Screening of cell-drug interactions using acoustic trapping and MALDI MS

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CONTENTS

1 INTRODUCTION

In the fields of biotechnology and analytical chemistry there is an increasing need for new, more efficient, cheaper and less time-consuming methods of analysis. Among the various applications of these methods is the field of drug development: better analytical methods may help reduce the increasing costs of drug development, eventually contributing to faster and more effective innovation of new pharmacotherapies. Miniaturized techniques are increasing in popularity due to their reduced reagent and solvent consumption, and potential for rapid analysis of large amounts of compounds (Whitesides 2006). In addition, cheap and disposable miniaturized platforms eliminate the carry-over effects that can be cumbersome in traditional larger scale methods.

This MSc (Pharm) thesis consists of two parts, the literature review and the experimental section. In the literature review, the topics to be discussed are general properties and applications of microfluidic systems, and acoustofluidics. In addition, brief background information is provided about serotonin transporter in platelets, MALDI mass spectrometry, and screening of cell-drug interactions. The experimental section describes the development of a new nanobiotechnology platform for mass spectrometric screening of cell-drug interactions using acoustic trapping. The miniaturized solid phase extraction procedure for the analytes to be used was optimized, and the performance of the acoustic trapping setup was assessed and modelled. The suitability of the platform was studied by investigating the interaction of selective serotonin reuptake inhibitor (SSRI) drugs fluoxetine and citalopram with their molecular target, the serotonin transporter, in human platelets and cell membranes from cells overexpressing serotonin transporter. A comparative SSRI binding assay was performed without using acoustic trapping.

2 LITERATURE REVIEW

2.1 Microfluidics and lab-on-a-chip systems

Microfluidics can be defined as "the science and engineering of systems in which fluid behavior differs from conventional flow theory primarily due to the small length scale of the system" (Fundamentals and Applications of Microfluidics 2006). Fluidic systems in microscale have unique physical characteristics compared to corresponding systems of conventional size, and thus microfluidics is not only about miniaturization of systems, but as well investigation and understanding of these microscale phenomena. Naturally, the understanding of the physics of microscale flows will improve the design and fabrication of microsystems.

The introduction of the fabrication technology called silicon micromachining in the mid-20th century and the subsequent revolutionary advance in microelectronics have paved the way for the application of miniaturization in various other contexts, one of which is microfluidics (Fundamentals and Applications of Microfluidics 2006). Other major factors contributing to the rise of microfluidics are the efficiency of miniaturized methods in chemical and biological analyses, and the development of portable devices for the detection of chemical and biological warfare agents (Whitesides 2006). Currently, microfluidic devices can be found in various fields and technologies, including chemical synthesis and analysis, as well as in countless applications in life sciences. Some of these applications are reviewed in the following chapter. Today, microfluidics is a major interdisciplinary field covered in many specialized journals, such as *Microfluidics and Nanofluidics*, *Lab on a Chip* and *Biomicrofluidics*, as well as international conferences. In addition, articles involving microfluidics are frequently published in numerous major scientific journals.

Characteristically for an emerging multidisciplinary field, the terminology used in microfluidics is broad, and occasionally inconsistency and confusion tend to occur. The following terms are associated with microfluidic technology in general; more specific terms used will be covered in concerning sections. The term microelectromechanical

systems (MEMS) refers to “devices that have characteristic length of less than 1 mm but more than 1 micron, that combine electrical and mechanical components and that are fabricated using integrated circuit batch-processing technologies” (Gad-el-Hak 1999). MEMS is commonly used to describe various kinds of microtechnology, but some argue that the limitation is that besides electrical and mechanical, it does not include any other techniques used, such as fluidics and optics (Fundamentals and Applications of Microfluidics 2006). A better umbrella term would thus be microsystem technology (MST). Sometimes nanofluidics, describing fluid flows in nanoscale, is considered as its own, separate field (Eijkel 2009). However, microfluidics can be thought to include also nanofluidics (Fundamentals and Applications of Microfluidics 2006). The term μTAS (miniaturized total chemical analysis system) was first used in 1990, referring to a total chemical analysis system performing all the sample handling steps in the immediate vicinity of the measurement (Manz et al. 1990). While μTAS only refers to analytical applications, the term lab-on-a-chip (LOC) is used for any kind of laboratory processes executed on a microfabricated chip.

As stated in the above definition of microfluidics, the fluid behavior in microscale differs fundamentally from that of macroscale systems. The mobile phase can be either liquid or gas, and even some supercritical fluid applications have been reported and reviewed (Marre et al. 2012). Due to the nature of the experimental section of this thesis, the main focus is on liquid systems. For liquids in microchannels, it is justified to assume liquid incompressibility, and the descriptions in the following chapters rely on that assumption (Squires and Quake 2005).

In fluid mechanics, it is common to make the continuum assumption: the fluid is considered as a continuum, indefinitely divisible, and its characteristics are assumed to change continuously between different points of the fluid flow (Gad-el-Hak 1999). The continuum assumption is indeed applicable for most situations in microfluidics, and consequently the laws of conservation of mass, momentum and energy can be used for the modeling of those situations. The partial differential equations called Navier-Stokes equations are the mathematical description of the flow behavior, and thus these

equations can be used in the estimation of the applicability of the continuum assumption.

However, when the size range of the fluid channels is decreased enough (generally below 10 nm), the continuum assumption tends to fail due to the channels being not wide enough to contain enough fluid molecules for continuous behavior (Squires and Quake 2005). In such situations, the intermolecular variations of fluid properties become significant relative to the size scale of the apparatus. Such deviation from the continuum assumption occurs also in the presence of high shear rates, when the Newtonian behavior of fluids becomes disturbed (Loose and Hess 1989). It is notable that the larger the molecular mass of the fluid, the lower shear rates are required for this phenomenon to occur. The applicability of the continuum assumption requires also that the system is sufficiently close to thermodynamic equilibrium (Gad-el-Hak 1999). In such cases of continuum assumption failure, molecular approaches are needed: instead of a continuum, the fluid is treated as a collection of molecules interacting with the channel walls and each other. In modeling, this obviously leads to more accurate results, since the fluid is modeled as it really is. However, modeling the velocities, locations and collisions of all fluid molecules separately makes the modeling process more complicated, and thus the molecular approach requires more computing power.

Boundary conditions are assumptions about the behavior of the fluid flow in the immediate vicinity of an interface with another phase, usually a channel wall (Gad-el-Hak 1999). Due to interactions between the fluid and the wall, altered fluid behavior in the terms of temperature, velocity and pressure can be expected near the wall. In systems of conventional size, it is commonly assumed that the fluid temperature, velocity and pressure right next to the wall equal those of the wall. Treating the fluid as a continuum leads to these assumptions: the characteristics change continuously within the fluid, and there is no reason to assume that the molecular interactions between two fluid particles radically differ from the same interactions between fluid particles and the channel wall; consequently, no discontinuity of those characteristics can occur between the fluid and the channel wall.

In microfluidic liquid applications, it can be expected that the large surface-to-volume ratio may cause confusion in the application of boundary conditions: the first two boundary conditions describing temperature and pressure are nevertheless valid in most of the cases, as the system size is still sufficiently large for the continuum assumption (Squires and Quake 2005). However, the failure of continuum assumption, i.e. the non-Newtonian behavior of fluid, leads to the inapplicability of the boundary condition describing fluid velocity, known as the no-slip boundary condition. For liquids, this happens e.g. in situations of high shear rate (Loose and Hess 1989), corner flows (Moffatt 1964) and the presence of two immiscible fluids on a solid surface (Thompson and Robbins 1989).

The most characteristic property of microscale flows is laminarity: the viscous forces dominate over inertial forces in the fluid (Squires and Quake 2005). As a result, no turbulence occurs, and the fluid flow has no velocity parallel to the channel. The most common parameter for the flow laminarity is the Reynolds number, defined as the ratio of inertial and viscous forces in the fluid:

$$\mathrm{Re} = \frac{f_{inertial}}{f_{viscous}} = \frac{\rho U_0 L_0}{\eta}$$

in which ρ stands for the density of the fluid, L_0 for the typical length scale of the system, U_0 for the velocity of the fluid movement and η for the shear viscosity of the fluid (Squires and Quake 2005). The borderline Reynolds number between laminar and turbulent flow depends on the fluid characteristics, but is generally between 2000 and 3000. Flows with low Reynolds numbers are laminar, and they are known as Stokes flows. It is notable that calculations of Reynolds number assume negligible gravity. The laminarity makes the flow behavior predictable, and enables unique applications, such as those utilizing parallel flows of immiscible liquids, between which solute exchange occurs via diffusion, in a single channel. On the other hand, the laminar flow has to be taken into account in cases such as mixing, which happens entirely by diffusion in the absence of turbulence.

The no-slip boundary condition leads to the typical flow profile of a fluid flow in a microchannel, which occurs also in cases in which slip takes place: since the fluid velocity at the immediate vicinity of the channel walls is zero or extremely small, there must be a velocity gradient present in the fluid flow (Squires and Quake 2005). The means of inducing the flow affects the flow profile in straight channels: for pressure-driven flow (Poiseuille flow) it is parabolic and for electro-osmotic flow flat (Figure 1). The difference in flow profiles has its effect on e.g. separation science, in which the parabolic flow profile is a known source of band broadening (Kutter and Welsch 1995). The effect responsible for this is known as Taylor dispersion (Taylor 1953, 1954). The same effect can be exploited in mixing applications (Chou et al. 2001).

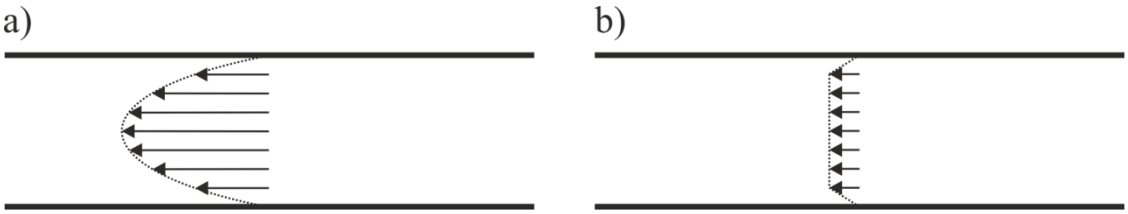


Figure 1: The fluid flow profiles in a microfluidic channel. a) pressure-driven flow; b) electro-osmotic flow.

Another typical characteristic of microfluidic systems is high surface-to-volume ratio, arising from the small size of the fluid channels (Fundamentals and Applications of Microfluidics 2006). In the contact surfaces between the fluid and the microchannel walls, the significance of surface tension is larger compared to macroscale systems. This results in capillary force being an important effect which can be greatly influenced by the choice of surface materials and surfactants. The surface tension can also be altered by electric fields (Lippmann 1875), in a process called electrowetting (Beni and Hackwood 1981). The increased importance of surface forces makes the use of electrokinetic techniques suitable for moving fluids and particles in microfluidic systems (Fundamentals and Applications of Microfluidics 2006).

When the distances within the fluid are small, the importance of diffusion is bigger (Manz et al. 1990). This can be both an advantage and a disadvantage: on the one hand, e.g. reactive species can be easily brought together and Brownian motion of particles can be used for temperature measurements (Chung et al. 2009), and the different

diffusion properties of molecules utilized in separation (Chou et al. 2000). On the other hand, Brownian motion can also be a source of uncertainty when measurements with tracer particles are done (Olsen and Adrian 2000).

A wide variety of materials and techniques is used for the fabrication and manufacture of microsystems (Chanmanwar et al. 2013). The intended purpose and desired outcome of the system to be produced affect the choice of material, and all these factors contribute to the choice of fabrication technique (Bruzzone et al. 2008). The differences in elasticity and chemical stability of materials are frequently utilized in the manufacture of microsystems through the use of molding masters as well as sacrificial and stable material layers. The materials available include glass, different forms of silicon, metals, a variety of polymers, and ceramics. An important property of the microfabrication technique used is resolution, which determines the possible size range and the finishing quality of the product. In small channels, minor changes in channel shapes can greatly influence the fluid flow properties within the channel. The objective is to make ideal-shaped channel systems with high speed and repeatability.

A wide variety of microfluidic applications exists, discussed in detail both in books and review articles (Fundamentals and Applications of Microfluidics 2006, Squires and Quake 2005, Whitesides 2006). Different microfluidic modules are often combined to form more complicated lab-on-a-chip systems. Numerous advantages are characteristic to the microfluidic applications: portability helps to bring the technology where conventional systems can’t be taken, and lower consumption of chemicals and energy decreases both the environmental burden and the price of operation. Given the possibility of affordable mass production, the price of the systems can be very low. The unique physical phenomena of microscale fluid behavior can be used of benefit, but on the other hand, they make the scaling-down of systems more challenging. Especially the connections between microscale and macroscale are particularly tricky: e.g. the significance of dead volumes increases. Narrow channels are more prone to suffer from clogging and bubble formation, and it may be difficult to combine several electrical instruments on a single chip without electrical disturbances.

The examples of applications of microfluidic systems in analytics include sampling (Tesař 2001), sample preparation (Fritz and Masso 2001), separation (Erxleben and Ruzicka 2005) and detection (O'Toole et al. 2006), as well as combinations of these (Cakal et al. 2010). Disposability of analytical devices decreases the risk of contamination (Ricco et al. 2002). Analysis with high sensitivity and resolution can be achieved using only minuscule amounts of samples, solvents and reagents (Whitesides 2006). Given the short fluidic distances, the microfluidic analysis is also very fast. The present and potential future applications of microfluidic analytic devices include the detection of biomarkers in diagnostics, the field of drug development, and scientific research in general, especially in the life sciences.

Besides applications in biochemical analysis in life sciences, microfluidic systems can be used in cell handling and cell cultures (Walker et al. 2004). The size of the systems is suitable for cells, and allows the modification of the microenvironment to produce more *in vivo* –like conditions. This has the potential to improve the *in vitro – in vivo* correlation of cell experiments. In a technology called organ-on-a-chip, cells are implemented on a specifically designed microchip, which is used to model the function of human organs (Polini et al. 2014). Models of separate organs can be combined into a model of entire organism, sometimes referred to as human-on-chip (Luni et al. 2014). Microfluidic devices are suitable also for chemical synthesis, protein crystallization and production of emulsions and foams (Whitesides 2006).

2.2 Acoustofluidics

It has long been known that acoustic fields exert forces on particles (King 1934). The term acoustofluidics refers to "ultrasound-based external forcing of microparticles in microfluidics" (Bruus 2011). This umbrella term includes different techniques, such as acoustophoresis and acoustic trapping, as well as their applications. Acoustophoresis is the moving of particles using acoustic waves, whereas acoustic trapping involves the immobilization of particles (Evander and Nilsson 2012). An excellent 23-part tutorial series on the topic of acoustofluidics has been published in *Lab on A Chip* in the years 2011-2013.

The first reference available on the technique of acoustic trapping dates back to 1996 (Lierke 1996). The technique involves an acoustic standing wave in a fluid-filled microchannel, generated by ultrasound of appropriate frequency, which depends on the measures of the channel (Evander and Nilsson 2012). The relation of channel width or height (x), frequency (f), speed of sound in the fluid medium (v), the wavelength of the sound (λ) and number of pressure nodes (n) is presented in the equation below. In most cases, a frequency generating one pressure node is used, leading to one cluster of acoustically trapped particles in the center of the microchannel (Figure 2).

$$x = n\frac{v}{2f} = n\frac{\lambda}{2}$$

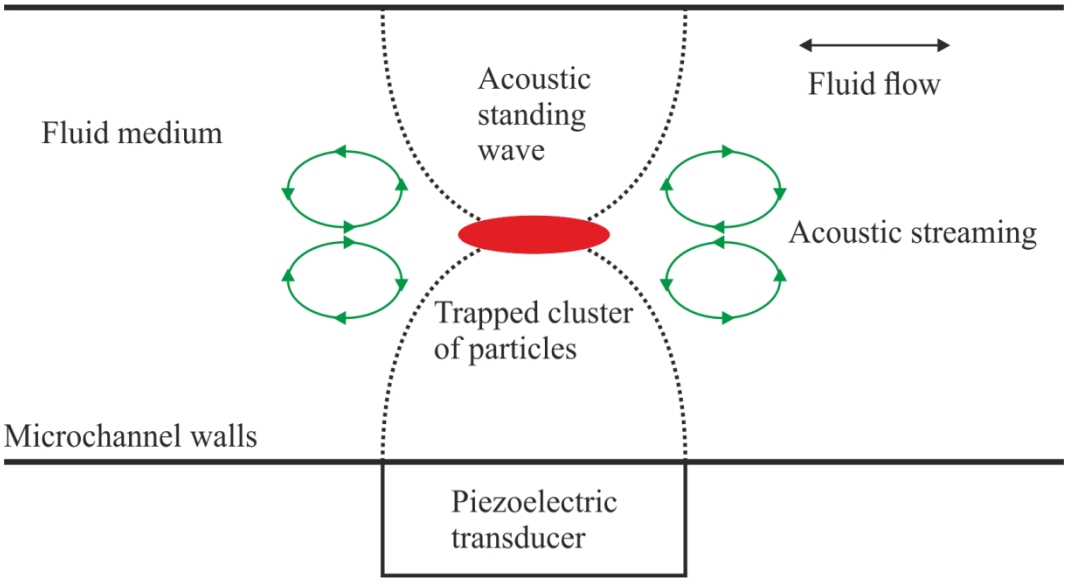


Figure 2: Illustration of the principle of acoustic trapping. Microparticles are trapped in the pressure node of the acoustic standing wave, generated in a microchannel by a piezoelectric transducer. Acoustic streaming causes vortical fluid movement around the cluster of trapped particles.

Points of acoustic potential minima and maxima are generated in the fluid by the acoustic standing wave. The objects, depending on the density and compressibility of themselves and the fluid medium, move either to these minima or maxima (Evander and Nilsson 2012). In water-based medium, cells and microparticles are typically gathered to the acoustic potential minima i.e. the pressure nodes. This is due to the fact that the

cells and particles usually have higher density and lower compressibility relative to water.

The forces moving the particles into the acoustic trap and keeping them trapped are called acoustic radiation forces (Evander and Nilsson 2012). The acoustic waves scattering on the particles are responsible for the force (Bruus 2012). For a particle in a fluid medium, the primary acoustic radiation force (F^{rad}) can be calculated using the following equations:

$$F^{rad} = -\nabla U^{rad}$$

$$U^{rad} = \frac{4\pi}{3} a^3 \left[f_1 \frac{1}{2} \kappa_0 \langle p_{in}^2 \rangle - f_2 \frac{3}{4} \rho_0 \langle v_{in}^2 \rangle \right]$$

$$f_1(\tilde{\kappa}) = 1 - \tilde{\kappa}, \qquad \tilde{\kappa} = \frac{\kappa_p}{\kappa_0}$$

$$f_2(\tilde{\rho}) = \frac{2(\tilde{\rho} - 1)}{2\tilde{\rho} + 1}, \qquad \tilde{\rho} = \frac{\rho_p}{\rho_0}$$

in which U^{rad} is the acoustic potential, a is the radius of the particle, κ_0 is the compressibility of the fluid, ρ_0 the density of the fluid, κ_p is the compressibility of the particle, ρ_p is the density of the particle, p_{in} is the incoming pressure field and v_{in} the incoming velocity field (the chevrons are used to indicate time average). Certain conditions are assumed: the particle is spherical with a radius significantly smaller than the wavelength of the standing wave, and the fluid is inviscid. A more thorough description of acoustic radiation force, including mathematical analyses and derivations, is presented earlier by Bruus (2012).

The description above concerns the situation of one particle in an acoustic field in a fluid medium. When there are two or more particles present, the acoustic waves scattered from the particles cause secondary acoustic radiation forces (Gröschl 1998). For particles residing in the acoustic trap, the forces are attractive, and thus contribute in

keeping them trapped. The secondary acoustic radiation forces, along with the lateral component of the primary acoustic radiation force, are the most significant forces resisting the force exerted on the particle by the fluid flow, known as Stokes' drag (Evander and Nilsson 2012). The magnitude of Stokes' drag on the particles depends on the particle radius and the flow rate of the fluid medium.

An important effect in acoustic trapping is the effect of acoustic streaming, introduced by Faraday in 1831 and in more detail described by Lord Rayleigh in 1884. In principle, the phenomenon occurs when the acoustic standing wave is attenuated by the viscosity of the fluid, a process in which energy is transferred to the fluid, generating movement in the fluid (Sadhal 2012, Wiklund et al. 2012). From the viewpoint of acoustic trapping, it is notable that acoustic streaming will create fluid vortices around the cluster of trapped particles (Figure 2).

As a relatively new branch of microfluidics, acoustofluidics is applied mainly in research, and commercial applications are not yet taken into frequent use. Numerous proof-of-concept publications demonstrate the potential of acoustofluidics as a technique suitable for a wide range of procedures. An acoustophoretic microchip has been successfully used to separate lipids from blood, a procedure that can be applied in cardiac surgery (Jönsson et al. 2004, Petersson et al. 2004, 2005). Examples of other applications include sample preparation (Norris et al. 2009), bioassays (Evander et al. 2007), and 3-dimensional cell cultures (Bazou et al. 2008).

2.3 Serotonin transporter in platelets

Serotonin, or 5-hydroxytryptamine (5-HT), is a neurotransmitter involved in a wide variety of functions in human body (Aghajanian and Sanders-Bush 2002). In addition to the importance of serotonin neurotransmission in the central nervous system and the gastrointestinal tract, it has major significance in the cardiovascular system as well, where it controls e.g. the vascular tone and platelet aggregation (Mercado and Kilic 2010). Elevated serotonin levels in blood are found in several cardiovascular diseases. Serotonin transporter (SERT), the primary target of many antidepressant drugs, is also

expressed on the plasma membranes of platelets, where it is structurally identical and genetically similar to the SERT in the central nervous system (Lesch et al. 1993). In platelets, the main function of SERT is to regulate the serotonin concentration in blood (Mercado and Kilic 2010).

Due to the similarity of SERT in platelets and central nervous system, platelets have been suggested to be used as a model for the function of serotonergic neurons (Da Prada et al. 1988). The suitability of the model has been studied by e.g. Uebelhack et al. (2006), but no explicit correlation between the serotonin uptake to platelets and its partitioning in the midbrain could be proven. Also the suitability of platelet SERT as a biomarker for illnesses such as such as obesity, epilepsy and depression has been investigated (Giannaccini et al. 2013, Cupello et al. 2008, Alvarez et al. 1999). The platelet SERT biomarker studies and their success have been reviewed by Yubero-Lahoz et al. (2013).

2.4 MALDI mass spectrometry

Matrix assisted laser desorption/ionization (MALDI) is an ionization technique for mass spectrometry, first referred to by Karas et al. (1985). Soon after, the technique was proven capable of ionizing large biomolecules intact (Karas and Hillenkamp 1988). Today, MALDI MS is a standard tool for the analysis of biological macromolecules, and also used sometimes in microfluidic applications (Lee et al. 2009). In 2002, one half of the Nobel Prize in chemistry was jointly awarded to Koichi Tanaka (MALDI MS) and John B. Fenn (ESI MS) for their work in the field of biological mass spectrometry.

In MALDI, the sample to be analyzed is mixed and co-crystallized on a sample plate with an organic compound called the matrix, and a pulsed laser beam is focused on the sample plate (Proteomics in Practice 2008). The matrix compound is chosen so that it absorbs light at the wavelength of the laser in use, thus leading to desorption and ionization of the matrix compound. The co-crystallized analytes are also desorbed from the sample plate along with, and ionized by, the matrix compound (Figure 3). Many of the commonly used matrix compounds are cinnamic acid derivatives, particularly α-

cyano-4-hydroxycinnamic acid (CHCA), but also basic compounds can be used (Fitzgerald et al. 1993). The desorption and ionization mechanisms in MALDI are complex processes, and especially for the ionization, multiple mechanisms have been suggested (Knochenmuss 2006). MALDI is predominantly operated under vacuum, but atmospheric pressure applications exist as well (Laiko et al. 2000).

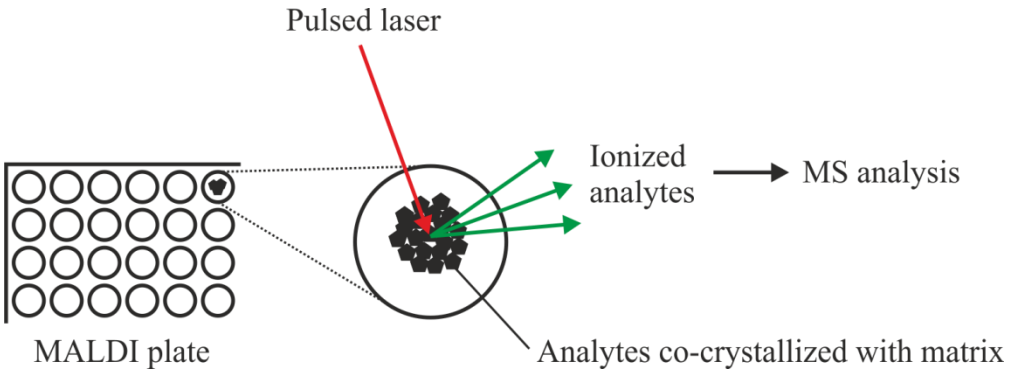


Figure 3: The MALDI MS process. A pulsed laser beam is used to desorb and ionize the co-crystallized analytes and matrix from the MALDI sample plate, followed by mass spectrometric analysis.

2.5 Screening of cell-drug interactions

The *in vitro* screening of cell-drug interactions, including both ligand binding assays on receptors and studies on the effects of drugs on cell cultures of viable cells, is vital for drug discovery and development (de Jong et al. 2005). Simple, reliable and fast methods capable of high-throughput screening (HTS) are required to scan through large chemical libraries in order to find molecules with desired pharmacological activity. In these assays, the detection can be done directly on the receptor-ligand interaction, a secondary messenger released as a consequence of the ligand binding to the receptor, or a functional cellular response to the receptor activation or deactivation.

For the investigation of a ligand binding to a receptor, the typical procedure is to label the ligand with a chemical entity enabling detection (Sittampalam et al. 1997). Commonly used labeling compounds contain either radioisotopes or fluorophores, detected by scintillation or fluorescence, respectively. Labeling a compound may, however, change the properties of the compound. In addition, high price and heavy

workload, and in the case of radioligand labeling, the safety issues, are characteristic of labeling techniques. For these reasons, label-free methods, such as surface plasmon resonance (SPR) and mass spectrometry (MS), are attractive alternatives to the labeling techniques (de Jong et al. 2005).

The typical binding assay involves exposing the target receptor to the test compound, the subsequent washing and elution steps (if necessary), and finally, detection (de Jong et al. 2005). The receptor can be in the form of organ tissue sections, whole viable cells, cell membrane preparations, or as such, produced by recombinant DNA technique. Specific receptor-ligand binding has to be differentiated from nonspecific binding of the ligand to the cell membranes. This can be done either by removing the nonspecifically bound ligand by suitable washing, or by quantifying the nonspecific binding, e.g. by using in excess another ligand for the target receptor, as done by e.g. Hess et al. (2011).

2.6 Aim of the study

In this MSc (Pharm) thesis, the performance of a new nanobiotechnology platform for the screening of cell-drug interactions was investigated. The system used involved acoustic trapping of cells in a borosilicate glass capillary with an ultrasonic standing wave generated by a piezoelectric transducer (Figure 2). This enabled the exchange of fluid medium in the capillary, with the cell cluster remaining stationary, which in turn permitted rapid aspiration and dispensing of sample solutions and eluents. Acoustic streaming (Figure 2) around the trapped cell cluster was expected to enable adequate fluid exchange (i.e. efficient washing) and the transport of sample to the close proximity of the cells. A membrane preparation from cells overexpressing serotonin transporter (SERT), used in typical ligand binding assays, and human platelets were used to screen the binding of the selective serotonin reuptake inhibitor (SSRI) compounds fluoxetine and citalopram to SERT. Prior to mass spectrometric analysis, the samples were subjected to solid-phase extraction using the Integrated Selective Enrichment Target (ISET, Figures 6 and 7), a miniaturized solid phase extraction method developed by Ekström et al. (2004, 2006). The analysis was done by MALDI MS (Figure 3), allowing the label-free detection of the analytes.

The experimental work started with the optimization of the ISET sample preparation for the analytes, after which the trapping capacity of the acoustic trap was investigated by trapping polystyrene beads and counting them with a Coulter counter. The system performance was modeled and the procedure optimized by trapping strong cation exchange (SCX) beads and incubating them with the analytes, followed by ISET sample preparation and MALDI MS analysis. After this, the binding assays using acoustic trapping, ISET sample preparation, and MALDI MS, were conducted with platelets, yeast cells (intended to be used as a model for nonspecific binding), and the cell membrane preparation from cells overexpressing SERT. Finally, a comparative mass spectrometric SSRI binding assay to the SERT in the cell membrane preparation was conducted without using acoustic trapping.

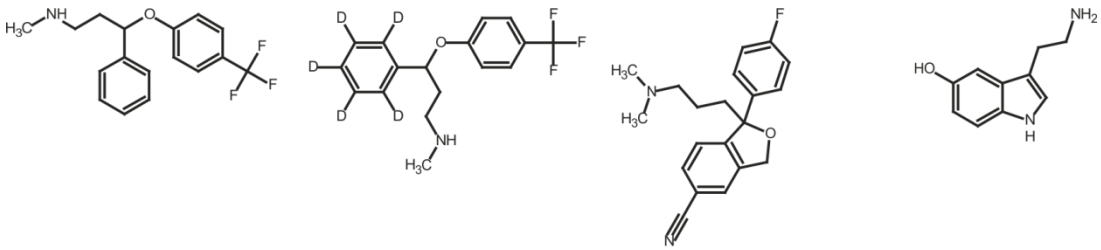
3 EXPERIMENTAL SECTION

3.1 Materials and methods

3.1.1 Chemicals

Water was purified with Milli-Q water purifying system (Millipore, Molsheim, France). Analysis grade hydrochloric acid, glycine, potassium chloride and Triton-X-100 were purchased from Merck KGaA (Darmstadt, Germany); analysis grade 25 % ammonia and ≥99 % sodium chloride from VWR International (Fontenay-sous-Bois, France); all other chemicals, LC-MS or HPLC grade when available, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The manufacturers of other materials and equipment used are stated in the corresponding chapters.

The compounds fluoxetine, deuterium labeled fluoxetine (fluoxetine-D5), citalopram, and serotonin (5-HT) were used as analytes for the experiments. Their chemical structures and monoisotopic masses are presented in Figure 4. These compounds were supplied as hydrochloride salts, except citalopram, which was as hydrobromide salt.



Fluoxetine	Fluoxetine-D5	Citalopram	Serotonin
309.134049	314.165433	324.163792	176.094963

Figure 4: The analytes used in the experiments, and the corresponding monoisotopic masses.

3.1.2 Sample preparation optimization

For sample preparation, the Integrated Selective Enrichment Target (ISET) platform was used; the system is described in detail elsewhere (Ekström et al. 2004, 2006). The platform consists of 95 pyramid-shaped nanovials on a silicon plate with thickness of

780 µm (Figure 5). At the tip of each pyramid-shaped nanovial, 9 quadratic holes, each 20×20 µm, provide an opening to the other side of the plate (Figure 5). The plates are fabricated by chemical wet etching and deep reactive-ion etching.

The ISET platform is suitable for various kinds of procedures, such as solid phase extraction (SPE) and protein digestion (Ahmad-Tajudin et al. 2014). The ISET SPE procedure (Figure 6) involves transferring solid phase beads into the nanovials with the suction provided by a vacuum pump (Vacuubrand GMBH, Wertheim, Germany), before or after introducing the beads to the sample which is to be subjected to SPE (Adler et al. 2012). The procedures are called direct ISET and indirect ISET, respectively (Figure 7). After this, the impurities are eluted (washed) and the plate bottom dried, followed by the elution of analytes. Suitable eluents are chosen according to the solid phase used. As the result of the elution, the analytes are co-crystallized and concentrated within the crystallized matrix around the nanovial outlet on the bottom side of the ISET platform, allowing direct MALDI MS analysis (Figure 6). Throughout the experiments, MS grade α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix compound.

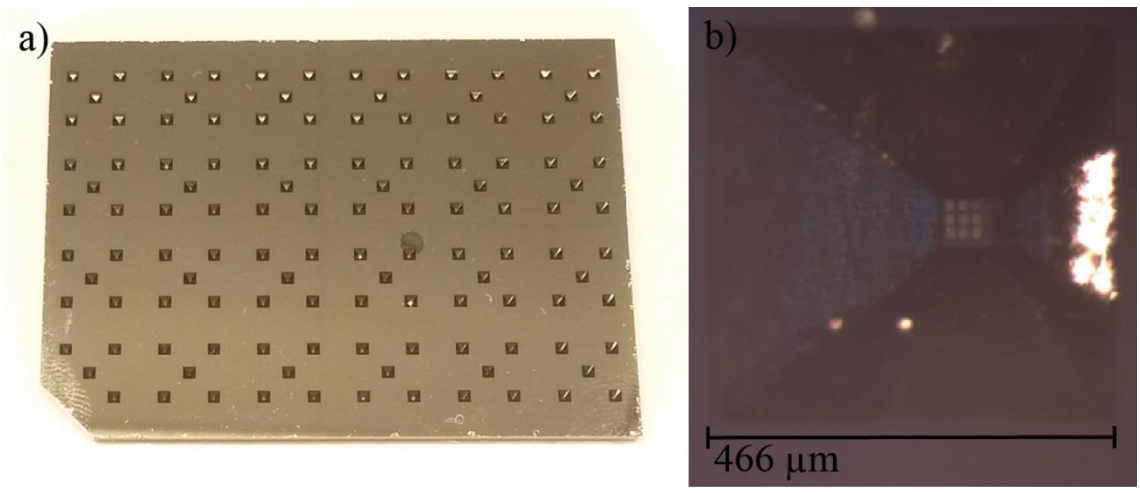


Figure 5: a) the ISET platform; b) close-up microscope image of a nanovial, showing the bead retaining grid at the bottom of the nanovial. The volume of a single nanovial is 600 nL.

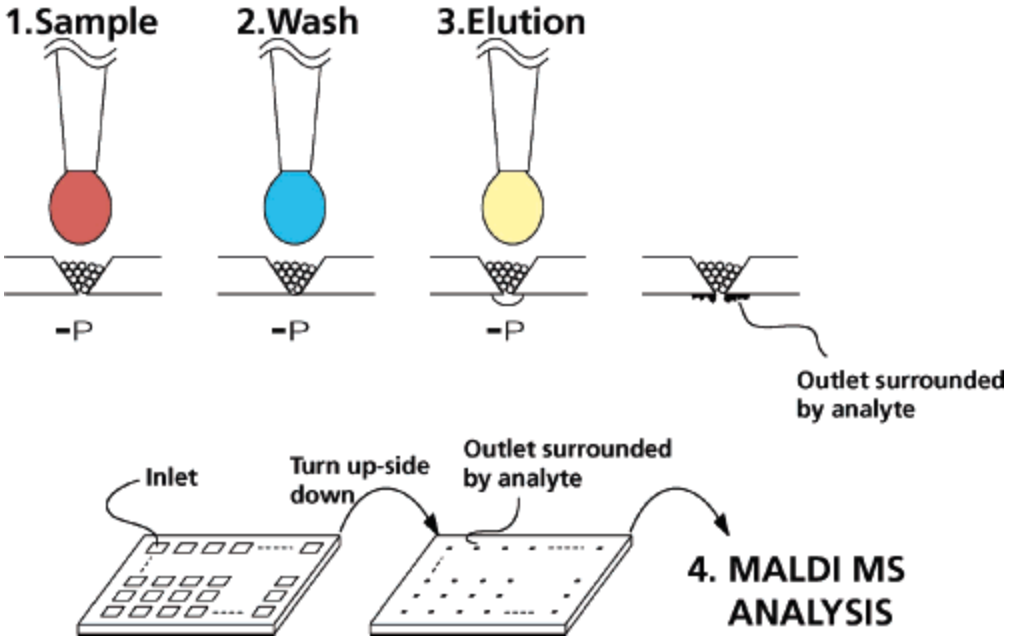


Figure 6: The SPE sample preparation procedure on the ISET platform. The sample is introduced to the beads already present in the nanovial (direct ISET), followed by washing away the impurities and the elution of the analytes. The analytes are co-crystallized with the matrix around the outlet, allowing direct MALDI MS analysis. Figure reprinted with permission from Ekström et al. 2006.

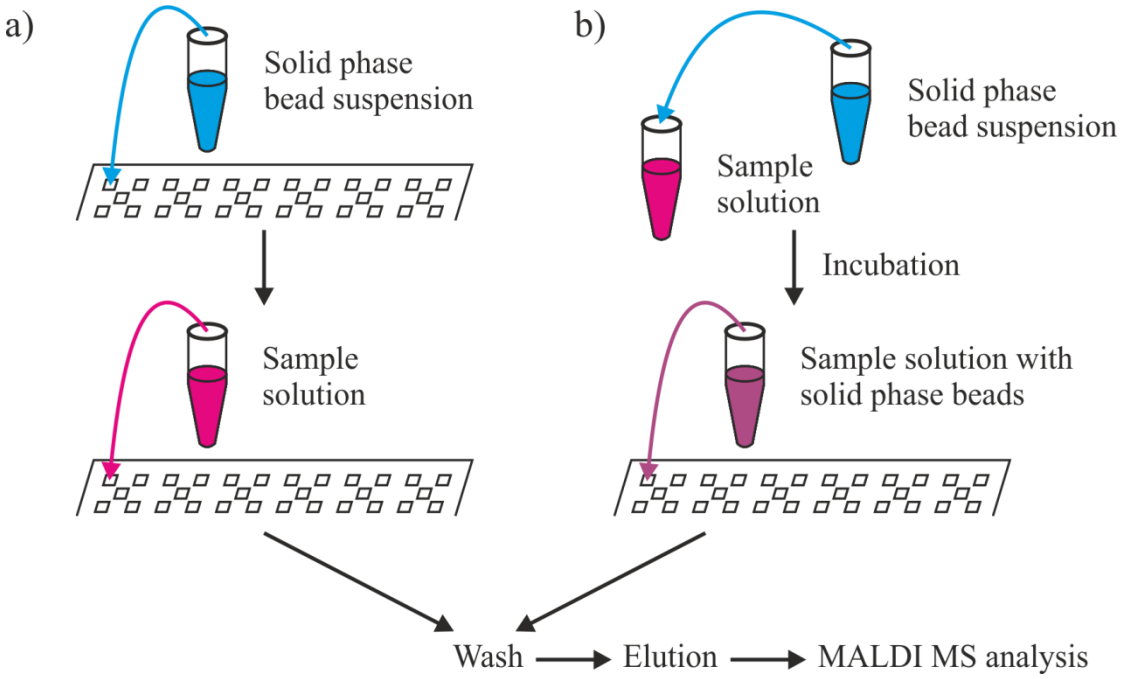


Figure 7: The ISET sample preparation procedure. a) direct ISET, in which the solid phase beads are transferred into the ISET plate before the introduction of the sample; b) indirect ISET, in which the solid phase beads are incubated with the sample solution prior to transferring into the ISET plate.

Experiment A. The performance of Poros R1 50 (C4 phase 50 µm) and Poros R2 50 (phenyl phase 50 µm) solid phase beads (Applied Biosystems Inc., Foster City, CA, USA) in direct ISET sample preparation of fluoxetine and fluoxetine-D5 was investigated. The sample solutions were prepared in 0.1 % TFA (trifluoroacetic acid) in MQ water and contained equal concentrations of fluoxetine and fluoxetine-D5, ranging from 0.25 to 50 µmol/L. 5 mg/mL CHCA in 60 % acetonitrile (ACN) was used as the matrix solution. The ISET sample preparation was done according to the procedure below. For liquid transport through the ISET, vacuum of 5 lnHg was used at phases 1-3 and 1.5 lnHg at phases 5-6.

1. Transferring solid phase beads into the nanovials.
2. Introducing 1 µL of the sample solution into the nanovials.
3. Washing with 2.5 µL of 0.1 % TFA.
4. Drying the ISET plate bottom with a laboratory wipe.
5. Elution with 0.5 µL of the matrix solution.
6. Drying for 15 minutes.

Experiment B. In addition to the direct ISET sample preparation presented above, indirect ISET sample preparation was tested: both R1 and R2 beads were preincubated with the sample prior to transferring onto the ISET plate. 15 µL of the sample and 10 µL of 20 mg/ml bead suspension were used, the incubation time being 45 minutes. After the incubation, 2.5 µL of the incubated suspension was transferred onto the ISET plate, the sample volume per nanovial being approximately equal to that of the corresponding experiments without incubation. Washing with was done like in **experiment A**, but to improve crystallization, two elutions with 30 minutes drying time in between were used.

After the ISET procedure, the samples from experiments A and B were analyzed with a Waters M@LDI-TOF MS mass spectrometer (Milford, MA, USA) with a 337 nm nitrogen UV laser, operated in reflector mode. The MALDI settings were optimized before data acquisition. The accumulated spectra of 100 laser shots were acquired per sample spot. Before reusing, the ISET plates were washed according to the following

procedure. The same procedure was used for ISET cleaning throughout all of the experiments.

1. Removing beads and crystals with a paintbrush under running tap water.
2. Cleaning with ethanol using a paintbrush.
3. Cleaning with MQ water using a paintbrush.
4. 2-propanol bath for 20 minutes.
5. Acetonitrile bath for 20 minutes.

3.1.3 Piezoelectric transducer testing and acoustic trapping capacity

The acoustic trapping setup used (Figure 8) consists of a piezoelectric transducer (Ferroperm Piezoceramics A/S, Kvistgaard, Denmark) on a microchip, placed on a plastic mount. A borosilicate glass capillary (VitroTubes, VitroCom, Mountain Lakes, NJ, USA) of the volume 20 µL, length 50 mm and external thickness 0.51 mm is connected to Tygon tubings (Saint-Gobain S.A., Courbevoie, France) with optical adhesive (Norland Products, Inc., Cranbury, NJ, USA). The capillary is placed on top of the transducer and tightened in place with a plastic holder. A glycerol film is used between the capillary and the transducer for acoustic coupling. A function generator (33120A, Agilent Technologies Inc., Santa Clara, CA, USA) is used to control the piezoelectric transducer; with a LabVIEW 2013 (National Instruments Corporation, Austin, TX, USA) frequency tracking software programmed in-house (Hammarström et al. 2014). The fluid flow in the capillary is controlled by a syringe pump (cetoni GmbH, Korbußen, Germany) with a 1 mL gastight syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland). In all experiments, the fluids were aspirated into the capillary from an Eppendorf tube cap.

Experiment C. 13 piezoelectric transducers, numbered #1 to #13, of a similar model were tested for their ability to hold 10 µm polystyrene beads (Sigma-Aldrich Co., St. Louis, MO, USA) in the acoustic trap with different flow rates (Figure 9). Flow rates ranging from 10 to 250 µL/min, with the intervals of 10 µL/min, were used, with MQ

water as the fluid medium. Microscope images were captured throughout the process. The overall procedure was as follows:

1. Trapping a cluster of beads by aspirating 10 µL of bead suspension (0.05 % m/V in MQ water) with the flow rate 50 µL/min.
2. Transferring non-trapped beads through the trapping site by aspirating 20 µL of MQ water with the flow rate 50 µL/min.
3. Stabilizing the amount of trapped beads by dispensing 30 µL of the fluid medium with the flow rate 10 µL/min.
4. Gradually increasing the flow rate until the bead cluster exits the trap.

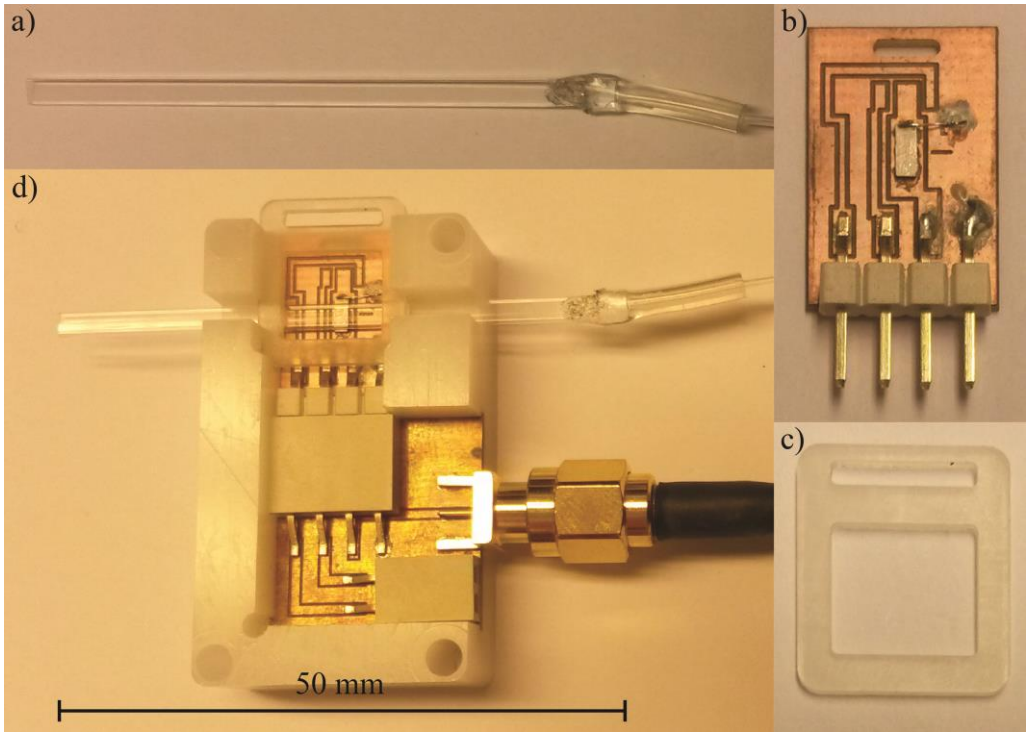


Figure 8: The acoustic trapping setup. a) capillary glued to tubing; b) piezoelectric transducer; c) plastic holder; d) the acoustic trapping setup on the plastic mount.

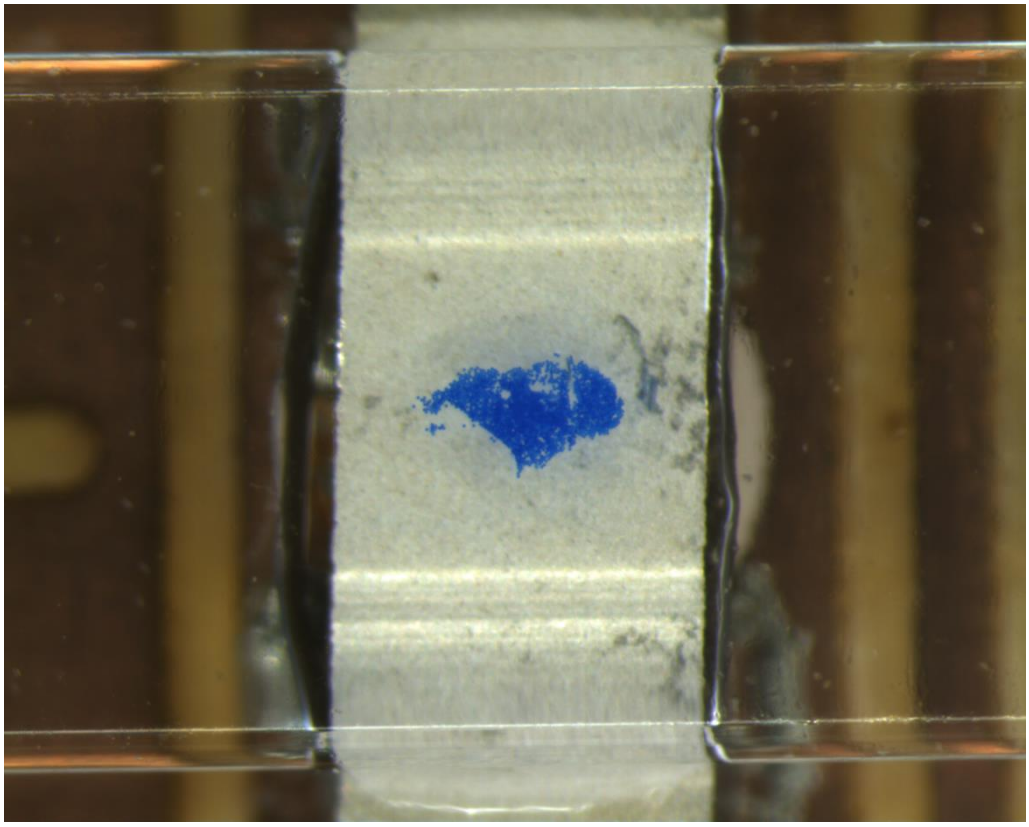


Figure 9: A microscope image of a small cluster of 10 µm polystyrene beads in the acoustic trap.

Experiment D. In order to estimate the sensitivity that can be achieved with the system, the trapping capacity of transducers #1, #5 and #8 was further investigated using 3 µm polystyrene beads (Sigma-Aldrich Co., St. Louis, MO, USA), which are approximately of the same diameter as human platelets (Paulus 1975). Flow rates of 20, 40, 60, 80 and 100 µL/min were used, with triplicate measurements for each flow rate. To avoid the adherence of the beads to capillary and container walls, 0.1 % Triton-X-100 in MQ water was used as the detergent to prevent bead aggregation and sticking to the walls. However, the syringe was filled with MQ water only, in order to avoid detergent contamination and carry-over effects in further experiments. The procedure was as follows:

1. Trapping a cluster of beads by aspirating 150 µL of bead suspension (1.25×10^7 beads/mL in 0.1 % Triton-X-100) with the flow rate 50 µL/min.
2. Stabilizing the amount of trapped beads by dispensing 150 µL of the fluid medium with the specified flow rate.

3. Turning off the acoustic trap.
4. Transferring the beads into an Eppendorf tube by dispensing 20 µL of the fluid medium with the flow rate 50 µL/min.
5. Adding 160 µL of 0.1 % Triton-X-100 in MQ water into the Eppendorf tube.
6. Adding 20 µL of bead suspension (1.25×10^7 beads/mL in 0.1 % Triton-X-100) into the Eppendorf tube.

A volume of 0.1 % Triton-X-100 in MQ water equal to the volume dispensed from the trap was used as the blank. Adding the bead suspension to the samples in phase 6 allowed the comparison of bead concentrations by subtraction. The sample bead suspensions were diluted into 100 mL of 0.9 % sodium chloride solution for Coulter counting with Multisizer 3 Coulter counter (Beckman Coulter Inc., Pasadena, CA, USA) using a 100 µm aperture. Triplicate measurements were made for each sample, and the amounts of trapped beads were calculated.

3.1.4 Experiment with strong cation exchange beads

Experiment E. In order to test the acoustic trapping system performance and optimize the procedure, 1 µm Dynabeads® strong cation exchange (SCX) beads (Invitrogen Dynal AS, Oslo, Norway) were incubated in-trap with sample solutions containing equal concentrations of fluoxetine and fluoxetine-D5, ranging from 5 to 50 µmol/L. The bead suspension and sample solutions were made in MQ water containing 0.1 % TFA, and the same solution was used as the fluid medium. Transducer #8 was used, and the flow rate was 20 µL/min. The eluent used was 30 % methanol, 5 % ammonia in MQ water. After dispensing the eluent, also the strong cation exchange beads were dispensed from the trap along with 20 µL of fluid medium, in order to investigate the efficiency of the elution. The following procedure was utilized:

1. Trapping a cluster of beads by aspirating 100 µL of 0.2 mg/mL bead suspension.
2. Stabilizing the amount of trapped beads by dispensing 200 µL of the fluid medium.
3. Aspirating 20 µL of sample solution.

4. Incubation for 30 minutes.
5. Removing unbound sample by dispensing 120 µL of the fluid medium.
6. Aspirating 20 µL of eluent.
7. Incubation for 30 minutes.
8. Dispensing 20 µL (eluent) into an Eppendorf tube cap.
9. Turning the acoustic trap off.
10. Dispensing 20 µL of the fluid medium, along with the SCX beads, into an Eppendorf tube cap.
11. Rinsing the capillary by dispensing 50 µL of the fluid medium.

1 µL aliquots of the eluent samples and fluid medium aliquots containing the SCX beads were pipetted as duplicates onto a MALDI plate for dried droplet analysis and 2×1 µL of matrix solution (5 mg/mL CHCA in 60 % acetonitrile) was added. The samples were analyzed with a Waters M@LDI-TOF MS mass spectrometer (Milford, MA, USA) with a 337 nm nitrogen UV laser, operated in reflector mode. The MALDI settings were optimized before data acquisition. The accumulated spectra of 100 laser shots were acquired per sample spot.

Experiment F. The efficiency of the indirect ISET sample preparation (Figure 7) was investigated. Into the remaining 16 µL of eluent or SCX bead residue from **experiment E**, 4 µL of 20 mg/mL Poros R2 50 solid phase bead suspension was added, followed by 20 minutes incubation. ISET sample preparation as triplicate sample spots was done as listed below. For liquid transport through the ISET, vacuum of 5 lnHg was used at phases 1-2 and 1.5 lnHg at phase 4. Before the ISET pretreatment, the fluid medium aliquots containing the beads dispensed from the trap were basified with 1 µL of 30 % methanol, 5 % ammonia prior to incubating for 30 minutes with 2.5 µL of 20 mg/mL R2 bead suspension. MALDI MS analysis was done as in **experiment E**.

1. Transferring 5 µL of sample with R2 beads into a nanovial.
2. Washing with 2.5 µL of 0.1 % TFA.
3. Drying of the ISET plate bottom with a laboratory wipe.

4. Eluting thrice with 0.5 µL of 5 mg/mL CHCA in 60 % ACN with 15 minutes drying time in between.

3.1.5 Drug binding experiments using acoustic trapping

Drug binding studies were done with yeast (*Saccharomyces cerevisiae*) cells (Kronjäst, Jästbolaget AB, Sollentuna, Sweden), human platelets from platelet rich plasma (PRP), kindly provided by Prof David Erlinge and colleagues, and cell membrane preparation from cells overexpressing human serotonin transporter ("SERT cell membranes") from PerkinElmer (Boston, MA, USA). 0.01 mol/L phosphate buffered saline (PBS) with the pH = 7.4 was used as the fluid medium for yeast cells and platelets, and 0.05 mol/L Tris-buffered saline (TBS) with the pH = 7.4 for the cell membrane preparation, according to the manufacturer's instructions. The sample solutions were made in the fluid medium used. In order to be aspirated into the acoustic trap, the yeast cells were suspended in PBS to form a 10 mg/mL suspension. PRP was used as such. In the cell membrane preparation, the SERT cell membranes were originally as aggregates in a fluid medium, and hence the preparation was diluted with TBS in a ratio 1:7 and vortexed before aspirating into the acoustic trap. All experiments were performed both with actual drug samples and solvent blank samples.

Since the magnitude of forces actuating acoustic trapping decreases along with the size of particles to be trapped, the use of seeding particles to facilitate acoustic trapping of small particles or cells may be necessary (Hammarström et al. 2012). The experiments described in this chapter were, however, performed without seeding particles. The piezoelectric transducer # 8 was used. Figure 10 shows yeast cells, platelets, and SERT cell membranes in the acoustic trap.

Experiment G. The yeast cell suspension, PRP, and SERT cell membrane suspension volumes required to fill the acoustic trap with cells or cell membranes were investigated. Since the operating frequency of acoustic trapping is proportional to the speed of sound in the fluid medium, and thus its density, abrupt changes in solvent proportions require a change in the operating frequency, and consequently may disrupt

the acoustic trapping. Therefore the succeeding of the acoustic trapping of yeast cells and platelets during the aspiration of different eluents was investigated. The eluents tested were solutions containing different percentages of acetonitrile (ACN) and methanol, prepared in 0.1 % TFA in MQ water, 0.5 % TFA in MQ water, and 10 mmol/L glycine in MQ water acidified to pH ≈ 2 with hydrochloric acid. With the SERT cell membranes, the succeeding of the acoustic trapping was tested only with the eluent 1:9 ACN:TBS.

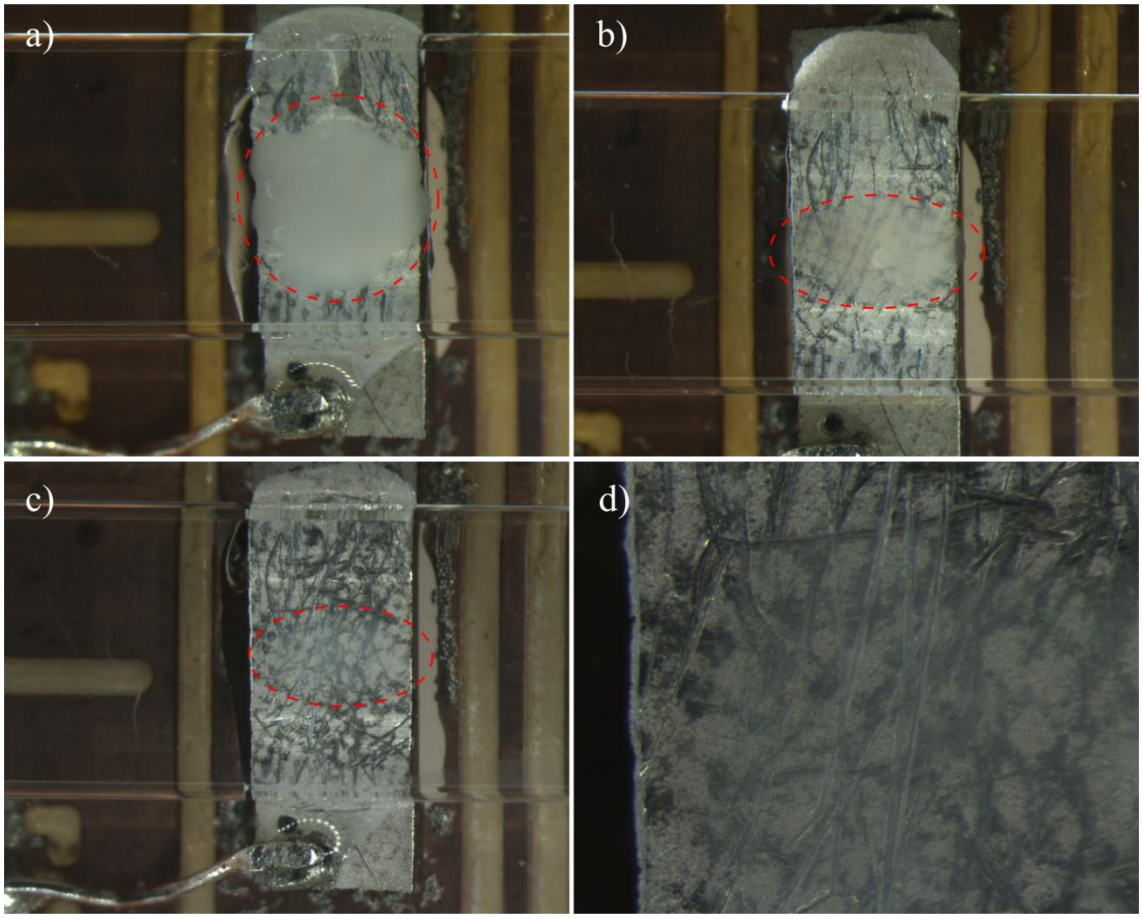


Figure 10: Acoustically trapped yeast cells (a), human platelets (b), and cell membranes (c), also in close-up (d). The cells or cell membranes in the acoustic trap are visible as the opaque above the piezoelectric transducer.

Experiment H. Three different eluents were chosen so that effective acoustic trapping with both yeast cells and platelets was possible. Their elution efficiency was investigated by incubating yeast cells or platelets in the acoustic trap with a sample solution containing 50 µmol/L of both fluoxetine and fluoxetine-D5, and eluting with

the different eluents. The overall procedure is presented in Figure 11. The following eluents were used: 1:9 ACN:PBS, 0.5 % TFA in MQ water, and 10 mmol/L glycine in MQ water acidified to pH ≈ 2 with hydrochloric acid. The same experiment was used to determine the suitability of yeast cells to be used as a model for nonspecific binding of the analytes to the cell membranes. In addition, the same experiment was done with platelets using 50 µmol/L citalopram as the sample solution and 1:9 ACN:PBS as the eluent. In addition to the eluents and cell residues, also the sample solutions after incubation were kept and pretreated, in order to verify the use of right sample solutions. The flow rate 20 µL/min was used unless otherwise specified; the trapping procedure was as follows:

1. Aspirating 40 µL of yeast cell suspension or PRP.
2. Stabilizing the amount of trapped cells by dispensing 60 µL of the fluid medium.
3. Aspirating 20 µL of sample solution.
4. Incubation for 10 minutes.
5. Dispensing 20 µL (sample solution after incubation) into an Eppendorf tube cap.
6. Removing unbound drug by dispensing 60 µL of the fluid medium.
7. Aspirating 20 µL of eluent.
8. Incubation for 10 minutes.
9. Dispensing 20 µL (eluent) into an Eppendorf tube cap.
10. Turning the acoustic trap off.
11. Dispensing 20 µL of the fluid medium, along with the cells, into an Eppendorf tube cap with maximum flow rate.
12. Rinsing the capillary by thrice dispensing 20 µL of the fluid medium with maximum flow rate.

The samples were stored overnight in a refrigerator and ISET pretreated according to the procedure below (direct ISET, Figures 6 and 7). The solid phase used was a 20 mg/mL suspension of Poros R2 50 reversed phase beads, and the matrix was 5 mg/mL CHCA in 60 % acetonitrile. For liquid transport through the ISET, vacuum of 10 lnHg was used at 1-4 and 1.5 lnHg at 6. The sample spots were analyzed with a MALDI-Orbitrap XL from Thermo Scientific (Waltham, MA, USA) with a 337.1 nm nitrogen

UV laser, operated in SIM mode with the resolution of 100000. The settings were optimized before data acquisition.

1. Transferring solid phase beads into nanovials.
2. Transferring 10 µL of sample per nanovial.
3. Incubating for 15 minutes.
4. Washing with 2.5 µL of 0.1 % TFA.
5. Drying the ISET plate bottom with a laboratory wipe.
6. Eluting thrice with 0.5 µL of matrix, with 15 minutes drying time.

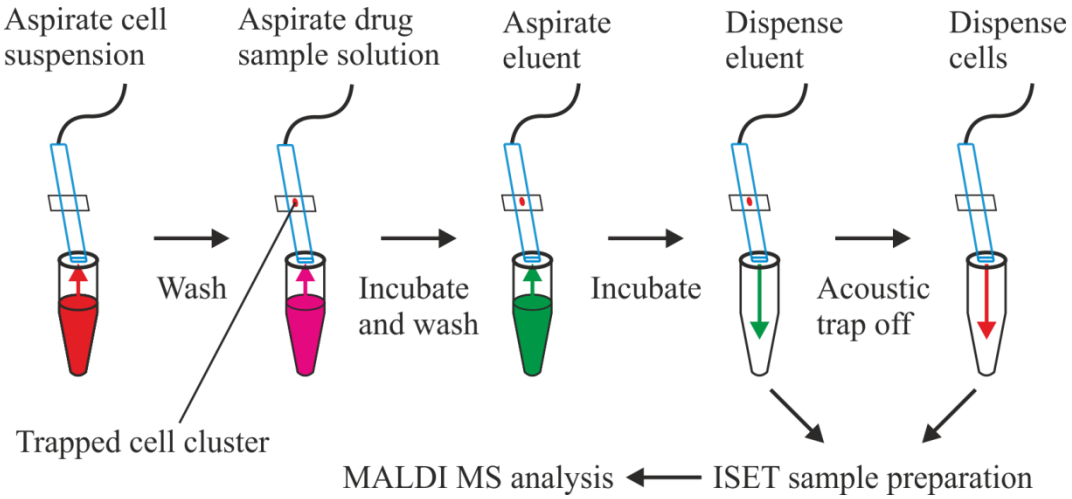


Figure 11: The overall procedure used for the screening of cell-drug interactions. A cluster of cells is acoustically trapped by aspirating cell suspension into the capillary and washing away the untrapped cells, followed by the aspiration of the drug sample solution, and incubation. After the incubation of the cells with the drug sample solution, the unbound drug sample is removed, and the eluent is aspirated. After the incubation with the eluent, the eluent and the fluid medium aliquot (along with the cells) are dispensed separately, followed by ISET sample preparation and MALDI MS analysis.

Experiment I. The same procedure as in **experiment H**, presented above and in Figure 11, was applied also without using any cells, using 50 µmol/L citalopram or 50 µmol/L fluoxetine, 50 µmol/L fluoxetine-D5 as the sample solution. This blank experiment was conducted in order to investigate the extent of drug adsorption to the capillary walls, and its effect on the analyte signal intensity in the mass spectra.

Experiment J. The binding of citalopram, fluoxetine and fluoxetine-D5 to serotonin transporter (SERT) in the cell membrane preparation from cells overexpressing SERT was investigated according to the procedure presented in **experiment H** (Figure 11), with the following alteration: the first of the three 20 µL fluid medium aliquots used to rinse the capillary was not discarded, and an additional 20 µL fluid medium aliquot was dispensed after the rinsing, and saved. Both of these aliquots were stored, ISET pretreated and analyzed in a similar manner as the actual samples. This was done in order to investigate the efficacy of the capillary rinsing procedure conducted between individual experiments. The sample solutions used were 50 µmol/L citalopram and 50 µmol/L fluoxetine, 50 µmol/L fluoxetine-D5. 1:9 ACN:TBS was used as the eluent.

Experiment K. The binding of citalopram and fluoxetine to serotonin transporter in the cell membrane preparation was investigated according to the procedure presented in **experiment H** (Figure 11), with the following alteration: after dispensing the cell membrane residue, the capillary was rinsed by dispensing 20 µL, aspirating and dispensing 20 µL of methanol, and dispensing 20 µL twice. Only the last aliquot was discarded, whereas the others were pretreated and analyzed in the same manner as the actual samples. Before the sample preparation and analysis, the methanol aliquots were evaporated to dryness and the residue dissolved into 20 µL of TBS. Five different sample solutions were used: 1 µmol/L fluoxetine; 1 µmol/L citalopram; 1 µmol/L fluoxetine, 1 µmol/L citalopram; 1 µmol/L fluoxetine, 100 µmol/L citalopram; 100 µmol/L fluoxetine, 1 µmol/L citalopram. The same procedure was performed also as a blank without any cell membranes, in order to investigate the extent of analyte adsorption to the walls of the capillary with each sample solution used.

Experiment L. The suitability of the acoustic trapping system for the screening of serotonin transporter activity in platelets was investigated according to the procedure below. The platelets were pre-incubated for 10 minutes in the acoustic trap with the sample solution, followed by 10 minutes incubation with either 50 µmol/L serotonin solution or blank, and cell lysis with 70 % formic acid in MQ water. The sample solutions used were 50 µmol/L citalopram and 50 µmol/L fluoxetine, 50 µmol/L fluoxetine-D5. The samples were stored, ISET pretreated and analyzed as described in

experiment H (Figure 11). In addition, the behavior of serotonin in MALDI MS was investigated by dried droplet analysis of 1 mg/mL standard solutions of serotonin and the SSRIs used. Sample volume was 0.5 µL and matrix (5 mg/mL CHCA in 60 % ACN) volume 1 µL.

Experiment M. The same procedure as in **experiment L** was repeated with the following alteration: before cell lysis, 20 µL of eluent was aspirated into the capillary, incubated for 10 minutes with the platelets, dispensed into an Eppendorf tube cap, and the remaining eluent was removed by dispensing 60 µL. This was done in order to investigate that if serotonin is visible in the spectra, whether it is really transported into the cells or merely bound to the cell membrane. The samples were stored, ISET pretreated and analyzed as described in **experiment H**.

3.1.6 Comparative drug binding experiments without acoustic trapping

Experiment N. For comparison with the drug binding studies using acoustic trapping, drug binding studies without acoustic trapping were performed using the cell membrane preparation from cells overexpressing SERT. Vacuum filtration with a SilentScreen 96-well filter plate (Nalge Nunc International, Penfield, NY, USA) with Nylon filtering membrane (pore size 0.45 µm) was used for the procedure (Figure 12). Similar sample solutions as in **experiment K** were used. With the 1 µmol/L fluoxetine, 1 µmol/L citalopram sample solution, no SERT cell membranes were used. This blank experiment was done in order to study the extent of drug adsorption to the filtering membrane. Before the experiment, the cell membrane preparation was diluted with TBS in a ratio of 1:7. The procedure was as presented below.

1. Transferring 40 µL of the SERT cell membrane suspension into a well.
2. Adding 100 µL of the drug sample solution into the well.
3. Incubation for 2 hours continuously shaking.
4. Removing the sample solution through the filtering membrane by vacuum suction.
5. Washing 5 times with 150 µL of Tris hydrochloride 50 mmol/L (pH = 7.4) in 0.9 % NaCl with similar vacuum suction.

6. Removing and discarding the washing waste.
7. Drying in 50 °C for 40 minutes.
8. Elution 3 times with 70 µL of methanol.
9. Evaporation of the methanol overnight in 30 °C.
10. Dissolving the residues in 40 µL of 0.5 % TFA in MQ water.

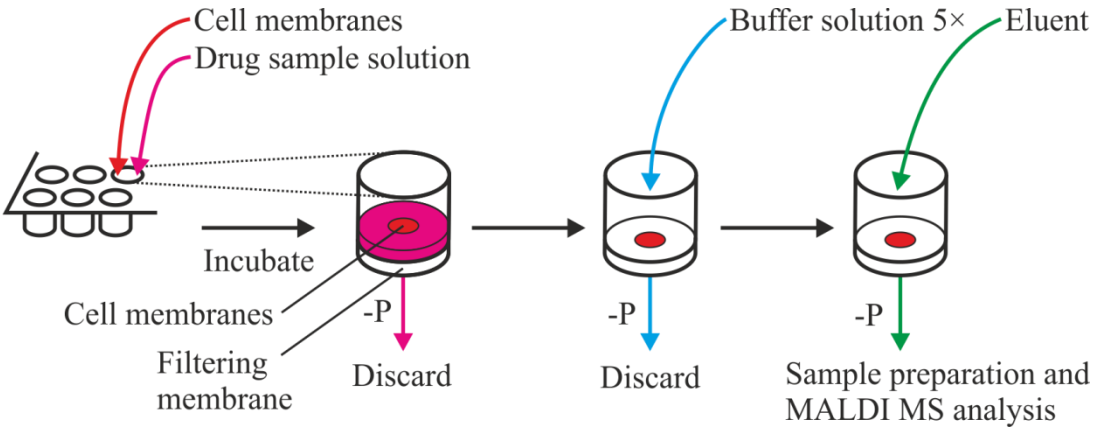


Figure 12: The vacuum filtration method for the screening of interactions between SERT cell membranes and SSRI drugs. The SERT cell membranes are incubated with the drug sample solutions in a microwell, followed by the removal of unbound drug by washing five times with buffer solution. After this, the analytes are eluted and the eluent sample is pretreated and analyzed.

The samples were ISET pretreated according to the procedure presented below (indirect ISET, Figure 7). For liquid transport through the ISET, vacuum of 10 lnHg was used at phases 3-5 and 1.5 lnHg at phase 6. The sample spots were analyzed with a MALDI-Orbitrap XL from Thermo Scientific (Waltham, MA, USA) with a 337.1 nm nitrogen UV laser, operated in SIM mode with the resolution of 100000. The settings were optimized before data acquisition.

1. Addition of 2 µL of Poros R2 50 bead suspension (20 mg/mL) into the wells.
2. Incubation for 1.5 hours.
3. Transferring the sample-bead suspension onto ISET plate as 20 µL duplicates.
4. Drying the ISET plate bottom with a laboratory wipe.
5. Washing with 2.5 µL of 0.1 % TFA in MQ water.
6. Eluting thrice with 0.5 µL of matrix solution (5 mg/mL CHCA in 60 % acetonitrile), with 15 minutes drying time.

3.2 Results and discussion

3.2.1 General

The performance of a new nanobiotechnology platform for the screening of cell-drug interactions using acoustic trapping and MALDI MS was studied. The core of the platform is the immobilization of cells with acoustic trapping in a fluid-filled borosilicate glass capillary (Figures 2 and 8). Acoustic trapping provides a means to hold the cells stationary while the fluid in the capillary is moved, allowing the in-trap incubation of trapped cells with different sample solutions, and the consequent elution of the sample from the cells (Figure 11). The technique of acoustic trapping utilizes an ultrasound standing wave, generated by a piezoelectric transducer, to create acoustic potential minima and maxima in the fluid medium (Evander and Nilsson 2012). In a water-based fluid medium, the cells gather to the acoustic potential minimum, the acoustic trap. A more thorough description of acoustic trapping is presented in chapter 2.2.

In this study, human platelets were used as the cells and selective serotonin reuptake inhibitors (SSRI) drugs as the ligands: serotonin transporter (SERT), the molecular target of the SSRI drugs, is naturally expressed on the platelets' cell membranes. Other experiments involved similar in-trap incubation of strong cation exchange beads, yeast cells, and cell membranes from cells overexpressing human SERT, with the SSRI drugs. Sample preparation was done with Integrated Selective Enrichment Target (ISET, Figures 6 and 7), a miniaturized solid phase extraction method developed by Ekström et al. (2004, 2006). The final analysis read-out was done with MALDI MS.

The ISET sample preparation procedure was optimized using fluoxetine and fluoxetine-D5 sample solutions of different concentrations. This was done in order to find a repeatable and effective method for sample preparation in the experiments to be done. Different kinds of solid phase extraction beads were tested, and procedures involving incubation of the beads with the sample solution before (indirect ISET) and after (direct ISET) transferring them onto the ISET plate were compared (Figure 7).

The next experiments involved testing of different piezoelectric transducers in the acoustic trapping of polystyrene beads with different fluid medium flow rates. The goal of these experiments was to investigate the functional differences of the piezoelectric transducers during acoustic trapping and to find a good transducer for the acoustic trapping experiments with cells.

Before experimenting with cells, the procedure involving acoustic trapping, ISET sample preparation and MALDI MS analysis was modeled using strong cation exchange beads instead of cells. Sample solutions with different concentrations of fluoxetine and fluoxetine-D5 were used. The sufficient in-trap incubation time of the beads with the sample solution and the eluent, and the suitable flow rate of the fluid medium were explored. The analysis involving ISET preparation was compared to analysis with dried droplet method from a conventional MALDI plate.

Finally, the corresponding experiments involving acoustic trapping, ISET sample preparation and MALDI MS analysis (Figure 11) were conducted with yeast cells, human platelets and cell membranes from cells overexpressing human serotonin transporter. The analyte elution efficiency of different eluents was compared, and the suitability of yeast cells to be used as a control experiment for nonspecific SSRI binding was investigated. Fluoxetine, fluoxetine-D5 and citalopram sample solutions of different concentrations were used. The role of analyte adsorption to the trapping capillary and the extent of sample carry-over effect were investigated by conducting the same experiments also with an empty acoustic trap (blank). Comparative SSRI binding experiments without acoustic trapping were conducted using the cell membrane preparation. The suitability of the system for the measurement of serotonin transporter activity in platelets was experimented: the platelets, pre-incubated in-trap with SSRI or blank, were incubated in-trap with a solution of serotonin, followed by cell lysis and MALDI MS analysis.

3.2.2 Sample preparation optimization

In order to optimize the ISET sample preparation procedure (Figures 6 and 7) for fluoxetine, the performance of two different solid phases, Poros R1 50 and Poros R2 50 reversed phase beads, in the ISET sample preparation of fluoxetine and fluoxetine-D5 standard solutions was compared. Direct ISET, in which the solid phase is transferred into the ISET chip before introducing the sample solution, was used (Figure 7); the experimental procedure is presented in **experiment A**. The lowest amount of analyte yielding signals in the mass spectra was 1.25 pmol for both fluoxetine and fluoxetine-D5. Slightly more intensive signals were achieved with Poros R2 50 reversed phase beads.

Comparison between Poros R1 50 and Poros R2 50 reversed phase beads was done also using the indirect ISET, in which the solid phase beads are incubated with the sample solution prior to transferring into the ISET plate (Figure 7). The experimental procedure was done according to **experiment B**. Like with the direct ISET protocol, the lowest amount of analytes yielding signals was 1.25 pmol, and the intensity of the signals was slightly larger with Poros R2 50.

In order to compare the performance of direct and indirect ISET (Figure 7) in the solid phase extraction of fluoxetine, the mass spectra from **experiment A** and **experiment B** were compared. More intensive signals were achieved with indirect ISET, in agreement with previous research (Adler et al. 2012). However, the comparison should be done with caution: longer incubation time was used with indirect ISET (45 minutes) than with direct ISET (15 minutes). In further experiments, indirect ISET was used when possible due to the higher signal intensities obtained. All further experiments were conducted using Poros R2 50 solid phase beads.

The amount and size of matrix-analyte co-crystals around separate sample spots on the backside of the ISET plate (formed during the elution with the eluent solution containing the matrix compound, Figure 6) varied, but the effect of the variation on the analyte signal intensity is not straightforward: some analyte signals obtained from

seemingly poorly crystallized spots were more intensive than other analyte signals from seemingly well crystallized spots. This phenomenon is to a large extent a result of the MALDI process where “sweet spots” and ion suppression effects easily can lead to signal variations of ± 50 % within the same spot (Dreisewerd 2003). It should also be noted that any solid phase extraction sample preparation without the use of an internal standard will not be quantitative. For these reasons, the ISET sample preparation method used is semi-quantitative at its best.

3.2.3 Piezoelectric transducer testing and acoustic trapping capacity

In order to investigate the acoustic trapping differences between piezoelectric transducers (Figure 8), 13 transducers of the same batch were tested by filling the acoustic trap with 10 µm polystyrene beads and inspecting the maximum tolerated fluid flow rate before the bead cluster was flushed out of the acoustic trap (**experiment C**). The testing revealed big variations between transducers in the fluid flow rate that could be applied before the bead cluster was lost, ranging from no trapping at all to successful trapping at a fluid flow rate 240 µL/min. No visual differences between the transducers could be observed. Three transducers (#1, #5 and #8) found to have intermediate to good fluid flow rate tolerance, were chosen for further experiments.

Transducers #1, #5 and #8 were further tested by filling the acoustic trap with 3 µm polystyrene beads and using Coulter counting to determine the amount of beads that could be retained in the acoustic trap at different fluid flow rates (**experiment D**). Figure 13 presents the amounts of 3 µm polystyrene beads trapped by transducers #1, #5 and #8 at different fluid flow rates (20-100 µL/min). These measurements revealed large variations in trapping capacities for the different transducers. The transducers used were manually manufactured, and it can be expected that an industrially manufactured kerfed transducer would significantly improve the situation (Hammarström et al. 2014). Also variability in the mounting of the capillary above the transducer may affect ultrasound transmission. Due to its superior trapping capacity according to this experiment, transducer #8 with a fluid flow rate 20 µL/min was chosen for the further experiments.

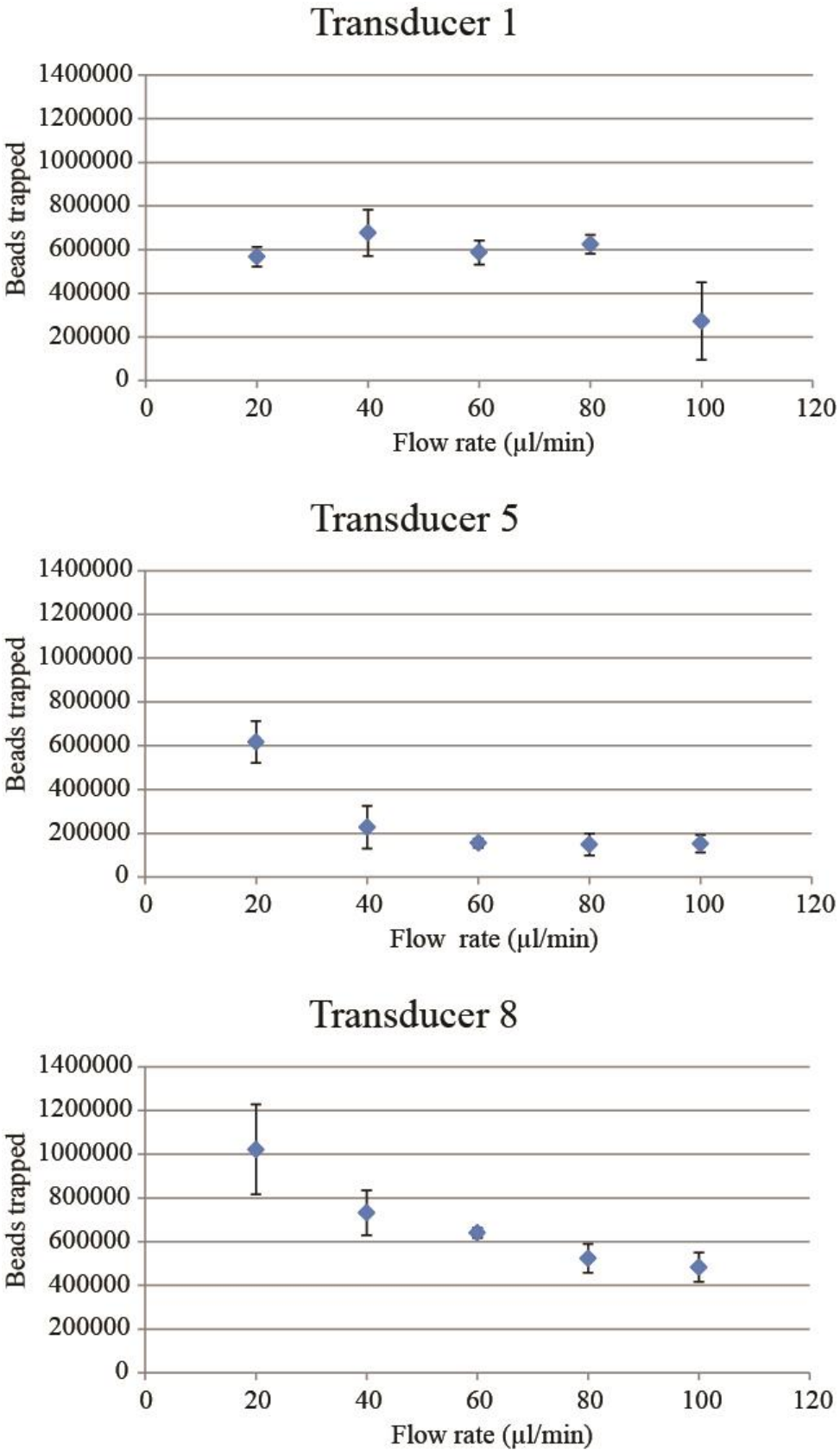


Figure 13: Trapping capacities of transducers #1, #5 and #8. The amounts of trapped beads at flow rates 20 to 100 µL/min were analyzed using a Coulter counter.

3.2.4 Experiment with strong cation exchange beads

The performance of the analytical system, involving acoustic trapping and MALDI MS analysis, was tested using strong cation exchange (SCX) beads according to the procedure presented in **experiment E**. SCX beads where chosen to emulate cells because they provide a negatively charged surface similar to the platelets (Seaman 1973). Both aliquots dispensed from the acoustic trap, the eluent aliquots and the fluid medium aliquots containing the SCX beads, were analyzed with MALDI MS from a conventional MALDI plate (dried droplet analysis). When the 5 µmol/L fluoxetine and fluoxetine-D5 sample solution was used, no signals of the analytes were observed. The use of 12.5 µmol/L sample solution lead to weak signals in the spectra from the eluent aliquots, the strongest signals having approximately S/N = 3 (Figure 14); further increasing the sample solution concentration did not increase the analyte signal intensities. No analyte signals from the fluid medium aliquots containing the SCX beads were observed with any sample solution concentration used.

The efficiency of the indirect ISET sample preparation (Figure 7) was investigated by ISET sample preparation and analysis of the remaining eluent aliquots and fluid medium aliquots containing the SCX beads from **experiment E**, according to the procedure presented in **experiment F**. With the eluent aliquots dispensed from the acoustic trap, the ISET sample preparation did not improve the sensitivity (comparison to the dried droplet analysis from a MALDI plate, described in **experiment E**, Figure 14). For the fluid medium aliquots containing the SCX beads, the analysis after ISET sample preparation yielded spectra similar to those from the eluent aliquots (Figure 14). This indicates that after the elution of fluoxetine and fluoxetine-D5 from the acoustically trapped SCX beads, analytes remain on the beads, and thus a more effective elution method should be investigated. The SCX beads have most likely been saturated with the analytes during the incubation, since the different analyte concentrations in the sample solutions (except the most dilute) lead to no difference in analyte signal intensities in the spectra. If bead saturation occurs, the sensitivity can't be improved by using a longer incubation time with the sample solution. Longer incubation times would also make the process inconveniently slow.

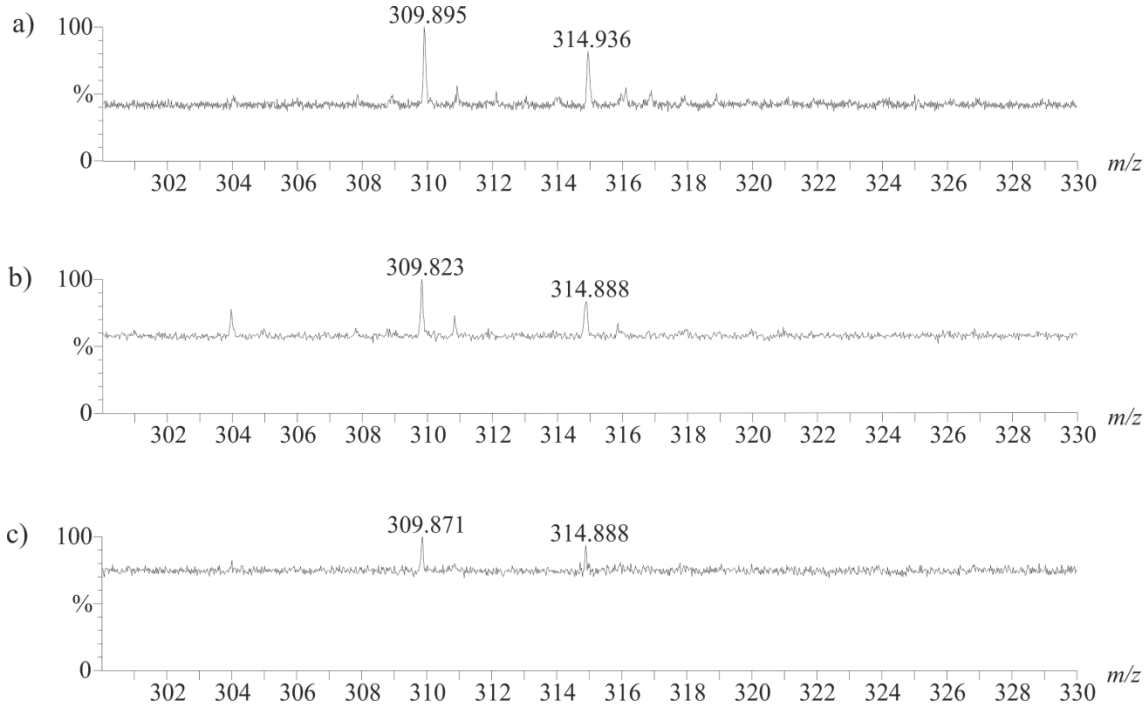


Figure 14: MALDI MS spectra from 1 µm SCX beads incubated for 30 minutes in-trap with 12.5 µmol/L of fluoxetine (*m/z* ≈ 310) and fluoxetine-D5 (*m/z* ≈ 315). The flow rate was 20 µL/min. a) eluent aliquot, dried droplet analysis from a MALDI plate; b) eluent aliquot, analysis after ISET sample preparation; c) fluid medium aliquot containing the SCX beads, analysis after ISET sample preparation.

3.2.5 Drug binding experiments using acoustic trapping

First, the yeast cell suspension, platelet rich plasma (PRP), and cell membrane suspension volumes needed to fill the acoustic trap with cells or cell membranes were investigated (**experiment G**). In addition, it was examined whether the acoustically trapped cluster of cells or cell membranes remains in the acoustic trap during the aspiration of different eluents. For yeast cell suspension, PRP, and cell membrane suspension, the aspiration volume of 40 µL was sufficient to fill the acoustic trap with cells or cell membranes. It was found that acetonitrile (ACN) or methanol percentage exceeding 25 % in the eluent caused the failure of the acoustic trapping of platelets even at flow rates as low as 10 µL/min, due to the large change in the acoustic properties of the fluid. The acoustically trapped yeast cells were more resistant to high organic solvent percentage in the eluent than the platelets, remaining trapped during the aspiration of eluents with up to 50 % organic solvent. With the other eluents (0.5 % TFA in MQ water and 10 mmol/L glycine in MQ water acidified to pH ≈ 2 with

hydrochloric acid), the acoustic trapping of both yeast cells and platelets was successful with all flow rates tested. The trapped cluster of cell membranes remained within the acoustic trap during the aspiration of the eluent 1:9 ACN:TBS (10 % acetonitrile in Tris-buffered saline) with the flow rate 20 µL/min. The organic solvent -containing eluent chosen for further experiments with yeast cells and platelets was 1:9 ACN:PBS (10 % acetonitrile in phosphate buffered saline), because successful acoustic trapping of both yeast cells and platelets with the same eluent was required. Also the ISET sample preparation step requires sufficiently low organic solvent percentage, as the presence of organic solvent would decrease the binding of analytes to the hydrophobic solid phase. For the cell membrane preparation, the eluent 1:9 ACN:TBS was used in further experiments.

In the following experiments, MALDI MS was used for semiquantitative analysis: the analyte signal intensities were compared to matrix signal intensities in the mass spectra (fluoxetine: *m/z* = 310.14 compared to *m/z* 310.07; fluoxetine-D5: *m/z* = 315.17 compared to *m/z* 315.08; citalopram: *m/z* = 325.17 compared to *m/z* = 324.32). In this paper, these ratios are referred to as matrix-relative signal intensities, and presented in the corresponding figures (Figures 15-21). It has to be kept in mind that due to the poor repeatability of the MALDI desorption and ionization process (Dreisewerd 2003), comparison should be done with caution, and the results be regarded as semiquantitative at best.

The elution efficiency of three different eluents (1:9 ACN:PBS, 0.5 % TFA in MQ water, and 10 mmol/L glycine in MQ water acidified to pH ≈ 2 with hydrochloric acid) was investigated by incubating yeast cells or platelets in the acoustic trap with a sample solution containing 50 µmol/L of both fluoxetine and fluoxetine-D5, and eluting with the eluent, according to the procedure presented in **experiment H**. The same experiment was used to determine the suitability of yeast cells to be used as a model for nonspecific binding of the analytes to the cell membranes. For both yeast cells and platelets, eluting with ACN:PBS 1:9 was found to lead to most intensive analyte signals (Figures 15 and 16). Also the approximate ratio between the intensities of the signals from the eluent aliquots and the signals from the fluid medium aliquots containing the

cells (dispensed from the capillary after the elution) was highest with the eluent ACN:PBS 1:9, suggesting the most effective elution of the analytes (Figures 15 and 16). Surprisingly, the analyte signals from platelets were less intensive than those from yeast cells, despite that the yeast cells lack serotonin transporter. This difference in the signal intensities was greater in the fluid medium aliquots containing the cells, implying more efficient analyte elution from the platelets than the yeast cells. Another SSRI drug, sertraline, has been reported to accumulate in cell membranes and other organelles in yeast cells (Chen et al. 2012), and this may be the case for fluoxetine as well. The fluoxetine accumulation to the cell membranes could explain the less efficient elution of fluoxetine from the yeast cells: analytes accumulated to cell membranes are less readily eluted than specifically bound analytes. According to these results, ACN:PBS 1:9 was the most effective eluent for eluting fluoxetine from both yeast cells and platelets. Yeast cells were abandoned as a control due to the high nonspecific binding observed.

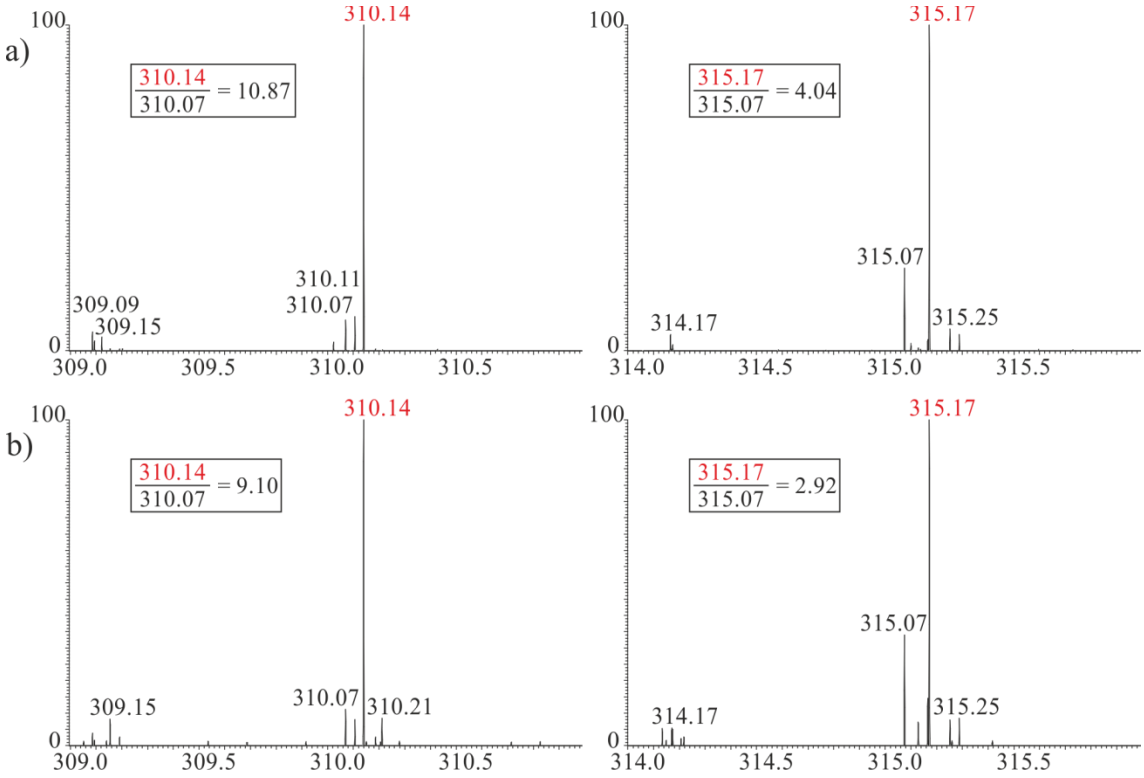


Figure 15: The mass spectra and the matrix-relative signal intensities from yeast cells incubated with an SSRI drug sample solution (50 µmol/L of both fluoxetine and fluoxetine-D5) and eluted with the eluent ACN:PBS 1:9. a) eluent aliquot; b) fluid medium aliquot containing the cells. The protonated molecular ions of fluoxetine (*m/z* = 310.14) and fluoxetine-D5 (*m/z* = 315.17) are written with red font.

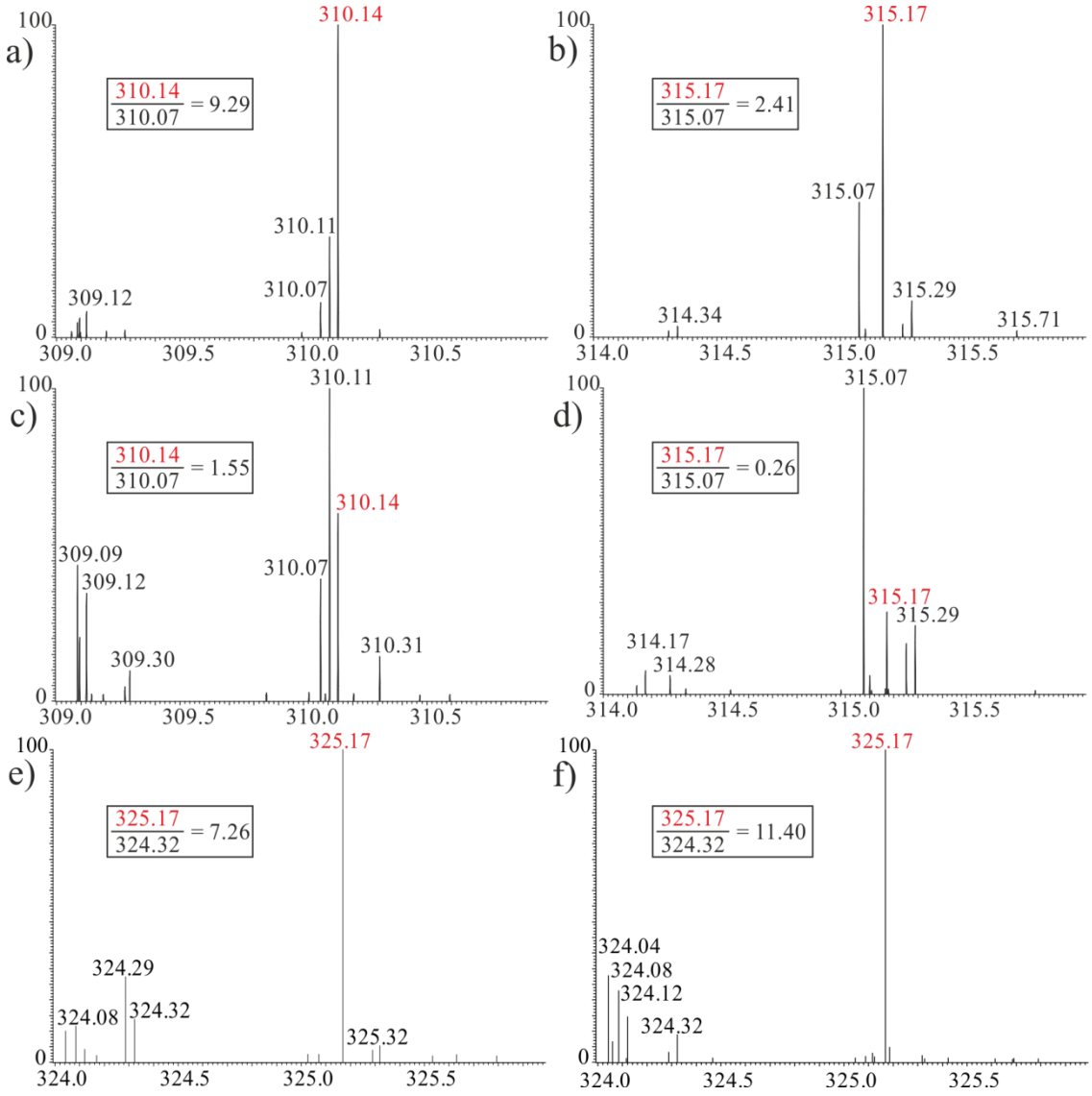


Figure 16: The mass spectra and the matrix-relative signal intensities from platelets incubated with an SSRI drug sample solution and eluted with the eluent ACN:PBS 1:9. a) and b) 50 µmol/L fluoxetine, 50 µmol/L fluoxetine-D5, eluent aliquot; c) and d) 50 µmol/L fluoxetine, 50 µmol/L fluoxetine-D5, fluid medium aliquot containing the platelets; e) 50 µmol/L citalopram, eluent sample aliquot; f) 50 µmol/L citalopram, fluid medium aliquot containing the platelets. The protonated molecular ions of fluoxetine (*m/z* = 310.14), fluoxetine-D5 (*m/z* = 315.17) and citalopram (*m/z* = 325.17) are written with red font.

In **experiment H**, the same procedure involving acoustic trapping, ISET sample preparation and MALDI MS analysis was used to investigate the binding of citalopram to platelets as well. The behavior of citalopram was confirmed to be similar to fluoxetine and fluoxetine-D5: clearly distinguishable signals of citalopram were present in the spectra from both the eluent aliquots and the fluid medium aliquots containing the

cells (Figure 16, e and f). According to the results of **Experiment H**, a more efficient elution method should be investigated. The high signal intensity of citalopram in the spectrum from the fluid medium aliquot containing the platelets suggests either poor elution of citalopram from the platelets or, more likely, citalopram carry-over.

The results from **experiment H** can't be used to estimate whether the analyte signals in Figures 15 and 16 arise from analytes bound to the cells and eluted from them, or from analytes adsorbed to the trapping capillary walls and eluted from them. In order to investigate the extent of drug adsorption to the walls of the capillary used for acoustic trapping, the same acoustic trapping procedure presented in **experiment H** was applied also with an empty acoustic trap, without using any cells (blank, **experiment I**). In the spectra from eluent aliquots dispensed from the trapping capillary, the analyte signal intensities relative to matrix signal intensities of the analytes were less intensive than in the experiments with cells (Figure 17). For fluoxetine and fluoxetine-D5, these analyte signal intensities were on the same scale as those from the fluid medium aliquots containing the platelets in **experiment H** (Figure 16), but for citalopram, the signal intensity was significantly higher, possibly due to either carry-over in the trapping capillary or contamination on the ISET plate used for sample preparation. These results suggest that a small portion of the analyte signals in the spectra in **experiment H** (Figures 15 and 16) arises from the analytes adsorbed to the capillary walls and desorbed during the elution process, but the analytes bound to the cells and desorbed from them are responsible for the majority of the signals. The capillary is manufactured of borosilicate glass, for which low tendency to adsorb drug compounds (compared to other types of glass) is typical (Marchal-Heussler and Barra 2006). However, the concentrations of fluoxetine, fluoxetine-D5 and citalopram in the sample solutions were so large (50 µmol/L) that adsorption can't be avoided. Lower drug concentrations in the sample solutions should thus be used, if the sensitivity of the MALDI MS detection allows.

Due to the analyte adsorption to the capillary in **experiment I**, lower drug concentration (1 µmol/L fluoxetine or citalopram) in the sample solution was used for similar blank measurements. This was done during **experiment K**. The mass spectra from the eluent

aliquots are presented in Figure 18. As expected, the signal intensity of fluoxetine was lower than with the 50 µmol/L sample, but still clearly detectable. The signal intensity of citalopram was extremely high, indicating excessive carry-over contamination.

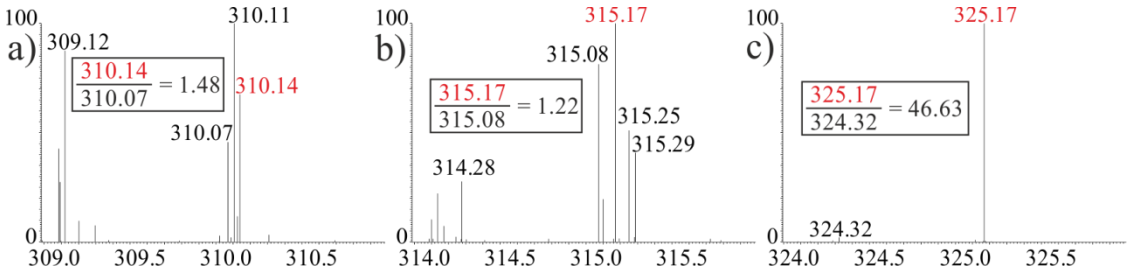


Figure 17: The mass spectra and the matrix-relative signal intensities from the empty acoustic trap (blank) incubated with an SSRI drug sample solution and eluted with the eluent ACN:PBS 1:9. a) and b) 50 µmol/L fluoxetine, 50 µmol/L fluoxetine-D5, eluent aliquot; c) 50 µmol/L citalopram, eluent aliquot. The protonated molecular ions of fluoxetine (*m/z* = 310.14), fluoxetine-D5 (*m/z* = 315.17) and citalopram (*m/z* = 325.17) are written with red font.

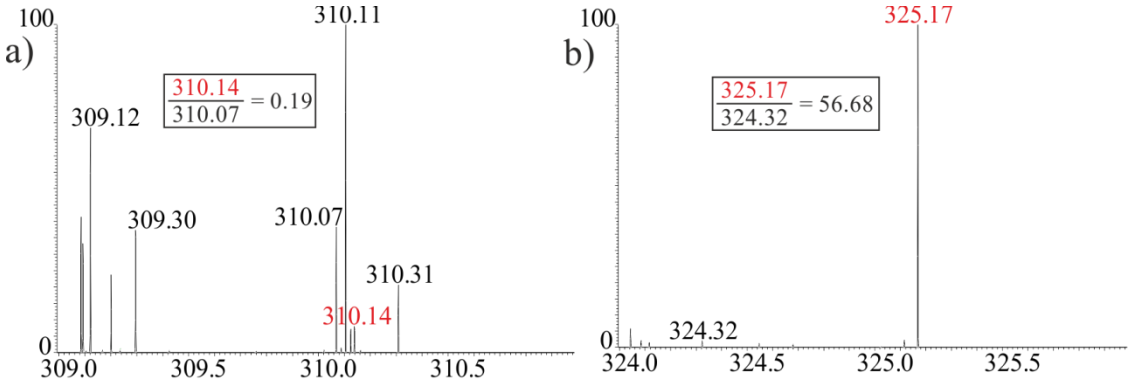


Figure 18: The mass spectra and the matrix-relative signal intensities from the empty acoustic trap (blank) incubated with an SSRI drug sample solution and eluted with the eluent ACN:TBS 1:9. a) 1 µmol/L fluoxetine, eluent aliquot; b) 1 µmol/L citalopram, eluent aliquot. The protonated molecular ions of fluoxetine (*m/z* = 310.14) and citalopram (*m/z* = 325.17) are written with red font.

In **experiment I**, 20 µL aliquots of the fluid medium (equivalent to the fluid medium aliquots containing the cells in experiments with cells) were dispensed from the capillary after dispensing the eluent. ISET sample preparation and MALDI MS analysis was done to these aliquots in order to investigate whether there are residual analytes adsorbed to the capillary after the elution. Signals of fluoxetine, fluoxetine-D5 and citalopram were present also in these aliquots (data not shown), which suggests that the

elution may not be enough to remove all of the adsorbed analytes from the capillary, leading to carry-over effects.

Due to the carry-over contamination effects appearing in **experiment I**, the efficiency of the capillary rinsing between individual experiments was investigated by ISET pretreating and analyzing the fluid medium aliquots dispensed from the capillary after rinsing the capillary between individual experiments. This was done during **experiment J**. The analysis revealed the inadequacy of the capillary rinsing process, as some residual analyte signals were visible in the mass spectra, citalopram with greater intensity than fluoxetine or fluoxetine-D5 (Figure 19). This result, implying citalopram carry-over, may explain the presence of citalopram signals in the spectra from the measurements in which no citalopram was used.

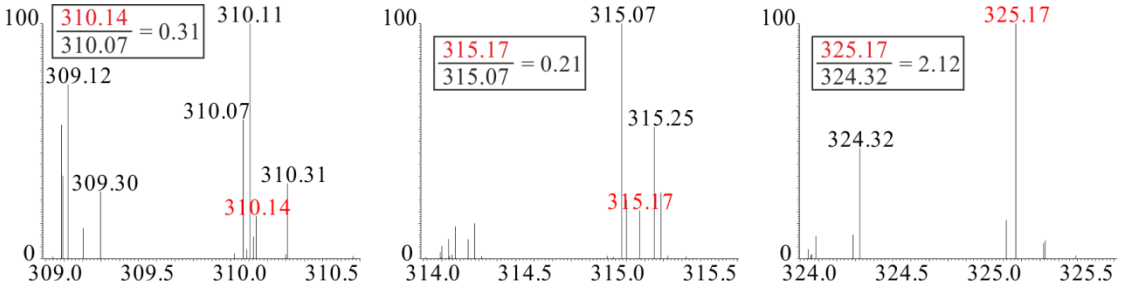


Figure 19: The mass spectra and the matrix-relative signal intensities of 20 µL fluid medium aliquots dispensed from the capillary after rinsing the capillary, showing the signals of fluoxetine (*m/z* = 310.14), fluoxetine-D5 (*m/z* = 315.17) and citalopram (*m/z* = 325.17).

Since the capillary rinsing with the fluid medium only was proven to be insufficient, it was investigated whether rinsing the capillary by aspirating and dispensing 20 µL of methanol between individual experiments would reduce the capillary contamination. After dispensing from the capillary, the methanol aliquots were evaporated to dryness, dissolved in 20 µL of the fluid medium, ISET pretreated and analyzed by MALDI MS. In addition, 20 µL of the fluid medium was dispensed from the capillary after the methanol rinsing (after-rinse aliquots), and these aliquots were ISET pretreated and analyzed by MALDI MS as well. This was done during **experiment K**. The mass spectra from the aforementioned methanol and fluid medium aliquots are presented in Figure 20. Some of the methanol aliquots, and all of the fluid medium aliquots dispensed after rinsing with methanol, lead to weak signals of the analytes in the mass

spectra (Figure 20). This suggests that the rinsing process applied between individual experiments is not sufficiently efficient. The absence of signals in some of the spectra obtained from the methanol aliquots can be explained by incomplete analyte dissolution after the evaporation of methanol. A more efficient rinsing procedure or a surface inactivation method for the capillary should be examined. Another alternative would be to replace the capillary between individual experiments.

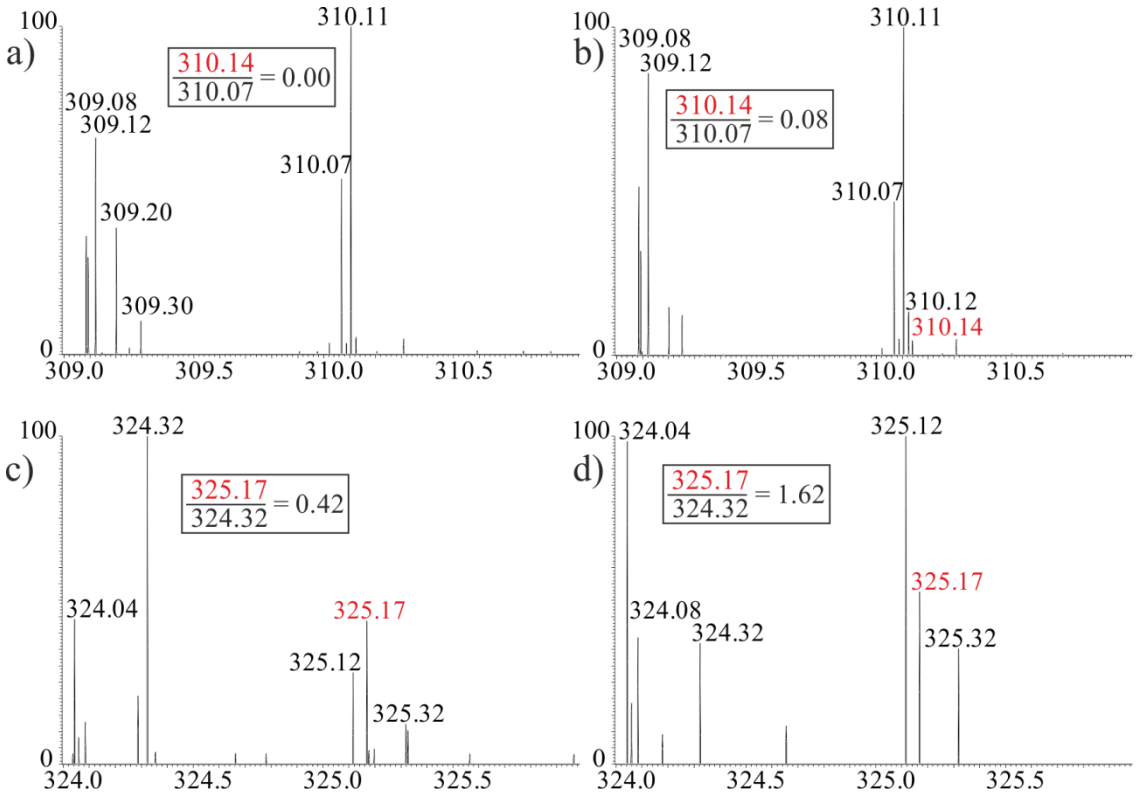


Figure 20: The mass spectra and the matrix-relative signal intensities of the methanol aliquots, evaporated to dryness and the residue dissolved in the fluid medium, and fluid medium aliquots, dispensed from the capillary after the methanol rinsing. a) methanol aliquot, sample solution 1 µmol/L fluoxetine; b) after-rinse aliquot, sample solution 1 µmol/L fluoxetine; c) methanol aliquot, sample solution 1 µmol/L citalopram; d) after-rinse aliquot, sample solution 1 µmol/L citalopram. The protonated molecular ions of fluoxetine (*m/z* = 310.14) and citalopram (*m/z* = 325.17) are marked with red font.

According to the results concerning analyte adsorption to the capillary (Figures 17 to 20), citalopram seems to have excessive tendency to adsorb to the capillary and cause carry-over contamination. Due to this, citalopram appears not to be well suited for this method, and other SSRIs should thus be used in future experiments.

The serotonin transporter (SERT) expression level in human platelets is 2.5 pmol/mg membrane protein (Wall et al. 1993). In order to achieve higher intensity analyte signals in the mass spectra, a cell membrane preparation with SERT expression level of 6.8 pmol/mg membrane protein ("SERT cell membranes") was used instead of platelets. The binding of citalopram, fluoxetine and fluoxetine-D5 (all 50 µmol/L) to SERT in the cell membrane preparation was investigated according to the procedure presented in **experiment J**. As expected, the analysis of the eluent aliquots and the fluid medium aliquots containing the cell membranes, both dispensed from the acoustic trap, showed analyte signal intensities larger than in the measurements with platelets in **experiment H** (Figure 21). The higher expression of SERT, and the consequent higher amount of specifically bound SSRI, in the cell membrane preparation than in platelets is the most likely explanation for the more intensive analyte signals observed with the cell membrane preparation. Comparison to platelets was done because of the possible difference in acoustic trapping capacity of SERT cell membranes and platelets. The corresponding mass spectra for citalopram are not presented, because the signal of citalopram was present in most of the mass spectra, due to the memory effect discussed above.

The specific and nonspecific binding of fluoxetine to the SERT cell membranes were investigated according to the procedure presented in **experiment K**: the acoustically trapped SERT cell membranes were incubated with the drug sample solution, followed by the elution of the analytes, and the consequent ISET sample preparation and MALDI MS analysis of the eluent aliquot. Five sample solutions, as listed in the Materials and methods section, were used, but due to the citalopram memory effect discussed above, only the following two of them were compared:

1. 1 µmol/L fluoxetine (total binding of fluoxetine)
2. 1 µmol/L fluoxetine, 100 µmol/L citalopram (nonspecific binding of fluoxetine)

With sample solution 1, fluoxetine binds to the SERT cell membranes both specifically and nonspecifically, whereas with sample solution 2, the citalopram present in excess was used to saturate the SERT in the cell membranes, leading to only nonspecific

binding in the case of fluoxetine. Similar approach to study nonspecific binding of SSRI drugs to SERT has been successfully used by Hess et al. (2011). The fluoxetine mass spectra of the eluents from SERT cell membranes incubated with drug sample solutions 1 and 2 are presented in Figure 22. Though there is a 4-fold difference in fluoxetine signal intensity between the spectra from the experiments with sample solutions with (Figure 22, a) and without (Figure 22, b) 100 µmol/L citalopram in them (representing the nonspecific binding and the total binding, respectively), it is not straightforward to estimate whether the difference is due to the fluoxetine binding to SERT or only uncertainty arising from the ISET sample preparation and the MALDI MS measurement. Fluoxetine adsorption to the capillary is a factor that complicates the assessment of the extent of nonspecific binding. From the data in Figures 16, 17 and 21, it seems like nonspecific binding and analyte adsorption to the capillary have similar signal intensities, accounting for approximately 10 % of the signals observed in the eluent samples.

The suitability of the system involving acoustic trapping, ISET sample preparation and MALDI MS analysis for the screening of serotonin transporter activity in platelets was investigated as described in **experiment L** and **experiment M**: the platelets were preincubated with SSRI solution or blank (fluid medium), followed by incubation with a serotonin solution and cell lysis. The approach was not successful, since serotonin could not be detected from the sample aliquots dispensed from the trapping capillary. The poor ionization properties of serotonin in MALDI MS were confirmed by analyzing 1 mg/mL stock solution of serotonin co-crystallized with a matrix solution on a MALDI plate. A different ionization method, such as electrospray ionization (ESI), should be tested for direct measurements of serotonin.

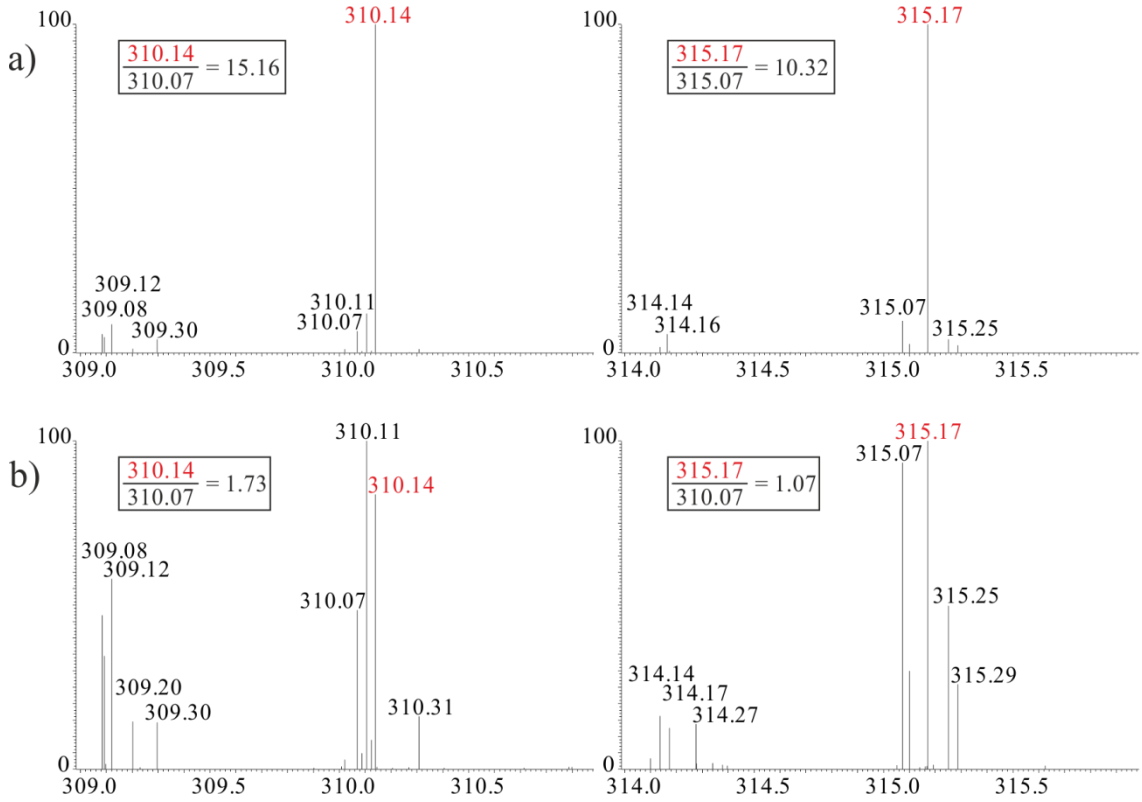


Figure 21: The mass spectra and the matrix-relative signal intensities of SERT cell membrane samples incubated with a solution containing 50 µmol/L of both fluoxetine and fluoxetine-D5 and eluted with the eluent ACN:TBS 1:9: a) eluent aliquot; b) fluid medium aliquot containing the cell membranes. The protonated molecular ions of fluoxetine (*m/z* = 310.14) and fluoxetine-D5 (*m/z* = 315.17) are written with red font.

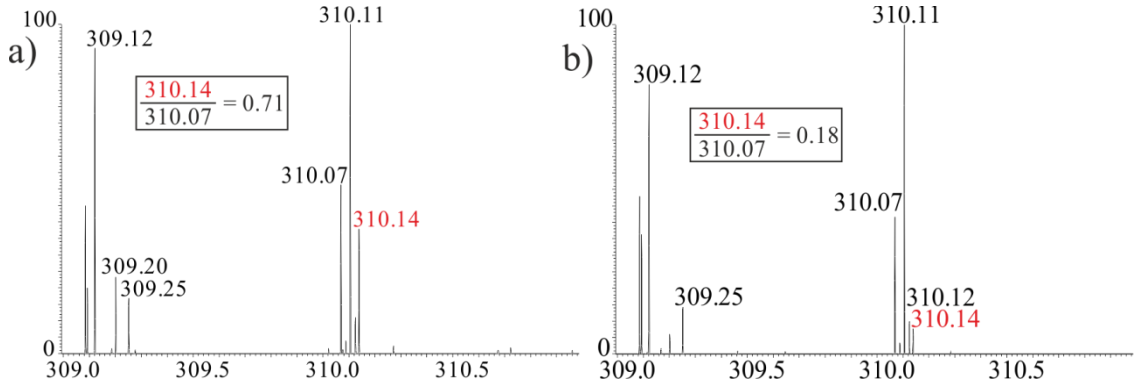


Figure 22: The mass spectra and the matrix-relative signal intensities of eluent aliquots from the **experiment K**: a) sample solution fluoxetine 1 µmol/ (total binding); b) sample solution fluoxetine 1 µmol/L, citalopram 100 µmol/L (nonspecific binding). The protonated molecular ion of fluoxetine (*m/z* = 310.14) is marked with red font.

3.2.6 Comparative drug binding experiments without acoustic trapping

For comparison with the drug binding studies using acoustic trapping, the binding of fluoxetine and citalopram to serotonin transporter (SERT) in the cell membrane preparation from cells overexpressing SERT ("SERT cell membranes") was investigated without acoustic trapping, using vacuum filtration (**Experiment N**, Figure 12). The following sample solutions were used: 1 µmol/L fluoxetine; 1 µmol/L citalopram; 1 µmol/L fluoxetine, 1 µmol/L citalopram; 1 µmol/L fluoxetine, 100 µmol/L citalopram; 100 µmol/L fluoxetine, 1 µmol/L citalopram. The choice of the sample solutions was done in order to investigate nonspecific drug binding to cell membranes, in a similar manner as in **experiment K**. Blank measurements without cell membranes were conducted in order to assess the binding of fluoxetine and citalopram to the filtering membranes. The sample preparation was done with indirect ISET (Figure 7). The mass spectra from the eluent samples are presented in Figure 23. The spectra of citalopram are shown, because for an unknown reason, the corresponding fluoxetine spectra showed no signals of fluoxetine, and could not be used for comparison with **experiment K** (Figure 22).

These results indicate that the magnitudes of both nonspecific binding (Figure 23, c) and analyte adsorption to the filtering membrane (Figure 23, d) are below the detection limit of the system, since no signals of citalopram are present in the spectra. Another alternative is the failure of the method, since the amount of SERT cell membranes used should lead to detectable amounts of citalopram. According to these results, the acoustic trapping method for screening of SSRI binding to SERT cell membranes was more successful than the vacuum filtration method, but the comparison should be made with care: significantly more optimization was done with the acoustic trapping method. In addition, a method involving vacuum filtration and mass spectrometry has been successfully used for a fluoxetine-SERT binding assay (Hess et al. 2011). It has to be noted that with these results, direct comparison of the acoustic trapping method in experiment K and the vacuum suction method is not justifiable, given the different cell membrane preparation and sample solution amounts used.

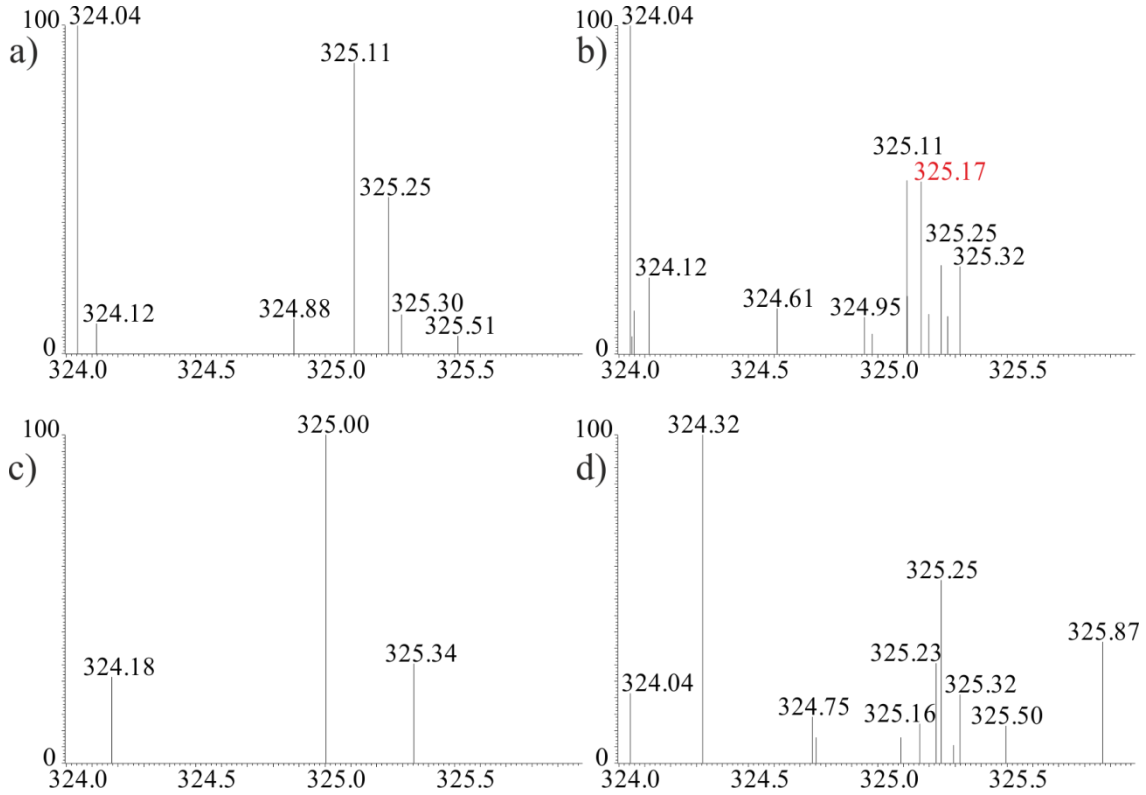


Figure 23: The mass spectra of citalopram from **experiment N**. a) SERT cell membranes, solvent blank; b) SERT cell membranes, sample solution 1 µmol/L citalopram (total binding); c) SERT cell membranes, sample solution 1 µmol/L citalopram, 100 µmol/L fluoxetine (specific binding); d) blank, sample solution 1 µmol/L citalopram, 1 µmol/L fluoxetine (adsorption to filtering membrane). The protonated molecular ion of citalopram (*m/z* = 325.17) is marked with red font.

3.2.7 Critical assessment of the method performance

Throughout the experiments, solvent blank experiments were conducted in addition to the experiments in which real fluoxetine, fluoxetine-D5 or citalopram samples were used. This was done in order to confirm that the buffer solutions and the eluents were free of the analytes. However, in many (but not all) of the mass spectra from the solvent blank experiments, weak signals of the analytes were present. According to the blank measurements in **experiment I** and **experiment J**, analyte adsorption to the walls of the borosilicate glass capillary used for acoustic trapping is detectable, and according to **experiment J** and **experiment K**, the procedure used for rinsing the capillary between individual experiments was not adequate. For these reasons, it can be assumed that analyte carry-over from the capillary is an issue of concern. To decrease analyte adsorption to the capillary, surface inactivation could be used. In addition, the signal of

citalopram was present in all spectra in **experiment J** and **experiment K**, which suggests ISET plate contamination, since the samples were pretreated with the same ISET plate. Thus, the main drawback of the analytical platform investigated was the carry-over effect. Mass-production of disposable capillaries and ISET plates would eliminate the problem, but also increase the price. More rounds of cleaning could be used, but this would make the method more time-consuming.

Another main drawback of the system investigated was sensitivity: in order to achieve analyte signals in the spectra, high analyte concentrations were required, which lead to high adsorption of the analytes to the capillary walls (**experiment I**, **experiment J**). According to the results of **experiment D**, the acoustic trapping capacity for transducer #8 is approximately 1000000 polystyrene beads of the diameter 3 µm. The platelet diameter being approximately the same (Paulus 1975), roughly the same value for trapping capacity can be assumed also for platelets. The SERT amount of 9 pmol/mg membrane protein corresponds to 500000 copies/cell (Tate and Blakely 1994). In platelets, the SERT amount is 2.5 pmol/mg membrane protein (Wall et al. 1993), giving roughly 200 fmol of SERT per 10^6 platelets in the acoustic trap. According to this calculation, the maximum amount of SSRI drug specifically bound to SERT in the acoustically trapped platelet cluster is approximately 200 fmol, which is challenging for the mass spectrometric detection. However, the actual amount of platelets trapped may differ from that of the polystyrene beads, given the different shape and compressibility of platelets. Consequently, even large uncertainty in the calculation can be assumed.

Improved sensitivity might be achieved using LC-ESI-MS/MS, as done by Hess et al. (2011). Alternatively, larger size of the apparatus could be used to increase the amount of cells trapped, and thus decrease the sensitivity requirement. However, this can be done only to some extent, because in overly large system, the advantages of microfluidic setup are lost: the flow will be turbulent instead of laminar, and sample and energy consumption will be larger. In addition, already with the equipment used, the fluid volumes were relatively large to be pretreated with ISET. Larger fluid volumes would require the utilization of a different sample preparation method.

In order to be useful in drug discovery, the analytical method needs to be fast enough to be able to go through large sample libraries in reasonable time. Clearly, with the method investigated, the way to achieve this would be parallel systems, as the incubation times per one measurement were relatively long. Parallel systems would require unfeasibly large numbers of the equipment. The technology required to achieve automatization, on the other hand, is not overly complicated: mounting the system on a *xyz*-stage and using pre-programmable syringe and vacuum pumps would permit the automatization of most of the phases.

4 CONCLUSIONS

In this MSc (Pharm) thesis, a new nanobiotechnology method, involving acoustic trapping, miniaturized solid phase extraction sample preparation, and analysis by MALDI MS, was described and tested for the screening of cell-drug interactions. The sample preparation using the integrated selective enrichment target (ISET) platform was optimized for fluoxetine, the selective serotonin reuptake inhibitor (SSRI) drug compound used as an analyte. The performance of the acoustic trapping setup was investigated both qualitatively and quantitatively. The method was modeled and the procedure optimized by using strong cation exchange (SCX) beads for emulating cells. The performance of the method in the screening of cell-drug interactions was investigated using yeast cells, human platelets, and a cell membrane preparation from cells overexpressing serotonin transporter (SERT), the molecular target of the analytes used. Comparative experiments without acoustic trapping were performed using vacuum filtration.

Within the scope of this work, the performance of the new nanobiotechnology method for the screening of cell-drug interactions could not be thoroughly substantiated, despite some promising results obtained. The major problems with the method were inadequate sensitivity and contamination issues: due to the low sensitivity, high analyte concentrations were required, which resulted in analyte adsorption to the platform and, consequently, carry-over contamination. The use of a more sensitive tandem mass spectrometric method combined with chromatographic separation (LC-ESI-MS/MS) could be used to enable the use of less concentrated analyte solutions, thus reducing the analyte adsorption to the platform, and the consequent carry-over contamination. Further research and method development is required to reliably prove whether or not acoustic trapping is suitable for the screening of cell-drug interactions.

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