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Microfluidics for High-Pressure Inertial Focusing

Focusing, Separation and Concentration of Micro and Sub-micron Particles

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Abstract
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The birth of microsystems set the ground for technologies never imagined before, for it is not only the small size what characterizes the miniaturized systems, but unique phenomena arise in the micro scale. This thesis relates to one such unique phenomenon, inertial focusing, a phenomenon that occurs in microfluidic systems if very special conditions are met and that allows for fine manipulation of particles in fluid samples. This ability is key in a bigger picture: the analysis of complex fluids, where rare particles of interest may be present in very few numbers amongst a myriad of others, making the task difficult – if not impossible. A system exploiting inertial focusing allows, for instance, to focus, separate, isolate and concentrate such rare particles of interest, and even to transfer them to another fluid, thereby enabling/facilitating their detection and analysis. Examples of rare particles of interest in complex fluids are circulating tumor cells in blood, that give away the presence of cancer, extracellular vesicles also in blood, that contain biomarkers with physiological and pathological information about the patient, or bacteria in natural water, where the species present and their numbers are to be monitored for safety reasons and/or biological studies. This thesis covers the state of art physical principles behind the phenomenon and extends the understanding both in theory and applications. Specifically, the technology is extended to allow for manipulation of sub-micron particles, a range of interest as it comprises bacteria, viruses and organelles of eukaryotic cells. This was possible by an analysis of the balance of forces in play and by the integration of inertial focusing in high-pressure systems (up to 200 bar). In a second block, a very special line of inertial focusing is introduced and developed; inertial focusing in High Aspect Ratio Curved (HARC) microfluidics. These systems, engineered to rearrange the force field responsible for the particle manipulation, not only achieve excellent performances for focusing and concentration of particles, but also extreme resolution in their separation (mathematically unlimited; demonstrated experimentally for differences in size down to 80 nm). Perhaps more important than the performance, the systems are stable, intuitive and simpler to design, attributes that we hope will make the technology and its outstanding benefits more accessible to the community. With its remarkable performance, it would not come as a surprise if, in the near future, inertial focusing makes a strong impact on how analyses are performed nowadays and opens up for possibilities beyond the current state of the art.

Keywords: Microsystems, Microfluidics, Inertial Focusing, Particle Manipulation

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Svensk Sammanfattning

Födelsen av mikrosystem erbjöd tekniska lösningar som inte tidigare var möjliga, eftersom det inte bara är den litenheten som kännetecknar mikrosystemen utan också de unika fenomen som uppstår när de blir tillräckligt små. Denna avhandling avser ett sådant unikt fenomen, tröghetsfokusering, som kan skapas i flödande mikrosystem med ett laminärt flöde (jag beskriver det lite längre ned) när en partikels tröghet är viktig. Tröghetsfokusering möjliggör olika typer av manipulering av partiklar i vätskeprover, som fokusering, separation och koncentration, och till och med att överföring till en annan vätska.

Dessa förmågor är nyckeln till ett svårlöst problem: analys av komplexa vätskeprover, där sällsynta partiklar av intresse kan finnas i ett mycket litet antal bland en enorm mängd av andra, vilket gör analysen svår – om inte omöjlig. Exempel på intressanta sällsynta partiklar i komplexa vätskor är cirkulerande tumörceller i blod som ger viktig information om cancer, extracellulära vesiklar i blod som innehåller biomarkörer med fysiologisk och patologisk information, eller bakterier och alger i vatten, där arten och dess antal kan behöva övervakas för biologiska studier eller av säkerhetsskäl.

Med tanke på dessa intressanta komplexa prover, hur kan de mest avancerade sensorerna idag upptäcka och analysera en partikel som är omgiven av miljoner eller miljarder andra i en matris full av molekyler? Svaret är väldigt enkelt. Det kan de inte. Även den mest känsliga och selektiva sensorn kämpar i en sådan miljö. När vi söker mer avancerad information räcker det inte med en fristående sensor utan den kräver flera andra integrerade komponenter till sin hjälp, för provpreparation, transport av provet och en kontrollerad miljö (t.ex. temperatur eller pH). Idag utförs detta oftast av flera olika instrument i ett laboratorium. Med hjälp av sin litenhet kan ett flödande mikrosystem erbjuda ett komplett laboratorium på en liten yta, ett lab på ett chip (eng. Lab-on-a-Chip, där chippet anknyter till mikroelektronikens chip med processorer, minnen eller andra avancerade integrerade kretsar). Det kan erbjuda stora fördelar i kostnad för såväl systemet som dess användning, som mindre behov av provmaterial och dyra analysvätskor.

När man väljer en teknik för att studera sällsynta partiklar behövs oftast hög kapacitet för att ta fram dem ur provet. Det är som att hitta en mycket liten nål i en liten höstack. En passiv teknik möjliggör detta samtidigt som den är enkel

och robust eftersom processen helt enkelt består av att föra provet genom flödessystemet med en kontrollerad hastighet. Dessutom är passiva tekniker märkningsfria, vilket minskar processens komplexitet och kostnader samt erbjuder sortering av okända partiklar. Som jämförelse kan aktiva tekniker ge mer flexibilitet, eftersom kraften kan justeras externt; och märkningstekniker kan vanligtvis erbjuda högre specificitet. Beroende på tillämpningen behövs olika tekniker för att förbereda ett prov. Vi startade detta projekt eftersom det var tydligt att en ny teknik behövdes för att erbjuda provpreparering med hög kapacitet av sällsynta ytterst små partiklar som bakterier, virus och organeller av eukaryota celler.

I mikrovärlden dominerar fenomen som relaterar till ytor över fenomen som relaterar till volymer. Ett index som ofta används för att återspegla krafter kopplade till massan och trögheten hos en kropp (relativt volymen) och dess ytkrafter är det dimensionslösa Reynolds-talet (Re, kvoten mellan tröghetskrafter och viskösa krafter i en vätska). För Re-värden över 2000 är vätskan turbulent, vilket kännetecknat av kaotiska och odefinierade flöden. Å andra sidan, för Re-värden långt under 2000 är vätskan laminär, vilket kännetecknas av väldefinierade och förutsägbara flödeslinjer som inte korsar varandra. I många flödande mikrosystem är Re mycket mindre än 1, vilket betyder att fenomen relaterade till ytor, som viskositet och ytspänning, dikterar systemets beteende, medan fenomen relaterade till volym, som tyngdkraft eller tröghet, betyder så lite att de i allmänhet är försumbara. I dessa förhållanden möjliggör analytiska uttryck exakta förutsägelser av systemens beteende. Att tyngdkraft eller tröghet inte spelar någon roll är en viktig konsekvens som jag anser nödvändig att betona för de läsare som inte är i fältet. När dessa krafter inte spelar en roll fungerar ett flödande mikrosystem oberoende av dess orientering eller lutning. Dess prestanda förblir även oförändrad om den placeras horisontellt, vertikalt eller vänds upp och ner. På samma sätt kan man inte förvänta sig att en partikel trycks ut mot ytterväggen i en kurva (som man gör när man till exempel tar en kurva med bilen). Istället, när man följer en kurva i ett flödande mikrosystem med mycket låg Re, flyttas inte partikeln i sidled. Faktum är att partiklar i sådana flödande mikrosystem följer vätskans strömlinjer så troget att de i praktiken inte kan gå över från en till en annan.

Tröghetsfokusering är ett fenomen där initialt slumpmässigt fördelade partiklar fokuseras i väldefinierade positioner när de färdas genom ett mikrofluidsystem. Rörelsen tvärs huvudflödet tillskrivs en kraft (lyftkraften) som läggs på partiklarna på grund av deras interaktion med vätskan när de färdas genom mikrokanalerna. Kraften har sitt ursprung i att ett tillräckligt kraftmoment förs över från vätskan till partiklarna, vilket bara förekommer i system där tröghet spelar en roll. Faktum är att fenomenet först observerades av Segré & Silberberg 1961, men de analytiska modeller som vanligtvis användes för beräkningar i flödande mikrosystem, där tröghet försummas, misslyckades med att

förklara partiklarnas experimentellt observerade beteende. Det var först 1974 som Ho & Leal gav en analytisk förklaring till fenomenet, vilket idag accepteras som grunden till vår förståelse av fenomenet.

Denna avhandling täcker fluidmekaniken bakom tröghetsfokusering och tillför ny förståelse i såväl teori som praktik. Vi använder för första gången höga tryck (upp till 200 atmosfärers tryck) för att med hög kapacitet möjliggöra fokusering, separation och koncentration av bakterier. Dessutom introducerar och utvecklar vi en ny och mycket speciell tröghetsfokuseringsteknik; tröghetsfokusering i krökta kanaler med större djup än bredd. Dessa system, ordnar om kraftfältet som är ansvarigt för partikelförflyttningen tvärs huvudflödet så att de fokuserade partiklarna hamnar i en rak symmetrilinje tvär kanalens väggar. Denna nya teknik uppnår inte bara utmärkta prestanda för fokusering och koncentration av partiklar, utan den kan ge en extrem upplösning i när man separerar partiklarna i förhållande till deras storlek (matematiskt obegränsat; demonstrerat experimentellt för skillnader i storlek ner till 80 nm). Det är kanske än viktigare att systemen är stabila, intuitiva och enkla att utforma – egenskaper som vi hoppas kommer att göra tekniken med dess enastående fördelar mer tillgängliga för ett större samfund än de som idag forskar i fältet.

Med dess anmärkningsvärt goda prestanda skulle det inte bli någon överraskning om tröghetsfokusering inom en snar framtid kan komma att ändra hur analyser utförs och öppna upp för möjligheter utöver vad som idag är möjligt.

To my family, for I would have never seen through the physics of inertial focusing without their contribution:

"Micro… what?? Well, in any case, we actually already know how to do that here, you just have to pass the fluid through a sock"

— Family

"*Nobody ever figures out what life is all about, and it doesn't matter. Explore the world. Nearly everything is really interesting if you go into it deeply enough!*"

— Richard P. Feynman

List of Papers

This thesis is based on the following Papers, which are referred to in the text by their Roman numerals.

I Cruz J, Hooshmand Zadeh S, Graells T, Andersson M, Malmström J, Wu Z G G and Hjort K (2017) High pressure inertial focusing for separating and concentrating bacteria at high throughput *J. Micromechanics and Microengineering* **27** 084001

II Cruz J, Graells T, Walldén M and Hjort K (2019) Inertial focusing with sub-micron resolution for separation of bacteria *Lab on a Chip* **19** 1257–66

III Cruz J, Hjort K and Hjort K (2020) Stable 3D inertial focusing by high aspect ratio curved microfluidics *J. Micromechanics and Microengineering* **31** 015008

IV Cruz J, Hjort K (2020) Fundamentals of Inertial Focusing in High Aspect Ratio Curved Microfluidics

Manuscript submitted to *Scientific Reports*

V Cruz J, Hjort K (2020) High-resolution Particle Separation by Inertial Focusing in High Aspect Ratio Curved Microfluidics

Manuscript to be submitted to *J. Microsystems and Nanoengineering*

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Author's Contributions

In all papers, the author contributed to most of design, experiment, characterization and analysis; and a major part of planning, discussion and writing.

Contents

1 INTRODUCTION

1.1 Microsystems

The field of microsystems relates to the science of miniaturized machines, both in the sense of their fabrication and their application. With components in the range of few to several hundreds of micrometers (µm), miniaturized systems offer very different properties and performances compared to those in the macro scale, opening a whole new world of opportunities. The size of several objects is shown in Fig. 1 to provide the reader with a notion of the dimensions of the micro-world.

The field of microsystems sprouted in the 1980s, little after the massive development of the silicon technology that took place motivated by the miniaturization of electronic systems [1]. With the ground for miniaturization developed, the technology included other physical phenomena and extended to other fields, like electro-mechanics, thermodynamics, fluidics or optics, to mention a few. The systems consist of microstructures, microsensors and microactuators, and a miniaturized system integrating one or multiple disciplines is referred to as a microsystem or a microelectromechanical system (MEMS). Today, the MEMS market has declined to US$ 11B due to the COVID pandemic, but it is estimated to have a health growth and be worth 18B in year 2025 [2].

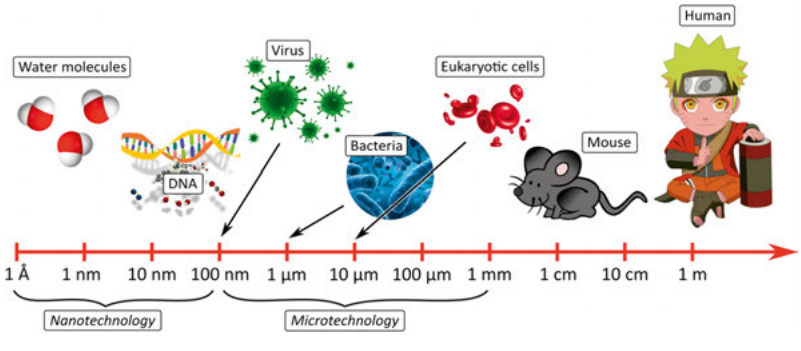

Figure 1. Scale of different systems: a human, a mouse, a eukaryotic cell, bacteria, virus, a molecule of DNA and water molecules.*

* The individual images are reproduced by courtesy of www.kindpng.com.

Since the technology was born, multitude of microdevices with exotic performances have been demonstrated, with many of them nowadays present in our lives integrated in technologies such as automobiles (micro-accelerometers with fast responses for the airbag release), aerospace technology (gyroscopes, ionic propulsion systems), biomedical applications (whole genome sequencing), inkjet printers (ink dispenser) and optical devices (micromirrors in projectors) [1,3,4].

The technology developed in this thesis relates more specifically to microfluidics; microsystems first presented in 1990 [5] and intended for fluid handling (very often combined with micromechanics, microelectronics or microoptics). With channel dimensions ranging from few to several tens of µm, the systems allow for very precise control of fluids and particles and make possible the miniaturization of standard laboratories while retaining the capacity to perform similar assays and analyses. Because of these characteristics, these systems are also often referred to as Lab-on-Chips (LOCs) or Micro-Total-Analysis-Systems (µTAS) [3,4]. An example of microfluidic system is shown in Fig. 2.

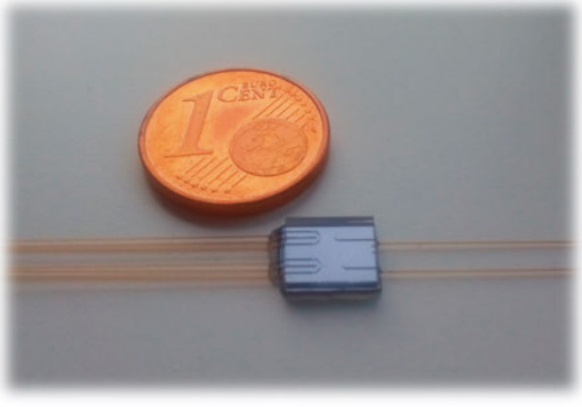

Figure 2. Microfluidic chip built in silicon-glass with glass capillaries as fluidic connections.

As for the special features that accompany microsystems [3,4,6], and more in particular microfluidics, the fact that they occupy a very small space makes the systems portable, allowing for analyses in situ (Point-of-Care systems; PoC systems), thereby eliminating the need of collecting and transporting samples to a central laboratory. The reduced size also translates into the need of very small amounts of sample and reagents, and the possibility of a massive parallelization at a small footprint. These features, together with, in many cases, an easy integration with other technologies and automation, lead to a significant cost reduction and simplification in the operation compared to large scale systems, as well as the possibility of performing a massive number

of simultaneous tests (crucial, for instance, in the search of new medical treatments). On the other hand, the physics in the micro scale allow for a well-controlled and homogeneous environment and faster responses of the systems than in the macro world. Last but not the least, the systems in the micro-world behave in very surprising and counterintuitive ways. This is to the point that, out of context, it may seem that Nature does not follow the physics we are used to! In reality, Nature does follow the same rules of the macro-world*; with the different effects simply being the result of a change in the relative relevance of the physics in play. This different behavior allows for phenomena that would not be possible in the macro world. In fact, one such unique phenomenon is inertial focusing in microfluidics, to which this thesis relates and that allows for, as the reader will understand as he progresses though this text, the discovery of micro-needles in micro-haystacks. Other unique applications made possible by miniaturized systems are single cell studies or Organs-on-Chips, with these last aiming at reproducing the conditions in a living body for more representative biological/medical assays.

1.2 The Micro-Needle in the Micro-Haystack – Rare Particles in Complex Fluid Samples

The birth of microfluidic systems revolutionized the means to perform experiments involving fluids, such as analyses and assays in fields like chemistry, biology and biomedical sciences. With the new possibilities and advantages aforementioned, microfluidics has nowadays become an indispensable technology in laboratories, yet its full potential lies far ahead in the future [3,4,6].

This thesis relates to inertial focusing, a phenomenon that arises within microfluidic systems when special circumstances are met and enables the manipulation of particles within fluid samples. This ability is a key piece in a much bigger picture: the extraction of information from complex fluid samples; samples that contain a myriad of particles (literally, millions or billions per mL), amongst which few may be of interest. Blood is a good example of such a fluid. Broadly speaking, 1 mL of blood contains 10^9 red blood cells (~6-8 μm), 10^6 white blood cells (~10-15 μm) and 10^8 platelets (~2-3 μm) suspended in plasma, a matrix composed of water, proteins and electrolytes. Apart from these particles and molecules, other less common species may be present in very low numbers. Typical examples are extracellular vesicles (EVs), circulating tumor cells (CTCs) in case of a cancer affecting any tissue of the body, or bacteria in case of blood infection.

* At least until you further reduce the size and enter the nano-world! Here, other physics not observed in the macro-world do come into play (quantum effects).

The EVs are small lipidic vesicles (~30 nm - 1 μm, excluding apoptotic bodies) secreted by cells that contain, amongst other molecules, lipids, proteins, mRNA and non-coding RNA. They act as cell-to-cell messengers, carrying information from the secreting cell and regulating the receiving one. Not surprisingly, EVs have been reported to contain biomarkers that may be used for diagnosis of various diseases and the evaluation of treatment responses [7–9].

The CTC are tumorous cells (~15-20 μm) released from a primary tumor that infiltrate the circulatory or lymphatic system. These cells are responsible for cancer metastasis; the extension of the tumor to other parts of the body. With CTCs being tissue specific, their detection in blood may reveal not only the presence of a cancer in early stages but also its possible location [7,10].

Bacteria may be present in the bloodstream after severe external infections or surgery, for instance. Blood infection can have important health consequences; not only may the original infection spread to other parts but the host's immune response may result in sepsis or septic shock, which presents a high mortality rate [11,12].

Surely, the reader agrees on the importance of being able to detect and analyze said rare particles of interest or any other possible target of interest. A blood sample is indeed a library containing extremely valuable physiological and pathological information from a subject. Noteworthy are the facts that, as blood is in constant circulation, it contains representative information from multiple parts of the body and that a blood analysis is much less invasive than other techniques. Other biofluids of interest are saliva and urine, as they also contain important physiological and pathological biomarkers.

Last but not least, yet another complex fluid of interest is natural water, populated by millions of microorganisms per liter whose identity and concentration are to be regularly monitored for human safety and biological studies such as the antimicrobial resistance, a growing problem in modern society [13,14].

1.3 The Need for Particle Manipulation

With the earlier description of a complex fluid in mind, how can the most advanced sensors of the XXI century detect and analyze a particle of interest if this is surrounded and heavily outnumbered by millions or billions of others in a matrix full of molecules? The answer is very simple. They cannot. Even the most sensitive and selective sensor struggles in such an environment. As we seek more ambitious information within challenging samples, a system cannot consist of a standalone sensor but requires the complement of multiple

other subsystems that make the task possible, such as for sample delivery, environmental control (e.g. temperature or pH) or sample preparation.

Nowadays, commonly used technologies for fluid sample preparation, each with different advantages, limitations and field of application, are centrifugation, filtration, flow cytometry and cell sorting (fluorescence or magnetically activated) and chromatography (including flow field fractionation) [15,16].

In the case of microfluidics, the ability to manipulate the position of particles within the fluid, i.e. particle focusing, separation, concentration or fluid exchange, facilitates the detection and posterior analysis of rare targets of interest while incorporating the advantages and vast potential from miniaturization described earlier. However, this ability is in fact one of the greatest challenges for the technology due to the particular fluid dynamics that govern the systems.

1.3.1 The challenge of particle manipulation in microfluidic systems

In the micro-world, phenomena that relate to surfaces largely dominate over phenomena that relate to volumes; fact to which microfluidics owe most of their characteristic behavior [4,6]. An index commonly used to reflect the relative importance of the body forces (relative to volumes) and the surface forces affecting the system is the dimensionless Reynolds number (Re = Inertial Forces/Viscous Forces). For Re values over ~2000, the regime of the fluid is turbulent; characterized for having chaotic, undefined streamlines (the trajectories of the fluid molecules) that result in different portions of the fluid mixing. On the other hand, for Re values below ~2000, the fluid behaves in a laminar regime; characterized for having well defined and predictable streamlines that do not cross each other (i.e., there is no mixing of different portions of the fluid). In the small scale of microfluidics, typically $Re << 1$, implying that phenomena related to surfaces, like viscosity and surface tension, dictate the behavior of the system, while phenomena related to volume, like gravity or inertia, mean so little that are generally neglected. The scenario resulting from these conditions is largely deterministic and analytical expressions describing the fluid dynamics make possible precise predictions of the behavior of the systems. The irrelevance of gravity or inertia is a key implication in this scenario that I consider necessary to emphasize for those readers not in the field. With those forces not playing a role, a microfluidic system works independently of its orientation or inclination; its performance remains invariant if positioned horizontally, vertically or flipped upside down. In the same way, one cannot expect a particle to be expelled towards the outer wall in a curve (as one does when turning a curve with the car, for instance). Instead, when

turning a curve in a microfluidic system with very low Re, the particle does not suffer any lateral displacement. In fact, particles in microfluidic systems follow the streamlines of the fluid so faithfully that in practice they cannot cross over from one to another, which brings us back to the challenge of making them migrate. Let us consider a fluid where a number of particles are initially randomly distributed, each one of them surrounded by their corresponding portion of the fluid. As described before, the flow through a microfluidic system is laminar and the fluid remains unmixed. If the different portions of the fluid remain unmixed, and particles follow exactly the same streamlines, it turns out that particles never abandon their neighbor fluid molecules. Surely the fluid parcels can be deformed (stretched or rotated, for instance), but they contain exactly the same particles before and after their journey throughout the system. In other words, the initial distribution of particles is preserved, with no particle migration from one portion of fluid to another*. Under these conditions, certainly the task to locate, separate and concentrate a rare target of interest or make it change medium seems unattainable. Nevertheless, there are known techniques to break the aforementioned behavior and induce particle migration across streamlines, thereby enabling particle manipulation and, with it, facilitating the analyses of rare targets of interest in fluid samples.

1.3.2 Technologies for particle manipulation

The technologies for particle manipulation within microfluidics rely on inducing a force on particles, which translates in an extra component in their velocity, thereby differentiating their trajectories from those of their neighbor fluid molecules. With it, migration of particles across streamlines is achieved and their initial distribution can be modified. Naturally, this is of special interest if the force affects the particles differently based on any or multiple of their attributes, as it opens up for the possibility to tailor the displacements and final positions accordingly.

These technologies are classified as active technologies if they require an external field to induce the force, or as passive technologies if they rely on the intrinsic properties of the fluid and geometry of the system. Examples of active technologies are acoustophoresis, thermophoresis, electrophoresis or magnetophoresis, where an acoustic, thermal, electric or magnetic field are employed, respectively. Examples of passive technologies are deterministic lateral displacement (DLD), viscoelastic focusing, hydrodynamic focusing and inertial focusing. Generally, a passive technique brings simplicity and robustness to the operation, as the process simply consists of running the sample

* There is, as a matter of fact, a random movement of the particles (diffusion) that may result in these moving across streamlines. However, being random, it does not contribute to particle ordering.

through the system at a controlled flow rate. On the other hand, an active technique may provide more flexibility, as the force can be externally tuned.

Another classification worthy of explanation segregates the technologies between those that require particle labelling in order to exploit the attribute of interest, and those where the attribute of interest is enough on its own (label-free). An example of the first is magnetophoresis, where magnetic beads are selectively bound to the targets based on specific attributes on their surface, thereby labeling them and allowing a differential manipulation with respect to the rest of the unlabeled particles by the use of a magnetic field. On the other hand, an example of label-free technology is inertial focusing, where the attribute that arises the force is mainly the size of the particle (complemented by others like its geometry and deformability) and therefore labelling the targets of interest is unnecessary. Generally, label-free technologies reduce the complexity and the costs of the process while offering relatively high throughputs. On the other hand, labelling technologies usually offer higher specificity.

This thesis focuses on the development of inertial focusing, a passive and label-free technique that allows for high throughput and high-resolution particle manipulation.

1.4 Inertial Focusing

Inertial focusing is a phenomenon where initially randomly distributed particles migrate and equilibrate at well-defined positions (focus positions) as they travel through a microfluidic system. The migration is attributed to a force (lift force) that is induced on the particles as a result of their interaction with the fluid as they travel through the microchannels. The force originates in the transference of momentum from the fluid to the particles, which can only occur in systems where inertia plays a role. Indeed, the phenomenon was first observed by Segré & Silbeberg in 1961 [17], and analytical models generally used for calculations in microfluidic systems, where inertia is neglected, failed to explain the experimentally observed behavior of the particles, see Fig. 3. It was not until 1974 that Ho & Leal provided an analytical explanation to the phenomenon [18]; today accepted as the foundation of the phenomenon and achieved by the inclusion of the inertial terms in the fluid dynamic calculations. As further proof of its inertial origins, the force was reported to grow quadratically as inertia gained relevance in the systems (as Re was increased) [17,18].

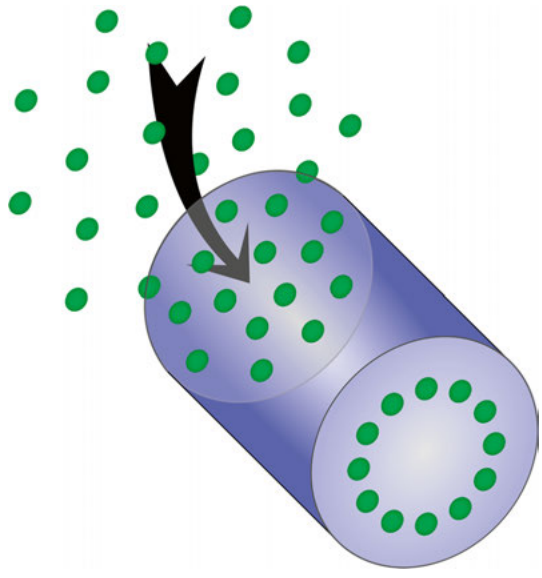

Figure 3. Schema of the unexpected phenomenon observed by Segré & Silberberg in 1961 [17], constituting the first scientific report about inertial focusing. Initially randomly distributed particles in a fluid focus forming a ring as they flow through a channel with circular cross section.

Inertial focusing can be exploited, for instance, to facilitate the interrogation of the particle content of a fluid by optical means (flow cytometry), Fig. 4A. In addition, the inclusion of multiple outlets to the systems allows for the concentration of different particles together, also Fig. 4A, or their separation and concentration based on their size*, Fig. 4B. Last, the use of multiple fluids in the system allows for any of the previous tasks combined with a particle transfer to another medium (purification of particles in case these are the target; filtration of a fluid if this is the target), Fig. 4C. Noteworthy is the fact that inertial focusing systems allows for manipulation of particles with similar density as the fluid, which may be out of reach for other technologies, like centrifugation.

* Technically, each particle has a specific *signature* that defines its behavior in a fluid. When writing *size* in relation to inertial focusing, we refer to the *hydrodynamic diameter* of the particle, mainly defined by the particle size, but other parameters also contribute to it, such as its shape and deformability.

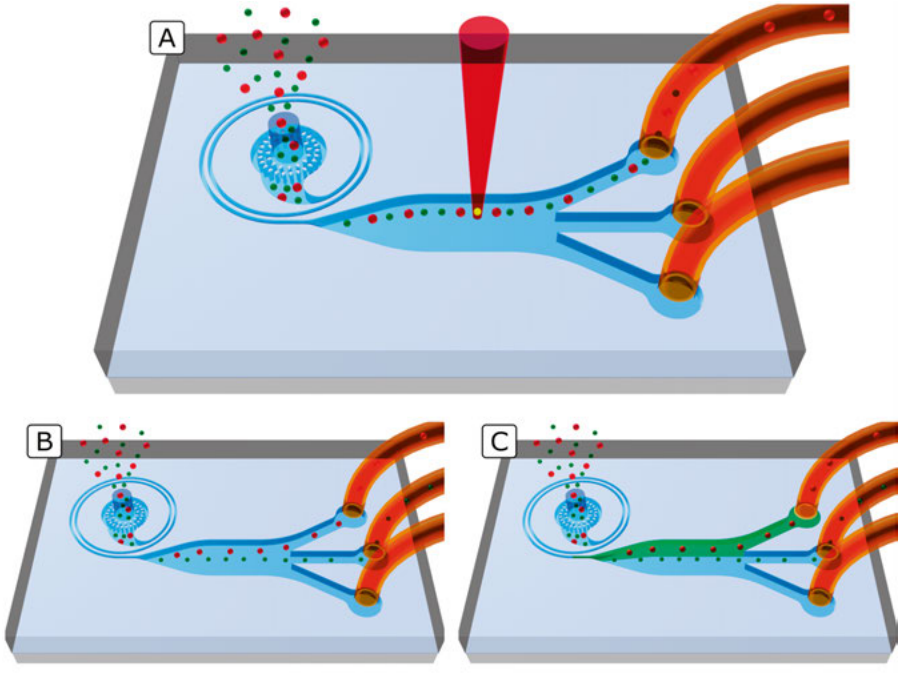

Figure 4. Inertial focusing system for (A) focusing and concentration of microparticles, (B) focusing, concentration and separation of microparticles and (C) focusing, concentration and separation with fluid exchange.

1.4.1 State of the art

In their work, Segré & Silberberg reported that, under certain flow conditions, initially randomly distributed particles in a fluid unexpectedly formed an annulus downstream a circular pipe [17], Fig. 3. In 1974, Ho & Leal provided the first analytical explanation of the phenomenon [18], which remains as the basis of the understanding nowadays. Naturally, evolved theories and calculations have been proposed by other researchers since then [19–23]. More technical details about the phenomenon are provided in the *Fluid dynamics* section. The technology has attracted much attention in the last decade as it has the potential to solve current challenges such as the analysis of complex samples, as described earlier. Microfluidic systems that exploit inertial focusing have been widely demonstrated for manipulation of inert and biological particles in the range of several micrometers, such as polystyrene microparticles, eukaryotic cells and algae [24–28]. As a remarkable example, label-free isolation of circulating tumor cells from blood has been reported in multiple instances [29–31], potentially allowing for cancer detection in early stages and physiological studies of the CTCs for therapy monitoring.

1.4.2 Scientific gap – Contributions of this thesis

The manipulation of particles becomes more and more challenging as smaller particles are targeted [32,33]. This trend is general for all mentioned technologies for particle manipulation, as the forces in play strongly depend of the particle size.

In this thesis, inertial focusing is extended to allow for manipulation of particles in the single-micrometer and sub-micrometer range; a scale of interest as it comprises bacteria, viruses and eukaryotic organelles, for instance. The technology was demonstrated for focusing, concentration and separation of bacteria (*E. coli, Klebsiella, Salmonella*) in an aqueous suspension in Papers I and II. Worth of mention is that, in a very innovative approach, Mutlu et al. have also recently demonstrated focusing of said small particles in what was presented as *oscillatory inertial focusing in infinite microchannels* [34]. As an alternative, the use of viscoelastic fluids is also showing promising results for handling particles in the sub-micron range [35,36], as well as DLD microfluidics [37–39].

On the other hand, despite the outstanding performance and the promises to provide solutions where other technologies hit a dead-end, inertial focusing has reached commercial applications in only a few cases [30]. With the understanding we gained in the journey to extend the technology for sub-micron particles, we realized about what we consider the main limitation of the technology, and possibly the reason preventing it to bloom outside dedicated research laboratories. Surely focusing of initially randomly distributed particles has been demonstrated, along with their concentration, separation and fluid exchange. However, the position of the focused particles shifts in a tortuous manner as a function of every single parameter of the system (the size of the particle, the flow rate, and the width, depth and radius of the channel). To make things worse, the influence of some of the parameters is extremely strong (cubic in case of the channel width, forth power in the case of the particle size). In a research laboratory, with the system under the microscope and the possibility to adjust the flow rate on demand, the potential applications are demonstrated. However, for a device that is meant to run unsupervised, small variations in the fabrication or imprecisions/fluctuations in the pumping system lead to shifts in the focus position of the targets, resulting in diminished performances and therefore entailing a strong limitation for commercial applications.

In this thesis, a particular line of inertial focusing that overcomes this problem is presented in Paper III (inertial focusing in High Aspect Ratio Curved (HARC) microfluidics). The microchannels are engineered to re-arrange the force fields responsible for the particle migration, achieving focus in a stable,

single position for a wide range of particle sizes and flow rates. In this case, the variations in the fabrication only impact the limits of the working range and, therefore, provided the systems operate with certain margin to the limits, the devices can run unsupervised and even absorb fluctuations from the pump without diminishing their performance.

The mechanism behind the HARC systems was presented and their performance demonstrated experimentally in Paper III. However, although an upper limit of operation was identified, it could not be described analytically due to the lack of a consolidated expression for the lift force in the literature.

In Paper IV, an expression for the upper limit is experimentally obtained, completing the toolbox to fully calculate and design the HARC systems for particle focusing. Two – fairly simple – equations must be fulfilled to succeed in engineering HARC systems, and we hope that this fact, together with the stability and high-quality performance, makes inertial focusing and its benefits more accessible for researchers in other fields. Also, the upper limit of operation is strongly related to the lift force, for which an expression is proposed based on experimental evidence.

While solving a major limitation, HARC systems unfortunately also lose one of the most valuable capacities of inertial focusing. While excellent for focusing and concentrating particles, by providing a single focus position for all particles, the systems lack the capacity of particle separation.

The last contribution of this thesis, Paper V, recuperates the lost ability of the technology and presents that HARC systems not only do offer the possibility to separate particles, but they can do so with mathematically unlimited resolution. The method is explained, together with the equations that govern the systems, and demonstrated experimentally by largely separating particles with a difference in size as small as 80 nm.

2 FLUID DYNAMICS

2.1 Fluid Flow in Microfluidics

This section was developed following mainly the book *Introduction to Microfluidics*, by Patrik Tabeling [6].

Equations describing the motion of a fluid (fluid flow) can be derived from the principles of mass, momentum and energy conservation. The Cauchy equation, constitutes, together with an equation for mass conservation, the general expression to describe the motion of a fluid:

$$\boldsymbol{\rho}\left(\frac{\boldsymbol{\partial u}}{\boldsymbol{\partial t}} + \boldsymbol{u}\nabla\boldsymbol{u}\right) = -\nabla\boldsymbol{p} + \nabla\boldsymbol{\tau} + \boldsymbol{f} \tag{1}$$

where ρ and u are the density and velocity of the fluid, t is the time, p is the pressure, τ is the deviatoric stress tensor and f are possible external forces per unit of volume. Inertial acceleration terms on the left (change of velocity with time and convective term) are related to forces on the right (pressure, viscous, and external forces).

Equation 1 cannot readily be solved; information related to the viscous behavior of the fluid is to be included to define p and τ. In the common case of Newtonian fluids (those where the stress tensor is linearly related with the velocity gradient), the resulting expression receives the name of the Navier-Stokes equation. For incompressible fluids, it becomes:

$$\boldsymbol{\rho}\left(\frac{\boldsymbol{\partial u}}{\boldsymbol{\partial t}} + \boldsymbol{u}\nabla\boldsymbol{u}\right) = -\nabla\boldsymbol{p} + \boldsymbol{\mu}\nabla^{\boldsymbol{2}}\boldsymbol{u} + \boldsymbol{f} \tag{2}$$

where μ is the dynamic viscosity of the fluid.

Although these considerations may seem restrictive, the Navier-Stokes equations for incompressible fluids represent most practical cases. Exceptions are non-Newtonian fluids such as molten polymers, some polymeric solutions, or common fluids such as honey, toothpaste and, in fact, blood*.

* In the case of blood handling, a common practice in microfluidics is to dilute it in pure water to a certain factor so it behaves like a Newtonian fluid.

Usually, Eq. 2 can be further simplified for microfluidics. With inertial forces not playing a significant role compared to viscous stresses (i.e., $Re << 1$), the terms on the left side of Eq. 2 can be neglected:

$$\mathbf{0} = -\boldsymbol{\nabla p} + \boldsymbol{\mu \nabla^2 u} + \boldsymbol{f} \tag{3}$$

this new equation (Stokes equation), together with that for mass conservation ($\nabla u = 0$), explains the fluid motion in cases where this is very slow, where the viscosity of the fluid is very large, or when the length scales of the system are very small. The flow governed by these equations receives the name of Stokes flow and possesses remarkable properties, which define the characteristic behavior of most microfluidic systems. These properties result in a laminar flow with well-defined streamlines that do not mix with each other, and particles that may be suspended are dragged with the fluid as if they were yet another fluid parcel. This enables, for instance, the transport of several fluids side by side with no exchange of matter except for that coming from diffusion*.

In the systems presented in this thesis, the Re is typically 20-300. Viscous forces still dominate the system; note that it is not until Re~2000 that the flow becomes turbulent, but inertia does play a role. The regime is laminar and the systems behave similarly to those with very low Re numbers, with mainly two exceptions that will be further detailed in dedicated sections:

-Suspended particles suffer a transversal force (lift force, F_L) that makes them migrate across streamlines.

-In a curved channel or in the presence of an obstacle, the inertia of the fluid generates a secondary flow.

2.2 The Main Flow

Figure 5 shows the flow in a typical microchannel with rectangular cross section, common in microfluidics due to the simplicity in the microfabrication. The relation between the height (H) and the width (W) defines the aspect ratio ($AR = H/W$). In this thesis, $AR < 1$ is referred to as low aspect ratio, and $AR > 1$ as high aspect ratio.

* Diffusion is the result of random displacements of the molecules and particles (Brownian motion).

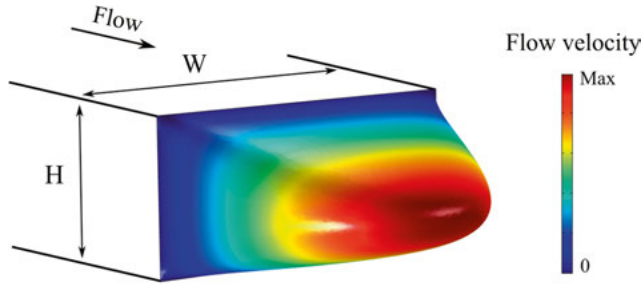

Figure 5. 3D flow in a microchannel with rectangular cross section. Reproduced from Ref. 40 with permission from the Royal Society of Chemistry.

Note that the profile of velocity is parabolic, with a maximum value at the center and zero at the walls (non-slip condition). This flow is referred to as main flow. Following the same notation as in the Papers, the velocity at any point is denoted by U and scales as:

$$\boldsymbol{U \sim \frac{Q}{WH}} \tag{4}$$

where Q is the flow rate; the amount of fluid running through the system. Often, it may be of interest to know the maximum and mean flow velocity; U_m and $\overline{U}$. In 3D flows, $U_m \approx \frac{2Q}{WH}$, and $\overline{U} = \frac{Q}{WH}$.

On the other hand, the flow rate is proportional to the pressure difference between its ends (ΔP) and its hydraulic resistance (R_H):

$$\boldsymbol{\Delta P = Q\, R_H} \tag{5}$$

with R_H depending on the viscosity of the fluid and the geometry of the channel. In channels with length L and a circular cross section with radius r:

$$\boldsymbol{R_H = \frac{8\mu L}{\pi r^4}} \tag{6}$$

In rectangular channels with $H > W$:

$$\boldsymbol{R_H \approx \frac{12\mu L}{HW^3(1 - 0.63\, {}^{W}/_{H})}} \tag{7}$$

If, on the contrary, $H < W$ like that of Fig. 5, the position of H and W must be swapped in the equation; the narrower dimension has a stronger contribution to the resistance.

In this thesis, the Reynolds number of the microchannels is defined as:

$$\boldsymbol{Re} = \frac{\boldsymbol{\rho U_m W}}{\boldsymbol{\mu}} \tag{8}$$

2.3 Inertial Focusing and the Lift Force

With inertial terms not playing a role in microfluidic systems, particles follow the fluid streamlines and do not migrate transversally within the channel. However, as inertia gains relevance, the interaction of the fluid with the particles and the channel walls results in a force field that induces a transversal migration on the particles towards certain equilibrium positions as they follow the microchannel. Such a phenomenon was first reported in 1961 by Segré & Silberberg [17] and is nowadays known as inertial focusing. Back then, initially randomly distributed particles flowing through a circular pipe were observed to accumulate in an annulus of approximately 0.6 times the radius of the pipe. This unexpected phenomenon was for the first time analytically explained in 1974 by Ho & Leal [18]. The force field in a straight system is the result of two main forces: the shear gradient-induced lift force and the wall-induced lift force. The first arises from the interaction of the fluid flow with a particle; as the particle is of finite size, it occupies a portion of the channel and induces a perturbation on the fluid flow. With a parabolic flow profile in microfluidics, this perturbation is not symmetric and results in a net transfer of momentum from the fluid to the particle; a force that pushes particles from the center of the microchannel towards the walls. On the other hand, the flow perturbation around a particle also interacts with the channel walls if these are in proximity, resulting in a repulsion from them. Figure 6 illustrates the interaction of the flow with a stationary particle and the channel walls. The force field composed by the summation of the two effects is referred to as the net lift force (F_L), for which Ho & Lean developed an analytical expression in 2D flows [18]:

$$\boldsymbol{F_L = C_L \rho U_m^2 a^4 / W^2} \tag{9}$$

where a is the particle diameter, W the width of the channel and C_L a coefficient that adjusts the value of the force according to the position of the particle in the channel and the Re number. The formula was developed for a two-dimensional flow (Poiseuille flow) with very low Re and particles much smaller than the channel ($a/W \ll 1$). Since then, the theory has been further developed for moderate [41] and high [19] Re numbers and particles with sizes in the order of magnitude of the microchannel in 3D flows [20,22,23]. Still, Eq. 9 is commonly used in the community to illustrate the phenomenon, given its relative simplicity.

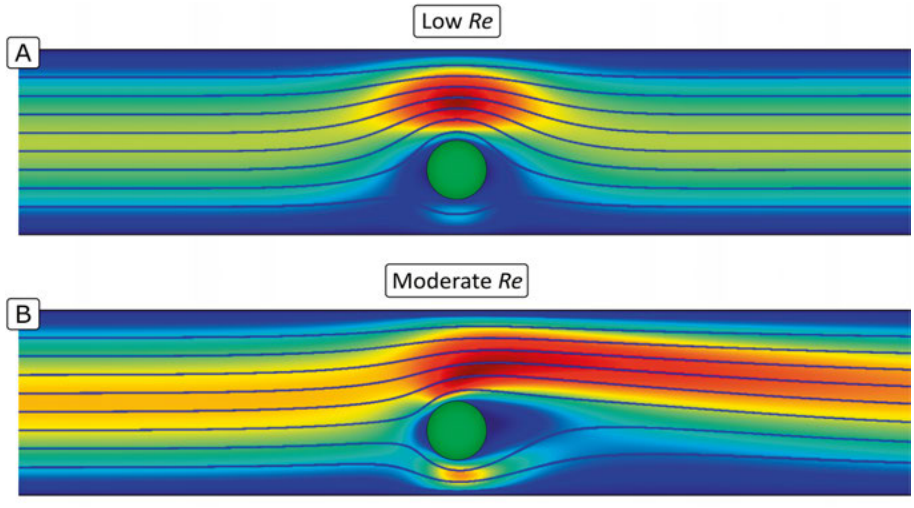

Figure 6. Perturbation of the fluid flow by a stationary particle. (A) At low Re numbers ($Re = 1$). Note the perturbation in absence of inertial contributions (B) At moderate Re numbers ($Re = 100$). Note the perturbation as inertia gains relevance.

The distribution of F_L in 3D flows results in randomly distributed particles migrating and focusing in an annulus in case of a microchannel with circular cross section, or in a discrete number of equilibrium positions located at the center of the faces in case of a microchannel with a polygonal cross section. Figure 7 illustrates the case of a circular and a rectangular cross section.

In case of non-circular cross sections, the effect of F_L in 3D flows can be broken down into two overlapping events [21,42].

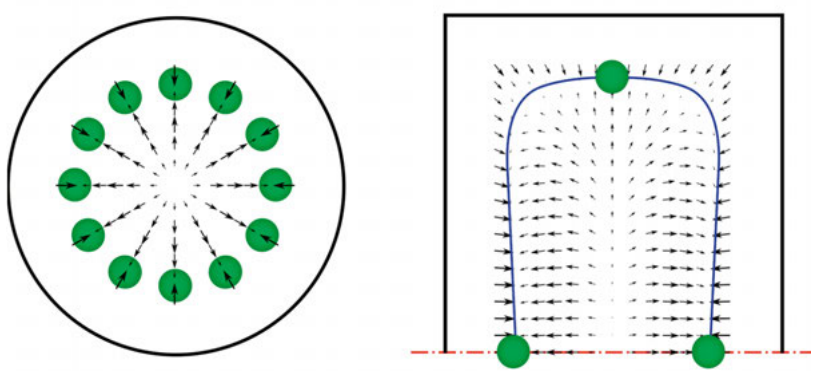

Figure 7. Lift force field in microchannels with circular and rectangular cross sections and their corresponding focus positions. The blue line represents the equilibrium perimeter, and the red dotted line a mirror symmetry line.

First, the randomly distributed particles migrate radially to an equilibrium perimeter that resembles the contour of the cross section. Then, born from the asymmetry of the system, a small component of F_L makes particles migrate tangentially to the equilibrium perimeter and gathers them at the center of the faces. These two migrations actually occur simultaneously, but the difference in magnitude of the radial and tangential component of F_L is such that the radial migration can be considered to occur first and be followed by a tangential slip over the equilibrium perimeter, as illustrated in Fig. 8. In other words, the force field of F_L in a 3D flow can be understood as the summation of a strong, radial F_L and a weak, tangential F_L.

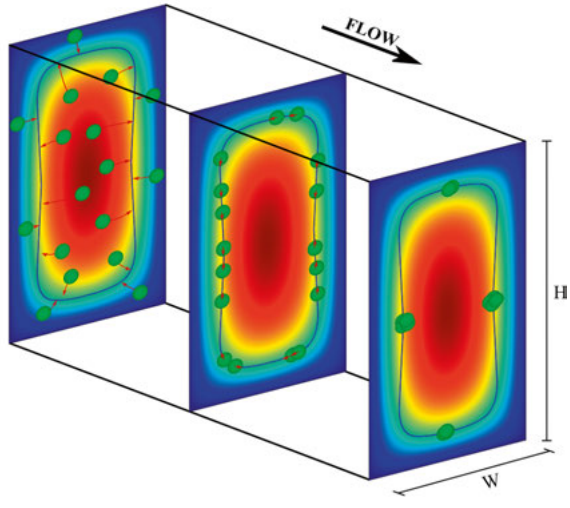

Figure 8. Particle migration by the lift force in a straight microchannel with rectangular cross section. Reproduced from Ref. 43 with permission.

There are other forces that naturally arise in microfluidic systems and induce a transversal migration to the particles, such as the Magnus force and the Saffman force. The migration is, however, dominated by the lift force, as the others are of a much smaller scale [24,44,45].

2.4 The Secondary Flow

In flows with relevant inertial terms, the addition of curvature to the microchannels induces a secondary flow, the Dean flow [46]. It is transversal to the main flow and takes the shape of two rotating vortexes that make particles circulate around the cross section, Fig. 9. The inclusion of this flow in the systems allows for more practical scenarios than those with only the lift force (straight channels). For instance, in channels with rectangular cross section and low AR, the number of equilibrium positions is reduced to two (perceived

as one under the microscope) or to one in particular circumstances [40,47–50]. In addition, the focusing may be achieved faster than in straight channels and thereby allow for shorter channels [47]. This comes from the fact that the tangential migration, with the role of gathering the particles along the equilibrium perimeter until the focus positions, is the limiting factor of the focusing in straight channels,. In curved systems, it is replaced by the sweep of the secondary flow [40].

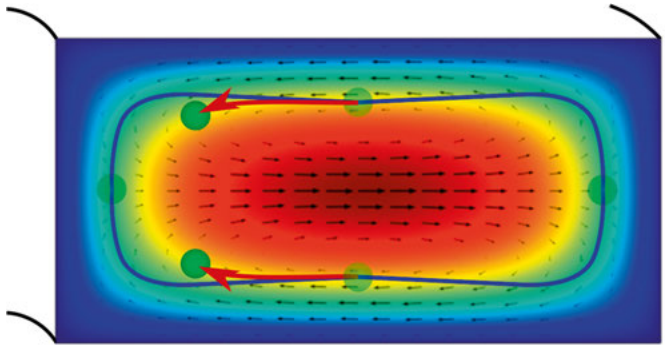

Figure 9. Secondary flow induced by the curvature of the microchannel and modified equilibrium positions. Reproduced from Ref. 43 with permission. © IOP Publishing. All rights reserved.

The velocity of the secondary flow (U_D), whose distribution strongly depends on the AR of the cross section, scales as [43,46]:

$$\boldsymbol{U_D \sim \frac{\rho}{\mu}\frac{U_m^2 W^2}{R}} \tag{10}$$

where R being the radius of curvature of the microchannel.

In practice, inertial focusing in a curved system may be understood as a superposition of a lift force similar to that of a straight channel with the same cross section (F_L, Eq. 9) and a Stokes drag by the secondary flow (F_D):

$$\boldsymbol{F_D = 3\pi\mu a U_D} \tag{11}$$

This scenario holds true for moderate secondary flows, where the main flow is around two orders of magnitude greater than the secondary flow and the main flow profile remains unchanged compared to a straight system [40]. However, as the secondary flow gains relative relevance, the main flow may be deformed and this approximation may lead to larger errors. Fortunately, the first scenario is generally the case in microfluidic systems. As an example, it is the case in all the systems presented in this thesis, except for those in some particular conditions in Paper II, with a very small radius of curvature and

high flow rates (note that the main flow grows linearly with the flow rate, while the secondary flow does so quadratically).

Given the distribution of F_L and F_D over the cross section of a 3D flow, Fig 7 and 9, and their very different scaling, Eq. 9 and 10, the force field resulting from the addition of both is strongly dependent on all the parameters defining the channel geometry, the particle size, and the flow conditions. Because of this, the equilibrium positions, where the vectors of both forces cancel each other, generally depend on the particle size and allow for a size-based particle separation. However, they also shift in a complex manner over the cross section as function of the rest of the variables [40,50], including the flow rate, as illustrated in Fig. 10, bringing complexity to designing the systems and practical limitations in their operation (diminished performances by small tolerances in the fabrication and drift of the pumping system).

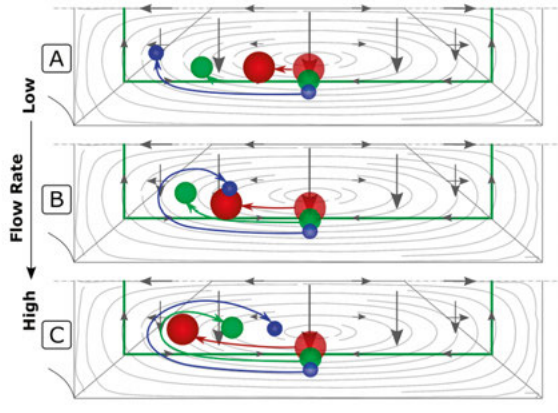

Figure 10. Evolution of the equilibrium positions in low aspect ratio curved channels as a function of particle size and flow rate. Reproduced from Ref. 40 with permission from the Royal Society of Chemistry.

2.5 The Physics of Inertial Focusing in HARC Systems

This section summarizes a particular line of inertial focusing that was introduced and developed in Papers III, IV and V, inertial focusing in High Aspect Ratio Curved (HARC) systems. It combines the physics explained in previous sections (main flow, secondary flow and lift force), with the noteworthy feature that, in this case, the force field resulting from of F_L and F_D provides a single, stable focus position, fairly insensitive to moderate changes in the flow conditions, channel geometry and particle size. As a result, the systems are simpler to design and overcome the practical limitations aforementioned – at least for focusing and concentrating a range of particles together. In the case of particle separation, HARC systems may achieve this with a mathematically unlimited resolution, and demonstrated down to tens of nanometers in Paper V. However, in this case, they suffer from the same limitations as other inertial focusing systems: the need for extremely precise fabrication and equipment for the operation. Figure 11 shows the force fields of F_L and F_D, and the combination of both in HARC systems.

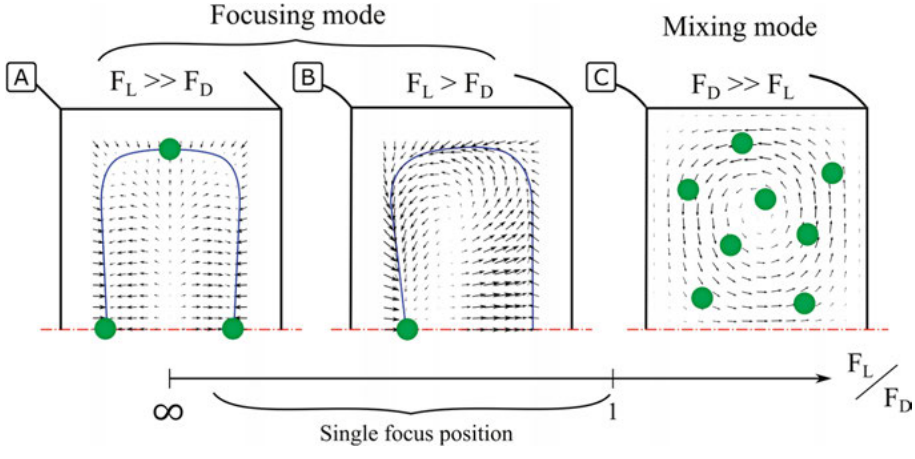

Figure 11. Generic force fields resulting from the summation of the lift force and the secondary flow in HARC systems. (A) The lift force dominates, similar scenario as a straight channel. (B) The adequate proportion of forces induces a single, stable equilibrium position. (C) The secondary flow dominates, particles remained randomly distributed.

Figure 11A illustrates a system dominated by F_L, with a performance like that of a straight channel. Figure 11C illustrates a system dominated by F_D, which cannot perform focusing nor separation. There is, however, an interesting regime resulting as a combination of both in the right proportion, that focuses particles at a single, stable position at the symmetry line, Fig. 11B. The existence of this regime is introduced, demonstrated and characterized in Papers III and IV. An example of performance is shown in Fig. 12. Note that the system has a lower and an upper limit of flow rate in the operation.

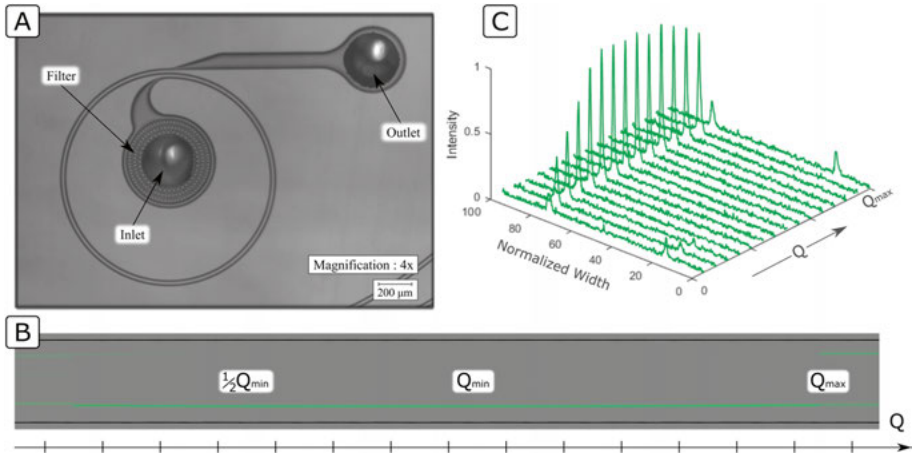

Figure 12. (A) HARC system and (B) its performance under the microscope with 1 µm fluorescent microparticles. (C) Intensity analysis of the performance.

To succeed in focusing with HARC systems, two conditions must be met:

1- The channel must be sufficiently long for all particles to reach the focus position. Mathematically, this is expressed as:

$$\boldsymbol{N_L \geq \frac{20AR^2}{Re}} \tag{12}$$

where N_L is the length of the microchannel expressed as a number of loops. For a fixed device, this sets a lower limit of operation ($\frac{1}{2}Q_{min}$).

2- F_L should be sufficiently strong to act as a barrier (Lift Barrier) and prevent particles from crossing the central part of the channel when following the secondary flow. Mathematically, this is expressed as a ratio of between F_L and F_D at such region:

$$\boldsymbol{\frac{J}{3\pi C_{ROI}}\frac{a^3R}{U_m W^5} > 1} \tag{13}$$

where $J = 3.6\,\pi\,10^{-6}\frac{m^2}{s}$ and $C_{ROI} = (6.55 - 1.87AR)\,10^{-3}$. This condition sets an upper limit of operation (Q_{max}).

HARC systems may also perform particle separation, as presented in Paper V. To achieve this, it is a pre-requisite that particles are initially focused by the inner wall, for which a HARC microchannel as described so far may be used, or a sheath flow. Once in such position, the Lift Barrier can be tuned to allow particles below a threshold to cross over to the outer wall, achieving a binary separation – larger than the threshold by the inner wall, smaller by the outer

wall. To allow a particle coming from a HARC focusing section surpass the Lift Barrier, the conditions in the microchannel must be changed so that Eq. 13 is no longer fulfilled. Many variables can induce such a change, with a modification in R or W being the most convenient. As an example, Fig. 13 shows the performance of a system consisting of two loops where the Lift Barrier is strong enough to focus 0.79, 0.92 and 1.0 µm particles together, followed by an extra loop where the Lift Barrier is weakened in relation to the secondary flow (by increasing W by 0.5 µm), allowing the smaller sizes to reach the outer wall. Note how the threshold depends on the flow rate, Fig. 13 B-C.

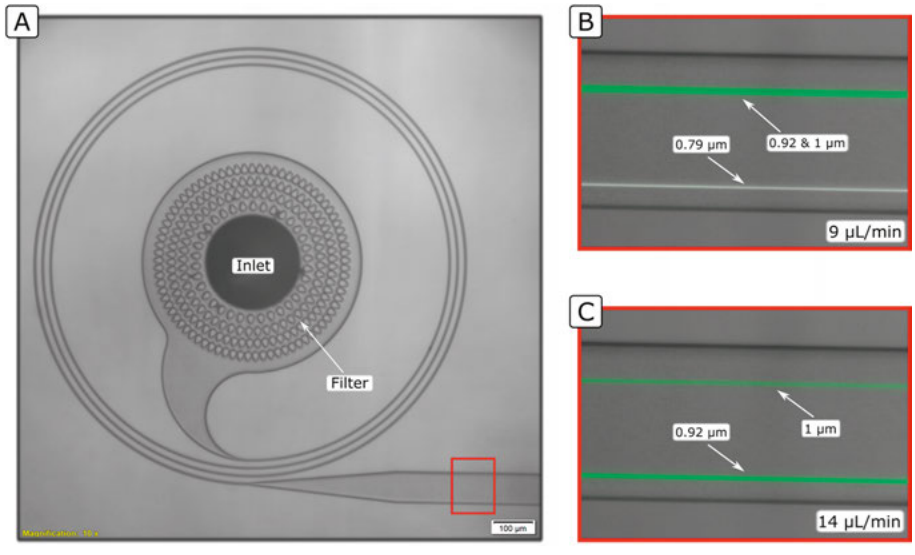

Figure 13. (A) HARC system consisting of a focusing section (first two loops) followed by a separation section (last loop, 0.5 µm wider). (B-C) Performance under the microscope with 0.79, 0.92 and 1.0 µm fluorescent polystyrene microparticles.

Further than that, when particles surpass the Lift Barrier, this acts as a size-dependent lag, which results in particles spreading in a rainbow of sizes while the migration towards the outer wall takes place. This situation differs from those previously presented in that particles are not in equilibrium but moving, and this movement is in fact a fairly complex function of all the parameters of the system. Therefore, a very precise fabrication and control of the flow are, once again, necessary to achieve the desired performances. The displacement of the particles is governed by:

$$\boldsymbol{dx = \frac{U_D}{U} R d\theta \left(1 + \frac{U_{FL}}{U_D}\right)} \qquad (14)$$

where the position along the symmetry line (x), is related to the angle rotated in the curved channel (θ). The first term corresponds to the trajectory of a fluid

molecule, and the second to the lag induced by F_L. Expressions for U, U_{FL}, and U_{FL} are provided in Paper V.

A relevant fact that I would like to capture and highlight is that Eq. 14 was achieved with important simplifying assumptions, yet proven to be remarkably accurate. In the model, freely suspended particles were assumed to move with the same velocity as the fluid (U in the axial direction, U_D in the transversal direction). Note that this is a fair assumption for very low Re numbers, but does not necessarily apply as inertia gains relevance. Then, expressions for U and U_D were obtained by simulating the fluid flow by COMSOL Multiphysics, solving Navier-Stokes for water.

Finally, if a particle is not freely suspended but has a force acting on it, this induces an extra component in the transversal velocity. In this case, F_L was assumed to induce a transversal migration $U_{FL} = F_L/(3\pi\mu a)$. The velocity of particles can thus be taken as U in the axial direction, $U_D + U_{FL}$ in the transversal direction. With the velocity of particles defined, an expression describing their trajectories, Eq. 14, can be developed. As an example, Fig. 14 shows the calculated and experimental trajectories for 0.79, 0.92 and 1.0 μm particles obtained in a system where the secondary flow was strengthened relatively to the Lift Barrier by decreasing R in the separation section.

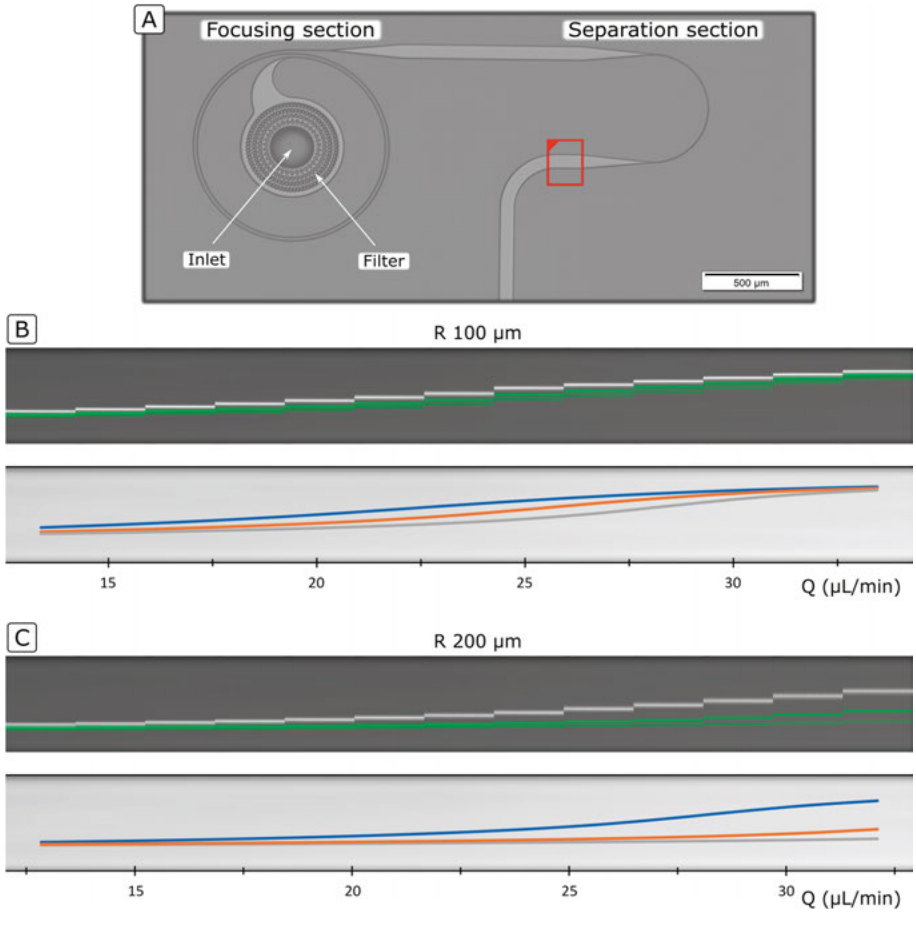

Figure 14. (A) HARC system consisting of a focusing section (two loops) followed by a separation section (half loop, smaller *R*). (B-C) Experimental trajectories (on top) and calculated ones (bottom) in different separation sections for 0.79, 0.92 and 1.0 µm particles.

3 SUMMARY OF PAPERS

Paper I. High pressure inertial focusing for concentrating and separating bacteria at high throughput

This paper extended the use of inertial focusing to particles in the single-micron size; range of bacteria and eukaryotic organelles such as nuclei or mitochondria.

The main forces responsible for particle focusing in curved channels were studied with the intention of finding the theoretical necessary conditions for a system to succeed in the focusing of particles in said range. Alternatively to calculating the systems from scratch, we proposed a table with scaling factors that allow for a transformation while maintaining the balance of F_L and F_D. With it, a system that works in certain particle range can be transformed to work in the desired range.

Table 1. Factors to scale a system and its working range.

Scaling Relations	Particle size	Height	Width	Flow rate	Focus length	Average speed	ΔP
Scale factor	X	X	X	X	X	X^{-1}	X^{-2}

As a conclusion from the table, it was predicted that focusing of 1 μm particles was feasible, but it would come at a cost of high pressures (tens of bar) to run the systems. Common fabrication techniques for microfluidics like soft replication were thus excluded, and we implemented instead a microfabrication technology on glass, reported to withstand pressures above 200 bar [51]. The microchannels were wet etched and consisted of a shallow, functional section (spiral, 10 μm) and a deep section (~200 μm) for the insertion of glass capillaries that served as fluidic connections to an HPLC pump.

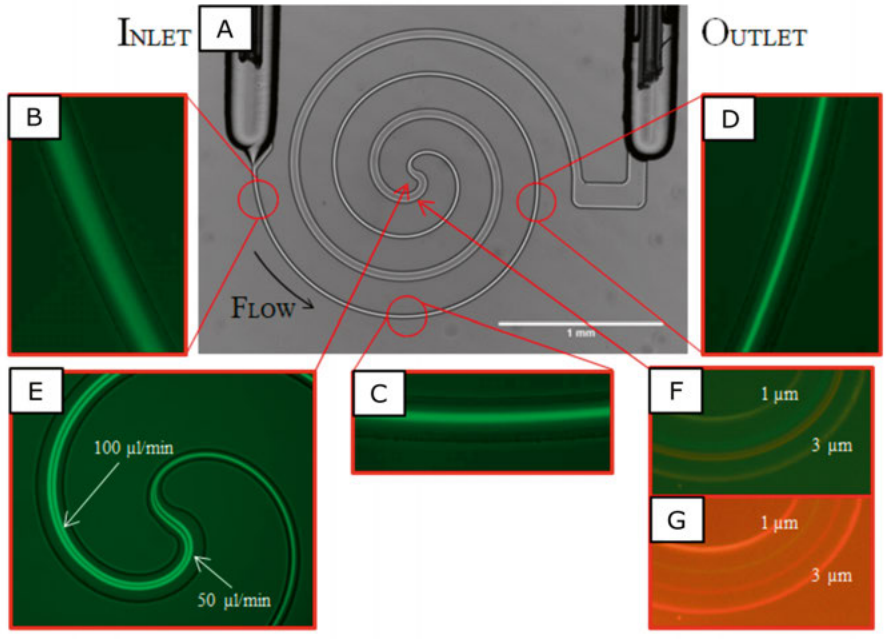

Figure 15. (A) Glass system under the microscope. (B-D) Performance with a suspension of *E. coli* at 100 µL/min at (B) the inlet (C) after ¼ loop (D) after ½ loop. (E) Focus position of *E. coli* at 50 and 100 µL/min. (F-G) Performance with 1 and 3 µm fluorescent polystyrene particles at (F) 100 µL/min and (G) 200 µL/min. Reproduced from Ref. 33 with permission.

The experimental tests showed positive results, and focusing of 1 µm particles and *Escherichia. coli* was demonstrated, Fig. 15.

Post Scriptum

In this paper, the radius of the microchannel (R) was not included in the scaling table as it was considered to play a role in the focusing position but not in the successfulness of the task. That may be the case for low aspect ratio curved systems, but for those with high aspect ratio that were developed later, R plays a critical role and should be scaled linearly with the targeted size.

Paper II. Inertial focusing with sub-micron resolution for separation of bacteria

In this work, the systems presented in Paper I were further developed:

-The pressure demand was reduced, allowing for focusing of particles down to 0.5 µm and particle separation with sub-micron resolution, as it was demonstrated experimentally.

-The technology was proven feasible for three bacteria species (*Escherichia coli, Salmonella typhimurium* and *Klebsiella pneumoniae*), with all species keeping their viability after the process.

Apart from an improved performance of the systems, new insights in relation to the process of particle focusing in low aspect ratio microchannels were presented based on experimental observations and simulations by COMSOL Multiphysics.

The systems were in this case fabricated on silicon-glass. The motivation to change from wet etched glass to dry etched silicon was to achieve well defined corners and a better control of the width. An example of such system can be seen in Fig. 16. The chips consisted of an initial straight segment, which gave particles a push towards the equilibrium perimeter, followed by a curved section that swept all particles to the focus position. Said curved section was limited to ~3/4 loop to avoid spiraling inwards, which would hinder the inclusion of multiple outlets in the system. If longer microchannels are required, multiple curves with a slight increase in R can be connected by a small turn, as in Fig. 16.

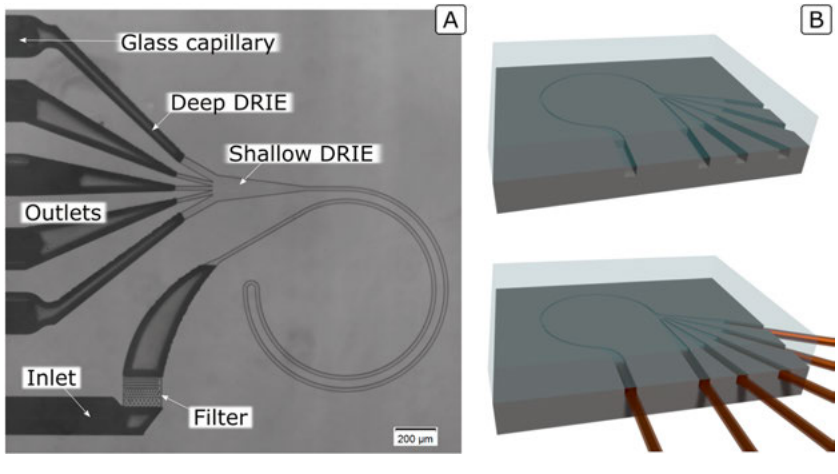

Figure 16. (A) Silicon-glass system under the microscope. (B) Sketch of the system and the connections in 3D. Reproduced from Ref. 40 with permission from the Royal Society of Chemistry.

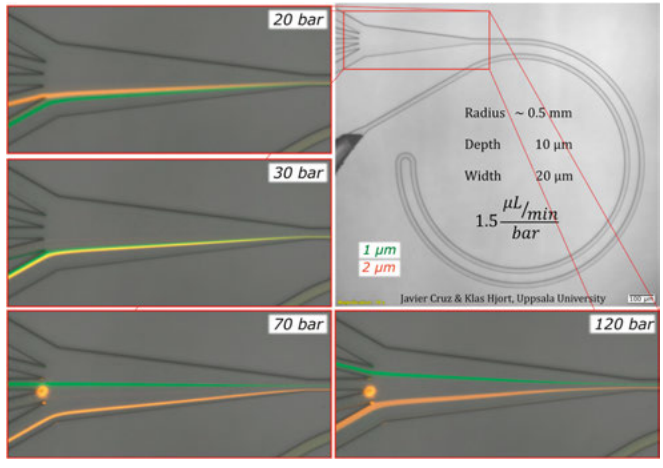

Figure 17. Focus positions of 1 and 2 µm fluorescent polystyrene microparticles as the pressure/flow rate increases in a microfluidic chip built in silicon-glass. Reproduced from Ref. 40 with permission from the Royal Society of Chemistry.

Figure 17 shows the performance of a silicon-glass system with 1 and 2 µm particles at different flow rates/pressures. The connection of multiple curved sections not only allowed for longer microchannels but also for the observation of the trajectories of pre-focused particles towards a new equilibrium position. Note that particles focused in one section may find themselves far away from the equilibrium position in the new one. In their journey towards the new focus position, particles were observed (top view) to approach it and leave it behind (!), until close to the inner wall they changed their migration direction towards the outer wall, this time to reach the equilibrium position and remain in it. These observations revealed information about the location of the focus position and the route that particles need to follow to reach it, providing new valuable insights about the process, see Fig. 18.

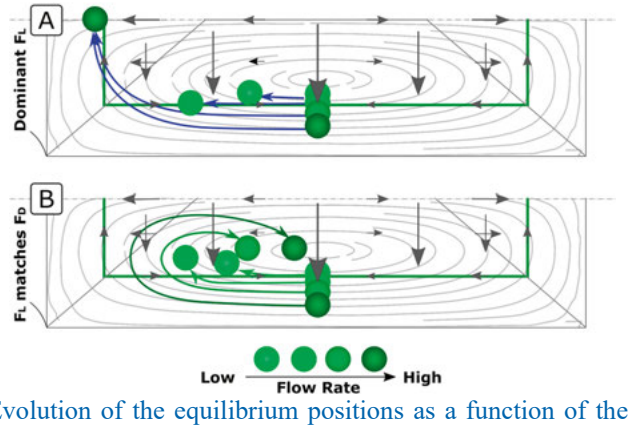

Figure 18. Evolution of the equilibrium positions as a function of the flow rate in rectangular low aspect ratio curved microchannels. Reproduced from Ref. 40 with permission from the Royal Society of Chemistry.

Paper III. Stable 3D inertial focusing by High Aspect Ratio Curved microfluidics

Stemming from the gained understanding in Paper I and II and the awareness of the limitation coming from the shifting focus positions, other microchannel configurations were studied. Paper III presents inertial focusing in High Aspect Ratio Curved (HARC) microchannels, systems where the resulting force field leads to a stable equilibrium position, common for a range of particle sizes and largely invariant with the flow rate and moderate variations in channel geometry, thereby overcoming the aforementioned limitation. Figure 19 shows a HARC system and its performance for a given particle size.

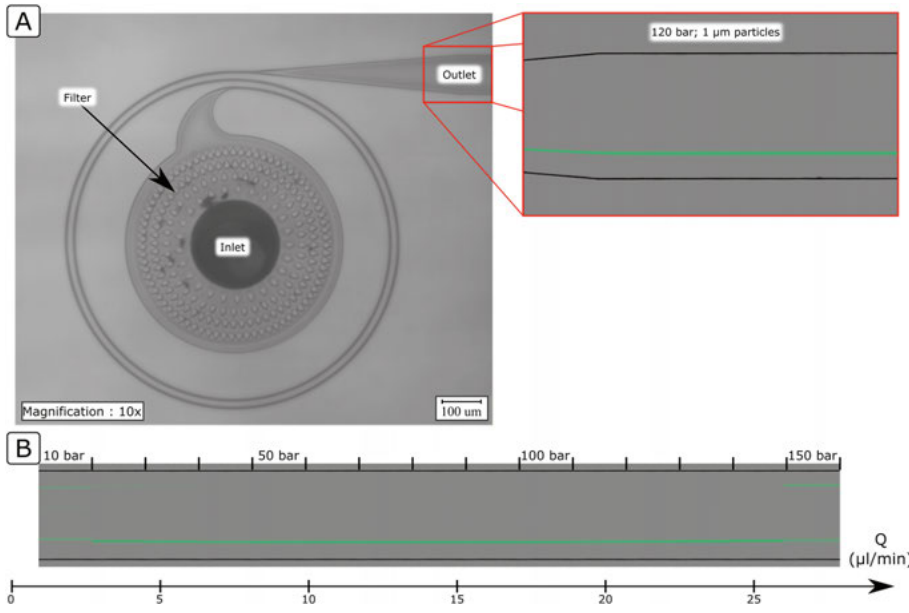

Figure 19. (A) View of a HARC system under the microscope and (B) its performance with 1 µm polystyrene fluorescent micoparticles in a range of flow rate. Reproduced from Ref. 43 with permission. © IOP Publishing. All rights reserved.

Similarly to previously presented technology for inertial focusing, HARC systems consist of a curved section where the focusing is achieved by a combination of the lift force and the secondary flow. However, the re-arrangement of the secondary flow in curved systems with rectangular cross sections and high aspect ratio results in a very different performance. The working mechanism is illustrated in Fig. 20. In essence, particles follow the secondary orbits until the inner wall, where the lift force opposes the migration and acts as a barrier (Lift Barrier). Provided that the Lift Barrier is strong enough, the particles cannot cross over to the outer wall and are focused at a single position by the symmetry line.

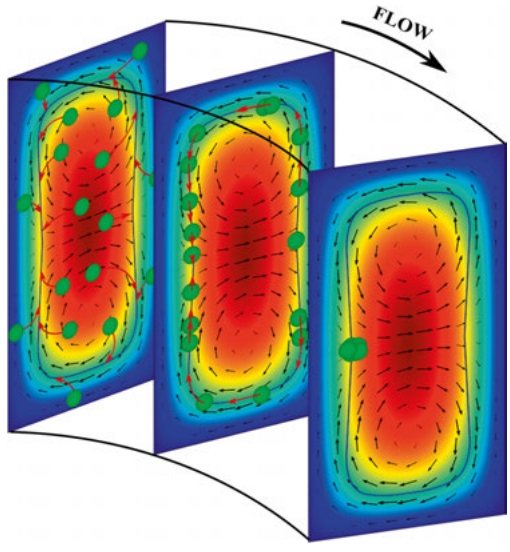

Figure 20. Inertial focusing mechanism in a HARC system. Reproduced from Ref. 43 with permission. © IOP Publishing. All rights reserved.

The success in focusing particles in HARC systems is defined by two conditions:

-Sufficient channel length (or flow rate). The system should be long enough for the initially randomly distributed particles to reach the focus position. Alternatively, for a given channel length, the flow rate should be sufficiently high. Note how, in Fig. 19, particles are not well focused for flow rates below ~7 µL/min. An expression to fulfil this condition is proposed:

$$\boldsymbol{Q \geq \frac{0.6AR^3W}{N_L}\frac{\mu L/min}{\mu m}} \tag{15}$$

-Sufficient strength of the Lift Barrier. The barrier is to be strong enough to retain particles at the inner wall against the drag of secondary flow. With the secondary flow growing faster with the flow rate than the lift force, there is an upper limit in Q where particles breach the Lift Barrier; see Fig. 19 over ~27 µL/min. An equation expressing this condition:

$$\boldsymbol{Q \leq ?} \tag{16}$$

may be obtained by solving the equation for $F_L \geq F_D$ at the focus region. However, different studies point at different conclusions regarding F_L and this question could not be solved in Paper III. Answering this question is the scope of Paper IV.

Paper IV. Fundamentals of inertial focusing in High Aspect Ratio Curved microfluidics

Inertial focusing in High Aspect Ratio Curved (HARC) microfluidics was shown to be a promising line of inertial focusing. However, the technology presents an upper limit of operation (Q_{max}) above which the systems lose the focusing capabilities, see Fig. 21 over ~27 µL/min, which remained analytically undefined. It is within the scope of Paper IV to solve this question and make inertial focusing and its benefits easily accessible to the community.

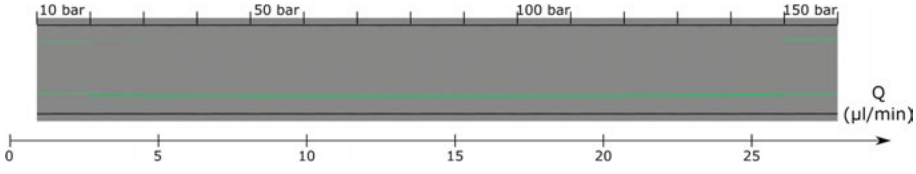

Figure 21. Typical focusing performance of a HARC system. Reproduced from Ref. 43 with permission. © IOP Publishing. All rights reserved.

The limit was first investigated experimentally. An example of the gathered data is shown in Fig. 22, where Q_{max} was mapped for different particle sizes, channel geometries and flow conditions.

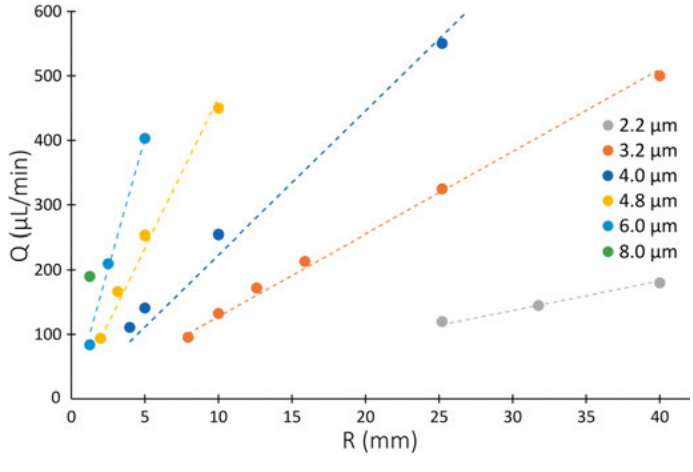

Figure 22. Mapping of Q_{max} in a HARC system (two loops, 41 x 84 µm (WxH)) with different particle sizes.

Complementing the experiments with a study of the strength of the secondary flow by COMSOL Multiphysics, an analytical expression for the limit was found:

$$\boldsymbol{Q_{max} = L\, k^3 R} \qquad (17)$$

where L is a coefficient that depends strongly on the AR of the microchannel and $k = a/W$.

With this work, together with the lower limit defined in Paper III, all necessary pieces to design HARC focusing systems were achieved. With the simplicity of design and the stability of the focus, we strongly believe that HARC systems may mean a step forward for inertial focusing to be widely implemented outside research laboratories.

Apart from completing the description of the HARC systems, the study in Paper IV revealed valuable information related to the lift force. The experimental conditions of Q_{max} were used to derive an expression for F_L at $W/3$ from the channel walls ($F_{L,ROI}$):

$$\boldsymbol{F_{L,ROI} \sim J\,\rho\,\frac{U_m a^4}{W^3}} \tag{18}$$

where $J = 3.6\,\pi\,10^{-6}\,\frac{m^2}{s}$ was introduced as the Lift Barrier constant.

Equation 18 not only expresses an experimentally measured strength of the lift force at a particular region of interest, but also reveals the scaling of F_L with no coefficient that hinders the calculations (as C_L does in other proposed formulas).

Paper V. High-resolution particle separation by inertial focusing in HARC microfluidics

Paper V extends the possibilities of inertial focusing in HARC systems, a technology developed in Paper III and IV. As presented, HARC systems provide the means for focusing initially randomly distributed particles in a single, stable position. While this facilitates engineering the systems for focusing and concentration of particles, it also implies the loss of separation capacity, one of the key features of inertial focusing. In this work, we present a method where HARC systems recuperate this lost feature. In fact, HARC systems not only do have the capacity to perform particle separation but can do so with a very high and modulable resolution (mathematically unlimited, demonstrated down to 80 nm).

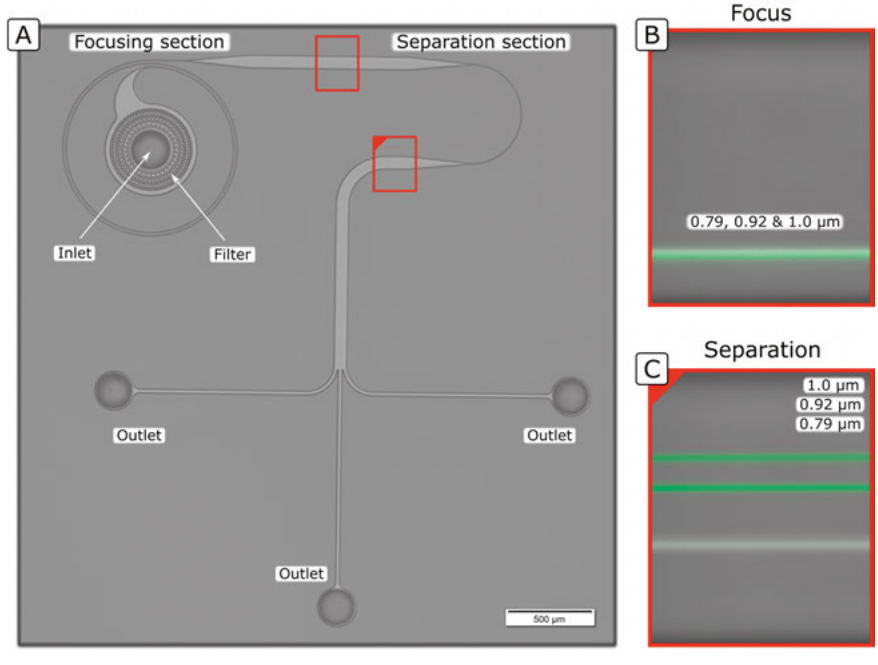

Figure 23. (A) HARC system under the microscope (B) Focus position of microparticles after the focusing section. (C) Particle separation achieved in the separation section. (Particle sizes 0.79, 0.92 and 1.0 µm and flow rate 32 µL/min).

The method exploits the concept of Lift Barrier introduced in Paper III and IV. An example can be seen in Fig. 23. First, initially randomly distributed particles can be focused together by inducing a sufficiently strong Lift Barrier in a focusing section, Fig. 23B. Once focused, the Lift Barrier may be weakened relatively to the secondary flow in a separation section to selectively al-

low particles to continue their journey towards the outer wall, thereby achieving a size-based separation of particles, Fig. 23C. A sketch of these processes is shown in Fig. 24.

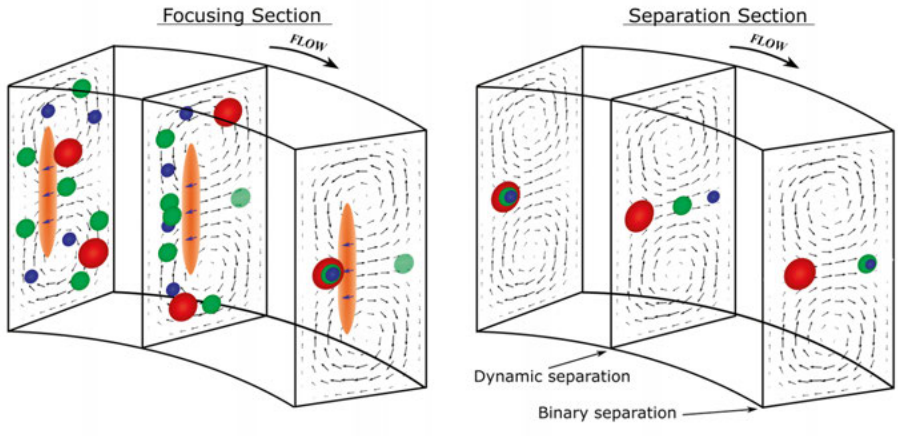

Figure 24. Mechanism behind the focusing and separation sections in HARC systems.

Interestingly, when particles surpass the Lift Barrier, F_L induces a size-dependent lag that results in particles spreading in a rainbow of sizes while the journey to the outer wall takes place. The phenomenon is fully disclosed in Paper V, including an equation governing the trajectories of particles that enables their prediction and the design of the systems:

$$dx = CU_m W^2 \frac{\rho}{\mu} \frac{f_{U_D}(x)}{f_U(x)} \left(1 + \frac{J}{3\pi} \frac{a^3 R}{CU_m W^5} \frac{f_{FL}(x)}{f_{U_D}(x)} \right) d\theta \qquad (19)$$

The model captures the phenomenon remarkably well, including when particles do or do not surpass the Lift Barrier and their trajectories thereafter. Figure 25 shows an example of the experimental results and the calculated trajectories for 0.79, 0.92 and 1.0 µm particles in different separation sections. The separation between the trajectories (resolution) can be modulated with the ratio F_L/F_D; see the different separation achieved in the trajectories shown in Fig. 25, for instance. In principle, one can amplify the separation with no physical limit. However, the resolution goes hand by hand with the pressure drop and, in the scale of the systems presented here, it quickly reaches the maximum tolerated by the devices (200 bar), setting a practical limit for the designs. In addition, achieving this resolution demands very precise fabrication tolerances and stability of the pumping system, which set another practical limitation.

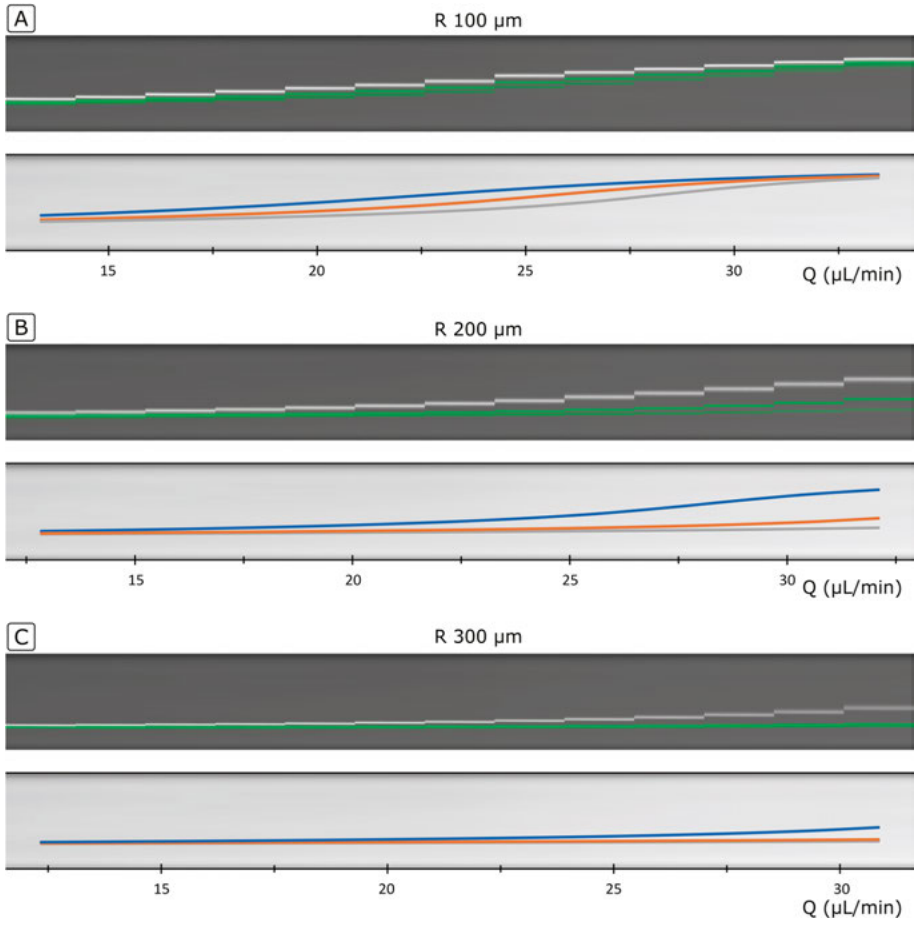

Figure 25. Experimental and analytically calculated microparticle trajectories after different separation sections (top and bottom, respectively).

4 FINAL REMARKS

4.1 Conclusions

Inertial focusing is a very promising technology for processing complex fluid samples prior to analyses; rare targets of interest may be focused, concentrated, separated and changed of medium, facilitating their posterior study.

The technology was successfully extended for manipulation of sub-micron particles, a range of biological relevance since it comprises multitude of bioparticles, such as bacteria, archaea, viruses and organelles of eukaryotic cells.

The systems must be carefully tailored to the range of particles of interest. The smaller the target, the smaller channels are needed and, with them, the higher pressure is demanded in the operation. In the range of sub-micron particles, robust systems are needed as tens to hundreds of bars are required in the operation.

Systems microfabricated in silicon and glass handle safely up to 200 bar and were shown to allow for focusing down to 0.5 μm particles.

The technology was experimentally validated for inert particles and for bioparticles, namely the three bacteria species *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae,* with these being unharmed after the process.

The behavior of bacterial cells in the system was in some cases analogous to that of inert, spherical particles. More specifically, *Escherichia coli* and *Salmonella typhimurium*, despite being rod-shaped bacteria, behaved like 0.95 and 0.9 μm spherical polystyrene particles, respectively. Polymeric particles may therefore allow for initial technological validations of systems, later intended for manipulation of bioparticles, in environments without biosafety restrictions.

The field is nowadays maturing but still growing rapidly. In this thesis, a new line of inertial focusing system is proposed: inertial focusing in High Aspect

Ratio Curved (HARC) systems, overcoming what we consider a major limitation of the technology: the tortuous shift of focus position as a function of multiple parameters, such as the particle size, flow rate and channel geometry.

HARC systems provide the means to focus a range of particles together in a stable, single position. The stability makes the systems easier to predict and reduces the diminished performances from possible fluctuations in the pumping system or small errors in fabrication, thereby making inertial focusing a robust technology for tasks involving focusing, concentration and fluid exchange of particles.

HARC systems also provide the means for a size-based particle separation with, in principle, mathematically unlimited resolution. This capacity was experimentally demonstrated by separating particles with a size difference down to 80 nm, which has the potential for separation of bacteria by species or by size within a same population. Alas, in case of particle separation with high resolution, the fabrication and operation of the HARC systems must be finely controlled.

Perhaps more important than the performance, the systems are stable, intuitive and simpler to design − attributes that we hope will make the technology and its outstanding benefits more accessible to a wider community.

With the remarkable performance of the technology and the possibilities enabled by such a high capacity of particle manipulation, it would not come as a surprise if, in the near future, inertial focusing makes a strong impact on how analyses are performed nowadays and opens up for possibilities beyond the current state of the art.

4.2 Limitations and Future Perspectives

The advances proposed in this thesis mean a small step forward for the technology of inertial focusing, yet I believe the phenomeno has much more potential waiting to be discovered.

Manipulation of sub-micron particles (down to 0.5 μm) and the possibility to resolve them based on fine size differences (down to 80 nm) was demonstrated, which opens the technology for a variety of applications with bioparticles such as eukaryotic organelles or bacteria. However, further development is to be done to extend the technology for smaller particles. Particles like viruses (with sizes of few hundreds of nanometers) or exosomes (~100 nm) still remain out of reach for the technology nowadays.

The means to fabricate the systems with the adequate dimensions for the manipulation of particles in the nanometer range are well within today's microfabrication capacities. The limitation to reach the nano-range comes, however, from the high pressure demanded to run the systems, which scales quadratically with the targeted particle [33]. As an example, if nowadays the technology allows for focusing of 0.5 μm particles at a cost of 100 bar, focusing 0.1 μm particles, would come at a cost of 25 times higher pressure; i.e., 2500 bar; a pressure that is far beyond the limit of the silicon-glass technology presented in this thesis.

A future perspective to extend the use of the technology towards the nanometer range may be the development of microsystems built in different materials, such as stainless steel. Alternatively, the pressure demand may be alleviated at a cost of sacrificing through-put by using a multi-phase flow. Even with these two options, the task entails a challenge of uncertain outcome.

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