Electronic Supplementary Information

Analyte Sensing with Unselectively Binding Synthetic Receptors: Virtues of Time-resolved Supramolecular Assays.

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2 Abbreviations

H	host
A	analyte
D	(indicator) dye
ADA	analyte displacement assay
*kin*ADA	kinetic ADA
*kin*ADA^{PFO}	pseudo first order *kin*ADA
IDA	indicator displacement assay
*kin*IDA	kinetic IDA
CB8	cucurbit[8]uril
CB7	cucurbit[7]uril
MPCP	(*rac*)-*N*-methyl-4-pyridinylium[2.2]paracyclophane
BC	berberine chloride
MDAP	*N,N'*-dimethyl diazapyrene
Tes	testosterone
Prog	progesterone
Nan	nandrolone
λ	wavelength
λ_{exc}	excitation wavelength
λ_{em}	emission wavelength
ε	molar extinction coefficient
I	emission signal (fluorescence intensity)
I^{norm}	normalized signal (fluorescence intensity)
I_{sim}	simulated signal (fluorescence intensity)

3 Materials

All solvents were used as received from Aldrich or Fluka without further purification. All chemicals were purchased and used as received unless stated otherwise. CB8[1] and CB7[2, 3] were synthesized according to literature procedures but can also be purchased from Strem or Sigma. MPCP[4] and MDAP[5, 6] were synthesized according to literature procedures. All stock solutions, unless stated otherwise, were prepared in Milli-Q water and kept in the fridge at +8°C for storage. Owing to the low solubility of the steroids, testosterone (solubility = 114 μM)[7] and progesterone (solubility = 33 μM)[7] in water, their respective stock solutions were prepared in ethanol and then diluted in the host-containing Milli-Q

water for the *kin*ADA and *kin*ADA^{PFO} measurements. The small amount of ethanol (< 0.15 % v/v ethanol) in the media (MilliQ water) did not affect the kinetics in all cases studies. The stock solutions prepared in ethanol were stored in the freezer at -20^{o}C. Nandrolone has a solubility of 810 µM[7] in water; hence, the stock solution was prepared in Milli-Q water. The concentration of MPCP, BC, and MDAP stock solutions were determined accurately by using their molar extinction coefficients, ε (MPCP[4]: 7112 $M^{-1}cm^{-1}$ at 335 nm, BC[8]: 22300 $M^{-1}cm^{-1}$ at 344 nm, MDAP[9]: 7800 $M^{-1}cm^{-1}$), by UV-Vis absorption titration measurements in Milli-Q water. For compounds featuring unreported molar extinction coefficient, the stock solutions were prepared by weighing in the required amount of the pure sample to attain the desired concentration. The concentration of CB8 stock solution was determined by fluorescence titration against a known concentration of MPCP dye by exciting the sample at 368 nm and collecting the emission intensity at 531 nm in Milli-Q water. The concentration of CB7 stock solution was determined by fluorescence titration against a known concentration of MDAP dye by exciting the sample at 339 nm and collecting the emission intensity at 454 nm in Milli-Q water.

4 Instrumentation

Absorption spectra were measured on a Jasco V-730 double-beam UV–VIS spectrophotometer and baseline corrected. Steady-state emission spectra and time-resolved emission profiles were recorded on a Jasco FP-8300 fluorescence spectrometer equipped with a 150 W xenon arc lamp, single-grating excitation, and emission monochromators. Emission and excitation spectra were corrected for source intensity (lamp and grating) and the emission spectral response (detector and grating) by standard correction curves. Stopped-flow kinetic experiments were carried out on a Jasco FP-8300 fluorescence spectrometer equipped with a water thermostated (25°C) SFA-20 stopped-flow accessory from TgK Scientific Limited, which was driven by a pneumatic drive. Microplate assays were performed on a PerkinElmer EnSight multimode plate reader in OptiPlate-96 microplates at 25°C. For measurements conducted in water, MilliQ water was used in all cases. For spectroscopy analysis in cuvettes, UV plastic cuvettes with a light path of 10 mm and dimensions of 10x10 mm from Brand with a spectroscopic cut-off at 230 nm were utilized.

The differential equations describing the kinetic process described in the paper have been solved numerically with Wolfram Mathematica 11/12. A best practice guide on conducting a *kin*ADA, *kin*ADA^{PFO}, and *kin*IDA can be found in our previous report, where we introduced the assays.[10] Unless stated otherwise, I^{norm} represents the normalized signal obtained upon dividing the data by the maximum value.

5 Mathematical Equations for Kinetic Assay

5.1 Kinetic Analyte Displacement Assay (*kin*ADA) & Kinetic Indicator Displacement Assay (*kin*IDA): with a single analyte

$$\mathrm{HA} + \mathrm{D} \rightleftarrows \mathrm{HD} + \mathrm{A}\ (\textit{kin}\mathrm{ADA}) \qquad \mathrm{A} + \mathrm{HD} \rightleftarrows \mathrm{HA} + \mathrm{D}\ (\textit{kin}\mathrm{IDA}) \qquad \text{Eq. S 1}$$

$$\mathrm{H} + \mathrm{A} \underset{k_{\mathrm{out}}^{\mathrm{HA}}}{\overset{k_{\mathrm{in}}^{\mathrm{HA}}}{\rightleftarrows}} \mathrm{HA} \qquad \text{Eq. S 2}$$

$$\mathrm{H} + \mathrm{D} \underset{k_{\mathrm{out}}^{\mathrm{HD}}}{\overset{k_{\mathrm{in}}^{\mathrm{HD}}}{\rightleftarrows}} \mathrm{HD} \qquad \text{Eq. S 3}$$

$$\frac{\mathrm{d}[\mathrm{HD}]_t}{\mathrm{d}t} = k_{\mathrm{in}}^{\mathrm{HD}} \cdot [\mathrm{H}]_t[\mathrm{D}]_t - k_{\mathrm{out}}^{\mathrm{HD}} \cdot [\mathrm{HD}]_t \qquad \text{Eq. S 4}$$

$$\frac{\mathrm{d}[\mathrm{D}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HD}} \cdot [\mathrm{H}]_t[\mathrm{D}]_t + k_{\mathrm{out}}^{\mathrm{HD}} \cdot [\mathrm{HD}]_t \qquad \text{Eq. S 5}$$

$$\frac{\mathrm{d}[\mathrm{HA}]_t}{\mathrm{d}t} = k_{\mathrm{in}}^{\mathrm{HA}} \cdot [\mathrm{H}]_t[\mathrm{A}]_t - k_{\mathrm{out}}^{\mathrm{HA}} \cdot [\mathrm{HA}]_t \qquad \text{Eq. S 6}$$

$$\frac{\mathrm{d}[\mathrm{A}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HA}} \cdot [\mathrm{H}]_t[\mathrm{A}]_t + k_{\mathrm{out}}^{\mathrm{HA}} \cdot [\mathrm{HA}]_t \qquad \text{Eq. S 7}$$

$$\frac{\mathrm{d}[\mathrm{H}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HD}} \cdot [\mathrm{H}]_t[\mathrm{D}]_t + k_{\mathrm{out}}^{\mathrm{HD}} \cdot [\mathrm{HD}]_t - k_{\mathrm{in}}^{\mathrm{HA}} \cdot [\mathrm{H}]_t[\mathrm{A}]_t + k_{\mathrm{out}}^{\mathrm{HA}} \cdot [\mathrm{HA}]_t \qquad \text{Eq. S 8}$$

$$I_t = I^0 + I^{\mathrm{HD}} \cdot [\mathrm{HD}]_t + I^{\mathrm{D}} \cdot [\mathrm{D}]_t \qquad \text{Eq. S 9}$$

$[H]_t$ – host concentration at time t, $[D]_t$ – dye concentration at time t,
$[A]_t$ – analyte concentration at time t, $[HD]_t$ – host⊃dye concentration at time t,
$[HA]_t$ – host⊃analyte concentration at time t,
$k_{\mathrm{in}}^{\mathrm{HD}}$ – rate constant for the association of the host⊃dye (HD) complex (complexation),
$k_{\mathrm{out}}^{\mathrm{HD}}$ – rate constant for the dissociation of the host⊃dye (HD) complex (decomplexation),
$k_{\mathrm{in}}^{\mathrm{HA}}$ – rate constant for the association of the host⊃analyte (HA) complex (complexation),
$k_{\mathrm{out}}^{\mathrm{HA}}$ – rate constant for the dissociation of the host⊃analyte(HA) complex (decomplexation),
I^0 – background signal, I^{HD} – constant proportional to the fluorescence efficiency of host⊃dye (HD) complex at the monitoring wavelength, I^{D} – constant proportional to the fluorescence efficiency of free dye (D) at the monitoring wavelength,
I_t – emission signal as a function of time

5.2 Kinetic Analyte Displacement Assay (*kin*ADA): with two analytes, A1 and A2

$$\mathrm{HA1} + \mathrm{D} \rightleftarrows \mathrm{HD} + \mathrm{A1} \qquad \mathrm{HA2} + \mathrm{D} \rightleftarrows \mathrm{HD} + \mathrm{A2} \qquad \text{Eq. S 10}$$

$$\mathrm{H} + \mathrm{A1} \underset{k_{\mathrm{out}}^{\mathrm{HA1}}}{\overset{k_{\mathrm{in}}^{\mathrm{HA1}}}{\rightleftarrows}} \mathrm{HA1} \qquad \mathrm{H} + \mathrm{A2} \underset{k_{\mathrm{out}}^{\mathrm{HA2}}}{\overset{k_{\mathrm{in}}^{\mathrm{HA2}}}{\rightleftarrows}} \mathrm{HA2} \qquad \text{Eq. S 11}$$

$$\mathrm{H} + \mathrm{D} \underset{k_{\mathrm{out}}^{\mathrm{HD}}}{\overset{k_{\mathrm{in}}^{\mathrm{HD}}}{\rightleftarrows}} \mathrm{HD} \qquad \text{Eq. S 12}$$

$$\frac{\mathrm{d}[\mathrm{HD}]_t}{\mathrm{d}t} = k_{\mathrm{in}}^{\mathrm{HD}} \cdot [\mathrm{H}]_t[\mathrm{D}]_t - k_{\mathrm{out}}^{\mathrm{HD}} \cdot [\mathrm{HD}]_t \qquad \text{Eq. S 13}$$

$$\frac{\mathrm{d}[\mathrm{D}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HD}} \cdot [\mathrm{H}]_t[\mathrm{D}]_t + k_{\mathrm{out}}^{\mathrm{HD}} \cdot [\mathrm{HD}]_t \qquad \text{Eq. S 14}$$

$$\frac{\mathrm{d}[\mathrm{HA1}]_t}{\mathrm{d}t} = k_{\mathrm{in}}^{\mathrm{HA1}} \cdot [\mathrm{H}]_t[\mathrm{A1}]_t - k_{\mathrm{out}}^{\mathrm{HA1}} \cdot [\mathrm{HA1}]_t \qquad \text{Eq. S 15}$$

$$\frac{\mathrm{d}[\mathrm{A1}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HA1}} \cdot [\mathrm{H}]_t[\mathrm{A1}]_t + k_{\mathrm{out}}^{\mathrm{HA1}} \cdot [\mathrm{HA1}]_t \qquad \text{Eq. S 16}$$

$$\frac{\mathrm{d}[\mathrm{HA2}]_t}{\mathrm{d}t} = k_{\mathrm{in}}^{\mathrm{HA2}} \cdot [\mathrm{H}]_t[\mathrm{A2}]_t - k_{\mathrm{out}}^{\mathrm{HA2}} \cdot [\mathrm{HA2}]_t \qquad \text{Eq. S 17}$$

$$\frac{\mathrm{d}[\mathrm{A2}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HA2}} \cdot [\mathrm{H}]_t[\mathrm{A2}]_t + k_{\mathrm{out}}^{\mathrm{HA2}} \cdot [\mathrm{HA2}]_t \qquad \text{Eq. S 18}$$

$$\frac{\mathrm{d}[\mathrm{H}]_t}{\mathrm{d}t} = -k_{\mathrm{in}}^{\mathrm{HD}} \cdot [\mathrm{H}]_t[\mathrm{D}]_t + k_{\mathrm{out}}^{\mathrm{HD}} \cdot [\mathrm{HD}]_t - k_{\mathrm{in}}^{\mathrm{HA1}} \cdot [\mathrm{H}]_t[\mathrm{A1}]_t + k_{\mathrm{out}}^{\mathrm{HA1}} \cdot [\mathrm{HA1}]_t - k_{\mathrm{in}}^{\mathrm{HA2}} \cdot [\mathrm{H}]_t[\mathrm{A2}]_t + k_{\mathrm{out}}^{\mathrm{HA2}} \cdot [\mathrm{HA2}]_t \qquad \text{Eq. S 19}$$

$$I_t = I^0 + I^{\mathrm{HD}} \cdot [\mathrm{HD}]_t + I^{\mathrm{D}} \cdot [\mathrm{D}]_t \qquad \text{Eq. S 20}$$

$[\mathrm{H}]_t$ – host concentration at time t, $[\mathrm{D}]_t$ – dye concentration at time t,
$[\mathrm{A1}]_t$ – concentration of analyte 1 (A1) at time t, $[\mathrm{A2}]_t$ – concentration of analyte 2 (A2) at time t
$[\mathrm{HD}]_t$ – host⊃dye concentration at time t,
$[\mathrm{HA1}]_t$ – host⊃analyte 1 concentration at time t, $[\mathrm{HA2}]_t$ – host⊃analyte 2 concentration at time t,
$k_{\mathrm{in}}^{\mathrm{HD}}$ – rate constant for the association of the host⊃dye (HD) complex (complexation),
$k_{\mathrm{out}}^{\mathrm{HD}}$ – rate constant for the dissociation of the host⊃dye (HD) complex (decomplexation),
$k_{\mathrm{in}}^{\mathrm{HA1}}$ – rate constant for the association of the host⊃analyte 1 (HA1) complex (complexation),
$k_{\mathrm{out}}^{\mathrm{HA1}}$– rate constant for the dissociation of the host⊃analyte 1 (HA1) complex (decomplexation),
$k_{\mathrm{in}}^{\mathrm{HA2}}$ – rate constant for the association of the host⊃analyte 2 (HA2) complex (complexation),
$k_{\mathrm{out}}^{\mathrm{HA2}}$– rate constant for the dissociation of the host⊃analyte 2 (HA2) complex (decomplexation),

I^0 – background signal, I^{HD} – constant proportional to the fluorescence efficiency of host⊃dye (HD) complex at the monitoring wavelength, I^D – constant proportional to the fluorescence efficiency of free dye (D) at the monitoring wavelength,
I_t – emission signal as a function of time

5.3 Pseudo First Order Kinetic Analyte Displacement Assay (*kin*ADA^{PFO})

$$k_{in}^{HA}\,[A]_0 \gg k_{in}^{HD}\,[D]_0 \qquad \text{Eq. S 21}$$

$$HA + D \xrightarrow{k_{out}^{HA}} H + A + D \xrightarrow{k_{in}^{HD}} HD + A \qquad \text{Eq. S 22}$$

$$k_{out}^{HA} \gg k_{in}^{HD}[D]_0 \qquad \text{Eq. S 23}$$

$$HA + D \xrightarrow{k_{out}^{HA}} HD + A \qquad \text{Eq. S 24}$$

$$\frac{d[HD]_t}{dt} = -\frac{d[HA]_t}{dt} = k_{out}^{HA} \cdot [HA]_t \qquad \text{Eq. S 25}$$

$$\frac{d[HA]_t}{[HA]_t} = -k_{out}^{HA} \cdot dt \qquad \int_{[HA]_0}^{[HA]_t} \frac{d[HA]_t}{[HA]_t} = \int_0^t -k_{out}^{HA} \cdot dt \qquad \text{Eq. S 26}$$

$$\ln[HA]_t = \ln[HA]_0 - k_{out}^{HA} \cdot t \qquad [HA]_t = [HA]_0\, e^{-k_{out}^{HA} \cdot t} \qquad \text{Eq. S 27}$$

$$I_t = I^{eq.} + Ae^{-k_{out}^{HA} \cdot t} \qquad \text{Eq. S 28}$$

$[D]_t$ – dye concentration at time t, $[D]_0$ – dye concentration at time 0 (initial dye concentration),
$[A]_t$ – analyte concentration at time t,
$[A]_0$ – analyte concentration at time 0 (initial analyte concentration),
$[HD]_t$ – host⊃dye concentration at time t,
$[HA]_t$ – host⊃analyte concentration at time t,
$[HA]_0$ – host⊃analyte concentration at time 0 (preequilibrated host⊃analyte complex),
k_{in}^{HD} – rate constant for the association of the host⊃dye (HD) complex (complexation),
k_{out}^{HD} – rate constant for the dissociation of the host⊃dye (HD) complex (decomplexation),
k_{in}^{HA} – rate constant for the association of the host⊃analyte (HA) complex (complexation),
k_{out}^{HA} – rate constant for the dissociation of the host⊃analyte(HA) complex (decomplexation),
$I^{eq.}$ – signal offset (at equilibration of HD), A – amplitude,
I_t – emission signal as a function of time

6 Binding Kinetics Analysis

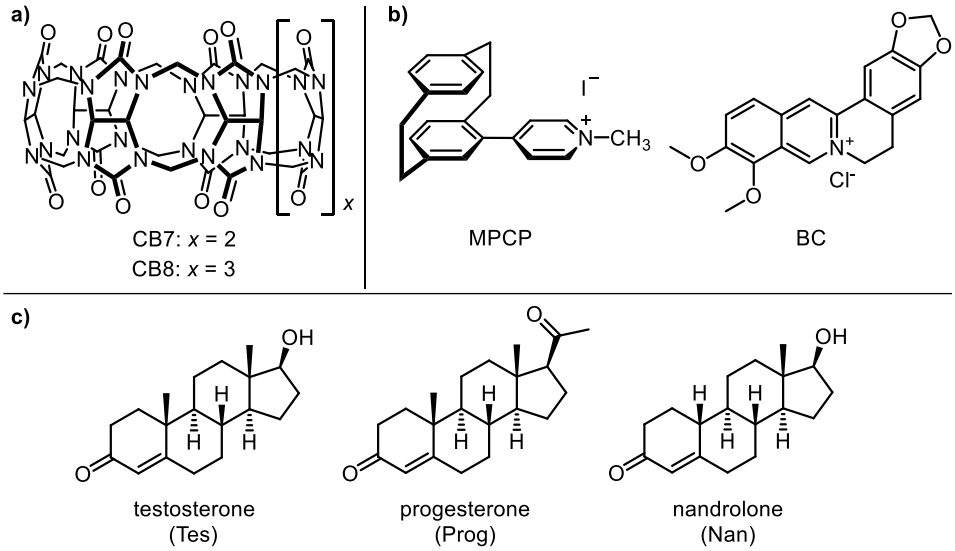

Fig. S 1: Chemical structures of (a) macrocyclic host CB7 and CB8, (b) indicator dye MPCP and BC, and (c) steroids utilized as the analytes in this study.

6.1 Analyte Identification

The applicability of *kin*ADA^{PFO} for achieving analyte identification in a functional assay with an unknown sample composition of unknown concentration was evaluated. In a *kin*ADA^{PFO} approach, the kinetic traces should be independent of the analyte concentration (see manuscript). This is advantageous for achieving analyte identification. Fig. S 2(a) presents the *kin*ADA^{PFO} traces of a spectroscopically silent solution containing CB8 host and Nan analyte upon spiked addition of MPCP. The red line depicts the fitted data following the *kin*ADA^{PFO} model (Eq. S 28). The kinetic host⊃analyte decomplexation ($k_{\mathrm{out}}^{\mathrm{HA}}$) and complexation ($k_{\mathrm{in}}^{\mathrm{HA}} = k_{\mathrm{out}}^{\mathrm{HA}} \cdot K_{\mathrm{a}}^{\mathrm{HA}}$) rate constants for the CB8⊃Nan binding interaction hence obtained is given in Table S 1.

Fig. S 2(b) displays the simulated kinetic traces according to the *kin*ADA model (Eq. S 9) for different concentrations of Nan as analyte in the presence of a substoichiometric amount of CB8 host. The addition of the MPCP dye initiates the assay. The kinetic and thermodynamic parameters listed in Table S 1 were used as input parameters for the simulations. The simulated kinetic traces show the desired analyte-concentration-independence. In addition, Fig. S 2 (c) displays the experimental *kin*ADA^{PFO} traces recorded for the same system that matches the simulations well. The time-resolved competitive assay is also applicable for a mixture of two steroids as analytes (see below). The *kin*ADA^{PFO} kinetic traces should be independent of the steroid concentration in the mixture, and the recorded traces should overlay at a given concentration ratio of one steroid over the other in the mixture (see Fig. S 9(a) below for the simulated results).

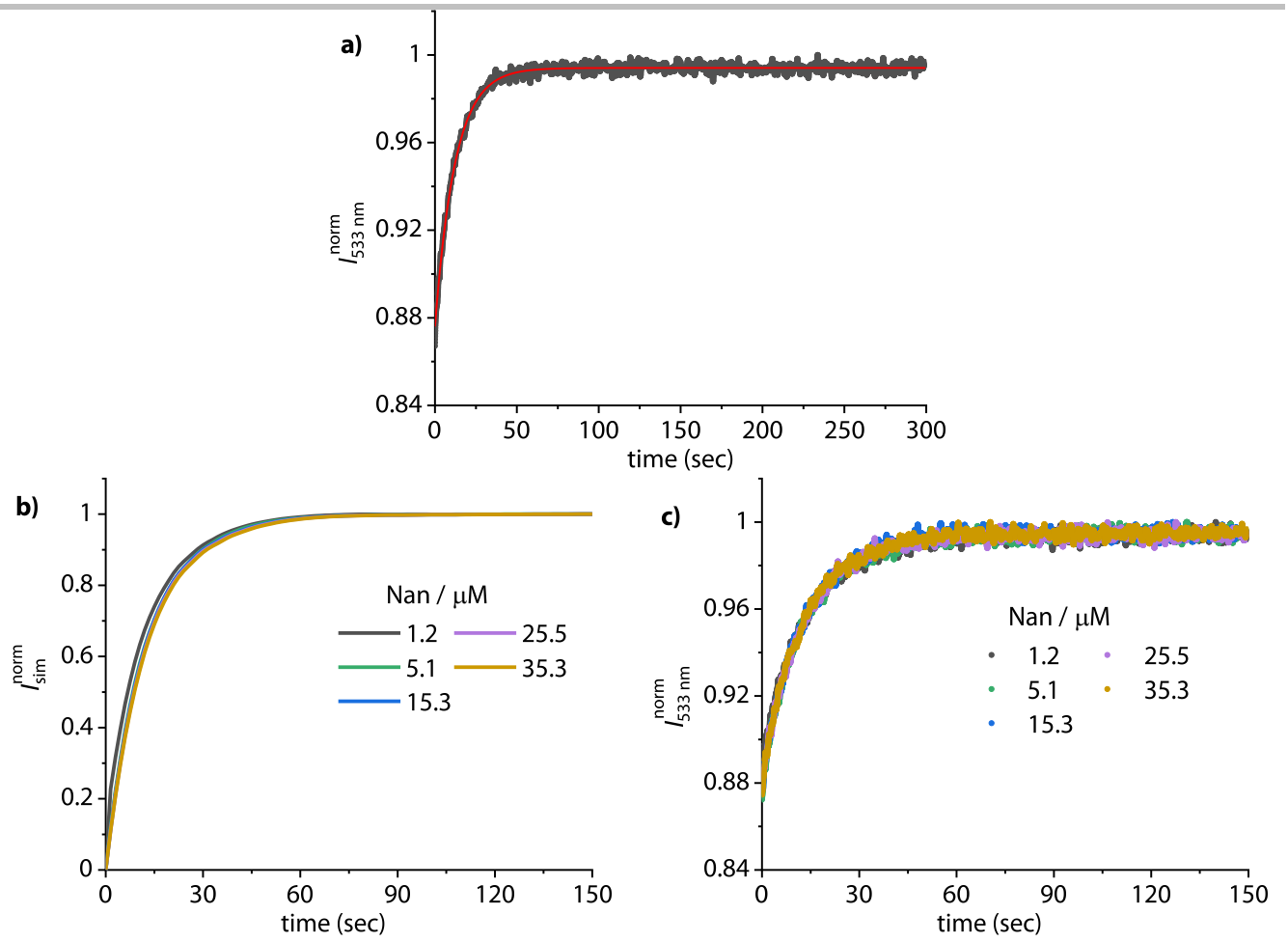

Fig. S 2: (a) Representative *kin*ADA^{PFO} traces (dotted line) determined by fluorescence intensity variations (λ_{exc} = 376 nm, λ_{ems} = 533 nm) of CB8 (1 µM) and Nan (1.2 µM) host⊃analyte complex upon spiked addition of MPCP dye (50 µM) in water at 25°C. The solid red line depicts the fitted data following the *kin*ADA^{PFO} model (Eq. S 28). The kinetic host⊃analyte decomplexation (k_{out}^{HA}) and complexation ($k_{in}^{HA} = k_{out}^{HA} \cdot K_a^{HA}$) rate constants for the CB8⊃Nan binding interaction obtained are given in Table S 1. (b) Representative simulated *kin*ADA traces (solid line) for CB8 (1 µM) with increasing concentrations of Nan (1.2 – 35.3 µM) upon spiked addition of MPCP dye (50 µM) in water. The kinetic and thermodynamic parameters listed in Table S 1 were used as input parameters in the *kin*ADA model (Eq. S 9) of the simulations. (b) Representative experimental *kin*ADA^{PFO} traces (dotted line) determined by fluorescence intensity variations (λ_{exc} = 376 nm, λ_{ems} = 533 nm) of CB8 (1 µM) with increasing concentrations of Nan (1.2 – 35.3 µM) upon spiked addition of MPCP dye (50 µM) in water at 25°C.

Table S 1: Experimental kinetic complexation (k_{in}^{HA}) and decomplexation (k_{out}^{HA}) rate constants for host⊃analyte complexes determined by *kin*ADA^{PFO} in water.

host⊃analyte[a]	K_a^{HA} [b]/ M^{-1}	k_{in}^{HA} [c]/ $s^{-1}\,M^{-1}$	k_{out}^{HA} [c]/ s^{-1}
CB8⊃Nan	2.1 x 10^7	1.71 x 10^6	8.14 x 10^{-2}

If not stated otherwise, all experiments have been conducted in Milli-Q water at 25°C[a]. See Fig. S1 for chemical structures. CB8 and Nan are present at a concentration of 1 µM each. MPCP (50 µM) was used as the indicator dye. For CB8⊃MPCP complex K_a^{HD}, k_{in}^{HD} and k_{out}^{HD} are 3.89 x 10^{12} M^{-1} ,1.2 x 10^7 s^{-1} M^{-1}, and 3 x 10^{-6} s^{-1}, respectively.[10] [b] ref[7] [c] Errors (StDev) from triplicate experiments are ≤20% in k_{in}^{HA} and k_{out}^{HA}.

6.2 Analyte Quantification

Once analyte identification is achieved through *kin*ADA^{PFO} method, the next step is to quantify the amount of analyte in the media. To achieve this, a *kin*IDA approach is utilized herein for different concentrations of Nan as analyte with CB7 as host and BC as the indicator dye. Fig. S 3 displays the *kin*IDA traces recorded under varying Nan concentrations. In addition, the kinetic rate constants obtained by fitting the kinetic traces to a *kin*IDA model (Eq. S 9) are shown in Table S 2.

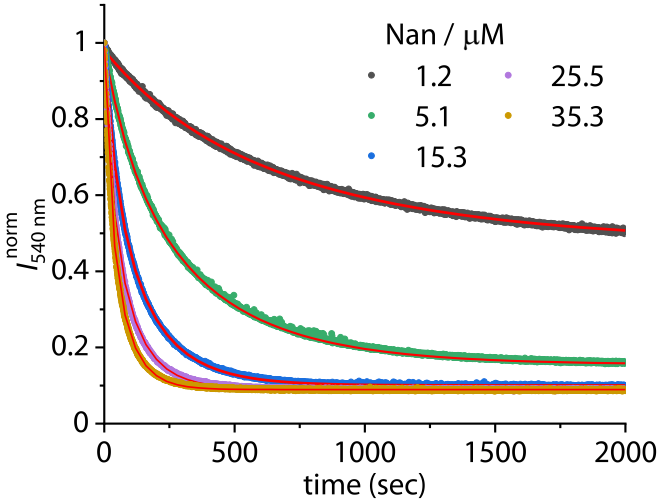

Fig. S 3: Representative *kin*IDA traces (dotted line) determined by fluorescence intensity variations (λ_{exc} = 462 nm, λ_{ems} = 540 nm) upon addition of CB7 (1 µM) and BC (1 µM) host⊃dye complex to solutions with increasing concentrations of Nan (1.2 – 35.3 µM) in water at 25°C. The solid red line depicts the fitted data following the *kin*IDA model (Eq. S 9). The kinetic host⊃analyte complexation (k_{in}^{HA}) and decomplexation (k_{out}^{HA}) rate constants for CB7⊃Nan binding interaction at the different Nan concentrations studied is given in Table S 2.

Table S 2: Experimental kinetic complexation (k_{in}^{HA}) and decomplexation (k_{out}^{HA}) rate constants for CB7⊃Nan complex at varying Nan concentrations determined by *kin*IDA in water.

host⊃dye [a]	**[Nan]$^{[b]}$ / µM**	k_{in}^{HG} [c] / $s^{-1}\,M^{-1}$	k_{out}^{HG} [c] / s^{-1}
CB7⊃BC	1.2	4.28 x 10^3	3.82 x 10^{-4}
(1 µM)	5.1	5.47 x 10^3	4.88 x 10^{-4}
	15.3	6.00 x 10^3	5.36 x 10^{-4}
	25.5	6.50 x 10^3	5.80 x 10^{-4}
	35.3	6.82 x 10^3	6.09 x 10^{-4}

If not stated otherwise, all experiments have been conducted in Milli-Q water at 25°C
[a] See Fig. S1 for chemical structures.
For CB7⊃ BC complex K_a^{HD}, k_{in}^{HD} and k_{out}^{HD} are 1.7 x 10^7 M^{-1} ,6 x 10^6 s^{-1} M^{-1} and 0.4 s^{-1} respectively.[10]
[b] See Fig. S1 for chemical structures. CB7⊃Nan K_a^{HA}= 1.12 x 10^7 M^{-1}
[c] Errors (StDev) from triplicate experiments are ≤20% in k_{in}^{HA} and k_{out}^{HA}.

The kinetic and thermodynamic parameters, as listed in Table S 2, were used to obtain the simulated kinetic traces upon the addition of CB7⊃BC to Nan according to the *kin*IDA model (Eq. S 9). The simulated and experminetally obtained kinetic traces are plotted in Fig. S 4.

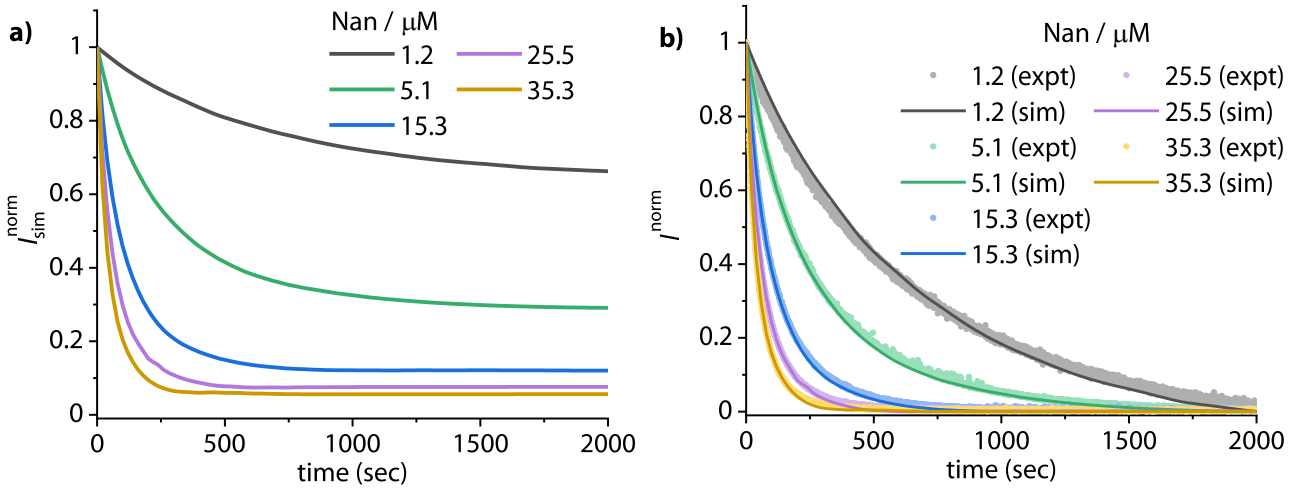

Fig. S 4: (a) Representative simulated *kin*IDA traces upon addition of CB7 (1 µM) and BC (1 µM) host⊃dye complex to solutions with increasing concentrations of Nan (1.2 – 35.3 µM) in water. The kinetic and thermodynamic parameters listed in Table S 2 were used as input parameters in the *kin*IDA model (Eq. S 9) to obtain the simulations. (b) A comparison of the experimental *kin*IDA traces as obtained in Fig. S 3 (dotted line) to the simulated data (solid line) shows a good agreement between both results. The data were normalized to [0, 1] for the comparisons and to obtain the overlaid simulated and experimental spectra.

6.3 Analyte Differentiation through Kinetic Selectivity

6.3.1 *kin*ADA^{PFO} for CB8⊃steroid using MPCP as an indicator dye

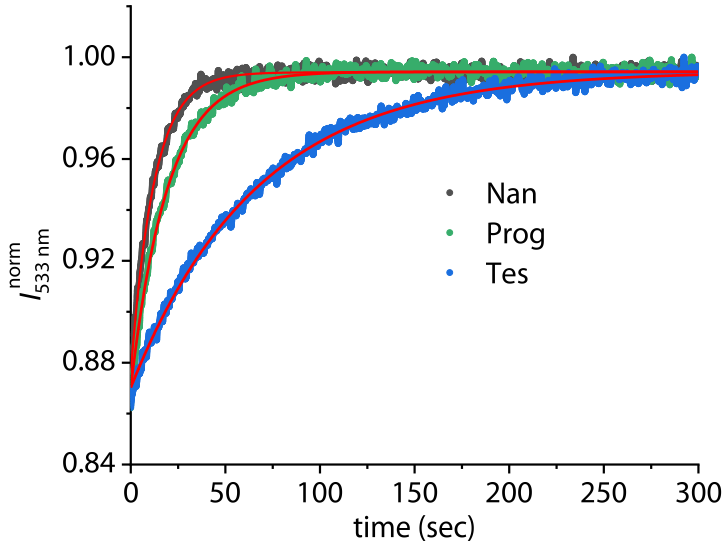

Fig. S 5: Representative *kin*ADA^{PFO} traces determined by fluorescence intensity variations (λ_{exc} = 376 nm, λ_{ems} = 533 nm) of CB8⊃Tes (1 µM, blue dotted line), CB8⊃Prog (1 µM, green dotted line) and CB8⊃Nan (1 µM, black dotted line) host⊃analyte complex upon spiked addition of MPCP dye (50 µM) in water at 25°C. The solid red line depicts the fitted data following the *kin*ADA^{PFO} model (Eq. S 28). The kinetic host⊃analyte decomplexation (k_{out}^{HA}) and complexation ($k_{in}^{HA} = k_{out}^{HA} \cdot K_a^{HA}$) rate constants for the CB8⊃steroid binding interaction obtained are given in Table S 3.

Table S 3: Experimental kinetic complexation (k_{in}^{HA}) and decomplexation (k_{out}^{HA}) rate constants for host⊃analyte complexes determined by *kin*ADA^{PFO} in water.

host⊃analyte[a]	K_a^{HG} [b]/ M^{-1}	k_{in}^{HG} [c]/ s^{-1} M^{-1}	k_{out}^{HG} [c]/ s^{-1}
CB8⊃Tes	1.1 x 10^{8}	1.62 x 10^{6}	1.47 x 10^{-2}
CB8⊃Prog	9.3 x 10^{7}	4.75 x 10^{6}	5.11 x 10^{-2}
CB8⊃Nan	2.1 x 10^{7}	1.71 x 10^{6}	8.14 x 10^{-2}

If not stated otherwise, all experiments have been conducted in Milli-Q water at 25°C

[a] See Fig. S1 for chemical structures. CB8 and the steroid are present at a concentration of 1 µM each. MPCP (50 µM) was used as the indicator dye. For CB8⊃MPCP complex K_a^{HD}, k_{in}^{HD} and k_{out}^{HD} are 3.89 x 10^{12} M^{-1} ,1.2 x 10^{7} s^{-1} M^{-1}, and 3 x 10^{-6} s^{-1}, respectively.[10]

[b] ref[7]

[c] Errors (StDev) from triplicate experiments are ≤20% in k_{in}^{HA} and k_{out}^{HA}.

The CB8 host exhibits kinetic selectivity for all three steroids studied.

6.3.2 Simulated and experimental data comparisons

The kinetic and thermodynamic parameters, as shown in Table S 3, were used to obtain the simulated kinetic traces for CB8⊃steroid upon addition of MPCP according to the *kin*ADA model (Eq. S 9) and is shown in Fig. S 6(a). The simulated results agree with the experimental *kin*ADA^{PFO} traces obtained (see Fig. S 6(b)). Hence several analytes in unknown samples can be differentiated by comparison of experimentally recorded data to simulations or to pre-recorded kinetic data.

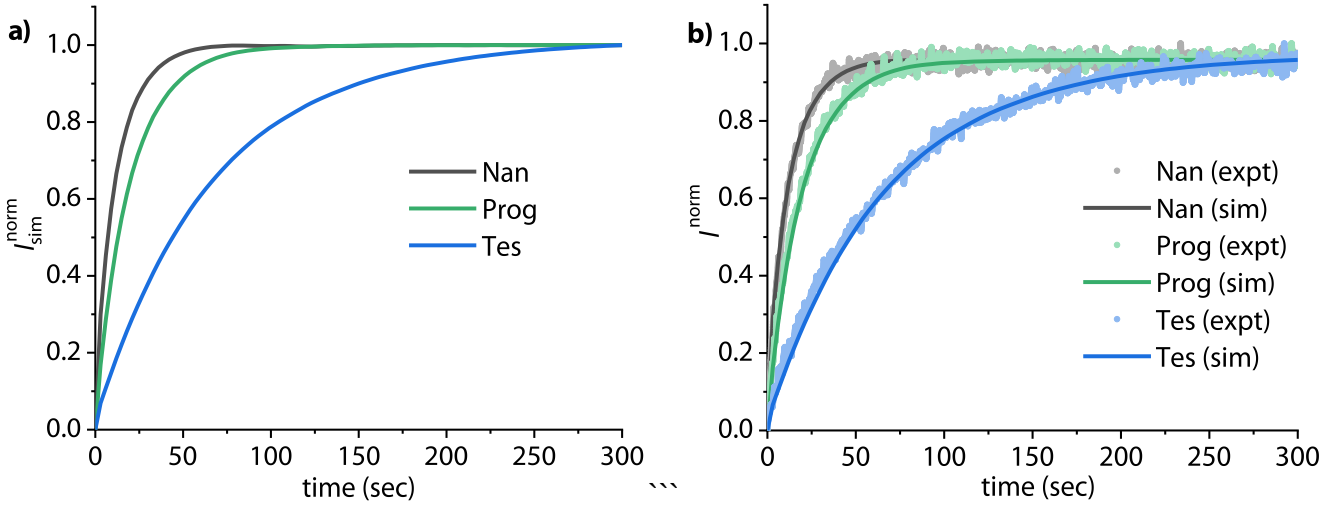

Fig. S 6: (a) Representative simulated *kin*ADA traces for CB8⊃Tes (1 µM, solid blue line), CB8⊃Prog (1 µM, solid green line), and CB8⊃Nan (1 µM, solid black line) host⊃analyte complex upon spiked addition of MPCP dye (50 µM) in water. The kinetic and thermodynamic parameters listed in Table S 3 were used as input parameters in the *kin*ADA model (Eq. S 9) to obtain the simulations. (b) Comparing the experimental *kin*ADA^{PFO} traces as plotted in Fig. S 5 (dotted line) to the simulated data (solid line) shows a good agreement between both results. The data were normalized to [0, 1] for the comparisons.

6.3.3 *kin*ADA^{PFO} for CB8 and a mixture of two steroids as analytes using MPCP as indicator dye

Fig. S 7 displays the *kin*ADA^{PFO} traces for a solution containing CB8 and an equimolar mixture of two steroids as analytes, followed by a spiked addition of MPCP. The kinetic traces obtained were compared to the *kin*ADA^{PFO} traces recorded for the individual steroids as analytes, which gave us an initial indication of the mixture's components.

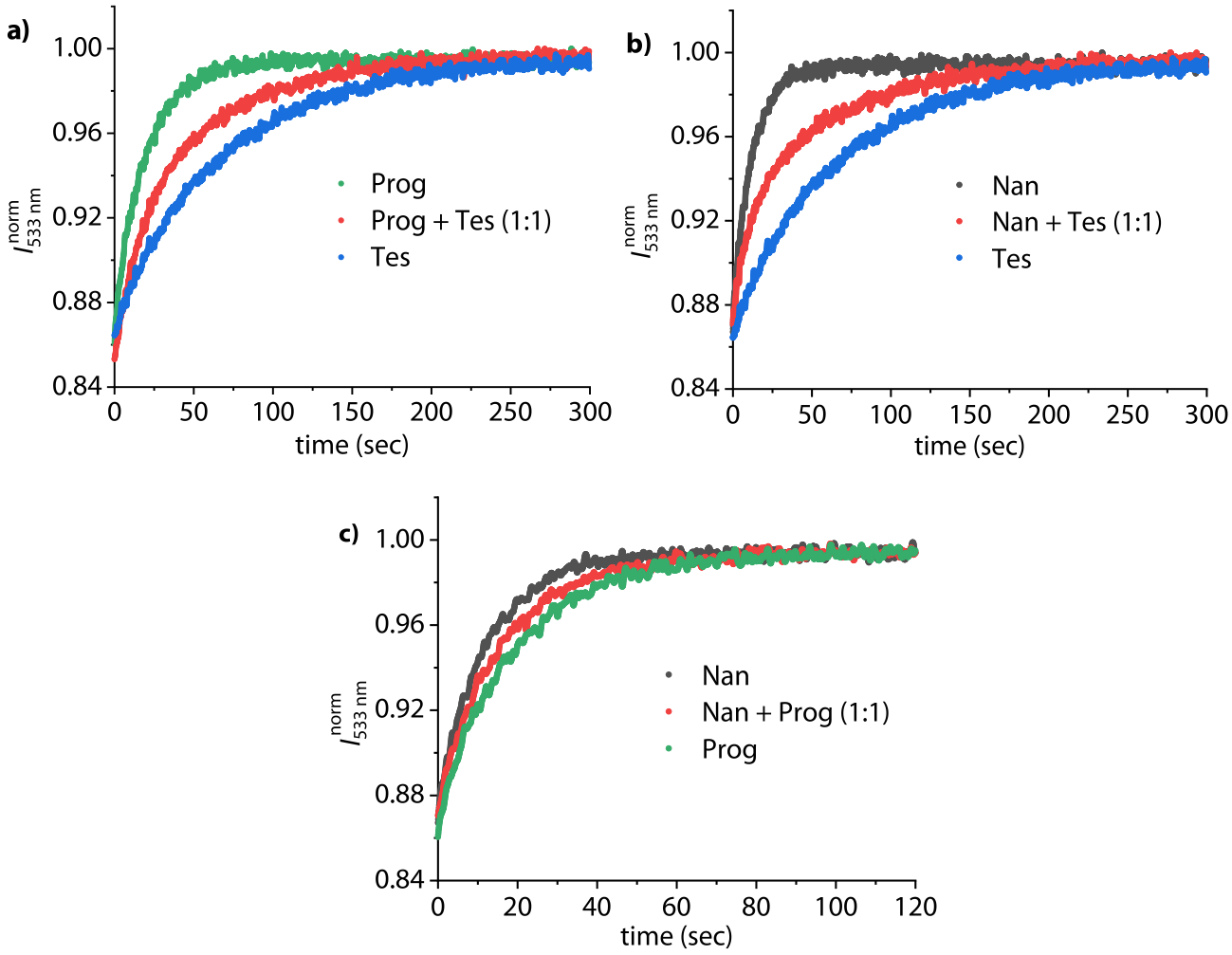

Fig. S 7: Representative *kin*ADA^{PFO} traces determined by fluorescence intensity variations (λ_{exc} = 376 nm, λ_{ems} = 533 nm) for a mixture of two steroids as analytes (red dotted line) in case of (a) CB8 (1 µM), Tes (0.5 µM) and Prog (0.5 µM), (b) CB8 (1 µM), Tes (0.5 µM) and Nan (0.5 µM) and (c) CB8 (1 µM), Prog (0.5 µM) and Nan (0.5 µM) upon spiked addition of MPCP (50 µM) in water at 25°C. The *kin*ADA^{PFO} traces were recorded for the individual steroids as analytes, CB8⊃Tes (1 µM, blue dotted line), CB8⊃Prog (1 µM, green dotted line), and CB8⊃Nan (1 µM, black dotted line). The assay was then started by the spiked addition of MPCP dye (50 µM), as shown in Fig. S 5 is given for a visual comparison.

Additional information on the components of the mixture was obtained by fitting the kinetic traces to a bi-exponential decay function (Eq. S 29) to yield the decomplexation rate constants k_{out}^{HA1} and k_{out}^{HA2} for the two analytes (analyte 1, A1 and analyte 2, A2) with the host (Fig. S 8). These parameters were then compared to the previously obtained kinetic parameters in Table S 3.

$$I_t = I^{eq.} + A_1 e^{-k_{out}^{HA1} \cdot t} + + A_2 e^{-k_{out}^{HA2} \cdot t} \quad \text{Eq. S 29}$$

I_t – emission signal as a function of time, $I^{eq.}$ – signal offset (at equilibration of HD), A – amplitude, k_{out}^{HA1} – rate constant for the dissociation of the host⊃analyte 1 (HA1) complex (decomplexation), k_{out}^{HA2} – rate constant for the dissociation of the host⊃analyte 2 (HA2) complex (decomplexation)

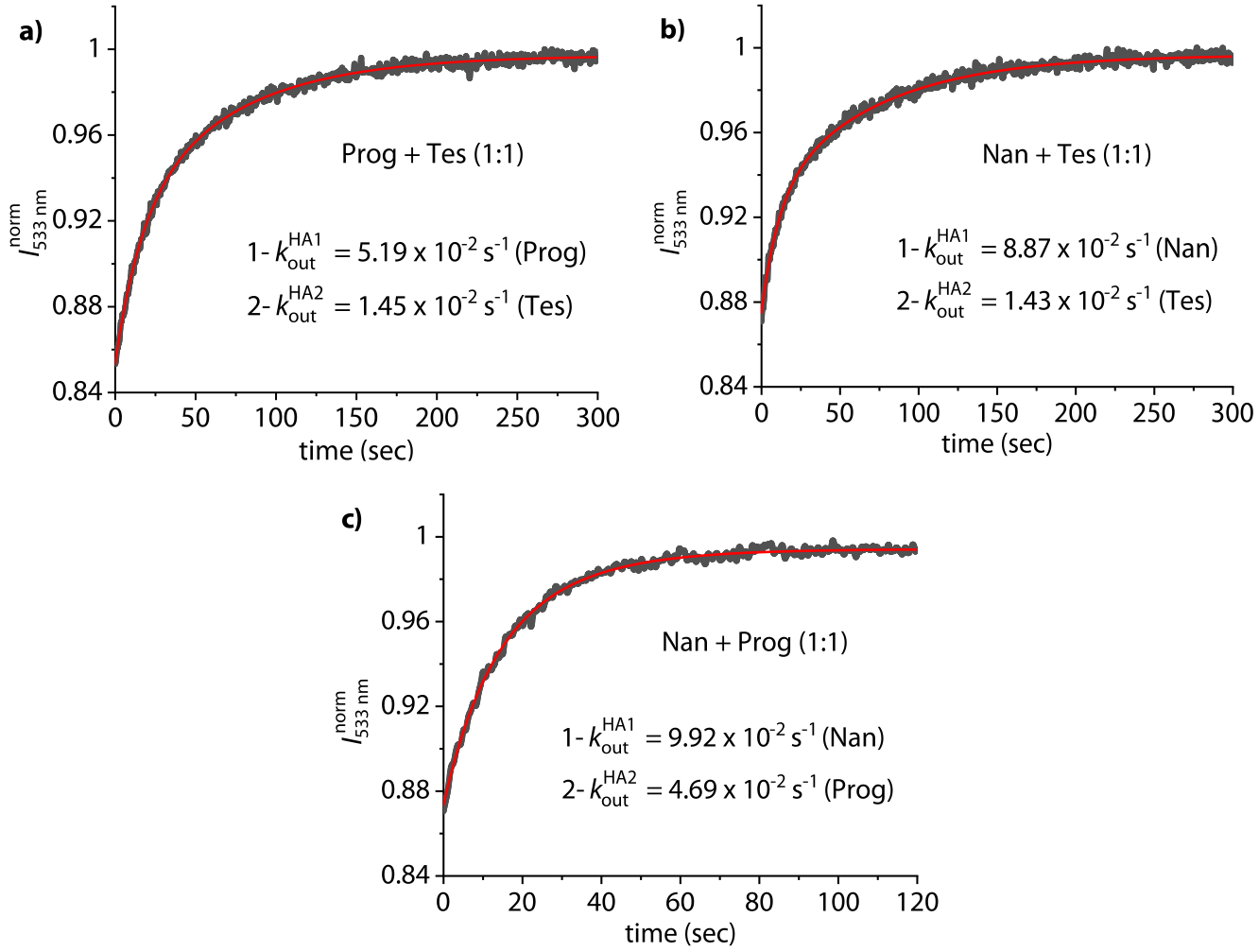

Fig. S 8: *kin*ADA^{PFO} kinetic traces (black dotted line) and the fitted data (red solid line) following a bi-exponential decay function (Eq. S 29) for a mixture of two steroids as analytes in case of (a) CB8 (1 µM), Tes (0.5 µM) and Prog (0.5 µM), (b) CB8 (1 µM), Tes (0.5 µM) and Nan (0.5 µM) and (c) CB8 (1 µM), Prog (0.5 µM) and Nan (0.5 µM) upon spiked addition of MPCP (50 µM) in water at 25°C (λ_{exc} = 376 nm, λ_{ems} = 533 nm). The fitted k_{out}^{HA1} and k_{out}^{HA2} kinetic parameters for the two analytes with CB8 are depicted in the respective graphs.

6.3.4 Simulated and experimental data comparisons for a mixture of two steroids as analytes

Fig. S 9(a) displays the simulated kinetic traces according to the *kin*ADA model with two analytes (Eq. S 20). The analytes Tes and Prog were present at varying concentrations while the concentration of the host CB8 was fixed. The addition of the MPCP dye initiated the assay. The kinetic and thermodynamic parameters listed in Table S 3 were used as input parameters for the simulations. Comparing the experimental *kin*ADA^{PFO} traces obtained in Fig. S 7 (a) to the simulated results enabled the differentiation of the components of the mixture along with calculating the concentration ratio of steroids in the mixture (Fig. S 9(b)).

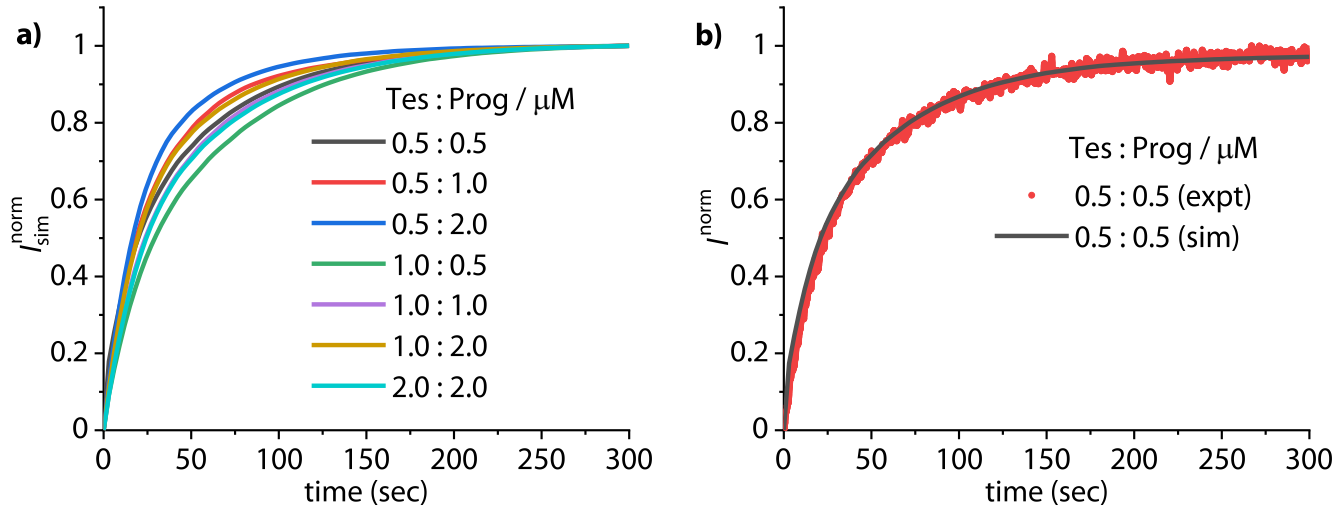

Fig. S 9: (a) Representative simulated *kin*ADA traces (solid line) for a mixture of two steroids as analytes in case of CB8 (1 µM) with varying concentrations of Tes and Prog upon spiked addition of MPCP dye (50 µM) in water. The kinetic and thermodynamic parameters listed in Table S 3 were used as input parameters in the *kin*ADA model with two analytes (Eq. S 20) to obtain the simulations. (b) Comparison of the experimental *kin*ADA^{PFO} traces as obtained in Fig. S 7 (a) for CB8 (1 µM), Tes (0.5 µM), and Prog (0.5 µM) upon spiked addition of MPCP (50 µM) (red dotted line) to the simulated data (solid black line) at the respective concentrations show a good agreement between both results. The data were normalized to [0, 1] for the comparisons and to obtain the overlaid simulated and experimental spectra.

6.3.5 *kin*ADA^{PFO} for CB8⊃steroid using MPCP as indicator dye (microplate reader assay)

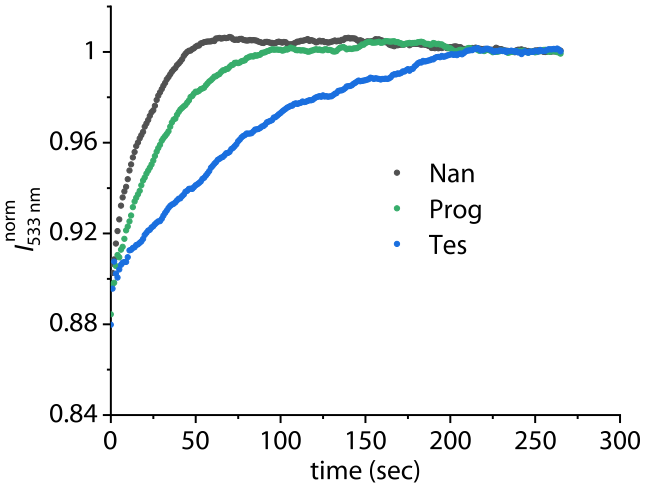

Fig. S 10: Representative *kin*ADA^{PFO} traces measured in a microplate reader (λ_{exc} = 376 nm, λ_{ems} = 533 nm) of CB8⊃Tes (1 µM, blue dotted line), CB8⊃Prog (1 µM, green dotted line) and CB8⊃Nan (1 µM, black dotted line) host⊃analyte complex in water at 25°C. The assay was initiated upon spiked addition of MPCP dye (50 µM).

7 References

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