

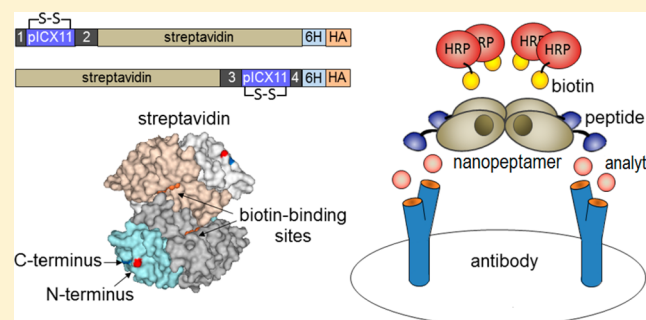
Recombinant Streptavidin Nanopeptamer Anti-Immunocomplex Assay for Noncompetitive Detection of Small Analytes

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Supporting Information

ABSTRACT: Short peptide loops selected from phage libraries can specifically recognize the formation of hapten-antibody immunocomplexes and can thus be used to develop phage anti-immunocomplex assays (PHAIA) for noncompetitive detection of small molecules. In this study, we generated recombinant chimeras by fusing anti-immunocomplex peptides selected from phage libraries to the N- or C-termini of core streptavidin and used them to setup phage-free non-competitive assays for the herbicide clomazone (MW 240 Da). The best conditions for refolding were optimized by a high throughput screening allowing to obtain tens of mg of purified protein per liter of culture. The noncompetitive assay developed with these chimeras performed with a 50% saturating concentration (SC_{50}) of 2.2 ± 0.3 ng/mL and limit of detection (LOD) of 0.48 ng/mL. Values that are 13- and 8-fold better than those obtained for the SC_{50} and LOD of the competitive assay setup with the same antibody. Apart from the first demonstration that recombinant peptide-streptavidin chimeras can be used for sensitive immunodetection of small molecules with a positive readout, this new assay component is a highly standardized reagent with a defined stoichiometry, which can be used in combination with the broad option of existing biotinylated reagents offering a great versatility for the development of conventional immunoassay and biosensors. The utility of the test was demonstrated analyzing the clomazone runoff during the rice growing season in northern Uruguay.



While macromolecular analytes are relevant targets in clinical diagnosis, small-molecules constitute the vast majority of the analytes of interest in environmental studies, toxicology, drug monitoring, biosecurity, etc. The classical two-antibody sandwich assay used for immunodetection of macromolecules cannot be applied to small-analytes, because of their small size that impedes the simultaneous binding of two antibodies. With few exceptions, such as the use of poly dentate ligands,¹ antimetatype antibodies,² or the open sandwich assay,³ the immunodetection of these analytes has been restricted to the use of competitive assays which use a competitor hapten either labeled with a tracer molecule or conjugated to a carrier protein for coating. Unfortunately, this competitive assay performs with inferior sensitivity, precision and kinetics range than the two-site noncompetitive format,⁴ and their adaptation into lateral-flow tests or biosensors is more difficult. In 2007, we introduced the use of small peptide loops that, upon binding of the hapten, specifically recognize the modifications of the antigen-binding site on the antibody, and therefore can be used to detect the formation of the immunocomplex (IC). These peptide loops are selected on the immobilized IC, from phage display libraries expressing 7–11 random residue peptides flanked by two cysteines that form a disulfide bridge and constrain the peptide structure. The initial application of these

anti-IC peptides for noncompetitive detection of small molecules was first described for the herbicides molinate and atrazine, and the drugs digoxin and cyclosporine,⁵ and later for the flame-retardant brominated diphenyl ether⁶ and the pyrethroid metabolite phenoxybenzoic acid.⁷ In this method, termed PHAIA (phage anti-immunocomplex assay), the IC was detected by the formation of a tertiary complex with the phage particle, which was subsequently revealed with an anti-M13 peroxidase conjugate, or by amplification of the phage DNA by real time PCR.⁷ In addition to a proportional signal, PHAIA also provides an increased sensitivity. Typically, using the same monoclonal antibody, the adaptation of any competitive assay into PHAIA is accompanied by a 10–20 fold increase in sensitivity, and this can be even higher in the case of polyclonal antibodies.⁸ Additionally, the formation of the antibody-analyte-peptide complex provides a double recognition of the analyte, which also contributes to a higher assay specificity.⁹ The phage particles are particularly robust and when the peptide is fused to the major phage coat protein, their filamentous nature provides a multivalent scaffold that allows

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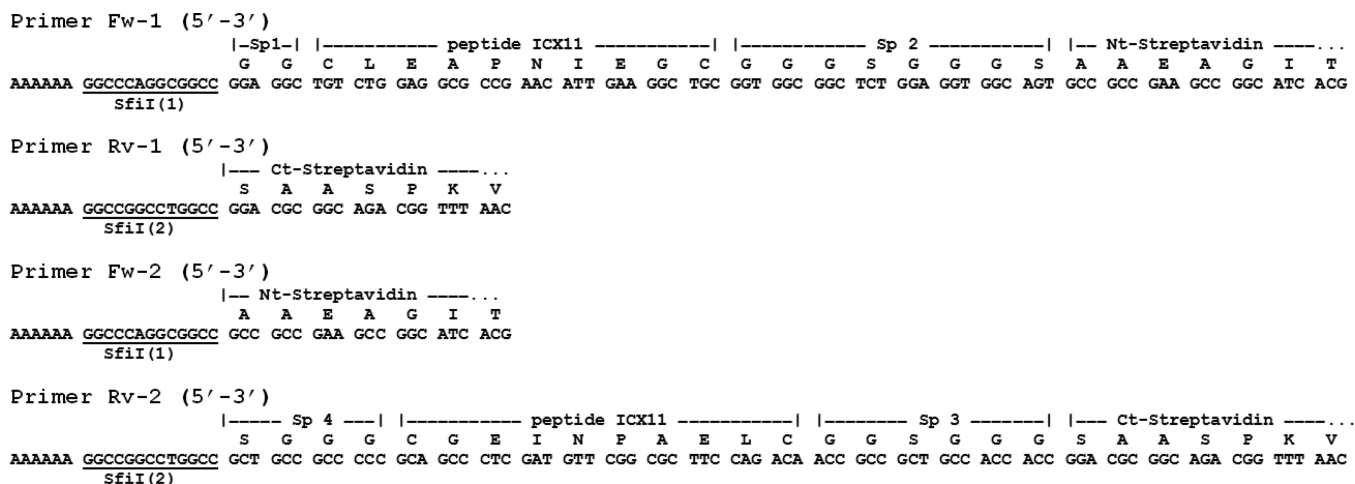


Figure 1. Primers used to assemble the anticlomazone IC cassettes. The regions coding for the peptide, spacers (Sp), and the annealing to the streptavidin gene are denoted on top of the nucleotide sequence. The SfiI sites further used for cloning are underlined.

the display of hundred copies of the peptide providing high avidity for the IC.¹⁰ However, the phage particles are “unconventional” reagents for the immunoassay industry, and they can confer antibiotic resistance to *Escherichia coli* laboratory strains, which can be a safety concern in molecular biology laboratories. To work-around these limitations, we recently demonstrated that the phage particles can be substituted by commercial conjugates of streptavidin or avidin loaded with synthetic anti-IC peptides that contain a biotinylated lysine in their N-terminus.¹¹ These complexes, that we termed nanopeptamers, could be used to develop two-site noncompetitive assays for small molecules, which performed with similar sensitivity and specificity than their parent anti-IC phage particles.

In this work, we present a further development of the streptavidin nanopeptamer technology optimizing their production as recombinant peptide-streptavidin chimeras. This not only dramatically reduces the production costs, but it also frees the biotin binding sites allowing the use of the full arrange of biotinylated reagents, including tracer enzymes, fluorophores, colloidal gold, magnetic beads, etc. which will facilitate the development of all sort of immunoassays, including optical, electrochemical and magnetic biosensors. Streptavidin (STR) is a homotetrameric protein naturally secreted by the actinobacterium *Streptomyces avidinii*, which has an exceptionally high affinity (K_d of 10^{-13} – 10^{-16} M) for biotin.¹² The monomers are synthesized as full-length proteins of 159 amino acid that are further processed to remove 12 and 20 amino acids from the N- and C-termini, respectively, yielding core streptavidin. There is one biotin binding site per monomer, but the binding of biotin by an isolated monomer has a K_d of 10^{-7} M, and only after assembling of the tetramer the affinity becomes dramatically higher.¹³ The protein has been expressed in *E. coli* either as a fusion protein or alone at good yields, though normally as an insoluble protein.^{14–17} As a model small analyte to develop the recombinant nanopeptamers we choose the herbicide clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone, MW 240 Da), one of the main herbicides used in rice culture, and the peptide CLEAPNIEGC which is specific for the IC formed by the herbicide and the monoclonal antibody MAb5.6.⁹ After assay optimization, the recombinant nanopeptamer was used to study the runoff of the herbicide during the rice growing season in Uruguay.

MATERIALS AND METHODS

Materials. Monoclonal antibody 5.6 (MAb5.6), a mouse IgG against clomazone, was prepared as described before (Rossotti 2010). Clomazone was purchased from Riedel-de Haën, Seelze, Germany. The BCA Protein Assay Kit for the quantification of purified antibody and protein G affinity columns for IgG purification, were purchased from Pierce (Rockford, IL). BSA, Tween 20, IPTG (isopropyl β -D-1-thiogalactopyranoside), polyethylene glycol 8000 (PEG), and 3,3',5,5'-tetramethylbenzidine (TMB), other common chemicals were purchased from Sigma (St. Louis, MO).

Assembling of the Anticlomazone IC Nanopeptamer Construct. The STR gene (amino acids 14–139) was optimized for expression in *E. coli* and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). To assemble the anticlomazone IC cassettes, the streptavidin-gene template was amplified by PCR employing the primers shown in Figure 1. The primers Fw-1 in combination with Rv-1, and Fw-2 with Rv-2 were used to amplify the nanopeptamer genes with the peptide CLEAPNIEGC (pICX11) at the N-terminus (pICX11-STR), or C-terminus (STR-pICX11), respectively. The cassettes carried two noncomplementary SfiI sites that were used to clone them into a modified pET28a+ vector (Novagen), between the OmpA signal peptide and the 6 x His and HA (hemagglutinin) tags. The ligated vectors were electroporated in competent BL21(DE3) *E. coli* cells (Life Technologies, Carlsbad, CA, USA).

Expression and Partial Purification of the Recombinant STR Nanopeptamers. Selected colonies of BL21(DE3) transformed with either the pICX11-STR or STR-pICX11 constructions, whose sequence were verified by DNA sequencing, were grown in 500 mL of LB ampicillin (50 μ g/mL) at 37 °C, with shaking at 250 rpm, with an absorbance of 0.4 AU at 600 nm. The cultures were then induced with 1 mM IPTG, incubated for 3 h and centrifuged at 5000 g for 15 min at 4 °C. The cells were sonicated in PBS (phosphate buffer saline) on ice and the inclusion bodies were purified by a detergent-washing protocol as described.¹⁸ The recombinant chimeras were dissolved in 10 mL of buffer 100 mM Tris-phosphate, 8 M urea, pH 9, by overnight rocking at 4 °C, the solution was clarified by centrifugation at 30 000g for 30 min, and the concentrations of the chimeras was estimated by densitometry from SDS-PAGE gels.

Protein Refolding Screening. A series of buffers covering a wide pH range were prepared: 200 mM acetate, pH 5.0; 100 mM phosphate, pH 6.0; 100 mM phosphate, pH 7.0; 200 mM Tris, pH 8.0; 200 mM Tris, pH 8.5; 200 mM Tris, pH 9.0; and 200 mM carbonate, pH 11. One hundred microliters of these buffers were dispensed into the wells of a 96-well microtiter plate and were combined with 100 μ L of the following additives to create a large arrange of refolding solutions: arginine (2.0 M, 1.0 M, 0.4 M), sucrose (3.0 M, 1.0 M, 0.4 M), glycerol (80%, 26%, 8%), and polyethylene glycol (PEG) 8000 (40%, 20%, 10%). Ten microliters of the urea solubilized chimeras were added into each well, mixed thoroughly and kept a 4 °C for 1 h.

Activity of the Refolded Chimeras. The biotin-binding activity of the refolded proteins was tested by ELISA. Polystyrene high-binding microtiter plates (Greiner, Germany) were coated with 100 μ L/well of 0.3 μ g/mL of biotinylated bovine serum albumin (BSA) in PBS, incubated overnight at 4 °C, blocked by incubation with 5% skimmed milk in PBS, 0.05% Tween 20 (PBS-T) for 1 h, and washed with PBS-T. The plates were then loaded with 95 microliters of 1% BSA in PBS-T and 5 μ L of each of the refolding solutions (about 150 ng of refolded protein), incubated for 1 h and washed. One hundred microliters of biotin conjugated to peroxidase (Pierce, Rockford, IL, USA) diluted 1:2000 in BSA 1%, after 40 min. After thorough washing, 100 μ L of the peroxidase substrate (0.4 mL of 6 mg/mL 3,3',5,5'-tetramethylbenzidine in DMSO, 0.1 mL of 1% H₂O₂, in 25 mL of 0.1 M citrate acetate buffer, pH 5.5) were dispensed into each well, and the reaction was stopped after 10 min by addition of 50 μ L of 2 N H₂SO₄. Absorbance was read at 450/650 nm in a microtiter plate reader (Multiskan MS, Thermo Labsystems, Waltham, MA). Similarly, the anti-IC specificity of the refolded nanopeptamers was also assessed by ELISA. Plates were coated with 5 μ g/mL of MAb5.6 (100 μ L/well), blocked, washed, and dispensed with 5 μ L of the refolded proteins diluted in PBS-T with or without clomazone (100 ng/mL), after incubation (1 h), the wells were washed and dispensed with an anti-HA antibody coupled to peroxidase (HRP) (Roche, Madison, WI) or alternatively with biotin-HRP (Pierce).

Nanopeptamer ELISA for Clomazone. Optimal concentrations of the coating antibody and detecting nanopeptamer were established by checkerboard titrations. Briefly, a microtiter plate was coated with 100 μ L/well of MAb5.6 in PBS), at concentrations of 10, 5, 2.5, and 0.5 μ g/mL that were dispensed in rows A and B, C and D, E and F, and H and I, respectively. After blocking, every other row was dispensed with 50 μ L of serial dilutions of the refolded protein in PBS-T, plus 50 μ L of PBS-T containing 100 or 0 ng/mL of clomazone, respectively. After they were incubated for 1 h and washed, the binding of the nanopeptamers was revealed using a 1/2000 dilution of the biotinylated HRP conjugate. After they were washed, the plates were incubated with substrate and read as described before. On the basis of the checkerboard titration, appropriate dilutions of coating antibody MAb5.6 and refolded protein were selected, to obtain a maximum difference between the absorbance obtained in the presence and absence of clomazone. The ELISA test was then essentially performed as described above. A 12-point internal standard curve (0–1000 ng/mL range) was performed by triplicates in each experiment. Absolute or normalized values were fitted to a four-parameter logistic equation using Genesis Lite 3.03 (Life Sciences, London) package software. For the analysis of field samples, the reaction mix was supplemented

with 10% of Interference Buffer (1 M Tris, 0.3 M NaCl, 0.3 M EDTA, 1% BSA, pH 7.4).

Metal Affinity Chromatography. The nanopeptamers were purified from the refolded extracts by immobilized metal ion affinity chromatography employing a Ni-NTA column (HisTrap HP 1 mL, GE Health Care, Pittsburgh, USA) equilibrated with buffer 50 mM Tris, 0.3 M NaCl, 20 mM imidazol, pH 7. After the sample was injected, the column was washed with 5 column volumes of 50 mM Tris, 0.3 M NaCl, 40 mM imidazol, pH 7, and the protein eluted (1 mL fractions) by increasing the concentration of imidazole to 500 mM. After dialysis against PBS, the recombinant nanopeptamers were supplemented with Complete Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN) and sodium azide 0.05%. The preparation was filtered (0.22 μ m) and stored in aliquots at 4 °C for short-term use or kept at –80 °C until used.

RESULTS AND DISCUSSION

Cloning and Expression of the Recombinant STR Nanopeptamers. We have recently showed that the Shiga like toxin (VTX) can be used as scaffold for the production of recombinant VTX nanopeptamers.¹⁹ However, they need to be conjugated to a tracer enzyme limiting the scope of their applications. The use of core streptavidin as scaffold for the display of recombinant anti-immunocomplex peptides will remove these limitations, because they can be combined with the broad options of biotinylated reagents that could serve as assay tracers. To produce recombinant STR nanopeptamers specific for clomazone we fused the peptide pICX11 (CLEAPNIEGC) to the N-terminus (pICX11-STR), or C-terminus (STR-pICX11), of a synthetic core streptavidin gene using the spacers shown in Figure 2. The coding sequences for these constructs were assembled by PCR using the primers

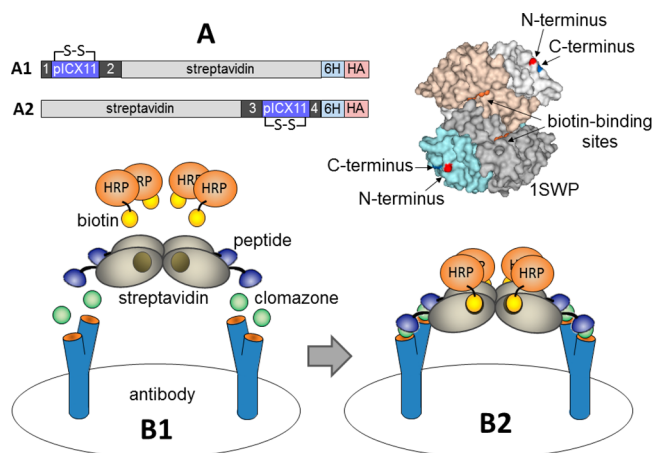


Figure 2. Schematic representation of the noncompetitive STR nanopeptamer assay for clomazone. (A) Outline of the core streptavidin (residues 14–139) nanopeptamer subunits containing the anticlomazone/MAb5.6 IC peptide (pICX11) at the N-terminus (A1 = pICX11-STR, MW 17 438 Da) or C-terminus (A2 = STR-pICX11, MW 17 438 Da). Spacers: (1) GG, (2) GGGSGGG, (3) GGGSGG, and (4) GGGSGQAGQ. Tags: 6H = 6 × His; HA = amino acid sequence YPYDVDPDYA. The N-terminus (red) and C-terminus (blue), as well as the biotin binding sites are shown on the 1SWP (PDB) structure of tetrameric streptavidin. The individual components of the assay are shown on the left (B1), and the antibody-analyte-nanopeptamer ternary complex is represented on the right (B2). HRP: Horseradish peroxidase.

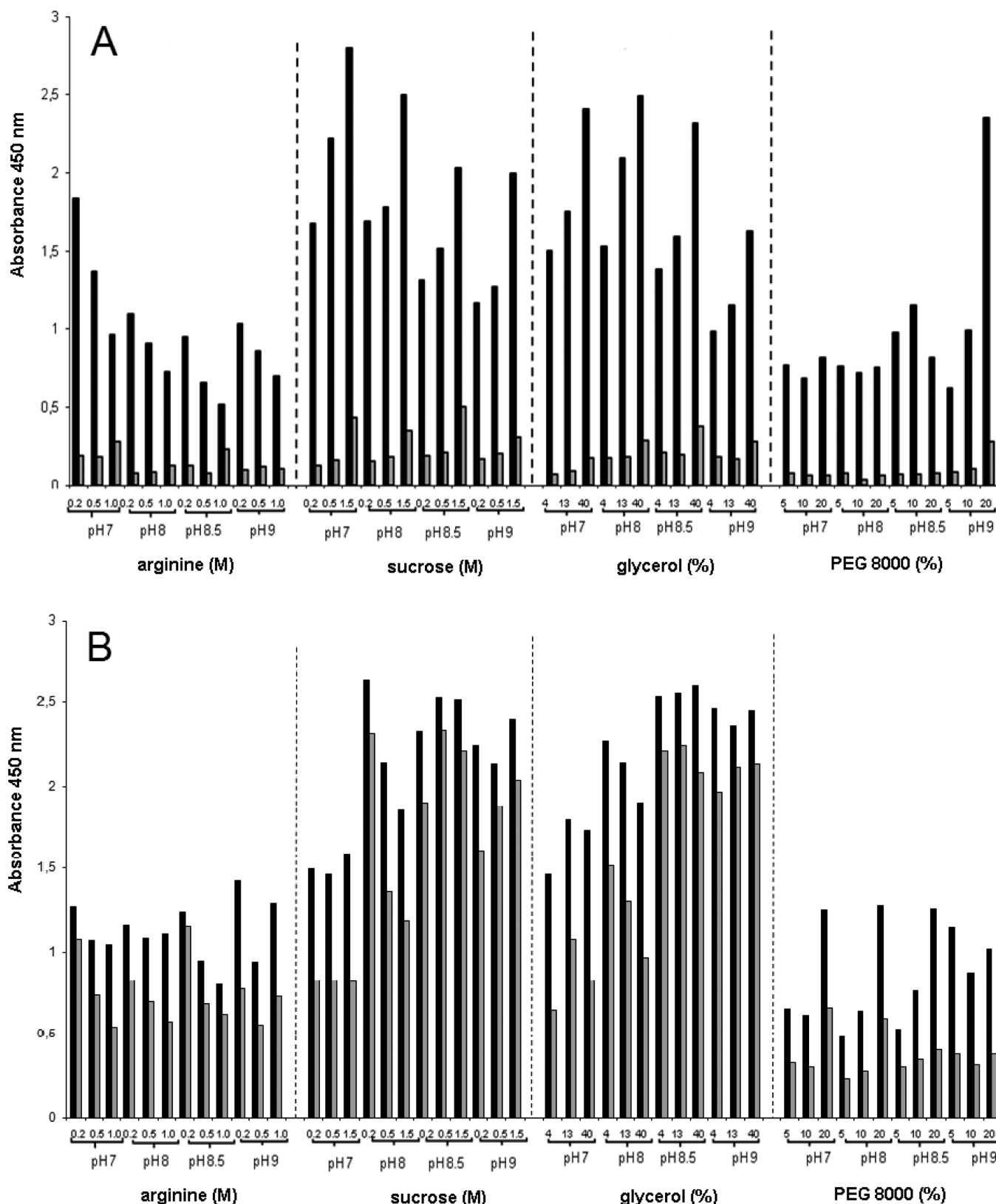


Figure 3. Specific recognition of the clomazone-MAb5.6 IC by the refolded nanopeptamers. The reactivity of the refolded chimeras was assayed on ELISA wells coated with the MAb5.6, in the presence (black) or absence (gray) of clomazone. Binding of the nanopeptamers was revealed with the anti-HA HRP conjugate using TMB as substrate. The concentration of the refolding agent is shown on top of the pH brackets. (A) Nanopeptamer pICX11-STR and (B) Nanopeptamer STR-pICX11.

described above, and were cloned into a modified pET28 vector (Novagen) between two noncomplementary SfiI sites, flanked

by the nucleotide sequences of the OmpA signal peptide and the C-terminal 6× His and HA tags. Initial experiments of

expression in *E. coli* BL21 showed that the streptavidin chimeras were only found in the insoluble fraction, which could not be reverted by modification of the temperature, IPTG concentration, or induction time of the cultures. Consequently, inclusion bodies were solubilized in buffer 100 mM Tris-phosphate, 8 M urea, pH 9.

Renaturation of Solubilized Chimeras. A large screening of refolding conditions was performed in microtiter plates as described above. Initially, the renatured proteins were tested for their binding to biotin using a sandwich ELISA, which showed that, with few exceptions, good activity was recovered in almost all tested conditions (Supporting Information, Figure S-1). Additional selection of the active proteins was performed on the basis of the specific recognition of the clomazone-MAb5.6 IC by the chimeras, using the HA tag for detection, Figure 3.

There was a marked difference between the N- and C-terminal chimeras. In general, all refolded pICX11-STR proteins reacted strongly and specifically with the IC, with little residual reactivity against the uncombined antibody. The difference in the readouts in the presence or absence of clomazone was even higher when the nanopeptamer was detected with the biotin-HRP conjugate (Supporting Information, Figure S-2). The strongest signals, and probably most efficient refolding, were found with the sucrose and glycerol buffers. In general, the C-terminal expression of the peptide gave place to a high background. This was unexpected because in our recent work describing the use of synthetic nanopeptamers, the peptides were tethered to the streptavidin tetramer through their N-terminus, and yet the reactivity with the uncombined antibody was negligible. The difference may arrive from the fact that in the recombinant chimera the peptide is fused to the $6 \times$ His and HA tags, but this issue was not further studied and the STR-pICX11 nanopeptamer was not further used. The performance of the most promising refolded pICX11-STR chimeras (minimal residual reactivity with the unbound antibody) was further studied by checkerboard titration (coating concentration of antibody versus concentration of nanopeptamer) using a fixed amount of analyte. In the development of competitive methods using phage (PHAIA) or synthetic nanopeptamers for detection, we found that the best sensitivity is obtained with the highest concentration of coating antibodies and detecting reagent, providing that they do not compromise the background of the assay.^{5,11} In this case, the recombinant pICX11 refolded in 100 mM phosphate, 1.5 M sucrose, pH 7.0 showed the best signal to background ratio over a long-range of concentrations (Supporting Information, Figure S-3) and this chimera was therefore selected for the development of the noncompetitive assay.

Nanopeptamer Assay for Clomazone. The pICX11 nanopeptamer was purified on Ni-NTA agarose and the formation of the tetramer was analyzed in SDS-gels, Figure 4. Under mild denaturing conditions (SDS at room temperature) streptavidin does not dissociate, and this was also the case for the nanopeptamer that occurred as a ~ 68 kDa band (lanes 1–3), which is in agreement with the theoretical size of 69 752 Da of the tetramer. After it was heated at 95 °C, the oligomeric structure of the complex dissociated and the monomer appeared as a band of ~ 18 kDa. The assay concentration of the purified nanopeptamer pICX11-STR was then optimized by checkerboard titrations and used to develop a noncompetitive test for clomazone, Figure 5. The midpoint of the titration curve, corresponding to the concentration of analyte giving 50% of signal saturation (SC_{50}) was 2.2 ± 0.3 , and the limit of

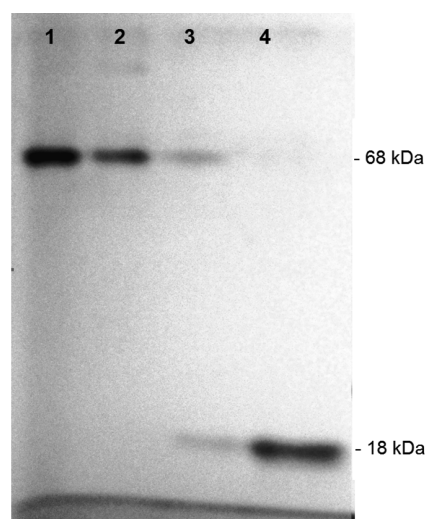


Figure 4. SDS-PAGE Analysis of the Purified Recombinant Nanopeptamer. Nonreducing 12% SDS-gel stained with coomassie blue. Lanes 1–3, fractions 1–3 eluted from the Ni-NTA column. These samples were mixed with sample buffer but were not heated before running. Lane 4, same as lane 1, but heated at 95 °C for 10 min which is necessary for the dissociation of the tetrameric structure of streptavidin.

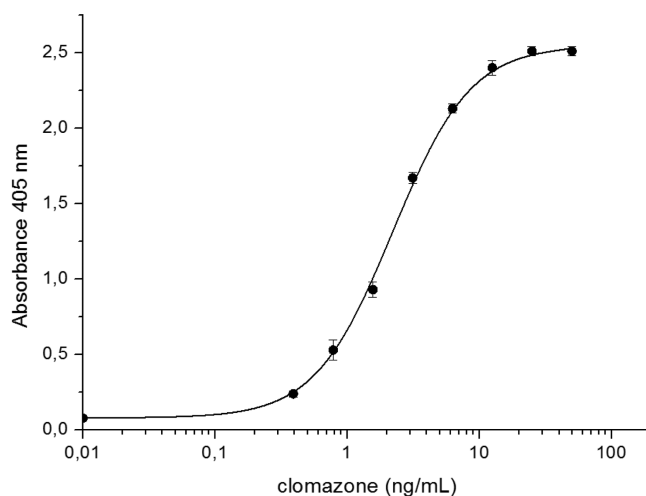


Figure 5. pICX11-STR noncompetitive ELISA for clomazone. The assay was carried out on wells coated with 2 μ g of MAb5.6 and using 1.5 μ g/well of the purified pICX11-STR nanopeptamer. Biotin-HRP was used for detection. The SC_{50} was 2.2 ± 0.3 ng/mL. Measurements were performed in triplicate; error bars correspond to the standard deviations.

detection (LOD = analyte concentration giving a 10% increase over the zero signal) was 0.48 ng/mL. These parameters are essentially the same that were obtained with the nanopeptamers prepared with streptavidin-HRP functionalized with biotinylated peptides,¹¹ showing that the variations in the attachment point to the streptavidin oligomer (Figure 1) do not have a major effect in the overall avidity of the complex for the IC. When these values are compared to the performance of the competitive assay set up with the same antibody, $SC_{50} = 28 \pm 1.1$ ng/mL and LOD = 4.0 ng/mL, it becomes evident that the recombinant nanopeptamer assay performs with increased sensitivity, representing an improvement of about 13 and 8 fold, respectively.

Streptavidin Nanopeptamer Analysis of Clomazone Runoff from Rice Cultivars. To study the potential utility of the test for the analysis of the runoff of pesticides from rice fields, we spiked water samples collected from areas with no record of clomazone application. Three water samples were spiked with various concentrations of the herbicide. To avoid the matrix effect caused by humic acids and other interfering compounds, the interference buffer was included, containing a bulk protein, a chelating agent to sequester metal ions, and high salt content to increase the ionic strength. The recoveries were good, even in the low concentration range, Table 1.

Table 1. Recovery of Clomazone from Spiked Water Samples

clomazone spiked (ng/mL)	mean recovery (% , n = 3)		
	water 1	water 2	water 3
1.0	110 ± 0.5	108 ± 3.8	105.4 ± 2.7
2.0	98 ± 7.0	110 ± 4.0	101 ± 8.1
3.0	89 ± 2.9	107 ± 12	99 ± 0.6
4.0		105 ± 17.5	104 ± 5.5
8.0	105 ± 4.3	73 ± 0.5	85 ± 1.3

The assay was then used to study the occurrence of clomazone in the basin of the Tala creek, a tributary stream of the Arapei river, in Salto, Uruguay. The Tala creek crosses a rice growing area, as well as soybean, sorghum, and raygrass fields. Clomazone is used exclusively in the rice area, being first applied in spring about 1 week before planting (0.86 kg/ha) and then about 15–20 days postemergence (0.38 kg/ha). After this second application, the fields were flooded and remained so for 50 days. The impact of the clomazone runoff was studied in seven sampling points along this basin, point 1 was upstream of the rice fields, points 2–4 and 6 were close to the fields, and point 5 and 7 were downstream of the planting area, as shown in Figure 6. Samples were taken regularly over a 55 day period (two contiguous (1 m) samples per point), and analyzed by triplicates using the nanopeptamer assay, Table 2. Overall, the concentration of the herbicide was very low, and below the LOD in most cases. Samples collected from sampling points 3, 4, 5, and 7 had measurable concentration of the herbicides at the end of the study, probably in connection with some heavy rain episodes. However, all of these samples but one had values that were below the recommended limit or 3 ng/mL for environmental surface water.²⁰

CONCLUSIONS

The substitution of synthetic nanopeptamers by recombinant peptide-streptavidin chimeras represents a significant step forward in the use of nanopeptamers for small analytes. On the one hand, there is a substantial reduction in cost, particularly because the chimeras avoid the use of expensive synthetic peptides, at the same time that can be robustly produced at high yields. Typically, after refolding and purification, about 10 mg/L of the recombinant nanopeptamer are obtained in shake flask cultures, being enough for about 500 × 96 well plate assays. Furthermore, the recombinant nanopeptamers leave the biotin-binding sites free, which are thus available to be used in combination with the broad variety of biotinylated reagents, including tracer enzymes, fluorophores, magnetic beads, etc., making them highly versatile reagents. As demonstrated here these chimeras can be

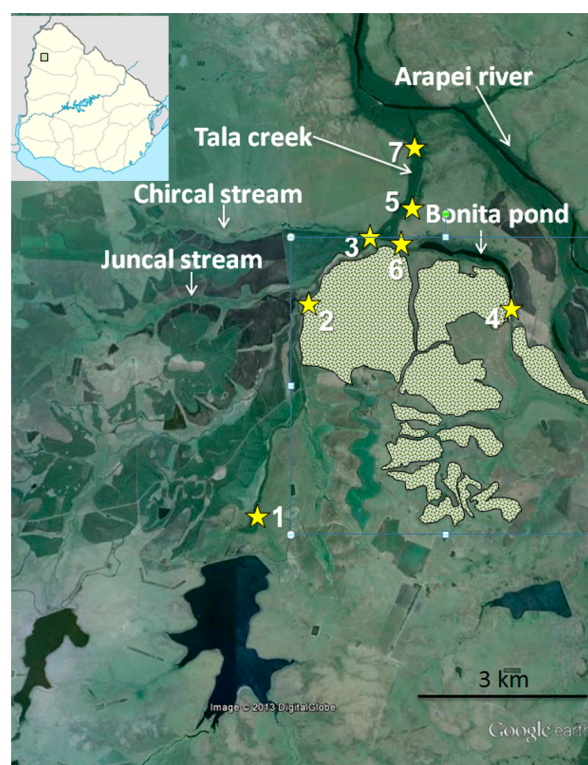


Figure 6. Tala brook basin in the north of Uruguay. The localization of the seven sampling points is denoted by stars, and the rice growing areas are shown in lighter color.

Table 2. Analysis of Water Samples from the Tala Basin^a

sampling point	day	clomazone (ng/mL)	sampling point	day	clomazone (ng/mL)
3	0	<LOD	5	0	ND
	13	<LOD		13	<LOD
	17	<LOD		17	<LOD
	28	<LOD		28	<LOD
	37	<LOD		37	1.3 ± 0.1
4	48	1.4 ± 0.0	7	48	1.4 ± 0.1
	55	1.7 ± 0.1		55	1.5 ± 0.2
	0	3.2 ± 0.0		0	<LOD
	13	1.0 ± 0.0		13	<LOD
	17	1.0 ± 0.0		17	<LOD
4	28	5.1 ± 0.1	7	28	<LOD
	37	1.7 ± 0.2		37	<LOD
	48	2.3 ± 0.2		48	1.1 ± 0.2
	55	1.2 ± 0.2		55	1.1 ± 0.2

^aValues represent the average of contiguous samples analyzed by triplicates ± SD. ND, nondetermined. Samples from sampling points 1, 2, and 6 were all <LOD.

developed into simple, cost-effective, and robust noncompetitive assays for environmental, food safety, or medical applications.

ASSOCIATED CONTENT

Supporting Information

Additional materials as described in the 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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