Rapid determination of enantiomeric ratio using fluorescent DNA or RNA aptamers†

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The natural chirality of DNA and RNA aptamers has been used to develop fluorescent agents to determine the enantiomeric ratio of adenosine and arginine, respectively. The quantification is based on structure-switching DNA or RNA aptamers labeled with fluorophore and quencher, allowing chiral detection down to 0.1 : 99.9 (L : D) for arginine after calibration. Such a method provides a general platform for simple, low-cost and high throughput detection and quantification of chirality of a broad range of molecules.

Introduction

Asymmetric catalysis is an important process in modern chemistry and biology, as it can generate a number of high value products such as chiral pharmaceutical drugs.[1] A critical step in asymmetric catalyst discovery and characterization is determination of the enantiomeric ratio (ER). Instrumental techniques such as chiral gas chromatography (GC) and chiral high pressure liquid chromatography (HPLC) can directly determine the ER. Nuclear magnetic resonance (NMR) is another option, although chiral solvents and chiral additives such as shift reagents may be necessary.[2] Even though chiral GC, HPLC, and NMR are very accurate and thus become workhorses of synthetic laboratories, they are not easily adaptable to high throughput analysis due to high sample handling requirements, leading to slow and costly analysis.

One effective way to improve the efficiency is to use optical methods for direct and simultaneous analyses of thousands of samples using arrays without any separation.[3] Many of the current optical methods, however, require the design and synthesis of a chiral receptor through trial and error processes, as well as asymmetric synthesis of optically active compounds. In addition, the selectivity of *de novo* designed systems may not be easily optimized due to the difficulties of testing a large number of designs. Therefore, a more general method for optical determination of the enantiomeric ratio is needed.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) has been established as a general method to obtain DNA or RNA aptamers that bind a wide range of molecules.[4] Since DNA and RNA are naturally chiral, it has been shown that these aptamers can discriminate between optical isomers by displaying different binding affinities.[5,6] This property has been used for chiral separations by HPLC and capillary electrophoresis,[7] and for asymmetric catalysis.[8,9] Therefore aptamers are an ideal choice as molecules upon which optical determination of ER can be designed. Although a number of aptamer-based sensors have been reported in the literature,[10] only recently has application towards chiral fluorescence sensing been demonstrated.[11] Herein we present a general platform for simple, low-cost and high throughput detection and quantification of chirality of a broad range of molecules by adopting the structural switching aptamer-based fluorescent sensing method,[12] and as a result, we have extended the best reported detection of the minor enantiomer from the 1% to the 0.1% level through calibration.

Experimental

Materials and reagents

Fluorophore (FAM) and quencher (DABCYL) labeled strands were synthesized and HPLC purified by Integrated DNA Technologies, Inc. (Coralville, IA). The adenosine aptamer was synthesized by Integrated DNA Technologies, Inc. and gel purified in house. The chimeric DNA/RNA arginine aptamer oligonucleotide was synthesized and purified *via* RNase-free PAGE by TriLink BioTechnologies (San Diego, CA). L-arginine and D-arginine were purchased from Chem-Impex International (Wood Dale, IL). D-adenosine was purchased from Sigma-Aldrich. L-adenosine was purchased from ChemGenes Corporation (Wilmington, MA). ACS reagent grade buffers and reagents were from either Fisher Scientific or Sigma-Aldrich. All solutions were made using water purified by a Millipore system.

Fluorescence measurements

Fluorescence experiments were carried out on a Fluromax-3 (HORIBA Jobin Yvon, Inc., Edison, NJ). The temperature of the sample was held constant at 25 °C using a temperature controller interfaced with the fluorimeter (LSI-3751 from Wavelength Electronics). Excitation wavelength was 490 nm and emission was monitored at 520 nm.

Sensor preparation

Fluorescence-based sensor solutions were mixed in the appropriate buffer using 40 nM fluorophore strand, 80 nM aptamer

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strand, and 120 nM quencher strand. The adenosine sensor buffer was 20 mM Tris-HCl at pH 8.3 containing 300 mM NaCl and 5 mM $MgCl_2$. The arginine sensor buffer contained 20 mM Bis-Tris at pH 7.0, 50 mM NaCl and 5 mM $MgCl_2$. Solutions were allowed to incubate for a minimum of 30 min at room temperature to anneal the DNA strands. Target was added 30 s after the start of data collection. Adenosine solutions were heated to aid in dissolution.

Results and discussion

As proof-of-concept, the structure-switching signaling DNA aptamer for adenosine[12] was tested for its ability to discriminate between D- and L-adenosine. As described previously,[12] the general design consists of three DNA segments (see Fig. 1): An aptamer with a nucleotide extension at the 5′-end (called the aptamer strand) that can hybridize to another DNA with a fluorophore (FAM) label at its 5′-end (called the fluorophore strand), and the third DNA strand which is labeled with a quencher (DABCYL) at the 3′-end (called the quencher strand). Sequences of the adenosine and arginine sensor components are shown in Fig. 1a and Fig. 1b, respectively. As illustrated in Fig. 1c, in the absence of the target, the fluorescence from FAM is suppressed by DABCYL through the hybridization. In the presence of the target, on the other hand, the binding of the target by the aptamer resulted in a weakened hybridization between the aptamer and quencher strands because of a decrease in the number of base pairs. Thus the quencher strand is released under ambient conditions, resulting in an increased fluorescence signal. At pH 8.3 in 20 mM Tris-HCl buffer containing 300 mM NaCl and 5 mM $MgCl_2$, addition of 1 mM D-adenosine to the DNA aptamers resulted in a normalized fluorescent intensity enhancement of ~9, due to structural switching of the adenosine aptamer. In contrast, 1 mM L-adenosine gave a fluorescence increase of ~1/3 that of D-adenosine (Fig. 2a), indicating that the fluorescent adenosine sensor can be used to discriminate between different optical isomers of adenosine.

The relative dissociation constants were determined to be 0.7 mM for D-adenosine and 2.6 mM for L-adenosine as determined by the ligand concentration at half of the maximal fluorescence increase.

To find out if the fluorescent signal can be used to quantitatively determine the percentage of D-adenosine in a mixture of D- and L-adenosine, the normalized fluorescent intensity at 520 nm was plotted against known different D-adenosine percentages while keeping the total concentration of the two optical isomers the same. At 1 mM adenosine, the response to both D- and L-adenosine independently is within the linear range and provides a high signal without reaching a maximum (Fig. 2b), therefore 1 mM total adenosine was chosen for the chiral determination. A linear response was obtained between 0% and 100% as shown in Fig. 2c, suggesting that the fluorescent DNA sensor can be used for chiral quantification. The size of the error bars in Fig. 1c are partially attributed to the low solubility of the adenosine stock solution.

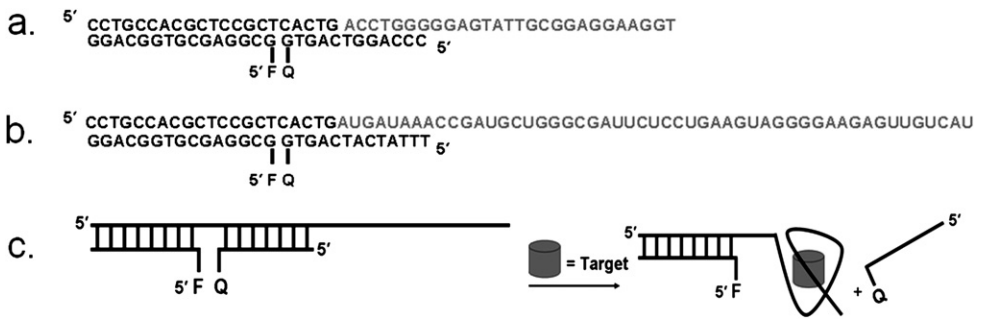

Fig. 1 Sequences and design of structural switching DNA and RNA aptamers for fluorescent determination of enantiomeric ratio of adenosine and arginine, respectively. (a) Sequences of the three components of structural switching DNA aptamer for adenosine (aptamer sequence shown in grey); (b) Sequences of the three components of structural switching RNA aptamer for arginine (aptamer sequence shown in grey); (c) Schematic illustration of structural switching method for both DNA and RNA aptamers shown in a and b. F and Q represents the FAM fluorophore and DABCYL quencher, respectively.

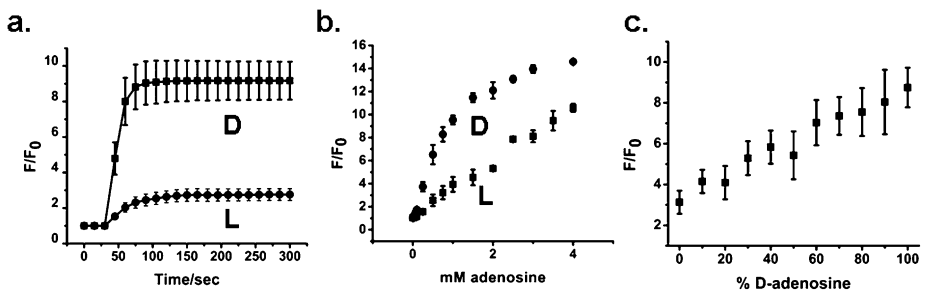

Fig. 2 Fluorescent response of the adenosine system to (a) 1 mM D- and 1 mM L-adenosine upon addition of target at 30 s, (b) varying concentrations of D- and L-adenosine, and (c) percentages of D-adenosine at a total adenosine concentration of 1 mM, 270 s after target addition. D- and L-adenosine samples in (a) and (b) were run separately. Samples in (c) are mixtures of D- and L-adenosine.

After the proof-of-concept experiment using the adenosine aptamer, we wished to apply the concept to a RNA aptamer and to the determination of a chiral amino acid, due to their ubiquity in biological systems. We chose a RNA aptamer for L-arginine due to its high selectivity over D-arginine.[6] The same structural switching design scheme as shown in Fig. 1c was used and the specific sequences are shown in Fig. 1b.

While the fluorophore strand remained the same, the quencher strand sequence was changed to ensure hybridization to the aptamer strand. The fluorophore strand sequence was kept the same to ensure continuity and to minimize cost. The arginine aptamer was truncated on both ends to remove primer regions and then extended on the 5′ end with the same extension sequence as in the adenosine system. The ratio of aptamer strand to fluorophore and quencher strand was maintained as for the adenosine system.

A range of buffers at different concentrations, pH, and Na^+ concentrations were tested. Unlike the adenosine system, the optimal buffer system for arginine detection was found to be 20 mM Bis-Tris pH 7.0 containing 50 mM NaCl and 5 mM $MgCl_2$. Shorter quencher strands were tested, although the response was not as high as for the strand shown above. Longer strands were not tested as very low Na^+ concentrations would be necessary to aid in destabilization which could interfere with target binding.

The response to D-arginine reaches a maximum three-fold increase whereas the response to L-arginine reaches a maximum five-fold increase at ~10 mM arginine as shown in Fig. 3a. The difference in response between the enantiomers is sufficient to allow for detection in a mixture. Concentrations of 2 mM and 10 mM total arginine were used to test varying percentages of L-arginine and the resulting data is shown in Fig. 3b, 4 and 5.

Unlike the linear fluorescent response of the adenosine system to % D-adenosine (Fig. 2c), the fluorescent response of the arginine

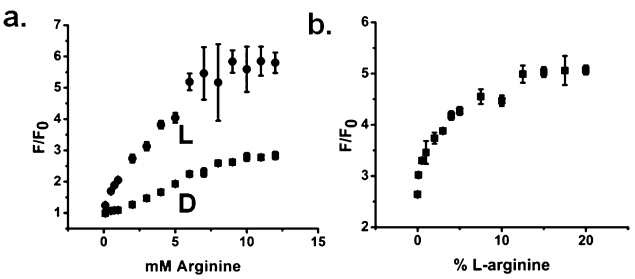

Fig. 3 Response of the arginine structure-switching system to (a) varying concentrations of L- and D-arginine and (b) percent L-arginine at a total arginine concentration of 10 mM.

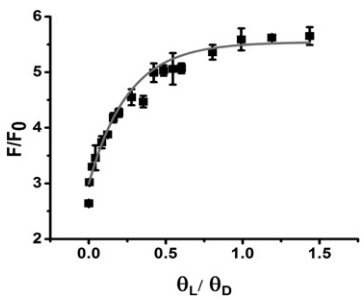

Fig. 4 Response to a range of θ arginine ratios at 10 mM total arginine with asymptotic fitting.

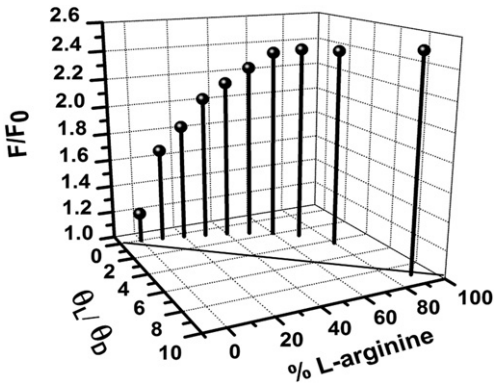

Fig. 5 Response to 2 mM total arginine *vs.* varying percentages L-arginine and the corresponding θ ratio.

sensor to % L-arginine at 10 mM total arginine first increases rapidly between 0–10%, with one relatively steep slope and then increases less dramatically between 10–20%. The rapid signal increase between 0% and 10% allows for greater sensitivity. The fluorescence response of the system in the absence of L-arginine (10 mM D-arginine) was 2.64 ± 0.02, whereas the normalized fluorescence response to 0.1% L-arginine at 10 mM total arginine was 3.02 ± 0.01, allowing detection at the 0.1% or 0.1 : 99.9 (L : D) ER level. Such high chiral discrimination is for the (L : D) ratio, not the (D : L) ratio because of the greater sensitivity towards L-arginine over D-arginine. By lowering the total concentration of arginine from 10 mM to 2 mM, the overall sensitivity towards L-arginine can be reduced, resulting in a larger detection range as shown in Fig. 5.

The relative dissociation constants were determined to be 3.4 mM for L-arginine and 5.0 mM for D-arginine. To allow determination of the ER from a plot accounting for the relative dissociation constants, the normalized fluorescence intensity was plotted against θ_L/θ_D as defined in eqn. (1).

$$\frac{\theta_L}{\theta_D} = \left(\frac{[L]}{([L] + K_d(L))}\right) \Big/ \left(\frac{[D]}{([D] + K_d(D))}\right) \quad (1)$$

θ as derived from the K_d equation, is equivalent to the number of ligand binding sites on the aptamer strand occupied by the target. Binding of target will cause release of the quencher strand. Therefore the θ ratio is the ratio of release of quencher due to L-arginine over the release of quencher due to D-arginine and is also equivalent to the signal due to L-arginine over signal due to D-arginine.

The fitting procedure was applied to the data for 10 mM total arginine (see Fig. 4) and 2 mM total arginine (Fig. 5). The difference in K_d between enantiomers is the reason for the noted enantioselectivity and the plot of F/F_0 *vs.* θ_L/θ_D yields a plot analogous to that of F/F_0 *vs.* % L-arginine.

As shown in Fig. 5 the relationship between % L-arginine and θ_L/θ_D is not linear due to the different K_d values for L-and D-arginine. By using the apparent K_d values and the known total concentration of arginine, one can calculate the ER.

The calibration curves given are for 10 mM and 2 mM total arginine. If one desires to use any other concentration of arginine a separate calibration curve must be fitted, which is quickly accomplished using a program such as OrginPro 8. The resulting asymptotic equations are shown in eqn. (2) and (3) for 10 mM total arginine and 2 mM total arginine, respectively. The asymptotic fit for 10 mM total arginine is shown in Fig. 4.

$$\frac{F}{F_0} = 5.54 - 2.55*(0.0199)^{\theta_L/\theta_D} \quad (2)$$

$$\frac{F}{F_0} = 2.41 - 1.18*(0.147)^{\theta_L/\theta_D} \quad (3)$$

In order to show that the curve fitting procedure is sensitive to small variations in the data over six months, the curve was regenerated and data points from concentrations not used to generate the curve were checked against the curve. The calculated % L-arginine was plotted against the known % L-arginine and the results indicated a reasonable fit (see Fig. S1, ESI†).

It must be emphasized that careful control of the total concentration of arginine is essential with this method as the calibration curve is developed at a specific concentration and will not apply at a significantly lower or higher concentration. In addition, although relatively large error bars are present for the adenosine system, the arginine system displays much smaller error bars, possibly due to the much greater solubility of arginine and the selectivity of the arginine aptamer.

Conclusions

A fluorescence-based chiral determination using RNA or DNA aptamers has been developed allowing detection of the enantiomeric ratio as low as 0.1 : 99.9 (L : D). Detection of each sample takes place optically, allowing detection using a standard fluorimeter in a matter of minutes. Fluorescence sensing is amenable to high-throughput format, which would allow increased productivity. No separation is required, therefore special equipment is not necessary and measurements can take place in disposable culture tubes. This method is rapid in that fluorescence-based detection takes place within several minutes without separation. Generation of a calibration curve at a known concentration takes place from which the ER can be determined. Since SELEX can be used to obtain aptamers for a broad range of molecules and the structural switching method has been demonstrated for fluorescent determination of a number of targets, the method presented in this work should be applicable for the determination of enantiomeric ratios in numerous asymmetric catalysis processes.

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