Characterization of heterogeneity of biomolecular interactions using 3rd generation biosensor

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1 Abstract

A new tool for kinetic evaluation of kinetic rate constants is enabled by a 3rd generation biosensor. The tool is developed to meet the need of reliably experimental information and communication between pharmaceutical companies and regulatory agencies to increase the productivity and decrease the associated risks. To obtain the necessary competences and resources for this, a project consisting of Attana AB, AstraZeneca AB, Waters Nordic AB and Karlstad University was established.

The main aim of the project is to achieve a comprehension understanding of interactions of different character e.g. fast and slow kinetics. This report concerns a fast interaction system. By analyzing a parathyroid hormone system using standard biosensor assays and single cycle kinetics with Attana Cell™ 200 instruments the fast interaction was characterized. The experimental data was analyzed using standard kinetic evaluation and an adaptive interaction distribution algorithm. The latter tool is developed at Karlstad university in order to describe the heterogeneity of interactions. The idea is to use the heterogeneity information as a decision support in drug development.

A sub aim was to investigate the feasibility of the single cycle kinetic assays compared to the standard biosensors assays. The results shows a decrease of experimental time by 70% for homogene interaction and the protocol enables assay without or with less regeneration.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PTH1R</td>
<td>Parathyroid hormone 1 receptor</td>
</tr>
<tr>
<td>PTH(1 – 34)</td>
<td>Parathyroid hormone (1-34), Teriparatide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>$k_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$k_a$</td>
<td>Association constant</td>
</tr>
<tr>
<td>$f_0$</td>
<td>Resonant frequency [Hz]</td>
</tr>
<tr>
<td>$\Delta f$</td>
<td>Frequency change [Hz]</td>
</tr>
<tr>
<td>$\Delta m$</td>
<td>Mass change [g]</td>
</tr>
<tr>
<td>$A$</td>
<td>Active crystal area [$cm^2$]</td>
</tr>
<tr>
<td>$\rho_q$</td>
<td>Density of quartz [$\frac{g}{cm^2}$]</td>
</tr>
<tr>
<td>$\mu_q$</td>
<td>Shear modulus of quartz [$\frac{g}{cm^2 s^2}$]</td>
</tr>
<tr>
<td>IRAS</td>
<td>Infrared reflection absorption spectroscopy</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal micro balance</td>
</tr>
<tr>
<td>KAU</td>
<td>Karlstad University</td>
</tr>
<tr>
<td>QbD</td>
<td>Quality by Design</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>KK</td>
<td>Swedish Knowledge Foundation</td>
</tr>
<tr>
<td>AED</td>
<td>Adsorption Energy Distribution</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>sNHS</td>
<td>N-hydroxysulfosuccinimide</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>SCK</td>
<td>Single cycles kinetics</td>
</tr>
<tr>
<td>LNB</td>
<td>Low non-specific binding</td>
</tr>
<tr>
<td>$K$</td>
<td>Equilibrium constant</td>
</tr>
<tr>
<td>AIDA</td>
<td>Adaptive interaction distribution algorithm</td>
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</table>
2 Introduction

The project is a collaboration between instances and companies and the focus of the report is to study kinetics between parathyroid hormone 1 receptor (PTH1R) and a human parathyroid hormone peptide (PTH(1-34)) both essential as regulators of calcium homeostasis and in bone physiology [1]. PTH(1-34) a.k.a Teriparatide, was approved as the first anabolic treatment of osteoporosis [2], a condition of generalized skeletal fragility which bone strength is sufficiently weak that fractures occur with minimal trauma [3]. The fast interactions between the hormones were fit for the project profile and the kinetics studies were enabled by a Attana Cell™ 200.

One reason for the high price in development of new medical drugs is the demanding and costly processes that drugs need to be able to complete in order to reach the market with an opportunity cost reaching from six hundred thousand to eight million USD per delayed day and the time for the drug to reach the market vary from 10-15 years [4]. The need for an improvement for the methods used in the experimental phase before costly and drawn-out processes like clinical trail is both needed in a economical and humanitarian aspect, due to Attana’s third generation biosensor these issues can be resolved in a more costly and faster approach than before.

Figure 1: The lengthy, costly and uncertain Bio pharmaceutical research and development process.

[5]

The birth of the biosensor technology started with the invention of the Clark electrode by Prof. Leland Clark in 1956, measuring the oxygen concentration in liquids [6]. Biosensor technology is based on a specific biological recognition element in combination with a transducer for signal processing which makes it a perfect candidate in the analytic role in pharmaceutical research [7]. Biosensors can be divided in three different type of generations.
The first generation of biosensor include flow cytometry technique and Enzyme-linked immunosorbent assay (ELISA). The first fluorescence-based flow cytometry was developed by Wolfgang Göhde in 1968 [8] and shortly after ELISA was invented by Peter Perlmann and Eva Engvall[9], both techniques uses labeling of the analyte and if and only if an binding has occurred a signal will be shown. With these techniques there no possibility for kinetic evaluation of the reaction but they can be used in both biochemical and cell-based assays.

The second generation biosensors like Surface plasmon resonance (SPR) and Bio-layer interferometry (BLI) uses different optical analytical techniques to measure real-time shift change in light on the surface without alternating the analyte (label-free). The real-time aspect provides the possibility of kinetics evaluation of reaction at the expense of not be able to conduct experiments on cell-based assays. The first SPR immunoassay was introduced by Bo Liedberg, Claes Nylander and Ingemar Lunström in 1983 at Linköping Institute of Technology[10].

Third generation biosensors is based on Teodor Aastrup’s PhD thesis from 1999[11] and are reagentless, characterized by the progression from use of a freely diffusing mediator to a system wherein the bio-catalyst and mediator are co-immobilized at an electrode surface, thereby making the bio-recognition component an integral part of the transducer [12]. This label-free technique measures the reaction real-time making it able for kinetic evaluation, target accessibility and off target interaction in both biochemical and cell-based assays.

Today calculations and formulas used to measure the affinity on binding and releasing are only meant to show results on plain surfaces and the calculations does not take into consideration that larger molecules will have a large spread, heterogeneity. The spread occurs due to multiple binding sites, more complex heterogeneity of interactions and risk of formation of aggregates between large
bio-molecules. When using this kind of calculation on first order interactions the
dissociation constant \((k_d)\) and association constant \((k_a)\) will be linear but in reality due to all the spread of large molecules the need for more complex algorithms are needed.

2.1 General aims of the study

- Deeper general understanding of molecular interactions at the biological interfaces, e.g. between an antibody and its receptor in the cell membrane surfaces.
- A new unity to describe the of heterogeneity of the interactions in a similar way as affinity association and disassociation rate.
- Improvement of methodology for analyzing and separation of valuable chemical components.
- Ease the communication between companies and regulatory agencies by implementation of a new concept “Quality by Design”.
- Develop new algorithms for interpretation of the data enabled by 3rd generations biosensors.

2.2 Specific aims of the study

- Deliver experimental data of the relative fast interaction between PTH1R and PTH(1-34) to the SOMI project.
- Evaluate single cycle kinetic (S.C.K) experiment with respect of data quality and assay time.
- Analytical separation of the two interactions between the PTH system.

2.3 Project background

2.3.1 Attana

Attana’s role in this project is to assist the development of a better understanding in interaction of molecules on surfaces and therefore helping quality assurance in the making of high purity bio molecular based medicine drugs. A better knowledge in this type of interactions will help in a more rapid process for drugs to reach the market and also ease the communication between the manufacturer of the medicine and the regulatory agencies.

Attana’s 3rd generation biosensors technology is based on Teodor Aastrup’s PhD thesis from 1990[11] whereas he could combine infrared reflection absorption spectroscopy technology (IRAS) with the piezoelectric effect discovered by P.Curie et al. in 1880.
Curie discovered that a piezoelectrical material responds to pressure resulting in a potential difference between two surfaces [13]. The potential that is exposed will make the material, e.g. quartz crystal start vibrating with a resonance frequency which is depended on the total mass of the crystal hence if material is added or removed from the crystal the frequency will change. By using this discovery Sauerbrey could find a relation to the frequency change and the mass change on applications and can be explained by the Sauerbrey equation, see equation 1 [14]. Change of 0.5 Hz - sensitivity threshold of Attana’s instruments - corresponds to a mass change of \(20 \frac{\text{ng}}{\text{cm}^2}\) for a 10 MHz crystal.

\[
\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q\mu_q}} \Delta m
\]  

(1)

Attana’s technology is now patented by five patents families including methods of performing label-free kinetics characterization of interaction of cells for all mass sensitive devices, patent modifications for QCM (Quartz crystal micro balance) systems that enables kinetic characterizations of bio-molecules in sera and with cells on surfaces.

Figure 3: Schematics of Assay available with Attana QCM, traditional biochemical assays, sera and crude assays and the unique cell-based assays. In a biochemical assays both receptor and pharma molecule are purified and basically the only interaction that can occur is the one between them. However, in-vivo, in the body several interactions occurs, e.g. off-target interactions with cell membranes. This interactions can be characterized with the Attana technology and are important to understand when developing new pharmaceuticals.
2.3.2 The Attana Cell™ 200

The Attana Cell™ 200 is a dual channel, continuous-flow system for automated analysis based on the QCM technology and can study interactions like specific binding in cells. To monitor binding interactions, one of the interacting molecules is immobilized on the sensor surface and the sample containing the other one is injected over the sensor surface. Binding data is displayed in real-time directly on a computer screen. The signal output is given in frequency (Hz) and is directly related to changes in mass on the sensor surface. The two sensor channels are referred to as channel A and channel B, where A is typically used for monitoring of the molecular interaction and B serves as a reference.

To setup the machine the C-fast list program is used. Data is collected by Attester Software and subsequently processed in the Evaluation Software, both parts of the Attacbé software suite.

2.3.3 INTERACT

INTERACT is collaboration between researchers at Karlstad University (KAU), the focus of the group is Molecular Interactions at surfaces and interfaces. The group is headed by three researchers, Ellen Moons (Material Physics), Lars Järnström (Surface treatment technology) and Torgny Fornstedt (Separation Science and Analytical Chemistry).

2.3.4 The SOMI project

The Studies of Molecular Interactions (SOMI) project is a co-operation between INTERACT research environment at KAU and three companies, Attana AB, AstraZeneca AB and Waters Nordic AB with the an common idea for a implementation of a concept called “Quality by Design” (QbD) which concentrate on scientific results instead of empirical knowledge [15][16]. The concept will work as a bridge of communication between companies and regulatory agencies like US Food and Drug Administration (FDA) [17].

“By practical implementation of a concept called “Quality by Design” (QbD) and easier communication between companies and regulatory agencies could be achieved. The concept has not been utilized by pharmaceutical companies due to lack of fundamental knowledge about molecular interactions and a key purpose of this study is to deepen the understanding of separation processes and the instrumentation.” [18]

One goal of the SOMI project is to further improve our understanding of molecular interactions and use this knowledge to strengthen the academic research in the area and for industrial applications. By new numerical algorithms there could be an improvement of the methodology for analyzing and separation valuable chemical components based on new scientific understanding rather than using existing knowledge and methodology for new applications.
“Much work has therefore been done in KK HÖG 2015 [The Knowledge Foundation] to develop new numerical algorithms that accounts for the increased degree of complexity, for example algorithms used to interpenetrate data from biosensor such as the ones manufactured by our KK-partner Attana AB.” [18]

Another goal is also to strengthen the competitiveness of Swedish industry regarding separation of valuable chemical components as well as improving INTERACT´s understanding of application and to place their fundamentally advanced theoretical studies in real industrial settings.

“...The key to understand modern analysis and purification methods is proper mechanistic modeling of the underlying molecular interactions, i.e. the interactions between the molecules to be separated and the separation surface media. We have previously developed numerical tools to help us determine the degree of heterogeneity in the thermodynamics called Adsorption Energy Distribution (AED) calculations. AED enables us to determine the number of interactions in the system before and thereby limit the number of possible mechanistic models; this has been used successfully used for smaller molecules.” [18]

3 Method

PTH1R was initially immobilized on a LNB (Low non-specific binding) carboxyl surface activated by a mixture of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysulsuccinimide (sNHS). To avoid frequency shift in the signal a HBST-buffer containing HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid), sodium chloride (NaCl) and a nonionic detergent (Tween20) with 7.4 pH was used throughout the whole experiment.

The experiments are divided into three sections, two of the experiments are experiments with regeneration of the surfaces but with different temperatures.
and the other one is a single cycle kinetics (SCK) experiment i.e without regeneration. The SCK is important for future experiments with cells and proteins with weak binding to the surface or without any possibility for regeneration. All three experiments uses same type of immobilization technique, materials and chemicals the only difference in the experiments is the configuration of the Attana C-Fast list. The interaction profile for the experiments is a 1:2 binding profile meaning that two values of each kinetics constant are obtained. A simulation can be made from these values and the results of the simulated sensograms will show both of the kinetics profile.

![Figure 5](image)

**Figure 5:** Left: C-fast list on SCK. Right: C-fast list with degradation. Comparison of SCK C-fast list and C-fast list with regeneration, six of seven concentration were injected on SCK the same time span as three of seven with regeneration.

### 3.1 Materials

<table>
<thead>
<tr>
<th>Article</th>
<th>Supplier</th>
<th>Art. number</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNB-Carboxyl chip</td>
<td>Attana</td>
<td>3623-3011</td>
<td>Sensor chip</td>
</tr>
<tr>
<td>HBS-Tween 10x</td>
<td>Attana</td>
<td>3506-3001</td>
<td>Running buffer</td>
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<td>EDC 0.4M</td>
<td>Attana</td>
<td>3501-3002</td>
<td>Pt. of Amine coupling kit</td>
</tr>
<tr>
<td>s-NHS 0.1M</td>
<td>Attana</td>
<td>3501-3003</td>
<td>Pt. of Amine coupling kit</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>Attana</td>
<td>3501-3004</td>
<td>Pt. of Amine coupling kit</td>
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<tr>
<td>PTH1R</td>
<td>NovoPro</td>
<td>202117</td>
<td>Receptor</td>
</tr>
<tr>
<td>PTH(1-34)</td>
<td>Biotechn</td>
<td>3011/1</td>
<td>Analyte</td>
</tr>
</tbody>
</table>
3.2 Experimental set-up (all three experiments)

3.2.1 Stabilizing Chip – Flow Rate Optimization

The binding study was initiated by docking the LNB chips in the instrument and setting the buffer flow at 100µl/min for 10 min to get a stabilized baseline. After reducing the flow to 25 µl/min a minimum frequency change (≤ 10Hz over 600s) had to be met before continuing with the experiments.

3.2.2 Immobilization of the PTH1R

The flow was set at 10µl/min and the temperature at 22°C. Following activation of the surfaces with EDC/sNHS (300s injection), PTH1R at a final concentration of 10µg/ml was diluted in acetic acid pH 4 and injected for 300s on surface A. Deactivation was performed by injection of ethanolamine. A total of circa 200Hz of PTH1R were immobilized on the surface A on all three experiments (exp. 1, 2 & 3). The reference surface was a LNB surface immobilized without PTH1R on channel B.

3.3 Exp. 1, degeneration with temp 22°C

For the first runs there will be a throughout showing of all data gathered, for the last runs only the merged graphs and the kinetics results will be displayed. Experiment one is divided in two different runs, “Run 1” and “Run 2”.

3.3.1 C-Fast setup exp. 1

The experiment has been performed at a flow rate of 25 µl/min with a temperature of 22°C. The PTH(1-34) were injected at seven different concentrations: 1.21 µM, 2.43 µM, 3.64 µM, 4.86 µM, 7.29 µM, 9.71 µM and 14.57 µM with a blank injection containing HBST before every concentration. At least one blank injection is needed for the kinetics evaluation.
3.3.2 Data results exp. 1, run 1.

Figure 6: Frequency (Hz) as a function of time (minutes), PTH(1-34) with 7 different concentration on surface PTH1R, starting from the lowest concentration, the higher signal peaks are the injection of PTH(1-34) and the peaks between are the response to degeneration procedures.

Figure 7: Frequency (Hz) as a function of time (seconds), PTH(1-34) with 7 different concentration on surface PTH1R, run 1. Left graph: Channel A, Middle graph: Channel B, Right graph: Channel A minus B.
3.3.3 Merged data results exp. 1.

The kinetics are evaluated on channel A minus blank on both runs.
3.4 Exp. 2, degeneration with temp 20 °C

Experiment two is divided in two different runs, “Run 3” and “Run 4”.

3.4.1 C-Fast setup exp. 2

The experiment has been performed at a flow rate of 25 µl/min with a temperature of 20 °C. The PTH(1-34) were injected at seven different concentrations: 1.21 µM, 2.43 µM, 3.64 µM, 4.86 µM, 7.29 µM, 9.71 µM and 14.57 µM with a blank injection containing HBST before every concentration.
3.4.2 Merged data results exp. 2.

Figure 11: Frequency (Hz) as a function of time (seconds), PTH(1-34) with 7 different concentration on surface PTH1R, run 3 (red) and run 4 (black). Channel A minus blank.

Figure 12: Kinetics evaluation, frequency (Hz) as a function of time (seconds), PTH(1-34) with 7 different concentration on surface PTH1R, exp. 2. Channel A minus blank (black), fitted kinetics OneToTwo (blue).

3.5 Exp. 3, Single Cycle Kinetics with temp 22°C

Experiment three is divided in two different runs, “Run 5” and “Run 6.”
3.5.1 C-Fast setup exp. 3.

The experiment has been performed at a flow rate of 25 µl/min with a temperature of 22 °C. The PTH(1-34) were injected without regeneration between the seven different concentrations: 1.21 µM, 2.43 µM, 3.64 µM, 4.86 µM, 7.29 µM, 9.71 µM and 14.57 µM. One blank was injected in the beginning of the run.

3.5.2 Data results exp. 3, run 6.

![Figure 13](image1.png)

**Figure 13:** Frequency (Hz) as a function of time (minutes), PTH(1-34) with 7 different concentration on surface PTH1R without regeneration, starting from the lowest concentration.

3.5.3 Merged data results exp. 3.

![Figure 14](image2.png)

**Figure 14:** Frequency (Hz) as a function of time (seconds), PTH(1-34) with 7 different concentration on surface PTH1R, run 5 (red) and run 6 (black). Channel A minus blank
The injection of 9.71 µM in run 5 is clearly some kind of error as viewed in the graph above, the second highest signal response (red). That injection is discarded in the kinetics evaluation.

Figure 15: Kinetics evaluation, frequency (Hz) as a function of time (seconds), PTH(1-34) with 7 different concentration on surface PTH1R, exp.3. Channel A minus blank (black), fitted kinetics OneToTwo (blue).

3.6 Simulations of interactions
Table 2’s data is collected from all three kinetics evaluations.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>22 °C</th>
<th>20 °C</th>
<th>22 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a\left(\frac{1}{M \times s}\right)$</td>
<td>$k_d\left(\frac{1}{s}\right)$</td>
<td>$K_{D_1}(nM)$</td>
<td>$k_a\left(\frac{1}{M \times s}\right)$</td>
</tr>
<tr>
<td>Exp. 1, 22 °C</td>
<td>$1.52 \times 10^4$</td>
<td>$5.70 \times 10^{-2}$</td>
<td>3750</td>
</tr>
<tr>
<td>Exp. 2, 20 °C</td>
<td>$1.55 \times 10^4$</td>
<td>$4.49 \times 10^{-2}$</td>
<td>2900</td>
</tr>
<tr>
<td>Exp. 3, SCK 22 °C</td>
<td>$1.10 \times 10^4$</td>
<td>$7.19 \times 10^{-2}$</td>
<td>6530</td>
</tr>
</tbody>
</table>
4 Discussion

The results of the experiment confirms that use of Attana Cell™ 200 is fit for further data gathering for the SOMI project hence strengthening the position of this technique in kinetics evaluation for label-free biological experiments and also making it possible for further collaboration of companies involved. In the final report of the SOMI-project only positive feedback towards Attana AB was mentioned and the data provided made it possible for an adaptive interaction
distribution algorithm (AIDA) algorithm to be developed. AIDA uses adaptive finite methods which is a fundamental numerical instrument to approximate partial differential approximations depending on discrete solution(s) and data to assess the approximation quality and improve it adaptively[19]. With help of this new algorithm the exact number of interactions between pharmaceutical molecules and its receptor can be established. The tool determines the heterogeneity of the drug-receptor interaction and can be used for e.g. improved selection, lead optimization exact number of interactions between pharmaceutical molecules and its receptors.

Collected data shown in table 3 and in figure 16 of the SCK (experiment 3) and the regular protocol (experiment 1) at the same temperature confirms that the SCK is possible for further experiments and is also backed up by previous studies using this technique.

The need for SCK is desirable when assays involves molecules that creates irreversible (or at least long-term) interactions e.g. nanoparticles which creates a hard corona with proteins [20] making it almost impossible to find a suitable regeneration.

The time and money saved by implementing the SCK into the huge amount of assays needed for quality assurance in this project is significant and standardization of the technique should be a centralized bullet point in upcoming project plans of the SOMI project at Attana. Worth to notice is also the time saved by implementing the AIDA algorithm into the calculation model, the AIDA is able to resolve the constant distribution in just second whilst commercial software requires hours of calculation.

5 Conclusion

- Full kinetics evaluation were delivered for the SOMI project to be used as raw data in the heterogeneity investigation. The data is included in a scientific manuscript in preparation.

- The experimental data confirms that the SCK data can be used for the PTH system interactions. The assay time was reduced with 70%. Consequently the throughput can be increased and it enables for analyze of more time sensitive biological interactions e.g. sensitive living cells. This potentially elucidates the need for regeneration scouting before the experiments are performed, this adds an extra time saving aspect.

- The two interactions could be detected and evaluated in the evaluation software this will also be further analyzed in the SOMI project.

References


