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Simple and Highly Enantioselective Electrochemical Aptamer-Based Binding Assay for Trace Detection of Chiral Compounds

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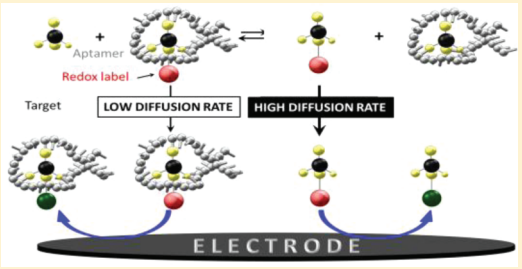
Supporting Information

ABSTRACT: A new electrochemical methodology is reported for monitoring in homogeneous solution the enantiospecific binding of a small chiral analyte to an aptamer. The principle relies on the difference of diffusion rates between the targeted molecule and the aptamer/target complex, and thus on the ability to more easily electrochemically detect the former over the latter in a homogeneous solution. This electrochemical detection strategy is significant because, in contrast to the common laborious and time-consuming heterogeneous binding approaches, it is based on a simple and fast homogeneous binding assay which does not call for an aptamer conformational change upon ligand binding. The methodology is here exemplified with the specific chiral recognition of trace amounts of L- or D-tyrosinamide by a 49-mer D- or L-deoxyribooligonucleotide receptor. Detection as low as 0.1% of the minor enantiomer in a nonracemic mixture can be achieved in a very short analysis time (<1 min). The assay finally combines numerous attractive features including simplicity, rapidity, low cost, flexibility, low volume samples (few microliters), and homogeneous format.



The development of rapid, simple, sensitive, and highly selective analytical methods for identifying and quantifying a trace amount of one enantiomer in a mixture containing its mirror image and many other unrelated compounds continues to be an important challenge in analytical chemistry. The development of such methods would greatly contribute to different pharmaceutical research areas such as pharmacokinetic/pharmacodynamic of racemic drugs, determination of enantiometric excess (ee), or investigation of the toxicological effects of particular enantiomers.[1] It would be also useful in the food industry for assessing food quality through the enantiomeric analysis of amino acids,[2] in forensic science for discriminating and quantifying illicit chiral substances,[3,4] or in the environment for monitoring the fate of emerging chiral pollutants.[5]

Among the current methodologies for rapid enantioselective analysis, many are based on synthetic chiral molecular receptors in combination with homogeneous host−guest binding assays.[6−8] The design of synthetic chiral receptors however requires extensive efforts of stereocontrolled syntheses. Moreover, the determination of ee with these systems is restricted to relatively high analyte concentrations (>0.1 mM) with enantiomeric ratio accuracies of only few %.[7] To overcome these drawbacks, the replacement of synthetic chemical receptors by the more highly affine and enantioselective tailor-made nucleic acid aptamers is an attractive alternative. These in vitro selected single-stranded oligonucleotides were shown to bind a wide range of molecules with an affinity and selectivity that can rival or even sometimes exceed those of antibodies. Because of their natural chirality and high conformational flexibility, they were also able to discriminate between enantiomeric isomers with a remarkably high resolution.[9−12] Another decisive criterion for the development of a rapid enantioselective analytical method is to select an appropriate readout device. By virtue of their simplicity, short detection time, high sensitivity, low-cost instrumentation, field-portable capability, and compatibility with direct analysis in biological fluids, electroanalytical methods appear as good candidates for developing a simple and fast enantioselective aptamer-based electrochemical assay. Coupling the distinct advantages of electroanalytical methods with the attractive properties of aptamers has received considerable recent attention, notably for the development of electrochemical aptasensors for which the detection strategy relies on a heterogeneous binding assay format.[13−15] However, the main

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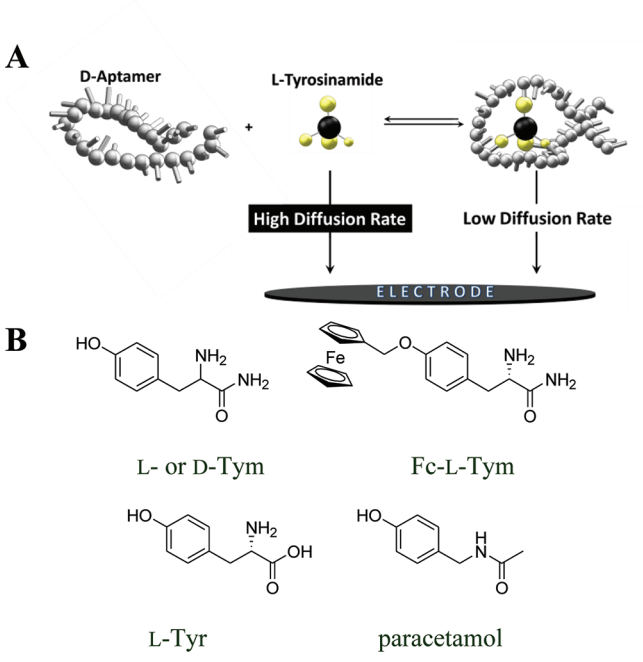
shortcomings of these approaches are the laborious and time-consuming preparation of aptamer-modified electrodes and the generally lower interfacial affinity binding constants as well as slower heterogeneous aptamer/target binding rates compared with homogeneous solution. In addition, many of these heterogeneous biosensing methods are based on a large conformational change of the aptamer which upon target binding influences the electrochemical response of a redox tag.[13−15] Although elegant, such an allosteric-based detection strategy is restricted to aptamer/target couples able to induce sufficiently high conformational change, a condition that cannot be always easily achieved.

Here we report a new, rapid, and simple electrochemical methodology that aims to monitor the enantiospecific binding of a small chiral molecule to an aptamer. The general principle relies on the difference of diffusion rates between a targeted molecule and the aptamer/target complex and thus on the ability to more easily electrochemically detect the former over the latter in a homogeneous solution (Scheme 1A). This electrochemical detection strategy is significant because, in contrast to the common laborious and time-consuming heterogeneous binding approaches, it is based on a simple and fast homogeneous binding assay in which the detection scheme does not depend on a target-induced conformational change of the functional nucleic acid. The methodology is here exemplified with the enantioselective molecular recognition of trace amounts of L- or D-tyrosinamide (Scheme 1B) by a 49-mer D- or L-deoxyribooligonucleotide receptor.

Scheme 1. (A) General Principle of the Homogeneous Electrochemical Aptamer-Based Enantioselective Binding Assay Involving an Electrodetectable Target and (B) Molecular Structures of L- or D-Tyrosinamide (L- or D-Tym), Ferrocene-Labeled L-Tyrosinamide (Fc-L-Tym), L-Tyrosine (L-Tyr), and Paracetamol



EXPERIMENTAL SECTION

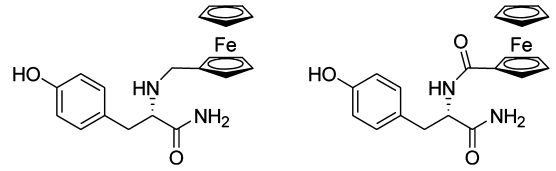
Reagents and Instrumentation. 2-Amino-1,3-propanediol hydrochloride (Aldrich), 2-amino-1,3-propanediol (Aldrich), NaCl (Fluka), $MgCl_2$ (Sigma), L-tyrosinamide (Aldrich), D-tyrosinamide (Iris Biotech GmBH, Germany), paracetamol (Aldrich), tyrosine (Sigma), triphenylphosphine (Aldrich), diethylazidocarboxylate solution (DEAD) (Aldrich), and ferrocene methanol (Aldrich) were used without further purification. All syntheses were performed with freshly distilled solvents (THF and MeOH). The oligonucleotides (l-DNA and d-DNA) were synthesized and HPLC-purified by Eurogentec (Angers, France), and their concentration was controlled by UV−visible spectroscopy using a Nanodrop 2000 spectrophotometer (ThermoFisher). The oligonucleotides sequences were (from 5′ to 3′): Apt49-T, AATTCGCTAGCTGGAGCTTGGATTGATGTGGTGTGTGAGTGCGGTGCCC; Scramble, CTATGACCCTACCTGCTGATGCGTAGCGAGACCCGGAATCTAACCCCCT.

All NMR spectra were recorded on a Bruker AC 400 MHz. Optical rotations were measured using a Jasco-P-1010 polarimeter.

Cyclic voltammetry measurements were performed with an AUTOLAB PGSTAT 12 potentiostat/galvanostat controlled by a computer, and the data were acquired using GPES 4.9007 software (EcoChemie, The Netherlands). Small electrochemical cells made of three screen-printed electrodes (a carbon-based working electrode of 0.071 cm^2, a carbon-based counter electrode, and a silver-based reference electrode) on a planar polyethylenterephtalate film were used for all experiments. An insulating dielectric layer was screen-printed over the three screen-printed electrodes in such a way to delimit a circular electrochemical cell, ideal for working in less than 50 μL of solution. The electrodes were printed on a DEK model-65 screen-printer using the commercial carbon-based ink Electrodag PF-470A and silver-based ink Electrodag 418SS. Unless otherwise stated, the electrochemical experiments were systematically performed in a 5 mM Tris buffer (pH 7) containing 10 mM $MgCl_2$ and 50 mM NaCl (TB). Before experiments, the screen-printed carbon electrodes were passively coated with a layer of bovine serum albumin (BSA) by immersing the electrodes in a 1 mg/mL BSA solution for 10 min at room temperature. The BSA-modified electrodes were next cycled by cyclic voltammetry between 0.0 and 0.8 V at v = 0.05 V s^{-1} until of the capacitive background current was stabilized.

Syntheses. The ferrocene-labeled compounds shown in Schemes 1B and 2 were synthesized as follows.

Scheme 2. Molecular Structures of 1 and 2



O-Ferrocenyltyrosynamide (Fc-Tym). The synthesis of Fc-Tym was inspired from the work of Baldoli et al.[16] with some modifications. L-Tyrosinamide (250 mg, 1.39 mmol,) and triphenylphosphine (361 mg, 1 equiv) were added under argon at room temperature to a solution of ferrocenemethanol (200 mg, 0.7 equiv) previously dissolved in 5 mL of dry THF. After cooling the solution to −30 °C, a 2 M solution of DEAD in THF (0.5 mL, 1 equiv) was slowly added. A volume of 20 mL

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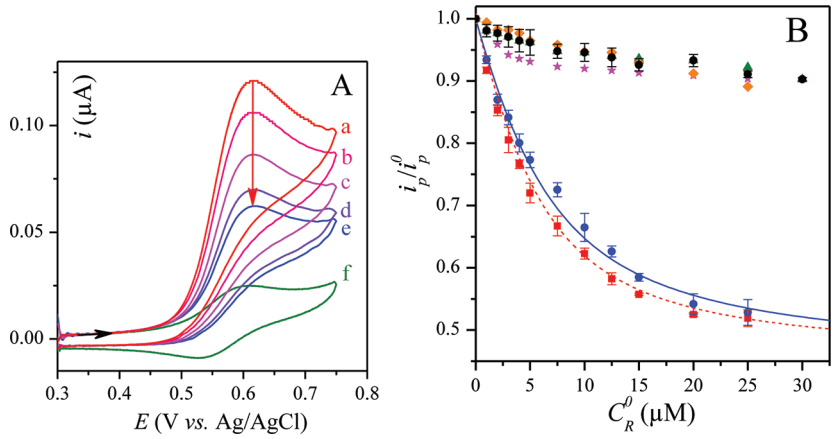

Figure 1. (A) CVs (ν = 0.05 V s^{-1}) of Tris buffer (5 mM, pH 7) solutions containing 1 μM $[Os(bpy)_3]^{2+}$, 5 μM L-Tym, 50 mM NaCl, 10 mM $MgCl_2$, and (a) 0 μM, (b) 2 μM, (c) 5 μM, (d) 10 μM (e) 20 μM Apt_{49}. (f) CV of 1 μM $[Os(bpy)_3]^{2+}$ alone. (B) Normalized catalytic peak current as a function of aptamer concentration for the ligand/receptor couples: (blue circle) L-Tym/D-Apt_{49}, (red square) D-Tym/L-Apt_{49}, (black circle) D-Tym/D-Apt_{49}, (pink star) L-Tyr /D-Apt_{49}, (gold diamond) paracetamol/D-Apt_{49}, and (green triangle) L-Tym/49-mer scramble oligonucleotide. Error bars: standard deviations from triplicates. Plain lines: fits of eq 5 to the experimental data.

of dry THF followed by 0.5 mL of dry methanol were then added to increase the solubility. The solution was stirred at room temperature for 2 days. The mixture was evaporated and submitted to column chromatography using a mixture of CH_2Cl_2 with 1−10% MeOH. The compound Fc-Tym was obtained as a pale yellow solid in 67% yield. ^{1}H NMR, MeOD, 400 MHz: δ (ppm)= 2.84 (dd, 1H, J_{AB} = 13.6 Hz, J_{AX} = 7.5 Hz, CH_2Ph), 3.05 (dd, 1H, J_{AB} = 13.6 Hz, J_{AX} = 5.8 Hz, CH_2Ph), 3.60−3.63 (m, 1H, CH), 4.27−4.28 (m, 7H, CH-Fc), 4.39 (t, 2H, J = 1.8 Hz, CH-Fc), 4.90 (s, 2H, CH_2O), 7.00 (d, 2H, J = 8.6 Hz, CH_{Ar}-C_q-O), 7.24 (d, 2H, J = 8.6 Hz, CH_{Ar}-C_q-CH_2). ^{13}C NMR, MeOD, 100.6 MHz: δ (ppm) = 41.6 (s, CH_2Ph), 57.4 (s, CH-NH_2), 67.9 (s, CH_2-O), 69.5 (s, CH-Fc), 69.6 (s, CH-Fc), 70.3 (s, CH-Fc), 84.1 (s, Cq-Fc), 116.0 (s, CH_{Ar}), 130.8 (s, Cq_{Ar}), 131.4 (s, CH_{Ar}−O), 159.3 (s, Cq_{Ar}-O), 179.5 (s, Cq-C(O)). $[\alpha]_D^{31}$ = +22.1 (c = 0.21, MeOH).

*NHCO-Ferrocenyltyrosinamide (**1**).* In ethyl acetate (30 mL), ferrocene carboxylic acid (100 mg, 0.43 mmol) is added. The solution is stirred at room temperature, and TBTU (154 mg, 0.97 equiv) and DIPEA (84 mg, 1.5 equiv) are added. The mixture is stirred during 30 min at room temperature. Then, L-tyrosinamide (78 mg, 1 equiv) is added to the solution. The resulting mixture is stirred at RT for 3 days. All the volatile materials are evaporated under vacuum. The orange solid is washed first by CH_2Cl_2 and second by Et_2O. The yield of the reaction is 60%. ^{1}H NMR, DMSO, 400 MHz: δ (ppm) = 2.89 (m, 1H, CH_2Ph), 3.01 (m,1H, CH_2Ph), 4.04 (s, 5H, CH-Fc), 4.36 (s, 2H, CH-Fc), 4.61 (m, 1H, CH), 4.81 (s, 1H, CH-Fc), 4.89 (s, 1H, CH-Fc), 6.73 (d, 2H, $^3J_{(H,H)}$ = 7.6 Hz, CH_{Ar}-C_q-O), 7.08 (s, 1H, NH_2), 7.22 (d, 2H, $^3J_{(H,H)}$ = 7.6 Hz, CH_{Ar}-C_q-CH_2), 7.45 (s, 1H, NH_2), 7.70 (d, 1H, $^3J_{(H,H)}$ = 8.4 Hz, NH), 9.15 (s, 1H, OH). ^{13}C NMR, DMSO, 100.6 MHz: δ (ppm) = 36.4 (s, CH_2Ph), 54.3 (s, CH-NH_2), 67.8 (s, CH-Fc), 68.5 (s, CH-Fc), 69.3 (s, CH-Fc) 69.8 (s, CH-Fc), 76.0 (s, Cq-Fc), 114.7 (s, CH_{Ar}-O), 128.7 (s, CH_{Ar}), 130.0 (s, Cq_{Ar}), 155.7 (s, Cq_{Ar}-O), 168.9 (s, Cq-C(O)-Fc), 173.9 (s, Cq-C(O)).

*NH-Ferrocenyltyrosinamide (**2**).* In MeOH (20 mL), L-tyrosinamide (200 mg, 1.1 mmol) and ferrocene aldehyde (261 mg, 1.1 equiv) were stirred at room temperature. Triethylamine (0.31 mL, 2 equiv) was added to the solution. After 2 h at room temperature, the mixture was cooled to −4 °C and $NaBH_4$ (46 mg, 1.1 equiv) was added. The solution was stirred at −4 °C for 2 h and then overnight at room temperature. All volatile materials are evaporated under vacuum. The solid was dissolved in dichloromethane and washed with water. The mixture was evaporated and submitted to column chromatography using ethyl acetate. The yellow solid is obtained with 40% yield. ^{1}H NMR, DMSO, 400 MHz: δ (ppm)= 1.83 (s, 1H, NH), 2.61 (m, 1H, CH_2Ph), 2.80 (m, 1H, CH_2Ph), 3.15 (m, 2H, CH_2Fc), 3.34 (m, 1H, CH), 4.04 (s, 5H, CH-Fc), 4.06 (s, 2H, CH-Fc), 4.10 (s, 1H, CH-Fc), 4.15 (s, 1H, CH-Fc), 6.67 (d, 2H, J = 8.3 Hz, CH_{Ar}-C_q-O), 7.03 (s, 1H, NH_2), 7.05 (d, 2H, J = 8.3 Hz, CH_{Ar}-C_q-CH_2),7.30 (s, 1H, NH_2). ^{13}C NMR, DMSO, 100.6 MHz: δ (ppm) = 38.4 (s, CH_2Ph), 46.7 (s, CH-NH_2), 63.3 (s, CH_2-Fc), 66.9 (s, CH-Fc), 67.0 (s, CH-Fc), 67.1 (s, CH-Fc), 67.4 (s, CH-Fc), 68.1 (s, CH-Fc), 87.3 (s, Cq-Fc), 114.9 (s, CH_{Ar}-O), 128.4 (s, Cq_{Ar}), 130.0 (s, CH_{Ar}), 155.7 (s, Cq_{Ar}-O), 175.6 (s, Cq-C(O)).

RESULTS AND DISCUSSION

To demonstrate the feasibility of the method, we have first taken advantage of the intrinsic electroactivity of tyrosinamide and therefore of its electrochemical detection by cyclic voltammetry (i.e., oxidation of the phenol function) in the presence of its 49-mer anti-L-tyrosinamide aptamer (D-Apt_{49}). The 49-mer aptamer we have used is a sequence similar to the one that was previously selected by Vianini et al.[17] and successfully applied to chromatographic-[18] and fluorescence-based L-Tym assays.[19,20] Since direct electrochemical oxidation of phenolic compounds generally leads to electrode passivation and thus to poorly reproducible anodic peak current in cyclic voltammetry (CV), the electrochemical detection of L-Tym was performed by redox-mediated catalytic oxidation of the phenolic function. For such a purpose, we have used the one-electron redox mediator couple $[Os^{III/II}(bpy)_3]^{3+/2+}$ which, according to its relatively high standard potential (E^0 = +0.58 V vs Ag/AgCl), is capable to efficiently catalyze the oxidation of phenolic compounds.

As illustrated in Figure 1A, for particular scan rates and concentrations of L-Tym and $[Os(bpy)^3]^{2+}$, we obtained in CV a well-defined irreversible anodic catalytic peak current at +0.62 V vs Ag/AgCl. The linear dependence of the CV peak current with the L-Tym concentration at ν = 0.05 V s^{-1} (Figures S1 in the Supporting Information), indicates conditions of total

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catalysis for which the catalytic peak current response, i_p, can be expressed by eq 1.[21]

$$i_p = 0.609nFSC_T^0\sqrt{D_T}\sqrt{Fv/RT} \quad (1)$$

where n is the number of electron involved in the catalytic reaction, F the Faraday constant, S the electrode area, C_T^0 the bulk concentration of L-Tym, and D_T the diffusion coefficient of L-Tym. After aptamer addition, the binding reaction (eq 2) is rapidly set at equilibrium (the binding equilibrium was observed to be reach after a few seconds).

$$\text{L-Tym/D-Apt}_{49} \overset{K_d}{\rightleftarrows} \text{L-Tym} + \text{D-Apt}_{49} \quad (2)$$

with K_d as the dissociation constant. On the basis of a classical law of action mass and mass balance (i.e., $C_T^0 = C_T + C_{RT}$, with C_T and C_{RT} the free and receptor-bound target concentrations) and assuming a 1:1 binding stoichiometry, the free mole fraction of target (χ_f) in the bulk solution can thus be estimated by eq 3.

$$\chi_f = \frac{C_T}{C_T^0} = 1 - \frac{K_d + C_T^0 + C_R^0 - \sqrt{\Delta}}{2C_T^0}$$
$$\text{with } \Delta = (K_d + C_T^0 + C_R^0)^2 - 4C_T^0C_R^0 \quad (3)$$

Here, C_R^0 is the total concentration of the aptamer receptor added to the solution. Under the assumption of a slow interconversion of free and bound target on the time scale of CV experiments, which is a reasonable assumption since dissociation kinetics of aptamer complexes is generally slow,[22] the catalytic peak current can thus be expressed by a linear combination of the catalytic currents related to the free and bound target through eq 4.[23]

$$i_p = 0.609nFSC_T^0\sqrt{Fv/RT}\left[\chi_f\sqrt{D_T} + (1-\chi_f)\sqrt{D_{RT}}\right] \quad (4)$$

where D_T and D_{RT} are the diffusion coefficients of the free and receptor-bound target, respectively. Because of its greater gyration radius, the deoxyribooligonucleotide receptor/L-Tym complex is expected to diffuse significantly slower that the free L-Tym target (i.e., $D_{RT} \ll D_T$). Consequently, according to eq 5, a decrease of the total catalytic current would result from increasing addition of D-Apt$_{49}$ in solution.

From the combination of eqs 3 and 4 and knowledge of the catalytic peak currents determined both in the absence of aptamer (i_p^0) and at extrapolated infinite aptamer concentration (i_p^∞), we can write eq 5 which allows one to predict the normalized peak current response decreases as a function of aptamer and target concentrations.

$$\frac{i_p}{i_p^0} = 1 - \frac{K_d + C_T^0 + C_R^0 - \sqrt{\Delta}}{2C_T^0}\left(1 - \frac{i_p^\infty}{i_p^0}\right) \quad (5)$$

Equation 5 is valid if i_p^∞ is entirely controlled by the diffusion of the target/aptamer complex to the electrode, a condition that has been verified with the linear variation of i_p^∞ with concentration at v = 0.05 V/s (see the Supporting Information).

Figure 1A shows the resulting asymptotic catalytic peak current response decrease as a function of D-Apt$_{49}$ concentration added to a fixed concentration of L-Tym. The nonlinear regression fit of eq 5 to the experimental plot (Figure 1B) allows for extracting the values of K_d = 2.9 ± 0.3 μM and i_p^∞/i_p^0 = 0.45. The value of K_d is in excellent agreement with the ones previously determined by isothermal titration calorimetry (1.75−3.2 μM)[24] and fluorescence polarization (1.7−2.2 μM).[20] From the ratio i_p^∞/i_p^0 and the experimental i_p^0 value, we can determine i_p^∞ as well as the diffusion coefficients of the free and bound L-Tym. Assuming one electron exchanged in the reaction, values of $D_T = 7 \times 10^{-6}$ cm^2 s^{-1} and $D_{RT} = 1.4 \times 10^{-6}$ cm^2 s^{-1} were found, respectively, which is in good agreement with the expected diffusion coefficient of an amino acid[25] and a small oligonucleotide[26] in water at room temperature.

To examine the specificity as well as the enantioselectivity of the assay, binding experiments with the D-Tym enantiomer and two structurally related ligands (i.e., the L-tyrosine and paracetamol, both containing an oxidizable phenol group, Scheme 1) were performed. As reported in Figure 1B, no significant catalytic current response decrease was observed upon the addition of D-Apt$_{49}$ to a solution of D-Tym, indicating that the cross-reactivity of the D-aptamer receptor with the D-Tym enantiomer is negligible. Similar results were also obtained with the noncognate L-tyrosine (L-Tyr) and paracetamol ligands (Figure 1B), demonstrating the strong discriminating properties of D-Apt$_{49}$ against closely related compounds and the relevance of the proposed methodology. Finally, no significant current response decrease could be observed when the aptamer was replaced by a 49-mer random DNA sequence (scramble oligonucleotide) containing the same GC ratio (Figure 1B). The small signal decrease observed during the titration curves of noncognate ligands and scramble oligonucleotide is related to a nonspecific response which can be avoided if the total concentration of DNA nucleotides is kept constant throughout the titration assay (i.e., $C_{Apt49} + C_{scramble}$ = constant).

We have next examined the possibility to perform the assay of D-Tym with the mirror image of the D-aptamer, i.e., the L-Apt$_{49}$. Similar to the titration curve of L-Tym by D-Apt$_{49}$, the catalytic peak current response of D-Tym was observed to rapidly decrease with the increasing addition of L-Apt$_{49}$ (Figure 1B), providing an equal binding curve to that of L-Tym. From the nonlinear regression fit of eq 5, a K_d value of 3.8 ± 0.7 μM was obtained, which is practically the same as the one determined for the mirror assay (i.e., 2.9 ± 0.3 μM). From the combination of the two specific responses of the D- and L-aptamer toward their respective L- and D-target, it becomes conceivable to determine trace amounts of D- or L-Tym in the presence of a large excess of the L- or D-enantiomer. However, with the present redox-mediated electrochemical detection strategy, such an idea cannot be easily achieved and it is also restricted to target analytes that are intrinsically electroactive. We have thus extended the methodology to a more general detection scheme based on an electrochemical homogeneous competitive binding assay. In this approach, the target is reacted in competition with a known amount of the redox-labeled target for binding to the aptamer, and the ratio of free to bound redox-labeled target is monitored electrochemically through the detection of the redox label.

The competitive assay can be described by the two equilibrated binding reactions 6a and 6b, in which Fc-L-Tym is L-tyrosinamide labeled by a redox-active ferrocene group. The ferrocenyl label was here incorporated on the -OH position of the phenolic group (Scheme 1B).

$$\text{L-Tym/D-Apt}_{49} \overset{K_d}{\rightleftarrows} \text{L-Tym} + \text{D-Apt}_{49} \quad (6a)$$

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$$\text{Fc-L-Tym/D-Apt}_{49} \overset{K_d^*}{\rightleftharpoons} - \text{Fc-L-Tym} + \text{D-Apt}_{49} \quad (6b)$$

with

$$K_d = (C_T C_R)/C_{RT} \quad (7a)$$

$$K_d^* = (C_{T^*} C_R)/C_{RT^*} \quad (7b)$$

where C_R is the concentration of free aptamer receptor. For successful achievement of such an assay, it is important that the labeling does not significantly affect the aptamer molecular recognition. Ideally, the two binding constants should be as close as possible ($K_d \approx K_d^*$). The value of K_d^* was determined by monitoring in CV the reversible response of the 5 μM Fc-L-Tym solution as a function of increasing concentrations of D-Apt_{49}. The simultaneous decrease of the anodic and cathodic peak currents upon the addition of D-Apt_{49} sequence (Figure 2A) and the absence of current change in the presence of L-

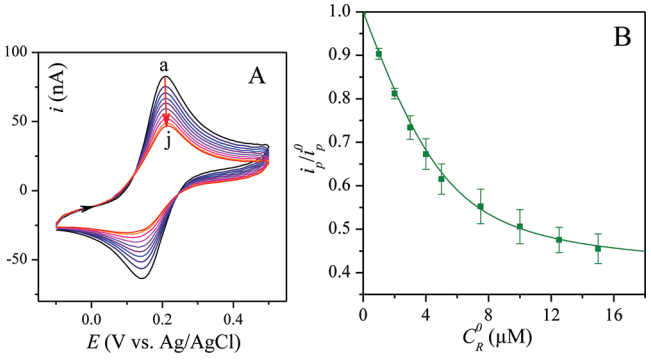

Figure 2. (A) CVs (v = 0.05 V s^{-1}) of 5 μM Fc-L-Tym in a Tris buffer (5 mM, pH 7.0) solution containing 50 mM NaCl, 10 mM Mg^{2+}, and (a) 0 μM, (b) 1 μM, (c) 2 μM, (d) 3 μM, (e) 4 μM, (f) 5 μM, (g) 7.5 μM, (h) 10 μM, (i) 12.5 μM, and (j) 15 μM of D-Apt_{49}. (B) Normalized anodic peak current response as a function of aptamer concentration. Each data is the average of three measurements. Error bars are standard deviations. Plain line: fit of eq 5.

Apt_{49} (not shown) clearly shows that the labeled target is well recognized by the aptamer and that the ferrocene label is less easily electrochemically detected when Fc-L-Tym is bound to D-Apt_{49} in solution.

Although the expression of the peak current is different from the one previously used for the catalytic response (it is given by $i_p = 0.443FSC^0_{Fc}(D_{Fc})^{1/2}\{(Fv)/(RT)\}^{1/2}$ [27]), the same eq 5 can be applied to fit the experimental plot of the anodic peak current decrease of ferrocene as a function of aptamer concentration (Figure 2B). From the data fitting, a value of $K_d^* = 1.2 \pm 0.4$ μM was determined, which is ~2.5-fold lower than the dissociation constant obtained for L-Tym. This slightly stronger affinity binding clearly shows that the labeling of L-Tym through the activable hydroxyl group does not hinder the aptamer binding (it is rather the opposite here), so that the hydrogen atom on the phenolic function does not significantly contribute to the molecular recognition of L-Tym. This is not the case of the primary -NH_2 function because when the ferrocene moiety was covalently linked to this position via an amide or a secondary amino group (Scheme 2), the aptamer was unable to bind these labeled L-tyrosinamides. From the fit in Figure 2B, a i_p^∞/i_p^0 value of 0.40 was also found, which is close to the one previously determined for L-Tym. This result was expected since the diffusion coefficients of free or bound Fc-L-Tym and free or bound L-Tym are nearly the same.

The competitive binding assay was achieved from two separate solutions containing a starting mixture of 5 μM Fc- L-Tym and 3 μM or 10 μM D-Apt_{49} and to which an increasing amount of L-Tym was added. On account of the competitive displacement of the aptamer-bound Fc- L-Tym by L-Tym, peak currents of the reversible voltammetric wave of ferrocene were observed to progressively increase with the L-Tym concentration, allowing for plotting the typical competitive calibration curves shown in Figure 3A. Solving the cubic algebraic equation

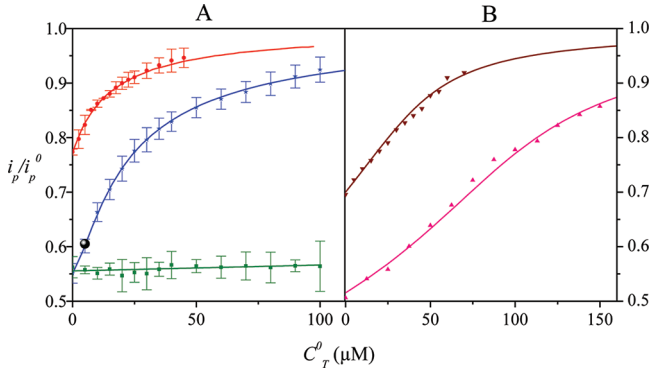

Figure 3. Competitive binding curves performed in (A) 5 mM Tris buffer (pH 7 + 50 mM NaCl + 10 mM $MgCl_2$) or (B) 20% fetal bovine serum (simply diluted with a 4 mM $MgCl_2$ aqueous solution) as a function of (red circle, blue star, brown triangle pointing down, pink triangle pointing up) L-Tym or (green square) D-Tym concentration. (black circle) Assay of 5 μM L-Tym in the presence of 5 mM D-Tym. The Fc-L-Tym concentration was always $C^0_{T^*}$ = 5 μM, whereas the D-Apt_{49} concentrations were C^0_R: (red circle) 3, (blue star, green square, black circle) 10, (brown triangle pointing down) 40, and (pink triangle pointing up) 100 μM. The plain curves represent the best fits of eq S13 in the Supporting Information to the experimental data using (red circle) $K_d^* = 1.2$ μM, $K_d = 2.8$ μM, and $i_p^\infty/i_p^0 = 0.44$; (blue star) $K_d^* = 1.2$ μM, $K_d = 2.2$ μM, and $i_p^\infty/i_p^0 = 0.44$; (brown triangle pointing down) $K_d^* = 75$ μM, $K_d = 9$ μM and $i_p^\infty/i_p^0 = 0.15$; (pink triangle pointing up) $K_d^* = 75$ μM, $K_d = 11$ μM and $i_p^\infty/i_p^0 = 0.15$. Error bars: standard deviations from triplicates.

that results from the combination of eqs 7a and 7b and equations of mass balance, an explicit analytical expression can be obtained (see the Supporting Information) and used to fit the experimental competitive binding calibration curves. From the best nonlinear regression fits and the predetermined value of $K_d^* = 1.2$ μM, the parameters K_d and i_p^∞/i_p^0 were extracted from the two competitive curves in Figure 3A. An identical i_p^∞/i_p^0 value of 0.44 was obtained, and similar K_d values of 2.8 $\pm$ 0.4 μM and 2.2 $\pm$ 0.6 μM were found, which is in very good agreement with the dissociation constant of L-Tym determined from redox-mediated catalysis. The calibration plots in Figure 3A show that as low as a few micromolar could be assayed with this method, which is competitive to the fluorescent methods previously used for the specific detection of L-tyrosinamide using the same aptamer.[19,20]

The enantiospecificity of the assay was next characterized using the same methodology but for solutions containing the D-Tym enantiomer. As shown in Figure 3A, no displacement of Fc- L-Tym from D-Apt_{49} by D-Tym could be observed over the 0−100 μM concentration range. Even concentrations as high as few millimolar D-Tym were tested but again without significant response change. These results once more demonstrate that the

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cross-reactivity with the enantiomer is negligible. To evaluate the enantiospecific performance of the aptamer, the assay of a trace amount of L-Tym in the presence of a large excess of D-Tym was examined and, as reported in Figure 3A, it has been found possible to detect as low as 5 μM L-Tym in the presence of 5 mM D-Tym. This remarkable performance means that as low as 0.1% of an enantiomeric impurity in a nonracemic mixture can be assayed in a very short analysis time (<1 min). This is competitive with conventional separation techniques[28] and 1 order of magnitude lower than the 1% currently attainable by NMR.[29]

Finally, the ability to perform an assay under realistic biological conditions was examined. For such purpose, homogeneous electrochemical competitive binding assays of 5-fold diluted fetal bovin serum samples spiked with known amounts of Fc-L-Tym were then performed (Figure 3B). The competitive binding plots under these conditions were observed to be slightly shifted toward higher concentrations, indicating a lower sensitivity assay in diluted serum than in pure buffer. From the nonlinear fit of the experimental curves, average values of K_d* = 75 μM and K_d = 10 μM were obtained, demonstrating a lower affinity binding of the aptamer toward both the L-Tym and the Fc-L-Tym in the presence of serum. Regardless of this minor issue, the applicability of the method in complex biological samples is finally demonstrated.

In conclusion, we have established a particularly straightforward electrochemical detection strategy for quantitatively discriminating trace amounts of a chiral compound in a complex mixture. To the best of our knowledge, this is the first example of homogeneous electrochemical binding assay based on an aptamer receptor and a redox-labeled target as well as the first enantioselective electrochemical aptamer-based assay. Such assays combine numerous attractive features including simplicity, rapidity (<1 min), low cost, flexibility, low volume samples (few μL), and homogeneous format. Since the in vitro selection can in theory allow isolating an aptamer receptor for any kind of target, the proposed electrochemical competitive binding assay could be extended to many other small chiral analytes but also to nonchiral targets. Because of the ease to mass produce the disposable low-cost screen-printed electrochemical cells used in this work, the methodology is also potentially amenable to high-throughput format.

■ ASSOCIATED CONTENT

Supporting Information

Further details about the evidence of total catalysis conditions and theoretical equations of the competitive binding assay. 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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