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**Author(s):** Line D. F. Nielsen, Malthe Hansen-Bruhn, Minke A. D. Nijenhuis, and Kurt V. Gothelf

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Protein Induced Fluorescence Enhancement and Quenching in a Homogenous DNA Based Assay for Rapid Detection of Small Molecule Drugs in Human Plasma

Line D. F. Nielsen,†‡ Malthe Hansen-Bruhn,†‡ Minke A. D. Nijenhuis and Kurt V. Gothelf*

Interdisciplinary Nanoscience Center (iNANO) and Department of Chemistry, Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark, *E-mail: kvg@chem.au.dk

KEYWORDS: homogenous assay, point-of-care, small molecule detection, immunoassay, quantification, DNA biosensor, PIFE, PIFQ

ABSTRACT: Homogeneous assays for determining the concentration of small molecules in biological fluids is of importance for monitoring blood levels of critical drugs in patients. We have developed a strand displacement competition assay (SDC) for the drugs dabigatran, methotrexate and linezolid which allow detection and determination of the concentration of the drugs in plasma, however, surprising kinetic behavior of the assay was observed with an initial rapid change in apparent FRET values. We found that protein induced fluorescent enhancement or quenching (PIFE/Q) were causing the initial change in fluorescence within the first minute after addition of protein, which could be exploited to construct assays for concentration determination within minutes in the low nanomolar range in plasma. A kinetic model for the assay was established and when taking the new finding into account the in silico simulations were in good agreement with the experimentally observed results. Utilizing these findings, a simpler assay was constructed for detection of dabigatran, which allowed for detection within minutes without any time-dependencies.

As healthcare services become increasingly patient-centered, there is a growing demand for inexpensive methods that allow for quick quantification of small molecules.1 In response to this demand, point-of-care (POC) testing has become increasingly present over the past decade. POC testing offers advantages over central laboratory testing, such as decreased turn-around time and reduced handling complexity.1–3 Furthermore, fast-readout POC tests for determining small molecule drug levels have vast potential for application in emergency settings, where the accurate detection and quantification of specific drugs underlies life-saving decisions. Finally, POC measurements enable patients to track and optimize the dosage of their drug therapeutics, thereby reducing side-effects and potentially improving therapeutic efficacy.4,5

The identification and rapid quantification of small molecule analytes in complex biological solutions is challenging. Most often, it is performed by liquid chromatography in combination with mass spectrometry techniques.6–8 Alternative techniques for small molecule analyte detection include enzyme-linked immunosorbent assay (ELISA).9 Although this heterogeneous detection method is applied successfully for identification of small molecules, it is often less suitable for rapid quantification.10 Other types of heterogeneous assays for small molecule quantification have been reported.11–13 Homogenous assays offer several advantages over heterogenous assays such as ELISA, as there is no need for immobilization- and washing-steps, thus decreasing the turnaround time. Furthermore, they are more suitable for quantification of the analyte, because there is no dependency on the quantity of immobilized species and on optical surface effects.10,14 Homogenous assays often employ aptamers,15,16 DNA-binding proteins,17,18, semisynthetic sensor proteins19,20 or antibodies including competitive antibody based assays21–25 where the readout is often based on fluorescence or Förster resonance energy transfer (FRET). Quenchbodies offer another approach to measuring small molecule protein interactions in solution, where the quenching of a dye linked close to the binding site of the protein is decreased upon analyte binding.26–27 Protein induced fluorescence enhancement (PIFE) is a related approach where a dye is linked to a protein-interacting molecule and upon protein binding, the protein enhances the fluorescence of the dye.28–30 PIFE has been extensively used for single molecule fluorescence studies, in particular for DNA binding proteins, but PIFE has also been used to study small molecule-protein interactions.31–33 Recently, protein induced fluorescent quenching (PIFQ) has been reported, where quenching is observed when a protein binds in proximity to a fluorophore (exhibiting the opposite effect of PIFE).33

We have previously reported on a homogeneous assay for overnight to 30-minute detection of small molecule-protein interactions, that utilizes a strand-displacement
competition (SDC) system.\textsuperscript{21,34} The mode of action of the SDC assay has previously been thought to be based on destabilization of a DNA-duplex by binding of a protein to a small-molecule ligand on the DNA strand. Herein, we report on the finding of surprising kinetic behavior of the SDC assays for detection of dabigatran (DAB), methotrexate (MTX), linezolid (LIN) and apixaban (APX). We present how the kinetic profile of the known SDC assay is dominated by protein induced fluorescence enhancement or quenching rather than destabilization of the DNA duplex, and based on in silico simulations a new mode of action of the SDC assay was determined. By exploiting this behavior assays for quantification of the clinically relevant drugs DAB, MTX and LIN in the low nanomolar range was constructed with a detection time of a few minutes (min) in human plasma, making these highly suitable for POC testing.

**EXPERIMENTAL**

**General.** All oligonucleotides were synthesized in-house by automated phosphoramidite oligonucleotide synthesis on a MerMade-12 RNA/DNA synthesizer using standard or modified phosphoramidites. All DNA strands were purified by reverse-phase HPLC, before utilization in an experiment, on a Hewlett Packard Agilent 1100 Series using Phenomenex Clarity 3μ Oligo-RP 50 \times 4.6 mm columns. Oligonucleotide concentrations were determined on a Thermo Fisher Scientific ND-1000 NanoDrop spectrophotometer. The commercially available 5-Aminoallyl-dU phosphoramidite was purchased from Berry & Associates. All reagents were commercially available and bought from Sigma Aldrich unless stated otherwise. The commercially available Amino C6 dT was purchased from Link Technologies. The Alexa dyes (Alexa555 and Alexa647) were purchased as NHS (N-hydroxysuccinimide) esters from ThermoFisher Scientific. Dabigatran was purchased from Cayman Chemical Company, the linezolid amine analogue ((S)-5-aminoethyl)-3-(3-fluoro-4-morpholinophenyl)oxazolidine-2-one) was purchased from Matrix Scientific, and the apixaban acid analogue (1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxylic acid) was purchased from Acesys Pharmatech. Methotrexate, linezolid and apixaban antibodies (aMTX, aLin and aAPX, respectively) were supplied as monoclonal antibodies by BioPorto Diagnostics A/S, while antidabigatran fab fragment (aDab) was bought as the antidote (Idarucizumab) from Boehringer Ingelheim. All reagents were used without further purification.

Fluorescence measurements were either performed in a quartz cuvette using a scanning spectrophotometer (FluoroMax 4 HORIBA Scientific) or in 96-well plates (COSTAR) using a plate reader (CLARIOstar, BMG Labtech). For full spectral scans on the spectrophotometer, excitation was performed at 550 nm and emission was measured from 560 nm to 700 nm with an integration time of 0.09 s and 1 nm wavelength interval. For single point measurements, excitation was performed at either 540 nm (spectrophotometer) or 522 nm (plate reader), with emissions measured at 567 nm and 667 nm. FRET was calculated, taking spectral leaking into account, by $FRET = \frac{F_{d} - 0.0815 \times F_{0}}{F_{a} + 0.0815 \times F_{0} - F_{0}}$, where $F_{0}$ is emission from the donor fluorophore (Alexa555) at 567 nm and $F_{a}$ is emission from the acceptor fluorophore (Alexa647) at 667 nm. FRET change ($\Delta$FRET) and apparent FRET change ($\Delta$aFRET) were calculated as the percentage change of (a)FRET relative to a zero-point reference, being the ABS strands pre-equilibrated in solution. All error-bars are standard deviations based on triplicates.

**DNA sequences.** The DNA sequences were adopted from our previous study with new ligands on the B strand.\textsuperscript{21,34} See Table S1 and S2 in supporting information for details.

**DNA-dabigatran and DNA-apixaban conjugation.** To a mixture of amine-modified DNA (10 μL, 2.0 mM), dabigatran (50 μL, 7.6 mM in DMSO with 10 % 0.1 M HCl) or apixaban acid analogue (20 μL, 10 mg/mL in DMSO) and Na$_2$CO$_3$ buffer (200 μL, 20 mM, pH 8.5) was added DMTMMCl (40 μL, 0.5 M in H$_2$O - freshly prepared) and incubated for 25 °C for 1.5 h. The mixture was purified by ethanol precipitation followed by RP-HPLC purification.

**DNA-linezolid conjugation.** To a mixture of amino-modified DNA (5 μL, 2.0 mM) in Na$_2$CO$_3$ buffer (60 μL, 20 mM, pH 8.5) was added acetonitrile (40 μL) and bis-NHS-ester (disuccinimidyl glutarate) (20 μL, 5 mg/mL in DMF). The reaction mixture was incubated at 25 °C for 30 min followed by ethanol precipitation. The pellet was redissolved in Na$_2$CO$_3$ buffer (50 μL, 20 mM, pH 8.5) and a solution of linezolid amine (25 μL, 5 mg/mL in DMF) was added and the mixture was incubated at 25 °C overnight. The mixture was purified by ethanol precipitation followed by RP-HPLC purification.

See Scheme S1 in supporting information for preparation of the bis-NHS-ester disuccinimidyl glutarate.

**DNA-methotrexate conjugation.** Based on a previously reported protocol,\textsuperscript{35} DEAE-Sepharose immobilization was employed. Briefly, a 250 μL suspension of DEAE-sepharose beads is added to a spin-column and washed three times with 200 μL DNA loading buffer (10 mM acetic acid, 0.005% Triton X-100). The column is spun dry, removing the loading buffer. DNA (5-20 nmol) in DNA loading buffer (200 μL) is added. After 10 min incubation the liquid removed. Methotrexate (10 mM, 384 μL, 3.8μmol) in DMF is added together with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide (each 500 mM, 8 μL, 4 μmol) in water resulting in a total volume of 400 μL. After overnight incubation at room temperature is the reaction mixture removed by centrifugation. The resulting slurry is washed with 200 μL DNA loading buffer. The product is eluted with 2 x 200 μL elution buffer (1.5 M NaCl, 50 mM Tris-HCl, pH 8.0). The mixture was purified by ethanol precipitation followed by RP-HPLC purification.

**DNA-fluorophore conjugation.** Performed according to a previous reported protocol.\textsuperscript{21,34} Briefly, the amine-modified DNA (25 μL, 100 μM) was mixed with Alexa555 and Alexa647 NHS ester (100 μg) dissolved in DMSO or DMF (50 μL). The resulting mixture was then added to 20 mM sodium carbonate buffer (20 mM, pH 8.5; 25 μL) and incubated overnight at rt. The mixture was purified by ethanol precipitation followed by HPLC purification.

**DNA-acetylation.** To an amine-modified DNA strand (9 nmole, 900 μL MQ) was added acetic anhydride (100 mM, 10μL) and TEA (1μL). The reaction was incubated for 2 h at rt. The mixture was purified by ethanol precipitation followed by RP-HPLC purification.

**FRET experiments.** The DNA strands A, B, and S were mixed in equal stoichiometric ratio in 1 x TAE-Mg (12.5 mM MgCl) buffer at pH 8 and incubated for minimum 2 hours. The
sample with or without target molecule was pre-incubated with the specific protein at rt for a selected time (e.g. 1 min). The mixture was added to the DNA strands and incubated at rt for a selected time (e.g. 30 seconds to 1 min). In the overnight studies all components were mixed and incubated at rt in the dark overnight.

For experiments in plasma EDTA-buffered human blood from a healthy unknown donor was acquired from the local blood bank (Aarhus University hospital, Denmark). The blood was centrifuged to separate the cells from plasma at 3,000 rcf for 15 min at rt. The plasma was then spiked with small molecule and incubated with protein, before addition to the DNA strands as described above.

**Limit of Detection (LOD)**. The LOD was calculated based on the equation below.\(^3^6\)

\[
\text{LOD} = \frac{3 \sigma_{\text{low}}}{\Delta \text{FRET change}}
\]

Slopes of the linear range of the concentration curves of dabigatran, methotrexate and linezolid were used as \(\Delta \text{FRET change} \). The standard deviation from the lowest concentration in each concentration curve was used as \(\sigma \). See Figure S1, S2 for graphs and calculations.

**Mathematical model.** The initial competitive strand-displacement equilibrium was characterized as a steady-state system based on the conservation of mass. In order to mechanistically evaluate the kinetic transition upon protein addition and eventual final equilibrium, a kinetic model based on the laws of mass-action was used. The model takes as input (i) the DNA-species concentrations calculated with the steady-state characterization, (2) the concentration of protein added, and (3) five parameter values. The latter are the strand displacement equilibrium constant \((pK^e)\), its change upon equilibrium shift \((\Delta pK^e)\), one rate of strand displacement \((k_B)\), and finally the dissociation and rate constants describing the protein’s affinity towards its ligand \((k^d)\) and \((k^o)\). The model describes the concentrations of each species changing over time until they settle in a new equilibrium, using a system of eight coupled ordinary differential equations (ODEs). Certain parameters are quantifiably restricted in order to model specific hypothesized mechanisms, e.g. \(\Delta pK^e > 0\) for protein-induced destabilization of BS: destabilization Finally, equation 4 describes the conversion from modeled concentrations to simulated \(\Delta \text{FRET}\), which may require the PIFE/Q parameter \(r\). The full derivation and ODE system is found in SI ‘model derivation’. The calibration and validation methods for comparison between simulated \(\Delta \text{FRET}\) and measured \(\Delta \text{FRET}\) are described in SI ‘model fitting’.

**RESULTS AND DISCUSSION**

In the SDC system, two DNA-strands A and B, dynamically compete for the binding of a third strand S through toehold-mediated strand displacement (Figure 1A). The equilibrium between the three strands depends on the relative stability of the resulting two duplexes (AS and BS): if one duplex is destabilized, the equilibrium will shift to favor the other. This competitive equilibrium is quantified by the equilibrium constant \(pK^e\) (Equation 1). Furthermore, its change \((\Delta pK^e)\) may be monitored through a fluorescent readout based on FRET. Specifically, the hybridization of the S and A strand brings the fluorescent labels in close proximity, resulting in increased FRET. The final FRET readout is a ratiometric representation of the AS and BS duplexes, thereby enabling direct quantification of these species.

\[
pK^e = \log \frac{[AS][B]}{[A][BS]} \quad (1)
\]

\[
\text{FRET} = \frac{FA}{FA + FD} \quad (2)
\]

In which [A] and [B] are the concentrations of the strands competing for the S-strand, [AS] and [BS] are the concentrations of their respective duplexes, and \(FA\) and \(FD\) are respectively the acceptor and donor emission intensity. To employ the SDC system in an assay for molecule-protein interactions, the B strand is conjugated to a small molecule of interest via a base in one of the nucleotides. This modification has little impact on the BS duplex stability, presumably because it is relatively small.\(^2^1\) However, once a protein (Y) binds the small molecule as its ligand, the stability of the BS duplex may be significantly affected. Specifically, the mode of action of the SDC-based assay was hypothesized to be as follows: the binding of a bulky protein Y to the B-conjugate creates steric repulsion that explicitly destabilizes the BS duplex (Figure 1A). Consequently, upon addition of the protein to the SDC system, the competitive equilibrium shifts in favor of hybridization between A and S. Since AS is more dominant in the final equilibrium, the final FRET signal is increased compared to FRET measured at the initial state. If the protein is pre-incubated with a sample containing an unknown amount of free small molecule ligand, and thereby the binding-site partly blocked, its addition to the SDC system is proportionally less effective in causing the anticipated equilibrium shift \(\Delta pK^e\). The final FRET readout can thereby also be used to quantify an unknown concentration of ligand in a sample of interest.

**Kinetic Behaviors of SDC Assays**

The hypothesized mechanism was in excellent agreement with the observed behavior of the first SDC-based assay, which studied the interaction between the ligand digoxigenin (DIG),\(^2^6\)\(^,^3^4\) a drug used to treat cardiac arrhythmia,\(^3^7\) and the anti-DIG antibody (Figure 1B, yellow). A slow increase in the FRET signal was observed after protein addition, consistent with the protein-induced destabilization of BS. The DIG assay fully settled into its final equilibrium after overnight incubation, although it should be noted that a fully settled equilibrium is not necessary for real-life application of the assay. In comparison, no significant increase was observed when the protein had been pre-incubated with an excess of free ligand.\(^3^4\) The readout obtained from the SDC assay allows for quantification of small molecules, such as digoxigenin and folate in plasma at therapeutically relevant range, with its fastest readout time being 30 min.\(^2^6\)\(^,^3^4\)

Surprisingly, very different kinetics were observed for some other protein:small molecule pairs (Figure 1B).
Looking for additional applications of the SDC assay, we were interested in monitoring of blood concentrations of a class of medication called direct oral anticoagulants (DO-ACs). The drug DAB belongs to this class and is a direct thrombin inhibitor,\textsuperscript{38,39} which is clinically used as an anticoagulant to prevent blood clots. For construction of the SDC assay, DAB was conjugated to the B strand and anti-DAB Fab was used as the protein. When the anti-DAB protein was added to the assay, however, a rapid decrease in FRET over 1-2 min was observed, followed by the anticipated slow increase over time (Figure 1B, teal).

Prompted by this unexpected finding, we decided to test the SDC system with three more targets: LIN, an antibiotic used in treatment of Gram-positive bacteria,\textsuperscript{40} MTX, an immunosuppressant often used as a chemotherapeutic agent,\textsuperscript{41} and apixaban (APX), which is a Factor Xa inhibitor anticoagulant also belonging to the DOACs.\textsuperscript{42} Custom raised monoclonal antibodies against LIN, MTX and APX were used as biomolecular binding partners. The LIN assay displayed a similar kinetic profile as the DAB assay (Figure 1B, light green). The MTX-assay displayed an even more pronounced initial decrease than the DAB-assay, followed by the similar gradual increase (Figure 1B, dark blue).

FRET measured in the final equilibrium state was lower than in that of the initial state; this was an especially peculiar observation, as it seems to imply that the BS duplex – which is supposedly destabilized by the protein Y – is more dominant in the final equilibrium. Perhaps even more surprising was the APX-assay, which showed a very rapid initial increase, followed again by the anticipated slow increase (Figure 1B, red). An overview of all five small molecules of interest is shown in Figure 1C. It should be noted that the FRET only changed when a B-strand was incubated with a compatible protein (Figure S4). Furthermore, any FRET changes were also fully inhibited by the presence of a compatible free ligand. The behavior thus requires binding specificity and does not result from an arbitrary protein or conjugate.

**Original Mechanism-of-Action of the SDC Assay**

In order to understand the unexpected kinetic behavior of the assay, a mechanistic model based on mass-action kinetics was drawn up in order to test the hypothesized mode of action (SI ‘model derivation’). A number of assumptions is made about the SDC system, which enables
the model to specifically simulate the protein-induced destabilization of BS mechanism. The prime assumption herein is that the change in $\Delta pK'$ must be positive, i.e. protein addition must cause a shift towards a more AS-dominant state. Equation 3 converts the simulated concentration of competing species to a predictive FRET change ($\Delta FRET$), at a certain timepoint $t$ (SI ‘model derivation’), which can then be compared to the experimentally observed ($\Delta FRET$).

\[
\Delta FRET_t = \frac{[AS]_t ([AS]_{t=0} + [BS]_{t=0})}{[AS]_{t=0} ([AS]_t + [BS]_t + [YBS]_t)} \cdot 100\% \quad (3)
\]

After calibration, the mechanistic model could reliably replicate the FRET change of the original DIG assay, which showed only the anticipated slow signal increase (Figure S15A). However, even with a loose interpretation of unknown parameters and uncertainties, this model was unable to simulate the fast signal decrease of the DAB assay (Figure S15B), let alone the entire behavioral variety observed over all five assays (Figure S15C). Therefore, the original hypothesis of the mechanism of the SDC assay is insufficient. Although all assays eventually show a slow signal increase consistent with protein-induced destabilization of BS, a different mechanism must dominate the assay’s readout in the first minutes of the kinetic transition.

**Extended Mechanism-of-Action of the SDC assay**

After unsuccessfully testing of several equilibrium-based hypothesized mechanisms (e.g. complexation of multiple species), we took a closer look at the raw fluorescence data forming the basis of the FRET readout. During the kinetic transition, a rather large initial change in donor intensity was observed, without concomitant change of acceptor fluorophore. This is inconsistent with the typical FRET measurement, in which the donor emission is inversely related to the acceptor emission. Addition of the relevant protein to the DAB or MTX assays caused a drastic increase in donor emission, while the acceptor emissions remained unchanged (Figure 2A). Thus, the observed FRET change is only an apparent FRET change ($\Delta aFRET$), resulting from the division of observed intensities rather than an accurate measure of the Förster energy transfer. In the BS duplex, the small molecule ligand of the B strand is situated closely to the fluorophore of the S strand (Figure 2E). We therefore hypothesized that the binding of the protein to the small molecule ligand will influence the emission of the nearby fluorophore, either enhancing or quenching the fluorescence (PIFE or PIFQ, respectively). The effects are caused by an increased local viscosity induced by the proximity of the protein may impair the fluorophore, which may result in increasing or decreasing fluorescent intensities when some of the non-irradiative excitation decay pathways are inhibited or promoted.33

To investigate the interaction of the protein with the fluorophore of the S-strand, we investigated the BS duplex in the absence and presence of protein (Figure 2B). In the case of DAB, LIN and MTX, the protein caused an enhancement of the fluorescence (PIFE). For APX addition of the protein caused a large quenching of the fluorescence (PIFQ) (Figure 2C, red). As control we added the various proteins to the BS duplex with acetylated B-strand, which did not give rise to a change the fluorescence, confirming the necessity of the specific binding of the protein to the ligand (Figure S5).

**Figure 2**

A) Donor and acceptor emission and calculated $aFRET$ values for the ABS system for DAB and MTX after addition of the respective protein. B) The setup for evaluating the impact of the binding of the protein to BS-strand (PIFE/Q) is shown. This impact is quantified with the $r$ parameter, which is the unitless ratio of the respective fluorescent intensities of BS and YBS. C) Results of the effect of protein on the BS duplexes (60 nM assay). BS reference is BS duplex with acetylated B-strand ($B_{ACET}$). Error-bars represent standard deviations D) Structure of donor fluorophore, Alexa555, and the DNA attachment site (black triangle). E) 3D rendering of the BS duplex with fluorophore and ligand. The ligand and fluorophore are placed on adjacent t-bases, which co-localizes the species (PDB 1BNA).
It is peculiar that some of the proteins cause an enhancement of fluorescence, while others cause quenching. Despite the photophysical model of PIFE,\textsuperscript{39} the relationship between protein structure and PIFE/Q largely remains a mystery. While it has been proven that different DNA sequences can explain PIFE/Q variability (e.g. due to intramolecular folding and/or guanine-induced quenching),\textsuperscript{33} the sequences of the ABS strands were kept constant – as were the positions of the protein and dye. It has been shown that even slight changes in molecular structure can impact the PIFE/Q effect.\textsuperscript{43} The difference between the observed effect of our investigated proteins could potentially be caused by different amino-acid composition of the parts of the proteins that are in proximity to the fluorophore.

The collective PIFE/Q effects of each individual ligand:protein pair are quantified with \( r \), defined as the ratio between emission intensity from protein-bound duplex (YBS) and unbound duplex (BS). The value of \( r \) is smaller than 1 if the protein has a quenching effect, and vice versa for an enhancing effect.

The findings on the donor emission manipulation form the basis of a new hypothesis on the SDC assay’s mechanism-of-action. The protein-induced destabilization of BS causing a shift in the strand displacement equilibrium is responsible for the gradual long-term signal increase observed in all assays. Additionally, the protein can induce significant and an almost immediate change in donor fluorescence when it is bound to the BS duplex. In order to test this hypothesis, we modified the mechanistic model that previously failed to replicate the experimental behavior. The only change was the introduction of the PIFE/Q parameter \( r \) into Equation 3, which is used to convert the concentration of species into a FRET signal. This parameter only affects the protein-bound species YBS (Equation 4).

\[
\text{ΔFRET}_t = \frac{[\text{BS}][\text{protein}]}{[\text{BS}][\text{protein}]+[\text{YBS}]+[\text{Q}]} \cdot 100\% \quad (4)
\]

The modified model was again fitted against all five assays simultaneously, to test whether it could explain the entire behavior variety. Both \( \text{ΔpK}^e \) and \( \#r \) are estimated uniquely for each assay, because these parameters are specific to the protein:ligand pair and essential to the hypothesized mechanism. Parameters and constants that are assumed invariant between distinct systems are elaborated on in SI ‘model fitting’. This fit shows a remarkable agreement between simulation and experiment (Figure 3A), even under the strict interpretation of the hypothesis and significant simplification of the overall system (as explained in SI ‘model fitting’). Furthermore, the standard error on the estimated parameter values is low, suggesting a quantitative certainty in the estimated values. Indeed, comparing the \( \#r \) estimates against the experimentally measured \( r \) reveals that, there is no significant difference between the theoretical model values and the assay observations (Figure 3B). As expected, the LIN, DAB, and MTX assays all have an \( r \) higher than 1, indicating the presence of PIFE. The APX assay has an \( r \) lower than 1, indicating PIFE/Q. Finally, the DIG assay has an \( r \) of 1, meaning that the protein has no effect on the fluorescence emission.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{A) Best fit of the mechanistic model with all five assay systems simultaneously. The fit shows a good agreement between wetlab data (markers with error bars) and simulation (lines). The model incorporates both protein-induced destabilization of BS (quantified by \text{ΔpK}^e) as well as the PIFE/Q effect (quantified by \( r \)). The model-estimated values for these parameters are shown in boxes. Remaining constants and parameters are reported in Table S4. Error-bars represent standard deviations. B) The model-estimated \( \#r \) values are quantitatively comparable to experimental \( r \) values measured in triplicates. P-values shown were calculated with a Welch’s t-test. Note that \( r \) is a unitless ratio. Error-bars represent 95% confidence intervals. C) Predictive simulation of possible assay behaviors, depending on the extent of protein-induced destabilization (\text{ΔpK}^e, brightness gradient) and PIFE/Q (\( r \), hue gradient). Plotting on a logarithmic timescale reveals that PIFE/Q effects dominate the assay behavior in the initial minute after protein addition. Protein-induced destabilization becomes apparent after the first minute, until the system settles in the final equilibrium. The final assay readout after a few hours is the culmination of both mechanisms. The DIG and APX traces are identical to the ones in subfigure A and was added to ease the comparison of the natural and logarithmic time scales.}
\end{figure}
The equilibrium shift $\Delta pK'$ also varies between assays, but in all cases it is relatively small (between 0.15 and 0.31) and never close to a full order of magnitude. Based on the estimated ranges of $\Delta pK'$ and $r$, a predictive simulation of possible assay outcomes of other ligand:protein pairs was performed (Figure 3C). Plotting kinetic traces on a logarithmic time scale cleanly reveals the two-phased behavior of the SDC-based assays: in the initial minute, the PIFE/Q effects are the main drivers behind the change in the apparent FRET signal, whereas afterwards the protein-induced destabilization of BS is the dominant mechanism. This two-phased behavior is the result of the on-rate of the protein binding being several orders of magnitude greater than the rates of the strand displacement reactions. The final apparent FRET readout after a few hours is the culmination of both mechanisms. Consequently, the final FRET readout may be decreased compared to the initial signal, even as the equilibrium shifts in favor of the FRET-active species AS.

Construction of Assays for Rapid Detection of DAB, MTX and LIN

Before realizing the true mechanism-of-action of the SDC assay, the rapid change in kinetics in the first few minutes caused by PIFE/Q was exploited to develop assays for rapid detection of DAB, MTX and LIN. Here, the biomolecular binding partner Y and the free analyte were incubated first, followed by the incubation with the equilibrated DNA strands, allowing for a detection system as seen from Figure 4A.

The incubation time needed for the protein to be fully inhibited with free analyte was investigated and found to be 1 min for DAB and LIN (Figure S6 and S7), and 30 seconds for MTX. Due to rapid detection time, these assays have high potential for POC applications.

For all three target molecules, PIFE is dominant in the first minutes (Figure 4A). As the timescale for detection in these assays are all within minutes, the effect from the shift in the DNA equilibrium is negligible (Figure 3C). When the analyte is not present, the protein will bind to the B-strand causing fluorescent enhancement of the donor fluorophore, which in turn appears as low aFRET (Figure 4A top). An increased amount of analyte in the first incubation step will occupy the protein to a larger degree, thereby resulting in less interaction of the protein with the modified B-strand.

In turn, there is less fluorescent enhancement of the donor fluorophore resulting in a higher aFRET (Figure 4A bottom). Thus, an increase in $\Delta aFRET$ occurs with increasing analyte concentration. Modulating the concentration of the analyte allows for the construction of dose response curves, as shown in Figure 4B, C and D. For DAB, dose response curves at 3 different assay concentrations (1:1 ratio between Y:A:B:S at 10 nM, 20 nM and 40 nM) were established, with a 2 min total detection time (Figure 4B, left).

As is evident from the curve, the linear range of the assay is tunable by the assay concentration. Therefore, we hypothesize that the system can be tuned to the desired range for any application only limited by the Kd of the protein. A similar setup was performed with the MTX assay with assay concentrations at 10 nM, 20 nM and 40 nM (Figure 4C, left). Here, the total detection time was 1 min consisting of 30 second incubation of protein with free analyte, and 30 seconds of the mixture with the DNA strands. A similar dose response curve is obtained as for DAB. Saturation of aDAB and aMTX with DAB and MTX is occurring at a 1:1 ratio and 1:2 ratio, respectively, presumably due to aDab having only one binding site (Fab fragment) and aMTX having two binding sites (full antibody). Both assays exhibited stable performance in the range of 5 °C to 35 °C (Figure S8 and S9).

Detection in Human Plasma

To advance the technology further towards POC applications, we investigated the ability of the assay to measure small molecules in human plasma. First, the impact of plasma on the kinetics of the DAB assay was investigated. The FRET minima were delayed by one min compared to the rate in buffer, which presumably is caused by the higher viscosity of plasma (shown in Figure S10). Therefore, the total assay time in the concentration study in plasma for DAB and LIN is 3 min; 1 min incubation with aDAB or aLIN, followed by 2 min incubation with the DNA strands. For MTX, no change in time of the minima in FRET was observed during measurement in plasma (Figure S11), hence a detection time of 1 min for MTX was maintained. Detection in buffer and detection in plasma are shown in Figures 4B, 4C and 4D for DAB, MTX and LIN, respectively. From the data it is evident that the systems perform similarly in buffer and plasma. The limit of detection (LOD) was calculated to be 2.25 nM and 4.75 nM for DAB and MTX in plasma, respectively (Figures S1 and S2) and 8.79 nM for LIN in buffer (Figure S3). Due to the lack of studies correlating plasma concentration with thrombic and hemorrhagic complications, DAB has no reported therapeutic range. The lowest concentration at which DAB is present in the blood, in patients taking a normal dose, has been determined to be above 38 nM. Studies show that a plasma concentration of MTX after treatment of 0.1-10 µM within 24-72 hours can be toxic. An optimal therapeutic range of LIN in critically ill patients has been determined to be between 5.9 µM to 29.6 µM. These assays are therefore capable of detecting both DAB, MTX and LIN at sub-therapeutic levels in plasma within minutes. This fast readout is highly desired for POC applications, e.g. an emergency setting or for therapeutic drug monitoring.

The cross reactivity between the three assays was investigated, to demonstrate the selectivity of these systems. No cross reactivity was observed between the antibodies and the three different small molecules (Figure S12).
Figure 4. The setup for detection and quantification of small molecules exploiting the PIFE effect. A) The protein is mixed with the sample that contains the small molecule analyte and allowed to react (30-60 seconds). To the mixture is added the pre-equilibrated ABS strands. Within the first min the PIFE effect is dominant. With small molecule present the no PIFE is occurring resulting in less fluorescent enhancement thus a higher aFRET. When no small molecule is present fluorescent enhancement is occurring, thus giving a lower aFRET. B) Left: Employing a 1 min pre incubation time, and a 1 min interaction with the DNA strands, a dose response in the low nanomolar range can be obtained. Using three different concentrations of the DAB assay, several distinct dose response curves can be constructed. Right: In plasma a 1 min incubation step followed by 2 min interaction with the DNA strands shows a similar response in 65% human plasma for detection of DAB as in buffer. C) Left: For detection of MTX a 30 sec incubation step was employed together with a 30 sec readout step, resulting in a dose response curve in the low nanomolar range within 1 min. Similarly, three different concentrations allow for tuning of the dynamic range. Right: Detection in 65% human plasma within 1 min shows similar performance as in buffer. D) Comparison of 20 nM LIN assay in buffer and in human plasma (88%). E) A PIFE DAB assay using only the BDAB and S-strand can be constructed measuring only donor emission. The PIFE effect can be reversed by addition of excess DAB (Figure 4E) and the PIFE assay can detect DAB in the nanomolar range in buffer as well as in plasma (Figure 4F). Removing the strand displacement equilibrium from the system increased the total signal change from 8% signal change in the original SDC assay (Figure 1B, DAB) to 20%.

PIFE-based Assay for the Detection of Small Molecules

To further confirm that the change in FRET within the first few minutes is derived from a PIFE/Q effect and that this can be utilized as assay readout, a simpler assay was constructed using only the S- and B-strand (setup is seen in Figure 4E). By omitting the A-strand the readout of the assay is only based on the influence of the protein on the donor fluorophore and the signal readout is not time-dependent as is the case for the SDC assay. Using the new PIFE assay a method consisting of 1 min incubation with the protein and the sample, followed by 1 min incubation of the mixture with DNA strands, provided a 2 min PIFE assay for detection of DAB. In a 60 nM setup it is possible to reverse the PIFE effect by 1 min incubation with DAB (Figure 4E) and the PIFE assay can detect DAB in the nanomolar range in buffer as well as in plasma (Figure 4F). Removing the strand displacement equilibrium from the system increased the total signal change from 8% signal change in the original SDC assay (Figure 1B, DAB) to 20%.
signal change in the novel assay. To our knowledge this is the first reported PIFE assay for small molecule quantification.

CONCLUSION

Based on our previously reported SDC-based assay, we developed efficient assays for the detection of clinically relevant drugs such as the direct oral anticoagulant DAB, the immunosuppressant MTX and the antibiotic LIN in plasma. Unexpectedly, the FRET changed rapidly in the initial minutes of sample incubation. In silico experiments elucidated that the apparent kinetic readout of the SDC assay is two-phased. On the hour-timescale of all assays, the biomolecular binding partner induced a slow signal increase through the thermodynamic destabilization of one of the competing DNA duplexes. However, the protein binding also had another effect; upon binding the DNA duplex, the nearby donor fluorophore emission is in most cases either enhanced or quenched through PIFE/Q. In such assays, the apparent FRET then responds immediately yet counterintuitively. It increases when the donor is quenched, and vice versa. The first minutes of these assays are thus dominated by a fluorescent artifact, rather than an equilibrium shift. This mode-of-action enabled the development of a fast-readout SDC-based assay that quantitatively detects the target analyte in less than 3 min. A simplified version of the assay, where the signal is purely generated using the PIFE/Q effect, detected nanomolar ranges of DAB in plasma. These assays show great potential for application in POC systems, as these are rapid, robust and are well-behaved in human plasma.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at http://pubs.acs.org.
DNA sequences, supporting figures for laboratory experiments, model derivation, model fitting and hypothesis testing with experimental data.

AUTHOR INFORMATION

Corresponding Author
* Kurt V. Gothelf - Interdisciplinary Nanoscience Center (iNANO) and Department of Chemistry, Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark
Email: kvg@chem.au.dk

Present Addresses
† MedicQuant ApS, Universitetsbyen 14, 8000 Aarhus C, Denmark

Author Contributions
All authors have given approval to the final version of the manuscript. †These authors contributed equally.

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ABBREVIATIONS

APX, Apixaban; aAPX, anti-apixaban antibody; DAB, Dabigatran; aDAB, anti-dabigatran fab fragment; LIN, Linezolid; aLIN, anti-linezolid antibody; MTX, Methotrexate, aMTX anti-methotrexate antibody; DIG, digoxin; aDIG, anti-digoxigenin antibody; rt, room temperature; FRET, Förster resonance energy transfer; ODE, ordinary differential equation; PIFE, Protein induced fluorescent enhancement; PIFQ, protein induced fluorescent quenching; POC, point-of-care; SDC, strand displacement competition.

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