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Development of a Microfluidic Device Coupled to Microdialysis Sampling for the Pre-concentration of Cytokines

Randy Francisco Espinal Cabrera
University of Arkansas, Fayetteville

DEVELOPMENT OF A MICROFLUIDIC DEVICE COUPLED TO MICRODIALYSIS SAMPLING FOR THE PRE-CONCENTRATION OF CYTOKINES

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By

Randy Francisco Espinal Cabrera
Autonomous University of Santo Domingo
Bachelor of Science in Chemistry, 2004

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University of Arkansas

ABSTRACT

A proof-of-concept microfluidic device combined with heparin-immobilized magnetic beads was created to concentrate cytokine proteins collected from microdialysis samples. Cytokines are known to be related to several diseases such as cancer, and Parkinson's diseases, so to be able to develop more effective diseases treatments their interactions have to be well understood. Amine-functionalized polystyrene and carboxyl-functionalized magnetic microspheres of ~6.0 µm in diameter were used to immobilize heparin. The amount of heparin immobilized on polystyrene beads was $5.82 \times 10^{-8} \pm 0.36 \times 10^{-8}$ M per 1.0×10^{6} beads and for magnetic beads was $0.64 \times 10^{-8} \pm 0.01 \times 10^{-8}$M per 1.0×10^{6} beads. The minimum initial heparin concentration needed to bind ~ 100% cytokines was 36.8 µM based on estimations for a fixed initial concentration (1.0 nM) of cytokines. For polystyrene beads, it was found that 0.1 and 1.0 nM ratCCL2 (MCP-1) bound to immobilized heparin at levels of 94.50 and 83.67%, respectively. For heparin immobilized magnetic beads, experimental percentages of cytokine bound to heparin were 70.38 ± 1.71 % (ratCCL2, 0.57 nM) and 11.07 % (ratTNF-α, 0.09 nM). The differences between experimental and estimated percentages of cytokine bound to heparin were 28.31 and 31.56% for ratCCL2 and ratTNF-α. A microfluidic system was designed and made of polydimethylsiloxane (PDMS) with soft lithography. The dimensions were as follows: a) Inlet channel width of 0.1 mm, b) circular trapping area of 3.6 mm in diameter, and c) outlet channel width of 0.2 mm. The equivalent circuit theory was used to estimate the pressure drop for each channel at a flow rate of 1.0 µL/min. Estimated Reynolds numbers for each channel were low (0.17, 0.01, and 0.11) in agreement with the theory. Estimated pressure drops were 112.2, 0.20, and 30.28 Pa. Using different flow rates, the infusion of magnetic microspheres into the device and their "spreading" behavior within circular channel was observed and quantified. "Spreading" behavior of magnetic microspheres on a circular channel could be controlled by changing their flow rate. Controlling the behavior of magnetic microspheres is very crucial for pre-concentration of cytokine proteins on bead-based microfluidic devices. This microfluidic device is now ready for testing of the trapping and preconcentration of cytokines in real microdialysis samples.

This thesis is approved for
recommendation to the
Graduate Council

Thesis Director:

_____

Julie A. Stenken, Ph.D.

Thesis Committee:

_____

Ingrid Fritsch, Ph.D.

_____

Christa Hestekin, Ph.D.

_____

Colin Heyes, Ph.D.

_____

David Paul, Ph.D.

_____

Steve Tung, Ph.D.

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1 CHAPTER 1. INTRODUCTION: Background and Significance

1.1 Cytokine

1.1.1 Overview and importance in biomedicine

Cytokines are vitally important signaling proteins generated during an immunological response. Whicher and Evans stated that "cytokines are peptides used by immune and inflammatory cells to communicate with each other and control the *milleu interieur* in which they operate".[1] Their molecular weight ranges between 8 to 80 kDa.[11] Cytokines or "chemical messengers" are released "by white blood cells and several other cell types in the body".[12,13] Several families of cytokines are released during an immune response.[13] These proteins are often dysregulated in diseases such as diabetes,[2] cancer,[1,3] and Parkinson's disease.[4] These cytokines act coordinately as a network.[13] As a result, a multiplexed analysis rather than one cytokine at a time analysis is necessary for determining the level of the network. There is an enormous interest in monitoring the concentration changes of cytokines in living systems during some disease stages.

Typically, cytokines are collected from blood and tissue samples. The problem with sampling cytokines from blood and tissue is that obtaining *in situ* and in real-time concentration changes of cytokines directly within the tissue is not possible. In contrast, microdialysis sampling methods can be used along with other techniques to measure and monitor cytokines concentration changes *in situ* and in real-time, see section 1.2. Cytokines are normally present in low concentrations (picomolar or femtomolar range).[6,7,8] According to Ao and Stenken most cytokines have a high biological activity and rapid concentration changes within living systems.[5] Therefore, quantitation of cytokine proteins is very challenging and requires highly sensitive detection methods such as antibody-based enzyme linked immunoassay (ELISA).

1.1.2 Cytokines detection methods or assays

Several research groups have worked on the development of detection methods to improve the quantitation of cytokines in living systems. Whiteside wrote a helpful review article about cytokines assays.[10] In this article she pointed out that immunoassays for cytokines such as ELISA are commonly

used because of “their acceptable specificity, sensitivity, rapid turnaround time, convenience, ease of performance, and a relatively low cost”.[10] Also, she classified the available methods for cytokines assessment in body fluids, cells, and tissues.[10] She mentioned that immunocytochemistry, immunofluorescence, and mRNA-based assays are the methods available for the detection of cytokines in tissues.[10] On the other hand, according to Remick, et al., the basic ELISA protocol takes approximately 19 hours to get the results.[9] This is one of the main issues of using ELISA to quantify biomolecules or cytokines along with the sample size requirement of at least 100 µL and the labor intensity or tediousness of the process.

Pre-concentration of biomolecules or proteins is widely used by many research groups and industrial processes.[14-19] As mentioned above, proteins such as cytokines can be present in picomolar to femtomolar concentrations. The problem is that commonly used analytical instruments or methods for protein quantitation such as ELISA can only measure down to 31.25 pg/mL or 2.39 pM (MCP-1, for example) (BD bioscience ELISA’s kit) without making any changes to the standard protocol.[20] Also, commonly used analytical methods for protein quantitation require mimumum volumes of at least 50 µL or 100 µL.[20] That is why it is very important to develop a method or system coupled to a sampling technique to be able to pre-concentrate cytokines and measure them using smaller sample volumes (1 µL) than the ELISA methods. In addition, the fact that some cytokines are present in low pg/mL concentrations and sampling techniques such as microdialysis has low extraction efficiencies when sampling cytokines represents a challenge for the development of the sampling system.

Monitoring concentration changes of cytokines over time is crucial to study the immune-related interactions of cytokines within living systems during disease states. Therefore, it is necessary to use minimally invasive sampling methods such as microdialysis in order to track the concentration changes over time. Finally, it is very important to understand the diffusive limitations of microdialysis sampling in order to overcome the challenges associated with monitoring concentration changes of cytokines over time.

1.2 Microdialysis Sampling

Microdialysis sampling is a minimally-invasive diffusion-based technique commonly used to sample from many different tissue spaces[21] and is starting to be used extensively in humans.[11, 22] The efficiency of microdialysis sampling during cytokine or protein collection is commonly represented as either relative recovery (RR) or extraction efficiency (EE).[23, 24] Relative recovery has been defined by Bungay et al. as the ratio of the biomolecule concentration of interest collected after microdialysis sampling (C_{outlet}) and the concentration of this biomolecule far away from the collection point ($C_{sample,\infty}$).[24] The latter statement can be simplified with the following equation:

Equation 1.1

$$RR = \frac{C_{outlet}}{C_{sample,\infty}}$$

Relative recovery (RR) or extraction efficiency (EE) is defined as:[25]

Equation 1.2

$$RR = EE = 1 - \exp\frac{-1}{Q_d(R_d + R_m + R_q)}$$

In this equation, $Q_d$represents the flow rate; R_d, the mass transport resistance of the quiescent medium external to the microdialysis probe; R_m, the mass transport resistance of the dialysate; and R_q, the mass transport resistance of the membrane. More specific terms are shown on Table 1.1. Equation 1.2 takes into account all the variables associated with influencing the amount of material recovered during the collection of any biomolecule using a microdialysis probe. To clarify, it is important to define and schematically represent each variable of Equation 1.2,[23,25] see Figure 1.1,[23] and Table 1.1.[22] The poor collection or EE performance of microdialysis sampling when used to collect certain biomolecules such as cytokines is due to several factors: a) mass transport resistances, see Table 1.1, b) biomolecular diffusion rate, c) the pore size of the microdialysis membrane as well as whether the membrane is hydrophobic or hydrophilic, and d) their low concentrations that change rapidly.[24] As a result, quantifying cytokines collected using microdialysis sampling method is challenging.

Cytokines are known to bind different biomolecules such as complex sugars (for example heparin),[26] antibodies,[26] and aptamers[27, 28] which brings the opportunity of creating affinity based methods to improve the collection of these proteins from tissue spaces during microdialysis sampling. Aptamers are oligonucleotides with similar properties than antibodies generated using a process called Selective Evolution of Ligands by Exponential enrichment (SELEX).[27, 29] According to Chris Le et al "aptamers are short nucleic acid sequences that are used as ligands to bind their targets with high affinity".[28]

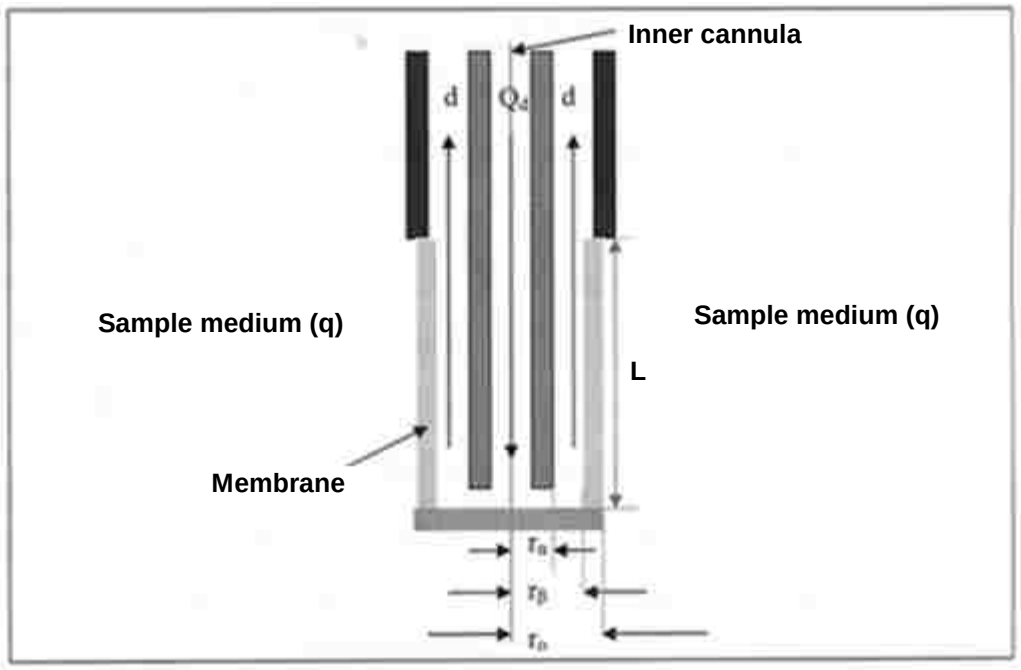


Figure 1.1 Schematic CMA/20 10 mm polyethersulfone microdialysis layout.[23]

Table 1.1 List of equations and variables of relative recovery equation, see Equation 1.2

Equations	Definition	Variables
$R_q = \frac{1}{2\pi D_q \Phi_q \sqrt{2 r_0 L}}$	Mass transport resistance of the quiescent medium external to the microdialysis probe[25]	Φ_q= Volume fraction for quiescent medium r_0 = Outer radius of membrane L = Effective membrane length
$R_d = \frac{13(r_i - r_\alpha)}{70\pi L r_i D_d}$	Mass transport resistance of the dialysate[25]	r_i = Inner radius of membrane r_α = Inner radius of cannula D_d= Diffusion coefficient in the dialysate
$R_m = \frac{\ln\left(\frac{r_0}{r_i}\right)}{2\pi L D_m \Phi_m}$	Mass transport resistance of the membrane[25]	Φ_m= Membrane volume fraction occupied by water
$D_d = \frac{6.85 \times 10^{-15}}{\eta \sqrt{*M^{\frac{1}{3}}.R_G}}$	Aqueous diffusion coefficient[25,30]	η = Dynamic viscosity M = Molecular weight R_G= Radius of gyration
$\lambda = \frac{r_s}{r_p}$	Ratio of analyte radius to pore radius[31]	r_s = Radius of analyte r_p = Radius of pore size
$H_m = K(1-2.1044\lambda + 2.089\lambda^3 - 0.948\lambda^5)$		Diffusion hindrance factor[32]
$K = (1 - \lambda)^2$		Partition coefficient[33]
$D_m = D_{aq}\ (1 - \lambda)^2(1 - 2.104\lambda + 2.089\lambda^3 - 0.948\lambda^5)$		Effective membrane diffusion coefficient using the diffusion hindrance factor[34]

Several researchers have created affinity based methods to improve the relative recovery of cytokines using microdialysis sampling technique.[5, 24, 35] Affinity-based methods could be defined as methods that take advantage of the binding affinities of two biomolecules or molecules. For instance, Duo et al. developed a method to improve cytokines relative recovery using affinity agents such as antibodies and heparin attached to polystyrene microspheres.[26] Their method consists of perfusing polystyrene

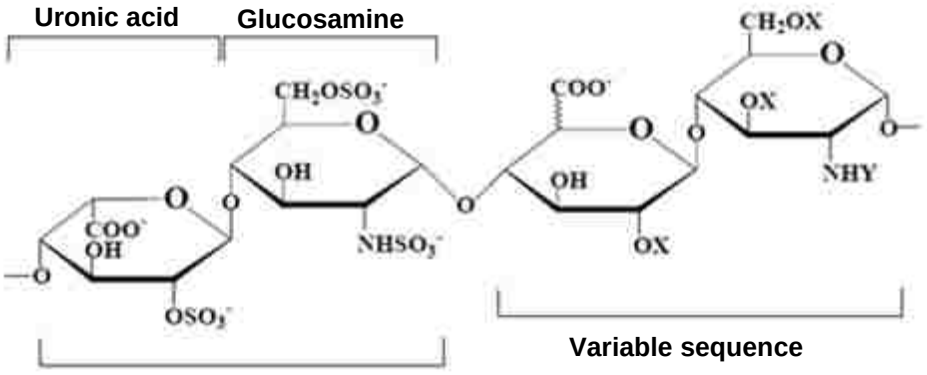
microspheres of 5.99 µm o.d. with an immobilized affinity agent, for example antibodies, through the microdialysis probe during cytokine protein collection to increase their mass transport across the membrane pores.[26, 35] According to Duo, heparin is an affinity agent more suitable for the capture and release of cytokines from functionalized microspheres than antibodies.[36] Antibodies have slower (10^{-5} s^{-1}) dissociation rate constant (k_{off}), are less chemically stable, and are more expensive than heparin (k_{off} = 10^{-2} to 10^{-4} s^{-1}).[36]

When antibody-immobilized beads are used as affinity agents during microdialysis sampling followed by direct measurements of dialysates containing the cytokines on the beads (flow cytometry) only one measurement can be performed if the concentration of the cytokine is too high (>5000 pg/mL). The problem with the flow Cytometry, immunoassay bead based detection, is that their design (Luminex or BD technology) only allows the beads or sample to be measured once, so further dilutions of a sample that is saturated or over range are not possible.[36] However, some research groups have developed methods or techniques that allow dilutions of samples if needed using microfluidic system. For example, Ligler and Kim developed a microfluidic system or "microflow cytometer" using bead-based techniques to measure multiple analytes at the same time.[37] The advantage of their system is that functionalized microspheres (having the analyte bound to them) can be recovered and measured more than once if necessary.

Another issue is that acidic (pH~4) dissociation reagents are required in order to release cytokines from antibody-immobilized microspheres which interfere with the assay performance and could denature the antibody.[36] In contrast, mild conditions (30% (v/v) acetonitrile) are used to dissociate cytokines from heparin functionalized microspheres.[36] This makes heparin a more "flexible" affinity agent than antibodies facilitating measurements, trapping and release of cytokines. However, using heparin as an affinity agent does not completely solve the quantitation issues when low concentrations of cytokines are collected. As a consequence, further pre-concentration of cytokines using heparin-microspheres is desired.

1.3 Heparin

Heparin, see Figure 1.2,[36] belongs to the family of glycosaminoglycans (GAGs). Highly sulfated glycosaminoglycans such as heparin and heparan sulfate are known to have affinity for several cytokines such as regulated upon activation, normal T-cell expressed and, secreted (RANTES) or C-C motif Ligand 5 (CCL5), monocyte chemotactic protein-1 (MCP-1) or C-C motif Ligand 2 (CCL2), macrophage inflammatory protein-1 (MIP-1), tumor necrosis factor-alpha (TNF-α), and interleukin-8 (IL-8).[38, 39, 40, 41] Moreover, according to Linhardt and Capila the interactions between heparin and proteins are mainly ionic.[39] They also pointed out that either carboxyl or sulfo groups on heparin form ion pairs with positively charged amino acids on proteins. In addition, hydrogen bonding or nonionic interactions between heparin and proteins are also present in some instances.[39] Finally, heparin is a suitable affinity agent commonly used in biomedical related areas, because it can be attached to amine or carboxyl functionalized surfaces or microspheres via reductive amination or end point attachment with well-established chemical procedures.[42, 43]



Heparin

Figure 1.2 Major and minor disaccharide repeating units in heparin (X = H or SO_3^-, Y = Ac, SO_3^-, or H).[36]

1.4 Microspheres

Magnetic microspheres are widely used both *in vivo* and *in vitro* in many research areas such as, analytical chemistry, biomedical,[44] chemical and bio-engineering, drug targeting,[45] microfluidics,[46] medicine,[47, 48] molecular biology,[49] nanotechnology,[48, 50] and proteomics. One of the most common applications of magnetic microspheres is as carriers to deliver specific biomolecules into biological systems or any other systems such as microfluidics. Also, magnetic microspheres can be used to extract or capture biomolecules, viruses or bacteria from different systems.[49, 51,52]

Another application in which magnetic microspheres have been used is in microfluidics devices. Veyret et al devised magnetic colloids to capture viruses.[49] They made magnetic colloids from oil in water magnetic emulsions in which poly (ethyleneimine) and poly (maleic anhydride-co-methyl vinyl ether) were adsorbed in two separated steps on the emulsion droplets. Veyret and co-workers could achieve a yellow fever virus capture efficiency of 90% from human serum. They performed several steps to isolate, purify, and detect the yellow virus from human or phosphate buffer saline using magnetic microspheres. Rittich et al functionalized magnetic microspheres with hydrophilic properties for molecular applications.[53] In their research, they developed magnetic nonporous hydrophilic microspheres by polymerizing poly (2-hydroxyethyl methacrylate-*co*-ethylenedimethacrylate)-(P(HEMA-*co*-EDMA)), poly(2-hydroyethyl methacrylate-*co*-glycidyl methacrylate)-(P(HEMA-*co*-GMA)), and poly(glycidyl methacrylate)-(PGMA) with a stable colloidal solution of magnetite on one step process. Also, they functionalized the magnetic microspheres with different enzymes such as RNase A, DNase I, proteinase K, and *Salmonella* antibodies to isolate *Salmonella* cells and degrade bacterial RNA, chromosomal, and plasmid DNAs. Choi et al developed a new magnetic bead-based a device for integrated bio-detection systems.[54] He and his colleagues devised a filterless bio-separator system to trap functionalized magnetic microbeads on a flat surface to separate specific biomolecules from a carrier fluid and treat them chemically to release and detect analytes of interest. However, magnetic microspheres approaches to pre-concentrate cytokines or proteins have not been reported before. Most of the research work found in the literature is about pre-concentration of viruses.[52] One of the novel aspects of this thesis was the used of magnetic microspheres to pre-concentrate cytokines or proteins.

1.5 Microfluidics

Studies and creation of microfluidic sytems is an interdisciplinary research area integrating scientists and non-scientists from different disciplines such as Mechanical, Electrical, Computer Engineering, Chemistry,Physics and Biology on the quest for faster, cheaper, and smaller devices able to solve many different types of problems. One of the principal advantages of this research area is that commonly used analytical instruments or techniques such as High-performance liquid chromatography (HPLC), mass spectrometry (MS), and microdialysis sampling can be coupled to microfluidic systems.[55,56,57] This makes microfluidic sytems a very versatile research area.[58] In contrast, most of the well-known fluid mechanics laws used for macrosystems cannot be applied to microsystem or microfluidics in some cases.[59,60,61] That is one of the main drawbacks of this research area. To explain, most research depends on experimental information to evaluate the flow behavior in microfluidic systems.[60,61,62]

This thesis had several goals. First, immobilization and quantitation of heparin onto either polymer or magnetic microspheres to capture cytokines and improve RR of cytokines after microdialysis sampling was pursued. Second, development of an equation to estimate the amount of heparin required (initial concentration) to be immobilized to beads to bind > 99% of cytokines at equilibrium. The amount of heparin included in the calculations was related to the amount of heparin-functionalized microspheres to be able to design a system based on the amount of heparin-functionalized microspheres necessary to pre-concentrate cytokines. Finally, design, fabricate, and test a microfluidic system to trap and release the heparin-functionalized microspheres after microdialysis sampling in order to further pre-concentrate cytokines.

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2 Chapter 2. Immobilization, Testing, and Pre-concentration Approach for Microfluidics Systems Applications of Heparin Functionalized Microspheres

2.1 Introduction

This chapter describes how heparin was chemically attached to amine functionalized polystyrene and carboxyl functionalized magnetic microspheres via reductive amination and EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) /NHS (*N*-Hydroxy-succinimide) chemistry, respectively. Two types of surface chemistry or functionalized microspheres were used due to the commercial unavailability of amine functionalized magnetic microspheres. Therefore, two immobilization procedures were used; one for polystyrene microspheres (NH_2-functionality) and one for magnetic microspheres (COOH-functionality). After immobilization the amount of heparin on the microspheres was measured following hydrolysis using a liquid chromatography with pulsed amperometric detection (LC-PAD) system. Following this measurement the heparin-functionalized microspheres were tested for their binding capacity with cytokines (MCP-1 and TNF-α) by measuring the interactions between them using two equilibrium methods, see below. In other to prove that heparin functionalized microspheres could be used to pre-concentrate cytokines and to determine the amount of heparin functionalized microspheres needed to reach ~ 100% of cytokines bound, a set of equations was derived and calculations were performed. These calculations were based on reported binding constant values for interactions between cytokines and heparin.

2.1.1 Immobilization and characterization of heparin

2.1.1.1 Magnetic microspheres

In order to trap and release cytokines from magnetic microspheres several experiments were conducted. First, heparin was immobilized onto carboxyl functionalized superparamagnetic microspheres of 6.3 µm mean diameter using the immobilization procedure described by Chung, et al.[1] Second, the amount of heparin immobilized onto the magnetic microspheres was quantified using a LC-PAD method similar to a method used by Duo[2] combined with a modified acid hydrolysis method.[3] The method used by Duo was based on the quantitation of glucosamine present in heparin after acid hydrolysis. The amount of glucosamine present in heparin after hydrolysis is approximately ~20% and varies depending on the

acid hydrolysis method used.[4] Moreover, according to Rohrer et al., glucosamine is the only monosaccharide present in heparin after acid hydrolysis.[5]

2.1.1.2 Polymeric microspheres

Heparin was attached to amine functionalized polystyrene microspheres of 6 microns via reductive amination.[2] The quantitation of heparin immobilized on the polystyrene microspheres was performed by using the same procedure mentioned in section 2.1.1.1 and more fully described in section 2.2.3.1. The idea of using heparin functionalized polystyrene microspheres was to demonstrate that cytokines could be pre-concentrated in a bead-based microfluidic system coupled to microdialysis sampling. In addition, heparin is cheaper than antibodies. However, antibodies are more specific than heparin. In other words, heparin can bind more than one biomolecule or cytokine. This can have a great impact during *in vivo* experiments and would certainly need to be tested as future work.

2.1.2 Testing of heparin functionalized microspheres

2.1.2.1 Polymeric microspheres

To test the heparin-functionalized polystyrene microspheres interactions with cytokines equilibrium dialysis was used. Equilibrium dialysis is a technique commonly used to evaluate binding interactions between biomolecules of different molecular weights (ligand and receptor).[6-8] Typically an equilibrium dialysis system is composed of two chambers and a membrane separating them, see Figure 2.1.[6,8]

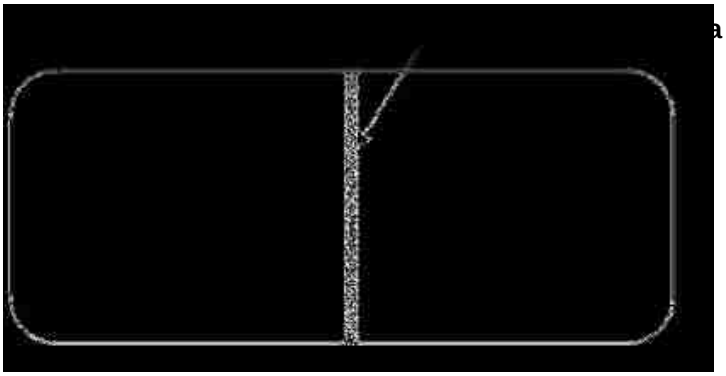

Figure 2.1 Schematic representation of the equilibrium dialysis system used to measure the heparin-cytokines interaction.[6]

In principle the idea of conducting equilibrium dialysis experiments is to allow a small ligand (smaller than membrane pore size) to diffuse across a membrane to interact with a receptor or other biomolecule that is retained due to the membrane pore size.[6-8] In other words, the ligand diffuses across the membrane and interacts with the receptor whereas the receptor cannot diffuse across the membrane to interact with the ligand due to its molecular weight being greater than the molecular weight cutoff of the membrane. At equilibrium, the concentration of the small ligand (free) will be the same in both chambers.[6-8] This allows calculation of the amount of ligand bound to the receptor by measuring the free concentration of the ligand and conducting a mass balance. Even though equilibrium dialysis experiments are commonly conducted by using biomolecules of different molecular weights, binding interactions between biomolecules of the same or similar molecular weights can be performed as well under certain conditions. For instance, the equilibrium dialysis system used for studying the binding interactions between heparin (~12 kDa) and cytokines (8 to 80 kDa) was composed of two chambers of 25 µL each and a cellulose acetate membrane of 100 kDa molecular cut off (MWCO).[6] In this instance both heparin and cytokines have similar molecular weights and are smaller than the membrane pore size. However, since heparin is attached to the 6 µm polystyrene microspheres, this prevents heparin from diffusing across the membrane. This way only cytokines can diffuse through the membrane pores.

2.1.2.2 Magnetic microspheres

To test the heparin-functionalized magnetic microspheres, ratMCP-1 and ratTNF-α were used. Heparin-functionalized magnetic microspheres and cytokines were incubated in plastic vials to test their binding interactions.[2] The method used to study this interaction was the same used by Duo.[2] His method consisted of placing equal volumes of a solution of heparin-functionalized microspheres in PBS and cytokine solution (in the same buffer) in a plastic vial and incubated it for two hours (i.e. MCP-1) at room temperature unless otherwise stated (see experimental section).[2]

2.1.3 Proof of principal pre-concentration approach using heparin functionalized polystyrene microspheres

To evaluate the limitations of using heparin immobilized on microspheres to trap and release and pre-concentrate cytokines and to have an estimate of the amount of heparin-immobilized beads needed

to achieve a particular pre-concentration, several calculations were conducted. These calculations were compared to the binding values obtained from the equilibrium experiments of section 2.1.2. The following set of calculations for the heparin-cytokines chemical interactions assuming a 1:1 binding interaction, which is commonly used for binding calculations,[9-12] and using reported binding constants of heparin-binding cytokines.[2]

2.1.3.1 Calculations set up

H = Heparin; **C** = Cytokine; **HC** = Complex (Heparin - Cytokine); $\mathbf{[H]_f}$ = Concentration of free heparin;

$\mathbf{[C]_f}$ = Concentration of free cytokine; $\mathbf{[H]_T}$ = Total concentration of heparin; $\mathbf{[C]_T}$ = Total concentration of cytokines; **[HC]** = Concentration of the complex

Chemical equilibrium

$$H + C \leftrightarrow^{k_{on}}_{k_{off}} HC$$

Equation 2.1 Binding constant

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[H]_f.[C]_f}{[HC]}$$

Equation 2.2 $[H]_T = [H]_f + [HC]$ ∴ **Equation 2.3** $\underline{[H]_f = [H]_T - [HC]}$

Equation 2.4 $[C]_T = [C]_f + [HC]$ ∴ **Equation 2.5** $\underline{[C]_f = [C]_T - [HC]}$

Substituting equation 2.3 and 2.5 in equation 2.1 ∴

Equation 2.6

$$K_D = \frac{([H]_T - [HC])\,([C_T] - [HC])}{[HC]}$$

Rearranging equation 2.6 ∴ $K_D\,[HC] = ([H]_T - [HC])\,([C]_T - [HC])$ ∴

$K_D\,[HC] = [H]_T\,[C]_T - [C]_T\,[HC] - [H]_T\,[HC] + [HC]^2$ ∴

$[C]_T[H]_T + [HC]K_D - [HC]\,[H]_T - [HC]\,[C]_T - [HC]^2 = 0$

Common factor [HC] ∴ **Equation 2.7** $\underline{[H]_T\,[C]_T + [HC]\,(K_D + [H]_T + [C]_T) - [HC]^2 = 0}$

∵ **Equation 2.7** has the form $ax^2 + bx + c = 0$ and we can apply the quadratic formula,

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

$x = [HC]$; $a = 1$; $b = -([H]_T + [C]_T + K_D)$; $c = [H]_T[C]_T$

∴ **Equation 2.8**

$$[HC] = \frac{([H_T] + [C_T] + K_D) \pm \sqrt{([H_T] + [C_T] + K_D)^2 - 4([H_T][C_T])}}{2(1)}$$

Equation 2.8 was used to estimate the percentage of cytokine bound to heparin at different initial heparin concentrations for a specific amount of initial cytokine concentration. The results calculated from this equation are shown in the results section, Table 2.3. The change on the percentage of cytokine bound to heparin as a function of the initial heparin concentration was evaluated for a fixed initial cytokine concentration of 1.0 nM (see Figure 2.7).

2.1.4 Hemacytometer

The microspheres concentration (magnetic or polymeric) used in all the experiments in this thesis were measured using a hemacytometer. This technique is commonly used to count blood cells in samples. According to Warren "a hemacytometer is a special type of microscope slide that is divided into squares of a defined area over which a defined volume of cell suspension is distributed".[13] To explain, microspheres in solution are placed in the hemacytometer chambers and counted one by one under a microscope.[13] Other methods for counting microspheres include ImageJ, a freeware software used to process images.[14] This software processes images coming from a microscope with a video system.

This chapter is going to focus on the immobilization, quantitation, and testing of heparin immobilized onto polymeric or magnetic microspheres and proof of principle of pre-concentration approach for microfluidics systems applications.

2.2 Experimental Section

2.2.1 Chemicals

Carboxyl functionalized superparamagnetic microspheres (3.45×10^8 beads/mL) of 6.3 µm mean diameter (COMPEL) were obtained from Bangs Laboratories, Inc. (Fishers, IN). Amine functionalized

polystyrene microspheres (6.00 µm o.do, stock concentration of 2.10 x 10^8 particles/ml in water) were purchased from Polysciences, Inc (Warrington, PA). *N*-Hydroxy-succinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 2- morpholinoethanoesulfonic acid (MES), albumin, from bovine serum (BSA), heparin sodium salt (from porcine intestinal mucosa), D-(+)-Glucosamine hydrochloride were from Sigma-Aldrich (St. Louis, MO). The hemacytometer was purchased from Hausser Scientific (Horsham, PA). A liquid chromatography instrument (LC) was used (Shimadzu LC-10AD, Japan). An anion-exchange column, Dionex CarboPac PA1 (250 x 2mm), with a CarboPac PA1 Guard column (50 x 2 mm) were used (Sunnyvale, CA). The detector was a Decade EC detector with a gold electrode (Antec Leyden, The Netherlands). Micro-Equilibrium Dialyzer of 25 µL and Cellulose Acetate membrane MWCO 100 kDa were purchased from Harvard Apparatus (Holliston, Massachusetts).[6] Standards (recombinant proteins) and ELISA kits for rat MCP-1 and rat TNF respectively were obtained from BD Biosciences (San Diego, CA). All other chemicals used were analytical grade unless otherwise stated.

2.2.2 Immobilization of heparin onto microspheres

2.2.2.1 Carboxyl functionalized superparamagnetic

Heparin was immobilized on carboxyl functionalized magnetic microspheres of 6.3µm using the same procedure that Chung, et al., reported for the immobilization of heparin on carboxyl porous poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres.[1] Typically 200 µL of stock solution of carboxyl functionalized superparamagnetic microspheres (3.45 × 10^8 beads/mL) were washed three times with 200 µL 0.1 M MES buffer (pH 5.5) and reconstituted with the same buffer. After that 47.9 mg of EDC and 28.8 mg of NHS were added to the solution followed by the addition of 30 mg/mL of heparin. This solution was incubated overnight under constant rotation.[1] After the incubation the amount of heparin immobilized was determined by using an acid hydrolysis method combined with a LC-PAD system, see section 2.2.3.

2.2.2.2 Amine functionalized polystyrene

The protocol for the immobilization of heparin was as follows: 200 µL of stock solution of amine functionalized polystyrene microspheres (2.10 x 10^8 beads/mL) was taken and washed three times with 1 mL of 0.2 M sodium phosphate buffer, pH 7.0 and then reconstituted in 1.5 mL of the same buffer having

30 mg/mL of heparin and 3 mg/mL of $NaCNBH_3$. After that the colloidal solution was incubated on a microplate shaker at 1000 rpm at room temperature for 2 days. To be able to compare the amount of heparin immobilized onto the microspheres control microspheres were prepared following the above procedure without heparin. The solution containing the microspheres was washed three times with 0.2 M sodium acetate buffer, pH 7.0 to eliminate any non-covalently bound components and reconstituted in 1 mL of the buffer. Then 1 mL of acetic anhydride was added to the colloidal solution and incubated for 1 hour at room temperature to deactivate the unreacted amine groups. Next, the microspheres were washed with HPLC water, 0.1 M NaOH, and 10 mM phosphate buffered saline (PBS) pH 7.4. The colloidal solution was stored in 10 mM PBS pH 7.4 containing 0.05% sodium azide.[2] Before and after heparin immobilization, microspheres were counted using a hemocytometer in order to determine if any microspheres were lost during the immobilization process. To clarify, 10 µL of the microspheres solution was placed in each chamber, placed under microscope, and counted. The heparin concentration immobilized to the microspheres was determined by hydrolyzing the microspheres solution and quantifying it as the amount of glucosamine present after acid hydrolysis. The standards were used based on the heparin lot used for immobilization to diminish any lot variation as heparin is very heterogeneous and can vary from lot to lot.

2.2.3 Characterization or quantitation of heparin immobilized

2.2.3.1 Polystyrene and superparamagnetic microspheres

The acid hydrolization process used was a modified version of the method for heparin quantitation found on the Pharmacopeial Forum.[3] A glucosamine standard solution was prepared by taking ~0.2 mg/mL of glucosamine hydrochloride RS in 4.8 M hydrochloric acid. An aliquot of ~625 µL of this standard solution was transferred to a 2.0 mL graduated plastic vial with attached lid, and heated for 6 h at 100°C in a sand bath. Then the heated solution place in a micro-vial rack at ambient temperature for one hour and then was quantitatively transferred to a 2 mL volumetric flask, and diluted with HPLC grade water to volume. The standard solution of heparin was prepared by transferring a known weight of heparin sodium (targeted ~1.5 mg) to a 2 mL graduated vial with attached lid, dissolved in 2 mL of 4.8 M hydrochloric acid, and capped. The standard solution of heparin was heated for 6 hours at 100°C in a sand bath, then the solution was placed in a micro-vial rack until it reached room temperature, and diluted with HPLC

grade water (3 in 50) unless otherwise stated (2 mL volumetric flask). Heparin-functionalized polymeric or magnetic microspheres were hydrolyzed and the concentrations of glucosamine obtained were compared to the standard solutions. After hydrolysis the microspheres were centrifuged and the supernatant was diluted (3:50) in HPLC-grade water. Several calibration curves were generated with the hydrolyzed standards prepared above. First, the hydrolyzed heparin was used to generate a calibration curve based on the amount of glucosamine. Second, the standard solution of hydrolyzed glucosamine of different concentrations was used to generate a calibration curve and compare analyzed amounts to the free hydrolyzed heparin, see Figure 2.2. The amount of glucosamine in the hydrolysate from the microspheres, and standard solution of glucosamine were quantified by ion-exchange chromatography with pulsed amperometric detection (LC-PAD). An anion-exchange column, Dionex CarboPac PA1, with a CarboPac PA1 Guard column was used. The detector was a Decade EC detector with a gold electrode in the pulse potentials and time durations of E_1 = +0.05 V, t_1 = 400 ms; E_2 = +0.75 V, t_2 = 200 ms; E_3 = -0.75 V, t_3 = 400 ms.[2] The mobile phase used was 95 mM sodium hydroxide with 10 mM sodium acetate, and the injection volume 10 µL. The flow rate for the LC was 0.25 mL/min using an isocratic mode.[2]

2.2.4 Testing of heparin immobilized

2.2.4.1 Polystyrene microspheres

A standard solution of rat MCP-1 (289 ng/mL according to the label) was used to prepare the standards for the ELISA, and solutions used in equilibrium dialysis experiments. An aliquot of 25 µL of heparin functionalized polystyrene microspheres (4.48 x10^{-7} beads/mL) with a total concentration of 5.22 x 10^{-7} M of heparin was placed in one of the equilibrium dialyzer chambers and 25 µL of 0.1 or 1.0 nM (reported MW 26.2 kDa)[2] ratMCP-1 diluted in 10mM phosphate buffer saline (PBS, pH 7.4) containing 0.05% BSA were placed on the other chamber (Figure 2.1).[6] Controls were prepared as follows: a) 25 µL of the same ratMCP-1 solution was placed in one chamber, and 25 µL of the same buffer was placed on the other chamber, and b) 50 µL of the same ratMCP-1 solution was placed in a plastic vial. Samples and controls were incubated for two hours at room temperature under constant rotation using a horizontal rotator to allow equilibrium to occur. The time frame of two hours were chosen based on the binding kinetics data reported by Duo for MCP-1 and heparin functionalized microspheres.[2] The control placed in the plastic vial was incubated with no rotation. After two hours 5 µL aliquot from each equilibrium dialyzer

chamber was taken and diluted in 126 µL of ELISA kit assay diluent. Also, 13 µL aliquot from the plastic vial was diluted in 327 µL of the assay diluent. Standards solutions of ratMCP-1 for ELISA kit were prepared according to the BD Biosciences procedure (standards were diluted in assay diluent as well).

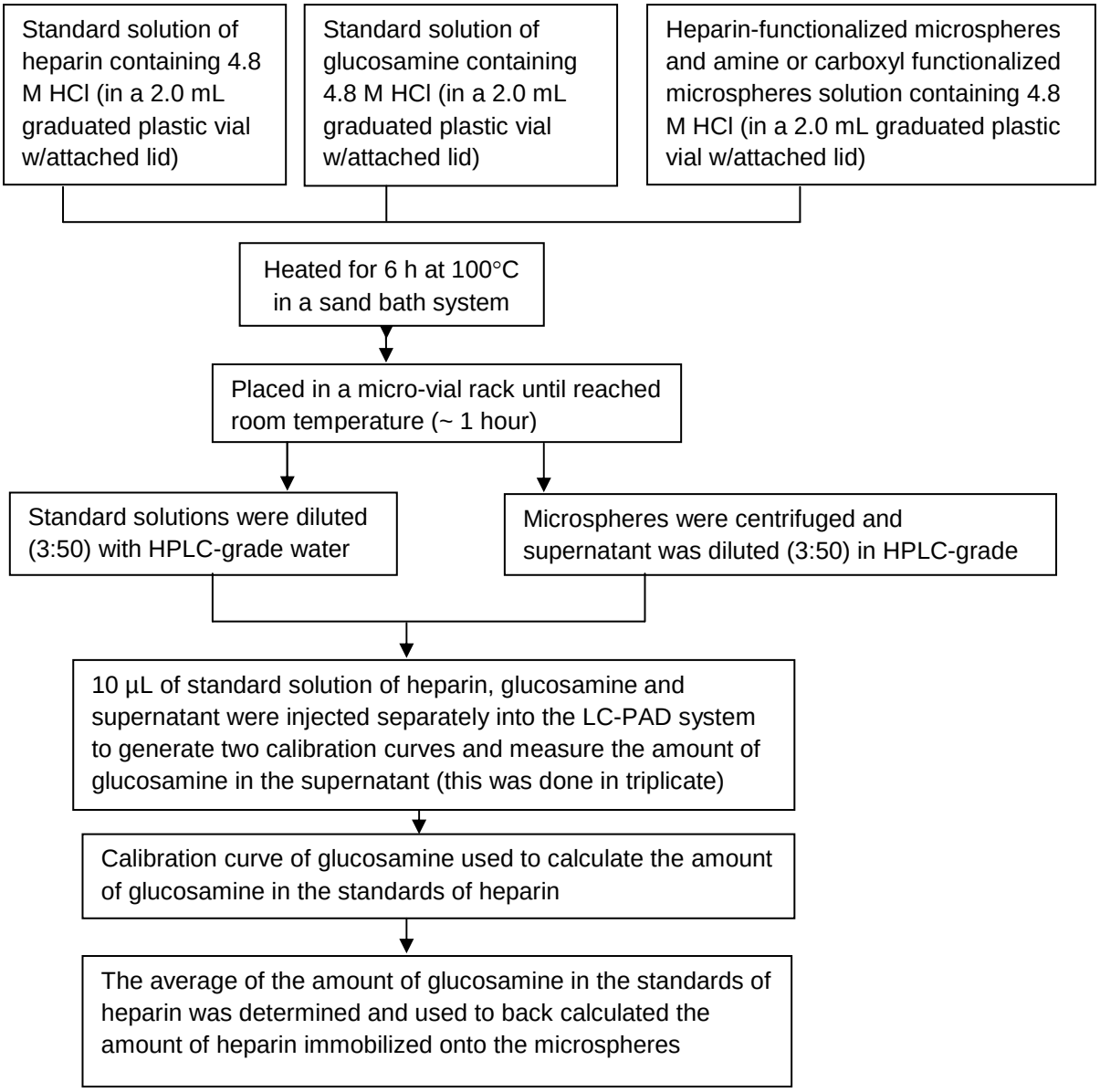

Figure 2.2 Flow chart of the method used to determine the amount of heparin immobilized onto the microspheres based on the glucosamine content.

A calibration curve of ratMCP-1 standards was used to calculate the concentration of samples and controls. These experiments were performed in triplicate.

2.2.4.2 Magnetic microspheres

The method for trapping and releasing cytokines was similar to the method used by Duo.[2] A solution, 100 µL, of heparin-functionalized magnetic microspheres (3.90×10^8 beads/mL) with a total concentration of 4.98×10^{-7} M of heparin in 10 mM PBS (pH 7.6) was placed in a plastic vial along with a 100 µL solution of either ratMCP-1 (15 ng/mL) or rat TNF-α (5 ng/mL) in the same buffer at different concentrations. These concentrations were selected based on the estimated values of the amount of cytokines bound to heparin, see section 2.1.3 and Table 2.3, and the volume (≥ 50 µL) and concentration needed to make three measurements using the ELISA kit within the kit's concentration range (31.3 to 2000 pg/mL).[15] The concentration of ratMCP-1 used was higher compared to rat TNF-α because MCP-1 has a higher affinity to heparin, see Table 2.3. In other words, in order to obtain a free concentration of MCP-1 within the kit's concentration range a higher initial concentration was used. The plastic vial containing both solutions was incubated at room temperature for 2 hrs. After incubation, the supernatant was removed and the cytokine content in the supernatant was determined using the corresponding rat MCP-1 or rat TNF-α ELISA kit.

A TecanSPECTRAFluor 96-wells plate reader was used to read the absorbance at 450 nm, wavelength correction was used to subtract absorbance at 570 nm from absorbance 450 nm according to the BD Biosciences technical data sheet.[15] The concentration of cytokines bound to the heparin-functionalized magnetic beads was determined by subtracting the initial or free concentration of cytokines that was placed in the vials before incubation to the free concentration of cytokines after incubation or centrifugation. In other words, the concentration of cytokines bound to the heparin-functionalized magnetic microspheres was calculated by difference (initial minus free = bound).

2.2.5 Proof of concept pre-concentration approach using heparin functionalized polystyrene microspheres

2.2.5.1 Estimations

Equation 2.8 was configured in a Microsoft Office Excel 2007 spreadsheet to facilitate the calculations. A set of calculations were performed manually or by hand to confirm the spreadsheet set up was working. The steps followed to estimate the amount of heparin functionalized microspheres require to achieve ~ hundred percent of cytokines bound to heparin were: a) The concentration of heparin

immobilized on 6 µm microspheres ([HB]) used was 15.7 x 10^6 heparin molecules per bead, b) the initial concentration ([B]) value used for "plain" microspheres or without heparin was 7.70 x 10^5 beads/mL, and c) the final concentration of heparin immobilized on the microspheres ([HB]) was calculated based on the following equation:

$$[HB] = \frac{[HB]\,[B]\,1000}{6.023 \times 10^{23}}$$

2.3 Results and discussion

2.3.1 Characterization or quantitation of heparin immobilized

The amount of heparin immobilized on either heparin-functionalized polystyrene microspheres or heparin-functionalized magnetic microspheres was calculated using the appropriate calibration curve, and the calculated glucosamine content ~35.0% and ~27.0%, respectively. Different heparin lots were used to make polystyrene and magnetic microspheres. That is why the glucosamine content was different, see section 2.2.2.2. Glucosamine content was calculated as follows: a) The peak areas of heparin hydrolyzed standards, were plugged in the equations generated by the calibration curves to determine the concentration of glucosamine, b) The concentrations of glucosamine from "a" were divided by the predicted concentrations of the heparin solutions or standards prepared (before acid hydrolysis), c) The results of "b" were averaged and used as the glucosamine content. In order to determine the amount of heparin measured (after hydrolysis) indirectly the concentration of glucosamine calculated using the calibration curve were divided by the glucosamine content. The concentration of heparin on heparin-functionalized polystyrene and magnetic microspheres after acid hydrolysis measured using LC-PAD, see Figure 2.3, and calculated as explained before is shown in Table 2.1.

Table 2.1 Amount of heparin on heparin-functionalized polystyrene and magnetic microspheres measured using LC-PAD after acid hydrolysis

Microspheres type	Microspheres concentration (beads/mL)	Heparin concentration (M)
Polystyrene	4.48 x 10^7	5.22 x 10^{-7} ± 0.32 x 10^{-7}
Magnetic	3.90 x 10^8	4.98 x 10^{-7} ± 0.08 x 10^{-7}

The amount of heparin-functionalized magnetic microspheres used was higher (3.90 x 10^8 beads/mL) than the amount of heparin-functionalized polystyrene microspheres (4.48 x 10^7 beads/ mL), but the heparin concentration on polystyrene and magnetic microspheres were approximately the same, see Table 2.1. The amounts of microspheres used were different, because each stock of microspheres (polystyrene and magnetic) came with different concentration. The reason why a 10 fold difference between the amount of heparin-functionalized magnetic and polystyrene microspheres yield approximately the same amount of heparin immobilized could be due to the efficiency of the coupling chemistry used due to the differences in available surface chemistries for the beads.

2.3.2 Testing of heparin immobilized

2.3.2.1 Polystyrene microspheres

The interaction of heparin-functionalized polystyrene microspheres and MCP-1 was used to test the binding capacity of the heparin beads. The method used was equilibrium dialysis. The experimental results were compared to the estimated values, see Table 2.2. The difference between experiment and theory were 4.25% for 0.1 nM and 15.08% for 1.0 nM. Since the reported K_D values were used for the estimations this could explain the difference between experiment and theory.

2.3.2.2 Magnetic microspheres

In order to test the heparin-functionalized magnetic beads a different approach was used. This method was used to minimize the non-specific binding of cytokines. The microspheres were incubated in a plastic vial with either MCP-1 or TNF-α as explained in section 2.2.4.2. The percentage of MCP-1 bound was 70.38% and for TNF-α was 11.07%, see Table 2.3. These values were expected because MCP-1 has a higher reported heparin affinity (K_D) than TNF-α, see Table 2.5.

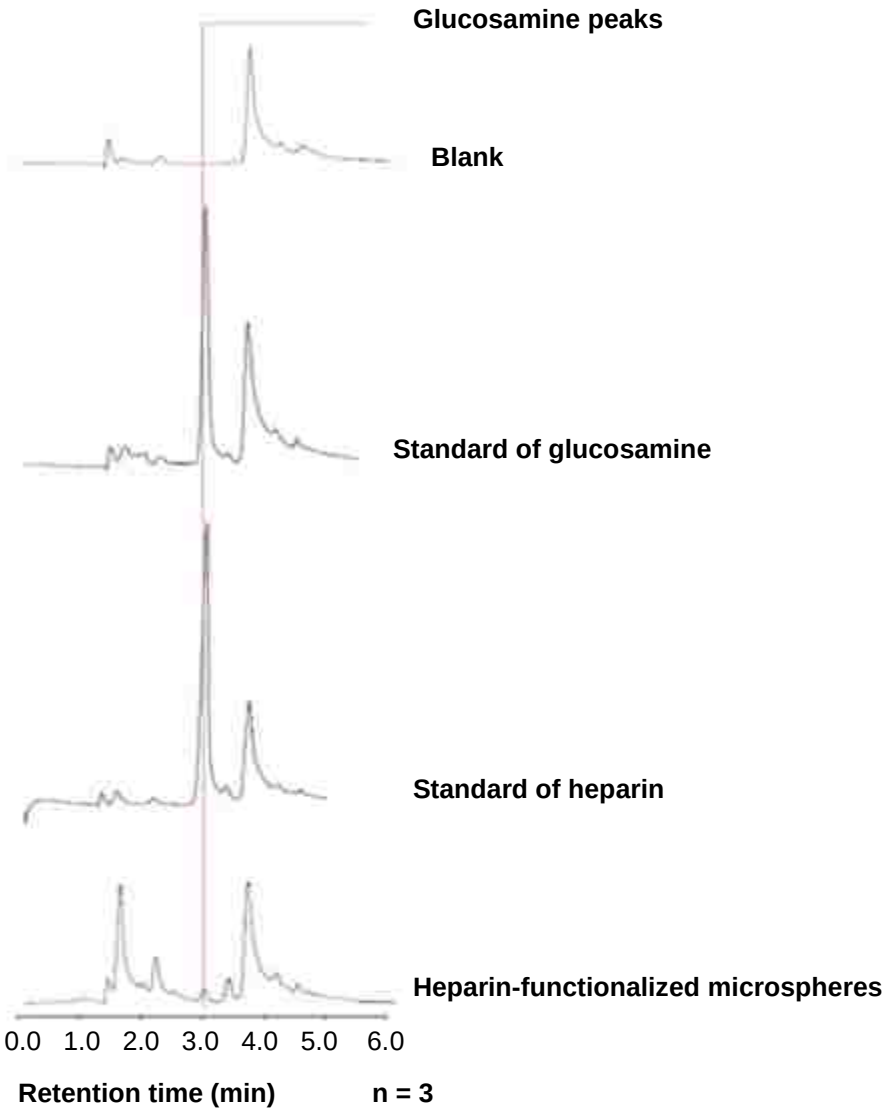

Figure 2.3 Chromatograms of standards of glucosamine, hydrolyzed heparin and heparin-functionalized microspheres after hydrolysis.

Table 2.2 Comparison of estimated and experiment of percentage of rat MCP-1 bound to heparin functionalized polystyrene microspheres

ratMCP-1			
Initial concentration of heparin (μM)	**Initial concentration of ratMCP-1 (M)**	***Reported binding constant (K_D, nM)**	**% of cytokine bound**
0.52 ± 0.03	1.00×10^{-9}	6.60 ± 0.80	98.75
	1.00×10^{-10}		98.75
		Calculated binding constant (K_D, nM)	
	1.00×10^{-9}	100.02	**83.67
	1.00×10^{-10}	30.40	***94.50

***[2], **n = 3, ***n = 2**

The estimated and calculated percentages of ratMCP-1 and rat TNF-α bound to heparin immobilized polystyrene and magnetic microspheres were compared see Table 2.4.The differences between estimated and calculated percentages for those cytokines were 28.31% and 31.56%, respectively. The difference between the calculated percentages (using measured values) of ratMCP-1 bound to heparin immobilized polystyrene and magnetic microspheres (1.0 nM and 0.57 nM respectively) was 13.29%. The estimated percentages (using estimated and reported values) difference was almost negligible (0.06%).

2.3.3 Proof of principal pre-concentration approach using heparin functionalized polystyrene microspheres

2.3.3.1 Estimations

Several calculations were performed using Equation 2.8. These estimations were conducted for MCP-1, TNF-α, IL-6, INF-γ, and IL-10 using reported K_D values measured using different techniques and conditions, see Table 2.5. The initial concentration of cytokines used was 1.0 nM and the initial concentration of heparin was varied from 3.68×10^{-10} M to 3.68×10^{-2} M. The reason why this range of concentration was used was that Duo[2] conducted similar calculations using 3.68×10^{-9} M. However, he did not show how the calculations were conducted. After conducting my calculations I decided to use his calculations as a reference and to be able to compare our results I used the same initial concentration that he used. I obtained similar as Duo.

Table 2.3 ratMCP-1 and ratTNF-α controls concentration in low binding plastic vials, in equilibrium dialysis chambers, concentration of free cytokines, and percentages of cytokines bound to heparin-functionalized magnetic microspheres or heparin after incubation at room temperature for 2 hours.

ratMCP-1			
Control in chambers (ng/mL)	**Sample (ng/mL)**	**% Bound (Exp.)**	**% Bound (Est.)**
13.58 ± 0.36	4.02 ± 0.23	70.38 ± 1.71	98.69
ratTNF-α			
Control in chambers (ng/mL)	**Sample (ng/mL)**	**% Bound**	**% Bound (Est.)**
*5.12	4.56 ± 0.25	*11.07	42.63

Table 2.4 Comparison of the estimated and calculated percentages of ratMCP-1 bound to heparin immobilized polystyrene and magnetic microspheres

Heparin-polystyrene beads			
Initial concentration of heparin (μM)	**Initial concentration of ratMCP-1 (M)**	***Reported binding constant (K_D, nM)**	**% of cytokine bound**
0.52 ± 0.03	1.00×10^{-9}	6.60 ± 0.80	98.75
	1.00×10^{-10}		98.75
		Calculated binding constant (K_D, nM)	
	1.00×10^{-9}	100.02	**83.67
	1.00×10^{-10}	30.40	***94.50
Heparin-magnetic beads			
Initial concentration of heparin (μM	**Initial concentration of ratMCP-1 (M)**	***Reported binding constant (K_D, nM)**	**% of cytokine bound**
0.49 ± 0.01	0.57×10^{-9}	6.60 ± 0.80	98.69
		Calculated binding constant (K_D, nM)	**% of cytokine bound**
		208.75	70.38 ± 1.71

***[2], **n = 3, ***n = 2**

After that I conducted a set of calculations as shown on Table 2.5. It was theoretically found (based on the calculations) that if an initial concentration of cytokine lower than 1.0 nM and any initial concentration of heparin is used the calculate percentage of cytokine bound to heparin is negligible or constant (data not shown). In other words, below concentrations of 1.0 nM the estimated % of cytokines bound to heparin for any initial concentration of heparin remains almost constant. This is a very important finding for the study of the interactions between heparin and cytokines of cytokine concentrations lower than 1.0 nM.

Several binding curves were generated to ensure that the estimations and derived equation were in agreement with the fact that for this type of calculations the K_D of a biomolecule correspond to the inflection point of its binding curve.[16] In Figure 2.5 can be seen how each cytokine K_D value correspond to its binding curve inflection point. The amount of heparin required to achieve ~ 100% of cytokines bound for a fixed initial concentration of cytokines (1.0 nM) was related to the amount of heparin-functionalized microspheres. This was done to estimate the size of the microfluidic trapping area needed to pre-concentrate the cytokines assuming the heparin content to the microspheres and the total microspheres per mL are known. In Table 2.6 the amount of beads highlighted in red represent the amount of heparin-functionalized microspheres necessary to obtain ~ 100% of cytokines bound. For MCP-1 and TNF-α, the amount of heparin-functionalized required is higher (7.70×10^9 beads/mL) compared to IL-6, INF-γ, and IL-10 (7.70×10^8 beads/mL). These estimations helped with the microfluidic system design, see Chapter 3.

Table 2.5 Estimated values of the percentage of cytokines bound to heparin at different $[H]_f$ values

Initial concentration of Heparin (M)	K_D (M)					
	6.70e-07[17]	2.00e-07[18]	7.47e-08[17]	5.40e-08[19]	6.60e-09[2]	
	TNF-α	IL-6	INF-γ	IL-10	MCP-1	
3.68e-10	0.05	0.18	0.48	0.66	4.65	% of Cytokine bound
3.68e-09[2]	0.55	1.8	4.64	6.28	33.63	
3.68e-08	5.20	15.49	32.81	40.26	84.49	
3.68e-07	35.43	64.75	83.09	87.18	98.23	
3.68e-06	84.59	94.84	98.01	98.55	99.82	
3.68e-05	98.21	99.46	99.80	99.85	99.98	
3.68e-04	99.82	99.95	99.98	99.99	100.00	
3.68e-03	99.98	99.99	100.00	100.00	100.00	
3.68e-02	100.00	100.00	100.00	100.00	100.00	

Note: Numbers in red indicate the initial concentration of heparin required to achieve ~100% of cytokines bound.

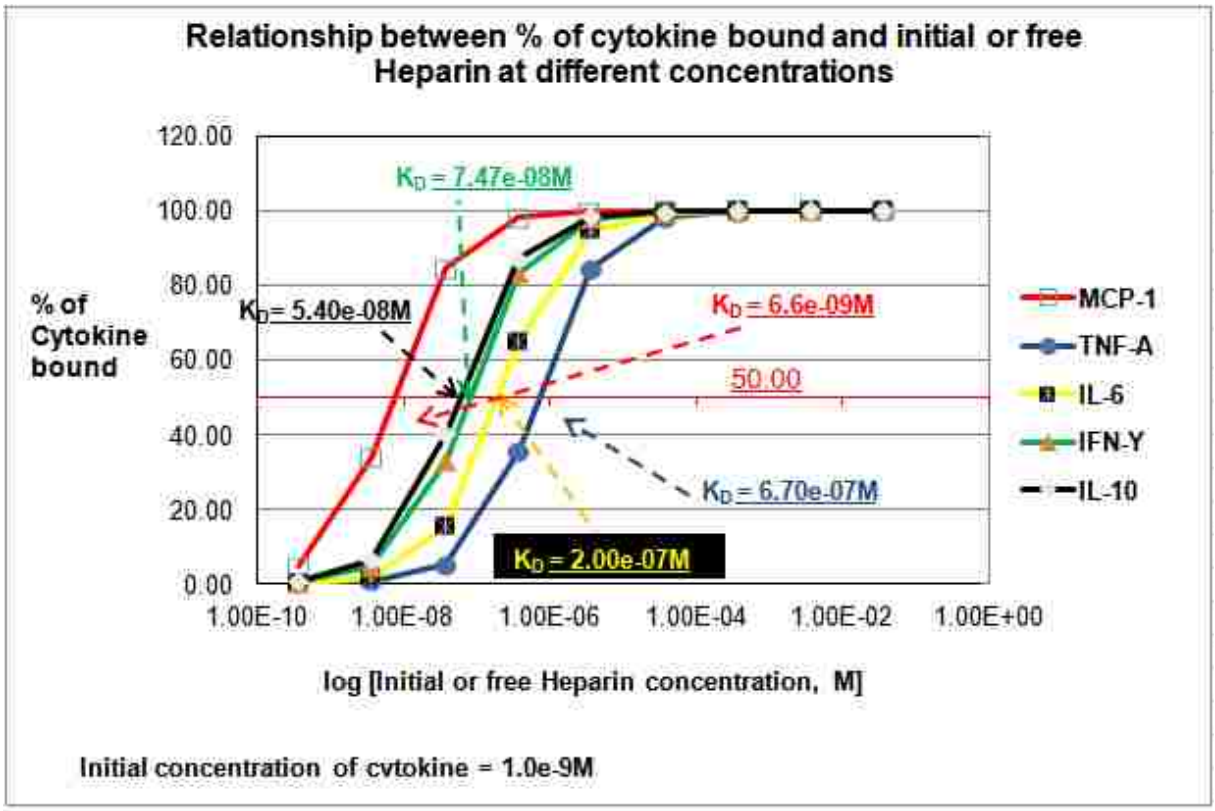

Figure 2.5 Relationship between percentages of cytokines bound and initial or free heparin at different concentrations

Table 2.6 Estimated amount of heparin, [HB], immobilized on amine functionalized microspheres of 6 microns, [B], based on a reported value of 15.7 x 10^6 heparin molecules per bead, [HB],[2] at different initial concentrations of heparin $[H]_f$.

[HB] (heparin molecules/bead)	[B] (beads/mL)	[HB] (M)	Estimated target $[H]_f$ (M)*
15.7x10^6	7.70x10^5	2.01x10^{-8}	
	7.70x10^6	2.01x10^{-7}	
	7.70x10^7	2.01x10^{-6}	
	7.70x10^8	*2.01x10^{-5}	*3.68x10^{-5}
	7.70x10^9	2.01x10^{-4}	3.68x10^{-4}
	7.70x10^{10}	2.01x10^{-3}	3.68x10^{-3}
	7.70x10^{11}	2.01x10^{-2}	3.68x10^{-2}

Note: Numbers in read mean the minimum amount of heparin-functionalized beads required to achieve ~ 100% of cytokines bound related to Table 2.5.*

2.4 Conclusion

Heparin was chemically immobilized on two different types of microspheres (amine functionalized polymeric and carboxyl functionalized magnetic). Heparin immobilized to each bead set was quantified using acid hydrolysis and LC-PAD. The binding affinity of cytokines with heparin-immobilized polymeric and magnetic microspheres was quantified using equilibrium dialysis and “plastic vial mixing” and incubation methods. The purpose of these studies was to relate the amount of heparin immobilized onto the microspheres to the microspheres concentration. In other words, I wanted to determine how many microspheres were required to obtain an “x” concentration of heparin to further capture an expected “y” concentration of cytokines. It was estimated theoretically based on experimental data how many microspheres with a known heparin content were necessary to achieve one hundred percent binding of cytokines. These estimations and experiments served as a fundamental base for the pre-concentration of cytokines. For the heparin immobilized polymeric microspheres after equilibrium dialysis (ratMCP-1) the difference between estimated and experimental values were 4.25% for 0.1 nM and 15.08% for 1.0 nM.

For heparin immobilized magnetic microspheres after “plastic vial mixing” and incubation (ratMCP-1 and ratTNF-α) the difference on the percentages were 28.31% and 31.56% respectively. The difference between the experimental values of the percentages bound between heparin immobilized polymeric and magnetic microspheres for ratMCP-1 was 13.29%. However, the difference between the estimated values was almost negligible (0.06%). These differences could be due to the fact that the cytokine K_D values used were obtained from the literature in which each value were obtain under different conditions as compared to the experiments performed in this thesis. Nevertheless, the difference was lower than expected. Finally, this work showed that cytokines could be pre-concentrated into a trap and release microfluidic systems using microspheres with chemical affinity for cytokines.

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binding&source=bl&ots=nyJownRunv&sig=xTl0VTkFCrzECypYWPi8nrNrShk&hl=en&ei=bxtxTtLhEaqNsQLI_u3ICQ&sa=X&oi=book_result&ct=result&resnum=3&ved=0CCkQ6AEwAjgK#v=onepage&q=binding%20curve%20reversible%20binding&f=false.

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3 Chapter 3. Design, fabrication, and testing of pre-concentrator microfluidic system

3.1 Introduction

In this chapter a simple microfluidic system was created to test the usefulness of heparin-immobilized magnetic microspheres for the pre-concentration of cytokines after microdialysis sampling. The microfluidic system created is the first system that have been developed using a circular trapping area to pre-concentrate cytokines. The designed microfluidic system used pressure differences as the driven force for the fluid. That played an important role on choosing the channels and overall dimensions of the device in order to facilate fluid flow in the system and to understand the limititaions of the system in term of flow rate. I was able to use relative high flow rate (10.0 µL per minute) without any leakage for a system (PDMS-glass) held together by weake forces or Van der Waals forces. Nevertheless, one of the issues with this new system is that circular-based systems have not been previously described in the literature. In other words lack of a well-understood and comprehensive fluid mechanics theories for microfluidics makes the design and characterization of such systems a challenge.

3.1.1 Theory and estimations

Several assumptions were made to be able to estimate the pressure drop of the developed microfluidic system based on the hydraulic resistance of connected straight channels, the Hagen-Poiseuille law, and Kirchhoff's laws or equivalent circuit theory, see Figure 3.1.[1] For pressure driven fluids microfluidic systems it is common to use the equivalent circuit theory to predict the pressure drop and analyse or modify designs using well understood electrical circuit theory.[1-5] The aim of this chapter was to explain how the microfluidic system was developed and tested.

3.2 Design and fabrication

The proposed microfluidic system consisted of several parts: a) A fluidic part having two straight channels of 0.1 mm (inlet) and 0.2 mm (outlet) having a circular trapping area of 3.6 mm (internal diameter) between them, see Figure 3.2. The height (0.1 mm) was the same for each channel and trapping area. The circular trapping area had a volume of ~1 µL equivalent to an estimated amount of ~9 x 10^6 magnetic microspheres of 6 µm in diameter. It was made of polydimethylsiloxane (PDMS), flexible

polymer commonly used for microfluidic fabrication,[6] and b) planar micro-electromagnetic trapping system. The latter is part of the future work.

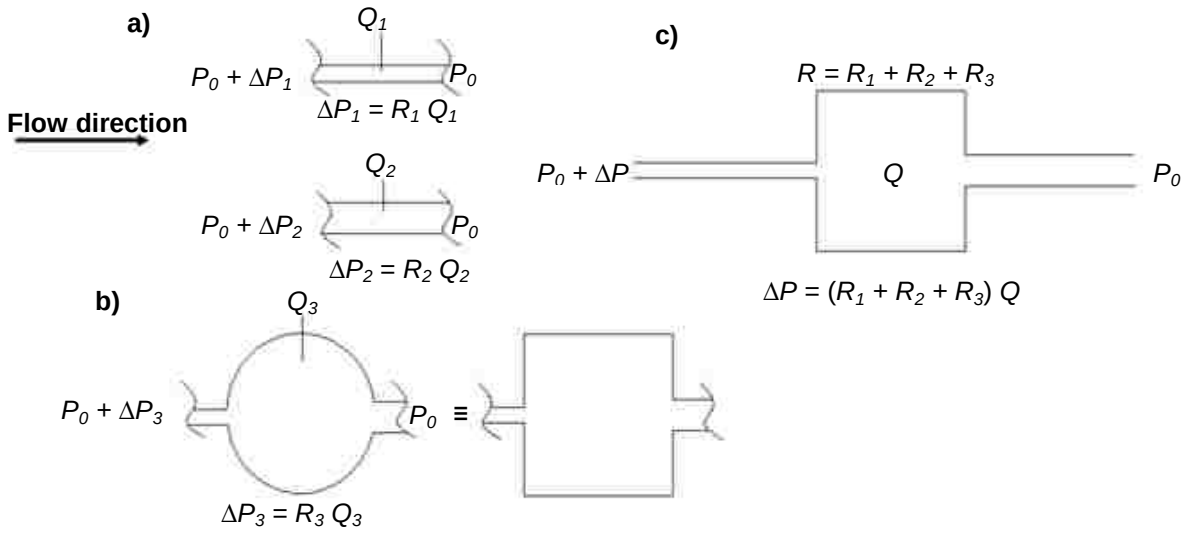

Figure 3.1 Equivalent circuit approach of three straight channels in series used to estimate the pressure drop in the designed microfluidic system, see 3.5.2. a) Inlet and outlet channels, b) circular trapping area showing the assumption that the cross sectional area of the"circle" was equal to the cross sectional area of a "square", c) the microfludic system. This figure was redrawn and adapted from reference 1.

Several steps were followed for the fabrication of the microfluidic system. First of all, a 2D sketch was drawn on AutoCAD 2011 provided by the University of Arkansas' computer lab, see Figure 3.2. The dimensions chosen for the design were based on the microfabrication capabilities of the University of Arkansas' High Density Electronics Center (HiDEC), and cost. The inlet and outlet microchannels dimensions were designed to minimize backpressure and to reduce leakage. The idea was to design and fabricate a system as straightforward, inexpensive, and fast as possible. Finally, the circular trapping area was designed to have a volume of 1 µL that was related to the minimum amount of heparin-functionalized magnetic microspheres estimated to reach ~100 % of cytokines bound as explained in Chapter 2.

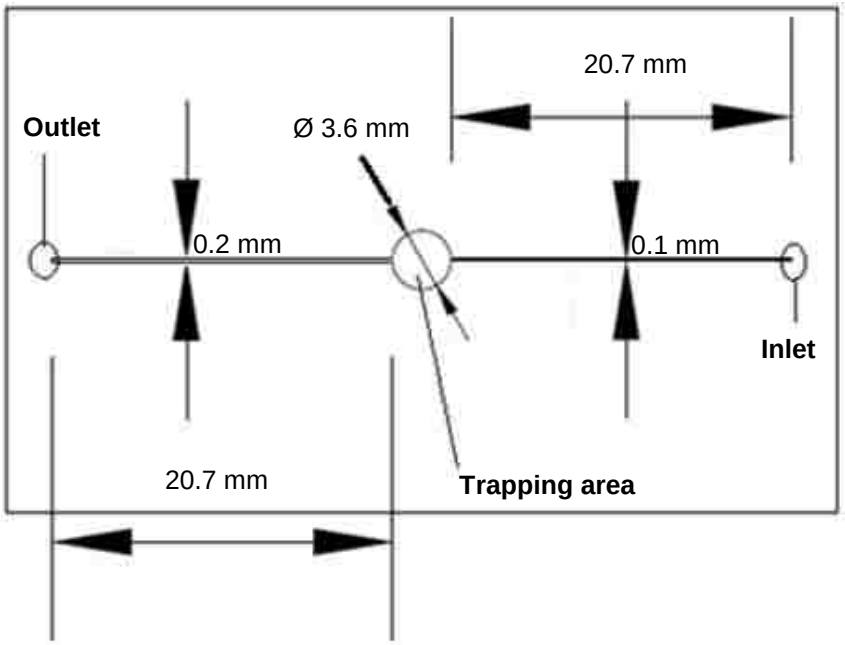

Figure 3.2 2D sketch of the photomask used to fabricate the microfluidic sytem (fluidic part).

The fabrication procedure used to build this system was a standard photolithography or soft lithography method (see below). After fabricating the system a glass-slide was used to cover the channels. This system was tested by recording the liquid filling and magnetic microspheres colloidal solution behavior inside the trapping area.

3.3 Materials and Methods

Carboxyl functionalized magnetic microspheres of 6.0 µm (COMPEL) were obtained from Bangs Laboratories, Inc. (Fishers, IN). Sylgard 184 silicone elastomer kit base or silicone (Dimethyl siloxane, dimethylvinyl-terminated major component) and curing agent or silicone resin solution (Dimethyl, methylhydrogen siloxane major component) was used (Down Corning, Midland, MI. Epoxy based photoresist or SU-8 was purchased from MicroChem (Newton, MA). Light microscope was purchased from Leica Microsystems, Inc. (Buffalo Grove, IL). The syringe pump was purchased from BASi (West Lafayette, IN). A digital camera ViviCam 7388s from Vivitar (New York, NY) was used. Fluorinated ethylene propylene (FEP) tubing (0.12 mm inner diameter) and tubing adaptors were purchased from CMA Microdialysis, Inc. (North Chelmsford, MA).

The microfluidic device in PDMS was fabricated using standard photolithography, see Figure 3.3. SU-8 was spin coated on a 4" silicon wafer to create a master mold. PDMS was prepared by mixing the base and curing agent at a 10:1 (w/w) ratio according to the DOW CORNING's product information sheet.[7] The mixture was degassed using an in-house made vacuum system, see Figure 3.4, and poured onto the SU-8 master mold. After that the SU-8 master mold was cured in a 75 ºC oven for 1 hour. The PDMS replica mold was cutted out using a razor blade, removed from the SU-8 master mold, and cleaned using isopropyl alcohol. After that the device was placed on a standard, previously cleaned using isopropyl alcohol, microscope glass slide to cover the channels. The interaction between the PDMS and the glass slide form a weak or reversible bond due to the Van der Waals forces.[6] According to McDonald, et al., this type of bond between PDMS and glass can withstand up to 5 psi, and the amount of residue left after the PDMS is peeled off is significantly low making the fabrication process easier.[6] The inlet and outlet of the device were made using a round punch having a nominal cutting edge diameter of 0.71 mm from Technical Innovations, Inc. (Angleton, TX). Finally, the inlet and outlet tubing were placed in the holes previously made by slightly pushing them in without using any fittings, see Figure 3.5.

3.4 Testing

3.4.1 Liquid filling

In order to test whether or not the system had any leakage and to observe the liquid filling behavior inside the circular trapping area, HLPC-grade water and 10 mM phosphate buffered saline (PBS), pH 7.6 were perfused at 1.0 µL/min flow rate in two different set of experiments. During each individual experiment a video was recorded at 1.0 µL/min flow rate starting at the "entrance" of the circular trapping area. The video was recorded using an in-house made micro-video recording system composed of a digital camera attached to a light microscope, see Figure 3.6. Two sets of snapshots were taken after the experiments were performed, see Figure 3.7, and 3.8.

3.4.2 Microspheres behavior inside circular trapping

Magnetic microspheres were taken from a stock solution (3.45×10^8 beads/mL) and washed three times with 100 µL of 10 mM phosphate buffered saline (PBS), pH 7.6. The microspheres were perfused into the microfluidic system using a BASi syringe pump. A video of the magnetic microspheres behavior was

recorded at different flow rates (1.0, 2.0, 5.0 and 10.0 µL/min) using the micro-video recording system made in our lab. Several snapshots from the video were taken as illustrated on Figure 3.9.

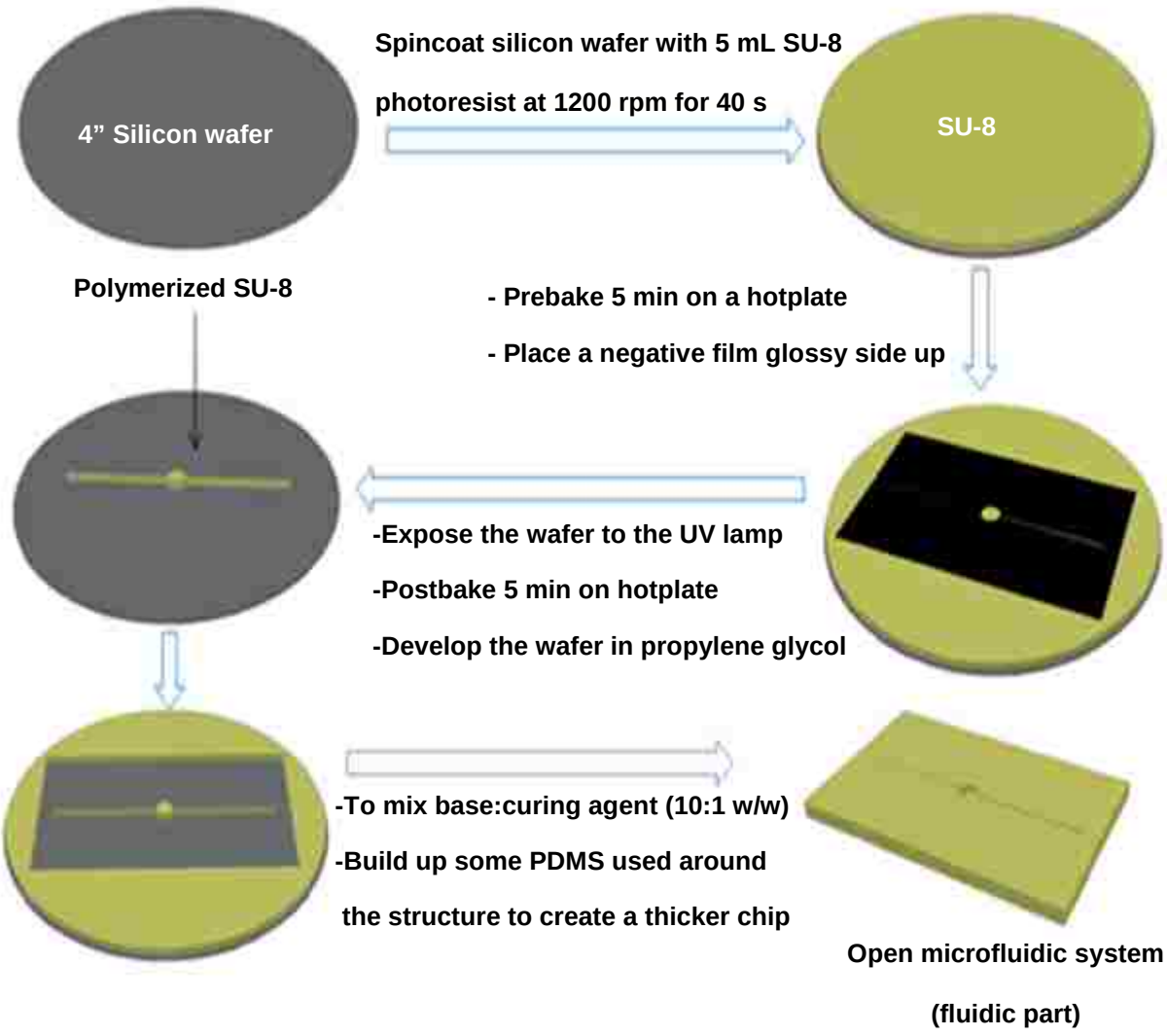

Figure 3.3 Simplified microfabrication process of the microfluidic system (fluidic part)

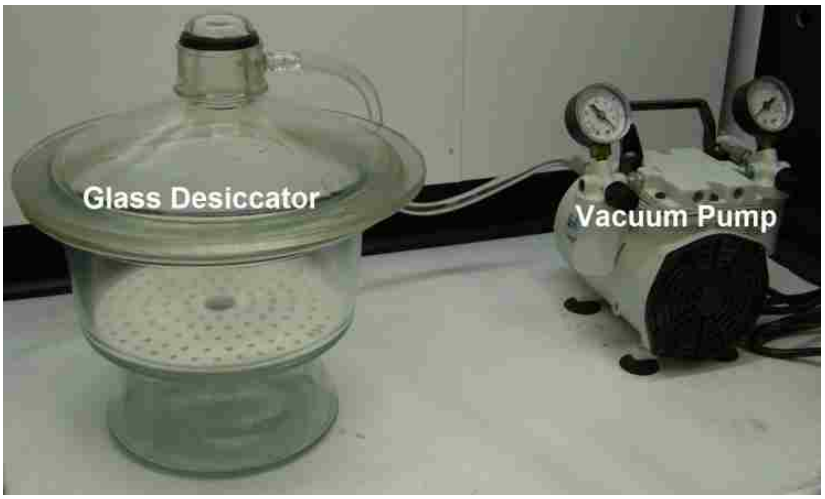

Figure 3.4 In-house made vacuum system

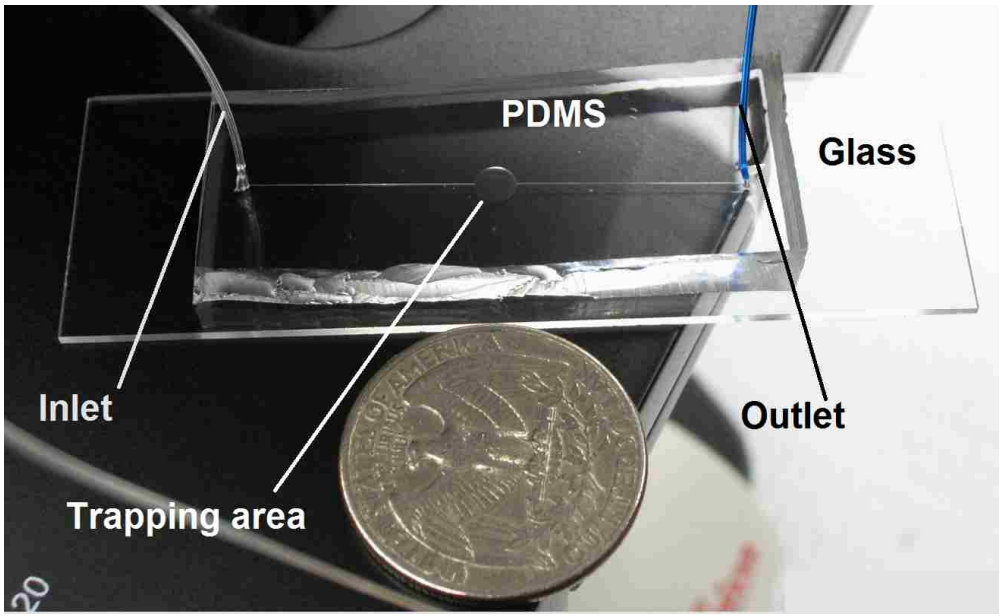

Figure 3.5 fabricated PDMS microfluidic system with inlet and outlet tubings connected

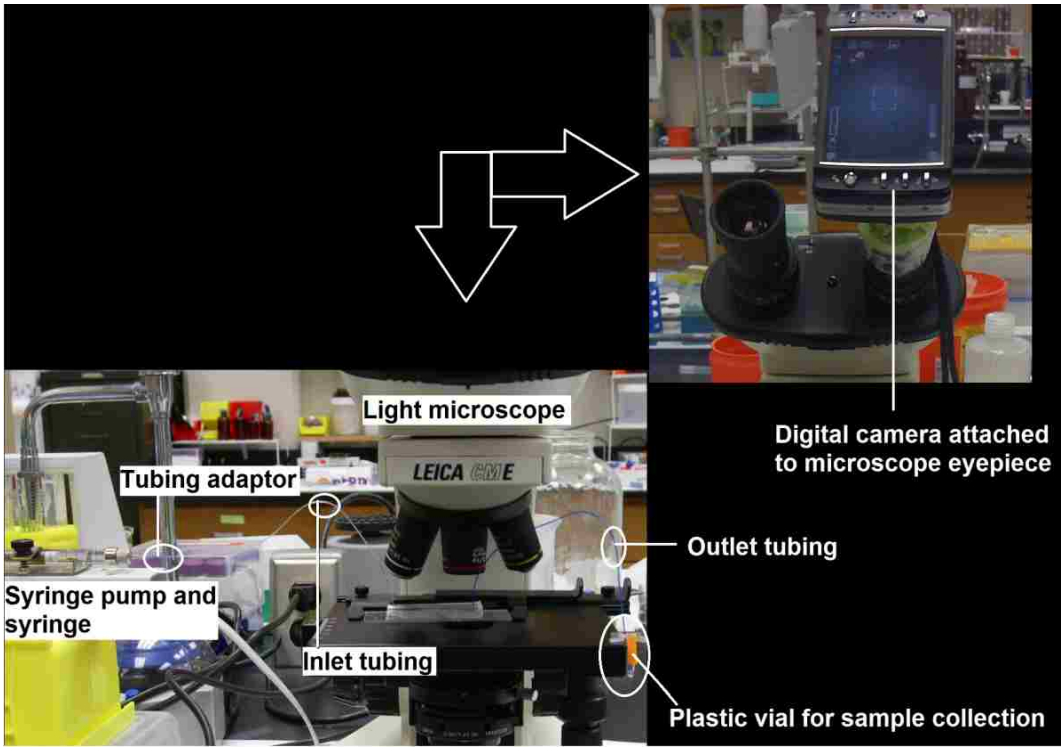

Figure 3.6 In-house made micro-video recording system

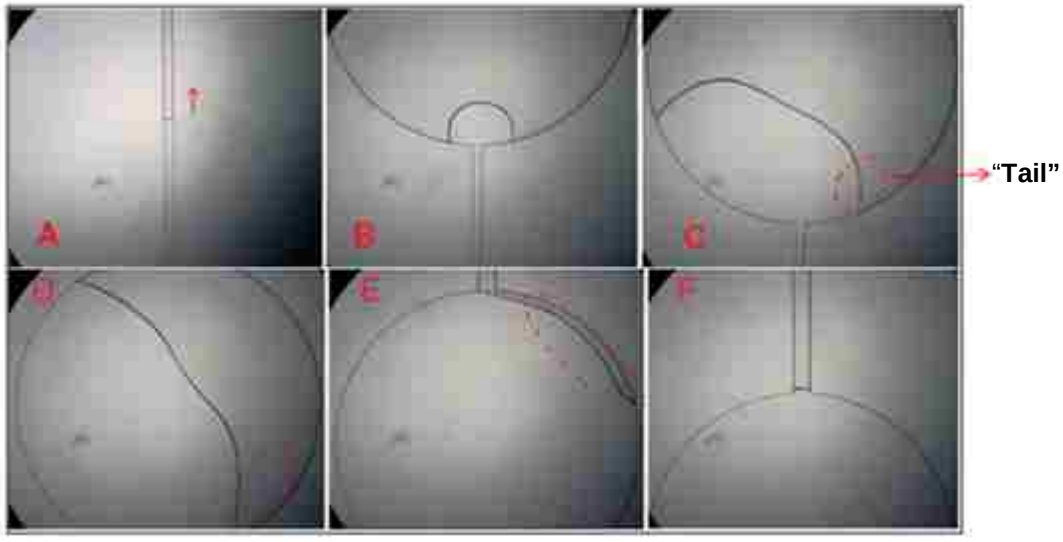

Figure 3.7 Snapshots of the liquid filling experiment for HPLC water pumped at 1.0 µL/min. The red arrow indicates the direction of the flow.

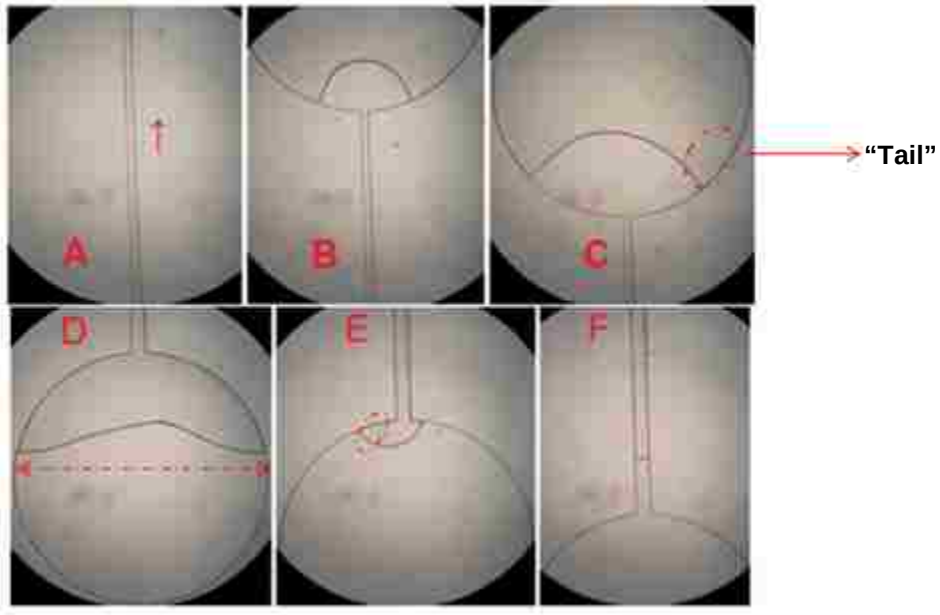

Figure 3.8 Snapshots of the liquid filling experiment for 10 mM PBS pH 7.6 pumped at 1.0 µL/min. The red arrow indicates the direction of the flow.

3.4.3 Online and offline collection of microspheres

To evaluate how coupling the microfluidic device to a BASi syringe system (rotator, syringe, and syringe pump) affected the concentration of amine functionalized polystyrene beads overtime at a fixed flow rate two set of experiments were conducted. An aliquot of 1.5 µL was taken from amine functionalized polystyrene microspheres of 6.0 µm from stock solution (6.57 x 10^8 beads/mL) and diluted in 998.5 µL of 10 mM PBS having 0.1% polysorbate 20 (Tween 20). The solution (~1.25 x 10^6 beads/mL) was split in two 1.0 mL solutions to be used in two different experiments. The amount of beads in these solutions was measured before and after collection using a hemocytometer. The set up for the experiments was as follows: First, BASi syringe was filled up with one of the 1.0 mL of amine functionalized polystyrene beads solution previously made for each experiment. After that the syringe was placed on a rotator to constantly shake the syringe and keep the amine functionalized polystyrene beads in suspension. A pumping flow rate of 5.0 µL/min was used for both experiments. Plastic tubing was attached to the syringe tip using a plastic adaptor for offline sampling or amine functionalized polystyrene beads collection.

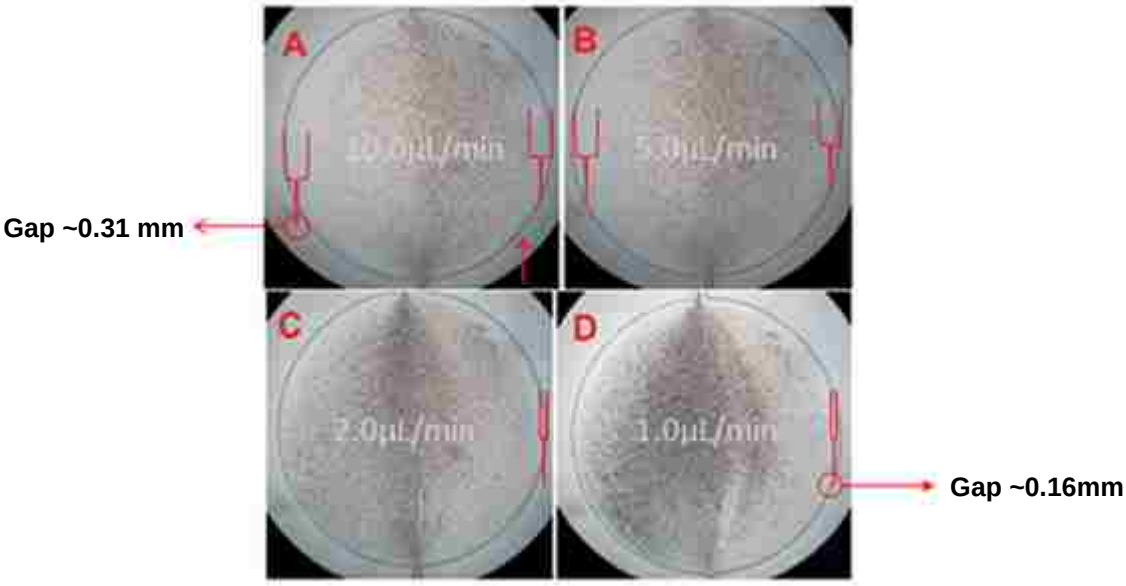

Figure 3.9 Snapshots taken from the video that was recorded during the perfusion of magnetic microspheres into the microfluidic system at different flow rates. The red arrow indicates the direction of the flow. A) Magnetic microspheres moving away from circular trapping area walls at 10.0 µL/min flow rate, B) magnetic microspheres moving toward circular trapping area walls at 5.0 µL/min flow rate, C) magnetic microspheres starting to spread evenly over the circular trapping area at 2 µL/min, and D) magnetic microspheres spreading evenly over the circular trapping area at 1.0 µL/min flow rate.

For online collection the syringe was coupled to the microfluidic device inlet and amine functionalized polystyrene beads were collected from the device outlet, see Figure 3.10. Amine functionalized polystyrene beads were collected in plastic vials every 15 min for 60 min from either the plastic tubing attached to the syringe tip or microfluidic device outlet. The concentration of amine functionalized polystyrene beads for each experiment was measured using a hemocytometer.

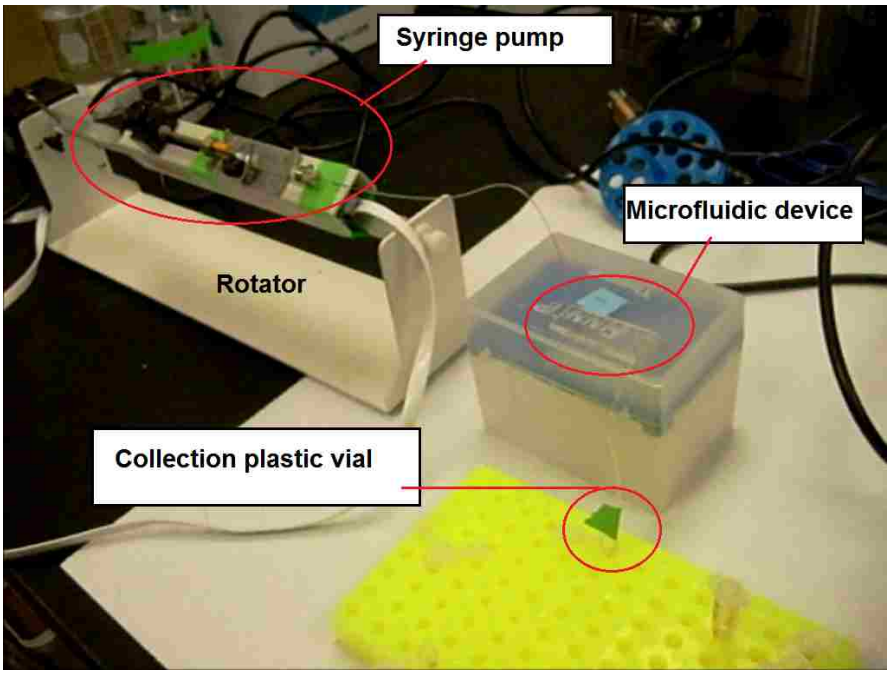

Figure 3.10 Experimental set up for the online and offline collection of amine functionalized polystyrene microspheres of 6.0 µm.

3.5 Results and discussion

3.5.1 Background of the design chosen

The possibility of using polymeric microspheres in the designed microfluidic system was studied. However, physical traps and hydrodynamic forces were necessary in order to trap and release polymeric microbeads from a trapping area after chemical analysis. For instance, one of the early ideas was to have a three channels microfluidic system with a trapping area, see Figure 3.11. The system was composed of one channel for the injection of the microspheres or inlet dialysate that would be coupled to the microdialysis outlet, another channel for injection of the dissociation buffer, and the outlet channel. To explain, the channel used for the injection of dissociation buffer, and the outlet channel would have widths smaller than 6 µm or any beads size chosen to trap the beads in the trapping area. The idea was to flush the microspheres out through the inlet (dialysate) channel by injecting a solution in both inlet channel

(dissociation buffer), and outlet at the same time. The drawbacks of this idea were the following: a) Channels with widths smaller than 6 µm are very expensive to fabricate (photomask), b) PDMS could collapse in such a narrow channel, c) Solution of microspheres and buffer must be injected at the same time (inlet dialysate and inlet dissociation buffer), d) dissociation buffer must be injected in both inlet channels as well to avoid bead loss, and e) automation was based on hydrodynamic flow control. It is good to point out that after reading Andersson et al article,[8] and evaluating the feasibility of using polymeric microspheres for the development of a trap and release microfluidic system using physical barriers magnetic microspheres were chosen. As stated on their article they used standard photolithography, bulk micromachining, deep reactive ion etching, and anodic bonding.[8] Compared to the two procedures used to make the microfluidic system (fluidic part) using magnetic microspheres on this thesis their system is more complex and difficult for this initial application. Even if the planar electromagnetic trapping system was added into the procedures, it would be easier to fabricate than the Andersson et al system. Also, using an electromagnetic system greatly facilitates the automation process. Furthermore, none of the previously mentioned research papers used a circular trapping area for their microfluidic systems. The advantages and disadvantages of using a circular trapping area for microfluidic applications can be seen on Table 3.1. Pant and his colleagues presented a poster called “System level Simulation of Liquid Filling in Microfluidic Chips”.[9] They used three different abrupt structures, sharp-rectangle, hexagon, and rounded-rectangle and studied the liquid filling behavior.[9] According to Pant, et al., sharp-rectangle abrupt structures or trapping areas tend to trap air bubbles at the corner.[9,10] In contrast, they found that hexagon, and rounded-rectangle abrupt structures did not trap air bubbles. Compared to sharp-rectangle trapping areas circular trapping areas do not trap air bubbles shown by this work.

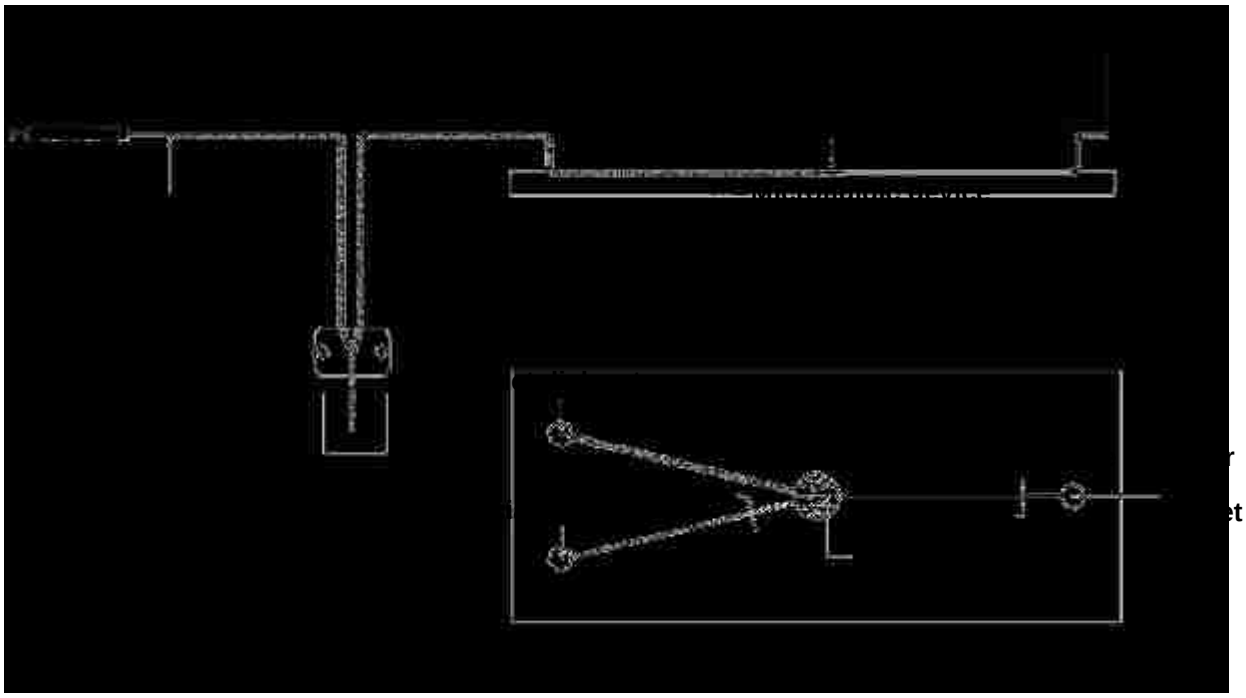

Figure 3.11 Earlier proposed microfluidic system with physical traps

Table 3.1 Advantages and disadvantages of the circular microfluidic system developed

Advantages	Disadvantages
Circular design avoid trapping air (sharp-rectangle chamber)[9]	Cannot withstand flow rates higher than 10.0 µL/min without leakage
Easy to fabricate and improve if needed	Only one injection port
Cheap and disposable	Channel walls have to be coated to avoid any non-specific absorption
Could be integrated to well-known magnetic trapping systems	Bonds between PDMS and glass could be broken when inserting the inlet or outlet tubing if care is not taken
Easy to automate or control via software	Not a well understood system
Biocompatible and autoclavable if needed	
Useful to study flow behavior in circular chambers	
Do not need thermal or oxygen plasma bonding	
Does not require complex fittings	

3.5.2 Pressure drop estimations

Channels and total pressure drop (ΔP) for the microfluidic system was calculated based on the Hagen-Poiseuille law, see Equation 3.1, for straight channels in series and analogy with the Ohm's, see Equation 3.2, and Kirchhoff's laws for circuit in series, see Figure 3.1, and Table 3.2. The following assumptions and constants were used: a) Water was flowing in the channels, b) Incompressible flow, c) trapping area cross sectional area was square, d) volumetric flow rate is constant, e) density of water = 1000 Kg/m^3, f) viscosity of water = 1 mPa.s, g) length of channel 1 and 3 = 20.7 mm, h) length of channel 2 = 3.6 mm, i) height of channels 1,2, and 3 = 100 microns, j) width of channel 1 = 100 microns, channel 2 = 3.6 mm, and channel 3 = 200 microns, and k) Q = 1.0 µL/min. Using equations 3.3, 3.5, 3.7, and 3.8 the total pressure drop was estimated, see Table 3.3. The Reynolds number for each channel was estimated using equations 3.4, 3.5, and 3.6, see Table 3.3. It was found that the total pressure drop is directly proportional to the flow rate (data not shown). For instance, the estimated total pressure drop for

0.5 µL/min was 71.13 Pa, and 1425.99 Pa for 10.0 µL/min. It is good to mention that if the cross section area of the trapping area (circular) were used on the pressure drop calculations the pressure would be slightly higher. Two sets of liquid filling experiments were conducted using HPLC water and 10 mM PBS pH 7.6 at a pumping flow rate of 1.0 µL. The snapshots of each set of experiments are shown on Figure 3.7 and 3.8. PDMS is hydrophobic, so it tends to reduce the fluid flow of water.

Table 3.2 List of equations and variables used for the estimations

Equation 3.1	Hagen-Poiseuille law	$\Delta p = R_{hyd} Q$	*Reference[1]
Equation 3.2	Ohm's law	$\Delta V = R\, I$	
Equation 3.3	Hydraulic resistance for a straight channel with rectangle cross section	$^{*}R_{hyd} = \frac{12\,\eta\, L}{1 - 0.63\left(\frac{h}{w}\right)} \frac{1}{h^3 w}$	
Equation 3.4	Reynolds number	$R_e = \frac{\rho V_0 D_h}{\eta}$	**Reference[11]
Equation 3.5	Mean velocity	$V_0 = \frac{Q}{A}$	
Equation 3.6	Hydraulic diameter of rectangular tubes	$D_h = \frac{2\,h\,w}{h + w}$	**
Equation 3.7	Conservation of flow rate	$^{***}Q = Q_1 + Q_2 + Q_3$	*
Equation 3.8	Simple additive law	$^{*}R_{hyd} = R_{hyd1} + R_{hyd2} + R_{hyd3}$	
Equation 3.9	Total pressure drop	$^{*}\Delta P_T = \left(R_{hyd1} + R_{hyd2} + R_{hyd3}\right) Q$	
Variables	R_{hyd} = Hydraulic resistance Q = Volumetric flow rate η = Dynamic viscosity *L = Length along the channel axis* *h* = Height of the channel *w* = Width of the channel A = Area of the channel	ΔV = Potential drop R = Electrical resistance *I* = Electrical current V_0 = Mean velocity ρ = Density L_0 = Length ***Subscripts refer to: Inlet = 1, Trapping area = 2, and Outlet = 3, see Figure 3.1	

Table 3.3 Results of the estimations for the microfluidic channels

Channel #	R_e	R_{hyd} (Pa.s.m^{-3}) x 10^{10}	ΔP (Pa)	ΔP_T (Pa)
1	0.17	671.35	112.2	142.60
2	0.01	1.22	0.20	
3	0.11	181.31	30.28	

Figure 3.7C to E show how the PDMS walls surface limit the fluid flow of water molecules due to the PDMS inability to form hydrogen bonds with water. To clarify, the flow path of the pumped HPLC water formed a “tail” that was diminishing as soon as it reached the outlet of the circular trapping area, see Figure 3.7. The hydrodynamic force generated by pumping HPLC water at 1.0 µL/min is high enough to break the resistance to fluid flow presented by PDMS (no data shown). In comparison, pumped PBS did show a slightly “tail” or lesser opposition to fluid flow by PDMS, see Figure 8. This could be explained due to the fact that typically PBS is composed of four inorganic salts (potassium chloride, potassium phosphate monobasic, sodium chloride, and sodium phosphate dibasic). This reduces the “repelling” effect that PDMS has for water molecules. In other words, the interaction between PDMS and PBS is higher than PDMS and water, because water molecules prefer the salt over the hydrophobic PDMS.

The video taken, see non-print materials, demonstrated that magnetic microspheres tend to focus on the middle of the circular trapping area at high flow rates (10.0 and 5.0 µL/min), see Figure 3.9–A and 3.9-B. On the other hand at lower flow rates (2.0 and 1.0 µL/min) magnetic microspheres tend to spread almost evenly over the circular area, see Figure 3.9-C and 3.9-D. The distance or gap between the walls (middle) of the trapping area and the stream of magnetic microspheres form next to it is inversely proportional to the flow rate, see Figure 3.9. Based on the diameter of the trapping area I was able to measure those gaps. For flow rates of 10.0 and 5.0 µL/min the gap remains approximately constant (~0.31mm). In contrast, when flow rates of 2.0 and 1.0 µL/min were used the same behavior was observed, but the gap was ~0.16mm.

These results helped to have a better understanding of how to control the magnetic microspheres behavior in the trapping area using hydrodynamic force and to determine how long it would take to have an evenly distributed magnetic microspheres layer. Similar behavior has been studied by several researchers for different applications such as beads separation ,[12,13] and flow behavior.[14] However, as mentioned before those studies used square channels. This kind of behavior has not been presented by any research group as far as the author knows.

In order to evaluate the variation of amine functionalized polystyrene microspheres concentration after coupling the microfluidic device to a BASi syringe system two set of experiments were performed.

The initial concentration of amine functionalized polystyrene beads counted before filling up the syringe for the offline collection was 1.25 x 10^6 beads/mL. For the online collection the concentration was 1.26 x 10^6 beads/mL. The total averages of the relative recovery of amine functionalized polystyrene beads collected were 66.60 and 66.90% for the offline and online experiments, see Figure 3.12. This indicated that there were not significant differences between the offline and online collection. To explain, coupling the microfluidic device to the BASi syringe system did not have any influence on the concentration of amine functionalized polystyrene beads. These experiments demonstrated that a uniform flow rate can be achieved when coupling the microfluidic system to a syringe system. Finally, it is very important to understand how magnetic microspheres behave in circular channels for the development of any trap and release systems for pre-concentration of biomolecules or other applications.

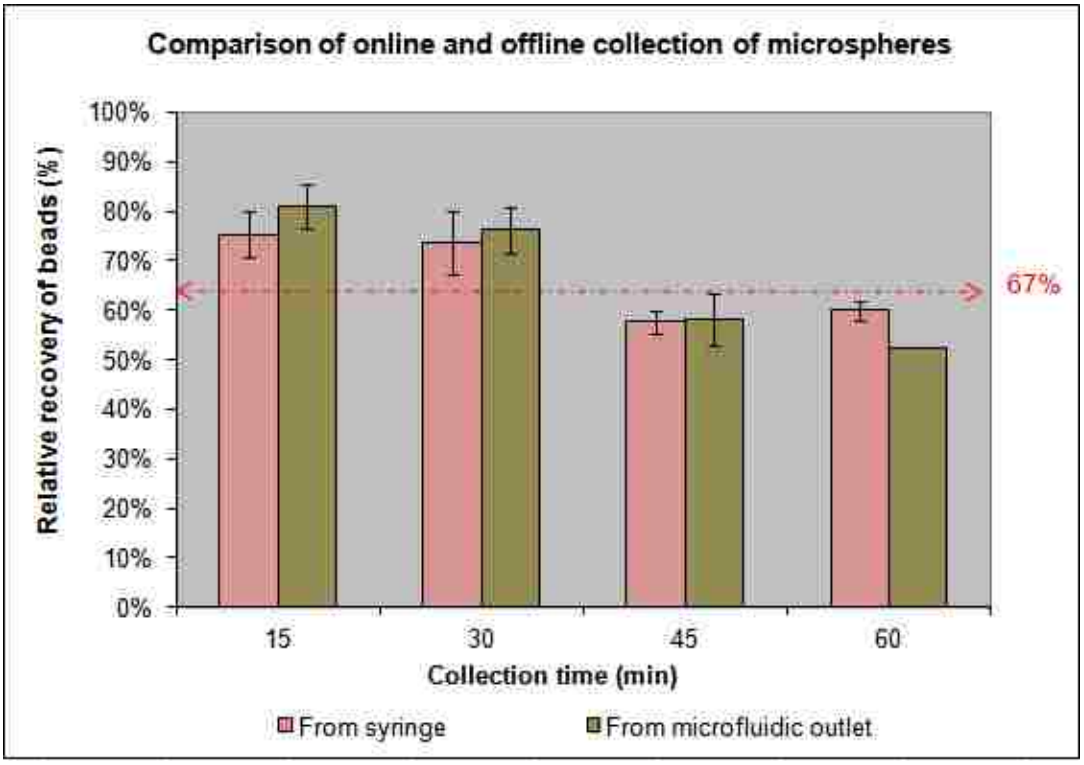

Figure 3.11 Comparison of the relative recoveries of online and offline collection of 6.0 µm amine functionalized polystyrene microspheres at 5.0 µL/min flow rate.

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4 Chapter 4. Conclusions and Future work

Cytokines play an important role in the immune response network generated during implants or prostheses, biosensors, and several disease states. The efficient collection and quantitation of cytokines during an immune response is crucial. This will allow the monitoring of cytokines concentration changes over time during any immune response. In other words, better understanding of the role that each cytokine has during an immunological response. However, this is a very difficult task due to the amount the variables involves in any biological system. Biological systems are very complex making the study of cytokines and collection of cytokines challenging. In order to study cytokines in their "native" environment during a specific state it is important to develop analytical tools that could facilitate the task. It is common practice, due to its simplicity, the collection and quantitation of cytokines from tissues and blood samples. The problem is that the concentration of cytokines measured from tissues or blood sample does not represent the "real" concentration of cytokines. It is more like an indirect measurement of the total concentration present within their biological environment. In their "native" biological environment the cytokine concentration is not constant due to their highly biological activity. If an ideal device or system is to be developed to collect and quantify cytokines it would have to have the following characteristics: a) Minimally invasive constant collection without disrupting their biological environment, b) constant measurement or quantitation of different cytokines (multiplexing) overtime with a lower limit of detection of picomolar and a upper of micromolar, c) easily to use, d) portable, and, e) low cost of fabrication. In reality some of these characteristic can be found on known systems. For example, microdialysis is commonly used to sample from tissue spaces. Microdialysis a diffusion based sample technique is limited to the diffusion properties of the cytokines in their biological environment and across the microdialysis membrane, the composition of the membrane (hydrophilic or hydrophobic), and the speed of the foreign body response caused by its "implantation" to mention a few. Research groups such as Dr. Stenken's group have been working on improving the collection time of cytokines when sampling with microdialysis and the recovery of cytokines. The bead-based method developed by her group increased the relative recovery of cytokines using heparin immobilized microspheres by two-fold *in vivo* and two to five fold *in vitro.*[1]

Quantitation of cytokines is commonly done by using ELISA. ELISA is a great tool to measure low concentration of cytokines (pg/mL range). However, ELISA is very labor extensive, expensive, time consuming (~19 hours), requires at least 100 µL of sample (longer collection time) and it is not robust. These facts add another problem to be solved in order to quantify cytokines efficiently.

Microfluidics is one of the fastest new research areas. This research area work on finding answers or developing tools to solve complex problems. It is of common knowledge that complex problems required sometimes more than one research area. For example, cytokines collection and quantitation requires people with knowledge in Biology, Analytical Chemistry, Engineering (fluid dynamics, microfabrication and electronics), and Physics (optics, binding interactions). However, researchers do not need to know the entire "book" if they only need one chapter to solve their problem. In other words, knowing what is needed of a specific area to solve a complex problem is what microfluidics brings. Microfluidic systems have several advantages: a) Low cost, b) easy to use, c) low sample volume, d) easy to fabricate, e) can be coupled to known analytical techniques, and f) well understood systems can be integrated into it.

The aim of this thesis was to use a bead-based method developed by Dr. Stenken's group and integrate it in a microfluidic system coupled to microdialysis sampling to pre-concentrate cytokines. The bead-based method improves the relative recovery of cytokines and the microfluidic system further pre-concentration cytokines to enhance their quantitation.

In Chapter 2 it was shown the ability to reproduce Duo's work by making heparin immobilized polystyrene and magnetic microspheres. Also, it was developed a system to determine the amount of heparin require to achieve ~100% of cytokine bound. This was related to the amount of heparin-immobilized microspheres needed as well based on the amount of heparin immobilized per bead. These beads were tested using equilibrium dialysis and a simple plastic vial incubation method used by Duo. It was estimated that the minimum amount of heparin required to achieve ~100% of cytokine bound was 3.68 µM. The comparisons of experimental and estimated values for ratMCP-1 (heparin immobilized polystyrene beads) were 4.25 and 15.08% difference for 0.1 and 1.0 nM. The percentages differences between experimental and estimated for ratMCP-1 and ratTNF-α were 28.31 and 31.56%, respectively.

The differences of the values could be due to the fact that reported binding constant values were used for the estimations. Part of the future work will be to measure cytokine binding constant to have a better “control” of the conditions.

A microfluidic system was developed and tested by pumping water and PBS. The hydrophobicity of PDMS was confirmed by looking at the opposition to fluid flow by the PDMS channels in the trapping area. Magnetic microspheres flow behavior at different flow rates was tested. The magnetic beads spread inversely proportional to the flow rate used.

The future work will be integration of a trapping, detection, and automation system to fully collect and quantify cytokines in real-time.

4.1 References

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