Towards time-resolved molecular interaction assays in living bacteria

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Abstract

Rare and neglected diseases such as multidrug resistant (MDR) tuberculosis, malaria and trypanosomiasis are re-emerging in Europe. New strategies are needed to accelerate drug discovery to fight these pathogens. AEGIS is a Pan-European project that combines different technologies to accelerate the discovery of molecules suitable for drug development in selected neglected diseases. This thesis is part of the AEGIS research area that considers time in a multidisciplinary approach, combining biology, physics and mathematics to provide tools to characterize biological events for improving drug development and information about the target diseases and lead compounds.

Real-time cell binding assays (RT-CBA) of receptor-ligand interactions are fundamental in basic research and drug discovery. However, this kind of assays are still rare on living cells, especially in the microbiology field. In this project, we apply the same high-precision assay type on bacterial systems and explored the interior of the cell with a time resolved assay.

The effect of temperature was evaluated in the RT-CBA using LigandTracer to ensure that it was possible to use the technology in a range of temperatures suitable for bacteria. A method for attaching Gram positive and negative bacteria on the surface of a normal Petri dish, showing a high reproducibly and a high cellular viability after 16 h. With these two key steps, an RT-CBA fit for microbiology is available.

Next, to answer biological questions, intracellular interactions were explored by expression and validation of intracellular proteins with fluorescent tags suitable for RT-CAAs. First, we used the subunit B from the Shiga toxin (STxB) as a model to understand different aspects about the internalization processes. RT-CBAs allowed to discovery new features of STxB binding and mechanism to deliver small molecules or small proteins into cancer cells. Then, for exploring intracellular interactions, insect cells were bioengineered for evaluating the ability of small molecules to internalize and bind to its target. Using Carbonic anhydrase II – sulfonamides as a model system, the molecular interaction in the cytoplasm could be measured using a quencher label approach. The development of this kind of novel RT-CBA tools provide new information about drug candidates for targets that are not properly expressed in bacterial cells.

The assays in this project can make drug design more efficient. Furthermore, the evaluation of binding activity of the new compounds developed by AEGIS, focusing on rare/neglected diseases, in a biological environment has the potential to accelerate drug discovery for the targeted emerging diseases.

Keywords: Intracellular Molecular Interactions, Real-Time Cell Binding Assays, Bacteria, LigandTracer, Kinetics

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To my late father
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III Encarnação, J.C., Napolitano V., Opassi G., Danielson, U.H., Dubin, G., Popowicz, G, Buijs, J., Andersson, K., Björkelund H. Revealing the dynamic features of STxB-Gb3 co-internalization mechanism of molecular cargo into cancer cells Manuscript


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Other papers by the author


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<tbody>
<tr>
<td>AEBSA</td>
<td>4-(2-Aminoethyl)benzenesulfonamide</td>
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<tr>
<td>AEGIS</td>
<td>Accelerate early stage drug discovery</td>
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<td>BiFC</td>
<td>Bimolecular fluorescence complementation</td>
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<td>BR</td>
<td>Binding region</td>
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<td>CD77</td>
<td>Cluster of differentiation 77</td>
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<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DUF</td>
<td>Domain of unknown function</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<tr>
<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>FCM</td>
<td>Fluorescence correlation microscopy</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPPS</td>
<td>Farnesyl pyrophosphate synthase</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<td>FS</td>
<td>Fragment screening</td>
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<tr>
<td>Gb3</td>
<td>Globo triaosyl ceramide</td>
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<tr>
<td>hCAII</td>
<td>Human carbonic anhydrase</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
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<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HTS</td>
<td>High-throughput screening</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute 2</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistant</td>
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<tr>
<td>MST</td>
<td>Microscale thermophoresis</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>PCA</td>
<td>Protein-fragment complementation assay</td>
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<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-protein interaction</td>
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<tr>
<td>PsrP</td>
<td>Pneumococcal serine-rich repeat protein</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal microbalance</td>
</tr>
<tr>
<td>RET</td>
<td>Resonance energy transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
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<tr>
<td>RT-CBA</td>
<td>Real-time cell-binding assay</td>
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<tr>
<td>SAR</td>
<td>Structure–activity relationship</td>
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<tr>
<td>Scar</td>
<td><em>S. carnosus</em></td>
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<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
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<tr>
<td>STEC</td>
<td>Shiga toxin-producing <em>Escherichia coli</em></td>
</tr>
<tr>
<td>STxA</td>
<td>Subunit A of Shiga Toxin</td>
</tr>
<tr>
<td>STxB</td>
<td>Subunit B of Shiga Toxin</td>
</tr>
<tr>
<td>UMPK</td>
<td>Uridine monophosphate kinase</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Introduction

Humans being' inherent curiosity for understanding life makes us wonder what moves life, how certain processes occur and what the basic variables responsible for it are. Following the so called “reductionism logic”, understanding the different basic variables of life has solved parts of this continuously evolving and extremely complex puzzle. The scientific community has tried to define different levels of complexity, dividing them into “units”. The fundamental unit of life is defined as a single cell, the basic structural, functional, and biological “building-block” of all known organisms. However, the cell is in itself an incredibly complex system where biological processes are ruled by other, smaller “units” such as proteins, DNA regulation and molecular metabolism.

The interest in the complexity of life is such that we can add another level of elaboration to explain a biological phenomenon. Setting our unit at macromolecular level we approach the regulation of biological processes within protein interactions. And if we push a little bit forward into the mechanism, we arrive at one of the final variables: a molecular interaction.

The reductionism of life processes to answer specific questions might seem an attractive and straightforward approach. However, it is when we start to dissemble the innumerable variables of a “simple” process that we truly realize how complex life is. From this perspective, multidisciplinary research approaching the different “units” can help to solve the big puzzle.

While going through different units, the time variable appears to be crucial for evaluating different processes, from a molecular interaction to an everyday activity. Even if usually neglected, time is crucial for characterizing protein interactions and biological events.

In this thesis, time is considered within a multidisciplinary approach, combining biology, physics and mathematics to provide information and tools to characterize biological events for improving drug development in non-mammalian cells. Real-time cell binding assays were used to provide high-precision information for bacterial systems and intracellular interactions. In addition, the study of protein carriers and the introduction of the time variable have provided novel information of molecular interactions with cell membranes.
Introduction to molecular interactions

The study of molecular interactions in biology can unveil how biological systems communicate with each other, among many other things. Understanding molecular interactions provides information about how processes occur, what happens if they are unbalanced, and how we can modulate them.

What is a molecular interaction?

Interactions between molecules are attractive or repulsive forces between molecules and between non-bonded atoms. Biological processes are activated or inhibited by various non-covalent molecular interactions. While a covalent binding is crucial in biology for the backbone of an organic molecule, these weaker interactions allow macromolecules to bind (associate) and release (dissociate) from each other. Weak interactions have indeed a significant contribution to the effect of biological structures, as for example protein conformation that can translate into function. A crucial role in both secondary and tertiary structure of proteins is given by hydrogen bonds, followed by van der Waals forces. Other non-covalent bonding such as metal–ligand interactions, hydrophobic forces, π–π interactions and electrostatic effects also contributes to the final and specific functionality of a macromolecule (1).

Principles of Molecular Recognition in life

All organisms consist of a set of networks between structures, that allows biological systems to interact through binding and dissociation, triggering signaling cascades or molecular reactions. The complexity of these molecular interaction networks is crucial for several processes.

Molecular interactions occur also at cellular level, when a bacterium, parasite or a virus interacts with a mammalian cell (2–4). In many cases, symbiotic relationships between different biological systems can be essential for the survival of the host, such as in the case of the human gut microbiota that plays a crucial role for the homeostasis of the gut (5–8). However, not all microorganisms have a symbiotic relationship. In this case we refer to microorganisms that colonize and cause a disease in the host known as pathogens. After the first interaction with the host, a pathogen finds the best conditions to multiply and ensure its successful transmission to a new host or secure its colonization. The pathogen is able to cause a disease if it is capable of infecting, multiplying and overcoming the host’s defenses (9–11).

As previously mentioned, molecular interactions are the basic of the signaling communication through macromolecules such as proteins. Different proteins have different functions. Some are responsible for catalyzing reactions by interacting with specific molecules or synthesizing/degrading com-
pounds (12). Other proteins are instead capable of interacting with other molecules that trigger biological processes, such as signaling growth cascades or death signaling pathways (13,14). In both these protein classes, molecular interactions are key to allowing life to happen.

The study of how proteins interact with other proteins or molecules is the field of molecular recognition, which refers to specific interactions between two or more molecules through non-covalent bonding. The target and molecule interact through molecular complementarity (15). This is described in most of the enzyme–substrate interactions by the “Lock and Key” binding model. Other common interactions include antibody-antigen interactions, DNA translation, or the binding of ligands to protein receptors. Molecular recognition occurs when the target protein contains molecular regions which the ligand can recognize and interact favorably with. The strength of the complex formation depends on the characteristics of the binding between the two molecules.

Some molecules bind specifically to a target as a crucial element in conveying the function of a protein or activation of a specific biological response. For this reason, molecular recognition plays an important role in the functional characterization of target-ligand complexes. It can give information about biological systems such as the interactions between two strands of DNA, how peptide backbones fold into secondary structures, antibody-antigen interactions, proteins and molecules binding to their receptor and triggering a response or how ribosomes can be powerful machines (16). All this is thanks to natural evolution that created selective and specific strategies for living systems to adapt to different environmental stimuli.

### Molecular interactions in therapeutics

Since most biological processes are triggered by molecular interactions, clearly the perturbation of cellular pathways by different interactions might be the origin of several cellular malfunction pathways. One example where a dysfunctional process can result in a malign effect can be found when studying transmembrane proteins. These proteins are involved in several crucial cellular processes, such as cellular signal transduction cascades or ion and small molecule transport. In cancer cells, certain protein receptors are commonly overexpressed in the cell membrane, increasing the likelihood of molecular interactions with growth factors and consequently cancer growth and survival (17,18). These effects created interest in the field of medical science, and lead to the development of molecules capable of interacting and targeting growth factor receptors. Therefore, a high percentage of drug targets are membrane-associated (19,20).

One molecule that can be tailored to recognize growth factor receptors with high efficiency and specificity is the antibody. For this reason antibodies are
still one of the major molecular classes considered for the treatment of cancer and other diseases (17,21). Moreover, antibodies are part of new clinical strategies when conjugated with toxins, radionuclides or nanoparticles to improve treatment efficiency (22,23). Developing such molecules – able to recognize and bind a specific cancer target – involves several biochemical and biophysical studies for potentially enhancing the binding behavior. The information about the likelihood of how molecules bind to a receptor and how stably and strongly they bind, is provided by the affinity and kinetic values. At clinical level these intrinsic characteristics of an antibody can explain the differences in cellular responses and facilitate the improvement of mAb design (23,24). Actually, affinity and kinetic information can allow the selection of the most promising clones for antibody maturation, which leads to an improved candidate for future studies (24).

When it comes to drug design for small molecules more information is needed. X-ray crystallography has made a crucial contribution to structure-based drug design since it provides information about the molecular structure of the crystal form of a protein. To quickly understand how the developed molecules can have a biological effect, companies invest heavily in high-throughput screening (HTS) technologies. In these kinds of technologies, the target can be tested at different biological levels, from proteins to cells or even embryos. HTS has its own limitations, such as low hit rates (which means that a low percentage of molecules has a biological effect during the screening), particularly with challenging targets or when promising compounds are not really suitable for a biological/clinical application because they are too large or too lipophilic (25,26). In order to limit problems arising from unsuitable compounds, a niche area of research has grown in the past 10 years: fragment-based drug discovery, based on fragment library screening (FS). Fragments are defined as low molecular weight (MW <300 Daltons), moderately lipophilic and highly soluble organic molecules, which typically bind with low affinity (mM to µM range) (27). Several techniques are used for fragment development, typically starting with virtual screening of molecules to find possible candidates that can bind and fit in the conformation of the protein interface. These weak interactions are then measured by sensitive biophysical techniques such as NMR (28) or X-Ray crystallography (29). The results from these techniques can give information about fragments that binds to different regions of the pocket. By joining such fragments, it is possible to create a potent ligand able to disrupt a protein-protein interaction (PPI). In the end, FS can often deliver a higher hit rate than HTS (27).

However, these kinds of molecular interactions should be confirmed in a proper cellular environment, since the activity of compounds might be strongly affected by cell permeability and competitive interaction inside or on the cell. Cell based methods can therefore help to optimize potential molecules by giving new information about the interaction, affinity and kinetics.
Protein–small molecule interactions and molecular design

Considering the extent of the network of proteins, the number of possible interactions between different proteins is enormous (30). The whole interface between PPIs is difficult to target with small molecules, but specific regions from the proteins have a higher, or even crucial, contribution for the protein functionality. These “hot spots” are responsible for most of the binding energy of the interaction and are possible to target with small molecules, that might compromise or stabilize the molecular binding (31,32).

Another strategy to target PPI is interfering with the conformation of the protein. Some small areas around the protein are crucial for their natural folding conformation, which means that small molecules able to interact with these areas might change protein stability (33). When reading about cancer therapy, and more specifically about PPIs in cancer, one of the most well-known PPIs is the interaction of murine double minute 2 (MDM2) with p53. The information obtained about the “hot spots” of the interaction between the two proteins has enabled the development of seven MDM2-p53 inhibitors that are already in clinical trials. Other approaches in cancer, such as the Bcl2/Bak have also recently allowed the development of new inhibitors for both proteins (33).

One of the strategies to find suitable molecules consists of finding natural compounds from a diverse number of organisms, plants or fungi, and studying their capacity against a specific target. Several compounds were found using this approach, such as acetylsalicylic acid (aspirin), morphine and even the anti-malarial drug quinine (34). A more logical and modern approach to discovering new molecules is the so-called rational drug design (35). This strategy is based on the design of molecules able to recognize a specific area of the protein target. Therefore, first efforts are directed towards the validation

![Fig1](image_url) Reversible binding of two proteins by the principal of molecular recognition. PPI interface is highlighted with light and dark red.
of a target, as crucial for the causative agent of the disease. Then, the molecular characteristics/interface are defined as hot spots on the target protein interface. Finally, new compounds are synthetized to react with the specific “hot-spots”. Hot spots that are confirmed to have biological activity relevant for interaction with the target and that are druggable, are usually called “pharmacophore” (25).

Rational drug design is a multidisciplinary approach performed in collaborative efforts between microbiologists, biochemists, computational analysts, synthetic chemists, pharmacologists, and physicians for finding new leads for future drugs. For example, biophysical chemistry provides important information about kinetics and thermodynamic data, which helps in the characterization of chemical groups that are crucial for the interaction, thus improving the rational drug design. Computational chemists can then use powerful in silico tools for predicting the best possible molecule structures. This iterative process leads to proposed molecules that biochemists and pharmacologists will evaluate, to learn about certain intrinsic characteristics and their efficiency in different biochemical and biological assays.

Neglected diseases and the role of AEGIS

Uncontrolled drug-resistant tuberculosis is increasing in Europe, as is malaria and trypanosomiasises in the southern hemisphere. The purpose of the Marie Curie project AEGIS (Accelerate Early staGe druG dlScovery) is to discover molecules suitable for drug development in selected rare diseases such as these. The AEGIS target portfolio addresses neglected and infectious diseases that are (re)emerging in Europe and are prevalent globally. The main focus of the consortium are molecular targets such as: Peroxisomal membrane protein Pex14, an essential membrane-bound protein required for the biogenesis of glycosomes in Trypanosoma parasites; the protein uridine monophosphate kinase from M. tuberculosis (UMPKmt) which does not exist in eukaryotes is therefore an interesting target for fighting tuberculosis; and parasite farnesyl pyrophosphate synthase (FPPS) which is a validated target to treat protozoan parasite diseases such as Chagas disease (American trypanosomiasis) or Leishmaniasis. One of AEGIS’s main aims is to improve the success of early stage drug development by combining different methods and new techniques against challenging but promising targets (i.e. PPIs), since these potential drug targets are often neglected due to the high risk associated for their validation. This is possible by combining computerized screening, biophysical screening, crystallography and recombinant protein assays to accelerate drug discovery on intended targets. However, these methods do not allow an understanding of the behavior of the new leads in a cellular environment. For that reason, one of the aims of AEGIS is also to get closer to the target organisms than purified/recombinant proteins. Using bacteria as model system in real-time assays is a balance between clean data and closeness to target environment
(target organism), allowing a verification of the binding capacity of the novel molecules in a living biological environment.

**Understanding interactions in vitro and in vivo**

For the discovery of the molecular binding site on the target, research focuses first on the structure–activity relationship (SAR) studies for the determination of the chemical group of the chemical molecular structure that will interact with the “hot-spots” of the target, disturbing the biological activity (36). In this scenario, crystallography plays a key role for understanding which forces are responsible for the binding between molecules and proteins. However, some proteins are not able to crystallize in a physiological or native form, which can require modification of the protein by mutation, or using a truncated form (26). Moreover, sometimes it is challenging to obtain co-crystals between the components. In such instances, techniques such as NMR or cryo-electron microscopy (cryo-EM) may provide more information about the complex itself (26). On the other hand, other assays can provide biophysical information about interactions, providing an important contribution on the forces involved. Techniques such as isothermal titration calorimetry (ITC) (37) or microscale thermophoresis (MST) (38) can provide thermodynamic information about the interaction, strength of binding (affinity-K_D), heat of binding
(or enthalpy, ΔH), entropy (ΔS) and, stoichiometry (n, number of binding sites) which in turn are valuable for understanding the mechanism of the interaction (39,40). Furthermore, other techniques such as surface plasmon resonance (SPR) or quartz crystal microbalance (QCM) can provide information about the kinetics of an interaction. It is clear that different techniques give different information about the interaction, and each and every one is limited, from the high precision and low throughput of ITC to the rapid screening of molecules (>100 Da) in SPR (26). The information obtained in this early stage drug discovery allows an iterative process for medicinal chemists to improve ligand potency by modifying the chemical structure of the tested molecules. However, in all in vitro techniques, proteins are in their pure form in a specific environment, far from the physiological crowded conditions in the target organism.

Over the years, the advance of microscopy methods has been used to explain protein interactions in living systems, mainly receptor signaling (41–44). These techniques are based on different principles, such as Förster resonance energy transfer (FRET), which is sensitive to the low proximity in the nanometer range and orientation between fluorophores (44); fluorescence correlation microscopy (FCM) (45), which can provide information about the stoichiometry and diffusion kinetics of large complexes; and bimolecular fluorescence complementation (BiFC) (46), which explains macromolecular complexes through the association of fluorescent protein fragments between the components of the complex. A common requirement for all microscopy methods is the use of fluorophores, i.e. the ligands or proteins are coupled with a fluorophore that can change the behavior of the real interaction. In addition, some of these approaches can have a relatively high ratio of false-positives, especially when applied in high-throughput screening (47). Nevertheless, these cell-based techniques can provide information about cell membrane complex where other biophysical techniques such as SPR and ITC have difficulties in providing answers. This is mainly because membrane proteins, which reside in a lipid environment with partially hydrophobic surfaces, are often difficult to purify and lack stability (48,49).

Measuring interactions on cells with label-free techniques is a desirable research area. However, working with a large and complex biological unit such as a cell can still highlight a lot of the limitations of these techniques. In some cases, the signal outcome is a response from an event and not the molecular binding itself. Moreover, time, cost or even mimicking biological conditions (such as metabolism, cell nature and incubation medium) during the measurement can still be an experimental challenge (50,51).

Interaction kinetics/thermodynamics

In thermodynamics, biological systems are open systems, which means that there is an interchange of energy and mass with the surroundings. In other
words, when we take into consideration the presence of a specific drug, concentration fluctuates between biological systems and therefore, kinetics and thermodynamics might have a relevant impact on drug design and efficacy. Thermodynamic and kinetic considerations around the most favorable compounds can be useful to study the independent variables ($k_a$, $k_d$, ΔH and ΔS), rather than a compound variable ($K_D$). Kinetics provide information about rapid (fast on fast off), to transient (medium on, medium off) or slow kinetics (slow on, slow off), whereas thermodynamics provide information about enthalpic hits that may facilitate the optimization.

In a biological system, when two molecules are attracted by different forces, the overall mechanism of the interaction can differ. To explain how strong and complex is an interaction, different binding models may be needed (52). When constructing different models to explain a biological process by a specific assay, several aspects should be considered, such as the equilibrium conditions of interaction, the ir-/reversibility of the reactions responsible for the ligand-target complexes, if a biological process is dependent on diffusion of molecules, and the possibility of more than one interaction between the ligand and target complex (53).

If we define a Ligand as L and its Target as T; and L forms a one-step complex with T, the simplest model to explain their interaction is the one-to-one model (1):

$$L + T \leftrightarrow LT$$

This model of molecular binding assumes that the interaction is fully reversible, where L can associate and dissociate from T at certain rates that are governed by weak attractive forces (54). Equilibrium of an interaction is reached when the number of complexes LT remains constant for given concentrations of L and T. Establishing equilibrium takes time.

Looking at equation 1, two important constants describe the interaction, the association rate constant ($k_a$) and the dissociation rate constant ($k_d$). Both constants give information about the interaction itself; the $k_a$ reflects the likelihood of the ligand to recognize the target (s$^{-1}$M$^{-1}$) and; $k_d$ reflects how stable the complex is (s$^{-1}$) (55). Moreover, as can be observed in equation 2, at equilibrium the $K_D$ can also be estimated by the ratio between the dissociation and association rate constants.

$$K_D = \frac{[L][T]}{[LT]} = \frac{k_d}{k_a} \quad \text{(At equilibrium)} \quad (2)$$

Depicting an interaction in real-time allows the estimation of the time needed to approach equilibrium and the values of the constants. Real-time binding
curves, which can be obtained by various methods, provide high precision in-
formation about $k_a$ (association rate) and $k_d$ (dissociation rate) of an interac-
tion. For a “one-to-one” model, the binding function is given by (55,56):

$$\frac{d[LT]}{dt} = [L] \times [T] \times k_a \times [LT] \times k_d$$

In drug design, the $K_D$ of an interaction is the most commonly used indicator of the strength of the interaction between the drug and the target. However, considering only the affinity to describe an interaction risks neglecting the real behavior in an *in vivo* environment (57,58). Biology is much more complex, and on cells, a series of different environments and mechanical events occur, which can modify how the molecule will interact with a target. For that reason, other models can compensate for certain biological events, but not all of them can explain more complex events.

Binding affinity has been traditionally used for optimizing new drugs in combination with *in vivo* pharmacokinetics. For the reasons stated above, awareness about using the kinetic study of molecular interactions for better predictions to discover powerful compounds has risen in recent years. A well-
known example is the muscarinic M3 receptor antagonists Tiotropium brom-
ide. One of the factors that probably contributed to it being the first long-
action bronchodilator was the fact that it has an incredible low $k_d$, a corre-
sponding half-life of 35h. This should be compared with the 2-30 min half-
life of other molecules from the same class (59). An interesting fact is that it shared a similar binding affinity for M3 receptors with other drugs. It should be considered at early stage drug discovery the kinetic profiling of different compounds against a target, before deciding which chemical components are preferable to a desirable kinetic profile.

In addition, thermodynamics is crucial for drug development to provide in-
formation about the core of the interaction, i.e., what is the nature of the interac-
tions responsible for the binding and dissociation of the ligand with the tar-
get. Thermodynamics provide a relationship between binding affinity and the thermodynamic properties enthalpy ($\Delta H$) and entropy ($\Delta S$). Enthalpy gives information about the non-covalent binding forces that contribute to complex formation, such as hydrogen bonds, van der Waals and electrostatic interactions. Entropy gives information about the number of degrees of freedom of the complex, providing information about conformational changes induced by the interaction between the ligand and the target. For example, native protein states have low conformational entropy, whereas a random coil has high con-
formational entropy.

$$\Delta G = \Delta H - T\Delta S$$

$$\Delta G = -RT\ln K_D$$
From the equation 4 and 5, it is clear that similar ΔG values may have differences in the ΔH and ΔS. Additionally, it is possible to correlate the thermodynamic data with the affinity value. The same is applicable for kinetic rates, since at equilibrium it is possible to obtain the $K_D$ by the kinetic rates (eq.2).

\[ \Delta G = -RT\ln K_D \]

Calculating the different kinetic rates at different temperatures can provide different values of the affinity and consequently the thermodynamic data.

One of the reasons as to why it is important to know the behavior of a biological complex at different temperatures, is the fact that some reactions may have a complex temperature dependency. This can be due to conformational changes, denaturation or degradation with the increase of temperature. However, in certain cases, lowering the temperature might have the same effect, where a protein can also lose its functionality at lower temperatures, since lowering the temperature might reduce the effect of entropy and the reaction can change from a spontaneous to a non-spontaneous reaction. In certain cases where the temperature is closer to 0ºC, we can observe this effect with specific proteins that dissociate in individual subunits (1). The importance of ΔH optimization was well observed on in the thermodynamic profiling of HIV protease drugs (60).

![Diagram](chart.png)

**Fig3** Relation between binding energy states and binding kinetics.
Measuring interaction kinetics in living cells

In biology, the state of equilibrium is rare because in a living system processes are dynamic, often linked in a chain or part of a cycle of fluctuations between reactants and products. In vitro research is usually performed in simpler systems with a low number of variables, based on equilibrium states and specific intervals of time. It is possible that the acellular conditions to study molecular binding are neglecting several biological events that might modify the interaction between the ligand and the target. For example, a ligand can face variations of the same target, such as post-translational modifications or mutations, conformational changes induced by the binding itself, or even face different internalization mechanisms (31,61). Moreover, the diffusion process for small molecules through cell membranes can vary due to differences in their hydrophilicity and size, changing their diffusion coefficients or their intracellular concentration (44,62–64).

Another issue to consider when measuring interactions on living material is the relevance of an experiment in a real biological environment, such as differences in metabolites, pH, cell origin and the impact of different temperatures. It can be challenging when binding is evaluated close to 37°C, where metabolism, internalization and degradation of ligands may occur (61,65,66). In cell-based assays, the effects of pH and temperature on binding characteristics are commonly ignored. However, it is known that variations in temperature (18°–35°C) or pH (5.5–8.5) can affect the measurement of the $K_D$ by a factor of 2 or 10, respectively (39,60), excluding effects due to metabolism and internalization.

Depicting interactions in cells can be done e.g. with fluorescence or luminescence. Many of the available techniques, such as enzyme linked immunosorbent assay (ELISA), are commonly done as end-point assays. A crucial requirement for end-point assays is the need to be in equilibrium for acquiring accurate results. Additionally, these methods rarely provide kinetic information.

Methods based on resonance energy transfer (RET), such as FRET can provide real-time data and are a good tool for detecting PPIs at subcellular level; however with the limitation on understanding special aspects of the PPI since the principle is related to distance-dependent phenomenon (43). Additionally, these techniques require engineered paired reporter proteins and optimization steps, which take time and effort to produce and which could even potentially disturb the functionality of the target molecule or the biological environment, resulting in misleading information about the nature of the interaction. Other techniques use the protein-fragment complementation assay (PCA) principle, where the two proteins of interest are linked to two fragments of a third protein that will be the fluorescent reporter. Currently, this principle has been used with more than 10 fluorescent proteins within BiFC assays, including GFP, YFP, CFP and Venus (67). An advantage of this assay is the fact that the PPIs
can be observed directly in living cells due to the intrinsic fluorescence of the reconstituted fluorescent protein.

Other approaches such as label-free methods have also been applied for measuring interactions in living cells (49,68). Clearly, one advantage of these methods is that the molecules of interest do not need to be labelled with fluorescent/radioactive/affinity molecules. The most prominent technologies able to provide real-time data and high throughput are based on either optical, electrochemical or piezoelectric approaches. Like other techniques, label-free techniques also have limitations. Label-free technologies have generally have lower sensitivity compared to label-based technologies (69). Due to instrumental limitations, it might be difficult to observe cellular reactions induced by the molecular binding, such as internalization or cell reactions as receptor recycling. Additionally, since extracting the kinetic rate constants from real-time interaction assays are dependent on the number of bound complexes, cells are usually fixed to minimize possible fluctuations (70). This might compromise the nature of the molecular binding in the living system or even miss dimerization of specific receptors. Based on the limitations of label-free techniques, using only these technologies for measuring interactions in cells might not be suitable for answering scientific problems.

Combining the advantageous elements of label-free and label-based approaches might therefore be the best option to achieve comprehensive and reliable data.

**RT-CBA/LigandTracer**

One of the methods to evaluate molecular interactions in living cells is the real-time cell-binding assay (RT-CBA). LigandTracer is a technology based on this method and is designed to study the interaction between ligands and living cells. It measures in real-time by continuously detecting ligands, labelled with fluorescence or radioactivity, in a target area and in a reference
area on a regular Petri dish, which is mounted on an inclined rotating support. This results in a reference-subtracted real-time binding curve that provides information about the affinity, kinetics and possibly also the binding mechanisms of the interaction between the ligand and the cells (71).

This technology has been used in an increasing number of studies, mainly with protein ligands and living mammalian cells expressing target receptors on the cell surface. Nevertheless, small molecular drugs, nanoparticles and antigens on magnetic beads have been also studied with this technology (68,72–75). Generally, a conventional LigandTracer measurement is performed with mammalian cells, and its potential for studying interactions in different living systems is wide. Recently, proximity ligand assays in suspension cells and living bacteria (where part of the work is present in this thesis) were shown to be possible (70,76).

Molecular interaction assays in living bacteria/non-mammalian cells

Bacteria, parasites or non-mammalian cells, such as insect cells, have differences in their biochemistry and molecular biology nature. Some cells have a higher metabolic rate compared to mammalian cells, differences in cell membrane composition or even mobility capacity. The nature of different cell types brings a wide number of challenges for measuring molecular interactions.

Some of these challenges start with the size of the cell. In general, a bacteria cell is only 1 µm wide and up to 5 µm long, which can bring difficulties for studying interaction in live-cell fluorescence imaging (77). Moreover, bacterial cells usually are suspension cells, unless they start to grow in biofilms. Biofilms are sensitive to washing procedures, which might make the step of washing any excess of dye more difficult, lowering the quality of the imaging acquired.

For measuring molecular interactions in bacteria, one of the most used techniques is still the conventional wide-field epifluorescence microscopy; however, the resolution is limited. Other techniques, such as laser scanning confocal microscopy can improve the axial resolution, but for measuring interactions with a higher resolution, super-resolution techniques are needed. Even if these techniques can reach up to 20 nm resolution in live-cell imaging, they still face challenges with the speed of data processing, high phototoxicity, image artefacts and that the rely on the availability of different tags for protein-protein interactions (77,78). Other approaches, such as label free biosensors are reaching the bacterial researchers. For example, in 2016, Rostova and colleagues showed one of the first examples of antibody-binding kinetics to the surface of living bacteria with a label-free optical biosensor, overcoming the small evanescent field depth into a sample medium characteristic for typical
optical biosensors (79). To evaluate intracellular interactions, several approaches have been performed as mentioned before. However, few methods can detect or give real-time information of dynamic protein-protein interactions in bacteria (80). Moreover, the general metabolic rate of bacteria is much faster than a mammalian cell, which results in a fast increase of the bacterial biomass. In real-time studies, the number of receptors should be constant for depicting the kinetic variables of the interaction. The bacterial nature can therefore bring challenges in depicting the kinetic profile in living bacteria.

In contrast, parasites have multifaceted life cycles, where they are submitted to different morphological stages. Additionally, parasites can adapt to different extra- or intracellular conditions, making them able to escape the immune system. The manipulation of these processes and pathways happens through molecular interactions that parasites use to their advantage. However, we still lack studies that have explored real-time measurements in living parasites. The need to efficiently study the complexity, heterogeneity, and dynamic processes from non-mammalian cells requires good temporal resolution, kinetic and thermodynamic measurements. A few methods can provide answers for some of these issues, however, we still do not have good solutions that combine all of them.
Aim

The aim of this PhD project was to develop, establish and apply methods for time-resolved molecular interaction analysis in non-mammalian cells. Using a living adequate model can bring new insights and platforms for evaluating new drugs targeting bacteria or parasites. The evaluation of the binding activity of the new compounds developed by AEGIS, focusing on rare/neglected diseases, in a biological environment has the potential to accelerate drug discovery for the targeted emerging diseases.

This was divided into following sub-goals:

- Understanding the impact of temperature on molecular interactions in living systems. Investigating the strengths and limitations of the RT-CBA at different temperatures.
- Developing a novel real-time method for the study of molecular interaction in living bacteria.
- Understanding the binding biology of specific molecules capable of delivering cargo in living cells.
- Exploring RT-CBAs ability to depict molecular binding in a living intracellular environment.

In brief, this project aimed to transfer the experiences from mammalian cells into a novel assay format suitable for non-mammalian cells so as to answer biological questions.
Summary of Findings

The purpose of AEGIS is to discover molecules suitable for drug development in selected rare diseases. Living bacteria is clearly closer to target organisms than purified/recombinant proteins but has traditionally challenged the scientific community its large variance in assays. This project aims to provide quality information, obtained by real-time data, with the development of a novel assay format suitable for prokaryotic and other non-mammalian cells.

Several approaches were needed for developing a suitable assay. First, the effect of temperature on RT-CBAs to ensure quality measurements with bacterial systems. Second, considering that the LigandTracer technology requires an attached target, an assay needed to be developed that allowed us to measure interactions on living immobilized bacteria. Third, expression and validation of intracellular proteins with fluorescent tags suitable for RT-CBAs were evaluated to answer biological questions.

Paper I

Impact of assay temperature on antibody binding characteristics in living cells: A case study

Aim and Background

Understanding receptor activation mechanisms and drug-receptor interactions are crucial for improving drug development. Some factors, such as binding affinity and kinetics of the ligand-receptor interactions, aid understanding of receptor activation mechanisms. Hence, real-time measurements can provide valuable information about the dynamics and mechanism of molecules in a living system. The thermodynamics of protein therapeutics should be similarly important, but in cell-based assays, the effect of temperature is rarely discussed. In manual end-point and real-time measurements most of the ligand binding studies are performed only at room temperature which does not allow estimation of thermodynamic coefficients such as enthalpy and entropy, that are dependent on temperature and that can provide additional important mechanistic information about the biological interaction between the ligand and the receptor. Additionally, challenges can occur when the binding is measured at body metabolic conditions where processes like internalization and degradation of the antibody can occur. In this study, the effect of temperature on the
binding of the monoclonal antibodies (mAb) to specific receptors on living cancer cells was evaluated with the LigandTracer technology, estimating the affinity and kinetics of the interactions at selected key temperatures.

Results
The interactions at different temperatures between two mAbs on two different human cancer cell lines were evaluated. First, the interaction between the mAb pertuzumab labelled with Texas Red (Texas Red-pertuzumab) with HER2 was studied at different temperatures on the human SKOV3 ovarian cancer cell line. Then, the interaction between the FITC labelled cetuximab (FITC-cetuximab) with EGFR were studied at different temperatures on A431 squamous carcinoma cell line. Both interactions were characterized performing a two-step incubation with two different mAb concentrations (Fig. 5 and 2).

When both antibodies were added to the cell lines at different temperatures, the time to reach equilibrium varied (Fig. 5 and 6). For example, at 37°C, when the Texas Red-pertuzumab was added to a final concentration of 4 nM, the interaction required approximately 3 h to approach equilibrium (Fig. 5C). However, at 15 and 21°C, with the same concentration, complexes were formed significantly slower and we could observe that an increase of incubation time is required to approach equilibrium (Fig. 5A-B). Increasing concentrations decreases the required time for the interaction to reach equilibrium, and when the concentration was increased to the final concentration of 12 nM.

**Fig.5** SKOV3 cells expressing HER2 were incubated with Texas Red-pertuzumab at the final concentrations of 4 and 12 nM (a step-wise increase, measured 3 h for each concentration). Measurements conducted at A) 15°C (n=2), B) 21°C (n=3) and C) 37°C (n=2). The black dotted curve depicts the study of the dissociation process (receptor saturation for 30 min with 100 nM Texas Red-pertuzumab, followed by a dissociation measurement over time at 0 nM). The different colors for the association curves are different independent experiments.
it was enough for approaching equilibrium also at 15 and 21°C. The interaction properties were estimated at different temperatures and it was noticed that the increase of temperature from 21°C to 37°C resulted in a five-fold increase of the association rate constant, $k_a$, of the interaction (data not shown). On the other hand, when increasing the temperature from 15°C to 21°C, no increase in $k_a$ was observed. For the experiments with Texas Red-pertuzumab it was not possible to study both association and dissociation processes in one run, since the dissociation process was too slow to be accurately detected during the time interval of a typical LigandTracer experiment. Thus, a non-typical LigandTracer experimental procedure was performed to study only the dissociation processes. This experiment aimed to provide more information through a longer measurement of the dissociation process, and by increasing the signal through saturation of the HER2 receptors with high concentrations of Texas Red⁸ pertuzumab. Afterwards, both curves were evaluated simultaneously through global fitting to estimate the association and dissociation rate constants at the different temperatures (Fig.5).

A similar temperature dependency in association rate was observed for the FITC-cetuximab – EGFR interaction with A431 cells. With the first concentration of FITC-cetuximab (3 nM), the time to equilibrium was detected within 1 h at 37°C (Fig. 6C). Instead, at 21°C or 15°C, this interaction required about 3 h incubation with a 3 nM concentration to approach the equilibrium (Fig. 6A-B). After 7 to 8 h, the incubation solution was replaced with new cell culture medium, allowing the detection of the dissociation process. No clear difference in dissociation rate was observed at different temperatures.

![Fig.6 A431 cells were incubated with FITC-cetuximab (3 and 9 nM added step-wise; the incubation time 3 h for each concentration) followed by the dissociation phase. Measurements were conducted at A) 15°C (n = 2), B) 21°C (n = 2) and C) 37°C (n = 2). The curves with different colors are different independent experiments.](image-url)
**Discussion**

This study provided tools for estimating an approximate level of influence of temperature on ligand-receptor interactions on living cells. In traditional cell-based assays, protein interactions are regularly studied at room temperature or even at 4-8°C. Still, in many cases, the effect of temperature is hardly considered or even neglected. By characterizing the behavior of a specific protein in *in vivo* studies, the kinetics at physiological temperatures might have a significant impact on the biological outcome compared with *in vitro* reported kinetics at lower temperatures.

The temperature effect illustrated in this paper also shows that it is important to consider the incubation time required to reach equilibrium when choosing incubation times for end-point measurements. Another issue when real-time experiments are performed at different temperatures, is the fact that we should consider that the kinetics usually change up to a factor of 10 when comparing the physiological temperature of 37°C with low temperatures (4-8°C) (81,82). Moreover, working with temperatures on different adherent living cell lines can be a challenge, since biological parameters such as viability, metabolism and cell adherence must be considered.

Finally, it is necessary to highlight that this study was also designed to provide fundamental tools for exploring intracellular interactions in living cells in a metabolic condition. Studying internalization processes requires experimental procedures at physiological temperatures, and since the project is aimed at non-mammalian cells the physiological temperature is not necessarily 37 °C.

**Paper II**

Detecting ligand interactions in real-time on living bacterial cells

**Aim and Background**

In this article, the application of real-time mammalian cell binding assay with LigandTracer technology was extended to cover Gram-positive and Gram-negative bacteria. Assays for studying interactions in real-time on living cells are still rare, especially in the field of microbiology field. The development of such assay to evaluate molecular interactions on bacteria with the Ligand-Tracer technology required a new immobilization method for bacteria, since this technology depends on cells being firmly attached to a Petri dish. For that reason, the Gram-negative *E. coli* CJ236 and BL21 strains as well as the Gram-positive *S. carnosus* TM300 strain were immobilized on polystyrene Petri dishes using antibody capture. With this assay it was possible to obtain kinetic binding traces with high precision, directed against surface-displayed bacterial proteins in living bacteria for as long as 10-15 hours.
Results

From different coating approaches, the most promising strategy was the use of an adsorbed anti-\textit{E. coli} antibody (ab31499, denoted Ab99). It was adsorbed to the surface of a Petri dish with a final incubation time of 3 h, enabling the immobilization of the \textit{E. coli} strains BL21 and CJ236 (with optical density of 1.0) to the Ab99-spots. FITC-Ab99 was then added to the dishes with both bacteria strains, and binding signals were detected with LigandTracer Green, confirming the presence of immobilized bacteria (Fig. 7A).

Based on the success of using adsorbed antibody for attaching Gram-negative \textit{E. coli}, a similar antibody-based immobilization strategy was applied to the Gram-positive bacterium \textit{S. carnosus}. This bacterium is a well-established bacterial model, which allows the display of specific protein receptors on the bacterial surface. Two \textit{S. carnosus} were constructed to display covalently coupled to the peptidoglycan, either a domain of unknown function (DUF) linker alone (Scar-DUF) or the DUF linker with the binding region (BR) 187–385 domain of the pneumococcal serine-rich repeat protein (PsrP). Both were immobilized with adsorbed anti-His antibody at high density on the target surface (Fig. 8A and B). The anti-His was used in these experiments since both constructs had a histidine tag. This setup was suitable to determine the affinity and kinetic binding curves of the interactions between the polyclonal FITC-labeled anti-BR antibodies and the monoclonal FITC-labeled anti-His antibody to both Scar-BR187-385 and Scar-DUF (Fig. 8C and D).
Fig 8 LT binding kinetics traces of the interaction between both FITC labeled polyclonal anti BR antibodies and FITC labeled anti-His to the surface displayed antigens of S. carnosus. A) Two different S. carnosus display strains were built with two different constructs on the bacterial surface with the DUF linker domain alone (Scar-DUF) or coupled with the BR domain of PsrP. For immobilization of S. carnosus, the anti-His antibody was adsorbed on three target areas, one control (without bacterial cells) and two loaded separately with Scar-DUF (grey) or Scar-BR (light grey). B) Phase-contrast microscopy images confirmed the presence of dense areas with bacteria Scar-DUF at the target areas and some few cells on the anti-His surface that possibly detached after the LT experiment. C) Binding traces of the polyclonal FITC labeled anti-BR antibodies to Scar-BR (black), with minimal or no binding to Scar-DUF (grey). Images after the binding experiment demonstrates the FITC-staining of immobilized Scar-BR, but not Scar-DUF or the anti-His control. D) Binding traces of the monoclonal FITC labeled anti-His to Scar-BR (grey) and Scar-DUF (black). Binding curves were fitted using a one-to-one binding model (light grey). After the experiment, phase-contrast microscopy images were taken, demonstrating FITC-staining of the immobilized Scar-DUF and Scar-BR bacteria, but not the antibody control.

Discussion
In this work, the RT-CBA technology LigandTracer was extended to cover Gram-positive and Gram-negative bacteria. We provide a method for precise analyses in relevant biological timescales in microbiology for the Ligand-Tracer platform.

Moreover, this method takes us one step closer to developing a method for analyzing intracellular interactions on non-mammalian cells, which is the main goal of the overall project. Since pharmaceuticals usually circulate in the treated organism (i.e. the human) for hours or even days it is of importance that long reliable measurements can be conducted.
Paper III
Revealing the dynamic features of STxB-Gb3 co-internalization mechanism of molecular cargo into cancer cells

Aim and Background
Shiga toxin is a toxin produced by intestinal pathogenic bacteria, such as Shigella dysenteriae and the shigatoxigenic serotypes of E. coli (STEC). These bacteria are responsible for triggering an acute infection of the intestine in humans, which is denominated as a Shigellosis. Shigellosis is a neglected tropical disease that has been estimated to have caused 270,000 deaths (2016), in particular affecting young children under the age of five in less developed countries. The reason as to why the Shiga toxin internalizes is the nontoxic B-subunit (STxB) that binds specifically to the Gb3 (also denoted CD77) receptor of mammalian cells. In some human cancers, such as lymphomas and colorectal carcinomas, the receptor Gb3 is strongly presented. One of the challenges of drugs to target efficacy is crossing different biological barriers. Since STxB has acquired the characteristics of an intestinal pathogen, such as being stable in different physiological environments and to efficiently penetrate tissue, it could be a promising cargo carrier for delivering small peptides or small molecules into cancer cells.

With the aim of evaluating the internalization process of the new STxB constructs, STxB was coupled with a FITC molecule (used as a model for small molecules in this study) or the enhanced green fluorescent protein (eGFP – used as a model for large molecules in this study) to understand its ability to internalize with coupled molecules and proteins at different conditions.

Results
The ability of LigandTracer to detect intracellular events with the StxB in the labeled forms of StxB-FITC or StxB-eGFP was explored. For this work, Daudi, Ramos (lymphoma) and HT-29 (colon cancer) cells were chosen since they have high levels of Gb3. Two different control cell lines were used for testing the binding of the StxB: a positive (Daudi) and a negative control (K562) for Gb3 presence (Fig. 9), where binding of StxB-FITC to Gb3 on Daudi was observed, but not to K562 that is devoid of Gb3.
Considering the previous results in Paper I, the binding of STxB constructs at different temperatures was explored: from low temperatures (refrigerated; 8ºC) to study a possible disruption of the internalization, to a physiological temperature for the investigation of metabolic processes (Fig. 10). In these first experiments it was possible to observe two events during the association process of STxB-eGFP at room temperature and 37ºC, with a fast binding with

![Graph](image)

**Fig 9** Determination of the specificity of StxB-FITC on Daudi cells. StxB-FITC binding on Gb3 positive Daudi cells (black) and Gb3 negative K562 cells (grey).

**Fig 10** Affinity determination of the interaction between StxB-eGFP on Daudi cells (A,B,C) and HT-29 cells (D,E,F) in LT experiments. Temperature assays were performed to understand the interaction with cells at refrigerated temperature (A,C), at room temperature (B,E) and during metabolism (37 ºC – C,F).
To validate these observations, a live-imaging confocal microscopy experiment was performed after 3 h of incubation with StxB-eGFP or STxB-FITC at 37°C, where a Z stack from the confocal suggests both constructs are being internalized after 3 h (Fig.11).

With the previous knowledge, the binding of the different constructs of STxB was explored in different conditions. When the STxB constructs are incubated for shorter times with Ramos cells, we can observe an association process that does not follow a conventional 1:1 kinetic model. This can be seen in Fig.12A-C where the binding does not reach an equilibrium level as predicted by the model. When the dissociation process is studied, an initial and considerable loss of labeled STxB is seen, followed by a small linear signal decrease. When STxB constructs are incubated for longer periods, the signal increases in a linear fashion after the initial more rapid association phase and the initial rapid
dissociation was less abundant than at shorter incubation times, reflecting the possibility of more internalized STxB (Fig. 12B-D).

A second approach to studying the behavior of the STxB constructs was the concentration dependency binding, HT-29 cells were incubated with a concentration series of STxB-eGFP. When normalizing the data after 30 minutes of association, it is clear that the curve shape is similar for the first 30 minutes of incubation (Fig13). At longer incubation times, it seems that an equilibrium binding level is reached within one hour at the low 3 nM concentration while at higher concentrations the signal increases linearly in a concentration dependent fashion after incubating for more than two hours. Internalization rates were estimated by relating the slope of the linear increase at different concentrations, with the surface-bound STxB-eGFP at equilibrium.

![Fig.12](image1.png)  
**Fig.12** Time dependency of StxB-FITC (A,C) and StxB-eGFP (B,D). StxB-FITC was incubated with concentrations of 30 and 90 nM at (A) short times of 30 min and 1h or at longer times C) 3h plus 3h. StxB-eGFP was incubated with concentrations of 30 and 90 nM at (B) short times of 30 min and 1h or at longer times D) 3h plus 3h

![Fig.13](image2.png)  
**Fig.13** Normalization of concentration series at 30 min (left). Percentage of STxB-eGFP internalized per hour related with the surface bound STxB-eGFP (right).
conditions at lower concentrations (3nM). The increase in concentration promotes the increase of internalization of STxB-eGFP.

It is known that STxB binds to Gb3 receptors and recruits more receptors leading to clustering and a cell membrane curvature (83). To study the proximity of STxB interaction binding to Gb3 overexpressing cells in real-time, -FITC or -eGFP STxB constructs were monitored for 1-3 h with (black) or without (grey) subsequent addition of a quencher labelled form (STxB-ATTO540Q) (Figure 14A-B). The addition of STxB-ATTO540Q resulted in an immediate decrease of fluorescent signal that deviates from the binding signal for STxB-FITC and STxB-eGFP, reflecting quenching of the fluorescent STxB constructs. For experimental controls, displacement assay was performed with unlabeled STxB, however, once unlabeled STxB was added to the cells, an unexpected increase of the signal was observed by the pre-incubated STxB-eGFP (Fig 14C-D). This reflects that by adding unlabeled STxB, additional binding of STxB-eGFP is promoted even though unlabeled STxB also displaces a portion of STxB-FITC (Fig 14A).

**Discussion**

Building cargo carriers for drug delivery to a specific target presents numerous challenges, such as life-time in the organism, crossing physiological barriers, penetrating tissue, pH and degradation by proteases. Finding strategies to fight physiological conditions to improve drug delivery, such as toxin-based con-
structs, can be a promising strategy. However, coupling molecules and peptides to a molecular cargo carrier can change the binding capacity of the cargo carrier itself when penetrating the cell. Methods that can provide rapid information about how newly designed constructs behave in living cells by detecting their binding kinetics at the surface and following internalization can help researchers to promptly deliver more efficacious therapies. In this study, with knowledge from Paper I, we can investigate in real-time how different properties, such as the temperature and the size of the molecule connected to STxB, can modulate the binding and internalization capacity of STxB construct in living cells. Moreover, we can understand that the binding of STxB constructs is a complex dynamic system that involves a fast binding, internalization and rapid recycling of receptors. Understanding new features from the STxB binding dynamics can bring new insights in the study of similar toxins on the cell membrane.

Paper IV
Bioengineering living cells for measuring intracellular interactions of small-molecules in real-time

Aim and Background
Nowadays, pharmaceutical companies face challenges to deliver approved drugs due to high investment costs of R&D and is estimated that the success rate of a small molecule to enter clinical trial is just 10%. One of the most important factors for drug efficacy is the exposure of a drug to its target. For that reason, pharma industry is joining efforts to study the molecular binding in intracellular targets with the current technologies. However, there is a need for methods to detect intracellular drug action and drug concentrations. Understanding how molecules are exposed and bind to intracellular targets can improve the success of new compounds and decrease R&D costs. One approach that has been used in the past is to bioengineer proteins to enable detection of interactions. In this study, we bioengineered human carbonic anhydrase II (hCAII) with the final aim of evaluating binding of sulfonamides in an intracellular environment in living cells. The aim of this study was to observe a small molecule binding to a fusion protein of hCAII with a green fluorescent protein (eGFP) fusion. For that purpose, a reference sulfonamide was created together with a quencher fluorophore.
Results
The hCAII was fused on the C-terminal with an eGFP protein. To increase the flexibility between both proteins, a GSGS tag was inserted between the hCAII C-terminal and the N-terminal of the eGFP. Protein expression by different virus titrations was evaluated in RT-CBAs and it was possible to observe a typical expression curve for more than 70h (Fig 15).

![Figure 15](image)

**Fig.15** Real-time expression of hCAII-eGFP in insect cells in normalized signals.

In addition, to understand the impact of the engineered protein on the cell localization, a confocal microscopy experiment was performed. A high expression of the construct is observed by the fluorescence of eGFP and an intracellular localization of the tagged protein (Fig16).

![Figure 16](image)

**Fig.16** Live imaging of Sf9 cells incubated with Bacmid virus for 48h. A) Detection of fluorescent from the plasma membrane dye B) Detection of the eGFP fluorescence in the middle layer of the Z scan. C) Overlap of the fluorescence images.
To understand the impact of eGFP on the functionality of the binding between sulfonamides and the hCAII, a first screen of isolated sulfonamides binding to isolated hCAII-eGFP was performed with SPR. The fusion protein hCAII-eGFP was immobilized at pH=7.4 on SPR biosensor surfaces for quantification of the interaction with the Q540-AEBSA and E67 sulfonamides (Fig.17). The kinetic rate constants and the equilibrium dissociation constant (K_D) for each sulfonamide were determined by global fit analysis of sensorgrams for a series of concentrations. A one-to-one fitting model from the sensorgrams showed a K_D of 36 nM for the E67 molecule and 562 nM for the reference quenching molecule AEBSA-Q540. From past experiments by collaborators, the control molecule E67 has shown a very similar binding to hCAII, with the affinity value of 42nM, indicating that the fusion of eGFP might not affect the binding site of hCAII (84). Interaction characteristics, obtained from fitting a 1:1 model to the curves, showed that the modification of the AEBSA with an ATTO-Q540 label in the amino tail does not affect the ability of the compound to bind hCAII-eGFP.

Thereafter, with a basic knowledge of the interaction between the molecules in a pure system, RT-CBAs were performed to understand the binding capacity of the reference molecule AEBSA-Q540 and other sulfonamides. Once hCAII-eGFP expression plateau was reached, AEBSA-Q540 was incubated with a final concentration of 25 µM. A decrease of the fluorescent signal was observed during the incubation of AEBSA-Q540 that resembles an inverted binding curve (Fig.18). The medium was replaced with fresh medium and the regain of the fluorescence was observed.

Following that, a competition in binding to hCAII-eGFP by unlabeled sulfonamides and the quencher labeled sulfonamide in an intracellular environment was studied. A second unlabeled molecule with a higher affinity then

Fig.17 Sensorgrams for interactions between AEBSA-Q540 (left) and E67 (right) to immobilized hCAII-eGFP at pH 7.4. The compound was injected in a 3-fold dilution series, where the highest concentration of AEBSA-Q540 is 10uM and the E67 is 3.33uM. Black lines represent fitted curves from nonlinear regression analysis using a 1:1 interaction model.
AEBSA-Q540 was used to compete. The sulfonamide E67 was incubated after AEBSA-Q540 had had the chance to interact with the target in living cells. Based on SPR data, E67 molecules have a fast-on, fast-off interaction, however, it was possible to observe a percentage of competition between E67 and AEBSA-Q540 (Fig19).

Fig.18 Real-time expression of hCAII-eGFP in insect cells and binding of AEBSA-Q540.

AEBSA-Q540 was used to compete. The sulfonamide E67 was incubated after AEBSA-Q540 had had the chance to interact with the target in living cells. Based on SPR data, E67 molecules have a fast-on, fast-off interaction, however, it was possible to observe a percentage of competition between E67 and AEBSA-Q540 (Fig19).

Discussion

In this study, hCAII was fused to an eGFP protein to study the ability of sulfonamides to internalize into a living cell and bind to hCAII in real-time. The strategy used to evaluate binding between the small molecules and hCAII was to develop a reference compound with a moderate affinity and conjugate the quencher ATTO-Q540 that can quench the fluorescence of excited eGFP to it. The aim of this strategy was to evaluate the capability of small molecules to internalize and compete with the AEBSA-Q540 for the binding site.
Expression pattern could be followed for more than 70 hours which indicates that both the biology and the measurement system are very robust.

One of the limitations of the assay is the use of a reference compound, in this case the sulfonamide with the quencher ATTO-Q540. With this approach, we introduced a big label the ATTO-Q540, which could greatly change the binding efficiency as well the internalization capacity. In contrast, the E67 molecule bound to both hCAII and hCAII-eGFP in a very similar manner, which gave information about the fusion impact on the interaction. The use of an eGFP inert tag with a flexible linker might minimize the effect of the fusion on the binding. According to our results, the concept of this time-resolved, proximity-based displacement assay using bioengineered targets might help researchers to follow biological events or concentrations required for observing intracellular effects with small molecules.

Currently unpublished findings
This chapter provides information about other strategies that were performed to attach bacteria and current efforts on intrabacterial interactions that are still being tested.

Attaching bacteria to a petri dish (related to Paper II)
During the experimental setup for immobilizing bacteria, other coating approaches were performed based on certain biological attachment mechanisms from *E. coli*, like the use of fibronectin (85) and, the use of the chemical interaction between the positively charged polymers from Poly-D-lysine (PDL) to the negatively charged bacterial surface.

Incubating *E. coli* with a 3h fibronectin coating allowed the attachment of *E. coli* and the measurement of the interaction between the FITC labeled Ab99 to the surface of bacteria, with similar kinetics as the adsorbed antibody method (Fig20B). Nevertheless, signal levels were similar to bacteria adsorbed to uncoated polystyrene dishes, which was another approach, which reflect on a non-improvement to the overall attachment. With confocal imaging it was found that fibronectin can boost bacteria attachment, but only in limited clusters (Fig20).

Chemical entrapment with PDL was also performed and the binding levels of Ab99 were even higher than to the bacteria attached with adsorbed Ab99. Unfortunately, unspecific binding of FITC-Ab99 to PDL were observed. Confocal images did however show many bacteria in the PDL coated areas (Fig20C) so this approach may be suitable for ligands that do not bind to PDL.
Chemical entrapments such as poly-dopamine and PDL are now being used for optimizing more affordable coatings in the product LigandTracer MultiDishes 2x2 for facilitating comparative studies in a bacterial intracellular environment.

First attempts with *E. coli* BL21 expressing eGFP were performed in the MultiDish, and cell attachment was not stable compared with coatings on polystyrene dishes. First results with suitable *E. coli* poly-dopamine coatings,

![Fig.20](image-url) **Fig.20** A, B) Normalized association and dissociation curves of FITC labeled Ab99 to *E. coli* strains CJ236 (black) and BL21 (grey) attached to cell culture dishes (A – only CJ236) or to fibronectin coated polystyrene dishes (B). n = 2-4 for each combination of strain, attachment approach and incubation variant (= with/without dissociation phase). C) Confocal images of living (green) and dead (red) cells after 16 h on a rocker, when attached to fibronectin or PDL.

![Fig.21](image-url) **Fig.21** Optimization of different PolyDopamine coatings on polystyrene PetriDishes (left) and LigandTracer MultiDishes (right).
show that the non-attached bacteria disperse from the target spot in the first minutes (Fig21-left), but the signal then remained stable. In contrast, cells were not properly attached, and the signal continued to decrease throughout the measurement on MultiDishes with a poly-dopamine coating (Fig21-right). In the future, optimization of a more suitable coating to these surfaces will improve comparative studies on bacteria.

**Monitoring an intrabacterial molecular interaction**

After validation, an hCAII-eGFP bacterial vector (cloned from the insect cell vector in Manuscript IV), was transfected on *E. coli* and different molecules are being studied in a prokaryotic intracellular environment. The hCAII-assay has also been used with insect cells and has helped to validate additional molecules from the AEGIS consortium, in an intracellular environment, but are currently in the patent process and cannot be displayed.
Conclusion

The overall goal of this thesis was to evaluate molecular interactions in an intracellular non-mammalian environment, such as bacteria, to Accelerate Early staGe druG diScovery (AEGIS) in neglected tropical diseases. In recent years, pharma industry has faced several challenges, such as the number of approved drugs comparing with the increase of investment in R&D. One of the big issues is the lack of target engagement due to the low exposure of the drug, the binding on the target environment or the functional biological activity of the drug. Moreover, several diseases caused by bacteria are rising, especially multidrug resistant bacteria. The need for rapid drug discovery requires rapid validation of possible new leads. A target engagement assay where it is possible to correlate drug efficacy in vitro to drug efficacy in vivo might ensure the appropriate drug behavior on the intended target.

For that purpose, developing new approaches with RT-CBA to improve the evaluation of target engagement in bacterial models, can accelerate drug discovery in bacterial diseases. In this thesis, efforts were made to explore the temperature influence on molecular interaction in living cells, interactions in bacteria cells, and the construction of strategies for evaluating intracellular interactions between a protein target and small molecules on non-mammalian cells.

The major findings in this thesis are as follows:

- When evaluating molecular binding in cell-based assays, there is a need to consider the assay temperature. Kinetics usually change up to a factor of 10 when comparing the physiological temperature of 37ºC with low temperatures (4-8ºC).
- RT-CBA technology can be applied to living Gram-positive and Gram-negative bacteria, producing reliable measurements for 10 h or more.
- STxB has a remarkable mechanism and capacity to efficiently bring molecules into the cell.
- The binding of a drug supplied in cell culture medium to its intracellular target in living cells can be monitored in real time.

These findings will be crucial for the AEGIS Pan-European project and similar projects. The ability to test new molecules developed against intended bacterial and parasitic targets in a biological environment has the potential to ac-
celerate drug discovery for the intended targets. Additionally, the development of these kinds of new RT-CBA tools can offer new information for targets that are not properly expressed in bacterial cells but, instead, can be expressed in insect or mammalian cells. For that reason, I believe that this new approach for evaluating internalization capacity and studying molecular interactions at the intracellular level can help research to better understand the real behavior of new molecules. The most important conclusion, in my opinion, is that time is crucial in understanding new features from the STxB binding dynamics, and that the importance of time should not be neglected in kinetic studies on living cells. Still, a couple of factors such as the native protein levels on the living systems or bacterial native growth, are a limitation to consider for future development of molecular interaction analysis within RT-CBAs.
Future at a glance

As an Early Stage Researcher from the AEGIS Innovative Training Network (ITN) my research had the aim to provide a tool for the AEGIS combined innovative multidisciplinary approach to accelerate and decrease cost in drug design. Moreover, one of the aims of this consortium is to tackle difficult but promising targets in parasites and MDR bacteria. However, RT-CBAs for bacteria or for analyzing intracellular interactions were few or non-existent when I started this project. For that reason, before setting a platform for the AEGIS targets, I had to validate the possibility of measuring interactions with real-time innovative and affordable technologies in non-mammalian cells, with the final aim of later evaluating intracellular interactions in prokaryotic (MDR bacteria) and single-celled eukaryotic organism (e.g. protozoans).

Therefore, a first approach was constructing intracellular constructs with well-known systems from the past to understand the possibility of exploring RT-CBAs for intracellular interactions in living non-mammalian systems. In this case, *E. coli* was used as the first system due to low difficulty in expressing the AEGIS targets. Furthermore, using hCAII and sulfonamides as a first intracellular model was also a well-known system in real-time measurements and can be translated for the validated target from the Carbonic Anhydrase from *Trypanosoma cruzi* (one of the AEGIS’s target).

In the future, time-resolved assays will gain in importance, especially in biology. For example, in my project, I have found that time was of crucial importance in characterizing the STxB binding dynamics. When using manual end-point studies from the past on the STxB binding for creating an initial experimental set up in RT-CBA, our discoveries did not match with other previous results. Even if some STxB published features were confirmed with the RT-CBAs discoveries in short time incubations, the possibility of measuring in real-time for longer times has brought new insight on the binding dynamics of STxB cargo carriers. For future research, time should be considered in order to fully understand the STxB mechanism. Moreover, the fact that temperature effects were quantified in RT-CBAs can contribute to better assay design and to measuring and exploring binding thermodynamics in living systems in the future.

In the future of drug design, the characterization of binding in living elements will gain in importance. *In vitro* recombinant protein models cannot fully represent all connected elements of a metabolism, receptor dimerization and receptor recycling that can happen when working with living systems. In
this thesis, we explored strategies for measuring binding of small molecules to intracellular targets. In our study with hCAII fused with an eGFP protein we provided strategies for intracellular evaluation of the kinetic binding of small molecules. I believe with the recent advances of new methods for expression control in cells, engineering living systems for different cell assays, especially for RT-CBAs, can significantly improve drug design.

Finally, the possibility to extend these approaches for a parasitic model where the targets are engineered for RT-CBAs could help to accelerate drug discovery for neglected tropical diseases by understanding the target engagement in a native intracellular environment. Biotechnology engineers can develop more controlled expression systems for these kinds of assays and evaluate new compounds in a more realistic biological system. In my point of view, these kinds of RT-CBAs combined with the AEGIS-ITN approach can revolutionize drug design bringing rapid solutions for neglected tropical and MDR diseases.
We are facing a new era where uncontrolled drug-resistant tuberculosis is increasing in the EU, as well as malaria and trypanosomiasis in the southern hemisphere – called neglected tropical diseases. The rapid evolution of resistant bacteria is a frightening concern and the development of new drugs to fight these diseases is still a slow process. It is therefore necessary to accelerate the drug discovery process to have a fast response against new pathogens. The combination of new strategies, new technologies and new methods can rapidly improve the success of finding a new drug.

In the early steps of the discovery of new drugs, scientists investigate the causes of a disease, the responsible pathogens or what makes a certain symptom occur. During this stage, they will find out how the pathogen interacts with the host (the one who gets sick, often humans and animals). They will also characterize a possible “target” that affects this host interaction or specifically the pathogen. Commonly, this target is a protein or a complex of proteins. Quite often, the target conveys their functions through specific binding, so called molecular interactions. The study

Estamos diante de uma nova era em que o contágio com tuberculose, resistente a diferentes tratamentos, está a aumentar na UE, assim como a malária e as tripanosomíases no hemisfério sul – designadas de doenças tropicais negligenciadas. A rápida evolução das bactérias resistentes é uma preocupação assustadora e o desenvolvimento de novos medicamentos para combater essas doenças ainda é um processo lento. Logo, é necessário acelerar o processo da descoberta de medicamentos para obter uma resposta rápida contra novos agentes patogênicos. A combinação de novas estratégias, novas tecnologias e novos métodos pode melhorar rapidamente o sucesso para a descoberta de um novo medicamento.

Nas etapas iniciais da descoberta de novos medicamentos, os cientistas começam por investigar as causas de uma doença, os agentes patogênicos responsáveis ou a causa um certo sintoma. Durante essas etapas, tenta-se perceber como o agente patogênico interage com o hospedeiro (aquele que fica doente, geralmente humanos e animais). Há também um esforço para caracterizar um possível "alvo" responsável por essa interação com o hospedeiro ou especificamente o agente patogênico. Geralmente, esse alvo é uma proteína ou um complexo
of molecular interactions allows scientists to understand which regions of the target protein are responsible for the interaction itself and develop molecules that will interfere with it. Most of the time, scientists perform these experiments in pure chemical systems. In other words, the target protein is isolated and exposed to the different potential binding partner. A pure chemical system is on the one hand clean and easy to work with, but it is also lifeless. Life is more complex than pure systems, and the same target that was expressed and isolated might not behave the same way in a living environment, as it can have different isoforms (variants) or conformations (assembly forms of a protein). Moreover, even the compounds developed might not behave the same way in a living system. Lead molecules discovered in these first pure chemical assays proceed to animal and later human studies, in which they might not present the desired biological effect initially foreseen. At this late step, it is difficult to identify which factor in particular is affecting the biological effect of the new drug. For this reason, increasing the reliability of the performance estimation of a lead molecule using early stage assays is crucial for improving drug development. Methods that can create a closer environment mirroring a more complex reality are one of the possible solutions.
One approach which allows understanding how these molecular interactions happen in cells, is the real-time cell binding assay. This method has been used already for different systems, in particular with human cancer cells. In this thesis, we explored the application of this real time assay to bacterial cells for accelerating drug discovery against neglected tropical diseases. We created methods that can allow understanding of the impact of time on molecular interactions in bacterial systems and revealed foundations for the use of living pathogens in earlier research steps, which will probably provide more information to better understand biological efficacy of new drugs. In the end, our assays provide more information that can help research to improve drug design before going to animal studies, saving animals, costs and time.

In more detail, we first explored temperature as a relevant factor for biological events. Studies for evaluating drug binding in cells are usually performed at room temperature that might neglect biological events that will later interfere with the drug’s behavior. We demonstrated that it was possible to evaluate the binding of different proteins on living cells in real-time at different temperatures. This can provide tools for better understanding the behavior of molecular interactions in a living system approaching a real-life scenario.

With article II, we explored the use of time resolved methods towards desenvolvimento do medicamento. Métodos que podem criar um ambiente mais próximo ao real, representando um ambiente mais complexo, poderá ser uma das soluções.

Uma abordagem que permite entender como essas interações moleculares acontecem nas células é, do inglês, real-time cell binding assays. Este método já foi usado para diferentes sistemas, em particular com células cancerígenas humanas. Nesta tese, exploramos a aplicação deste ensaio em tempo real a células bacterianas, com o objetivo de acelerar a descoberta de medicamentos contra doenças tropicais negligenciadas. Desenvolvemos métodos que permitem compreender o impacto temporal nas interações moleculares em sistemas bacterianos, e partilhamos bases para o uso de agentes patogênicos vivos em etapas iniciais de investigação, que poderá fornecer mais informações para uma melhor compreensão da eficácia biológica dos novos medicamentos. Os nossos métodos poderão dar informações para melhorar o design de medicamentos previamente aos estudos com animais, o que permite reduzir os custos e tempo.

Em particular, primeiro exploramos a temperatura como um fator relevante para fenômenos biológicos. Geralmente, os estudos para avaliar a interação do medicamento com as células são realizados à temperatura ambiente, o que pode negligenciar o estudo de fenômenos biológicos que, posteriormente têm um papel no comportamento do medicamento. Demonstrámos que era possível ava-
bacterial cells. For this kind of assay the targets normally need to be attached to the surface. Many bacteria such as *E. coli* grow better in suspension especially with the increase of motion in their environment. We therefore investigated different techniques with chemicals or antibodies that capture bacterial cells and can be used to immobilize bacteria on the surface without altering their viability. This method can be a useful tool for characterizing different molecules or antibodies against bacterial targets. During our studies, we also explored a virulence factor of certain bacteria such as the *S. dysenteriae* and some serotypes of *E. coli* (STEC). This virulence factor is called Shiga toxin and is a protein with two parts (subunits), one responsible for binding to a receptor and following the receptor to the inside of the cells (STxB), and the other for the toxic effect (STxA). A curious fact is that the receptor that Shiga toxin binds to is present in higher levels in certain types of cancer, which creates interest in using STxB in cancer therapy as a “drug-transporter” targeting specifically the cancer cells. We studied how the STxB binds to cancer cells and its capacity to bring big and small molecules inside. During these studies, we have learned new features of STxB binding mechanisms that can provide new information to the field and possible approaches to bring molecular cargo inside cancer cells.
As mentioned before, many pharmacologically interesting targets are proteins located inside the cells. Molecules developed in pure systems against these targets might have certain binding limitations once in a living environment, such as diffusion capacity or other internal cell environment factors. The lack of knowledge about the amount of drug that reaches its therapeutic target was pointed out as one of the main causes for clinical drug failure. For example, one thematic on drug development is that intracellular drug free concentration has been considered as the same as the free drug concentration in the plasma. However, it is known that recent studies have shown that different factors can change these concentrations and ignoring this might bring misleading results about the drug efficacy. For these reasons, a method that allows understanding of the diffusion and target engagement in an intracellular environment can improve drug development and bring a rapid and less costly drug against MDR bacteria. In the article IV, we explored the use of a bioengineered protein to develop a suitable tagged-target protein to study intended molecular interactions inside cells. In this assay, we used insect cells as a first easy model to learn the different variables to consider in a eukaryotic system. Besides the fact that is an extremely explorative approach, we gained important information that might contribute to better drug design.

estudo, investigámos como STxB se liga às células cancerígenas e a sua capacidade de trazer moléculas grandes e pequenas para o meio intracelular. Durante estes estudos, aprendemos novos mecanismos da interação entre STxB e recetores celulares que podem fornecer novas informações para algumas áreas de investigação como possíveis abordagens para conduzir carga molecular para dentro das células cancerígenas.

Como mencionado anteriormente, muitos dos alvos moleculares interessantes para a indústria farmacêutica são proteínas localizadas dentro das células. Moléculas desenvolvidas em sistemas puros contra esses alvos podem ter certas limitações uma vez num ambiente vivo, como capacidade de difusão ou outros fatores do ambiente celular. O desconhecimento sobre a concentração de medicamento que chega ao seu alvo terapêutico foi apontado como uma das principais causas de falha terapêutica do medicamento. Por exemplo, uma temática sobre o desenvolvimento de medicamentos é que a livre concentração intracelular de fármacos ser considerada a mesma que a livre concentração no plasma. No entanto, sabe-se que estudos recentes mostraram que diferentes fatores podem alterar essas concentrações e ignorá-los pode trazer resultados errados sobre a eficácia do medicamento. Por esses motivos, um método que permita entender a difusão e a interação com o alvo terapêutico num ambiente intracelular pode contribuir para o desenvolvimento do medicamento e levar ao rápido desenvolvimento de fármacos
This project is a step forward to being closer to a real scenario and to understand the interaction between a drug and a target inside a bacterial cell. To attempt that, we used a multidisciplinary approach that combines biology, chemistry, mathematics and time, with the final aim of creating measurement methods as well as understanding the biological interactions that have been studied in this thesis. Many kinetic processes have a biologically relevant time-scale as you learned from the STxB binding. Thus, the initial biological effect of a molecule can be explained by kinetic properties, not only affinity. In drug development, we are slowly adapting to the “kinetic era” and this thesis brings new steps for why kinetics, especially in living systems, are important to consider in the development of new drugs.

contra as bactérias multirresistentes com menor custo. No artigo IV, explorámos o uso da bioengenharia de proteínas para desenvolver uma proteína alvo com um marcador fluorescente adequado para o estudo de interações moleculares dentro das células. Neste método, usámos células de inseto como um primeiro modelo para aprender as diferentes variáveis a serem consideradas num sistema eucariótico. Além do facto de ser uma abordagem extremamente inovadora, obtivemos informações que podem contribuir para um melhor design de medicamentos.

Este projeto é um passo em frente para nos aproximar de um cenário real e entender a interação entre um fármaco e um alvo molecular dentro de uma célula bacteriana. Para isso, usámos uma abordagem multidisciplinar que combina biologia, química, matemática e temporal, com o objetivo final de criar métodos de medição, bem como entender as interações biológicas que foram estudadas nesta tese. Muitos processos cinéticos têm uma escala temporal biologicamente relevante como a interação da proteína STxB. Assim, o efeito biológico inicial de uma molécula pode ser explicado por propriedades cinéticas e não apenas pela afinidade. No desenvolvimento de medicamentos, a adaptação à “era cinética” é lenta, e esta tese traz novos dados sobre a importância de considerar a cinética, especialmente em sistemas vivos, no desenvolvimento de novos medicamentos.
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References


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