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Stochastic Detection of Enantiomers

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Living organisms respond differently to the enantiomers of various drugs.[1−3] Accordingly, analytical techniques for the rapid quantification of the enantiomers of small chiral molecules are important. A currently favored approach is chromatography on a chiral support.[4,5] Additional possibilities include binding to chiral host molecules bearing fluorescent reporter groups,[6] mass spectrometry of diastereomeric adducts,[7] and chiral discrimination with antibodies.[8] Single-molecule detection might also prove useful for the detection of chiral molecules. Scanning tunneling microscopy has been applied to determine the chirality of individual organic molecules on metal and semiconductor surfaces under ultrahigh vacuum.[9] However, STM is not a practical technique for routine analytical work. By contrast, stochastic sensing is a powerful tool for the identification of analytes in solution.[10] Engineered versions of the transmembrane pore α-hemolysin (αHL) have been used as sensing elements for the detection of metal ions, anions, DNA, proteins, and small organic molecules. In the latter case, detection was mediated by cyclodextrin (CD) adapters lodged within the protein pore.[11] In the present work, chiral discrimination, which was not achieved in the earlier studies, is effected by this means.

In previous work, cyclodextrins were applied to αHL pores from the trans side of the lipid bilayer (Figure 1); cyclodextrins do not bind from the cis side to the natural (wild-type, WT) αHL pore or to tight-binding mutant pores.[12] In the present work, we used a mutant pore $(M113F/K147N)_7$ that binds βCD from both the trans and the cis sides (see Supporting Information), allowing the screening of a wide variety of binding conditions. The design of the pore was based on the αHL mutant $(M113F)_7$, which binds βCD tightly from the trans side[12] [$K_f = (8.3 \pm 0.1) \times 10^6$ M^{-1}; see Supporting Information]. Molecular modeling based on the structure of the WT pore[13] suggested that truncation of the side chain of Lys-147 (Figure 1) would permit the binding of βCD from the cis side, which was indeed the case [in homoheptameric pores formed from M113F/K147N: $K_{f-trans} = (1.3 \pm 0.2) \times 10^5$ M^{-1}; $K_{f-cis} = (1.0 \pm 0.2) \times 10^4$ M^{-1}]. The mean dwell times of βCD were similar whether the adapter was applied from the trans ($\tau_{off} = 1.2 \pm 0.1$ s) or the cis side ($\tau_{off} = 2.5 \pm 0.1$ s), but these values differ sufficiently to suggest that the mode of binding is different in each case. A lower association rate constant largely accounts for the weaker binding from the cis side (for details, see Supporting Information). The use of the $(M113F/K147N)_7$ pore carried an additional bonus in that the appearance of substates (partial closures) during occupancy by βCD was greatly reduced by comparison with $(M113F)_7$.

The interactions of the drug molecules ibuprofen [2-(4-isobutylphenyl)propanoic acid] and thalidomide [2-(2,6-dioxo-3-piperidinyl)-1*H*-isoindole-1,3(2*H*)-dione] with βCD lodged within the

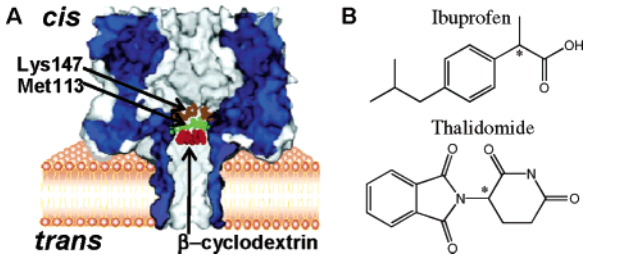

Figure 1. (A) Sagittal section through the $(M113F/K147N)_7$ pore showing the sites of mutation and the proposed location of the βCD adapter. Lys-147, brown; Met-113, green; βCD, red. (B) Structures of ibuprofen and thalidomide. ∗, chiral center.

$(M113F/K147N)_7$ pore were examined in detail. (*S*)-Ibuprofen is the isomer that is active as an anti-inflammatory, antipyretic, analgesic drug.[3,14] Recently, new therapeutic properties of ibuprofen have been discovered. For example, ibuprofen protects neurons from glutamate toxicity in vitro[15] and is active against certain strains of *Candida*.[16,17] Thalidomide was introduced in 1956 and used as a sedative and anti-nausea agent, especially during early pregnancy. However, the drug was withdrawn due to associated birth defects. Nevertheless, thalidomide is being reintroduced for the treatment of leprosy, various cancers, and AIDS based on its immunomodulatory and antiangiogenic properties.[18,19] There is evidence that the *R*-enantiomer is responsible for the sedative activity of thalidomide[20] and the *S*-enantiomer is associated with teratogenic and antitumor properties,[21] although such studies remain controversial because racemization occurs readily in vivo.[22]

With the availability of the $(M113F/K147N)_7$ pore, four conditions of application were possible for each enantiomeric analyte (Xy = Cc, Ct, Tc, Tt, where X is the side of βCD application and y is the side of analyte application; C or c, cis; T or t, trans; see Figure 1A). Further, each condition could be examined over a range of applied membrane potentials. After an exhaustive analysis, we found specific sets of conditions under which the enantiomers of ibuprofen and thalidomide could be distinguished based on their extents of current block (see Supporting Information). For ibuprofen, the combinations Tc and Ct were best, and for thalidomide, Tt and Ct were the most useful. For example, in the Tc configuration at −80 mV in 10 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl, (*S*)-ibuprofen produced a larger block (1.7 pA residual current) of the $(M113F/K147N)_7$•βCD complex than did (*R*)-ibuprofen (4.4 pA residual current, Figure 2). The components of a mixture of the enantiomers of ibuprofen at ∼20 μM could readily be distinguished and quantified after ∼100 ms of recording and correction for the significantly different association rate constants of the two forms (k_{on} values, see Supporting Information).

We further showed that stochastic detection could be used in kinetic studies of racemization. Above pH 6.0, thalidomide undergoes racemization and hydrolysis.[23,24] Racemization is cata-

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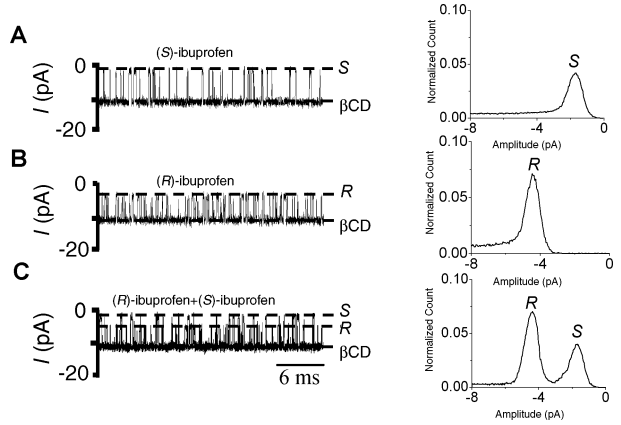

Figure 2. Current traces showing the interaction of (*R*)- and (*S*)-ibuprofen with the (M113F/K147N)$_7$•βCD complex: (A) 20 μM (*S*)-ibuprofen; (B) 20 μM (*R*)-ibuprofen; (C) 20 μM (*S*)-ibuprofen and 20 μM (*R*)-ibuprofen. βCD (40 μM) was added from the trans side and ibuprofen from the cis side (Tc configuration, see the text). Recordings were made at −80 mV (cis at ground) in 10 mM sodium phosphate buffer, pH 7.4, containing 1 M NaCl. The corresponding amplitude histograms are shown (total counts =1, unoccupied (M113F/K147N)$_7$•βCD state omitted from plot).

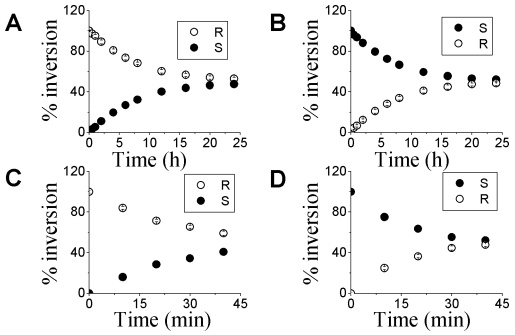

Figure 3. Racemization of (*R*)- and (*S*)-thalidomide as detected by stochastic sensing. (A) (*R*)-thalidomide, no HSA; (B) (*S*)-thalidomide, no HSA; (C) (*R*)-thalidomide, with HSA; (D) (*S*)-thalidomide, with HSA. (*R*)- or (*S*)-Thalidomide (5 mM) was incubated in 67 mM sodium phosphate, pH 7.4, in the presence or absence of 590 μM HSA (Sigma, A3782) at 37 °C. At various timepoints, the thalidomide was extracted with ethyl acetate.[24] The hydrolysis products of thalidomide are anionic at pH 7.4 and were not extracted. The solvent was evaporated, and the residue was taken up in DMSO and diluted 100-fold in phosphate-citrate buffer, pH 3.5, containing 1 M NaCl. A portion was added to the trans chamber for stochastic detection at −80 mV in the Tt configuration in phosphate-citrate buffer, pH 3.5, containing 1 M NaCl. The mean inter-event intervals (τ_{on} values) for pure (*R*)- and (*S*)-thalidomide were obtained for a range of concentrations from residence time histograms.[12,30] The slopes of plots of $1/\tau_{on}$ versus [thalidomide] then revealed k_{on} values for the *R*- and *S*-forms. These values were required to derive the concentrations of the *R*- and *S*-forms in mixtures. Because the *R*- and *S*-enantiomers gave distinct current blocks, the events analysis for each form in a mixture could be carried out separately. For the *R*-form, the periods of occupancy by the *S*-form were deleted, and vice versa.[31] The rate constants for enantiomerization in the absence of HSA were obtained by using $(R_t - S_t)/(R_t + S_t) = \exp(-2kt)$, which assumes that $k = k_{R\rightarrow S} = k_{S\rightarrow R}$, where $k_{R\rightarrow S}$ is the rate constant for inversion of the *R*-form, and $k_{S\rightarrow R}$ is that for inversion of the *S*-form. R_t and S_t are the concentrations of each enantiomer at time *t*. For experiments done in the presence of HSA, $k_{R\rightarrow S}$ and $k_{S\rightarrow R}$ were estimated by nonlinear regression analysis of the experimental data using the equation $(R_t - S_t)/(R_t + S_t) = (k_{S\rightarrow R} - k_{R\rightarrow S})/(k_{R\rightarrow S} + k_{S\rightarrow R}) + [(2k_{R\rightarrow S}/(k_{R\rightarrow S} + k_{S\rightarrow R})]\exp-(k_{R\rightarrow S} + k_{S\rightarrow R})$-*t*. Values for $k_{R\rightarrow S}$ and $k_{S\rightarrow R}$ were obtained by averaging the results from three experiments conducted in each direction, *R* to *S* and *S* to *R*.

lyzed by human serum albumin (HSA).[23,24] We used the (M113F/K147N)$_7$•βCD sensor to monitor the kinetics of thalidomide racemization in 67 mM sodium phosphate buffer, pH7.4, in the presence and absence of 590 μM HSA (the physiological concentration in plasma) at 37 °C. At various time intervals, the ratio of the *R*- to *S*-isomers of the remaining thalidomide was determined by stochastic detection (Figure 3), yielding the following rates: $k_{R\rightarrow S} = (1.8 \pm 0.2) \times 10^{-5}$ s^{-1}, $k_{S\rightarrow R} = (1.9 \pm 0.1) \times 10^{-5}$ s^{-1} without HSA; $k_{R\rightarrow S} = (3.0 \pm 0.8) \times 10^{-4}$ s^{-1}, $k_{S\rightarrow R} = (6.8 \pm 0.3) \times 10^{-4}$ s^{-1} with HSA. In principle, $k_{R\rightarrow S}$ should equal $k_{S\rightarrow R}$ for the reaction catalyzed by HSA, but the difference in rates has been observed previously[23,24] and can be explained if most of the thalidomide is bound to the multiple enantioselective sites on the protein before extraction and analysis.[25,26]

In summary, we have demonstrated that chiral drug molecules can be rapidly distinguished by stochastic detection with the (M113F/K147N)$_7$•βCD pore. Chiral discrimination by cyclodextrin as determined from K_f values is generally weak.[27−29] By contrast, our approach generates a distinct current signal from each bound enantiomer, which can be used, for example, to monitor racemization. Because many combinations of engineered pores and modified cyclodextrins can be made, the approach should be readily extendable to a wide variety of chiral analytes.

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Supporting Information Available: Further details are given about reagents, mutagenesis, preparation of the (M113F/K147N)$_7$ pore, current recordings, and the interaction of the pore with βCD. This material is available free of charge via the Internet at http://pubs.acs.org.

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