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Article

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Concentration Related Response Potentiometric Titrations to Study the Interaction of Small Molecules with Large Biomolecules

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Abstract

In the present article, the utility of a special potentiometric titration approach for recognition and calculation of biomolecule/small molecule interactions is reported. This approach is fast, sensitive, reproducible and inexpensive in comparison to the other methods for the determination of the association constant values (K_a) and the interaction energies (ΔG). The potentiometric titration measurement is based on the use of a classical polymeric membrane indicator electrode in a solution of the small molecule ligand. The biomolecule is used as a titrant. The potential is measured versus a reference electrode and transformed to a concentration related signal over the entire concentration interval, also at low concentrations, where the mV (y-axis) versus $\log c_{analyte}$ (x-axis) potentiometric calibration curve is not linear. In the procedure, the K_a is calculated for the interaction of cocaine with a cocaine binding aptamer and with an anti-cocaine antibody. To study the selectivity and cross-reactivity, other oligonucleotides and aptamers are tested, as well as other small ligand molecules such as tetrakis (4-chlorophenyl)borate, metergoline, lidocaine, and bromhexine. The calculated K_a compared favorably to the value reported in the literature using SPR. The potentiometric titration approach called "Concentration related Response Potentiometry", is used to study molecular interaction for 7 macromolecular target molecules and 4 small molecule ligands.

Keywords: Potentiometry; titration; aptamer; interaction analysis; cross-reactivity.

1. Introduction

A myriad of label free methods exist to obtain quantitative information on the affinity between two different biomolecules or between biomolecules and small molecules. These include (but are not restricted to) surface plasmon resonance (SPR),[1] isothermal- and differential scanning calorimetry (ISC, DSC),[2] quartz microbalance (QMB),[3] evanescent wave sensors,[4] affinity chromatography,[5] and circular dichroism (CD)[6] Studying molecular interactions is a vast area of research in many biological, biomedical and analytical processes such as cell signaling pathways, proteomic analysis, antibody development, drug discovery, biosensors etc.[7] The market for biosensors is expected to grow rapidly, especially in point of care diagnostics, home diagnostics, and environmental areas. Measuring the interaction characteristics of a (bio)molecule, used as a receptor in a (bio)sensor, is therefore an important area of application. Biomolecules such as aptamers and antibodies are very promising elements in such biosensor constructions,[8,9] as they selectively interact with the targeted analyte. Yet, still very little is known on the selectivity of aptamers and antibodies, especially if they were selected to recognize small (organic) molecules.[10] Also quite unknown is the comparison of association constants K_a (or its inverse, K_d) of e.g. aptamer/analyte interactions to K_a values of receptor molecules/analyte combinations which are also used abundantly. This is especially true for potentiometric sensors. One the important successes of potentiometric techniques was achieved with a biomolecule (valinomycin) as a recognition element, around 1970.[11,12] As already shown as early as 1974 [13,14] for this valinomycin receptor molecule, the selectivity and sensitivity depend strongly on K_a values, which were determined with circular dichroism titrations. The performance of this field of electrochemistry has dramatically improved.[15] Recently, many attempts were noted for the renewed use of biomolecular recognition elements, and for the determination of biomolecular analyte targets.[16-19] It is to be expected that the use of biomolecular recognition elements can strongly improve potentiometric sensing devices. Therefore, also this area of research has a need for practical methods to estimate bioreceptor/analyte interaction to select the best candidates rapidly.

Most of the techniques mentioned above for studying biomolecular interactions are expensive, time consuming and complicated. They can quantify the affinity from concentration based assays, or by measuring the kinetics of association and dissociation. In the concentration based assays, the formation of a biomolecule/small molecule complex is studied with binding curves. These are titration curves where the ligand is added as a titrant to a solution of the biomolecule, and the extent of formation of the complex is plot on the y-axis. A

potentiometric approach to calculate not only equivalence points but also equilibrium constants (K_a values of weak acids, solubility products, complex formation constants) was introduced already as early as 1952 by Gran.[20] In the Gran approach, equilibrium constants are derived from data obtained before the equivalence point. However, molecular interactions in the biomolecular world cover a large range of K_a values, often in regions where equivalence points are difficult to detect in classical potentiometric titrations. Around the same period, Scatchard published his famous approach[21] for the study of the interaction of biomolecules with small molecules. The method was also applied with potentiometric titration in several publications, e.g., to determine association constants between small molecules and macrocyclic receptors.[22] Dürüst and Meyerhoff[23-25] successfully used potentiometric titrations and Scatchard plots to study the interaction between large biomolecules. When working in Molecular Biology conditions however, the low amounts of bio-molecules not only reduce sample size, but also require to work in the low analyte concentrations region where the potentiometric signals are not linearly related to the logarithm of the analyte concentration/activity. Using the complete (non-linear) potentiometric calibration curve, and Hill analysis, Meyerhoff and Rechnitz[26] were the first authors to use potentiometric titrations for the study of biomolecule/small molecule binding events.

In the present work, analytes such as cocaine, lidocaine, metergoline and bromhexine were titrated by different oligonucleotides and aptamers, and by an antibody as a titrant. The mV y-axis of the potentiometric titration curve was transformed to a concentration related signal, also covering the lower concentration range. This transformation of the y-axis takes into account that potentiometric calibration curves (mV versus $\log c_{analyte}$ plots) are not linear in that low concentration range. Limits of detection are defined totally differently in potentiometry as compared to other analytical techniques. These LOD's are often mentioned in the literature as being by definition much too high in comparison with other techniques which use a multiple of the Gaussian background noise as the detection limit. There is actually no objection to work accurately beyond the artificially defined potentiometric limit of detection (LOD) values.[14,26] This creates perspectives for fast screening of molecular interaction not only for biosensors use, but also for use in Molecular Biology and Biomedicine.

2. Experimental Section

2.1. Chemicals

All the chemicals were of analytical reagent grade. 2-(N-morpholino)ethanesulfonic acid (MES) hydrate and high-molecular mass polyvinylchloride (PVC) was purchased from Acros Organics (Geel, Belgium). The other membrane components such as tetrakis chlorophenyl borate (TCPB), 2-nitrophenyloctylether (NPOE) and tetrahydrofuran (THF) as solvent for the membrane's components were of the highest quality and from Fluka (Bornem, Belgium). Metergoline, lidocaine, cocaine and bromhexine were obtained from Sigma-Aldrich (Bornem, Belgium). Anti-cocaine antibody was purchased from Abcam (Cambridge, England).

Because TCPB is not soluble in water media, a stock solution of 10^{-3} M TCPB was prepared in MES buffer/Ethanol 9:1 (v:v). This solution was diluted 10x with MES buffer (1 mM, pH 7.0) to use TCPB as a titrant in titration experiments.

2.2. Oligonucleotides

The sequence of different oligonucleotides that were used in this research are as shown below:

Cocaine Binding Aptamer (CBA, 43-mer)[28]

5'-TCTCGGGACGACAGGATTTTCCTCAATGAAGTGGGTCGTCCC-3'

Non-specific oligonucleotide (25-mer-DNA)

5'-AAAATATCATCTTTGGTGTTTCCTA-3'

43-mer and 80 mer Random Primer (43-mer-RP, 80-mer-RP)

5'-NNNNNNNNNNNNNNNNNNNNNNNNNNNNNN……N-3'

Metergoline Binding Aptamer (MBA, 80-mer)[28]

5'-
AGCAGCACAGAGGTCAGATGCCGTCAGCCCCGATCGCCATCCAGGGACTCCCCCC
TACTGCCTATGCGTGCTACCGTGAA-3'

Chloramphenicol Binding Aptamer (CAPBA, 80-mer)[29]

5'-
AGCAGCACAGAGGTCAGATGACTTCAGTGAGTTGTCCCACGGTCGGCGAGTCGGT
GGTAGCCTATGCGTGCTACCGTGAA-3'

The 25-mer-DNA and random primers were obtained from Integrated DNA Technologies (IDT, Gent, Belgium) and the others from Eurogentec (Liège, Belgium).

2.3. Instrumentation

Potentiometric working electrodes. The indicator electrode was made of a PVC cylinder (5 mm OD × 40 mm length). It contained a cylindrical substrate electrode (3 mm diameter × 1 mm length), which is an electronically conducting graphite/PVC composite material.[30] The composite substrate electrode was polished with Carbimet grit 600 (Buehler Ltd, USA). The rubber phase sensing membrane was coated on the composite substrate electrode. The electrodes with a composite substrate had the important property that the coated membrane and the composite were tightly mixed at the interface.[31]

The membrane coating used for the detection of positively charged analytes contained 2% (w/w) TCPB, 33% (w/w) PVC and 65% (w/w) NPOE. 100 mg of this mixture was dissolved in 1mL THF and 2 × 40 μL of this solution were applied on the electrode surface (dip-coating) by 2 sequential steps. After evaporation at room temperature (1 h), the resulting ionically conductive rubber polymeric phase coated membrane had a thickness of 100 μm. The electrodes were kept in buffer solution (1mM MES pH 7.0) for at least 3 h to obtain a stable baseline for the potentiometric titration.

Potentiometric setup. The potentiometric titrations were performed using an indicator electrode and a reference electrode, in a 10 mm diameter beaker. The buffer solution (1 mM MES pH 7.0) was stirred during the entire experiment with a magnetic stirrer (5 × 2 mm). After obtaining a stable baseline, the first injection was done by addition of the analyte (5 μL) to 500 μL of the buffer solution. Always, an exponential increase in potential could be seen in this step, reaching a plateau value. After obtaining a stable signal, the titrant was added using a micropipette at constant time intervals. The usual volume of the titrant was 8 μL and pipetting was done every 30 s, unless otherwise stated. The membrane potential was measured against an homemade miniaturized (1.4 mm OD × 40 mm length) reference electrode (Ag/AgCl) using a high impedance (10^{13} Ω) homemade amplifier. The detection signals were recorded on a data station composed of a PC equipped with a 6013 NI DA converter and LabVIEW 7 (National Instruments, US) based software. The overall time constant of the high impedance amplifier plus data station was set to 200 ms. All the measurements were done at least three times on three different working electrodes after conditioning and stabilization in the MES buffer solution.

3. Results and Discussion

3.1. Potentiometric detection of cocaine in a batch system

Figure 1A shows the potentiometric signal obtained after injecting different concentrations of cocaine in the batch system.

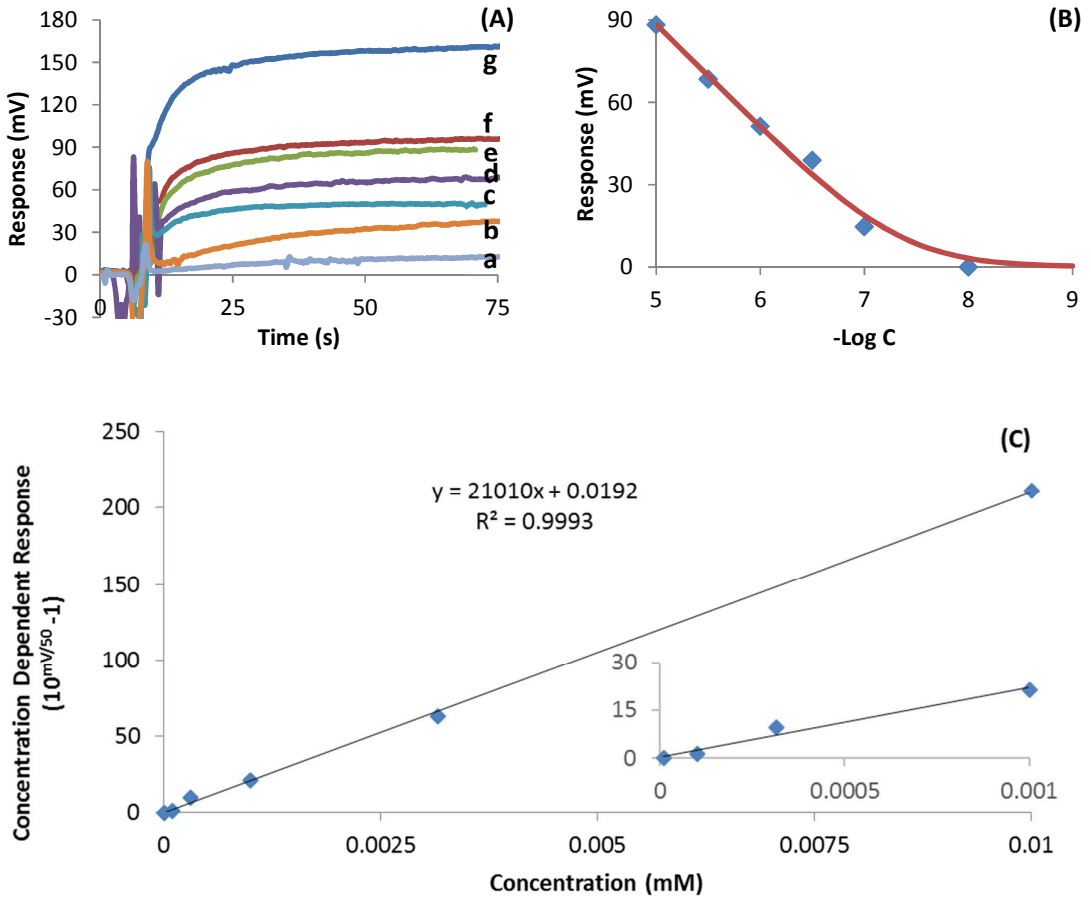

Figure 1: Potentiometric signals in batch for cocaine. (A) Typical potentiometric recordings in batch after cocaine injection. The concentration of cocaine varied from 10^{-7} M (curve a) to $10^{-6.5}$ M (b), 10^{-6} M (c), $10^{-5.5}$ M (d), 10^{-5} M (e), $10^{-4.5}$ M (f) and 10^{-4} M (g) in 1 mM MES buffer (pH: 7.0). (B) Potentiometric responses of 10^{-5} to 10^{-8}M cocaine injections in relation to log concentration. The measured potentiometric responses in figure 1A (◊) and equation 1 fitted by "Solver" (red curve) are shown. (C) Potentiometric responses of 10^{-7} to 10^{-5}M cocaine injections after transformation to a concentration related signal (see also Eqn. 3). *Insert:* Cocaine concentrations from 10^{-8} to 10^{-6}M.

The potentiometric indicator electrode used was of the coated wire type, containing TCPB as an ionophore. Some initial spiking phenomena are observed (figure 1A) as we inject in a miniaturized (500 µL) system equipped with a large indicator electrode. The signal heights at the plateau values plotted versus the logarithm of the analyte concentrations follow calibration curves of the type shown in figure 1B and expressed by Eqn. 1:

$E = E^0 + S \text{ Log } (c_{analyte} + Cst)$ Eqn. 1

E values (mV) are the responses at the plateau values in the potentiograms for each analyte concentration (figure 1A). E^0 is the value of the potential, obtained by extrapolating the analyte concentration, $c_{analyte}$, to a concentration of 1 M. S is the slope (RT/zF) of the calibration curve (figure 1B), in the linear part. zF is the charge per mole of analyte present in the potential-forming process, here 1 faraday. Cst is a constant, which depends on the degree of interference which is provoked by the buffer solution. It is equal to the LOD as it is usually (and typically) defined in potentiometry.[27] The 3 variables E^0, S and LOD are related: $LOD = 10^{-\frac{E^0}{S}}$. Analogous calibration curve equations were used and discussed by other authors, e.g.[27] This function has also been extensively used by our group in analytical applications of these potentiometric sensors.[33, 34]

The parameters for the calibration function as exemplified by figure 1B and Eqn. 1 were obtained for cocaine in the conditions of figure 1B, by curve fitting via "Solver" (Microsoft Excel 2007) as E (mV) = 353 + 50.4 [Log ($c_{analyte}$) + 98.3 × 10^{-9}]. The obtained slope (S = 50.4 mV) is necessary to convert the potentiometric response (E, in mV) to a concentration dependent response by equation 2, as derived in an earlier publication[35]:

$c_{analyte} = (10^{E/S} - 1) \times LOD$ Eqn. 2

The mV signal of the potentiometric sensor was transformed to $c_{analyte}$. Equation 2 is the transpose of equation 1. It is valid when E (eqn. 1) is set to zero for $c_{analyte} = 0$. In practice, this is simply done by offsetting the output voltage (the baseline) of the high impedance voltmeter to zero for $c_{analyte} = 0$ (i.e. when measuring or recording in pure buffer solution). Or by offsetting the recorded data to a zero baseline in e.g. Excel. The expression $(10^{E/S} - 1) \times LOD$, or any expression linearly related to it will be called a transformed response, tR, in what follows:

$tR \approx 10^{E/S}-1$ Eqn. 3

In figure 1C, tR is plot against the analyte (cocaine) concentration. This yields a linear calibration curve, also in the lower concentrations range (see insert of figure 1C). In this case, S was equal to 50 mV.

3.2. Interaction analysis with transformed response potentiometric titration.

In the potentiometric titrations, we measured the potential of a working electrode in a beaker containing the small molecule, in relation to the added titrant (biomolecule) volume. Injections were done at constant time intervals (see experimental), and it was more practical to plot the titration curves with a time related x-axis. Two different methods were explored: one method measures the concentrations of the complexing species and the complex at the equivalence point (EP). Another method measures these concentrations at an arbitrarily chosen point largely surpassing the equivalence point (post-EP: PEP). The reason to do this, is that equivalence points are easy to localize when K_a values are e.g. in the 10^{10} M^{-1} region, but can be difficult to localize when they are around 10^6 M^{-1}. A "classical" titration curve (mV y-axis) is shown in figure 2 for the titration of cocaine with a strongly binding molecule, i.e. TCPB. As the amounts of biomolecule material available are mostly very small, the analyte volume is kept small (0.5 mL in our case) and the number and quantity of titrant additions has to be kept to a minimum. This is another restriction for locating equivalence points easily. Therefore, equivalence point determinations were done in this study with the Gran method. Exact location of equivalence points is not the aim of these titrations. The concentrations of analyte and titrant are mostly known, so we can calculate the position of the equivalence point. To calculate K_a, we can work at an arbitrarily chosen point after the equivalence point (post-ep: PEP). The titration curves then will not have the classical mV ("E": see eqn. 1) y-axis but a concentration related tR (see eqn. 3) y-axis.

The study of the interaction starts with the definition of the equilibrium, and its equilibrium constant, K_a, via Eqn. 4. We assume 1:1 complex formation between analyte (A) and a titrant (bio)molecule (M):

$$A + M \rightleftharpoons A.M$$

$$K_a = \frac{[A.M]}{[A][M]} \qquad \text{Eqn. 4}$$

The dissociation constant ($K_d = \frac{1}{K_a}$) and the Gibbs free energy of interaction ($\Delta G^0 = -RT\ln K_a$) can be calculated from K_a.

The first approach, which will be called in the text "Equivalence Point Method", uses the equivalence point of the potentiometric titration curve to determine K_a. In the case of the titration of cocaine with CBA or TCPB (figure 2), the equivalence points were clearly visible and could be determined by the Gran method.[20, 36, 37] Eqn. 5 is used to calculate the association constant. $[A]_0$ is the concentration of the analyte (cocaine in this case) in the titration beaker at the start of the titration. $[A]_{EP}$ is the analyte concentration at the

equivalence point. $[M]_{EP}$ is the concentration of the titrant molecule (TCPB in this case) at the equivalence point. For a 1:1 complex, $[A]_{EP} = [M]_{EP}$. V_0 and V_{EP} are respectively the volumes in the titration beaker at the start of the titration, and at the equivalence point.

$$K_a = \frac{\frac{([A]_0 V_0 - [A]_{EP} V_{EP})}{V_{EP}}}{[A]_{EP}.[M]_{EP}} = \frac{\frac{([A]_0 V_0 - [A]_{EP} V_{EP})}{V_{EP}}}{[A]_{EP}^2} \qquad \text{Eqn. 5}$$

$[A]_{EP}$ can be calculated from tR at the equivalence point (see eqn. 2), tR_{EP}, and from tR at the beginning of the titration, tR_0, as tR_{EP} and tR_0 are linearly related to $[A]_{EP}$ and to $[A]_0$ respectively. $[A]_0$ is chosen so as to have a good compromise between sufficient sensor response (mV), and analyte concentrations which allow to reduce the quantity (the cost) of the titrant biomolecule. Using 500 µL of a 10^{-5} M cocaine analyte solution was a good compromise. In the future, smaller indicator electrodes (e.g. 1 mm diameter) will allow to work in more miniaturized and automated systems, which are available on the market. Working with 10^{-5} M analyte solutions also implies that, during titration, we will approach (or reach) the non-linear part of the potentiometric mV versus $\log c_{analyte}$ calibration graph. Therefore, it is needed to work with the "Transformed Response", tR, as outlined above.

$[A]_0$ being known, we have to derive $[A]_{EP}$ via eqn. 6:

$$[A]_{EP} = [A]_0.\frac{tR_{EP}}{tR_0} = [A]_0\left(\frac{10^{(E_{EP}/S)}-1}{10^{(E_0/S)}-1}\right) \qquad \text{Eqn. 6}$$

E_0 being the potential at the start of the titration (not to be confused with E^0), i.e. at titrant volume equal to zero, and E_{EP} being the potential at the equivalence point. As mentioned above, these potentials are offset to a zero value at zero analyte concentration (pure buffer solution). The second approach, which will be called in the text "Post-EP Method", PEP, uses a point after the equivalence point of the potentiometric titration curve to determine K_a. The "Post-EP Method" is used to calculate the association constant (Eqn. 7), as a function of the concentration of the analyte at the start, $[A]_0$, (which is known) and at the post-equivalence point (PEP). For the latter point we took the point at the end of the titration in the present conditions:

$$K_a = \frac{\frac{([A]_0 V_0 - [A]_{PEP} V_{PEP})}{V_{PEP}}}{[A]_{PEP}\left[\frac{\left([M](V_{PEP}-V_0)\right)-([A]_0 V_0 - [A]_{PEP} V_{PEP})}{V_{PEP}}\right]} \qquad \text{Eqn. 7}$$

with $[A]_{PEP} = [A]_0(\frac{(10^{(E_{PEP}/S)}-1)}{(10^{(E_0/S)}-1)})$

The titration of cocaine with TCPB and CBA is used as an example to explain the two different models (figure 2).

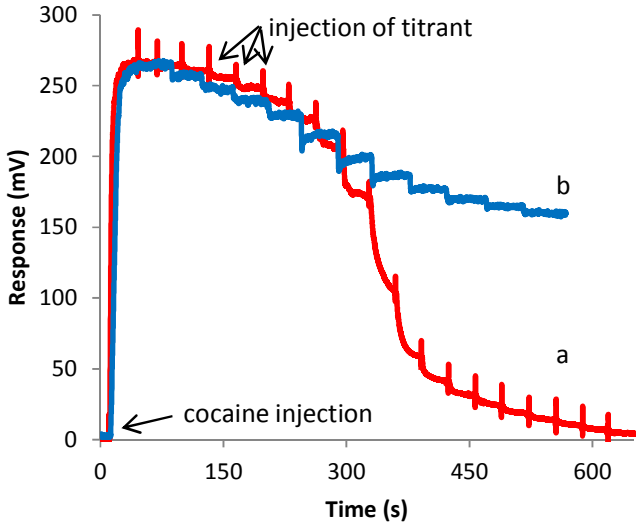

Figure 2: Potentiometric titration of 10^{-5}M cocaine in 500 µL MES buffer 1 mM with 5 µL additions of 10^{-4} M concentrations of respectively TCPB (a) and CBA (b) every 30 s.

As shown in table 1, the obtained K_a values using either the "Equivalence Point Method" (Eqn. 4) or the "Post-EP Method" (Eqn. 6) both are in the 10^9 M^{-1} range for TCPB. ΔG^0 values are completely comparable as calculated by the two suggested methods. Similar results were obtained for potentiometric titrations of cocaine with CBA, yielding a ΔG value of 8.6 ± 0.1 kcal mol^{-1} with the EP Method and 8.0 ± 0.1 kcal mol^{-1} with the PEP Method.

Table 1: Comparison of the potentiometric titration of cocaine with TCPB between the two suggested methods based on the Equivalence Point (EP) and Post-EP (PEP). Mean values (n = number of different electrodes tested) of K_a and ΔG values are presented.

Method	K_a (M^{-1})	ΔG^0 (kcal mol^{-1})	n
EP	3.4×10^9	12.8 ± 0.2	3
PEP	9.0×10^9	13.3± 0.1	3

All the results given later in this work are calculated with the "Post-EP Method", because the latter method is much more suited for working in biomolecular conditions. These conditions include firstly the µg availability of biocomponents at high cost, leading to small numbers of

titrant injections. Secondly, the K_a values of the biomolecule/small molecule complex are often in the order of 10^6 or lower. This is at the limits of obtaining clear equivalence points in classical potentiometric titrations. In titration methods, equivalence points are needed for quantitative determinations of analyte concentrations by addition of known concentrations of titrant. To determine K_a values however, estimating their approximate position (the "analyte" concentration and the titrant biomolecule concentrations are known) is sufficient. Furthermore, if the described transformed response potentiometry titration method is used, we can work with a ligand concentration related y-axis. Equivalence points will become difficult to localize, but the resulting curves will give a much better eye check evaluation of the binding event (see further in this text).

3.3. Specificity of the small molecule/biomolecule complex formation

3.3.1. Titration of cocaine (as ligand) with different oligonucleotides (as titrants)

Four different oligonucleotides were used for the selectivity study. The specific selected anti-cocaine aptamer (CBA) was compared with an anti-chloramphenicol aptamer (CAPBA) and with two randomly chosen oligonucleotides (25-mer-DNA and 43-mer-RP). Figure 3A shows the potentiometric titration of cocaine with CBA (a), CAPBA (b), 43-mer-RP (c) and 25-mer-DNA (d). Here, the concentration related transformed response, tR, is plot on the y-axis. As can be seen, the titration curve of cocaine with CBA almost completely goes back to the baseline in comparison to the curves where other oligonucleotides were used. This type of plot allows visual indication of the binding tendency, which is clearly in the order CBA > CAPBA > 34-mer-RP > 25-mer-DNA. That means that CBA has a better interaction with cocaine because of the specificity of the selected biomolecule. As seen in figure 2 and 3, the potential increased immediately after injection of analyte to the MES buffer solution. Following stepwise injections of titrant to the solution, the potential gradually came back to the baseline step by step.

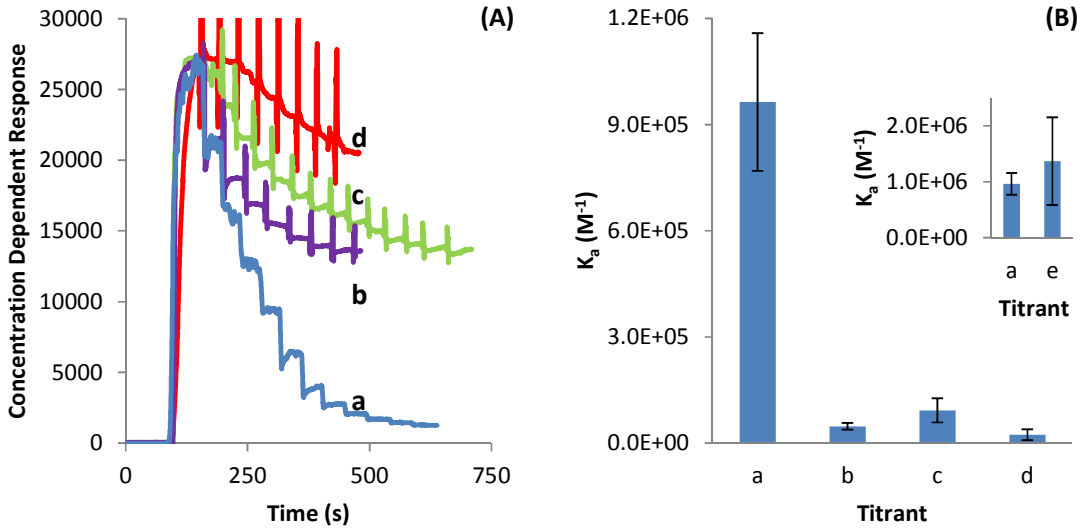

Figure 3: Potentiometric titration of cocaine with different titrants.(A): Potentiometric titration of 10^{-5}M cocaine in 500 µL 1 mM MES buffer with 8 µL injections of 10^{-4} M CBA (curve a), CAPBA (b), 34-mer-RP (c) and 25-mer-DNA (d) every 30 s. (B): Association constants (K_a) of cocaine with CBA (a), CAPBA (b), 43-mer-RP (c) and 25-mer-DNA (d) and anti-cocaine antibody (e, see insert). Standard deviations are show in the histogram (with n = 6 different electrodes tested for a and n = 3 for b, c, d and e).

Because CBA had 43 bases, a 43-mer-RP was chosen for the investigation of the selectivity. In the series of titrants, the CAPBA has the second largest response, followed by the 43-mer-RP and the 25-mer-DNA. Therefore, the 43-mer-RP and the 25-mer-DNA have little or no specific interaction with cocaine. This illustrates that the synthesized aptamer for cocaine has good recognition properties for its target, as shown by Hilton et al.[28]

The calculated association constants for interaction between cocaine and different titrants shown in figure 3B are: 960 × 10^3 M^{-1} for CBA, 47 × 10^3 M^{-1} for CAPBA, 93 × 10^3 M^{-1} for the 43-mer-RP and 24 × 10^3 M^{-1} for the 25-mer-DNA, respectively. The calculated K_d for CBA in our approach (1.1 ± 0. 2) × 10^{-6} M (n = 6, n is the number of different electrodes tested) is in good agreement with the reported value for K_d (between 0.4 and 10 µM) in the paper describing the synthesis of this CBA.[38] These K_a values correspond with a ΔG of 8.0 ± 0.1 kcal mol^{-1} (n = 6). The results show that we can use this new potentiometric titration approach for fast evaluation of newly synthesized aptamers at low-cost and low biomolecule expenditure. It is also interesting to note that CBA had a K_a value around 10^6 M^{-1}, as compared to a K_a value of around 10^{10} M^{-1} for TCPB (see above results). This would mean that this aptamer receptor molecule could not yet compete with TCPB for e.g. use in potentiometric sensors. TCPB, with its negative charge and stongly lipophilic character, clearly has a strong interaction (high K_a value) with positively charged lipophilic analyte

molecules. TCPB doped potentiometric sensors will respond sensitively but non-specifically to such analytes. It is to be expected that aptamers (or other receptor molecules) with such high K_a values are needed to develop selective potentiometric sensors with the same sensitivity as TCPB doped sensors.[33]

3.3.2. Titration of cocaine with a cocaine antibody

In order to validate the suggested model for potentiometric titrations, cocaine was also titrated with another type of recognition element, i.e. an anti-cocaine antibody (see figure 3B, histogram e). The obtained titration curve was comparable to the one obtained for the titration of cocaine with CBA. The calculated association constant for CBA, $(9.6 \pm 0.2) \times 10^5$ M^{-1}, was also comparable to the value obtained for the anti-cocaine antibody, $(1.4 \pm 0.8) \times 10^6$ M^{-1}, which results in similar ΔG^0 values for CBA (8.0 ± 0.1 kcal mol^{-1} , n = 6), and anti-cocaine antibody (8.1 ± 0.4 kcal mol^{-1}, n = 3).

As the response of a potentiometric sensor is related to the interaction energy of the analyte with the sensor's components, these two types of biomolecules can be expected to contribute comparably to sensitivity. Remark again however, that none of the two comes even close to the affinity obtained with a simple (lipophilic) organic molecule such as TCPB. In our opinion, the biomolecules may add selectivity, but they clearly still lack the high K_a values needed to obtain sensitivity.

3.3.3. Titration of different ligands with CBA (as biomolecular titrant)

For studying the cross-reactivity of the specific biomolecular interaction, positively charged molecules (in MES buffer solution pH 7) were titrated with CBA. Fig. 4A illustrates the titration curve of cocaine (a), metergoline (b), bromhexine (c) and lidocaine (d) with CBA as the titrant. The calculated K_a values are 960×10^3 M^{-1}, 3300×10^3 M^{-1}, 570×10^3 M^{-1} and 100×10^3 M^{-1}, respectively.

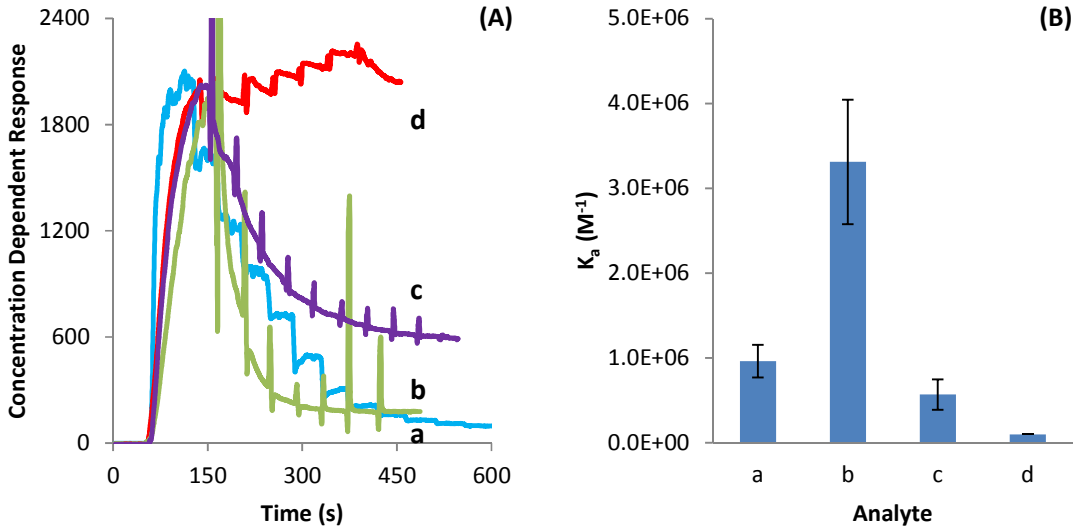

Figure 4: Potentiometric titration of different analytes with CBA. (A): Potentiometric titration of 10^{-5} M solutions of cocaine (a), metergoline (b), bromhexine (c) and lidocaine (d) in 500 µL 1 mM MES buffer with 8 µL 10^{-4} M CBA every 30 s. Y-axis units are normalized. (B): Association constants (K_a) of cocaine (a), metergoline (b), bromhexine (c) and lidocaine (d) with CBA. Standard deviations are shown in the histogram with n = 6 electrodes for a, n = 4 for c, n = 3 for b and d.

Fig 4B shows the histogram of the K_a values. The selected aptamer has better interaction with metergoline than with cocaine. Little is known on the cross-reactivity in the papers describing this anti-cocaine aptamer. A strong point of the present study is that such cross-reactivities can be studied much faster than with the presently available methods. All calculated K_a and ΔG values are summarized in tables 2 and 3.

Table 2: Titration of a 10^{-5} M solution of cocaine as an analyte, with of different titrants (8 µL injections of 10^{-4} M solutions) in 500 µL 1 mM MES buffer pH 7.

Titrant	K_a (M)	ΔG (kcal mol^{-1})	n
CBA	9.6×10^5	8.0 ± 0.1	6
25-mer-DNA	2.4×10^4	5.7 ± 0.3	3
43-mer-RP	9.3×10^4	6.7 ± 0.2	3
CAP-APT	4.7×10^4	6.3 ± 0.1	3
TCPB	9.0×10^9	13.3 ± 0.1	3
Anti-Coc Antibody	1.4×10^6	8.1 ± 0.4	3

Table 3: Titration of 10^{-5} M solutions of different analytes with CBA as a titrant (8 µL injections of a 10^{-4} M solution) in 500 µL 1 mM MES buffer pH 7.

Analyte	K_a (M)	ΔG (kcal mol^{-1})	n
Cocaine	9.6×10^5	8.0 ± 0.1	6
Lidocaine	-	-	3
Metergoline	3.3×10^6	8.7 ± 0.1	3
Bromhexine	5.7×10^5	7.7 ± 0.1	4

3.3.4. Titration of metergoline with different oligonucleotides

The binding constant between metergoline and metergoline binding aptamer (MBA) was also studied, and compared to binding with CBA, and with an 80-mer-RP oligonucleotide (figure 5). The calculated K_a and ΔG values were respectively 4.5 x 10^7 and 10.2 kcal mol^{-1} for MBA and 3.1 x 10^6 and 8.74 kcal mol^{-1} for CBA. No interaction was observed for the 80-mer-RP. The MBA shows stronger binding for its metergoline ligand than the other biomolecule/small molecule interactions investigated in this study.

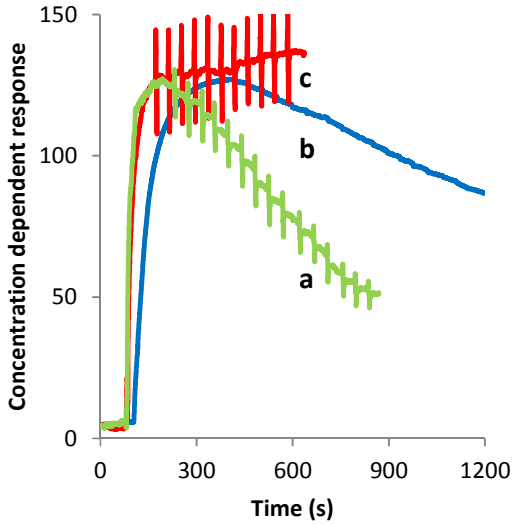

Figure 5: Potentiometric titration of metergoline as analyte (10^{-6} M) with different titrants: MBA (a), CBA (b) and 80-mer-RP (c) (5 µL 10^{-6} M injections) in 500 µL 1 mM MES buffer pH 7.

4. Conclusions

This report shows that potentiometric titration recordings with an analyte concentration related y-axis yield very useful information on molecular/biomolecular interactions. This is especially so if this is done also in the region of low ligand concentrations, where the sensor's calibration curve deviates from linearity. This transformation is simple, and it is unproblematic to apply to measured potentiometric data with the use of a non-linear least squares fitting program. Very interesting is the feature that a simple eye-check of the described "Concentration related response potentiometry" curves gives an immediate indication of the binding efficiency. This practical method therefore lends itself to rapid high throughput screening of receptor (bio)molecule candidates. Miniaturized potentiometric titration equipment is best suited for this application. Use of the small molecule as a titrant, and the large (charged) biomolecule in the titration beaker is also possible, but requires extra study. Extension of the method with a (more labour intensive but more informative) binding curves/Scatchard approach can be very interesting.

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