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Aptamer-Based Enantioselective Competitive Binding Assay for the Trace Enantiomer Detection

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The development of highly enantioselective assays and sensors has received much attention for the determination of enantiomeric impurities at a low level. For chiral compounds, the efficient monitoring of the in selection procedure has allowed the isolation of nucleic acid aptamers which are able to strongly discriminate the target enantiomers. In this paper, we demonstrated for the first time that an aptamer can be successfully used to design a highly enantioselective tool for the trace enantiomer detection. The aptamer-based stereoselective assay was developed using an affinity capillary electrophoresis-based competitive, homogeneous format and an on-capillary mixing approach. Detection of as low as 0.01% of the minor enantiomer in a nonracemic mixture can be achieved, in a short analysis time (<5 min).

As a consequence of the chiral nature of living systems, metabolic and regulatory processes mediated by biological systems are sensitive to stereochemistry, and different responses can be observed when comparing the activities of enantiomers. Drug enantiomers often differ in their pharmacological and toxicological activity, resulting in stereoselective clinical responses. In some cases, even minor enantiomeric impurities can cause severe toxic side effects.[1] In addition, it is now well-established that D-amino acids, found at a low level in higher order organisms in the form of free amino acids, peptides, and proteins, can play a preponderant role in biochemistry and physiology. Notably, D-serine functions as an important neuromodulator, D-aspartate is implied in developmental and endocrine functions, and D-arginine plays a role in the urea cycle.[2] The detection of D-amino acids is also of importance to estimate the chronological age in forensic sciences, assess food quality, or analyze materials of extraterrestrial origin.[3−5] Therefore, it appears of great interest to design efficient analytical methodologies which are able to detect traces of one enantiomer in the presence of a high excess of the other enantiomer, and the development of assays and sensors, based on highly enantioselective synthetic molecular receptors or antibodies, has received much attention for the determination of low enantiomeric impurities.[6−8]

Nucleic acid aptamers are single-stranded oligonucleotides with binding properties originating from in vitro selection experiments (SELEX methodology). They are able to bind the target molecules with a very high affinity, equal or sometimes even superior to those of antibodies, and their use as affinity reagents in bioanalytical techniques constitutes an interesting alternative to immunoassays and immunosensors.[9−12] For chiral compounds, the efficient monitoring of the SELEX methodology has allowed the isolation of aptamers which are able to strongly discriminate the target enantiomers. Extreme enantioselectivities have been reported for various compounds including oligopeptides, amino acids and derivatives, nucleosides, or drugs.[13−17]

Due to these binding features, aptamers appear to be excellent potential elements for the development of new highly stereoselective assays and sensors, dedicated to the trace enantiomer detection. In this paper, we describe for the first time an aptamer-based enantioselective assay which allows the detection of the minor enantiomer in a nonracemic mixture at a very low level. The analytical methodology was established using affinity capillary electrophoresis (ACE) through a competitive binding format and an on-capillary mixing approach.

EXPERIMENTAL SECTION

Synthesis of the Dansyl-Labeled D-Arginine. Briefly, the synthesis of the labeled D-amino acid was accomplished in six steps: (i) reaction of dansyl chloride with 2-aminoethanol, (ii) alkylation of the hydroxyl function introduced with 2,2′-dichloro-

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diethylether for obtaining the chloro derivative in which (iii) the chlorine atom was substituted for an iodine atom, (iv) reaction of the iodo derivative obtained with the amino group of a D-arginine derivative carrying protected guanidine and carboxylic acid functions (2,2,4,6,7-pentamethylbenzofuran-5-sulfonamide, Pbf, and allyloxycarbonyl groups, respectively), (v) deprotection of the carboxylic acid function under basic conditions and (vi) removal of the Pbf group in TFA (14% yield for the six steps). Details on the synthesis and characterization of the label can be found in the Supporting Information.

Capillary Electrophoresis Experiments. The D- AND L-arginine enantiomers, Tris−HCl, KCl, and $MgCl_2$ were supplied by Sigma Aldrich (Saint-Quentin, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. Electrophoretic experiments were carried out on a CE Agilent capillary electrophoresis system (Waldbronn, Germany) equipped with a diode array detector. The running buffer (25 mM Tris−HCl, 25 mM KCl, 10 mM $MgCl_2$, pH 7.0) was prepared daily and degassed using an ultrasonic bath. The solutions of single enantiomers or nonracemic mixtures (sample) and labeled D-arginine were prepared daily in the buffer. The truncated 53-mer L-RNA oligonucleotide (5′-CAUGAUAAACCGAUGCUGGGCGAUUUC-CUGAAGUAGGGGAAGAGUUGUCAUG-3′) was synthesized and HPLC-purified by CureVac (Tubingen, Germany). A 380 μM L-RNA aptamer stock solution was prepared in the buffer and stored at −20 °C. All solutions were filtered prior use through 0.20 μm pore size membranes. The working aptamer solutions were obtained by dilution of the filtered stock solution with the buffer to the final concentration (100 μM). Prior to the first utilization, the aptamer was renaturated by heating at 90 °C for 10 min and left to stand at room temperature for 30 min. When not in use, the working aptamer solutions were stored at 4 °C. Under these storage conditions, the working solutions were stable during more than 1 month due to the mirror image strategy.

The coated PVA capillary (Agilent, 50 μm i.d. with extended light path (total length, 64.5 cm) was conditioned at the beginning of the day using the following sequence: (i) 10 min of water and (ii) 15 min of running buffer at 1000 mbar. A "short-end" injection method was developed (effective length, 8.5 cm; negative polarity, cathode at the inlet and anode at the outlet, applied voltage, −25 kV; temperature, 12 °C; UV detection at 250 nm). The species solutions were injected hydrodynamically (−50 mbar) as follows: 20 s aptamer (100 μM) plug (∼24 nL), 32 s sample plug (∼41 nL), 4 s label (400 μM) plug (∼5 nL). Between runs, the capillary was conditioned with the running buffer for 5 min at 1000 mbar. The working aptamer solutions were put on ice between each electrophoretic run in order to maintain the L-RNA oligonucleotide in its fully folded tertiary structure.[18]

The injected solution volume (see above) was determined from the time required for a given species, introduced in the capillary under the constant pressure of −50 mbar, to reach the detector window. The presence of D- or L-arginine enantiomers at different concentrations in the running buffer did not affect significantly the injected sample volume, even for the samples containing L-arginine at 2 mM and 20 mM concentrations (less than 5% of difference).

RESULTS AND DISCUSSION

Design of the CE-Based Competitive Binding Assay. A truncated 53-mer RNA aptamer, previously identified by a SELEX procedure directed against L-arginine,[17] was used as a model enantioselective aptamer. The aptamer−target association displays high affinity and stereospecificity, characterized by a dissociation constant K_d of 330 nM, a slow dissociation rate due to the selection procedure based on a heat denaturation/renaturation step, and an enantioselectivity of ∼12 000.[17] As previously reported,[13,18,19] the L-RNA aptamer, i.e., the mirror image of the "natural" D-RNA aptamer, was used as a biostable receptor. In accordance with the principle of chiral inversion, the mirror image of the "natural" aptamer is expected to recognize with the same affinity and specificity the mirror image of the target, i.e., D-arginine.[13] Moreover, previous partial-filling CE experiments have shown that, over a low-moderate temperature range, D-arginine formed with this L-RNA aptamer a very stable complex, while the L-arginine−aptamer association was negligible.[18]

As an alternative to the classical solid-phase techniques, ACE-based assays represent a powerful technology for the detection and quantification of analytes. For example, ACE immunoassays offer several clear advantages over the commonly used immunoassays: they consume less sample and reagents, eliminate washing steps, are compatible with automation and online analysis, do not require antibody or analyte immobilization on a solid support, avoid nonspecific binding of antibody or analyte to the surface, and present a wide analyte applicability.[20]

Taking into account all these attractive background features, an ACE-based assay was designed using the anti-D-arginine L-RNA aptamer as a target-specific receptor. A number of recent works have focused on the use of aptamer-based ACE for the quantification of various proteins such as thrombin, IgE, or HIV type 1 reverse transcriptase.[21−23] These electrophoretic experiments involved, in most cases, a direct (noncompetitive) format where the aptamer was labeled and mixed with the target sample during a sufficient incubation time to allow equilibrium between the interacting species. The electrophoretic separation of the mixture produced typically two distinct peaks corresponding to the free labeled aptamer and the labeled complex, allowing the quantification of the protein. In the present work, as arginine is a low molecular mass species bearing only one net positive charge while RNA is much heavier and highly negatively charged, the charge-to-mass ratio of the analyte−aptamer complex is close to that of the free aptamer.[18] So, a direct ACE assay based on the separation of the free aptamer from the complex cannot be easily achieved so that a competitive binding format was developed. Furthermore, an on-capillary mixing approach was carried out, where the reaction and the separation occurred simultaneously in the same capillary by introducing solutions of sample and reagents in the

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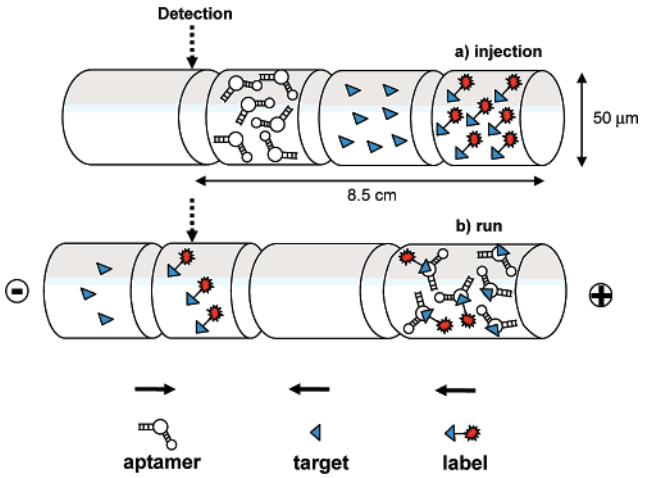

Figure 1. Principle of the CE-based competitive binding assay using the on-capillary mixing of the different species. Arrows indicate the migration direction of the interacting species when the electric field is applied.

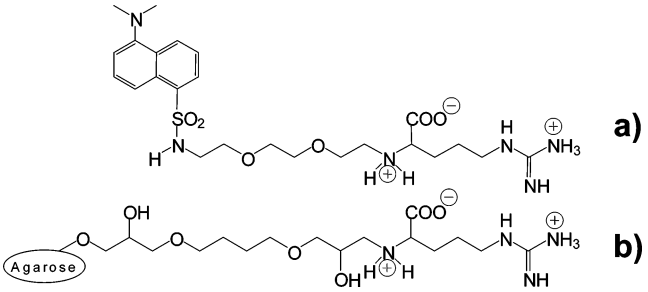

Figure 2. Structures of (a) dansyl-labeled D-arginine used in the CE-based competitive binding assay and (b) immobilized arginine, obtained by derivatization of epoxy activated agarose, used as target in the SELEX methodology.

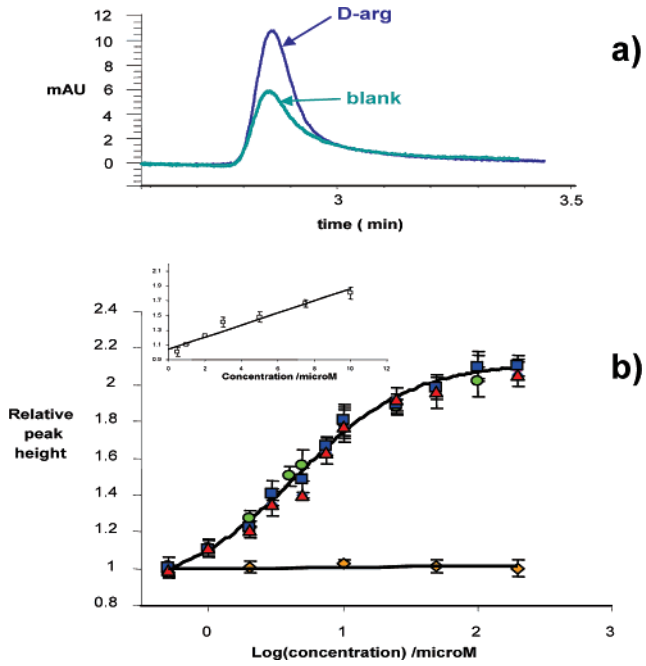

Figure 3. (a) Typical electropherograms showing the free label peaks obtained for blank (no enantiomer in the sample plug) and 10 μM D-arginine in the sample plug. (b) Standard curves obtained for D- (square) and L- (diamond) arginine enantiomers over the 0.5−200 μM concentration range. The target curve was fitted by Table curve 2D (SPSS Science Software GmbH, Erkrath, Germany) to the data plot using a sigmoidal equation. Additional standard curves obtained for the D-enantiomer in the presence of 2 (circle) and 20 mM (triangle) L-enantiomer are presented. Inset: assay response at low concentrations of D-arginine (0−10 μM) in the sample plug. Error bars represent standard deviations (n = 5).

form of individual zones (plugs).[24] The general scheme used is illustrated in Figure 1.

The electrophoretic experiments were conducted using a polyvinyl alcohol (PVA)-coated capillary (i.d., 50 μm). This coating shields the silanol groups of the fused silica and eliminates the electroosmotic flow (EOF). The capillary was first prefilled with the background electrolyte, and three different plugs were introduced hydrodynamically using the "short-end" injection method: (i) a plug containing a known amount of the L-RNA aptamer, (ii) a sample plug containing an unknown amount of target (D-arginine), and (iii) a plug containing a known amount of the labeled target (referred as label).

For use as label, D-arginine was tagged with a dansyl group (Figure 2a, see the Supporting Information). The linker, its attachment position, and the chemistry used for the labeling were chosen in order to mimic the structure of the immobilized arginine (obtained by derivatization of epoxy activated agarose) that was used as target in the SELEX methodology (see Figure 2b).[17] This allowed avoiding possible alteration of the binding properties of the labeled target. More, the label remained positively charged under the running buffer conditions used.

When the electric field was applied, both the target and the label moved toward the cathodic end, while the negatively charged aptamer migrated in the opposite direction (Figure 1). The target was first in contact with the aptamer zone, causing the formation of the D-arginine−aptamer complex. The slow complex dissociation kinetics[17,18] allowed separation of free from complexed target. The unbound D-arginine migrated toward the cathode, while the target−aptamer complex and the free aptamer moved at a close mobility toward the anodic end. Then, labeled D-arginine passed through the aptamer zone and bound to the free aptamer binding sites. As evoked above for the target, the unbound fraction of label was separated from the label−aptamer complex zone. The free labeled D-arginine band, moving toward the cathodic end, was detected at the UV detector window (Figure 1).

D-Arginine was injected as a sample plug over a 0.5−200 μM concentration range. Figure 3a shows a representative example of electropherograms obtained for a blank (sample plug without D-arginine) and a sample plug containing 10 μM of D-arginine. The effect of the D-arginine concentration on the assay response is presented in Figure 3b. The curves were constructed by plotting the relative peak height (ratio of free label peak height for the sample to the free label peak height for the blank) versus the solute concentration, as reported previously.[25] The label free fraction was enhanced with the sample target concentration increasing, as a consequence of the fewer aptamer binding sites available. The calibration curve presents a sigmoidal shape typical for competitive assays. At low D-arginine concentrations, the assay response increased linearly as the target concentration varied from

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0 to 10 μM (see the inset in Figure 3b; linear interpolation: $y = 0.083x + 1.038$ with a regression coefficient $R = 0.98$). The detection limit of D-arginine was calculated to be 1.5 μM through the statistical analysis of the regression function.

Stereoselectivity and Trace Enantiomer Detection. The binding curve for L-arginine was subsequently determined using the same methodology, i.e., with a sample plug containing L-arginine. As can be seen in Figure 3b, no binding of the nontarget enantiomer to the aptamer was obtained over the 0.5−200 μM concentration range. Furthermore, higher concentrations of L-arginine in the sample plug (2 and 20 mM) were analyzed and did not cause any significant response. This demonstrated that the cross-reactivity of the aptameric receptor with the opposite enantiomer was negligible.

The enantioselective properties of the anti-D-arginine L-RNA aptamer were accounted to analyze enantiomer mixtures of arginine. The D-arginine assay was performed in the presence of high L-arginine concentrations (2 and 20 mM) in the sample plug. As can be seen in Figure 3b, no significant changes were observed in the standard curves of the target enantiomer. Moreover, the statistical limits of detection for D-arginine were found to be equal to 1.5 and 1.2 μM in presence of 2 and 20 mM of L-arginine, respectively. So, as little as 2 μM of D-arginine in the presence of 20 mM of L-arginine can be detected. The assay allows the detection of 0.01% of the minor enantiomer in a nonracemic mixture, 1 order of magnitude lower than the 0.1−1% detection limits which are typically attainable with currently available stereoselective analysis methods using NMR or conventional separation techniques.[26−28] In addition, the present sensing system compares very well with the very sensitive methods based on other target-specific bioaffinity systems (such as enantioselective immunosensors and immunoassays).[6,29,30]

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CONCLUSION

In conclusion, we have demonstrated that an aptamer can be successfully used to design a highly enantioselective tool for the trace enantiomer detection. To the best of our knowledge, this work reports the first example of an ACE-based assay dedicated to the enantioselective analysis. Such assay combines a number of attractive features including simplicity, flexibility, minute consumption of material (only some nanoliters, see the Experimental Section), and a homogeneous format. More, the on-capillary mixing approach does not require any incubation time and then allows very little time to conduct the assay relative to that needed by a preincubated method.[19−22] An analysis, including the plug introduction in the capillary and the detection of the free label peak, is completed in less than 5 min.

The present assay is potentially applicable to a laser-induced fluorescence detection system for improving sensitivity. In addition, for a high-throughput chiral analysis, parallel runs on a chip or capillary array electrophoresis are possible.[31,32] As aptamers are able to recognize virtually any target of interest with high affinity and specificity,[33,34] it can be anticipated that this type of CE-based enantioselective competitive binding assay could be adaptable, by modifying some experimental settings, to any chiral analyte that presents a charge-to-mass ratio different to that of aptamers.

SUPPORTING INFORMATION AVAILABLE

Details on the synthesis and characterization of the dansyl-labeled D-arginine. This material is available free of charge via the Internet at http://pubs.acs.org.

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