor protein pVIII arranged in a fish-scale pattern, and the use of a phagemid system for the generation of the virions yields phage particles with approximately 200 copies of pVIII expressing the peptide, meaning an average distance between peptides close to 10 nm.¹⁹ In addition, the peptide is tethered in a different way in both systems. For the sake of simplicity, the peptides were synthesized with a biotin residue added to their N-terminus, while they are expressed as pVIII N-terminal fusions on the phage. Considering the differences in valences and display features of both systems, it seems that the specific recognition of the IC by the peptide can be transferred out of the “phage context” with a considerable degree of flexibility.

As demonstrated here, the availability of phage-free reagents for noncompetitive assays could have a major impact in the development of rapid two-site tests for small molecules with a positive visual end point, a feat not possible with the available technology. Since the strategy for the selection of anti-IC phage borne peptides is well established, and considering the varied offer of commercially available streptavidin conjugates, nano-

peptamers may be the long-sought detection reagent for the development of fast noncompetitive assays for small analytes. In that sense a major proof of concept contributed by our work is their use in the development of lateral flow test for small molecules with a positive reading improving the sensitivity and interpretability of these tests.

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>.

AUTHOR INFORMATION

Corresponding Author

*E-mail: ggonzal@fq.edu.uy. Phone: +(598) 2487 4334.

Author Contributions

L.V. and A.G.-T. contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

- Hage, D. S. *Anal. Chem.* **1995**, *67*, 455R.
- Ng, A. H.; Uddayasankar, U.; Wheeler, A. R. *Anal. Bioanal. Chem.* **2010**, *397*, 991.
- Schneider, R. J. *Anal. Bioanal. Chem.* **2003**, *375*, 44.
- Jackson, T. M.; Ekins, R. P. *J. Immunol. Methods* **1986**, *87*, 13.
- Piran, U.; Riordan, W. J.; Livshin, L. A. *Clin. Chem.* **1995**, *41*, 986.
- Pradelles, P.; Grassi, J.; Creminon, C.; Boutten, B.; Mamas, S. *Anal. Chem.* **1994**, *66*, 16.
- Ueda, H. J. *Biosci. Bioeng.* **2002**, *94*, 614.
- Gonzalez-Techera, A.; Vanrell, L.; Last, J. A.; Hammock, B. D.; Gonzalez-Sapienza, G. *Anal. Chem.* **2007**, *79*, 7799.
- Rossotti, M. A.; Carlomagno, M.; Gonzalez-Techera, A.; Hammock, B. D.; Last, J.; Gonzalez-Sapienza, G. *Anal. Chem.* **2010**, *82*, 8838–8843.
- Kim, H. J.; Rossotti, M. A.; Ahn, K. C.; Gonzalez-Sapienza, G. G.; Gee, S. J.; Musker, R.; Hammock, B. D. *Anal. Biochem.* **2010**, *401*, 38–46.
- Gonzalez-Techera, A.; Kim, H. J.; Gee, S. J.; Last, J. A.; Hammock, B. D.; Gonzalez-Sapienza, G. *Anal. Chem.* **2007**, *79*, 9191.
- Inaba, J.; Nakamura, S.; Shimizu, K.; Asami, T.; Suzuki, Y. *Anal. Biochem.* **2009**, *388*, 63.
- Tanaka, F.; Hu, Y.; Sutton, J.; Asawapornmongkol, L.; Fuller, R.; Olson, A. J.; Barbas, C. F., 3rd; Lerner, R. A. *Bioorg. Med. Chem.* **2008**, *16*, 5926.
- Kim, H. J.; McCoy, M.; Gee, S. J.; Gonzalez-Sapienza, G. G.; Hammock, B. D. *Anal. Chem.* **2011**, *83*, 246.
- Rufo, C.; Hammock, B. D.; Gee, S. J.; Last, J. A.; Gonzalez-Sapienza, G. *J. Agric. Food Chem.* **2004**, *52*, 182.
- Srisa-Art, M.; Dyson, E. C.; deMello, A. J.; Edel, J. B. *Anal. Chem.* **2008**, *80*, 7063.

(17) Carlomagno, M.; Matho, C.; Cantou, G.; Sanborn, J. R.; Last, J. A.; Hammock, B. D.; Roel, A.; Gonzalez, D.; Gonzalez-Sapienza, G. J. *Agric. Food Chem.* **2010**, *58*, 4367.

(18) Hendrickson, W. A.; Pahler, A.; Smith, J. L.; Satow, Y.; Merritt, E. A.; Phizackerley, R. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2190.

(19) Martens, C. L.; Cwirla, S. E.; Lee, R. Y.; Whitehorn, E.; Chen, E. Y.; Bakker, A.; Martin, E. L.; Wagstrom, C.; Gopalan, P.; Smith, C. W.; Tate, E.; Koller, K. J.; Schatz, P. J.; Dower, W. J.; Barrett, R. W. *J. Biol. Chem.* **1995**, *270*, 21129–21136.