Microdevice-integrated immunoassays for Point-of-Care Therapeutic Drug Monitoring

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Abstract

A significant number of drugs such as antibiotics, immunosuppressants or antiepileptics exhibit narrow therapeutic windows and high inter-individual pharmacokinetic variability. In such cases, Therapeutic Drug Monitoring (TDM) is compulsory in order to optimize the efficacy of the drug while avoiding adverse effects. Concentration measurements required for dose-adjustments are however complex and are therefore only performed at specialized facilities. The development of a device for accurate whole-blood quantification of critical drugs at the Point-of-Care (POC) would enable fast time-to-result and provide a more convenient solution to patients.

This thesis aimed at the design of sample-to-result devices comprising microstructures that allowed for the quantification of small molecules in blood. The designs were based on the development and use of miniaturized fluorescence polarization immunoassays (FPIA). The main feature offered by the FP assay format was the ability to perform homogeneous measurements with no separation or washing steps required, making it particularly suitable for automation.

An important result of this thesis is the demonstration that the concentration of small drugs can be quantified in whole blood within paper-like membranes using Fluorescence Polarization Immunoassay (FPIA). Different types of paper-like materials such as glass microfibers, cellulose and filter paper were screened for artefacts such as scattering or autofluorescence. Accurate determination of the fluorescence polarization of red-emitting fluorophores at sub-nanomolar concentrations was found possible within glass fiber membranes. This enabled the development of a competitive immunoassay for the quantification of the antibiotic tobramycin using only 1 µL of plasma in glass fiber micro-chambers. Furthermore, the same membrane was used for transversal separation of blood cells followed by accurate FPIA read-out at the bottom part of the micro-chamber. Within the therapeutic window, coefficients of variation were around 20% and recoveries between 80-105%, demonstrating the ability to run quantitatively both clinical chemistry and sample preparation by incorporating FPIA in glass fiber membranes.

Another important step towards a POC device was the design of a compact, bench-top FP-based analyzer. In this case, glass capillaries were used as detection chambers as they cause little light scattering and for reasons of convenience. Once assembled, the performance and functionality (i.e. ease of alignment of the optical parts, sensitivity) of the analyzer were assessed by measuring total fluorescence intensity as well as anisotropy of the prototypical fluorescent dye fluorescein. Furthermore, the new optical system was employed for tobramycin quantification previously spiked in plasma samples showing good analytical performance in terms of coefficients of variation and recoveries within the therapeutic range of the drug.

A more challenging class of drugs are the immunosuppressants, administered in cases of organ transplantation. For tacrolimus, for instance, due to its distribution within red blood cells and its very low concentration, an assay can become laborious in terms of sample preparation and implementation into microstructures. Here, several fluorescent derivatives of tacrolimus at different wavelengths were synthesized. Their affinity for various biorecognition molecules were tested allowing the choice of a ligand-receptor pair for the development of a FPIA for tacrolimus quantification in buffer. Next, attempts were undertaken to adapt the immunoassay for analysis in whole blood samples. Considerable challenges due to the complexity of the matrix were revealed and initiated efforts to improve the whole blood preparation techniques with the aim of quantifying tacrolimus within its therapeutic range.

With the aim of paving the way towards personalized therapies, this thesis makes the realisation of a POC for TDM a realistic goal.

Sommario

Un significante numero di farmaci come gli antibiotici, gli immunosoppressori o gli antiepilettici, presentano una stretta finestra terapeutica e gradi variazioni farmacocinetiche inter-individuali. In questi casi, il monitoraggio dei farmaci terapeutici è necessario per l'ottimizzazione dell'efficienza del farmaco e nello stesso tempo per evitare gli effetti avversi. Le misure di concentrazione necessarie per l'adattamento delle dosi sono comunque complesse e di conseguenza sono eseguite nelle strutture specializzate. Lo sviluppo di un dispositivo portatile e prossimo al paziente, per l'accurata quantificazione dei farmaci critici consentirebbe un risultato veloce e una soluzione più conveniente per gli pazienti.

L'obiettivo della tesi è stato il design di piattaforme analitiche con microstrutture incorporati, che hanno permesso la quantificazione di piccole molecole in sangue. Lo sviluppo di queste piattaforme è stato possibile con l'utilizzo di metodi miniaturizzati basati sulla fluorescenza polarizzata (FPIA). La caratteristica principale offerta da questo formato FP è stata l'abilità di eseguire misure omogenei, facendo questa tecnica particolarmente adatta per l'automatizzazione.

La tesi ha dimostrato che la concentrazione dei farmaci di basso peso molecolare può essere quantificata in sangue dentro le membrane di carta utilizzando il metodo di Fluorescenza Polarizzata. Diversi tipi di carta e microstrutture simili come per esempio le, cellulosi e carta da filtro sono stati analizzati in quanto riguarda la dispersione della luce ma anche l'autofluorescenza. Questo ha permesso lo sviluppo di un metodo competitivo per la quantificazione dell'antibiotico tobramicina, usando soltanto 1 µl di plasma nelle microfibre di vetro. Inoltre, le stesse membrane sono state utilizzate per la separazione trasversale del sangue competo, seguito da lettura accurata della fluorescenza polarizzata nella parte inferiore della micro-camera di misura. Nella finestra terapeutica, i coefficienti di variazioni sono stati intorno a 20% e la concentrazione ricoverata tra 80-105%, dimostrando l'abilità di eseguire quantitativamente chimica clinica e preparazione del campione tramite l'incorporamento della Fluorescenza Polarizzata dentro le microfibre in vetro.

Un significante passo verso un dispositivo POC è stato il design e sviluppo di un dispositivo analitico miniaturizzato e compatto basato sulla fluorescenza polarizzata. In questo caso, i capillari di vetro sono stati utilizzati come contenitori di misura per via della loro bassa dispersione della luce e motivi di convenienza. Una volta assemblato, la funzionalità, è stata valutata misurando l'intensità di fluorescenza totale cosi come l'anisotropia della molecola fluorescente prototipo, fluoresceina. Inoltre, il nuovo sistema ottico, utilizzato per la quantificazione della tobramicina, in precedenza aggiunta in campioni di plasma, mostra buona caratteristica analitica in termini di coefficienti di variazioni entro la finestra terapeutica del farmaco.

Una classe di farmaci più esigenti sono gli immunosoppressori, somministrati in casi si trapianto di organi. Per tacrolimus, a causa della sua distribuzione nelle cellule rosse e la sua concentrazione molto bassa, un metodo per la sua quantificazione può diventare laboriosa in termini di preparazione del campione e implementazione nelle microstrutture. Qui, diversi derivativi fluorescenti a diverse lunghezze d'onda sono stati sintetizzati. La loro affinità verso varie molecole biocompatibili è stata testata permettendo la scelta della coppia ligando-recettore adatta per lo sviluppo di FP per la quantificazione di tacrolimus in buffer. In seguito, tentativi sono stato fatti per adattare il test per l'analisi del farmaco in sangue.

Con lo scopo di aprire la strada verso le terapie personalizzate, questa tesi prevede che la realizzazione di una piattaforma analitica miniaturizzata per il monitoraggio dei farmaci è un obiettivo realistico.

Parole chiave: *monitoraggio terapeutico dei farmaci; dispositivo point-of-care; sistemi microfluidici, carta, tobramicina, tacrolimus, Fluorescenza Polarizzata;*

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List of abbreviations:

TDM Therapeutic Drug Monitoring

ADME Absorption Distribution Metabolism Elimination

PK/PD Pharmacokinetics/Pharmacodynamics

IVD *In Vitro* Diagnostics

POC Point-Of-Care

RBC Red Blood Cells

FPIA Fluorescence Polarization Immunoassays

EMIT Enzyme Multiplied Immunoassay Technique

MEIA Microparticle Enzyme Immunoassay

ATTO655-Tob Fluorescent derivative of Tobramycin

TAC Tacrolimus

TOB Tobramycin

FK BP Binding Protein

ISDs Immunosuppressants

CV Coefficient of Variations

LOD Limit of detection

LOQ Limit of quantification

UV   Ultraviolet

NIR Near Infra-Red

PDMS Polydimethylsiloxane

DBS Dried Blood Spots

PBS phosphate buffered saline solution

TBS Tris-buffered saline

SEM Scanning Electron Microscope

DMSO Dimethyl Sulfoxide

Contents

Aim and outline of the thesis

Blood tests for monitoring therapeutic drugs are nowadays exclusively performed in central laboratories, even when therapies demand for immediate decisions. This requires significant amounts of sample while being expensive and introducing significant delays that may affect the clinical outcome. Rapid and accurate whole-blood quantification of critical drugs at the point-of-care would enable fast turnaround time and provide a convenient solution for patients. The central aim of this PhD project, matured within the NanoTera Program, is to study and design immunoassays based on optical detection suitable for the implementation into a fully integrated Point-of-Care device for personalized therapeutic drug monitoring for various classes of small drugs.

In particular, this thesis presents new results on:

- assay designs with reduced and simplified sample pre-processing by exploring the ability of paper and paper-like membranes, such as glass fiber microstructures, to accommodate measurements of small drugs with optical techniques;

- incorporation of whole blood quantitative assays for small (therapeutic) molecules into paper-based microstructures while preserving the required performance of the overall test;

- development of an immunoassay format that can be universally extended to various types of microstructures such as glass capillaries;

- design and test of a novel optical bench-top system for drug monitoring with improved performance of the overall test;

- development of a simplified, novel immunoassay for an immunosuppressant used in organ transplantation;

Considering the various facets of the research, the thesis is structured in the following way:

Chapter 1 will introduce the clinical practice of TDM and key parameters like pharmacokinetics (PK) and pharmacodynamics (PD) will be defined. Moreover, classes and characteristics of drugs requiring TDM will be described as well as the analytical methods implicated in their quantification. Furthermore, by looking at gaps and opportunities in the state of the art for POC devices, challenges will be highlighted that should be considered in the development of a Point of Care test for the quantification of small molecules. The first chapter will conclude by

describing the principles of competitive immunoassays, their analytical features and the principle of FPIA.

Chapter 2 introduces a novel approach for a quantitative test for *in vitro* Diagnostics (IVD) based on a competitive optical fluorescence polarization immunoassay (FPIA), taking advantage of the features of paper and paper-based microstructures. Based on several developments to perform blood separation and drug quantification, the feasibility to measure small drugs within micro-chambers made of paper-like materials such as glass fiber microstructures was demonstrated. The choice of the fluorophore seemed to be an important factor in order to allow for measurements in whole blood and further implementation into microstructures.

Chapter 3 will describe the analytical performance of a compact FP analytical device designed by the group of Prof. Martial Geiser at the HES-SO Valais-Wallis. Here, an important aspect consists in the fact that the immunoassay is incorporated within squared glass capillaries in replacement of the standard well-plates, and thereby requires a minimal volume of reagents and sample (up to 1 μL), allowing for a minimally invasive approach for blood collection. The performance achieved with the demonstrator will be described and discussed by comparison with a commercial reader. Finally, the results will be presented of the same immunoassay carried out using serum separated from whole blood using a passive microfluidic PDMS device designed by David Forchelet from the laboratory or Prof. Phillipe Renaud, at EPFL.

Chapter 4 will focus on immunosupressants, a particular class of drugs used in organ transplantation. Here, the goal is to design a sensitive FPIA for tacrolimus (TAC) with simplified sample preparation and rapid quantification. For this reason, different fluorescent derivatives of TAC were synthesized and tested with regards to different biorecognition molecules. Moreover, different sample preparation procedures were explored and applied to whole blood samples. The current format of the assay requires further optimization for the quantification of tacrolimus within the therapeutic range in real samples.

Chapter 5 concludes the thesis with an overview on the achievements of this thesis. Future perspectives such as integration of additional sample preparation steps, multiplexing, and validation of the demonstrator in clinical settings, will also be discussed.

Chapter 1:

1. General Introduction

The research community nowadays has a great opportunity to develop novel analytical techniques, chemicals and materials that will shape the future of diagnostics. A great change in clinical practice is driven by the emergence of Point-of-Care (POC) devices, novel diagnostic and therapy tools, that will further enable the screening and monitoring of patients and lead to personalized and controlled therapy [6]. These novel developments rise a series of questions such as:

- Could fast, accurate and minimally invasive Point of Care devices improve the clinical outcome of Therapeutic Drug Monitoring and have a positive impact on the overall costs?
- How healthcare would benefit from these novel medical devices?
- What are the most appropriate approaches to develop quantitative assays of small molecules in biological samples that could be integrated within devices?

I review here some potential answers to these questions by looking at the specific needs of drug monitoring and recent progresses in the development of Point-of-Care testing.

1. 1 Therapeutic Drug Monitoring

1.1.1 Principles of Therapeutic Drug Monitoring

Quantification of drug concentrations in blood can be traced back to 1932, when lithium and digoxin were the first therapeutic agents recognized to be worth monitoring in this way [1]. Since then, a wide number of different therapeutics have been assessed for monitoring.

Therapeutic Drug Monitoring (TDM) is a multi-disciplinary clinical speciality aimed at improving patient care by individually adjusting the dose of drugs for which clinical experience or clinical trials have shown it improved outcome in general or specific populations. It can be based on *a priori* pharmacogenetic, demographic and clinical information, and/or on the *a posteriori* measurement of blood concentrations of drugs (pharmacokinetic monitoring) and/or biomarkers (pharmacodynamic monitoring)[1]. The type of drug and treatment, the metabolism, a narrow therapeutic range as well as variability between patients due to age and/or

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[1] Definition of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (https://www.iatdmct.org/about-us/about-association/about-definitions-tdm-ct.html)

physiological state (i.e. gender, pregnancy) are just some of the parameters that can strongly influence the pharmacokinetic and pharmacodynamics properties of the drug. This is illustrated in Figure 1.1, which highlights that TDM is based on the assumption that there is a defined relationship between dose and plasma or blood drug concentration.
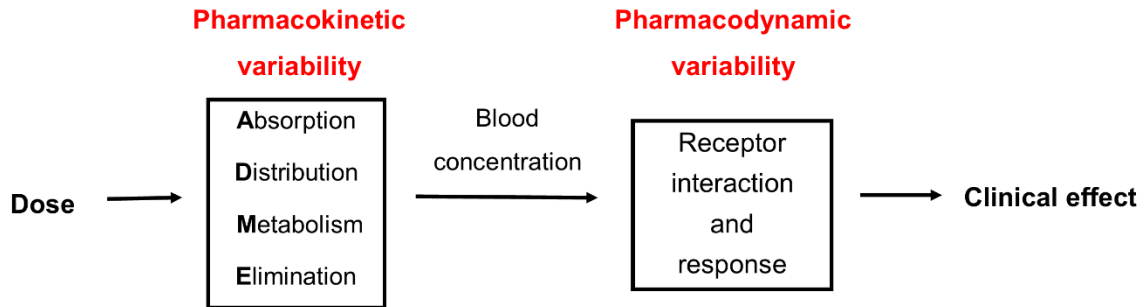
Figure 1.1: Schematic representation of the interrelationship between pharmacokinetics and pharmacodynamics, (adapted from [3]).

The course of a drug in the body is defined as **pharmacokinetics** and it combines a multitude of processes like Absorption, Distribution, Metabolism and Excretion (ADME), being described as follows:

- **Liberation**: the release of a drug from the dosage format (tablet, capsule, extended-release formulation);
- **Absorption:** movement of the drug from the site of administration (e.g. for drugs taken orally) to the blood circulation. Many factors affect this stage, including gastric pH and presence of food particles, as well as the efflux mechanism if present in the gut. First-pass metabolism plays an important role in determining the bioavailability of a drug given orally;
- **Distribution**: the movement of a drug from the blood circulation to tissues/target organs. Drugs may also be bound to serum proteins, ranging from no protein binding to 99% protein binding;
- **Metabolism** chemical transformation of a drug to the active and inactive metabolites. The liver is responsible for the metabolism of many drugs, although drugs may also be metabolized by a non-hepatic path or be subjected to minimal metabolism;
- **Excretion:** the elimination of the drug from the body via renal, biliary or pulmonary mechanisms [2], [3].

For a significant number of drugs, a given dose does not lead to identical concentration-time profiles in all patients[4]. Figure 1.2 shows an example of inter-patient variability when

voriconazole, an antifungal medication used to treat several fungal infections, is administered to new-borns.
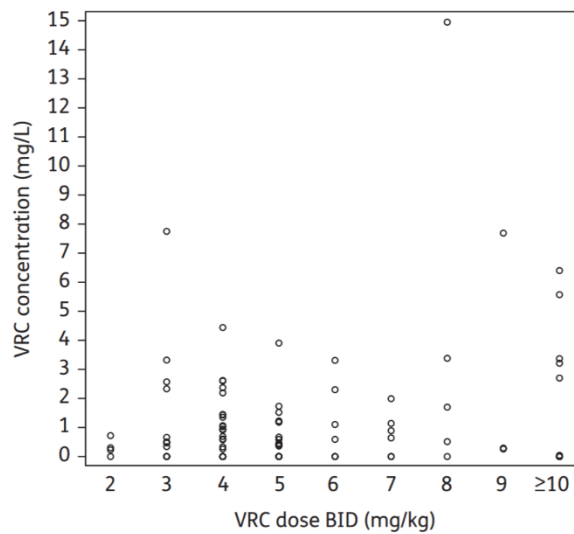
Figure 1.2: Inter-patient variability of voriconazole: drug concentration in dependence of the administered dose of voriconazole; Adapted from [5]*.*

High pharmacokinetic variability can make it difficult to predict how a patient will respond to a drug. Moreover, with decreased predictability comes increased risks of both toxicity and subtherapeutic dosing in the individual patients. To decrease inter-individual pharmacokinetic differences, doses should ideally be tailored to the individual patient (individualized dosing)[6].

As represented in Figure 1.3 and 1.4, in a dosing study it is important to consider peaks (highest concentration) and troughs (the lowest concentration) of drug levels following dose administration. The aim of administering multiple doses is to maintain the drug concentration within the therapeutic window for the entire duration of the therapy. The samples are collected at a prescribed time after the dose is administered in order capture the peak concentration while another sample must be collected prior to the next dosage to capture the trough level. Therefore, precise timing of dose administration, sample collection and concentration measurement is of critical importance to fine tune patient's next dosage.
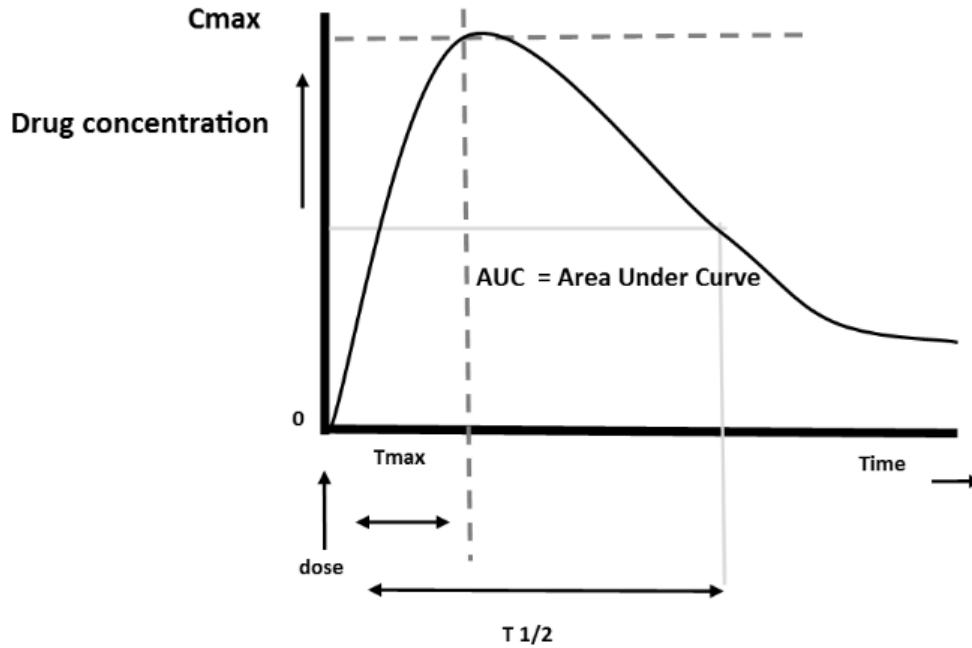
Figure 1.3: Variation of the plasma concentration of a drug with time;

The influence of the drug on the body is referred to as **pharmacodynamics** and it investigates the correlation between the drug concentration and the intensity and duration of the pharmacological effect. It considers also the biological effect of drugs, drug exposure as well as drug mechanism of action [11]. The relation between the biological effect and drug concentration is represented by dose-response curves, which enable the calculation of two main properties: the potency and the efficacy:

- **Potency** is a measure of the drug concentration needed to produce a response of given intensity and it is often quantified by the Effective Concentration (EC50) or the amount of drug that produces 50% of the maximal response
- **Efficacy** is the maximal biological effect that a drug can produce when interacting with a receptor.
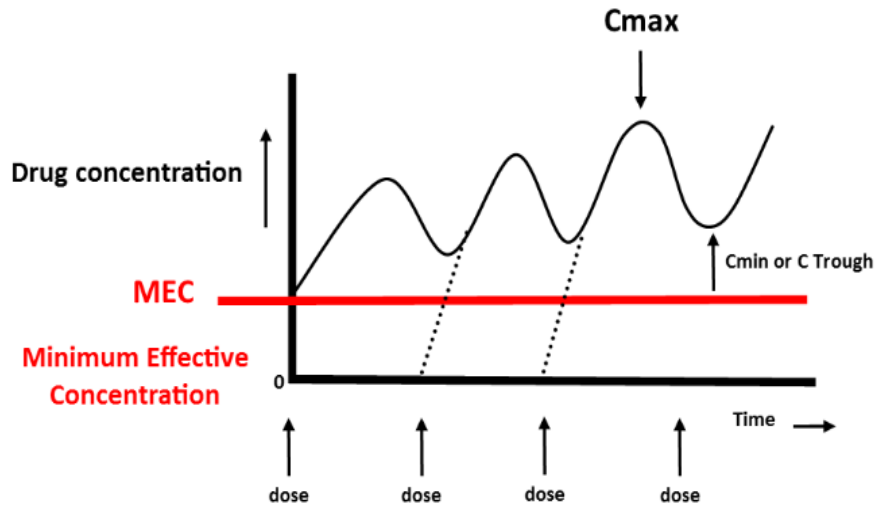
Figure 1.4: Time profile of the concentration after multiple doses where C_{max} is the maximum (or peak) serum concentration that a drug achieves in a specified compartment or test area of the body after the drug has been administrated while C_{min} is the minimum or trough concentration that a drug achieves before the administration of the next dose; MEC is the minimum concentration of a drug required to produce a desired pharmacological effect in most patients.

Therapeutic drug monitoring is required in several situations such as the following:

- **Toxicity**

  - Diagnosis of toxicity when the clinical syndrome is undifferentiated (e.g. unexplained nausea observed for a patient taking digoxin)
  - Prior adjustment of the dose to avoid toxicity (e.g. aminoglycosides, cyclosporin)

- **Dosing**

  - Optimization of the dosage (usually after reaching a steady state)
  - Assessment of adequate loading dose (e.g. after starting phenytoin treatment)
  - Dose forecasting to help predict patient's dose requirements (e.g. aminoglycosides)

- **Monitoring**

  – Assessment of compliance (e.g. anticonvulsant concentrations in patients having frequent seizures)

  – Diagnosis under treatment (particularly important for prophylactic drugs such as anticonvulsants, immunosuppressants)

  – Diagnosis of failed therapy (therapeutic drug monitoring can help distinguish between ineffective drug treatment, non-compliance and adverse effects that mimic the underlying disease) [7].

In the current context, a point-of-care device would enable drug measurements at the right time allowing for a personalized dosage and enabling maximum precision in drug therapies.

1.1.2   TDM flow nowadays

At present, TDM relies on concentration measurements performed in large medical laboratories and requires the collection of mL of blood, which might become uncomfortable for the patient on a long-term therapy. The samples need to be transferred to the specialized laboratory where the measurements are performed. After examination by the laboratory staff, the measurement results are sent to the medical doctor who is in charge of the patient. The clinical interpretation of the results represents a key challenge as the medical doctor is responsible for translating a drug concentration value into an appropriate decision of dose adjustment [8]. Figure 1.5 describes the classical procedure from sample collection to clinical interpretation and therapeutic decision, process that can sum up to several hours in a non-emergency setting, having potential detrimental consequences for patients in a critical condition.
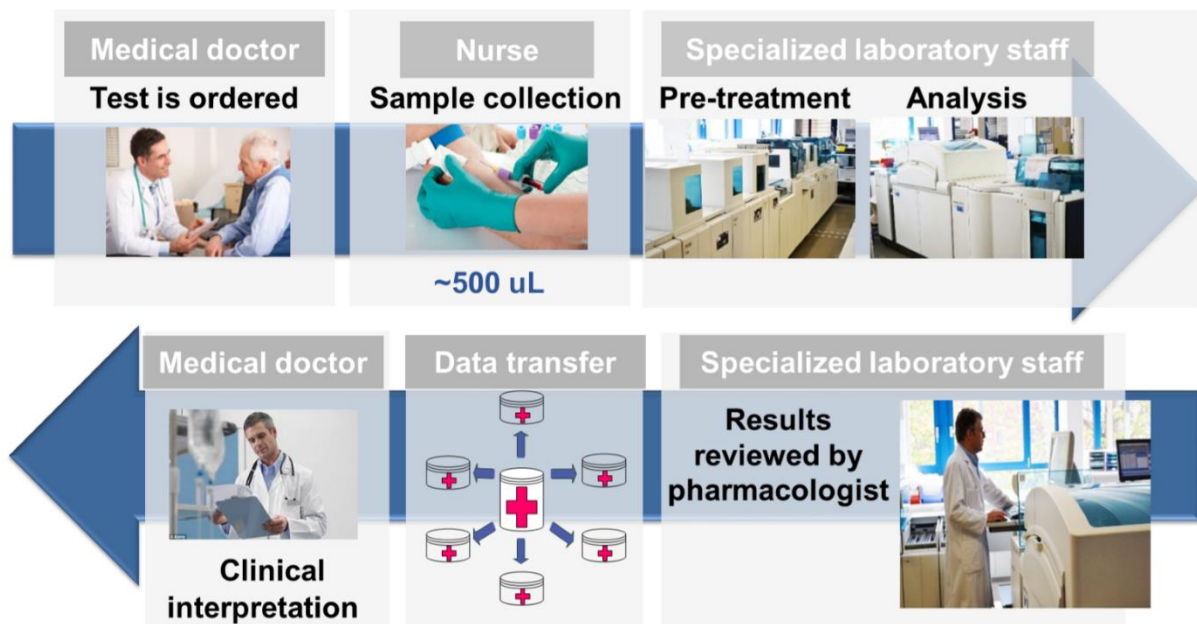
Figure 1.5: Therapeutic Drug Monitoring process today

TDM results are strongly dependent on the following factors:

- **Time sampling:** sample collection should occur at the correct time after drug administration (errors in time sampling are probably most responsible for mistakes in interpreting results);

- **Type of sample and pre-treatment:** the reagents used in the pre-treatment must be compatible and adapted to the properties of the molecules to be quantified; the pre-treatment can be complex with extraction, centrifugation, etc.
- **Method used:** the method or assay applied should be reliable, sensitive and specific, preferably with a response in less than 24 hours from the sample collection; tubes, preservatives, storage and handling conditions can impact the results;
- **Clinical interpretation of the results**: into consideration should be taken not only the concentration of the drug but also other clinical features (pharmacogenetics, age, previous analysis); food, concomitant medication, supplements can also have an influence on the results;
- **Management of the results:** drug dosage decisions [8], [9].

Therefore, the availability of an automated device to measure drug levels near the patient would simplify the time-consuming procedure by allowing medical doctors to promptly act based on the results.

1.1.3 Classes of drugs requiring TDM and current techniques

 A drug should satisfy certain criteria to be suitable for therapeutic drug monitoring and these include:

- a narrow therapeutic range
- significant pharmacokinetic variability
- a reasonable relationship between plasma concentrations and clinical effects
- an established target concentration range
- availability of cost-effective drug assays [7].

Several classes of drugs are commonly monitored to ensure a correct blood concentration, including antiepileptics, antiarrhythmic, antibiotics, antineoplastics, bronchilators, and immunosuppressants. In addition, many modern drugs involved in therapies requiring a long term administration, like HIV [10] or cancer [11], [12], are good candidates for TDM.

In this thesis, two prototypical small therapeutics belonging to two different classes of drugs were used: an antibiotic and an immunosuppressant.
Tobramycin (TOB), (Figure 16 A), a 465 Da aminoglycoside antibiotic, was employed as a prototypical drug for the design of immunoassays implemented within paper-based and capillary microstructures. Used against gram-negative bacterial infections, in particular caused

by Pseudomonas aeruginosa, its mechanism of action relies on binding to a site on the bacterial 30S and 50S ribosome, preventing formation of the 70S complex. As a result, mRNA cannot be translated into protein leading to bacterial death. This drug may be prescribed to neonates and children so that it requires special attention to control variations of its concentration in the body[13], [14]. At concentrations above the therapeutic range (>10 µg/ml), it might cause nephrotoxicity and ototoxicity [15]. Although various methods of monitoring and dose adjustment have been proposed for Tobramycin, the most common is to measure a 24-hour trough concentration and to adjust the dose to maintain the trough concentration below a value of 2, 1 or 0.5 mg/L while the requirements for peak levels are between 2-12 µg/mL (1 mg/mL of tobramycin represents 2.14 mM) [16]. Currently Tobramycin is quantified using immunoassays (FPIA and EMIT) [17] but also using chromatography methods like HPLC [18].

Tacrolimus (TAC), (Figure 1.6 B), known as FK506, is a macrolide immunosuppressant that is used for primary immunosuppression after organ transplantation [19]. With a molecular weight of 804.031 g/mol, 1mg/mL of tacrolimus represents 1.243 mM. Therapeutic drug monitoring in case of Tacrolimus is essential in order to avoid toxicity while maintaining its efficacy. As it has a large inter and intra-variability (20-80 %), Therapeutic Drug Monitoring should be performed frequently, every day until 14 postoperative days, which requires to monitor with confidence the administered dose [20], [21]. This task is particularly challenging due to its narrow therapeutic range: levels above 20 ng/mL can cause serious side effects like nephrotoxicity and neurotoxicity while levels below 5 ng/mL induce a real risk of organ rejection [22]. Moreover, Tacrolimus is strongly bound to erythrocytes and therefore whole blood must be used for quantification measurements [23]. A table summarizing the drug characteristics, the monitoring requirements and the methods employed in the analysis of immunosuppressants will be reported in Chapter 4.
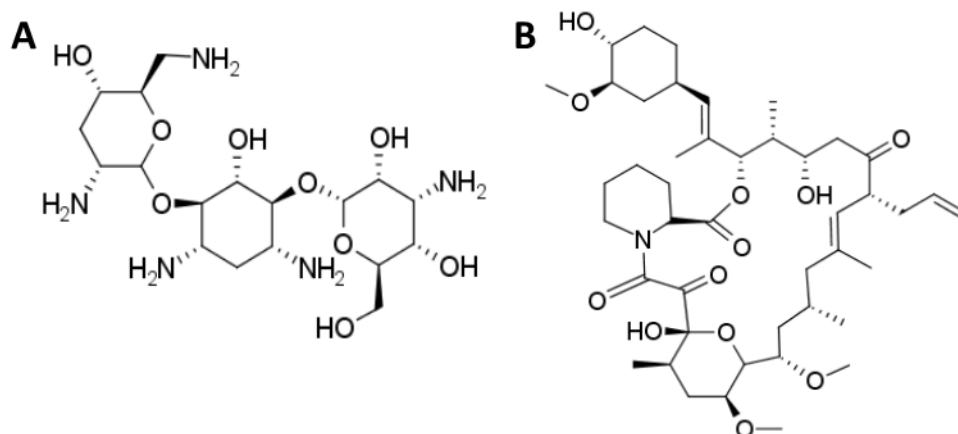
Figure 1.6: Chemical structure of A) Tobramycin and B) Tacrolimus

Selection of the right analytical method for a therapeutic drug requires the consideration of several aspects including the chemistry, the metabolism, the required precision in relation with the intended clinical use, the sample volume and the throughput as well as testing costs. The LC-MS/MS analytical platform is versatile and specific assays for almost any drug can be established. While LC-MS/MS holds the potential for greater specificity compared with immunoassays, interference issues induced by the blood matrix are a persistent problem. Failure to recognize false results caused by interference in an analysis, especially for critical drugs, could lead to serious problems for patients [24].

Immunoassays have the advantage of easy performance on multiplexed, automated, high-speed clinical analyzers, existing in most hospitals laboratories with fairly good precision; however, they are not always available, especially for newer drugs. Immunoassay developments and improvements like Florescence Polarization Immunoassay (FPIA), Microparticle Enzyme-Linked Immunoassay (MEIA), Enzyme Multiplied Immunoassay Technique (EMIT), Cloned Enzyme Donor Immunoassay (CEDIA) play a major role in Therapeutic Drug Monitoring.

1.2 Point-of-care Testing

Point-of-Care Testing (POCT) is defined as a medical diagnostic tool that supports patient diagnosis in the physician's office, an ambulance, the home, the field, or in the hospital and is typically performed by non-laboratory personnel. The results of the test are timely and allow for rapid treatment of the patient. Therefore, empowering clinicians to make decisions at the "point-of-care" has the potential to significantly impact health care delivery and to address the challenges of health disparities [25]. In limited-resource or non-existent healthcare settings, or where it is very hard to physically access relevant facilities, POC approaches can save hundreds of thousands of lives every year [26].

The benefits of POC diagnostic devices include minimal sample volume (a drop of whole blood obtained from a finger prick, up to 25 μL), short turnaround time (TAT), minimal manual input, portability, low cost, immediate clinical decision-making, and "ease-of-use" formats so that they can be successfully deployed at the primary care level [27],[65]. Based on immunoassays, they are often called one-step assays, referring to the fact that results can be obtained simply after the introduction of sample into the device.

The turn-around time (TAT) it is one of the most important benefit of POCT. For several therapies, the time of collection is critical for correct dosage interpretation. For instance, in the case of some immunosuppressants, where blood samples should be drawn within a 15-min time frame at 2 h post-dose, [28], a POC would offer flexibility in terms of timing for sample collection and no anticoagulants would need to be used [29], [30], [31].

Another reason for which a POC is preferred are the pre-analytical factors. It has been estimated that pre-analytical errors account for more than two-thirds of all laboratory errors, while errors in the analytical phase and post-analytical phase account for only one-third , and range from mistakes of filling tubes with an incorrect blood to anticoagulant ratio for coagulation tests to the use of empty or inadequately filled tubes [32]. Unfortunately, this critical area is often neglected in laboratory investigations and overlooked in quality control procedures and troubleshooting investigations [33].

The traditional limitations of POCT are related to the technologies used for performing the assay. Adequate accuracy and precision are sometimes difficult to achieve on these devices. Moreover, the methodologies employed in POCT differ from those employed in the central laboratory. Therefore, standardization across POCT sites or comparison of the results with those obtained at a reference laboratory are important measures to ensure quality. Moreover, proper training is key to make sure the test is used correctly and delivers the greatest benefit. The lack of POCT device for TDM practice might be predominantly due to the high challenges linked with the implementation of a technology for small-molecule quantification in a compact and reliable miniaturized analytical system.

1.2.1 State-of-the art of existing POCT solutions

In the last 20 years or so, the rise of large-capacity automated systems for analytical (bio)chemistry has stimulated the emergence of centralized laboratories performing a wide spectrum of diagnostic and genetic analyses. In parallel, many of new POC diagnostic tests have been introduced each year [34]. The success of glucose meters, used nowadays for measuring glycemia in diabetic patients and which are representing over 50 % of the POC worldwide, has motivated people to opt for self-testing. The number of assays available on POC platforms continues to grow and nowadays tests cover a broad spectrum of categories including thyroid, fertility, cardiac, anemia, tumor markers, drugs of abuse, toxicology, adrenal/pituitary, reproductive, allergy, infectious disease, transplant, bone metabolism, cytokines, and other special proteins[35].

To date, besides the glucose meters, the most common POC devices are the Lateral Flow Immmunoassays (LFA) test strips, which rely on capillary-force-driven, passive fluidic flow. LFA strips move samples and analytes using the wetting properties of capillaries within porous substrate materials or integrated wicking pads. Their advantages include simplicity in design, compact form, low cost, disposability, absence of moving parts, and no need for external power. Their limitations are variations in flow rate due to sample viscosity variations, site temperature, changes in the surface properties of the device over time, and poor batch- to-batch reproducibility of substrate materials such as nitrocellulose [36].

One of the first Point-of-Care devices using microfluidics and micro-fabricated platforms was the iSTAT device developed in 1983, bought by Abbott in 2004 and used for a wide range of blood chemistries like ions, chloride, glucose, gases, coagulation and cardiac markers such as troponin I. Figure 1.7 illustrates some of the commercially, hand-held POC devices, used nowadays mainly for the cardiac marker troponin, among other targets.

Figure 1.7: Examples of Point-Of-Care platforms:

A. i-STAT is a cartridge-based analyser with all the analytical reagents for the test contained within an individual cartridge. The test sample and reagents never enter the 'analyser' which transforms electrical signals from the test cartridge into readable results [37]*.*

B. Cobas h 232 point of care launched in 2007 by Roche Diagnostics, allowing the multiplex quantitative determination of Troponin T, creatinine kinase MB, myoglobin, NT-proBNP and D-dimer.

C. *Handheld Minicare I-20 from Philips launched in May 2016; troponin I quantification at sub-picomole/L concentrations in few minutes. The immunoassay is based on actuated magnetic nanoparticles; the quantification is performed optically through frustrated total internal reflection (f-TIR); The device performs whole blood separation on a single-use disposable cartridge;*

D. *Digital microfluidic cartridge based on a magnetic beads immunoassay performing multiplexed tests for quantitative troponin.*

Another field where POC devices are of high interest is illicit substance screening. Examples of commercial POCT for drugs of abuse (DOA) with molecular weight lower than 1000 Da (small molecules) are highlighted in Table 1. However, these devices are mainly qualitative and/or semi-quantitative.

Table 1: Commercially available Point-of-Care devices for small-molecule quantification

Type of POC	POCT device	Manufacturer	Application	Product features	References
Unit-use (hand-held)	Point-of-Care Diagnostic System	GenPrime	Multiplex-DOA	*High-resolution flatbed scanner *Lateral flow tests *Colour charged Coupled Device	[38]
Bench-top (sensor-based)	Xprecia Stride Coagulation Analyzer	Siemens Healthineers	Coagulation Monitoring (Warfarin)	*Tests PT/INR with lab accuracy *Fresh capillary whole blood	[39]
	Evidence, Evidence Evolution and Evidence Investigator	Randox Laboratories	Multiplex-DOA	*Up to 2000 tests/h - 2640 tests/h - 702 tests/75min	[40][41][42]
	RX daytona, daytona+, monaco, Benchtop Clinical Chemistry Analyzer	Randox Laboratories	Multiplex-DOA	*180 photometric tests/h - 270 photometric tests/h - 170 photometric tests/h	[43][44][45]
	RX monza Semi-Automated Clinical Chemistry Analyzer	Randox Laboratories	Multiplex-DOA	*128 programmable tests *192 programmable channels	[46]
	Altair™ 240 Automated Chemical Analyzer	EKF Diagnostics	Chemical Analyzer	*Dual reagent probes *240 tests/h	[47]

1.2.2 Towards point-of-care devices for small-drug quantification

The growing need for quantitative POC devices for small therapeutic drugs has been emphasized in the literature [48]. Nowadays, in the research community, efforts are being made to design novel strategies for small-drug quantification (Table 2). These efforts are either

focused on designing novel bio recognition molecules for the therapeutic drugs, or on exploring alternative materials for immunoassay implementation. Research in miniaturization is mainly focused on limited reagent consumption, decreased analysis times, increased (separation) efficiency and on enabling automation.

Table 2: Efforts in designing new technologies for small-drug quantification to be implemented into miniaturized analytical devices.

Group	Drugs targeted	Methods description	Analytical performance	Sample used	Features	Ref
Griss. et. al. Nature 2014	Methotrexate Tacrolimus Sirolimus CsA Topiramate	Bioluminescent Resonance Energy Transfer (BRET)	100 nM - 1.5 nM C_{50}=500 ± 90 nM	Diluted human serum	Lucid biosensors: * A receptor protein *A luciferase *A synthetic molecule containing a fluorophore and a ligand for the receptor protein;	[49]
Ferguson et al. Sci. Transl. Med 2013	Doxorubicin Kanamycin	Electrochemical detection	0.1-10 µM 0.13-2.5 µM	*Live rats *Whole blood	MEDIC: an electrochemical aptamer-based sensor	[50]
Zhao et al. Biosensors and Bioelectronics 2014	Methotrexate	Surface Plasmon Resonance (SPR)	28–500 nM	Human serum	Folic-acid-Au NP competition with the bioreceptor, immobilized on the SPR sensor chip;	[51]
Cappi et. al.	Tobramycin	Surface Plasmon Resonance (SPR)	10-80 µM	Human serum	Portable T-LSPR with aptamer-functionalized Nano-islands	[52]
Fornassaro et al. Faraday discussion 2016	Methotrexate	Surface Enhanced Raman Spectroscopy (SERS)	0.1-20 mM	Human serum	Au nanoparticles deposited on paper	[53]
Murakami et.al Analytical Biochemistry 2004	Tacrolimus	Enzymatic immunoassays using micro-flow antibody chip	LOD = 1 ng/mL	Whole blood Extraction with ethylacetate: hexane 1:1	Polystyrene beads coated with mouse anti-FK506 antibody	[54]
Berrettoni 2014	Tacrolimus	Internal reflection fluorescence (TIRF)	0-10 ng/mL	buffer	An optical biochip	[55]
Tachi Lab on a chip 2009	Theophylline	Fluorescence Polarization Immunoassay (FPIA)	0-40 µg/mL	Buffer	Pyrex glass substrates using standard photolithographic and wet chemical etching techniques	[56]
Menotta et al. Chimica Acta 2015	Tacrolimus	Atomic Force Microscopy (AFM)	0-30 ng/mL	Whole blood followed by extraction	AFM based- biosensor	[57]
Chang et al. Biosensors and Bioelectronics 2014	Cyclosporine A	LSAW (Leaky Surface Acoustic Wave)	LOD: 0.89 ng/ml LOQ: 2.96 ng/ml 1-1000 ng/ml	Whole blood followed by extraction	LSAW immunosensor immobilized with protein A from Staphylococcus aureus and monoclonal anti-CsA antibody on the gold electrode surface of piezoelectric crystals, which then captured the CsA	[58]

For instance, the group of K. Johnsson et al. developed a novel type of molecular biosensor able to quantify different molecules in serum, using Bioluminescence Resonance Energy Transfer (BRET) as a quantification method [49]. As quantification targets, they focussed especially on immunosuppressants (sirolimus, tacrolimus, CsA) but also on anticancer agents such as methotrexate enabling this way the possibility of multiplexing strategies.

A very ambitious approach in this sense is *in vivo* monitoring of small therapeutic molecules for TDM proposed by Ferguson et al. using the MEDIC device. [50], [59]. They use aptamers as detection molecules. Aptamers are DNA or RNA molecules selected using a procedure called SELEX and which are specific for different types of antigens. The advantage of aptamers is that they can be screened via *in vitro* processes against a synthetic library. Therefore, even for small inorganic ions or intact cells, specific DNA sequences can be found. No cell lines or animals have to be used in the production process. As soon as the aptamer is selected, a subsequent amplification by polymerase chain reaction (PCR) can produce a large amount with good purity [60]. Another promising approach using aptamers for quantification of drugs for TDM in human plasma with a hand-held SPR-based device is described by Cappi et al. [52].

Other approaches include miniaturization of already existing assays like for example Tachi et al. with FPIA within PDMS micro-channels [61]. Berretoni et al. [55] designed a novel TDM POCT biochip for the detection and quantification of calcineurin inhibitors cyclosporin A and tacrolimus and the inhibitors of the mammalian mTOR proteins, rapamycin (sirolimus) and everolimus, as well as mycophenolic acid. Peculiarly, only data for tacrolimus were obtained and although the concept proved to be quite promising, no further work was conducted.

1.2.3 Requirements and benefits of a POC for TDM

Despite the clinical importance of TDM with a wide range of different molecules requiring analysis and with time-dependent dosage decisions, clinical diagnostics continues to rely entirely on central laboratories as unique testing facilities. In an ideal situation, drug monitoring would be performed on site (or near-the patient). However, despite of all the recent efforts and advances, this is still far, predominantly due to the high challenge that represent the implementation of a technology for small-molecule quantification in a compact and reliable miniaturized analytical system.

A POC-TDM device should meet the performance requirements in terms of accuracy, precision, limit of quantification, reproducibility, repeatability and robustness Engineering a POCT must focus on providing appropriate health outcome and cost - effective data while the

tests must be reliable and easy to use. Integration, portability, low power consumption, automation are just several of the most important requirements that a Point of Care device should meet [62].

By comparison with the flow of TDM today, Figure 1.8 envisions the future of TDM using an on-site sample-to answer device.

Figure 1.8: Schematic scenario of Therapeutic Drug Monitoring using a point-of-care analyser.

In the scenario of TDM performed using a POC analyser, immediately after the physician takes the decision of measuring the drug concentration, the nurse promptly collects a fingerpick drop of blood and add it on a microchip implemented with specific sample preparation. Ideally, the device should have sensitivity within the pM range for drugs available at low concentrations but should also present high specificity for the different metabolites.

A device that allows for point-of-care testing (POCT) for TDM in real-time will help medical doctors avoiding toxic side effects and enhancing drug efficacy. The availability of such devices would allow:

- to perform the analysis within minutes in order to supply rapid results to physicians;
- to complete analytical tests without involving traditional central laboratories, thus improving the effectiveness of the process with a corresponding decrease of costs (benefiting the Health Care System) [36];
- to improve the effectiveness of some particular drugs, such as anticancer agents, while reducing their toxicity by adjusting the administered therapy in real-time based on the real plasma concentration, thus leading to an improved outcome for the patients [63].

Therefore, reducing cost, time, sample volumes, while maintaining the efficiency, are key milestones of a Point of Care design. In general, in a classical laboratory assay for drug quantification, addition of an anticoagulant such as EDTA is required to preserve the integrity of the sample. With a POC device, steps such as sample collection in tubes or storage and transportation would be avoided as the analysis would be performed on site.

The cost saving advantages of POCT are a key benefit to help promote drug monitoring so that it can reach an important position in the medical device market [37]. There are expectations that accelerating patient diagnosis and treatment could improve outcomes, which could result in substantial savings [64], [65], [66]. However, even if many studies have tried to assess the costs of POC devices versus central laboratories for different applications, there is no standardized manner to calculate costs for POCT and their estimation uses traditional approaches (fixed vs variable; direct and indirect) [67], [65]. Most of the analyses do not take into account, for instance, the financial impact of the reduced volume of blood required for POCT versus central laboratory testing [68]. The perspectives on cost of POCT may vary depending on whether you are a patient, insurance company, employee, laboratory employee, or hospital employee. The billed amount the patient or insurance company sees for POCT will usually be less than for central laboratory testing. Billing for POCT is less complete, and payment rates for a glucose meter test are less than for a central laboratory test. Therefore, the savings for patients and payers for health care are probably many millions of dollars annually, while this represents a decrease in revenue for the providers [68]. By choosing the proper sample type and reducing the size, the costs and the blood volume loss can be reduced as was shown in a study on new-borns with the introduction of a multiplexed POC-blood analysis [69]. Finally, yet importantly, the benefit of a POC device for small molecules would bring comfort to the patient. In some situations, such as organ transplantation, the drugs are administered for life-time and the patient needs to be periodically monitored. This includes traveling to the clinical laboratory and invasive sample collection.

Expanding TDM to broader populations of patients by making it available via a POC device would improve the outcome of drug therapies with less adverse effects and play a very important role in improving treatment efficacy, increasing survival and quality of life. Despite all these obvious advantages, currently, there is no robust, sample-to-result device on the market enabling quantification of small molecules for therapeutic drug monitoring. This is predominantly due to challenges in the implementation of an appropriate technology in a compact and reliable miniaturized analytical system. These challenges will be described in the following sub-chapter.

1.2.4 Challenges and opportunities

As shown in previous paragraphs, drug monitoring in combination with a POC device can have significant benefits. However, for the design of such a device, several challenging aspects have to be taken into account.

One of the most important requirements is to maintain the analytical performance after the integration into the microstructures. The Federal Drug Administration (FDA) advises that the imprecision determined at each concentration level should not exceed a coefficient of variation (CV) of 20% except at the Lower Limit Of Quantification (LLOQ), where it should not exceed 25% CV [70]. However, this might be a difficult challenge for the drugs which are administered at very low concentrations. For instance, in the case of tacrolimus, which is in the low nM range in the blood after administration, dilutions with reagents required for the assay decrease the analytical concentration down to the pM range. Hence, maintaining the required analytical performance within a miniaturized system in such case can become extremely challenging.

The ability of an assay to be specific towards the target of interest is highly desired. Some drugs have metabolites presenting similar potencies as the parent drug and their quantification is required for a correct dosage decision. For instance, significant cross-reactivity between tacrolimus and its metabolites has been reported for several immunoassays [71]. Therefore a POC device with a high degree of specificity would be highly desirable.

An interesting attribute of a POC - TDM would be a multiplexing capability meaning the capability to measure two or more analytes. Multiplexing is often preferred as it can decrease the cost per test. While in a clinical laboratory, the operator runs on the same platform protocols for different analytes using specific reagents and thus specific assays, this might be challenging to implement in a miniaturized device with drugs that exhibit various chemical and biochemical properties [72], [73].

Quality control and standardization among various testing locations and the central laboratory are ongoing issues and subject of debates for the applicability of POC devices [74]. These open issues include also the data management and the possibility to connect all the POCT from different testing locations to each other and to a central medical institution.

1.3 Immunoassays

1.3.1 Principles and classification

The term "immunoassay" is understood to refer to one of a series of methods that take advantage of the antigen – antibody reaction for detecting an analyte (for example, a hormone, a protein, or a drug) [75].

This reaction requires from a few seconds to many hours to achieve equilibrium, depending on a range of different factors. The pH, ionic strength, and temperature all affect the reaction time. The nature of the reaction can vary considerably and is of profound significance to the development of an effective assay. Early immunoassays often had overnight incubations to allow the reaction to fully reach equilibrium. However most current immunoassays involve comparatively short incubations and do not allow the reaction to reach a state of equilibrium. In these assay designs, it is even more important to understand a few simple concepts and consequences of the kinetics of the antibody–analyte reaction.

The theory of immunoassays is excellently described in the Immunoassay Handbook [76] and I summarize here the most important aspects citing some excerpts from this reference.

Immunoassay success depends on three important properties of the key reagents in their design, the antibodies:

- Their ability to bind to an extremely wide range of natural and man-made chemicals, biomolecules, cells, and viruses. This is because antibodies are proteins, and the binding sites are derived from a huge number of potential combinations of amino acid sequences. Each of the 20 amino acids has its own unique properties for binding and orientation, and chains of amino acids can twist and fold to provide binding at multiple sites.

- Exceptional specificity for the substance to which each antibody binds. The remarkable specificity of antibodies enables minute concentrations of analyte to be assayed in the presence of many closely related substances.

- The affinity between an antibody and its target by creating strong non-covalent bond that can survive the processing and signal generation stages.

The strength of the antigen-antibody reaction is referred to as the binding affinity. Binding affinity is the probability of association of a ligand (antigen) with a binding molecule (antibody). The reaction between antibody and antigen may be simplistically described by the Law of Mass Action:

$$[Ag] + [Ab] \leftrightarrow [Ag - Ab] \qquad \qquad (Equation\ 1)$$

[Ag] represents the antigen concentration,

[Ab] represent the antibody concentration,

[Ag-Ab] is the antigen-antibody complex

The equilibrium constant K_{eq}, also known as the affinity constant, can be described:

$$K_{eq} = K_a/K_d = [Ag\text{-}Ab] / [Ag][Ab] \qquad \qquad (Equation\ 2)$$

K_a is defined as the association rate constant,
K_d is the dissociation rate constant [76].

High affinity depends positively on the rate of association of the two molecules and negatively on the rate of dissociation of the complex formed.
The antibodies can be either polyclonal or monoclonal. However, for immunoassay development for drug analysis, monoclonal antibodies are more advantageous than polyclonal and this is attributed due to the higher degree of affinity and specificity towards the analyte.

Based on the labeling specifications, immunoassays can be subdivided in:
- **Label-free:** the assay does not need any label for quantification;
- **On-labelled:** meaning the use of a label, such as a fluorescent label, for the detection.

The immunoassays can be further classified in two main groups:
- **Heterogeneous:** involves a physical separation of bound and free moieties[77] as for example the Enzyme Linked Immunosorbent Immunoassay (ELISA).
- **Homogeneous:** requires only mixing of a sample and immunochemical reagents followed by detection [72].

Immunoassays have four main inter-related characteristics whose performance needs to be assessed:

- **Sensitivity**: ability to quantitatively measure minute concentrations of the analyte; the sensitivity of competitive assays is governed by three factors:
    - the equilibrium constant;
    - the error of signal measurement;
    - the level of non-specific binding, where this forms a significant proportion of the total binding [76].
- **Specificity:** ability to discriminate between closely-related molecular structures. No need for prior purification. Specificity is needed to be demonstrated since the analyte of interest is critical because most immunoassays are not preceded by extraction of the analyte from the matrix of interest[78]
- **Accuracy**: ability to provide meaningful quantification (through the use of standards);
- **Precision:** according to International Federation of Clinical Chemistry (IFCC) is minimal variation between measurements so that single or duplicate results can be trusted. Within-run precision is defined as the precision of the same sample run on

several occasions within the same assay while between run is the ability to reproduce the same result from run to run and from day to day [79].

1.3.2 Analytical aspects regarding competitive immunoassays

Competitive design uses only one antibody and is mostly applied for analytes with small molecular weight. These class of immunoassays are preferred for small-molecule quantification mainly due to the technical difficulty involved in creating an antibody 'sandwich' with relatively small analytes. Predetermined amounts of labelled drug and antibody are added to the specimen followed by incubation. In the basic design, the analyte molecules present in the specimen compete with labelled molecules for the antibody binding sites molecules. If the signal is generated when the labelled xenobiotic binds to an antibody molecule, the signal is inversely proportional to analyte concentration in the specimen (e.g., fluorescence polarization immunoassay [FPIA]). On the other hand, if the signal is generated by an unbound labelled xenobiotic, the assay signal is directly proportional to the xenobiotic concentration (e.g., enzyme multiplied immunoassay technique [EMIT]).

The advantage of homogeneous assays is that after incubation, a separation step is not required to distinguish the signal produced by antibody-bound and free label. Nevertheless, extraction of the analytes with solvents may be useful before to the immunoassay to increase selectivity[80].

Among the most commonly employed homogeneous immunoassays are:

- **Fluorescence Polarization Immunoassay (FPIA):** the method uses a fluorescent-labelled drug and an antibody specific for the drug. When the drug from the patient sample is present, it will compete with the fluorescent derivative for the binding sites of the antibody. Polarized light is then emitted in certain directions depending on whether the fluorescent derivative is bound to the antibody or not. The greater amount of the drug in the sample, more fluorescent labeled drug will rotate free in solution generating a signal inversely related to the concentration of the drug.
- **Enzyme Multiplied Immunoassays Technique (EMIT)** is an enzymatic technique based on the competition for the target analyte antibody binding sites. Free analyte analog molecules labeled with an enzyme, for instance glucose-6-phosphate dehydrogenase enzyme, are added to the test solutions to compete to the analyte to be tested. The active enzyme reduces NAD (no signal) to NADH (absorbs at 340 nm), so that absorbance is monitored at 340 nm. When labeled analyte binds to the Ab, the enzyme becomes inactive, and so the signal is generated by the free label, and signal

intensity is directly proportional to the analyte concentration [48]. It is used mainly for quantification of therapeutic drugs such as immunosuppressants or antibiotics but also for quantification of recreational drugs and different proteins.

- **Cloned Enzyme Donor Immunoassay (CEDIA):** an enzyme is genetically engineered into two inactive fragments: a small one called enzyme donor (ED) conjugated with the drug analogue, and a larger fragment enzyme acceptor (EA). When the two fragments associate, the full enzyme converts a substrate into a cleaved coloured product. If drug analyte molecules are present, they will compete with the ED-labelled drug in solution for the limited antibody sites, so that free ED-labelled drug analogue will bind to EA generating a colorimetric signal directly proportional to the amount of analyte. analysis[81].

- **Particle enhanced turbidimetric inhibition immunoassay (PETINIA):** the principle relies on the fact that, in the absence of analyte, free antibodies bind to drug microparticles conjugates to form aggregates that absorb in the visible range. When the analyte is added, the antibody binds to the free analyte preventing the aggregation of microparticles. Therefore a reduction in absorbance (or turbidimetry) is observed and the signal is inversely proportional to analyte concentration [48].

In the following paragraph theoretical aspects of FPIA principle as well as advantages will be described.

1.3.3 Fluorescence Polarization Immunoassay principle

Fluorescence polarization/fluorescence anisotropy (FP/FA) is a versatile solution-based technique that has been widely used to study molecular interactions, enzymatic activity, and nucleic acid hybridization. After its first theoretical description in 1926 by Perrin, the application has evolved from obtaining binding isotherms under carefully controlled settings to the study of small molecule-protein, antigen-antibody, and hormone-receptor binding in miniaturized automated settings. It was not until the mid-1990s that FP was adopted in high throughput screening to facilitate the drug discovery process, with its use being extended from direct interaction studies to complex enzymatic assays [82].

In particular, Fluorescence Polarization Immunoassay (FPIA) is widely used in central clinical laboratories for therapeutic monitoring of small molecules and drug of abuse testing [72]. The principle of FP derives from the fact that the degree of polarization of a fluorophore is inversely related to its molecular rotation (Figure 1.9), itself being largely driven by Brownian motion. Quantitatively, FP/FA is defined as the difference of the emission light intensity parallel (I||)

and perpendicular (I⊥) to the excitation light plane normalized by the total fluorescence emission intensity [83] [82].

Polarized light describes light waves that are only present in a single plane of space. After absorption of polarized light, small fluorescent molecules in solution rotate rapidly before the light is emitted as fluorescence. The emitted light will be released in a different plane of space from that in which it was absorbed and is therefore called unpolarized light.
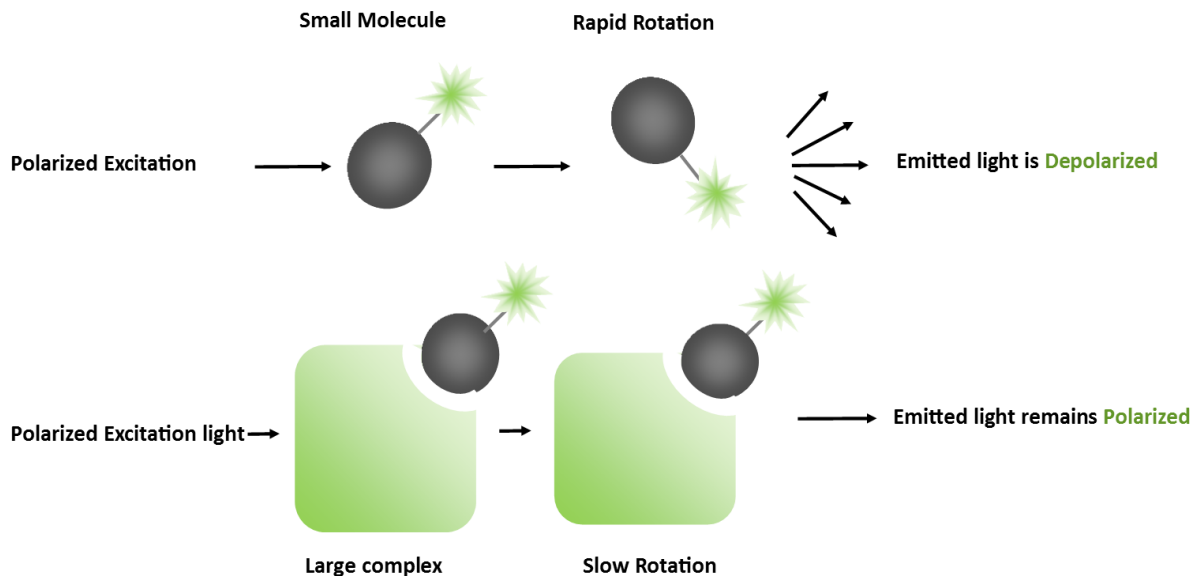
Figure 1.9: Fluorescence Polarization Immunoassay principle: fluorescein-labelled drug competes with unlabelled drug for antibody sites. The small, free drug-fluorescein rotates faster leading to less polarized emission. Larger antibody-drug-fluorescein complexes rotate slower and produce more polarized emission.

Fluorescence anisotropy is usually determined by the measurements of fluorescence emission in parallel and perpendicular planes. The degree of polarization (*P*) or anisotropy (r) is calculated according to the following equations [84]:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad \text{and} \quad r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}), \qquad (Equation\ 3,\ 4)$$

where I∥ is the fluorescence emission measured in the plane parallel to the plane of excitation and I⊥ is the fluorescence emission measured in the plane perpendicular to the plane of excitation.

The polarization (P) and anisotropy (r) values can be integrated as follows [85]:

$$P = 3r/(2 + r) \text{ and } r = 2P/(3 - P), \qquad (Equation\ 5,\ 6)$$

FA and FP, both depend on the intensity ratio, involving the same type of measurement. The dependence of the FP/FA on the molecular motility can be described using Perrin's model [86]:

$$1/P - 1/3 \ = \ (1/P0 \ - 1/3)\,(1 + \ \tau/\theta) \qquad (Equation\ 7),$$

where P_0 is the fundamental polarization of the dye, τ is the excited-state lifetime of the dye while θ is the rotational correlation time of the fluorophore. For a hydrodynamic sphere, these equations can be translated as:

$$\theta = \ \frac{\eta V}{RT} \qquad (Equation\ 8),$$

where η is the solvent viscosity, V is the molecular volume of the fluorescent dye, R is the gas constant and T is the absolute temperature.

Equation 8 completes the connection between molecular size and FP/FA, leading to the following conclusions:

- FP increases with molecular size of the dye
- FP increases with the viscosity
- FP decreases with the life-time of the dye

The difference in the way polarization and anisotropy is defined is the presence of a second perpendicular intensity term in the denominator. The anisotropy of a mixture (free tracer and tracer bound to antibody) is the weighted average of the anisotropies of the individual components, i.e. the anisotropy varies linearly with the amount of free tracer (in percentage) and for this reason fluorescence anisotropy is more used in biochemical and biophysical research.

It is important to note that the fluorescent probes must be designed to give maximum polarization/anisotropy change upon binding to the target receptor. For this reason the linker between the fluorophore and the pharmacophore must be as short and rigid as possible [87].

Furthermore, when designing competitive immunoassays, limiting concentrations of antibody and tracer are required to reach the necessary analytical sensitivity. In particular, tracer concentrations dictate the range of concentrations in which an effective competition occurs with the analyte and therefore the ultimate assay sensitivity. Therefore, the lowest possible tracer concentration that allows for reliable detection of label and does not affect competition is desirable for highest sensitivity [88].

FPIA results in an **inverse dose-response curve** (Figure 1.10) so that low levels of analyte result in a high anisotropy while high concentrations correspond to low anisotropies.
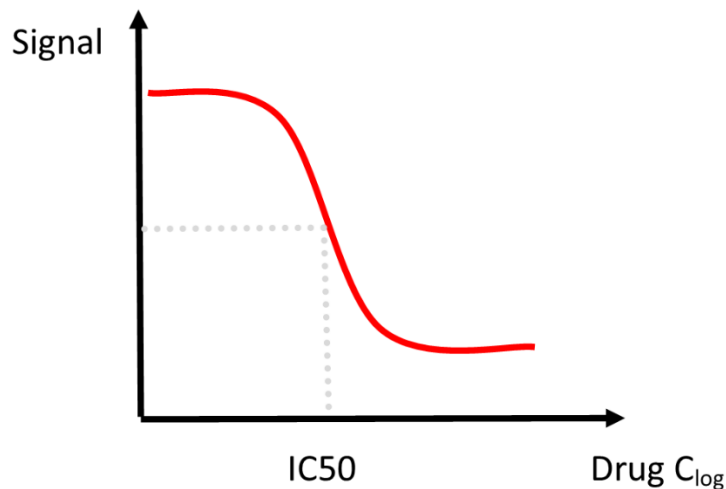
Figure 1.10: Typical FPIA dose-response curve describing the relationship between assay signal and analyte concentration, where IC50 is the concentration required to produce 50% inhibition, in this case, corresponding to 50% displacement of tracer from antibody binding. The fit is performed using a 4-parameter equation (Hill equation) and the slope is described by the rate. For ligand-binding studies, with no cooperativity in the direct binding mode, the rate is 1 while for competitive assays, the rate is different than 1 and depends, among others, on the concentration of the tracer.

FPIA is suitable for small molecules, but not large ones, for two main reasons:

- First, binding of a large antigen to an antibody that may be of similar mass produces a smaller relative change in the tumbling rate and thus a smaller change in polarization;

- Secondly, most fluorescent labels have excited state lifetimes in the range of 10^{-9}–10^{-7} s, which is too short to permit rotational reorientation of larger biopolymers [72]. It is important to note that IgG antibodies have a calculated rotation between 10^{-7} and 1.2×10^{-7} s, with the assumption that the molecule is rigid. However, several experiments showed that the observed polarization is determined by Brownian rotation, not of the whole IgG molecule but of his parts, which is substantially smaller (under 10^{-7} s), being interconnected by flexible bonds [89].

The utility of FP/FA in the clinical and biomedical sciences is large providing accurate and sensitive measurements for a variety of drugs like toxicology analytes, drugs of abuse and some hormones [90], [91].

While FP assays are relatively simple to perform with good precision and sensitivity, they require specialized instrumentation that is not routinely available or commonly used. In addition, the FPIA tests marketed by market leaders in clinical analyzers such as Roche Diagnostics with the Cobas series (Figure 1.11 A) or by Abbott Laboratories with the TDx or AxSYM analyzer (Figure 1.11 B) can only be run on their specific analyzers, further restricting the application of this platform, particularly in automated clinical laboratories[24].

A   **B**

Figure 1.11: Examples of clinical analyzers used nowadays in clinical settings: A) Cobas Integra from Roche Diagnostics B) AxSYM from Abbott Laboratories.

Chapter 2

2. Incorporation of Fluorescence Polarization Immunoassays into paper microstructures

In this present chapter, I demonstrate the feasibility of directly determining the concentration of small therapeutics in whole blood within paper-like membranes using Fluorescence Polarization Immunoassay (FPIA).

2.1 Paper-like substrates for sample preparation and quantification of drug concentration

The first paper-based sensor can be considered the invention of paper chromatography by Martin and Synge, who were awarded with the Nobel Prize in chemistry in 1952[2]. Another achievement in the field was the commercialization of the pregnancy test, which can be considered as one of the most used point-of-care biosensors. After the pregnancy test, other diagnostic point-of-care paper-based devices for diabetes and for the detection of biomarkers. More recently the development in this field has been extended towards the microfluidic paper analytical devices (µPADs) [92].

Paper and paper-like microstructures are excellent supports for diagnostics due to several key properties such as: biocompatibility, three-dimensional fibrous structure[93], high surface area [94] and facilitated storage of dried reagents [95]. Paper is also used as a support for qualitative tests for analytes with applications covering clinical diagnostics, organic and inorganic chemical analysis, environmental and geochemical analysis, and pharmaceutical and food chemistry. Lateral-flow immunoassays are a highly engineered and broadly used as paper-based diagnostic assays; they can provide "yes/no" detection of a wide range of analytes using labelled antibodies. Paper also is used in other areas such as: chemistry for synthesis of peptides and small molecules, as a platform for microarrays and lately, from drug of abuse quantification in the lateral flow assay format[96] to direct synthesis of DNA in paper [97]. [98].

Recently, Cate and al. [99] published a comprehensive review regarding the efforts in designing paper-based devices focusing on the fabrication, the incorporation of functionalities, the detection and read-out approaches within µPAD's. Figure 2.1 illustrates

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[2] https://www.nobelprize.org/nobel_prizes/chemistry/laureates/1952/press.html

some of the applications and designs of the paper and paper-based platforms in current research.

Figure 2.1: Examples of two-dimensional paper-based devices: A) Wax stamping with movable type printing; B) Wax dipping. C) Screen-printed wax device and electrodes. D) Wax drawing through a stencil. (E) Wax printing. (F) Inkjet etching of polystyrene in paper with toluene (G) Inkjet printing of AKD (H) Flexographic printing of polystyrene (I) Photoresist patterning with screen-printed electrodes. (J) Computer controlled knife cutting in nitrocellulose. (K) Laser-cut hollow channels. (L) Vapor-phase polymer deposition. (M) Chemical modification with alkylsilane self-assembling and UV/O$_3$ patterning. Adapted from [99].

The trend towards functionalization of µPAD's begins with the integration of multistep processing, preferably automated and extends to chemical surface modification or printing conductive materials for paper-based electrodes. For the detection of the analytes, the most common methods employed are: electrochemical, chemiluminescence or the colorimetric-based detection. In order to achieve quantitative analysis in paper-based diagnostics, cameras or scanners are used to record the colour intensity due to its relationship with the amount of analyte and Figure 2.2 shows examples of such devices.
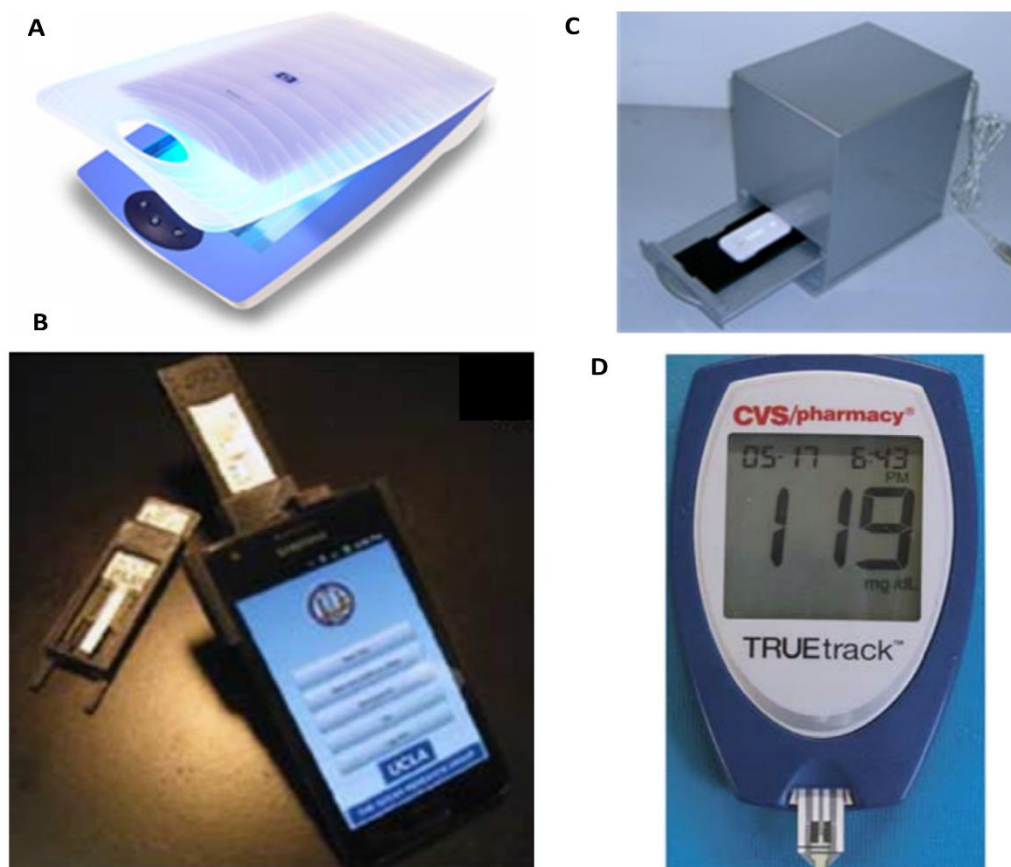
Figure 2.2: Typical methods to achieve quantitative detection of analytes (i.e. glucose) within paper. Optical-based analysis: A) Scanner-based analysis, able to obtain images of the assays results followed by image processing analysis; B) Mobile-phone based analysis; C) Reader-based analysis; D) Electrochemical based-analysis [100]

Paper-based platforms continue to progressively develop into complex devices, with a wide variability of functions and assays incorporated, in a simple-to-use format. However, it is desirable to explore and extend the applicability of paper-based microstructures also to small-therapeutics quantification.

2.2 Glass fiber-based assay for the characterization of drug content in whole blood

Other types of microstructures used in diagnostic tests are fiber-glass membranes [101]. While in a standard Lateral flow (LFA) test, glass fibers are mainly used as support for the reagents[92], or as sample pre-processing platform, recently, a model of fibre-glass membrane has been involved in the detection of protein, glucose, nitrite, pH and ketone bodies in urine[101]. The applicability of fiber-glass sheets were also previously used for C-reactive protein (CRP) quantification through fluorescence[102].

Glass fibers are well known as blood separator keeping the RBC on the surface but due to the challenge raised by the relatively rough surface characteristics [95] it has never been coupled with direct quantitative measurement in paper. For this reason, glass fiber membranes may be a good candidate as a matrix for paper-based microfluidics, as it is a material which consists of numerous extremely fine fibres of glass, with a fine capillary structure and is biochemically/ chemically inert. Even though paper is extensively used in diagnostic tests, the direct quantification of small molecules combined with sample preparation is still limited.

Herein, we demonstrate the applicability of FPIA within fiber-glass membranes for drug quantification in human samples using as model the small molecule antibiotic, tobramycin.

The assay was optimized for tobramycin quantification using whole blood. The design was characterized with respect to the fluorescence background between different types of paper chosen and the possibility to measure polarization within them. Firstly, we synthesized a novel tracer whose fluorescence is not impacted by the biological samples and we compared it with a green fluorescent dye. Due to the low fluorescent background and ability to host FP, fibres-glass membranes were fitted for our application and further chosen for Tobramycin quantification.

The proof-of-concept for drug quantification within fiber-glass membranes consists in a single-step procedure: a sample processing system (i.e. separation of red blood cells) with simultaneous small molecule quantification measurements through a competitive Fluorescence Polarization Immunoassay.

2.3   Materials and methods

2.3.1   Chemical Reagents

Tobramycin (99 % purity), kanamycin sulfate, dimethylformamide, phosphate buffered saline (PBS) were purchased from Sigma Aldrich (St-Louis, USA), Atto 655 – NHS ester from ATTO-TEC (Siegen, Germany) and paraffin from Droguerie de la Majorie (Sion, Switzerland). Fluorescently-labelled tobramycin and antibodies specific for tobramycin were from a commercial kit for FPIA on Roche Cobas Integra 400 (Roche Diagnostics GmbH, Basel, Switzerland).

Blood samples:

All blood samples were purchased from the Interregional Transfusion Centre (Bern, Switzerland). They were collected from healthy volunteers in K_3EDTA tubes, shipped and

stored at 4°C and used within a week from collection date in order to avoid the lysis of red-blood-cells. Plasma was obtained by centrifuging blood samples at 2000 rpm for 20 minutes at 4°C, was stored at -20°C and used within a month.

Spiked samples:

For the FP quantitative analysis in plasma or whole blood, a 2 mg/mL stock solution of tobramycin was prepared in Milli-Q water. From this stock solution, diluted solutions of tobramycin at 0, 5, 15, 45, 135, 405 and 1215 µg/mL, were prepared in 0.1 M phosphate buffer at pH 7.4. 10 µL of these diluted solutions were added to 90 µL of whole blood or plasma in order to obtain standard solutions with concentrations at 0, 0.5, 1.5, 4.5, 13.5, 40.5, 121.5 µg/mL in tobramycin.

2.3.2 Measurement micro-chambers in paper and paper-like materials:

Grade 1 filter paper (Whatman, USA), cellulose diagnostic paper (C083 Cellulose Fiber Sample Pad Strips, Merck Millipore, Darmstadt, Germany) and unmodified glass microfiber sheets with PVA binder (G041 Glass Fiber Conjugate Pad Strips Merck Millipore, Darmstadt, Germany) were cut in 1 cm x 1 cm squares. Micro-chambers with a diameter of 7 mm were patterned with molten paraffin on the various paper and paper-like pads using a micropipette as a mask. Once in contact with the paper material, the paraffin directly solidified. The micro-chambers were used only once for quantification. The volume of solution added for screening the different types of paper materials was 30 µL while for drug quantification in glass fiber micro-chambers, 10 µL was deposited. Once added into the detection micro-chamber, the liquids were driven by capillary action and occupied the whole chamber.

Scanning electron micrographs were taken with a Field emission scanning electron microscope ZEISS LEO 1525 (Carl Zeiss, Oberkochen, Germany) with Everhart Thornley SE detector, Inlens SE detector and Oxford EDX Xmax 20 detector.

2.3.3 Synthesis of the fluorescently-labelled tobramycin derivative:

In order to test the FPIA in paper in the near-infrared range, an Atto655 tobramycin derivative (Atto655-Tob) was synthesized by formation of an amide bond between primary amine 11 of tobramycin and a succinimidyl ester of Atto 655 following protocols developed for the preparation of tobramycin haptens used to elicit antibody responses [103]. The derivatization is based on the differential basicity and nucleophilicity of the different amines of tobramycin with amine 11 being the most basic and reactive [104]. 10 mg of tobramycin (21 µmol, 20 equivalents) were dissolved in 50 µL milliQ water and 50 µL DMF, while 1 mg of Atto655 NHS ester (1.1 µmol, 1 equivalent) was dissolved in 500 µL dry DMF. The two solutions were mixed and stirred using a magnet for 1h on an ice bath at 5°C. 50 µL of the reaction mixture were

then purified by Reverse Phase HPLC (1220 Infinity LC; RID detector 1260 Infinity and fraction collector 1260 –AS, Agilent Technologies, California, USA) on a 5 µm Luna C18 column (25 cm length, 4.6 mm internal diameter, 00G-4252-E0, Phenomenex, California, USA) at ambient temperature using a constant flow rate of 1 mL/min. The mobile phase consisted in a gradient acetonitrile / H_2O (gradient 32% ACN - 49 % ACN in 10 min). A molecular mass of $[M+H^+] =$ 977.46 (calculated: 977.5) was measured for ATTO 655-Tob ($C_{45}H_{68}N_8O_{14}S$) using an Accurate-Mass Q-TOF LC MS (6530, Agilent Technologies, Santa Clara, California, USA). Confirmation that Atto655-Tob was recognized by the antibodies was performed by measuring changes in fluorescence anisotropy in absence and presence of antibodies in 96-glass bottom well plates.

2.3.4 Instrumentation for fluorescence-polarization measurements:

A custom made optical setup was used for the measurement of the degree of polarization of the fluorescein derivative (Fluo-Tob) and Atto 655 derivatives (Atto655-Tob) of tobramycin in 96-well titer plates and in paper and paper-like materials.

The light from a diode laser at 470 nm (LDH-D-C-470, 20 mW PicoQuant, Germany) for Fluo-Tob or from a He-Ne laser at 632.8 nm (PN 05-LHR-91, 1.5 mW, Melles Griot, USA) for Atto655-Tob was directed either directly for diffraction-limited illumination or through a lens (500 mm focal length) for expanded illumination into the free entrance back port of an inverted optical fluorescence microscope (Olympus IX71, Olympus Corporation, Japan) equipped with a 10x/0.30 objective (PLNFLN, Olympus, Japan). Excitation intensities were adjusted using optical density filters so that fluorescence intensities were within the dynamic range of the detectors. The excitation light of the 470 nm diode laser was filtered using an excitation filter (MF 475-35, Thorlabs, Germany). Laser light was reflected into the objective using an appropriate dichroic mirror (for excitation at 470 nm: MD499, Thorlabs, Germany; for excitation at 632.8 nm: ZT633RDC, Chroma, USA). When using the lens, the area of illumination was around 90 µm in diameter as determined using a camera (Pco-edge sCMOS camera, PCO, Kelheim, Germany). Fluorescent solutions were either dispensed in paper and paper-like micro-chambers or in a 96-well microtiter plate with transparent glass bottom (Greiner Bio-One International, Austria). Alignment along the z-axis was performed manually based on transmission microscopy of the micro-chambers.

Fluorescent light was collected by the same objective, passed through the dichroic mirror, the filter (MF525-39, Thorlabs, Germany) and was reflected out of the microscope through a lateral port into a polarizing beamsplitter (Thorlabs, Germany), which separated parallel and perpendicular polarization. Both beams were detected using low-noise photodiode detectors PDF 10 A, 200-1100 nm, Thorlabs, Germany). Acquisition time was 15 seconds. The samples were illuminated only during acquisition to minimize photobleaching.

The signal in mV of both diode detectors was read using a NI controlled by LabView (National Instruments, Austin, Texas). Background was subtracted from the signals and anisotropies were calculated using the following formula [90]:

$$r = \frac{(I_{\mathrm{par}} - G \times I_{\mathrm{per}})}{(I_{\mathrm{par}} + 2G \times I_{\mathrm{per}})} \qquad\qquad (\textit{Equation 9})$$

where *r* is the anisotropy, I_{par} and I_{per} the intensities recorded for the parallel polarized and perpendicularly polarized beams and *G* = 0.92 is the "G"-factor accounting for the difference in detection efficiency for the two beams. The "G"-factor was determined by comparing measurements of anisotropies of 7 nM standard fluorescein solutions using the custom-made reader and a commercial SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Diagnostics, California, USA) operated with an excitation filer selected from 485 to 550 nm, an integration time of 150 ms and a temperature of 25°C with 60 µL of the solution dispensed in a 384 flat bottom microtiter plate with non-binding surface (Corning Incorporated, USA).

2.3.5 FPIA assays in serum and in whole blood

One µL of whole blood or plasma previously spiked with tobramycin was mixed with 2 µL of Atto655-Tob in PBS at a concentration of 70 nM and 18 µL of tobramycin-antibody at the concentration provided by the supplier (Figure 2.3). After gentle manual mixing, reagents were left to incubate for 3 to 5 minutes at room temperature. 10 µL of this solution were then added onto the micr o-chamber, the focus was adjusted and the anisotropy was recorded at five different locations with the micro-chamber within a time span of typically two minutes.
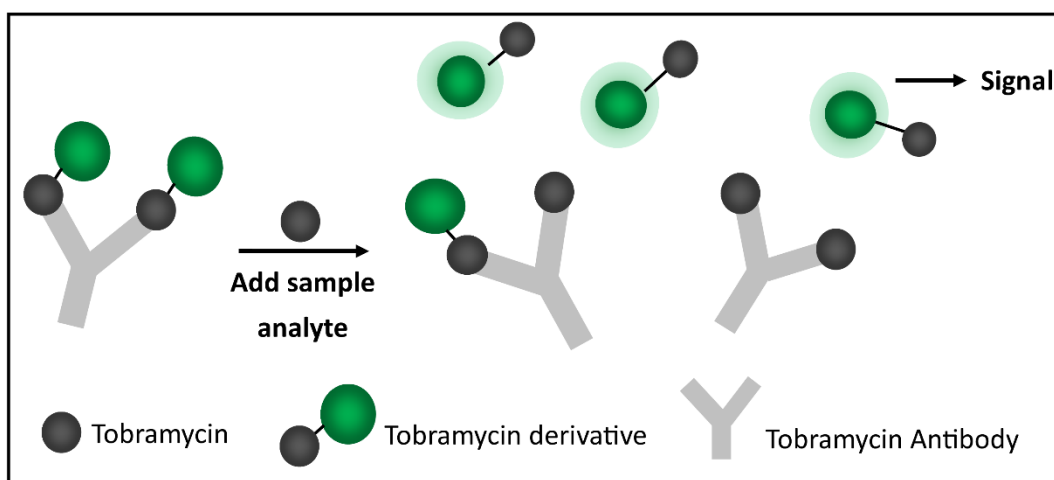

Figure 2.3: Scheme describing the competitive concept of FPIA using tobramycin antibody

The calibration curve was obtained by using serum or whole blood spiked with tobramycin at increasing concentrations encompassing the therapeutic range. The resulting calibration points were fitted in IgorPro 6 (WaveMetrics, Oregon) using a 4-parameter logistic equation:

$$r = r_{min} + \frac{(r_{max} - r_{min})}{\left\{1 + \left(\frac{IC_{50}}{c}\right)^{rate}\right\}}$$ (Equation 10)

where r is the measured anisotropy, c the concentration, r_{min} the lowest anisotropy measured in presence of an excess of unlabelled tobramycin, r_{max} the highest anisotropy measured in absence of unlabelled tobramycin, IC_{50} the concentration value corresponding to the intermediate anisotropy and $rate$ a parameter accounting for the slope of the calibration curve. Calibration curves were recorded on every measure
ment day. Concentrations were back-calculated from anisotropies using the 4-parameter logistic equation.

Statistics

All error bars in figures and graphs are standard deviations obtained from measurements performed at least in triplicates (i.e. at least 3 independent solutions obtained by mixing and incubating the reagents and which were dispensed in different micro-chambers). Triplicates were measured at different times within the same day.

2.4 Results and discussion

2.4.1 Feasibility of fluorescence polarization measurements within paper and paper-like materials

Three different types of paper and paper-like materials were investigated for their ability to accommodate accurate FP measurements: Whatman Grade 1 filter paper, cellulose diagnostic pads and glass microfiber sheets. These different materials were chosen as they strongly differed in terms of chemical properties, thickness, wettability, porosity and microscopic structure as displayed in scanning electron micrograph images (Figure 2.4 B, C, D). To simplify and standardize sample deposition, small 7 mm-wide circular micro-chambers for single-use measurements were created using a mask and wax impregnation (Figure 2.4 A).
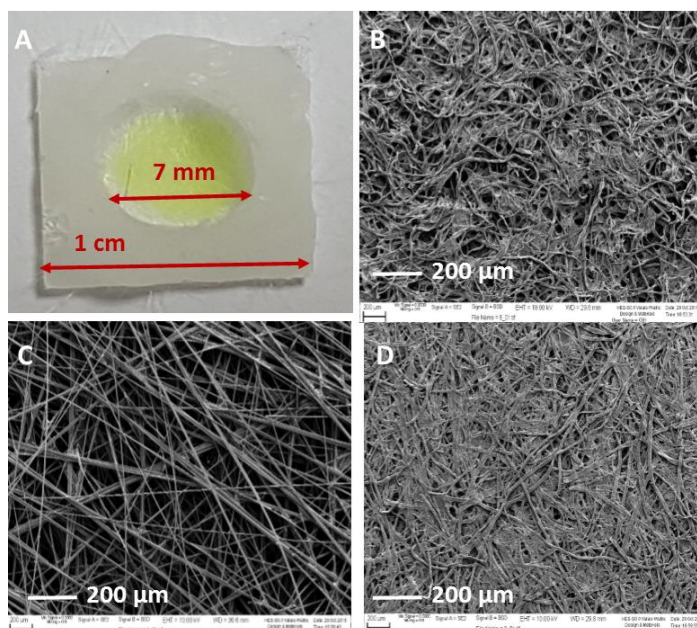
Figure 2.4: Micro-chambers made out of paper and paper-like materials used for fluorescence-polarization measurements: A) Image of a typical 7 mm-large round glass fiber micro-chamber used for FPIA made by patterning a 1 cm square glass fiber sheet with wax using a circular mask. 10 µL of a fluorescein solution was added for better visualization. B)-D) Scanning electron micrographs of the paper and paper-like materials used in this study. The microscopic structure differed considerably between diagnostic-grade cellulose pads (B), glass microfiber sheets (C) and Grade 1 Whatman filter paper (D).

The micro-chambers were prepared manually, but could easily be manufactured using standard printers [105].

Paper and paper-like matrices could adversely affect the measurement of fluorescence polarization in several ways. First, optical artifacts caused by light scattering induced by the µm-size paper fibers but also by refraction and total reflection at the water-fiber interface, in particular for glass fibers, could lead to depolarization of the excitation and the fluorescence light. On the other hand, inelastic Raman scattering at the fibers would produce red-shifted polarized light that would be transmitted through the detection filters. Second, autofluorescence of the paper materials, in particular due to additives, would contribute a signal with fixed polarization independent of the analyte. Finally, adsorption of the fluorescent reporters on the fiber would artificially increase the degree of polarization.

Deciphering which contributions impact FP measurements in the different papers required screening using different fluorescent reporters. As the effects were likely depending on the illumination and emission wavelength, two fluorescent tobramycin derivatives were employed: a commercial fluorescein derivative absorbing in the blue (λ = 470 nm) and emitting in the green (λ = 505-545 nm) and a novel Atto - 655 derivative (Atto655-Tob) with absorption in the

red (Λ = 633 nm) and emission in the near-infrared region (Λ = 650-750 nm) (Figure 2.5). FP measurements in 96-well microtiter plates and in micro-chambers made of different paper and paper-like materials were performed using a custom-made reader developed around an optical microscope.
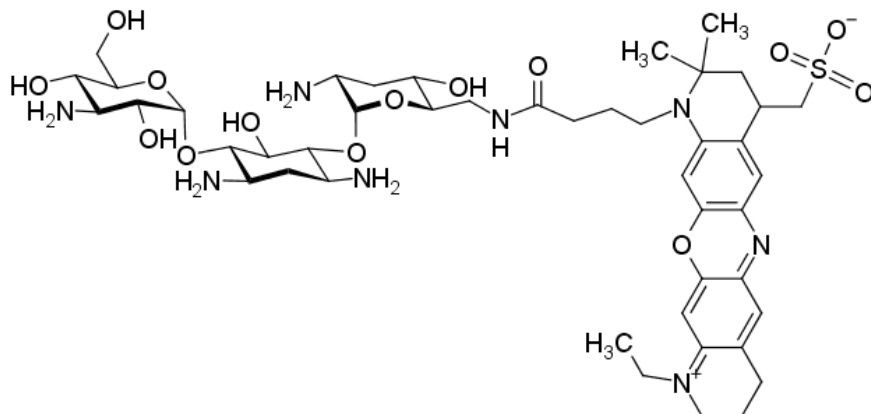
Figure 2.5: Tracer used for tobramycin quantification. Chemical structure of Atto655-Tob.

To assess the fluorescent background and the Raman scattering caused by the different paper-like materials, the signals recorded for a clear phosphate buffered saline solution (PBS) were compared with the signals obtained when using the same solution in a 96-well microtiter plate with glass bottom (Figure 2.6 A-B). Background signals were similar in microtiter plates and in glass fiber membranes, but higher in cellulose and in filter paper. This was observed both for an excitation in the blue at 470 nm (Figure 2.6 A) and for an excitation in the red at 632.8 nm (Figure 2.6 B). The effect was much more pronounced in the green, which might indicate that the background was predominantly due to autofluorescence but also to the light-scattering effect that interferes more at shorter wavelengths, as shown previously [85]. The lower fiber density in the glass fiber membranes compared to cellulosic materials and the absence of some additives might explain why the effect was hardly noticeable in the former.

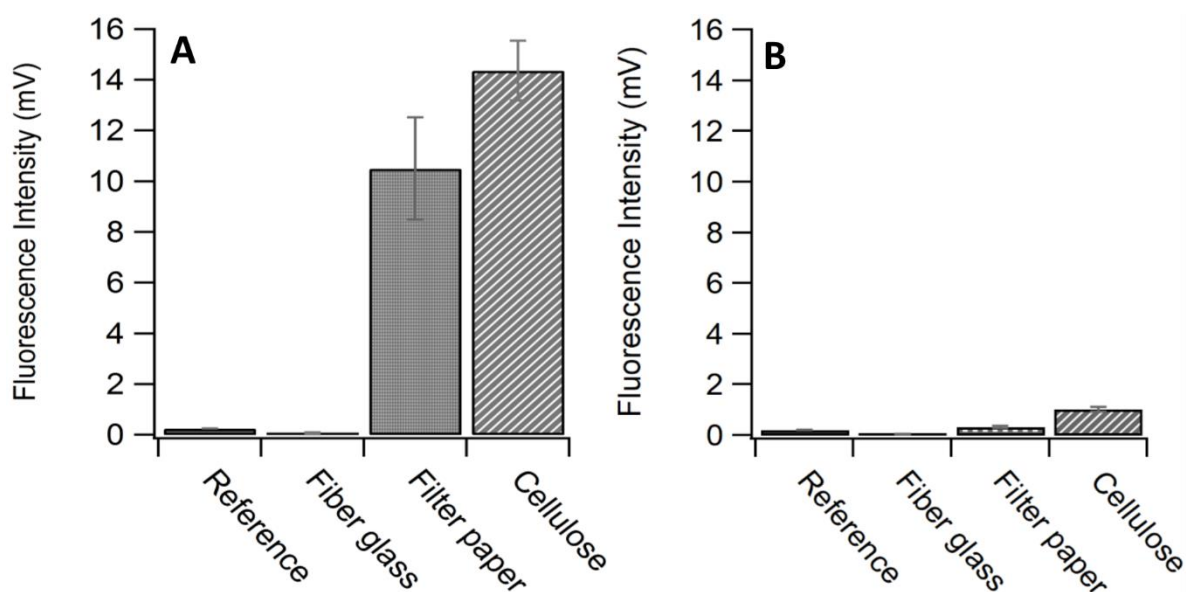*Figure 2.6: A) Fluorescence intensity of a PBS solution measured in a 96-glass bottom well plate (reference measurement) as compared with the fluorescence intensity of the same solution dispensed in micro-chambers made out glass fiber membranes, cellulose pads or Grade 1 Whatman filter paper. Excitation was performed at 470 nm and detection using filters corresponding to fluorescein emission. B) Fluorescence intensity of a PBS solution measured in a 96-glass bottom well plate (reference measurement) as compared with the fluorescence intensity of the same solution dispensed in micro-chambers made out glass fiber membranes, cellulose pads or Grade 1 Whatman filter paper. Excitation was performed at 633 nm and detection using filters corresponding to Atto 655 emission.*

In a second step, we compared the anisotropies obtained for both tobramycin derivatives when measured in the three types of paper-like materials or in a 96-well microtiter plate. Anisotropies observed in cellulose and filter paper differed in general from the reference values recorded in wells, in particular in the red. Autofluorescence as well as scattering, both effects partially compensating each other, likely affected the observed anisotropies. Values obtained for measurements in glass fiber membranes in the blue (Figure 2.7 A) were slightly higher than in wells, possibility due to some adsorption of the fluorescent derivative on the fibers. By contrast, anisotropies obtained in the red correlated very well with values obtained in microtiter plates blue (Figure 2.7 B) and could be accurately measured down to 400 pM of Atto655-Tob in glass fiber sheets (Figure 2.8 B).
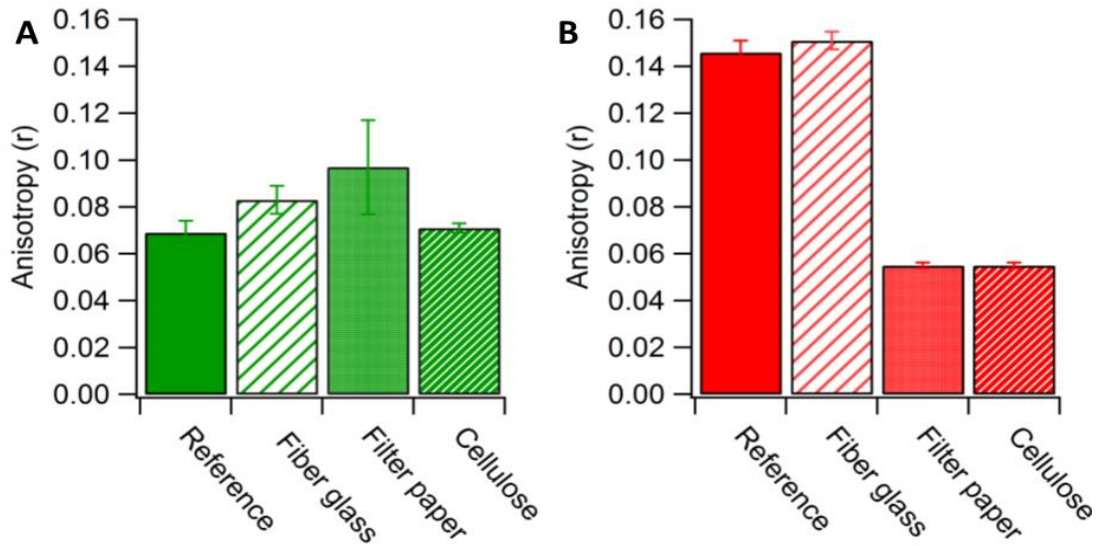
Figure 2.7: A. Anisotropy value measured for a 7nM solution of a fluorescein derivate of tobramycin in a 96-glass bottom well plate (reference measurement) compared to the anisotropy values measured for the same solution in micro-chambers made out glass fiber membranes, cellulose pads, and Grade 1 Whatman filters. B. Anisotropy value measured for a 7 nM solution of an Atto 655 derivate of tobramycin in a 96-glass bottom well plate (reference measurement) compared to the anisotropy values measured for the same solution in micro-chambers made out glass fiber membranes, cellulose pads, and Grade 1 Whatman filters.
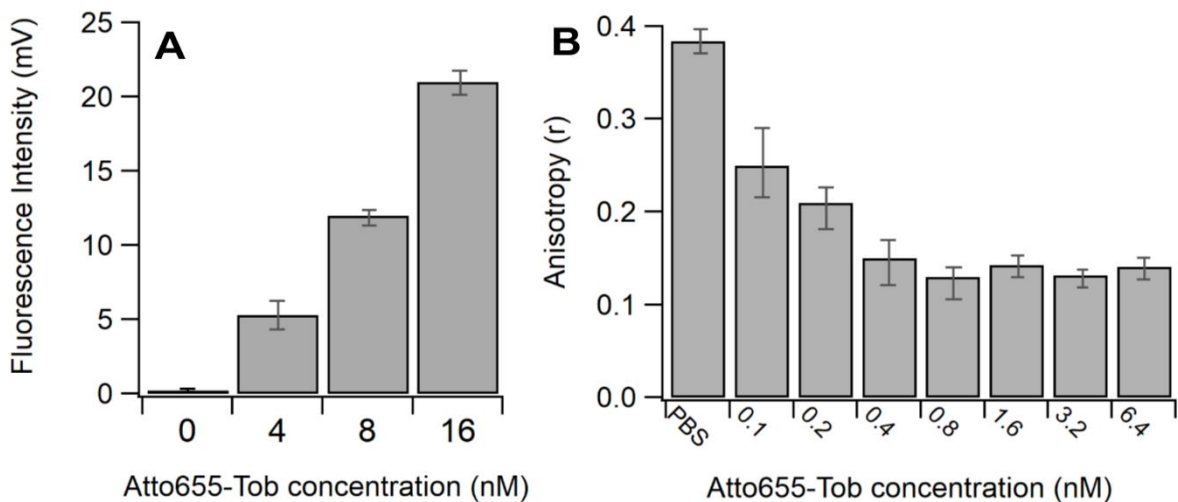
Figure 2.8: A) Fluorescence intensity of the tracer Atto655-Tob at different concentrations, measured in paper; B) Anisotropy values measured for low concentrations of Atto-655 derivate compared to the anisotropy values measured in PBS in fiber-glass micro-chambers;

Overall, glass fiber membranes with an excitation in the red exhibited the best properties for accurate determination of fluorescence polarization with very low background and anisotropies comparable to microtiter plate measurements and were therefore chosen for

quantification of tobramycin using FPIA. For the design of the competitive fluorescence polarization immunoassay in glass fiber membranes, we chose a low tracer concentration (7 nM) that satisfied the fluorophore/receptor stoichiometry and at which the background signal did not interfered with the measurements (Figure 2.8 A).

2.4.2 Precision of FP measurements in glass fiber micro-chambers

While measurements of fluorescence polarization in glass fiber membranes were accurate, the precision using a diffraction-limited excitation spot was poor with large variation coefficients of typically 10.89 %CV (Figure 2.9 A). Optical visualization of the fluorescence images indicated that it was at least partly due to inhomogeneous illumination caused by the glass microfibers in particular due to wave guiding effects. In order to improve homogeneity, the illuminated region was enlarged by adding a lens in the excitation path. Our experiments show that an excitation spot size of around 100 µm diameter corresponding to the typical dimensions of structural features in glass fiber sheets yielded the best performance in terms of reproducibility (Figure 2.9 A)
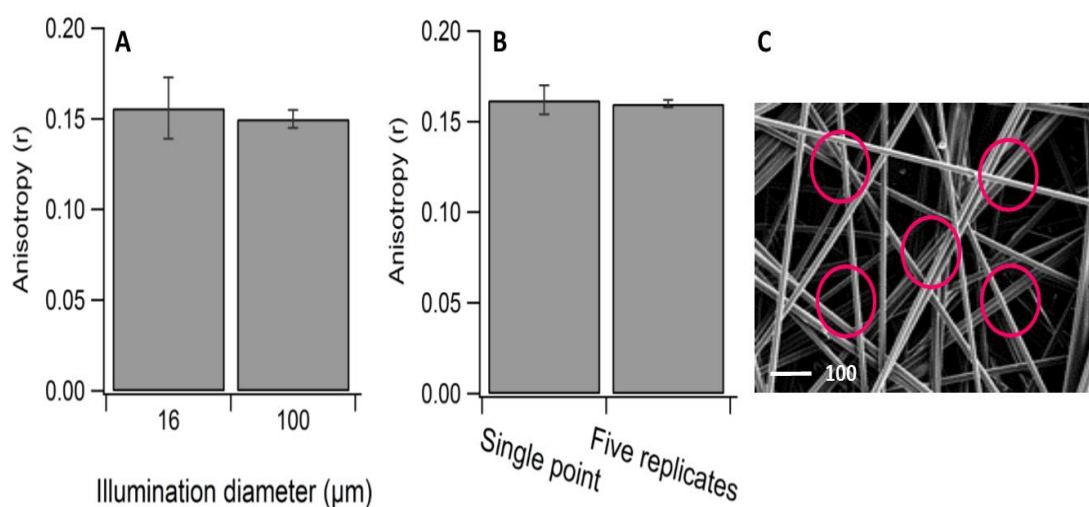
Figure 2.9: A. Extension of the diameter of the illumination spot from 16 to 100 µm improved the precision of the anisotropy measurement of a 7 nM solution of ATTO655-Tob as seen by the smaller standard deviation; B. By replicating anisotropy measurements at five different locations within the same micro-chamber and calculating the mean of these replicates, the precision was considerably improved compared to using only one single measurement per micro-chamber as seen by the reduced standard deviation. A 7 nM solution of ATTO655-Tob was employed; C. Scanning electron micrograph showing the size of the illumination spot (red circle) compared to the dimensions of the structural features within the glass fiber micro-chamber. Five replicates were recorded per measurement within a micro-chamber.

In order to further improve the precision of FP measurements, five replicates were recorded in the same micro-chamber by randomly moving the excitation spot (Figure 2.9 C) and were averaged. The resulting coefficients of variation of typically 1% for these average values were similar to the imprecision of measurements in 96-well microtiter plates (Figure 2.9 B).

2.4.3 Fluorescence-polarization immunoassay of tobramycin in plasma using glass fiber micro-chambers

As a next step, we investigated the feasibility to use glass fiber micro-chambers for the quantification of tobramycin in plasma using FPIA. Atto655-Tob was used as a tracer and mixed with specific antibodies and only 1 μL of plasma spiked with tobramycin in order to perform a competitive immunoassay. Mixing and incubation occurred before addition of a drop of the solution into the glass fiber micro-chamber and read-out. Concentrations of reagents were set so that the calibration curve spanned the entire therapeutic range (Figure 2.10 A).
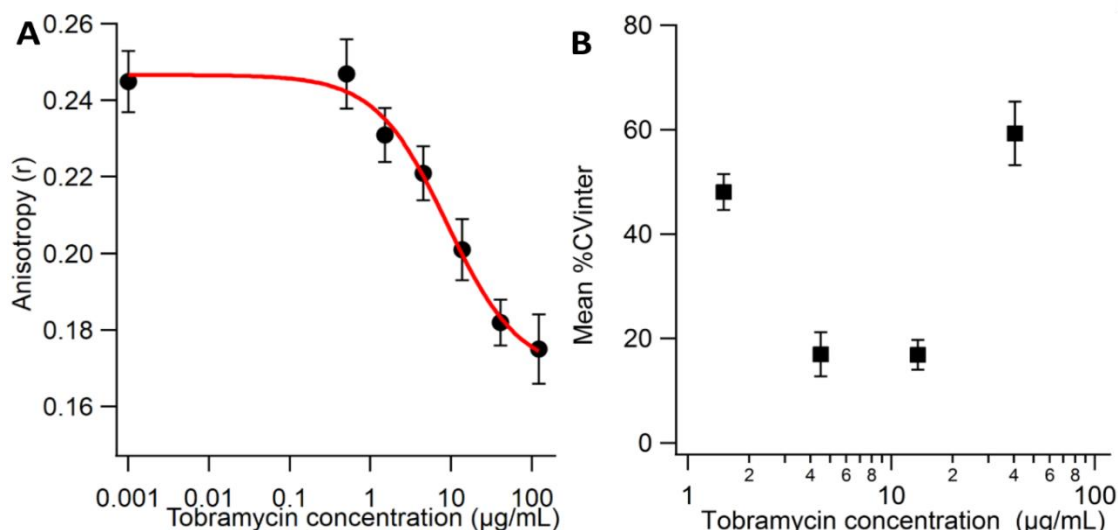
Figure 2.10: Performance of a fluorescence-polarization immunoassay in glass fiber micro-chambers for the quantification of tobramycin in human plasma: A) Representative calibration curve obtained using plasma samples spiked with increasing concentrations of tobramycin and measured in triplicate within the same day. Tobramycin concentrations were chosen to encompass the therapeutic relevant range between 1 and 10 μg/mL. The red line is a fit of the calibration points using a four-parameter logistic equation yielding the following parameters: $IC_{50} = 7.5 \pm 3.3$ μg/mL, $r_{min} = 0.162 \pm 0.017$, $r_{max} = 0.258 \pm 0.017$, rate = 0.7 ± 0.3.

B) Concentrations of the standard solutions were back-calculated from the anisotropy values using the four-parameter logistic equation and $\%CV_{inter}$ were estimated. Mean $\%CV_{intra}$ were obtained by averaging $\%CV_{intra}$ recorded on 3 different days and were found to be typically less than 20% within the therapeutic range.

In order to obtain a first rough estimate of the quantification range and the precision of this yet non-optimized assay, standard solutions were measured in triplicates on several days. Typical percentage coefficients of variation, %CV_{intra}, assessed from back-calculated concentrations were determined to be below 20% within the therapeutic range (Figure 2.10 B). Analytical performance goals for TDM can be estimated by different ways [106]. For aminoglycosides, imprecision requirements expressed as %CV were recommended to be at least below 18% [107]. The FP immunoassay in glass fiber micro-chambers meets therefore preliminary analytical performance needs, even though if current clinical assays show %CV below 10% [24].

2.4.4 Transversal separation of plasma and erythrocytes within glass fiber micro-chambers

One of the challenges in implementing new diagnostic assays for point-of-care testing is sample pre-treatment, in particular the separation step used to isolate plasma from whole blood, which is normally performed by centrifugation in a laboratory. Optical measurements are impaired in whole blood samples due to absorption and scattering by blood cells [108]. Blood separation techniques have already been exhaustively investigated on Whatman filter papers [109]. In our study however, the glass fiber micro-chambers are made out of glass fiber sheets with large pores used for conjugate release in lateral flow immunoassays. Yet we observed experimentally that separation occurred across the chamber with retention of blood cells on the upper side (at the site of deposition) while cleared plasma was apparent at the bottom of the micro-chamber (Figure 2.11 A).
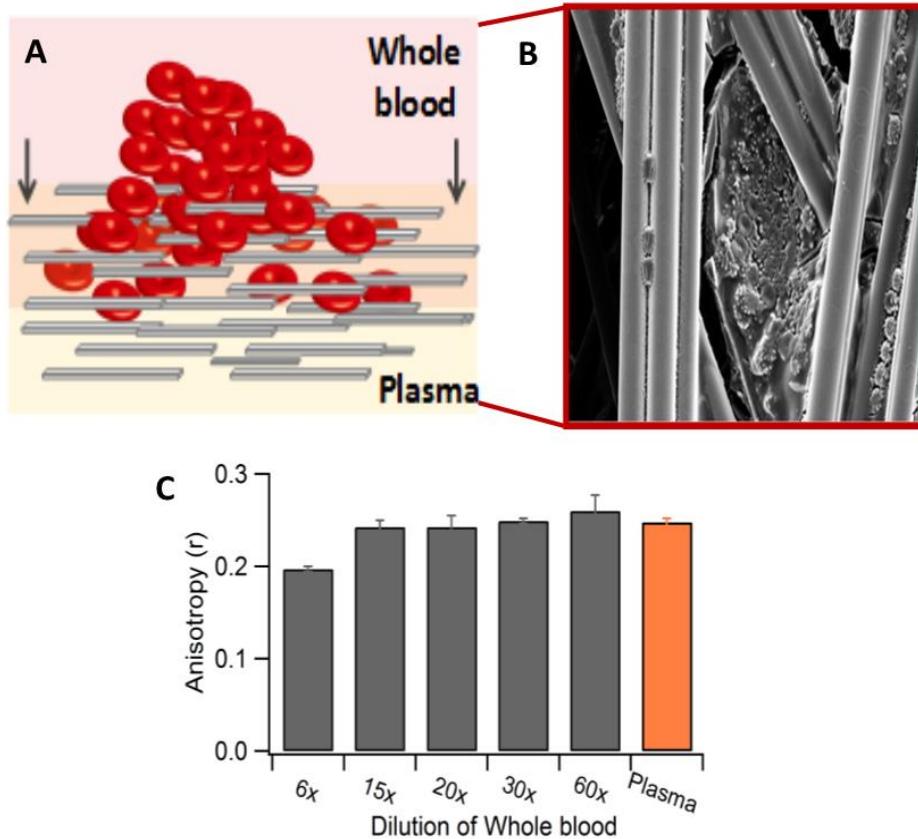
Figure 2.11: Blood separation within glass fiber micro-chambers Figure: Blood separation within glass fiber micro-chambers: A) Schematic illustration of axial separation of plasma and blood cells: red blood cells accumulated at the upper part of the micro-chamber while plasma diffused down to the bottom allowing for accurate anisotropy measurements; B) Scanning electron micrographs showing that red blood cells became entrapped at site of connections between fibres where the mesh is dense; C). A slight prior dilution of the blood was required so that accurate fluorescence polarization measurements could be performed. Tobramycin-specific antibodies and the tracer Atto655-Tob were added to whole blood at various dilutions and anisotropies were measured within micro-chambers and compared to reference measurements in plasma obtained in 96-well microtiter plates. At dilutions of 15x or higher, no difference between anisotropies measured in whole blood and in plasma were observed.

Scanning electron microscopy images suggested that red blood cells become entrapped at site of connections between fibres where the mesh is dense (Figure 2.11 B). Blood separation was not due to coagulation as blood was pre-treated with potassium EDTA. Plasma flowed vertically across the membrane. The removal of cellular components eliminated interferences on fluorescence polarization given that whole blood was previously slightly diluted, demonstrating that the plasma separation was effective, but not complete. Variable dilutions of whole blood were evaluated

and it was found that a 15X or higher dilution of blood enabled accurate FP measurements with anisotropies identical to measurements in pure serum performed in 96-well microtiter plates (Figure 2.11 C). At a similar blood dilution, it was not possible to determine FP accurately in a well of a microtiter plate demonstrating that separation using the glass fiber membrane was critical to perform the FP measurements.

2.4.5 Fluorescence-polarization immunoassays in glass fiber micro-chambers using whole blood

The previous findings opened the possibility to perform a quantification of tobramycin from a drop of whole blood using straight forward steps as illustrated in Figure 2.12.

Figure 2.12: Tobramycin quantification within glass fiber micro-chambers at the point-of-care: 1 µL of whole blood, which might be obtained from a finger prick and collected using a glass capillary, was mixed with the tracer Atto655-Tob and antibodies against tobramycin. The solution was incubated for 3 minutes before addition of 10 µL to the glass fiber micro-chamber and anisotropy read-out at its bottom part.

A drop of blood containing tobramycin, which could be typically collected using a fingerprick, was simply mixed with the reagents (antibody and tracer) and incubated for three minutes prior to addition to the glass fiber micro-chamber, where blood separation occurred at the upper part while drug quantification was performed at the bottom part.

We investigated the feasibility of accurately quantifying Tobramycin using this procedure. Calibration curve could be recorded using standard solutions of whole blood spiked with known amounts of tobramycin (Figure 2.13 A).
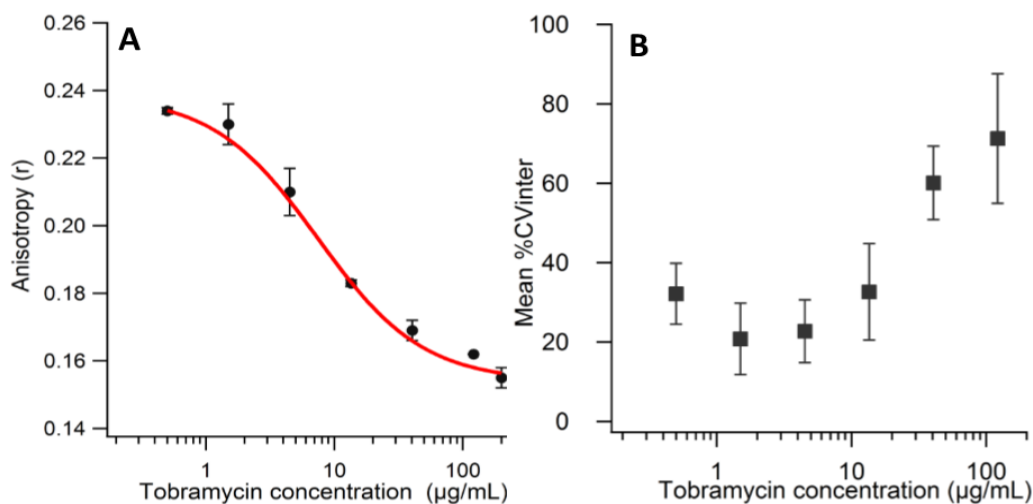
Figure 2.13: Performance of a fluorescence-polarization immunoassay in glass fiber micro-chambers for the quantification of tobramycin in whole blood;

A. Representative calibration curve obtained using whole blood samples spiked with increasing concentrations of tobramycin and measured in triplicate within the same day. Tobramycin concentrations were chosen to encompass the therapeutic relevant range between 1 and 10 µg/mL. The red line is a fit of the calibration points using a four-parameter logistic equation yielding the following parameters: $IC_{50} = 7.8 \pm 1.6$ µg/mL, $r_{min} = 0.160 \pm 0.004$, $r_{max} = 0.239 \pm 0.006$, rate = 0.6 ± 0.2. B. Concentrations of the standard solutions were back-calculated from the anisotropy values using the four-parameter logistic equation and $\%CV_{intra}$ were estimated. Mean $\%CV_{intra}$ were obtained by averaging %CV recorded on 3 different days and were found to be typically around 20% within the therapeutic range.

In order to obtain preliminary estimate of the quantification range, standard curves were obtained for several days and average $\%CV_{intra.}$ were obtained by back-calculating concentrations. In general, a higher variability in terms of CV was observed with longer storage of the samples even if whole blood was used within the week (Figure 2.13 B). This might be due to haemoglobin release from red blood cells, that might interfere by absorbing light and by autofluorescence even if the strong absorbing bands are predominantly in the range between 500-600 nm [110]. $\%CV_{intra.}$ that would be obtained using fresh blood are therefore likely to be smaller. Currently, within the therapeutic range, $\%CV_{intra.}$ are between 20% and 30% with a performance similar to the measurements in serum, which is quite remarkable. The limit-of-detection lies around 0.2 µg/mL, which is a clinical relevant concentration.

To obtain first estimates for the performance in terms of precision and accuracy, control solutions were prepared by spiking tobramycin at low, medium and high concentrations compared to the therapeutic window and measured for several days. For the three controls,

imprecision is lower than 20% and recoveries are better than 20% range, within 100±20% range (Table 3), except for the high control

Table 3: Analytical performance of the FP immunoassay in whole blood: Whole blood was spiked at low, medium and high concentration with respect to the therapeutic range of tobramycin. Average recovery and standard deviation of the recovery were assessed by performing measurements on three consecutive days. Recoveries remained within ±20%.

Range	Nominal Concentration	Mean %Recovery (±SD)
Low	1.48	92 (17)
Medium	3.51	105 (7)
High	7.24	77 (14)

The FPIA meets therefore the requirements for TDM of tobramycin even if a large potential for further improving performance exists. In particular, incorporation of the glass fiber micro-chambers into a point-of-care device would significantly improve sources of imprecision caused by evaporation, temperature fluctuations and variations in incubation durations.

2.5 Conclusions and perspectives

With the advent of microfluidics in paper and paper-like materials for point-of-care applications, alternative detection formats are required. Here, we enlarged the arsenal of possible detection technologies by demonstrating that FPIA can be accurately performed within glass fiber micro-chambers. The possibility offered by these micro-chambers to separate plasma from whole blood before quantification within the same chamber could prove to be an important asset in the development of solutions for point-of-care testing. Our study demonstrates that this novel combination of glass fiber chambers with FPIA makes realistic the development of a point-of-care device for therapeutic drug monitoring using a single drop of blood. We speculate that the assay could be further simplified by incorporation and drying of the reagents within the micro-chambers and that the performance could be further improved by stabilizing temperature and environmental conditions inside a dedicated device. We envision that such a POCT device would allow for the transfer of therapeutic drug monitoring out of clinical laboratories directly to the physician's office and even to the patients themselves

Chapter 3

3. Fluorescence polarization immunoassays within micro-channels using compact optical readers

Over the last decade, the miniaturization of analytical instrumentation has become an increasing trend among the biological and chemical sciences [111], bringing along advantages as small size, faster turn-around times and decreased costs [112]. The small sample size together with sensitive quantification assay, should prove useful for drug monitoring. However, when discussing about designing POC devices, integration of all components is fundamental. Microfluidic elements for sample collection, reagent mixing and blood separation as well as the detection chamber must be in such way interconnected that they do not interfere on the measurement result. This mutual compatibility can prove to be extremely challenging as more components are being integrated, because the technical requirements can vary greatly for each component or assay step [113].

Although optical methods are the most used methods in analytical chemistry, there is a lack of miniaturized optical systems for small-molecule quantification[114], [115]. This might be attributed to the fact that optical devices require careful alignment, which might present challenges when translated into POC devices. In general, fluorescence remains the most widely used method of optical detection. Fluorescent detection has a number of advantages over other techniques, namely high sensitivity and a wealth of available fluorophores and labelling chemistries [116]. For fluorescence measurements, laser excitation is often used due to the fact that lasers have a low divergence and can easily be focused into a small illumination region. The ability to easily focus a laser beam into a small-spot delivers the advantages of high intensity and optical efficiency.

As mentioned in previous chapters, in clinical settings, Tobramycin is mainly quantified via FPIA. FPIA instruments have been commercialized by Roche Diagnostics and Abbott Laboratories [107] [117], among others, and have been applied to TDM for various small molecules, including tobramycin. Due to its simplicity and speed, FPIA is amenable to miniaturization and automation [118]. Up to date, only one miniaturized FP system was described, however, using the standard well-plates for the measurements [119]. The possibility to use FPIA for quantification of the small drug theophylline directly within a microfluidic channel was recently demonstrated: Tachi et al, implemented this technique to

perform immunoassays within microchannels, but using a custom, non-compact optical setup [120].

In this chapter, a miniaturized analytical device based on Fluorescence Polarization Immunoassays (FPIA) is described, which could be used for Therapeutic Drug Monitoring at the point of care. The compact optical device enables measurement of fluorescence polarization within glass microchannels, which are here mimicked with glass capillaries. The compact analytical system was applied to specifically quantify tobramycin from spiked plasma samples, directly within glass capillaries.

To validate our demonstrator, different concentrations of Tobramycin were measured within a dynamic range covering the effective therapeutic window of the drug (2-12µg/ml). In this approach, the standard 384-well-plates measurements are replaced by the introduction of a new measurement chamber, glass capillaries, with a section of 1 mm^2, which are prototypical of micro-fluidic channels made out of glass. Choice of glass capillaries was done considering mostly that, in the contrary to most polymers, light polarization is preserved while transmitted through the material. Polymers are not adapted for FP measurements directly as they are depolarizing light, but likely, paper-based micro-chambers might be used.

For one measurement, 10 µl of mixed reagents were added into the glass capillary. The values obtained with our demonstrator agreed well with the commercial assay showing that tobramycin could successfully be quantified with a miniaturized optical device with reduced number of steps, volume of sample and reagents, allowing measurements in less than one minute for one analysis.

3.1 Materials and methods

3.1.1 Chemical Reagents

All blood samples were bought from Interregional Transfusion Centre (Bern, Switzerland). They were collected from healthy volunteers in K_3EDTA tubes, in order to prevent coagulation and they were shipped and stored at 4°C and used within a week from collection date. Plasma was obtained from whole blood centrifuged at 2000 rpm at 4°C for 20 minutes and it was kept at -20°C and used in less than a month. Fluorescently-labelled tobramycin and antibodies specific for tobramycin were from a commercial kit for FPIA on Roche Cobas Integra 400 (Roche Diagnostics GmbH, Basel, Switzerland). Solutions of plasma samples were prepared by spiking Tobramycin at a concentration of 0-1210 µg/ml, followed by a dilution 10x in PBS. One µl of these solutions was incubated with 6 µL of Tobramycin derivative and 46 µL of

antibody for 5 minutes at RT. The calibration curves were prepared three times within a day and each sample was performed in triplicates. Furthermore, for inter-run reproducibility, the same tests were performed on 3 consecutive days.

3.1.2 FPIA measurements

The miniaturized optical device was validated by spiking human plasma. The concentrations used were: 0, 0.5, 1.5, 4.5, 13.5, 40.5, 121.5 µg/mL, covering the therapeutic range of the drug (1-12 µg/ml).

All the measurements were performed at RT, in the dark, in order to reduce the background. The reagents were mixed previously in an eppendorf and added to the capillary with a pipette. A SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Diagnostics, California, USA) was used for reference fluorescence polarization measurements under the following conditions: excitation filer selected from 485 to 550 nm, integration time set at 150 ms while the temperature was set at 25°C. The fluorescence measurement readings in the UV region were performed in 384 flat bottom with non-binding surface microtiter plates (Corning Incorporated, USA), using a final volume of 80 µl per well.

The capillaries were bought from C&M Scientific Ltd. (Livingston, UK). ID square 1* 0.20 mm thickness wall. Each capillary was manually placed horizontally in a previously manufactured (3D print) capillary holder attached above the microscope objective. Ten µl of human plasma was used for each measurement.

3.1.3 Design of the miniaturized FP analytical device

The miniaturized FP device, schematically represented in Figure 3.1, is divided in two parts: excitation of the sample and collection of the fluorescence signal.

In order to have a stable polarized source, a non-polarized and pigtailed LED (Thorlabs - MF470) was used with a Glan Thompson Polarizer PGT (Thorlabs - GTH5M). The beam was collimated with the lens L1 (Thorlabs – (A375 –B (TL)). The LED emits on a wide spectrum (470 +/- 55 nm), thereby, an excitation filter Fs (Thorlabs – MF475) selected a smaller band width (475 +/-15 nm). In order to focus the beam in the center of the capillary, a microscope objective L2 (Olympus - UPLFLN 10X2) was placed.

The excited sample emitted fluorescence at another wavelength and unknown polarization. This beam was again collimated by the microscope objective L2. The dichroic mirror MD (Edmund Optics - 67-079) separated the emission and excitation beam. The dichroic mirror reflected the wavelength from 435 to 488 nm and transmitted from 520 to 730 nm. The fluorescence was furthermore filtered using an excitation filter (Thorlabs - MF530).
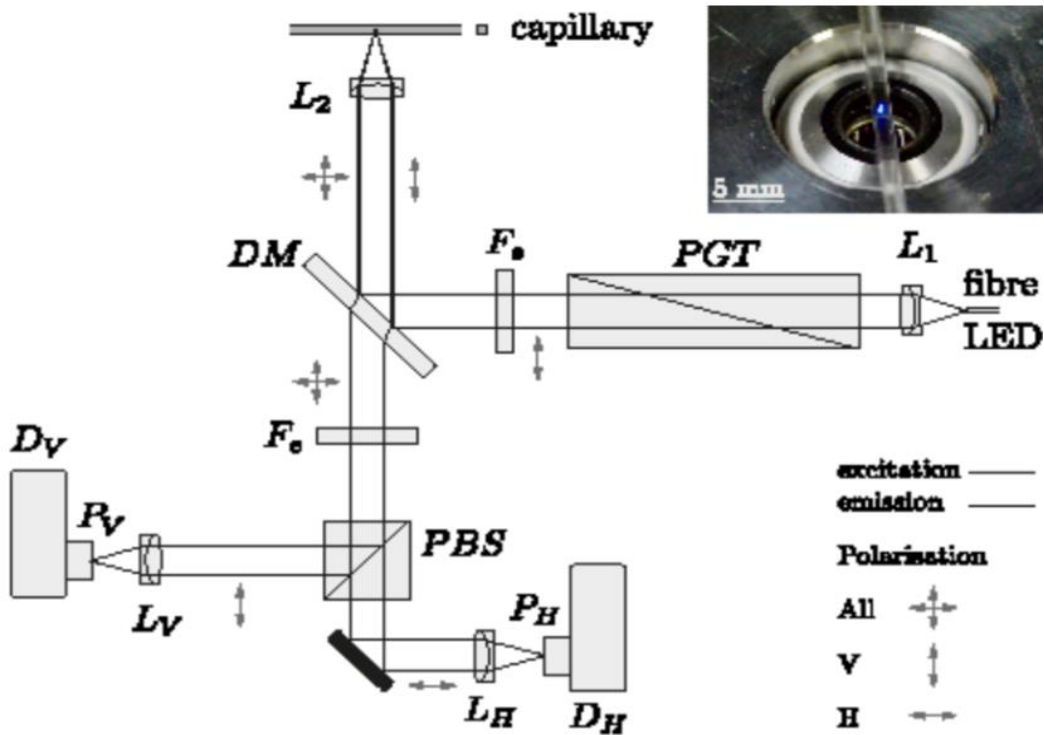
Figure 3.1: Scheme of the FP analyzer with a picture of the measurements within capillaries

The polarizing beamsplitter cube PSB (Thorlabs – PBS201) reflected S-Polarization and transmitted P-Polarization. The lens L_V (Edmund Optics 20mm) and pinhole P_V (Ø500um) made a confocal system between the capillary and the detector D_v (Thorlabs – PDF10A/M) respectively L_H, D_H and P_H.

3.1.4 Detection unit including controlling software

The signal was registered in mV and quantified by LabView software, which directly calculated the anisotropy (r), from the 2 measured intensities, parallel ($I_\parallel$) and perpendicular ($I_\perp$), at the respective detectors. The G factor was calculated using, as reference, the anisotropy measured on a reference device (SpectraMax Paradigm Multi-Mode Microplate Reader), according to Equation 11. Anisotropy values were corrected using the G value. The G value was 0.92.

$$Gfactor = \frac{I_\parallel(1-r)}{I_\perp(2r+1)}$$
(*Equation 11*),

where r is the anisotropy, $I_{\parallel}$ is the fluorescence emission measured in the plane parallel to the plane of excitation and $I_{\perp}$ is the fluorescence emission measured in the plane perpendicular to the plane of excitation.

3.2 Results and discussion

3.2.1 Compact FPIA analyzer using glass capillaries as detection chambers

The benefits of assay miniaturization are now coming into focus because of the reported cost advantages of changing assay volumes from 100 μL down to 5 μL [121]. Although on chip measurements using FPIA have been previously studied [120], there has been however little effort as of yet towards the miniaturization of this technique.

A compact, benchtop FP analyzer (Figure 3.2 A and B) was developed by Prof. Martial Geiser and his team and this setup makes use of an objective for the excitation and collection in order to focus the excitation beam within the microstructures. After an initial assembly of the setup, the functionality and optimization (i.e. alignment of the optical parts, sensitivity) of the analyzer were assessed by measuring different concentrations of fluorescein. Total fluorescence intensity as well as anisotropy were evaluated by comparison with a commercial device and the minimum concentration detected was 1 nM of fluorescein. The integrated intensities were comparable between the two devices, after correction with the G–factor and allowed us to proceed with drug concentration measurements.

In terms of assay, the glass capillary could only accommodate very low amounts of sample and therefore, as a first step, the possibility to perform accurate FP measurements using low sample volumes was investigated. The assay was simplified and the volume of plasma was reduced to a minimum (1μL). The results obtained using the downsized assay were compared to measurements using standard sample preparation and volumes and performed in 386 well plates. Tobramycin could be quantified with the downsized FP assay with high precision (within-run CV < 10%) and accuracy (recoveries between 90 and 110%) in the therapeutic range of 1 to 12 μg/ml. LOQ and LOD were 0.2 and 0.6 μg/ml, respectively. Under these conditions, the required sensitivity of the assay was achieved. Miniaturization was clearly an inherent capability of FP. The possibility that the FP assay for tobramycin could be performed with volumes of ≤ 10 μL opened the way to fully integrate it. For this, the assay was transferred from microplates to glass capillaries.
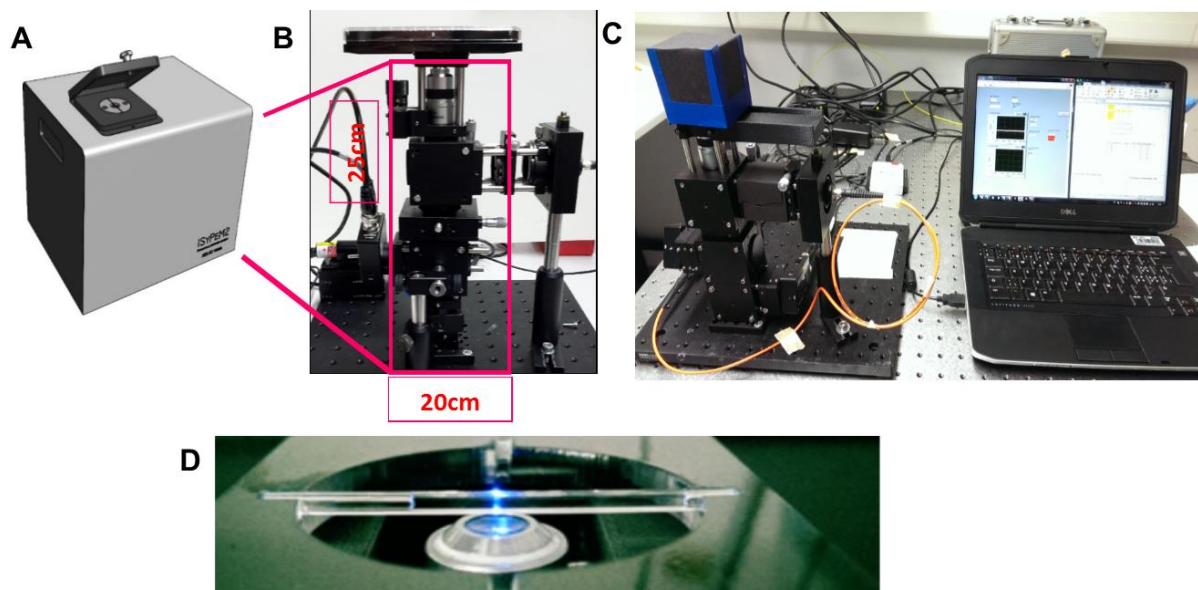
Figure 3.2: A) Compact optical device for FP quantification; B) picture of interior of the device with the different optical elements; C) Connection of the reader to the control PC; D) capillaries used as measurement chambers;

Square glass capillaries were chosen as it was found experimentally that round capillaries deviated the illumination beam due to their shape. By contrast, when using tested squared capillaries, the shape of the illumination spot remained unmodified.

For the following measurements, a volume of reagent mixture of 10 μL was used, which was10x fold less when compared with well plates. For one measurement, it typically required only 10 μL of plasma, previously diluted 10x with PBS, which demonstrated its aptitude for point-of-care therapeutic drug monitoring.

3.2.2 Small-drug quantification in human plasma within glass capillaries

The quantification of tobramycin concentrations in plasma samples by FPIA in capillaries was evaluated. Results showed that the performance obtained using the compact optical system were comparable with the results obtained using a commercial reader. Figure 3.3 plots the dose-response curves for Tobramycin quantification performed within glass-capillaries using plasma samples.
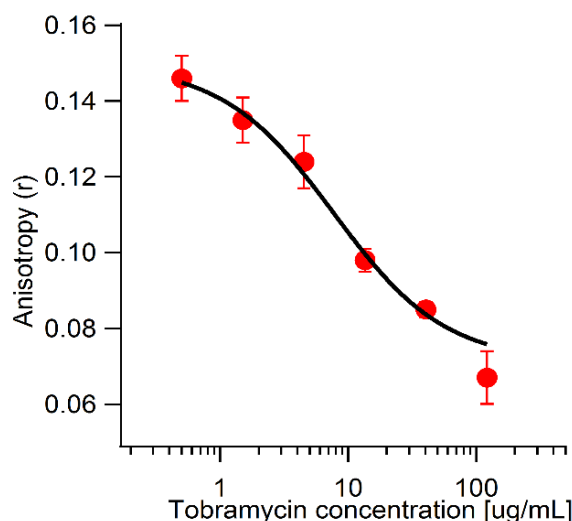
Figure 3.3: Dose-response curve for Tobramycin quantification within glass capillaries, obtained with the FP analyser; The black line is a fit of the calibration points using a four-parameter logistic equation yielding the following parameters: $IC_{50} = 7.704$ µg/mL, $r_{min} = 0.071 \pm 0.004$, $r_{max} = 0.149 \pm 0.006$, rate $= 0.89 \pm 0.252$.

The analytical performance of the miniaturised FP optical system was evaluated in terms of reproducibility and sensitivity. The results show that the performance of the demonstrator is comparable with a commercial FP device in a range of concentration from 0-120 µg/ml Tobramycin.

To determine the intra-run precision of the demonstrator, each of the standards containing tobramycin were tested 3 times within the day. In this case, the coefficient of variation (CV_{intra}) varies within the triplicates from 15% to 30% within the therapeutic range of the drug. To measure each solution three times, each capillary was manually shifted to another position. It is not possible to know, whether it is measured on the same spot. The inter-run coefficient of variation (CV_{inter}) was calculated using measurements performed in triplicates within the same capillary but at different locations, within the sample. However, the three different measurements spots within the same capillary were reproducible, with a coefficient of variation of 2%.

The results of the measurements showed that, while the commercial FPIA device had a precision of 20%CV, the miniaturized analyser was slightly less precise, with a CV going up to 30% (Figure 3.4)
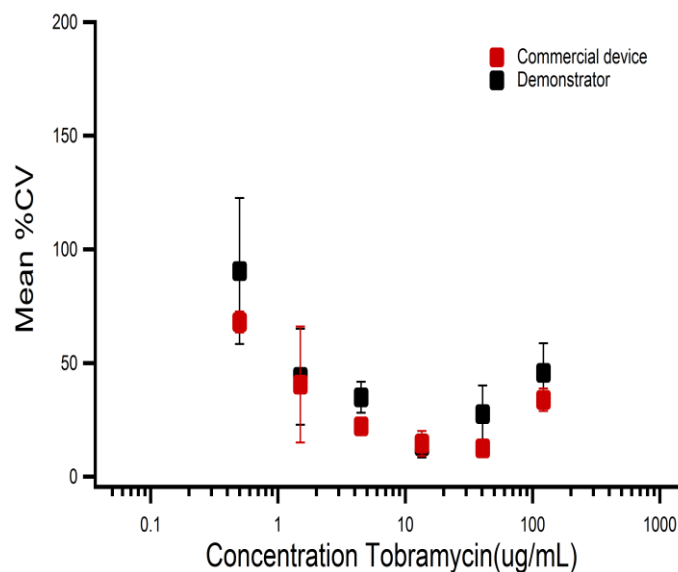
Figure 3.4: Coefficient of variation comparison between the commercial device and the miniaturized FP analyser showing that the difference between the two is not considerable;

Herein, if we compare coefficients of variation of the two systems results indicated that the analyzer was slightly less precise than the commercial one, the potential causes for imprecision can be attributed to the fact that the system is not automatized. The sample being placed manually handling errors might appear like for instance during the mixing step of sample with other reagents. Also, the system is not yet integrated with temperature and humidity control, key parameters for fluorescence polarization measurements.

3.2.3 Integration with microfluidics: a whole-blood PDMS separator

Microfluidic chips for immunoassays are commonly fabricated from silicon, glass, and polymer materials. The first generation of microfluidic devices were made from silicon and glass by photolithography and etching in a clean room based on well-established microfabrication techniques. However, polymers such as polymethylmethacrylate, cyclic olefin copolymer, polystyrene, and polydimethylsiloxane (PDMS) have emerged as alternative materials to silicon and glass due to the simple fabrication process (e.g., molding, embossing and printing) and low cost [122], [123]. Among the polymers, PDMS is the most popular material used for microfluidic immunoassays because of its flexible, optically transparent, biocompatible, and low-autofluorescence properties. Moreover, PDMS can be easily bonded to silicon, glass, or another piece of PDMS through treatment with oxygen plasma [114], [124] [125], [126].

Herein, we used a PDMS chip, designed by David Forchelet under the coordination of Prof. Philippe Renaud, from Microsystems Laboratory, EPFL. The microdevice is able to perform

blood separation at the point of collection. The capillary-driven microdevice processes fingerprick blood micro-samples without the need for external equipment. The device relies on sedimentation as a simple and spontaneous driving force for the separation of undiluted whole blood. The chip device has two main parts: the blood inlet and the plasma outlet. In addition to the separation channel, an ejection mechanism is present on the chip. A defined volume of 2 µL is ejected from the chip by using an air bubble and capillary valve. The ejection of the plasma allows for using the generated plasma in any on-chip or off-chip operation or characterization.

The proof-of-concept, expressed in Figure 3.5, consists in the separation of whole blood, previously spiked with tobramycin, the collection of resulted plasma, mixed with the assay reagents and followed by FP measurements for the assessment of recovered drug.

Initially, Tobramycin was spiked in whole blood and then added on the PDMS separation chip. After 15 minutes, when the separation was complete, 12.5 % of plasma was recovered. The flow through the microfluidic channel did not cause any visible haemolysis of RBC. Furthermore, the aim was to see if the concentration of tobramycin, previously spiked, could also be recovered.

Plasma resulted from the separation was mixed manually with the reagents and then added to the measurement chamber. Mixing the assays reagents is another important step that can be further implemented onto the FP device.
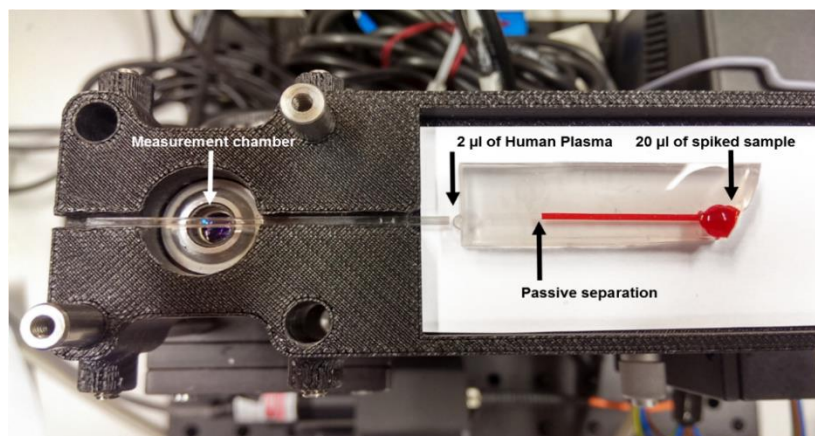
Figure 3.5: Proof-of-concept for separation and integration with the FP device: fresh whole blood was used for testing the PDMS microfluidic. 20 µL of spiked whole blood was added with the micropipette into the reservoir designed for sample collection. The separation was passive requiring 15' to be fully completed. After the separation, 2 µL of clean plasma was

obtained. Then, the plasma was mixed with the tracer and antibody and anisotropy was measured.

Figure 3.6 shows a dose-response curve for Tobramycin. The assay was performed in triplicates and standard deviation was calculated.
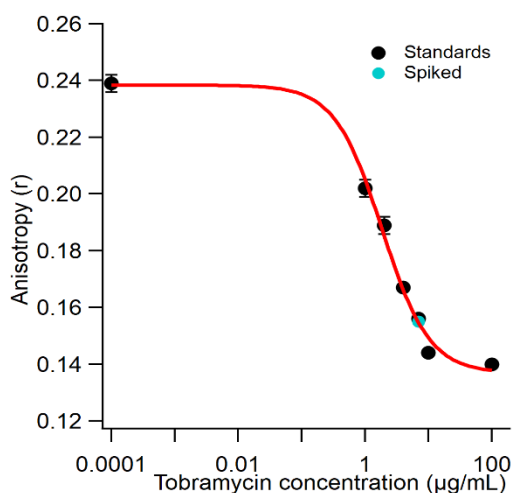
Figure 3.6: Dose-response curve for the quantification of Tobramycin in whole blood using separation with the PDMS microfluidic device. The red line is a fit of the calibration points using a four-parameter logistic equation yielding the following parameters: $IC_{50} = 1.854 \pm 0.284$ µg/mL, $r_{min} = 0.136 \pm 0.0048$, $r_{max} = 0.238 \pm 0.004$, rate = 0.708 ± 0.191.

The back calculated concentration of the tobramycin obtained after the whole blood separation was expressed in terms of recovery and had a value of 94.2 %, which is in the range of required recoveries for standard FP immunoassays (80 -120 %).

3.3 Conclusions

This study shows that is possible to develop a miniaturized and compact integrated optical system without using the standard well-plates for the fluorescence polarization measurements. As proof-of-concept, the analyser demonstrated its clinical adaptability when using human plasma samples. Moreover, measuring in glass capillaries holds the advantage of using small amounts of samples and reagents. The analytical performance obtained with the new system were comparable with those obtained on a conventional reader.

Chapter 4

4. Measuring challenging drugs: the immunossupressant case

The following chapter will relate efforts in designing a FP immunoassay in order to determine the concentration of an immunosuppressant drug (ISD) within blood samples and, in particular, describe an improved drug extraction procedure. The aim of the following study was to develop a simple and fast method for tacrolimus quantification.

Initially, chemical and biochemical properties of ISDs will be discussed highlighting the critical parameters that make their quantification challenging. Furthermore, we will overview the analytical methods used for their quantification, highlighting the needs for developing a rapid device for ISD qualification. To this reason, we will describe our efforts in designing an FP technique for Tacrolimus, one of the most used ISDs.

4.1 Biochemistry of immunosupressants

Immunosuppressant drugs such as sirolimus, everolimus, cyclosporine A and tacrolimus are effective for the treatment of organ or tissue rejection following transplant surgery [127] [128]. During the ISD therapy, monitoring the blood concentration levels is an important aspect of clinical care because insufficient drug levels lead to organ rejection while excessive levels lead to toxicities. Unfortunately, although all are very potent, some have a narrow therapeutic window and large inter-patient variability [129].

They are categorized according to their mechanism of action as seen in Table 4:

Table 4: Immunosupressive agents classified according to their mechanism of action [127][130]*.*

Immunosuppressant Drug Classes	Mechanism of action (target)	Drugs
Corticosteroids	Regulation of gene expression (Glucocorticoid receptors)	Prednisolone, methylprednisolone, and dexamethasone
Antimetabolite/antiproliferative agents	Inhibition of purine and pyrimine synthesis (inosine-5′-monophosphate dehydrogenase)	Azathioprine, cyclophosphamide, mycophenolate mofetil, and mycophenolate sodium
Calcineurin inhibitors	Kinase and phosphatase inhibitors (Calcineurin, JNK/p38 kinase)	Cyclosporine, voclosporin*, and tacrolimus
Mammalian target of rapamycin (mTOR) inhibitors	Kinase and phosphatase inhibitors (Cyclin kinase cascade)	Sirolimus and everolimus
Costimulatory blocker	Inhibition of CD28 mediated costimulation of T cells (CD 80 and CD 86 on antigen presenting cells)	Belatacept

For nearly three decades, calcineurin inhibitors (CNIs) have been the most widely used form of immunosuppression in organ transplantation. CNIs have led to dramatic reductions in both acute rejection rates and early graft loss in renal transplantation. Long-term outcomes, however, are likely affected by CNI-associated allograft nephrotoxicity as well as cardiovascular risks such as diabetes, hypertension, and dyslipidaemia [131].

The lymphocyte blood cells host the immunosuppressive site of action for calcineurin inhibitors (CNIs), proliferation signal inhibitors (PSIs) and mycophenolic acid (MPA). Due to the fact that the intracellular compartment of lymphocytes is the target site of drug action, whole blood or plasma concentrations can only serve as substitute markers [132].

Tacrolimus is a very lipophilic compound with a molecular weight of 804 Da, existing as a monohydrate in the solid state. It is highly soluble in methanol, chloroform, acetone and ethyl acetate, soluble in ethyl ether, propylene glycol and polyethylene glycol, but insoluble in water and n-hexane [4]. The rate of absorption of tacrolimus is variable with peak blood or plasma concentrations being reached in 0.5 to 6 hours; approximately 25% of the oral dose is bioavailable. Tacrolimus is completely metabolized prior to elimination. The mean disposition half-life is 12 hours and the elimination of tacrolimus is decreased in the presence of liver impairment and in the presence of several drugs. Tacrolimus has reported nephrotoxicity cases in 52% of the kidney transplants and 40% of the liver transplants [133], [134], [135], [136], [137].

4.2 Current analytical methods for TDM of immunosupressants and their limitations

Various techniques are involved in the quantification of ISDs, all applied based on their chemical properties, bioavailability, target, metabolism, therapeutic range and protein binding. The main methods involved in today's TDM of ISDs are semi-automated or automated immunoassays (IAs) and liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS). Tacrolimus cannot be quantified by HPLC-UV because it lacks a significant chromophore [138]. Table 5 summarizes all the characteristics of the methods used for ISD's quantification.

Table 5: Immunosupressants specific considerations, requirements for TDM and characteristics of main analytical methods involved in their quantification

Drug	Therapeutic range	TDM Sample matrix	Protein binding	Required TDM after RTx	Existing methods	Analytical range (ng/mL)	Instrument and manufacturer	Volume and sample matrix	Throughput (tests/hour)	TAT	Calibration	Ref
Tac	*1st year after RTx* 5–10 ng/mL *Follow-up* 3–8 ng/mL	WB	88%	*Month 1* 3x/week *Month 2-3* 1x/week *Month 4-6* 1x/2 weeks *Month >6* Any outpatient visit	ACMIA	1.0-30	Dimensions® EXL 200 Integrated Chemistry System (Siemens Healthcare Diagnostics)	15 µL EDTA WB	167	15 min	*Assay-specific time interval *With new reagent lot	[139]
					CEDIA	2.0-30	Hitachi 912 or 917 analyzer (Roche)	3-50 µL S,P, U, SF	*360 *720 opt. ISE	*6 min *12 min	*Daily	[140], [141]
					CMIA	2.0–30	Architect i1000SR (Abbott Lab.)	10-150 µL S, P, WB,U	100	1.6 min	*If controls are out of range * If specified otherwise	[142], [143]
					MEIA	3.0–30	Abbott IMX® Analyser (Abbott Lab.)	150 µL S	Specified: 24/40min	40 min	With new reagent lot	[144], [145], [146]
					EMIT 2000	2.0–30	V-Twin System (Siemens Healthcare Diagnostics)	*R_1 110-400 µL *R_2 0-180 µL *R_3 0-180 µL EDTA WB	*260 with 2 R *130 with 3 R	N/F	With new reagent lot	[147]
					QMS	1.0–30	Indiko™ Clinical and Specialty Chemistry System (Thermo Fisher)	V: N/F WB	1200	N/F	With new reagent lot	[148]
					LC-MS/MS	0.5-50	Numerous manufacturers	WB, DBS, OF	N/A	N/A	N/A	[149], [150], [151]
CSA	*Month 0–3* 120–200 ng/mL *Month 4* 80–160 mg/mL	WB	91.95%	*Week 1-3* 1x/day *Month 1* 3x/week *Month 2-3* 1x/week *Month 4-6* 1x/2 weeks	ACMIA	CSA 30–500 CSAE 350–2000	Dimensions® EXL 200 Integrated Chemistry System (Siemens Healthcare Diagnostics)	15 µL EDTA WB	167	15 min.	*Assay-specific time interval *With new reagent lot	[152], [153]
					CEDIA+	LA 25–450 HA 450–2000	Automated clinical chemistry analysers* (Thermo Scientific)	V: N/F EDTA WB	N/F	N/F	*Performed with two levels of quality control everyday	[154]

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4.2 Current analytical methods for TDM of immunosuppressants and their limitations

Drug	Therapeutic range	TDM Sample matrix	Protein binding	Required TDM after RTx	Existing methods	Analytical range (ng/mL)	Instrument and manufacturer	Volume and sample matrix	Throughput (tests/hour)	TAT	Calibration	Ref
				Month 6-12 1x/4 weeks	CMIA	30–1500	Architect i1000SR (Abbott Lab.)	10-150 µL S, P, WB,U	100	N/F	*If controls are out of range * If specified otherwise	[142], [153]
				Month >12 Any outpatient visit	EMIT 2000	CSA 40–500 CSAE 350–2000	V-Twin System (Siemens Healthcare Diagnostics)	R₁ 110-400 µL R₂ 0-180 µL R₃ 0-180 µL EDTA WB	*260 with 2 R *130 with 3 R	N/F	With new reagent lot	[155]
					ADVIA Centaur	30–1500	ADVIA Centaur/XP/XPT (Siemens Healthcare Diagnostics)	10-200 µL S, P, U	240	18 min. then 15 s.	Available automatic QC ordering by test, control, date, and time	[156], [153]
					LC-MS/MS	10-2000	Numerous manufacturers	WB, P, DBS, OF	N/A	N/A	N/A	[149], [150], [151], [157]
MPA	1.0–3.5 mg/L	P, EDTA P	97%	*Week 1-3* 3x/week	Roche total MPA	0.4–15	Cobas 311/501/502 analysers (Roche)	3 µL S, P, U	300	10 min.	*Start-up	[158], [159], [160]
				Month 1-2 1x/week								
				Month >2 1x/month	EMIT 2000	100-15000	V-Twin System (Siemens Healthcare Diagnostics)	R₁ 110-400 µL R₂ 0-180 µL R₃ 0-180 µL EDTA, HP	*260 with 2Rs *130 with 3Rs	N/F	With new reagent lot	[161]
				At any outpatient visit in the long-term run	PETINIA	0.2-30	Dimensions® EXL 200 or LM Integrated Chemistry System (Siemens Healthcare Diagnostics)	2-60 µL P	167	N/F	*Assay-specific time interval *With new reagent lot	[162], [163]
					CEDIA	300-10000	Hitachi analysers* (Roche)	V: N/F P	N/F	N/F	*As required *Reagent bottle change *Calibrator or reagent lot change *After monthly instrument maintenance	[164]
					LC-MS/MS or HPLC/UV	100-50000	Numerous manufacturers	OF	N/A	N/A	N/A	[165]
EVR	3–8 ng/L	WB	74%	Adapted to thorough level	QMS	1.5–20	Indiko (Thermo scientific)	V: N/F WB	1200	N/F	*Full 6-point procedure *With new lot number	[153], [166]

4. Measuring challenging drugs: the immunossupressant case

Drug	Therapeutic range	TDM Sample matrix	Protein binding	Required TDM after RTx	Existing methods	Analytical range (ng/mL)	Instrument and manufacturer	Volume and sample matrix	Throughput (tests/hour)	TAT	Calibration	Ref
				monitoring of CNI								
					LC-MS/MS	NF	Numerous manufacturers	WB, DBS	N/A	N/A	N/A	[149], [150]
SIR	4–12 ng/mL		92%	*Month 1* 1x	CMIA	2.0–30	Architect i1000SR (Abbott Lab.)	10-150 µL S, P, WB,U	100	N/F	*If controls are out of range * If specified	[142]
				Month >1	MEIA	2.5–30	Abbott IMX® Analyser	V: N/F	N/F	N/F	N/F	[145],
				Every other month			(Abbott Lab.)	WB				[145], [167]
					ACMIA	2.0–39	Dimensions® EXL 200 Integrated Chemistry System (Siemens Healthcare Diagnostics)	15 µL EDTA WB	167	15 min.	*Assay-specific time interval *With new reagent lot	[152]
					EMIT 2000	3.5-30	V-Twin System (Siemens Healthcare Diagnostics)	R$_1$ 110-400 µL R$_2$ 0-180 µL R$_3$ 0-180 µL EDTA WB	*260 with 2 R *130 with 3 R	N/F	With new reagent lot	[168]
					LC-MS/MS	NF	Numerous manufacturers	WB, DBS	N/A	N/A	N/A	[149], [150]

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4.3 Towards a POC device for ISDs

Although LC-MS/MS method presents higher analytical specificity, lower reagent costs and multiplexing ability in measuring ISDs, they however require more complex sample pre-treatment procedures, with longer turn-around times which may delay dose adjustment, especially during the early phase of organ transplantation procedure and thus, with higher risks of allograft rejection [169]. Immunoassays involved in ISD's quantification also require significant amount of sample and present high variability among the laboratories.

A POC device would enable the rapid turn-around time, especially in case of emergency or immediately after post-transplantation, when the dosage needs to be tightly controlled. Moreover, since the treatment with ISD lasts for a lifetime, a sample-to-answer device would simplify the life of patients by allowing less-invasive measurements. Moreover, the considerable amount of resources and expertise will not be required in case of a POC for ISD's. However, some of the factors that may influence the cost-effectiveness of a new diagnostic tool for ISD's over the current analytical process are the prevalence of the organ transplantation, cost of the treatment, cost of the test, and throughput [170].

For Tacrolimus, for instance, due to its distribution inside human red blood cells, the very low concentration and the narrow therapeutic window, an assay can become laborious in terms of sample preparation and implementation into microstructures.

Although various methods are applied for tacrolimus quantification, this chapter presents efforts in designing a novel FPIA for Tacrolimus.

Two fluorescent derivatives for tacrolimus were synthesized, which emit at different wavelengths. With both of these derivatives, a specific antibody (IgM) and the physiological protein target of tacrolimus, FK-BP12, were tested in affinity studies. One of the main results obtained using the fluorescence and anisotropy properties of the two tracers, is that the precipitation reagents used in extraction of tacrolimus highly influence the drug quantification efficiency.

4.4 Experimental procedures

4.4.1 Chemical reagents

Tacrolimus (99 % purity), fluorescein-NHS (λ_{ex} 490 nm – λ_{em} 525 nm), $ZnSO_4$ and the organic solvents were purchased from Sigma Aldrich (St-Louis, USA) while Atto 655 – NHS ester (λ_{ex} 648 nm – λ_{em} 668 nm) was purchased from ATTO-TEC (Siegen, Germany). IgM for Tacrolimus was purchased from Abcam and Biorbyt (Cambridge, United Kingdom) stored in Tris-buffered saline with as a preservative 0.1 % sodium azide.

4.4.2 Preparation and handling of whole-blood samples

1 mg of tacrolimus was dissolved in 1mL of methanol and the solution was then diluted in TBS until a concentration of 20 µg/mL was achieved. Tacrolimus was further spiked in whole blood samples within a concentration range of 0-160 ng/mL. The extraction from blood samples was accomplished by mixing 400 µl of whole blood with 100 µl of $ZnSO_4$ (300 mM) and 400 µl MeOH (50 %), respectively, into an Eppendorf. The Eppendorf tube was then rigorously vortexed for 15 seconds.

The next step involved centrifugation for 10 minutes at 30k x G, at 4°C.The final stage consisted in decanting the clear supernatant in a new Eppendorf. This final solution was considered as the Whole Blood Extract (WBE).

4.4.3 Synthesis of novel fluorescent derivatives for Tacrolimus:

Synthesis of Fluorescein -Tacrolimus

The derivatization procedure of tacrolimus has been performed by modification on C22 with amino-methyl-fluorescein. 29.4 mg (36.5 µmol) of tacrolimus was dissolved in 3.5 mL of anhydrous methanol and 16.7 mg (204 µmol) of sodium acetate and 40 mg (183 µmol) of O-carboxymethyl hydroxylamine were added. The reaction was stirred at room temperature for 12 hours under argon. Solvent was removed at reduced pressure to give crude solid residue. Furthermore, 6 mg of tacrolimus mono-oxime (6.85 µmol) were dissolved on 0.15 mL of anhydrous DMF. 2 mg of EDAC and 1.35 mg of N-Hydroxysuccinimide was added. The reaction was stirred at room temperature for 1h under argon. The product was further mixed with 5µL of trimethylamine and 0.5mL of 5-(aminomethyl) fluorescein in anhydrous DMF.

The product was confirmed by the Accurate-MassQ Tof LC-MS, 6530 (Agilent Technologies, Santa Clara, California, USA) obtaining for Fluorescein-Tac ($C_{67}H_{85}N_3O_{18}$), (Figure 4.1 A), the nominal mass of M= 1219.58 g/mol, [M+H$^+$] = 1220.58 g/mol**.** The purity obtained was 96%. For the analysis 0.91 mg of Fluorescein-Tacrolimus were dissolved in 1 mL of methanol and the stock solution was further diluted with TBS to the working concentration.

Synthesis of ATTO655-Tacrolimus

The synthesis in case of the red-emitting dye with tacrolimus was similar with the synthesis in of the green-emitting derivative, until the intermediate tacrolimus mono-oxime was formed. Furthermore, 1 mg of tacrolimus mono-oxime was dissolved in 0.05 mL anhydrous dymethylformamide. 0.33 mg of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) and 0.22 mg of N-hydroxysuccinimide were added and the reaction was stirred at room temperature for 1h. The resulting compound was mixed with 0.25 mL of ATTO655 amine (1 mg of ATTTO655 amine previously dissolved in 0.25 ml) and 1µL of triethylamine. The reaction

was stirred at room temperature for 3h under argon. The success of the synthesis was confirmed by the Accurate-MassQ Tof LC-MS, 6530 (Agilent Technologies, Santa Clara, California, USA) obtaining for ATTO 655-Tac ($C_{75}H_{109}N_7O_{18}S$), (Figure 4.1 B), the nominal mass of M= 1427.75 g/mol, $[M+H^+]$ = 1428.78 g/mol.
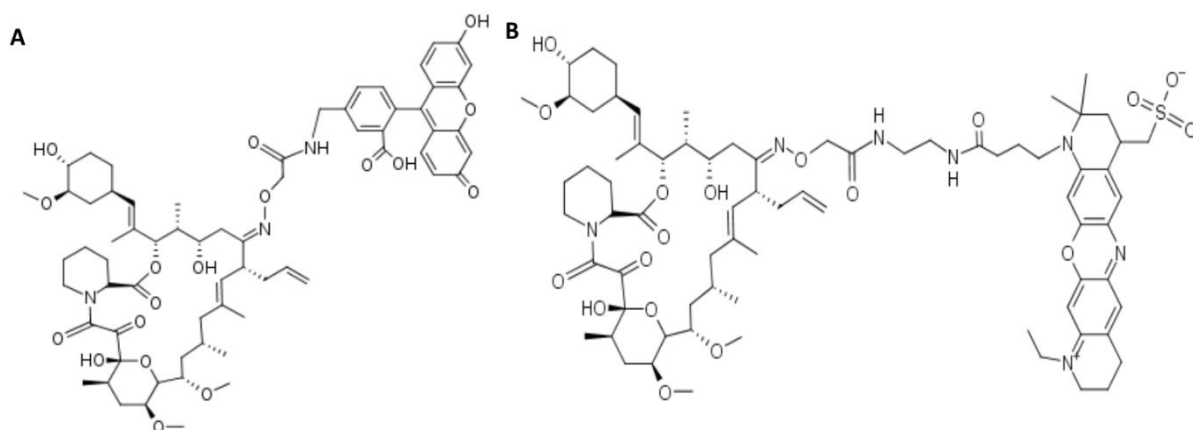

Figure 4.1: Structures of tacrolimus derivatives: A) Fluorescein-Tacrolimus; B) ATTO655-Tacrolimus;

4.4.4 FPIA analysis

FPIA measurements were performed by mixing 20 µl of IgM (2.22 nM) with 20 µl of Fluorescein-tacrolimus (7.5 nM) and 20 µl of tacrolimus with concentrations ranging from 0-100 ng/mL. With the intent to mimic the sample preparation procedures for the drug extraction, 1 mg of tacrolimus was previously dissolved in a mixture composed of 39.6% of methanol, 30% of Tris-buffered saline (TBS) and 20.4% of milliQ water. The reaction mixture was incubated for 10 minutes at room temperature. FP measurements were performed on a SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Diagnostics, California, USA) operated with an excitation filer selected from 485 to 550 nm, an integration time of 150 ms and a temperature of 25°C with 60 µL of the solution dispensed in a 384 flat bottom microtiter plate with non-binding surface (Corning Incorporated, USA). Then binding affinities with the red-emitting fluorophore were tested using the custom-made optical setup described in 2.3.4.

4.4.5 Data analysis

The interaction of the fluorescent labeled tacrolimus with the biorecognition molecules could be treated as a simple association 1:1. The apparent equilibrium constant (k_{app}) could be calculated as described previously for tacrolimus derivatives[171] :

$$Kapp = \frac{[FP]}{[F][P]},$$

(*Equation 12*)

where [FP] is the equilibrium concentration of the tracer/protein complex and [P] and [F] are the equilibrium concentrations of free protein and free tracer.

For the competitive assays, the resulting calibration points were fitted in IgorPro 6 (WaveMetrics, Oregon) using a 4-parameter logistic equation, as described in section 2.3.5:

$$r = r_{\min} + \frac{(r_{\max} - r_{\min})}{\left\{ 1 + \left(\frac{IC_{50}}{c} \right)^{rate} \right\}} \quad , \qquad \text{(Equation 13)}$$

where r is the measured anisotropy, c the concentration, $r_{\min}$ the lowest anisotropy measured in presence of an excess of unlabelled tacrolimus, $r_{\max}$ the highest anisotropy measured in absence of unlabelled tacrolimus, IC_{50} the concentration value corresponding to the intermediate anisotropy and $rate$ a parameter accounting for the slope of the calibration curve.

4.5 Results and discussion

4.5.1 Affinity and kinetic studies of Tacrolimus derivatives with bio-recognition molecules

Fluorescence anisotropy is widely used for measuring high-affinity protein-protein interactions – among others and has been used for characterization of binding affinity studies for immunosuppressants [172]. However, an FP assay for tacrolimus quantification currently do not exist.

Herein, for the design of FP assay for tacrolimus, two fluorescent derivatives were synthesized. Fluorescein-NHS was chosen for the derivatization of tacrolimus for excellent fluorescence quantum yield, and good water solubility. A red-emitting fluorophore, Atto655-tacrolimus, was also synthesized and would be preferred due to lower interferences with human samples, as showed in the case of tobramycin measurements in Chapter 2.

The green and red-emitting derivatives of tacrolimus were explored in terms of binding affinities using a standard saturation experiment with the drug's specific biorecognition molecules. Initially, FKBP12 protein (Figure 4.2 A) was chosen as is the tacrolimus natural binding protein [172], [173], [174]. The Atto655-tacrolimus was titrated with the drug's target, the FKBP12 in buffer and 20% methanol. The organic solvent was used to simulate the extraction conditions and to understand the effect on the binding affinity.
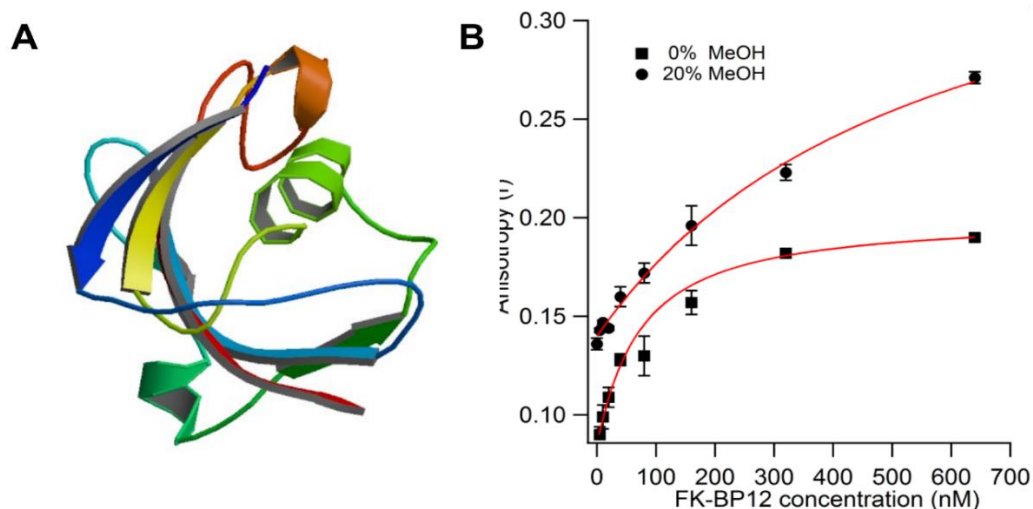
Figure 4.2: A) Cristal structure of FK BP 12 protein, the specific binding protein for Tacrolimus; B) FK-BP titration simulating the plasma sample preparation conditions: the round points represent the titration in 20% MeOH and 80 % TBS while the squared ones represent the titration in 100% TBS;

The curve illustrated in Figure 4.2 B shows that, when a fixed concentration of Atto655-Tacrolimus was titrated with different concentrations of FK-BP12 protein, the K_{app} in the case of clean target (buffer only) was 70 nM. Furthermore, the addition of 20% of MeOH increased the K_{app} to 500 nM, indicating that the addition of organic solvent significantly affected the binding affinity of the fluorescent derivative. This low affinity does not allow for the successful implementation of immunoassays in the nM range.

The second biorecognition molecule was an IgM antibody, the only commercially available for tacrolimus. Atto655-Tacrolimus showed no binding with IgM and was therefore discarded as potential tracer for the tacrolimus immunoassay.

To assess the utility of Fluorescein-tacrolimus as fluorescent probe, we then determined K_{app} of the derivative binding to FK BP12, as well as IgM and human serum albumin (HAS) as a negative control (Figure 4.3).
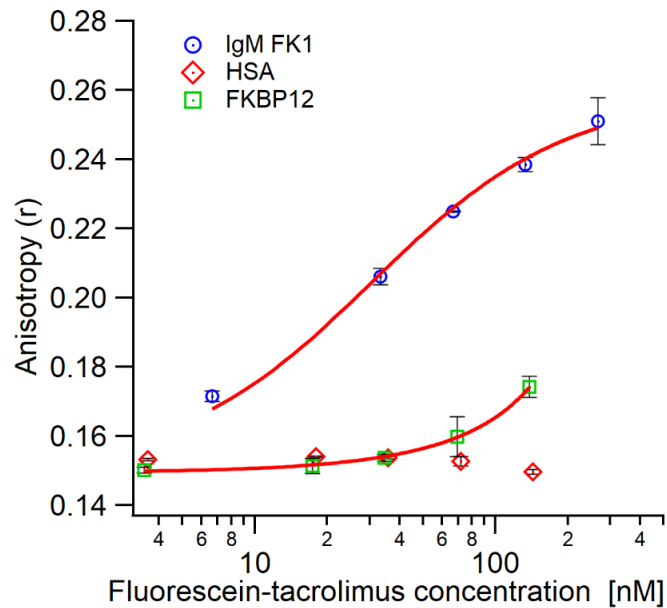
Figure 4.3: Fluorescence anisotropy changes observed during titration of Fluorescein-tacrolimus with IgM (K_{app} = 50 nM), human serum albumin (HSA) and FKBP 12;

While the titration of Fluorescein-tacrolimus did not lead to any response when FK-BP12, or HSA was added, the formation of complexes between Fluorescein-tacrolimus/IgM led to an increase of fluorescence emission anisotropy, having a K_{app} of 50 nM. This result allowed to further design and optimize the assays with this antigen/antibody pair, although the affinity was low.
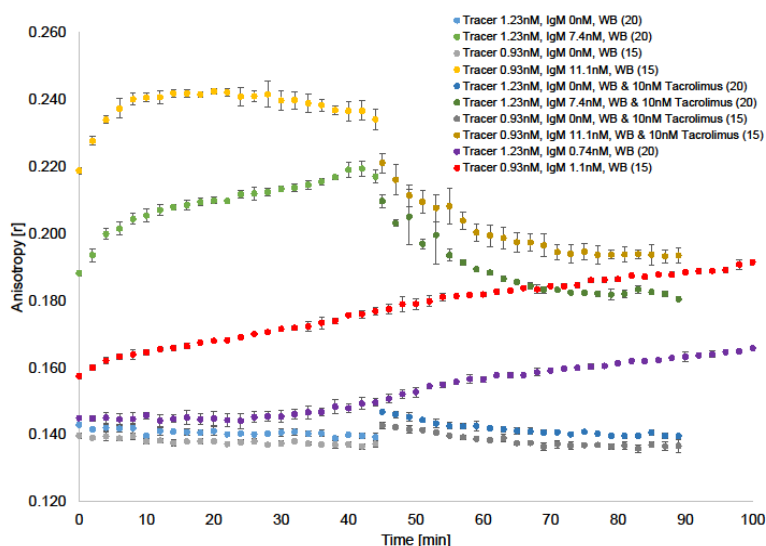

Figure 4.4: Kinetic studies for the binding of IgM with the fluorescein derivative of tacrolimus; Different concentrations of tracer/IgM were tested and the pairs that showed a minimal binding (yellow, green, blue and grey markers) where then titrated with a fixed concentration of tacrolimus (10 nM) showing the displacement of the tracer from the antibody binding sites by

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the drug (dark yellow, dark green, dark blue and dark grey); Standard deviations were calculated from triplicates;

Kinetic studies of IgM with Fluorescein-tacrolimus were also performed (Figure 4.4) by testing and titrating different concentrations of the tracer and IgM antibody. After an equilibrium reached after approximatively 10 min, the addition of 10 nM of tacrolimus to the tracer/IgM complex led to a reduction of the observed anisotropy values.

4.5.2 FPIA for tacrolimus quantification in clean target

A new FP assay for tacrolimus quantification was designed in TBS buffer. Different concentrations of tacrolimus within the range of 0-100 ng/mL were spiked in TBS. The dose-response curve illustrated in Figure 4.5 shows that the assay format might be suited for the concentration measurements of tacrolimus within therapeutic range, but with limited performance.
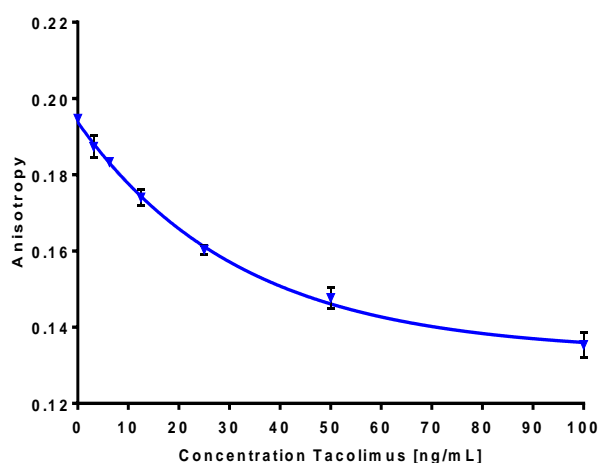
Figure 4.5: Drug-response curve of tacrolimus quantification using Fluorescein-tacrolimus derivative, in TBS; each point was performed in triplicates.

The assay showed a dynamic range at concentrations slightly higher compared with the therapeutic range but with good reproducibility among the replicates with standard variations between 0.001 – 0.005.

4.5.3 FPIA for tacrolimus in real samples

Whole blood preparation represents a key step for tacrolimus quantification, as it will condition the correct quantification of the drug. In general, the immunosuppressants must be extracted and separated from the other components of the patient blood sample [175].

In general, sample preparation procedure for tacrolimus implies several steps such as:

- **Red-blood-cell lysis**: usually performed with methanol;
- **Protein precipitation**: is performed with addition of $ZnSO_4$ and/or an organic solvent such as methanol or acetonitrile;
- **Separation:** which is usually performed by centrifugation;

Extraction of FK 506 from its binding proteins from whole blood is usually achieved by addition of organic solvents [176] and methanol has been typically employed to extract tacrolimus and other ISD's[177]. However, a careful balance must be achieved such as the methanol is sufficient to liberate the drug from the binding protein but not so high as to interfere with the immunoassay.

In our approach, different ratios of organic solvents for cell lysis and precipitation agents were evaluated with regard to the extraction efficiency and the matrix effects. Figure 4.6 B shows an image of the fresh whole blood samples after treatment with different sample preparation mixtures used for the protein precipitation and drug extraction. Initially, we tested the influence of methanol in 80% and 20% water only on the whole blood sample, which resulted in a highly heterogeneous solution indicating the lysis of RBC. After an optimization of sample preparation procedures, we transposed the assay to real whole blood samples. A concentration range (between 0-160 ng/mL) of tacrolimus spiked in whole blood was used (Figure 4.6 A).
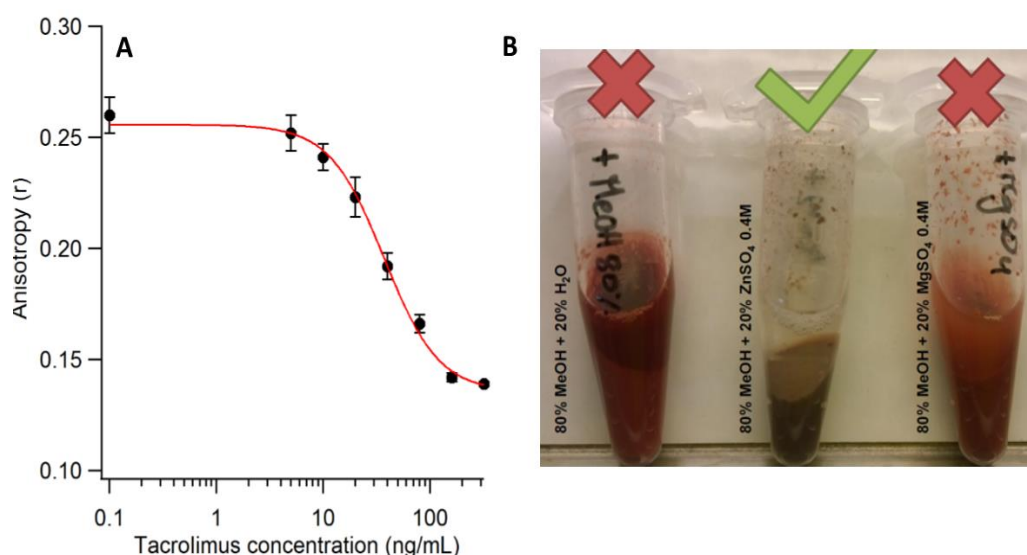

Figure 4.6: Dose-response curve of tacrolimus quantification using spiked whole blood samples; B) picture of whole blood extraction after different sample preparation strategies; The red line is a fit of the calibration points using a four-parameter logistic equation yielding the

following parameters: $IC_{50} = 32 \pm 3.3$ ng/mL, $r_{min} = 0.148 \pm 0.007$, $r_{max} = 0.252 \pm 0.016$, rate = 0.9 ± 0.3.

When using spiked whole blood samples for tacrolimus quantification, by comparison with clean target, the dynamic range of the assay was shifted within a range of concentration of 10-100 ng/mL. This might be explained by the fact that the binding affinities properties were changed using blood samples and the drug extraction procedures. However, although organic solvents are implied and which might affect the reproducibility of the assay, here the standard deviations (STD) among the triplicates measurements showed good reproducibility (0.001 – 0.006). Additional improvements should be performed in order to be able to quantify tacrolimus within the therapeutic range (10X fold reduction in concentration).

4.6 Conclusions

Herein efforts in designing an FP method for tacrolimus have been described. FP could be an advantageous method for tacrolimus quantification due to its sensitivity but also mix and measure properties. For the assay design, two derivatives for tacrolimus at different wavelengths were synthetized and tested in terms of binding affinity with the biorecognition molecules. The green-emitting fluorophore/IgM was the pair that allowed the assay design in clean target for tacrolimus quantification within the therapeutic range. Our results suggest that, although FPIA is feasible for quantitative analysis for tacrolimus, the binding affinities but also extraction of the tacrolimus from RBC can be strongly influenced by the sample preparation procedures of whole blood. Having a significant impact on the quantification of the drug, sample preparation procedures could be further optimized by avoiding using organic solvents, for instance, for the lysis of the red blood cells in order liberate the tacrolimus/protein complexes but also to precipitate the proteins.

Chapter 5

5. Conclusions and further perspectives

This thesis presents various approaches to implement FPIA into microstructures with the aim to quantify small drug molecules in whole blood. While this assay format is well established in microtiter well plates or in cuvettes, the approach to perform FP measurements within microstructures remained unexplored to a large extent.

As a first approach, paper and paper-based microstructures were chosen due to their low cost, biocompatibility but also ability to perform sample preparation. The goal was to test the feasibility of using paper and paper-based microstructures to host FP measurements. This study highlighted the importance to screen the different types of materials available like cellulose, filter paper and glass fibers and the various fluorophores in order to choose the best pair for anisotropy measurements. Glass fiber microstructures were selected for their low fluorescent background and scattering effect in combination with a red-emitting fluorophore. After optimization of the spot size and the number of measurements per detection chamber, the measurement errors significantly decreased. This enabled the accurate quantification of tobramycin within glass fiber microchambers in human plasma sample with CVs below 20% within the therapeutic range.

The ability to measure FP in plasma in glass fiber microchambers was combined with the capacity of this membrane to separate whole blood. With prior optimization of whole-blood dilution, the anisotropy measurements in glass fiber sheets yielded values similar to the anisotropies obtained for plasma. Therefore, analysis were performed for the quantification of tobramycin previously spiked in whole blood. The results were very promising and indicated that, using fresh whole blood (i.e. within 24 h), tobramycin could be quantified with coefficients of variations between 20-30 % in the range 1-12 µg/mL, the therapeutic domain of the drug.

The assay performance in paper still suffers from large coefficient of variations and this might be due to several different factors: temperature and humidity, which can affect the kinetic of the binding reaction, uncontrolled evaporation of the reagents once added to the paper and, possibly, also variations in the migration speed of the reagents. In addition, errors in pipetting minute amounts of sample might have influenced the coefficients of variation.

Two major advantages were achieved by employing this approach of performing FP within glass fiber micro-chambers: the ability to use paper - based microstructures for the pre-processing steps, using this way minute amounts of whole blood samples and, simultaneously, the ability to perform sensitive quantification of tobramycin within its therapeutic range. This last finding holds great promises for using the present technology as an alternative to clinical analyzers available in centralized institutions.

Another motivation for selecting fluorescence polarization as a read-out was triggered by the possibility to perform quantitative assays on a compact detection system. In this approach, glass capillaries were used as detection chambers. Even if often reduction in size comes at the price of compromised performance, this was not the case for the novel optical device, which exhibited a preliminary performance similar to standards methods with coefficients of variation of 30% within the therapeutic range of tobramycin.

Opportunities and challenges linked to the use of FPIA were revealed when implementing the assay for tacrolimus. On the one hand, a novel FP assay for tacrolimus could be developed that was able to quantify the drug in buffer within the therapeutic window 2-15 ng/mL. This was possible by synthesising and testing different fluorescent derivatives of tacrolimus emitting at different wavelengths. On the other hand, drug extraction from whole blood was found to be of critical importance and to adversely impact the performance of the assay so that further optimization procedures should be undertaken for the efficient extraction of tacrolimus.

Possible further perspectives of this PhD thesis are described below:

- Taking advantage of the results provided by the proof-of-concept study on the ability of FPIA to quantify molecules within paper, it would be very interesting to apply this methodology on samples from patients that are taking the drug.

- Additional research would be desirable to make full use of the multi-functionality of paper and an improvement could be to further simplify the sample preparation procedure. An alternative for the simplification of sample preparation could be achieved, for instance, with the immobilization of the reagents through drying in the different microstructures (i.e glass fiber sheets or glass capillaries). However, in this format it might be difficult to control the incubation time after the resuspension of the antibodies or antigens, therefore this step should be kept in consideration as this might cause a lower sensitivity and may give false positive signals [178]. Another procedure

would be to re-design the paper platform so that the separation, mixing and dilution could be integrated, like is described by Osborn et. al.[179].

- It would be interesting to integrate the paper-based micro-chambers into a miniaturized analytical device: miniaturized medical devices require the combination of sample preparation platforms with sensitive read-out techniques. By carefully selecting the sample preparation procedures, the types of microstructures, multiple detection and quantification could be achieved within adequate concentration ranges.

- The described FP system still requires further optimization in order to fully work as a POC test. A necessary improvement is to integrate the miniaturized analytical system with temperature and humidity sensors that would be able to measure and regulate these parameters. Moreover, at present, the mixing of reagents and their addition to capillaries is performed manually. Since it was demonstrated that it possible to use a PDMS chip to separate whole blood, it could be considered in a new design to incorporate supplementary microchannels in order to perform also the mixing of reagents and the transfer into the detection capillary.

- Simultaneous measurements of multiple drugs, i.e. multiplexing, would be a possible and desirable direction for future work.

In conclusion, the capability of achieving sample-in answer-out within paper-based platforms would make a great contribution to the development of paper-based diagnostics for quantification of low-molecular weight therapeutics and it would represent a big step forward in improving patient care at the POC. Also, the design of a rapid and compact device like the one described herein, could open the path towards the development of a POC device for TDM.

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Curriculum Vitae

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Education

2013- 2017   École Polytechnique Fédérale de Lausanne (EPFL)

PhD Student in Chemistry and Chemical Engineering,
Laboratory affiliated: Diagnostic System Unit, HES-SO Valais, Wallis
- Design of miniaturized assays for small-molecule drugs within whole-blood followed by their implementation into a Point-of-Care system for Therapeutic Drug Monitoring
- Thesis: Microdevice-integrated immunoassays for Point-Of-Care Therapeutic Drug Monitoring

2010- 2013   University of Turin, Turin, Italy

Master's in Advanced Chemical Methods
- Designed a novel 'theragnostic' agent (therapy and diagnosis) able to ameliorate hepatic injuries in mice through Magnetic Resonance Imaging (MRI)
- Thesis: 'The use of apoferritin as carrier of GdHPD03A and curcumin for the treatment of hepatic pathologies guided of MRI'

2010   University of Turin, Turin, Italy

B.Sc. in Chemistry
- Thesis: Application of electroanalytical techniques for the study of chemical and biochemical systems.

Projects and internships:

- 2016 CTI Entrepreneurship Training - Business Concept - EPFL, Switzerland
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Publications:

Journal papers related to this thesis:

- E.-Diana Bojescu, Denis Prim, Marc E. Pfeifer, Jean-Manuel Segura, *Fluorescence-polarization immunoassays within glass fiber micro-chambers enable drug quantification in whole blood for point-of-care therapeutic drug monitoring,* (to be submitted);

- Diana Bojescu, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *Small-drug quantification from whole-blood within paper-based microstructures for Point-Of-Care Therapeutic Drug Monitoring*, The 20th International Conference on Miniaturized Systems for Chemistry and Life Sciences (MicroTAS), Conference paper; 9-13 october 2016, Dublin, Ireland

- E.-Diana Bojescu, Denis Prim, Marc E. Pfeifer, Jean-Manuel Segura, *Towards Point-Of-Care devices for immunosupressants monitoring: challenges and opportunities,* (review under preparation);

Journal papers not related to this thesis:

- Juan Carlos Cutrin, Simonetta Geninatti Crich, Diana Burghelea, Silvio Aime: *Curcumin/Gd loaded Apoferritin: a novel "theranostic" agent to prevent hepatocellular damage in toxic induced acute hepatitis*, Molecular pharmaceutics, april, 2013

Oral presentations:

- Diana Burghelea, D.Prim, M. Pfeifer, J.-M. Segura: '*Drug quantification in blood, within microsturtures for Point-Of-Care Therapeutic Drug Monitoring'*, 1ere Journée de la Recherché (HEI), 24th of August 2015, Sion, Switzerland;

- D.Burghelea, D.Prim, M. Pfeifer, J.-M. Segura: *Drug quantification in blood, within microstructures for Point-Of-Care Therapeutic Drug Monitoring*, Summer School Biotechnet, 1st of September 2015, Wädensvil, Switzerland;

- D.Burghelea, D.Prim, M. Pfeifer, J.-M. Segura: *Drug quantification in blood, within microstructures for Point-Of-Care Therapeutic Drug Monitoring*, Swiss Chemical Society Fall Meeting, 4th of September 2015, Lausanne, Switzerland;

- Diana Bojescu, Denis Prim, Frederic Truffer, Nicolas Malpiece, Marc Pfeifer, Martial Geiser, Jean-Manuel Segura, *Micro-device integrated platforms for Point-Of-Care Therapeutic Drug Monitoring,* Swiss Chemical Society Fall Meeting, 21-22 August 2017, Bern, Switzerland;

- Diana Bojescu, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *Microdevice-integrated immunoassays for Point of Care Therapeutic Drug Monitoring*, MediLaboTech, 30th of August-1 September 2017, Lausanne, Switzerland.

Posters:

- Diana Bojescu, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *A miniaturized Fluorescence Polarization Immunoassay for Point-of-Care Therapeutic Drug Monitoring* 23-25th of June 2014: The European summit for Clinical Nanomedicine and targeted Medicine – Basel, Switzerland

- D.Bojescu, D.Prim, M. Pfeifer, J.-M. Segura: Miniaturized Fluorescence assays for Point-Of-Care Therapeutic Drug Monitoring, Summer School in Translational Biology(EPFL), 6-10th July 2015, Interlaken, Switzerland;

- D.Bojescu, D.Prim, M. Pfeifer, J.-M. Segura: '*Drug quantification in blood, within microstructures for Point-Of-Care Therapeutic Drug Monitoring*', 1ere Journée de la Recherché (HEI), 24th of August 2015, Sion, Switzerland;

- D.Burghelea, D.Prim, M. Pfeifer, J.-M. Segura: 'Drug quantification in blood, within microstructures Point-Of-Care Therapeutic Drug Monitoring**,** Euroanalysis 2015, 6-10th September 2015, Bordeaux, France;

- Diana Burghelea, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *Small-drugs quantification from whole-blood within paper-based microstructures for Point-Of-Care Therapeutic Drug Monitoring***,** Swiss Chemical Society Fall Meeting, 15th of September 2016, Zurich, Switzerland;

- Diana Bojescu, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *Small-drug quantification from whole-blood within paper-based microstructures for Point-Of-Care Therapeutic Drug Monitoring*, The 20th International Conference on Miniaturized Systems for Chemistry and Life Sciences (µTAS), 9-13 october 2016, Dublin, Ireland, poster presentation;

- Diana Bojescu, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *Single-step whole-blood bioassays for small-drug quantification within paper-based microstructures*, NanoTera final event, 31st March 2017, Lausanne, Switzerland, poster presentation;

- Diana Bojescu, Denis Prim, Frederic Truffer, Marc Pfeifer, Martial Geiser, Jean-Manuel Segura, Micro-device integrated platforms for Point-Of-Care Therapeutic Drug Monitoring, Swiss Chemical Society Fall Meeting, 21-22 August 2017, Bern, Switzerland;

- Diana Bojescu, Denis Prim, Marc Pfeifer, Jean-Manuel Segura, *Microdevice-integrated immunoassays for Point of Care Therapeutic Drug Monitoring*, MediLaboTech, 30th of August-1 September 2017, Lausanne, Switzerland;

Prizes:

- 2015 Best poster award, 1ere Journée de la Recherché (HEI), 24th of August 2015, Sion, Switzerland

- 2016 Chemistry Travel Award offered by SCNAT, SCS and SSFC

- 2016 Best Poster Award at Swiss Chemical Society (SCS), Fall Meeting, Zurich, Switzerland