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Development of a Novel Reactor for removing Heparin during Extracorporeal Procedures

Manju Sunil Varghese

A dissertation submitted to the faculty of Engineering, University of Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD).

Johannesburg, 2007

DECLARATION

I declare that this dissertation is my own work. It is being submitted for the degree of Doctor of Philosophy (PhD) in the University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

_____

M. Sunil Varghese (nee Joshua)

_____ Day of _____ 2007.

DEDICATION

This work is dedicated in loving memory of my father, Mr. Joshua P. Abraham, who during his life was an inspiration to everyone who crossed his path.

ABSTRACT

Heparin administration during extracorporeal procedures such as kidney dialysis and heart surgeries is a challenging problem, as under heparinisation leads to clotting and over heparinisation may cause excessive bleeding complications. Various reactor designs including the use of heparinase and poly-L-lysine.HBr hollow fiber have been proposed, however none of them have been clinically approved yet.

This work presents the possibility of designing a packed bed reactor with poly-L-lysine/alginate beads. The poly-L-lysine/alginate beads have been used widely in fields of microencapsulation of cells and can be made using techniques such as the Initech encapsulation and layer by layer approach. In some cases ginipin has been used for microcapsule preparations to provide stronger cross linking. In this study, the poly-L-lysine beads are made using the encapsulation procedure patented by Chang[18] (1992). Batch experiments, using saline, fetal calf serum and blood were performed to investigate the efficiency of the beads. The absorption of heparin was determined to be a first order absorption process and fits the Freundlich Isotherm. The beads were determined to be relatively safe in blood. The absorption of heparin was linked to the poly-L-lysine content in the membrane. Thus the rate of the amount of heparin absorbed could be increased by increasing the membrane thickness or by increasing the number of poly-L-lysine/alginate beads. The results were used to investigate the feasibility of using a packed bed reactor for the absorption process by adjusting the specifications of the reactor and analysing simple flow models.

ACKNOWLEDGEMENTS

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CHAPTER 1: INTRODUCTION

1.1. INTRODUCTION

Exposure of blood to foreign objects in extracorporeal devices such as the kidney dialyser usually leads to the formation of clots. Anticoagulants such as heparin are injected into the patients' blood during such procedures to prevent clotting. Heparinisation is a major problem as under-coagulation may result in clotting while over-heparinisation increases the risk of haemorrhage. This problem arises from the difficulty of monitoring the adequacy of anticoagulation during the short period of hemodialysis. Approximately 20 million extracorporeal procedures are performed each year, of which between 8 to 30% of bleeding complications arises due to systematic heparinisation.

Heparin is not prescribed to patients with low platelet numbers or with bleeding disorders. Patients with some source of bleeding such as recent surgery, injury or ulcer are also in danger of excess bleeding when administered with heparin. Side effects of heparin include unusual bleeding or bruising, blood in urine or stools, abdominal or back pain, heavy menses and skin irritation. Alternate modalities, instead of the standard heparin anticoagulation, are used; however all of them have disadvantages and complications. It would be useful to have a system that would eliminate heparin after the standard anticoagulation procedure is administered. Various reactor designs including the use of heparinase and poly-L-lysine.HBr hollow fiber have been proposed, however none of them have been clinically approved yet.

1.2. AIM OF PROJECT

The aim of this project is to perform experiments for the development of a reactor that would permit full heparinisation of blood entering any extracorporeal device, but enable the elimination of the heparin before the blood returned to the patient

This work presents the possibility of designing a packed bed reactor with poly-L-lysine/alginate beads. Batch experiments, using saline, fetal calf serum and blood were performed to investigate the efficiency of the beads.

1.3. THESIS CONTENTS

The thesis comprises of three articles. Each chapter (article) comprises of a literature review, an introduction, apparatus and methods, results, discussion and a conclusion.

Chapter 3 (Article 1) investigates the possibility of using poly-L-lysine/alginate beads to extract heparin from saline solution. Chapter 3 presents the results and discussions of mass transfer experiments that were performed to test the feasibility of using poly-L-lysine/alginate beads to remove heparin. The experimental data was fit to a first order absorption model. The experiments were repeated several times and the average standard deviation of the results were less than 2%.

Chapter 4 (Article 2) investigates ways of optimizing the absorption procedure. Chapter 4 presents the results and discussion of experiments where the physical parameters, like the number of the beads, volume, surface area and the size of the beads were varied. The findings were related to the membrane thickness of the beads. Methods of increasing the membrane thickness and thereby optimizing the process were investigated.

Chapter 5 (Article 3) explores the feasibility of the proposed reactor by utilizing theoretical models for the flow process and the results obtained from the previous chapters. Various feasible reactor options are investigated for optimum heparin removal.

CHAPTER 2: APPARATUS AND METHODS

2.1. INTRODUCTION

The apparatus used for the project can be divided into three main categories: calibration, formation of poly-L-lysine/alginate beads and the mass transfer experiments. All the experiments were performed in the chemical pathology lab at the University of Witwatersrand Medical School. The required chemicals were supplied by Sigma. The concentration range of heparin used in the experiments varied from 0U/mL to 3U/mL, which is higher than the expected clinical values. Heparin was supplied by the renal unit of Johannesburg General Hospital.

2.2. CALIBRATION

The concentration of unfractionated heparin in saline and fetal calf serum was determined by using the calibration method of Ameer et al[26]. A spectrometer (Figure 2.1) was used to obtain the absorbency of a required sample of heparin at a particular wavelength. The absorbance of unfractionated heparin corresponding to the concentration ranges of 0U/mL to 3U/mL is linear (Ameer et al[26]., 1999).

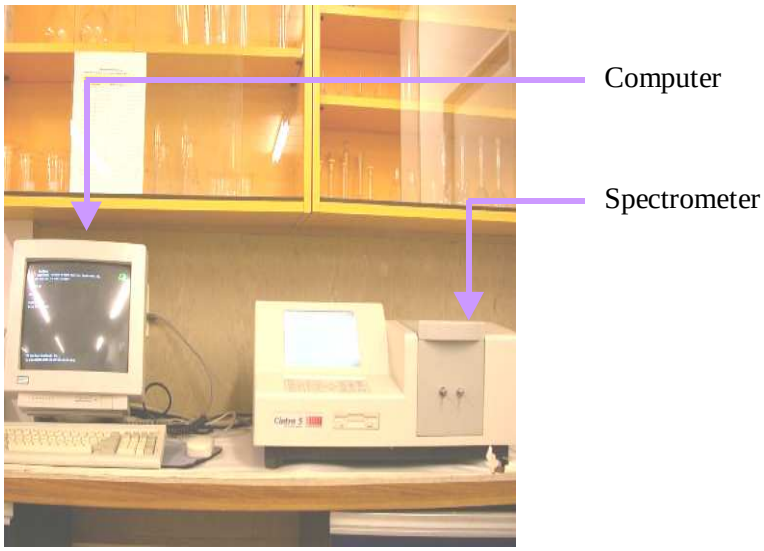

Figure 2.1 Cintra 5 UV-VIS Double Beam Spectrometer

1 mL quartz cuvettes were used to obtain a 1cm path length. Two of these cuvettes were used for the duration of all the experiment to minimize the variables. One of the cuvette was used as a blank, while the other was used to obtain the required absorbance.

2.2.1 Method for calibration

Standard solutions of 3U/mL, 1.5U/mL and 0.75 U/mL of unfractionated heparin were prepared in 100mM MOPS and 5mM-calcium acetate (pH of 7.4) saline solution. A solution of 0.01mg/mL of Azure II dye was also prepared using distilled water (Figure 2.2). 900 µL of Azure II dye was mixed with 100 µL of the heparin sample solution in a 1 mL quartz cuvette (path length of 1cm). The mixture was incubated at room temperature for 1 minute. The absorbance of the mixture was then measured using the spectrometer at the selected wavelength. The known concentrations of heparin were plotted against the obtained values of the absorbance (Figure 2.3) to obtain the expected linear plot.

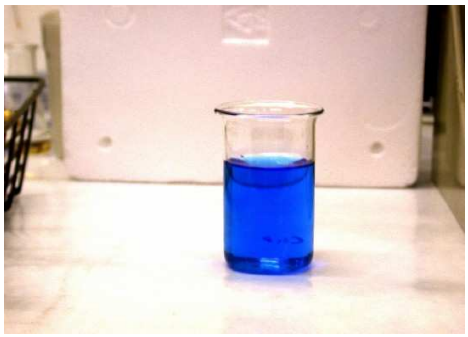

Figure 2.2 A beaker containing 0.01 mg/mL of Azure II dye solution

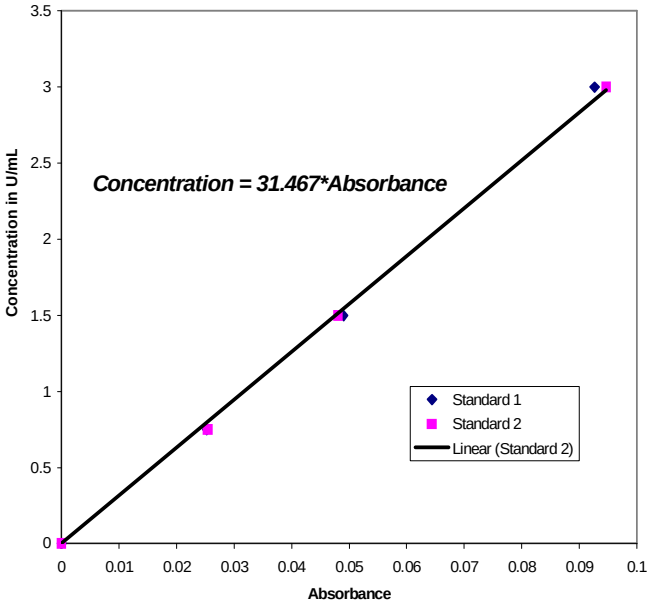

Figure 2.3 Calibration Curve of unfractionated heparin in saline

Refer to Appendix A1 for the tabulated values of the above calibration curve.

2.2.2. Determination of the required wavelength

A sample of known concentration of heparin was mixed with azure II dye as described previously in section 2.2.1. The wavescan of the sample was obtained using the spectrometer. The wavescans obtained for 0U/mL, 1.5 U/mL and 3U/mL are illustrated in Figure 2.4.

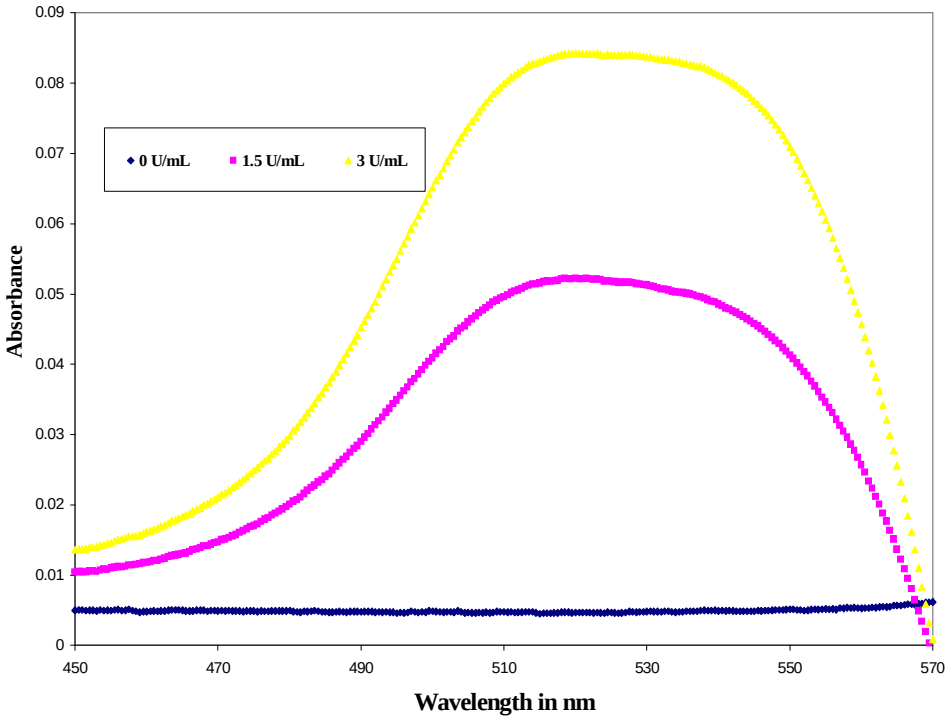

Figure 2.4 Wavescans of 0U/mL, 1.5 U/mL and 3 U/mL of unfractionated heparin in Saline

From Figure 2.4, a wavelength of 520 nm was selected to measure the absorbance of the heparin samples.

Plastic cuvettes could be used for analysis, because the selected wavelength is in the visible range; however these cuvettes were easily scratched and damaged causing drastic changes to the absorbance values. Quartz cuvettes were used to avoid these problems and for consistency.

2.2.3. Timescan of unfractionated heparin in saline

A timescan for a known concentration of heparin was performed to observe whether the absorbance was time dependent. The timescan was done for a period of 0 to 300 seconds

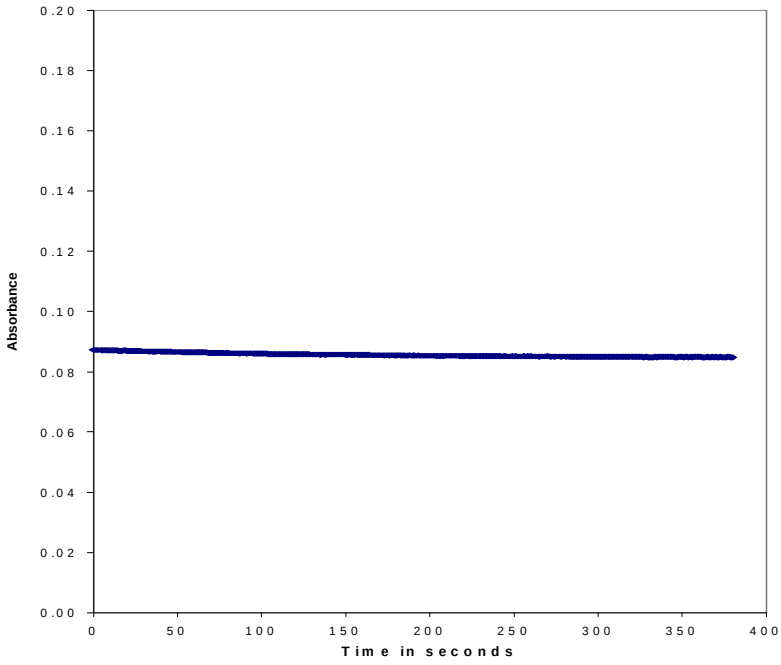

Figure 2.5 Timescan of a sample of unfractionated heparin in saline

The absorbance of the mixture of heparin and Azure II dye was determined to be independent of time from the above time scan.

2.3 FORMATION OF POLY-L-LYSINE/ ALGINATE BEADS

The standard encapsulation procedure patented by Chang[29] in 1992 was used to produce poly-L-lysine/alginate beads. The apparatus used is illustrated in Figure 2.6.

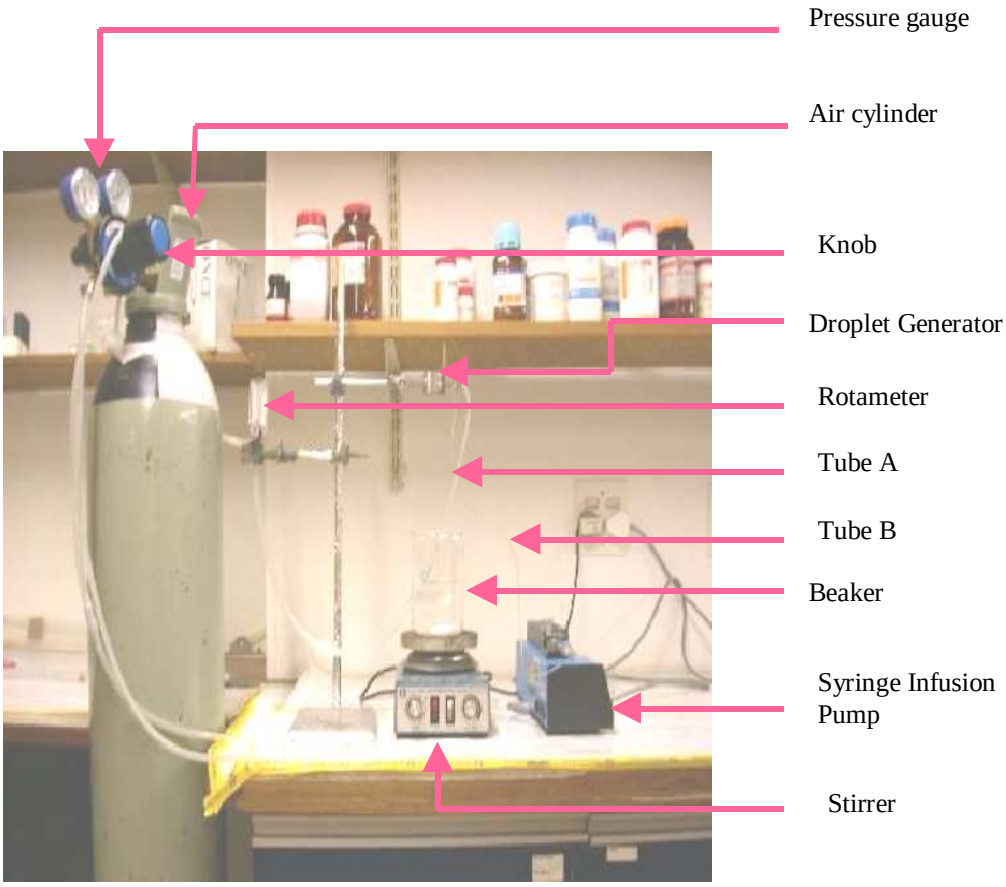

Figure 2.6 The apparatus used to produce the poly-l-lysine/alginate beads

Flow of air from the air cylinder was controlled by a manifold dial, and was measured in L/min using a rotameter. Air flowed into the droplet generator through tube A. Sodium alginate was pumped by the syringe infusion pump through tube B to the droplet generator. The droplet generator allowed contact of air with sodium alginate solution and produced beads in spherical form.

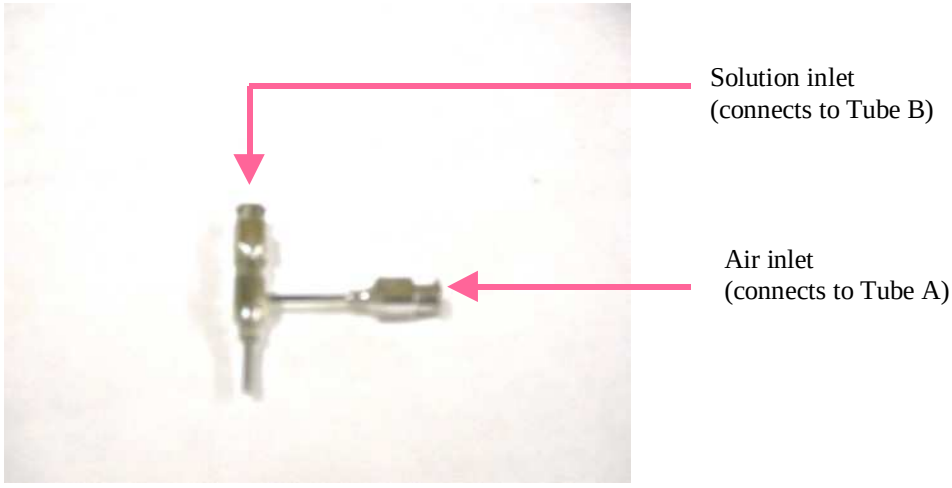

Figure 2.7 Droplet generator

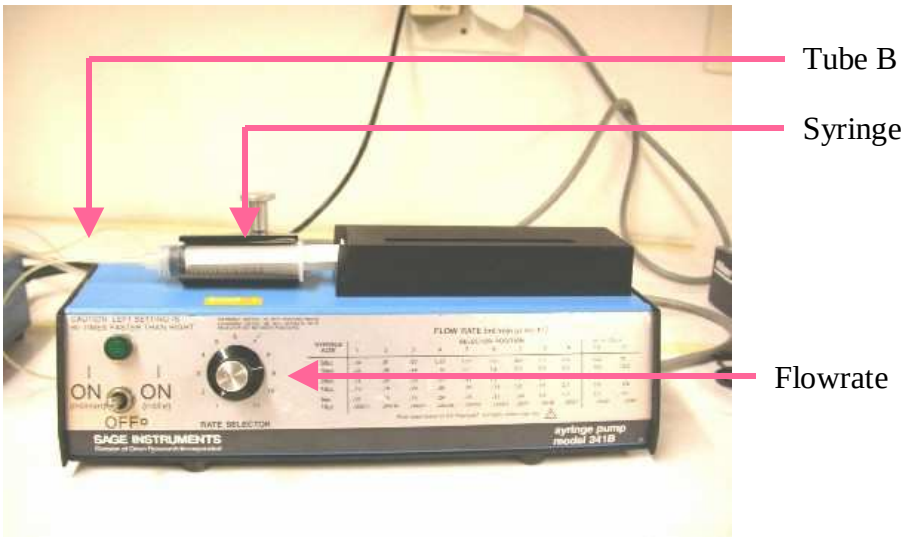

Figure 2.8 Front view of the syringe infusion pump

2.3.1 Method to form poly-L-lysine/alginate beads *(Chang[29]., 1992)*

Equal amounts of saline and 4% sodium alginate (Appendix A2) solution were mixed in a 10mL syringe. The solution was extruded with the syringe infusion pump through the droplet generator. The flowrate of the alginate solution was in a range of 0.1mL/min to 0.4mL/min, while the air from the cylinder was between 0.5L/min and 4L/min, depending on the bead size that was required. The droplets of beads were

allowed to fall into 300 mL of 100mM calcium chloride solution (Appendix A2), where calcium alginate, a spherical gel-like substance, was formed. The beads were then left in the calcium chloride solution for 15 min with continuous stirring. The beads were strained and washed with saline, and placed in 80 mL poly-L-lysine solution (Appendix A2) for 20 min. This was the stage where the membrane of the beads was formed. The beads were then strained and washed and placed in 200 mL of 0.2% sodium alginate solution (Appendix A2) for 5 min to form cross-links with the poly-L-lysine membrane. The beads were strained and washed and placed in 200mL of 50mM sodium citrate solution (Appendix A2) for 6 min to remove the gel like substance (Calcium alginate) from the beads, thereby producing "empty" poly-L-lysine/alginate beads.

The beads were then stored in saline solution until they were used in experiments. If the beads were required to encapsulate an enzyme, the initial stage of mixing was altered by the addition of 4% sodium alginate solution to an equal amount of the enzyme solution (instead of the saline solution). The size and number of beads can be altered by changing the air and solution flow rates. The number of beads was estimated by counting the droplets that fell out of the droplet generator, while the size of the bead was measured using a light microscope, fitted with a ruler.



Figure 2.9 Poly-L-lysine/alginate beads in a drop of saline solution.

The beads formed were spherical with a uniform size distribution.

2.4. BATCH MASS TRANSFER EXPERIMENTS

Mass transfer experiments were performed on a batch of beads using the following apparatus.

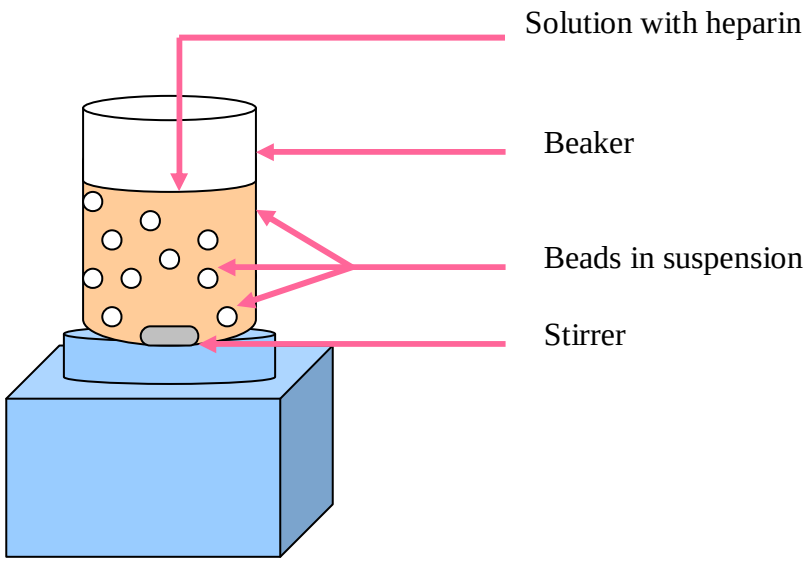

Figure 2.10 Schematic Diagram of the apparatus used for mass transfer experiments

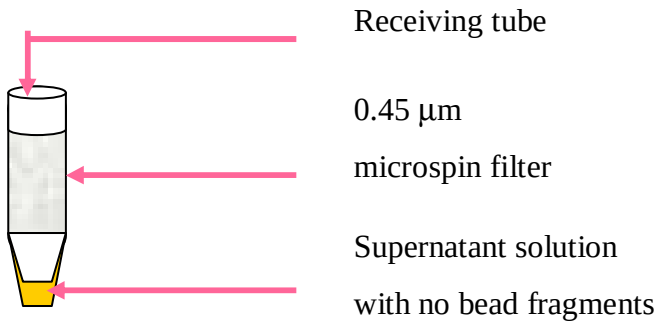

Figure 2.11 Schematic diagram of the microspin filter and the receiving (centrifuge) tube

2.4.1. Method for the mass transfer experiments

A known volume of saline or blood solution containing heparin was added to a known volume of uniformly sized beads. The contents in the beaker were then stirred to ensure that the beads were in suspension and to maintain uniform exposure of heparin to the beads. Samples of 300 μL of the supernatant solution were taken out at time intervals from 0 seconds to 1.5 hours, using a pipette. The samples were placed in centrifuge tubes containing 0.45μm microspin filters. These samples were centrifuged at 4000 rpm at $25^{O}C$ for 5 min to filter out the fragments of beads from the supernatant solution. The samples were then analyzed using the spectrometer. Absorbencies obtained from the spectrometer were used to obtain the concentration of heparin in the samples from the calibration curve (Figure 2.3).

The calibration curve was analyzed and plotted prior to all the experiments to check for variances.

2.4.2. Efficiency of pipettes

An experiment to check the accuracy of the pipettes was performed. Ten samples of 900 μL of water were taken out using the 900 μL pipette. These samples were then weighed. The expected mass was 900g since the density of water is $1000kg/m^3$. A curve of the expected mass versus the mass obtained was plotted as shown in Figure 2.12.

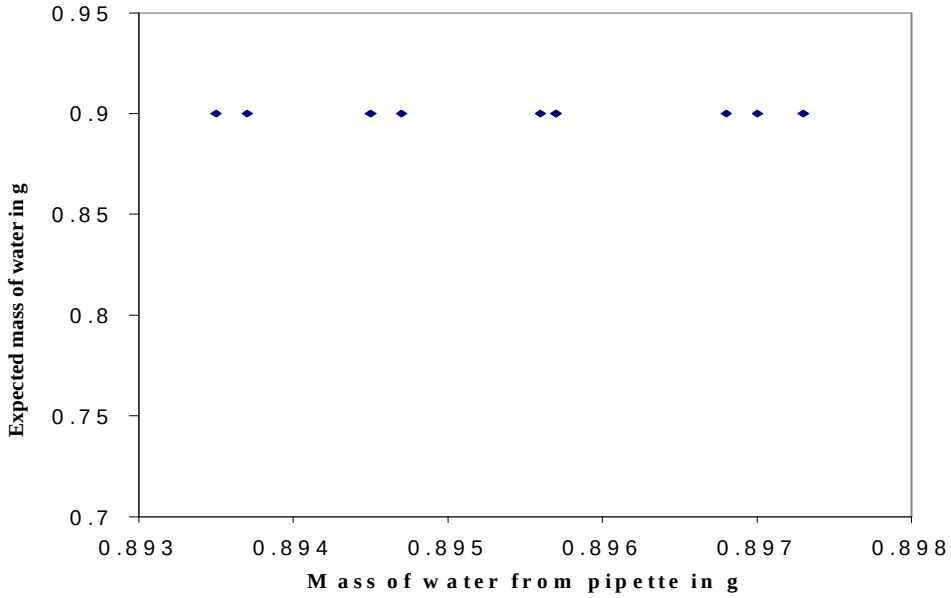

Figure 2.12 A curve of the expected mass of water versus the weighed mass of water for a 900 µL pipette

Refer to Appendix A3 for the tabulated values of the above curve.

From the above curve, it is evident that the 900 µL pipette is accurate. The standard deviation for the above values is approximately 0.13%.

Similarly a curve for the 100 µL pipette was obtained using the same procedure described above. (Appendix A4). The standard deviations for the 100 mL pipette were 0.025%.

The pipettes were therefore reliable and accurate.

2.5. CONCLUSION

The above analytical techniques and the technique used to form the poly-L-lysine/alginate beads were followed throughout the duration of this project. Repeatability and reliability of the techniques was good.

It is recommended that the apparatus used to form the beads must be improved as a slight change in the position of the droplet generator; or the flowrates of the solution and air changed the shape and size of the beads. The beads' size was obtained using a microscope after the beads were formed. If the beads were not of the required size, the entire procedure had to be repeated. It would be beneficial to design an apparatus to produce a constant amount of beads with the required size automatically.

CHAPTER 3: EFFICIENCY OF POLYMER BEADS IN THE REMOVAL OF HEPARIN

The following article has been submitted to the Journal: "Artificial Cells, Blood Substitutes and Biotechnology" and is currently being reviewed. This paper focuses on the possibility of using poly-L-lysine/alginate beads to remove heparin. The experiments were performed to measure the removal rates of heparin, test the efficiency of the beads, determine the repeatability and to identify the factors that could influence the removal rate of heparin.

EFFICIENCY OF POLYMER BEADS IN THE REMOVAL OF HEPARIN: TOWARD THE DEVELOPMENT OF A NOVEL REACTOR

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3.1. ABSTRACT

Administration of heparin during extracorporeal procedures increases the risk of haemorrhage. Various reactor designs, including the use of heparinase and poly-L-lysine.HBr hollow fiber have been investigated for the removal of heparin prior to the blood being returned to the patient; however none of them have been implemented clinically. In this paper it is proposed that beads made from poly-L-lysine/alginate can be used to remove the heparin. The aim of this work is to perform the necessary experiments in order to get the information required to design a heparin removal reactor which uses these beads. The experiments are aimed at measuring the removal rates of heparin by the beads, testing the efficiency of the beads to remove heparin, determining repeatability and identifying factors that could influence the removal rate. Batch rate experiments using poly-L-lysine/alginate beads in saline solutions were performed to investigate the removal rate of heparin. The results, which indicate that heparin is efficiently removed, may lead to improved bioreactor designs.

3.2. INTRODUCTION

Heparin is introduced into the blood during the use of extracorporeal devices such as renal dialysis, to prevent clotting resulting from the blood contacting foreign surfaces such as the dialyser membrane. However, hheparinisation during extracorporeal procedures is a challenging problem since under-coagulation may result in clotting within the extracorporeal circuit, while over-heparinisation exposes the patient to risks of prolonged bleeding (Swartz[1]., 1990).

Heparin is a heterogeneous group of straight-chain anionic mucopolysaccharides called glycosaminoglycans having anticoagulant properties.

The main sugars occurring in heparin are (Elkins Sinn [2].,2004):

- α-L-iduronic acid 2-sulphate,
- 2-deoxy-2-sulphamino-α-D-glucose 6-sulphate,
- β-D-glucuronic acid,
- 2-acetamido-2-deoxy-α-D-glucose, and
- α-L-iduronic acid.

Heparin is strongly acidic because of its content of covalently linked sulphate and carboxylic acid groups.

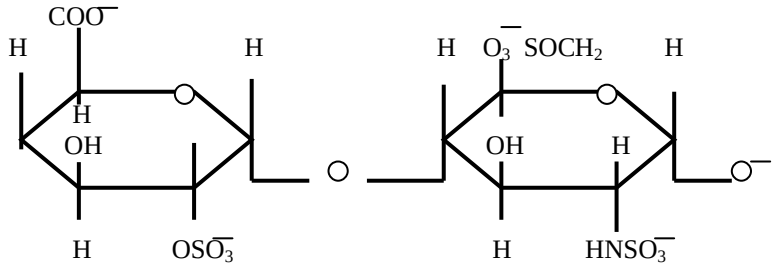

Figure 3.1: Chemical structure of a single unit of unfractionated heparin (adapted from Quader et al[3]., 2002)

Heparin is heterogeneous with respect to molecular size, anticoagulant activity and pharmacokinetic properties. The molecular weight of heparin ranges from 3000 to 30000. The anticoagulant activity of heparin is heterogeneous because only one third of the heparin molecules administered to the patients have an anticoagulant profile (Hirsh et al[4]., 2001).

Heparin administration is the standard therapy for the treatment of deep vein thrombi (Hemostasis & Thrombosis Center[5]., 2002).

The main clinical uses of heparin (Rxmed [6]., 2004) are:

- Acute phase myocardial infarction;
- Prevention and treatment of venous and arterial thrombosis;
- Treatment of pulmonary embolism;
- Prevention of pulmonary embolism and deep vein thrombosis in pregnancy;
- Management of complications of arterial diseases such as atherosclerosis;
- Extra-corporeal therapies, such as heart-lung oxygenation and renal dialysis;
- Open heart surgery;
- Deep phlebitis (inflammation of the walls of the vein);
- Vitreoretinal surgery;
- Topical application in pruritus (itching); and
- Topical application for leg ulcers.

The basic analytical method to measure heparin in saline was demonstrated by Jaques et al[7]. A 2mL aliquot of saline, 2mL of phosphate buffer (pH 7.3) and 1 mL of Azure A solution (100mg% Azure A diluted to 1:8.5) was added to a given volume of heparin solution. The solution was mixed and transferred to a glass cell. The colour of the cell solution was matched immediately in a lovibond tintometer using the red and blue glasses. *A lovibond tintometer is a colorimeter, which uses colour measurement for analysis of substances.* The heparin content of the sample was determined from the red reading to provide a standard curve of the following shape.

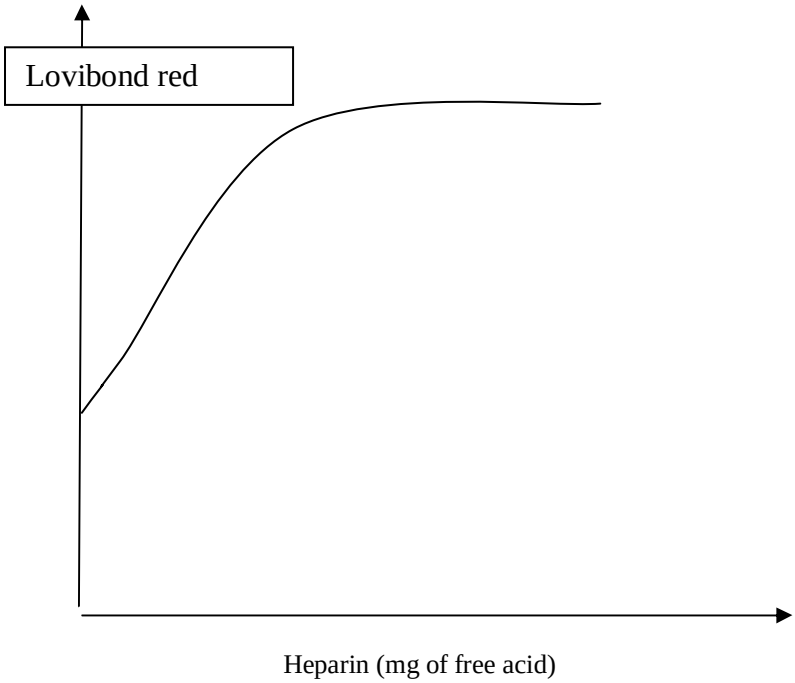

Figure 3.2: Schematic diagram of the standard curve obtained from Jaques[7]

A modification of the above method was developed by Ameer et al[8] in 1999 to measure the concentrations of unfractionated heparin in saline. A 4.5 mL volume of Azure II dye solution (0.01mg/mL) was added to 0.5 mL of the heparin solution to be tested. The azure II dye measures the negative sites of heparin. The sample was mixed and incubated at room temperature for 1 min before measuring the absorbance at 500nm. A standard curve for this assay was prepared by using solutions of known heparin concentrations ranging from 0 to 3 U/mL. The standard curve was linear in the above range.

Attempted solutions to anticoagulation during hemodialysis include systemic heparinisation, regional heparinisation, low-dose heparinisation, heparin-free hemodialysis in patients with high risks of bleeding, (Swartz[1]., 1990), administering protamine to neutralize heparin and administering antithrombic drugs other than heparin (Langer et al[9]., 1982). However the safety and efficiency of these methods

remain debatable. One of the major barriers to the optimal dosing of heparin arises from the difficulty of monitoring the adequacy of anticoagulation during the short (usually three-to-four hour session) period of hemodialysis. (Low et al[10]., 1996) The use of heparin and its antagonist protamine has been associated with potentially fatal complications in high-risk patients such as those suffering from acute renal failure or those who have undergone recent surgery. (Broyer et al[11]., 1986)

The development of a safe and efficient bioreactor design for heparin removal has remained a challenge. Such a reactor would permit full heparinisation of blood entering any extracorporeal device but would enable the elimination of the heparin before the blood returned to the patient (Langer et al[9]., 1982). Immobilized heparinase I specifically degrades heparin resulting in non-toxic products (Larsen et al[12]., 1986). Another alternative is to bound protein to the walls of a hollow fiber dialyser to enhance the whole blood compatibility of the system, but the surface area requirements remain impractical (Vallar et al[13]., 1996). Porous particles such as agarose or cellulose beads have been proposed as a way to optimize the protein or absorbent loading per volume of device (Gejyo et al[14]., 1993; Langer et al[9]., 1982). Heparin can also be neutralised by addition of polyanions, enzymes or resins to clinical samples (Wenz et al[15]., 1991). Immobilised protamine (Yang et al[16]) and poly-L-lysine ligand coupled to agarose substrate (Rodger et.al[17]., 1994) are other methods employed to remove heparin from blood. However according to the authors, additional research is required to use these methods for clinical purposes.

In order to expand on the above possibilities, it is proposed to use poly-L-lysine beads to remove heparin from saline solutions. The poly-L-lysine beads are made using the encapsulation procedure patented by Chang[18] (1992), and utilized by Vally et al[19] (2005) in South Africa to encapsulate liver cells. Batch rate experiments using the poly-L-lysine/alginate beads will be performed to obtain the necessary parameters required for the design of the reactor. The experiments in this article are designed to test issues like the efficiency of the beads to remove heparin, repeatability and some of the factors that could influence the rate.

3.3. APPARATUS AND METHODS

3.3.1 Calibration

Concentration of unfractionated heparin was determined using an Azure II dye assay as described by Ameer et al[8]., 1999a. A cintra 5 UV-VIS double beam spectrometer was used to measure the absorbency of heparin. A mixture of 900µL of Azure II dye (0.01mg/mL) and 100µL of the heparin solution under test was placed in a 1mL-quartz cuvette. The absorbency was then measured at a wavelength of 520 nm. A standard curve for this assay was prepared by using solutions of known heparin concentrations ranging from 0 to 3U/mL. The standard curve was linear for this concentration range.

3.3.2 Formation of poly-L-lysine/alginate beads

The standard encapsulation procedure patented by Chang[18] in 1992 was used to produce poly-L-lysine/alginate beads.

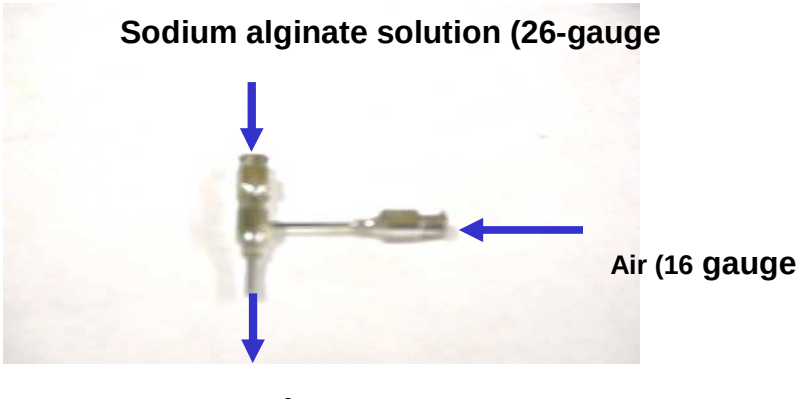

Figure 3.3: Droplet Generator

The droplet generator (Figure 3.3) allowed contact of air with sodium alginate solution and produced beads in spherical form.

Equal amounts of saline and 4% sodium alginate solution were mixed in a 10mL syringe. The solution was extruded from the syringe by a syringe infusion pump (Harvard apparatus; Millis, Mass) and pumped through the 26-gauge needle of the droplet generator. Air was allowed to flow through the 16-guage needle of the droplet generator. The flowrate of the alginate solution was in a range of 0.1mL/min to 0.4mL/min, while the air from the cylinder was between 0.5L/min and 4L/min, depending on the required bead size. The droplets were allowed to fall into 300 mL of 100mM calcium chloride solution and cured in the calcium chloride solution for 15 min. The beads were strained using a 0.45 μm mesh filter, washed and placed in 80-mL poly-L-lysine solution for 20 min. The beads were then strained and washed and placed in 200 mL of 0.2% sodium alginate solution for 5 min. The beads were strained and washed for a final time and placed in 200mL of 50mM sodium citrate solution for 6 min. The beads were then stored in saline solution until they were used in experiments.

3.3.3 Batch rate experiments

The aim of the rate experiments is to illustrate the efficiency of the poly-L-lysine beads and to fit a model to obtain the intrinsic rate. Although the rate limiting step cannot be identified, it could be expected that the magnitudes of rates measured in the batch experiments would be achieved in a well designed reactor. The rate will depend on parameters such as concentration of heparin in the solution, time and the number of beads or the ratio of the volume of beads to the volume of solution. The experimental apparatus will be designed to reduce, and if possible eliminate, the mass transfer limitations between the beads and the solution by stirring the solutions. In this way the intrinsic removal rate that will be achieved in a reactor can be measured.

Saline solution containing a known concentration of heparin was added to a known volume of uniformly sized beads. The contents in the beaker were then stirred to ensure that the beads were in suspension and to maintain uniform exposure of heparin to the beads. Samples of 300μL of the supernatant solution were taken out at time intervals from 0 seconds to 1.5 hours. The samples were centrifuged to separate the

fragments of beads from the supernatant solution. The supernatant samples were then analyzed using the spectrometer.

As the experiments are aimed at measuring data that would be needed for the reactor design, the experimental programme is set up so that the measurements, analysis and interpretation are iterative. This approach is called process synthesis and the advantage of it is that one can gain relevant data for the design more quickly and efficiently.

3.4. RESULTS

3.4.1 Initial Measurement of Rate of Removal of Heparin

A fixed number of beads were used in a fixed volume of saline containing heparin. The aim of this experiment was to get an estimate of the magnitude of the removal rate of heparin by the beads and thereby establish whether the beads can be used for the purpose of removing heparin from a reactor. Approximately 8000 $\pm$ 1000 poly-L-lysine/alginate beads of size 900μm $\pm$ 100μm were placed in 40-mL saline solution with a heparin concentration of 3 U/mL, which was stirred continuously at room temperature. Aliquots of 300 μL of the heparin solution were pipetted out at intervals from zero seconds to one and half hours. The absorbency of the samples was analyzed at a wavelength of 520 nm using the spectrometer. The results obtained from this experiment are shown in Figure 3.4 and tabulated in Appendix B1.

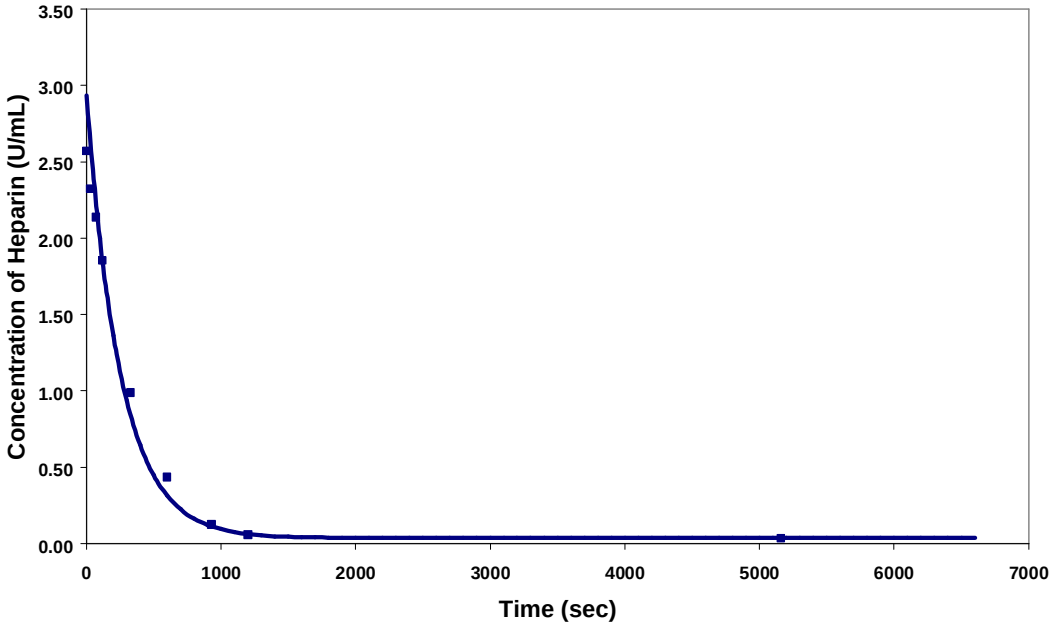

Figure 3.4: Graph of heparin concentrations during the rate experiment using 8000 beads and 40 ml of 3U/mL heparin solution.

The results indicate that the beads can efficiently remove the heparin from the saline solution. The concentration of heparin in solution was reduced by half in about 100 seconds which implies that the removal rate is fast enough for a practical design. Equilibrium was attained after approximately 1000 seconds. The average standard deviation for the results obtained was less than 2%.

The next set of experiments were performed to test the consistency of the results The rate experiments were repeated using constant variables, such as the number of beads (850 $\pm$ 100 beads), volume of the heparin solution (23-mL), concentration of the heparin in the solution (3U/mL) and the size of the beads (900µm $\pm$ 100µm). The number of beads used in this set of experiments was decreased from 8000 (used in the previous experiment) to 850 in order to try and saturate the beads. The experiments were repeated at room temperature. The results (Appendix B2) are shown in Figure 3.5.

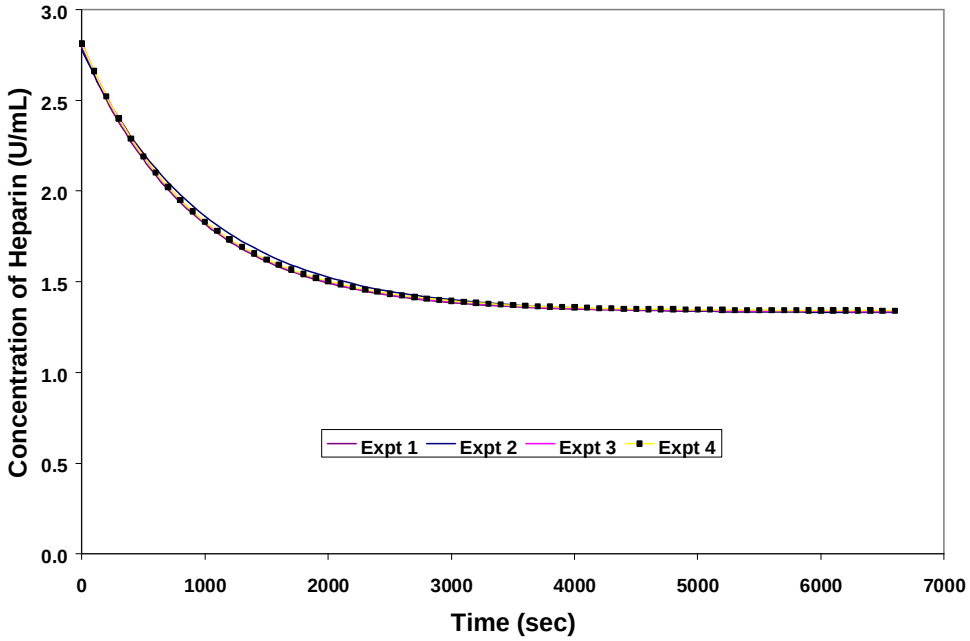

Figure 3.5: Repeat experiments for 850 beads of 900 υm diameter and 23 mL of 3U/mL of heparin solution.

Figure 3.5 indicated that the results were consistent and repeatable. The standard deviation for the results was less than 2%, which was similar to the previous experiment.

A control experiment to prove that heparin was not absorbed onto the glass surface of the cuvettes and the beakers was performed. A saline solution of 40 mL with 3U/mL of heparin, containing no beads, was stirred for 30 minutes. Samples of 300 µL of the solution were pipetted out from the stirring solution at different time intervals. The samples were then centrifuged as before and analyzed at 520 nm using the spectrometer (Figure 3.6 and Appendix B3).

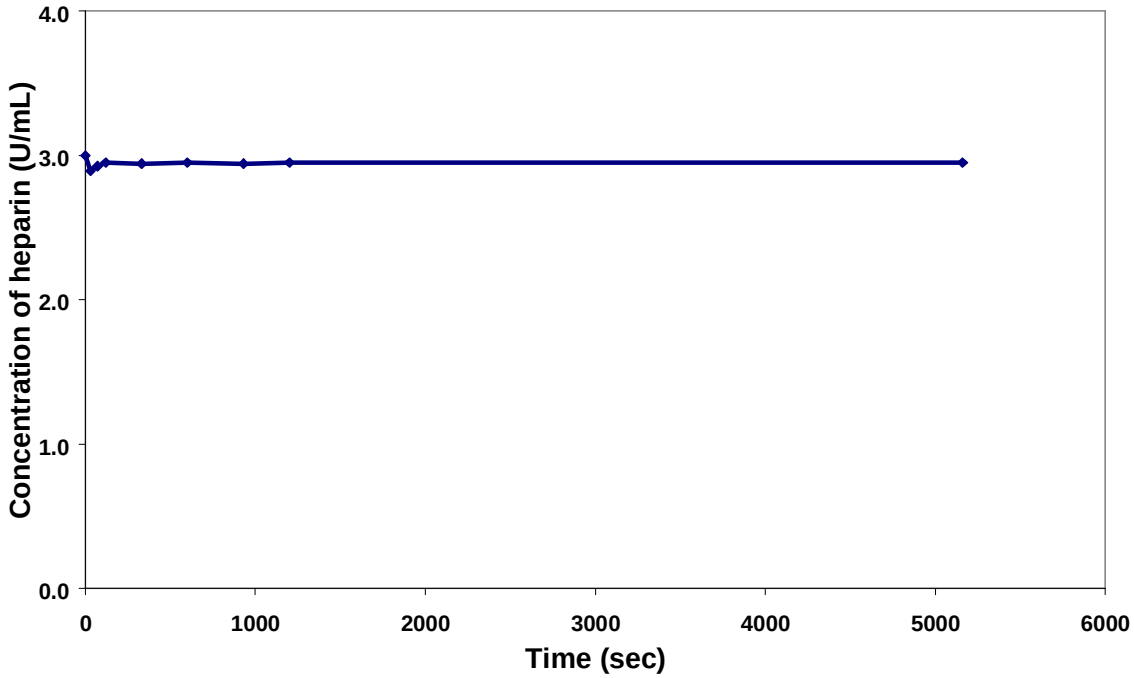

Figure 3.6 Concentration of heparin in saline solution containing no beads

The results obtained from this experiment indicated that a negligible amount of heparin was absorbed onto the glass surface. The results therefore indicate that the heparin is removed by the beads only and the rates measured are the rates of the absorption rate of heparin on the beads.

The next experiment was performed to check if any of the heparin was released back into the solution after some time. Poly-L-lysine/alginate beads that had been loaded with heparin were incubated in saline for one week at room temperature to determine if the absorbed heparin was released back into the solution. Samples of the saline solution were taken out every day and analyzed to measure the concentration of heparin.

After analysis, no heparin was found in any of the samples, which implies that the absorbance of heparin on to the beads is an irreversible process.

3.4.2 Estimation of the order of the absorption process

In order to fit a model to the rate process, as a first approximation, assume that the rate can be described by a simple first order absorption model.

For a first order absorption process:

$$\frac{dx}{dt} = K(x - x_e) \qquad \text{……..……..……Equation 3.1}$$

where:

x is the concentration of heparin in solution (liquid phase) in U/mL;

t is the time in seconds;

K is the rate coefficient in seconds^{-1};

x_e is the concentration of heparin in U/ml in solution (liquid phase) at infinite time (equilibrium concentration)

Equation 3.1 can be integrated and rearranged to give a linear equation.

$$\ln(x - x_e) = Kt + \ln(x_o - x_e) \qquad \text{……..…….Equation 3.2}$$

where

x_o is the initial concentration of heparin in the solution in U/mL

Since the concentration of heparin towards the end of the experiment decreased very slowly, the final concentration was assumed to be the concentration of heparin at infinite time or the equilibrium concentration, which was obtained from the experiment. The initial concentration of heparin was 3U/mL.

The curve of $\boldsymbol{ln\ \{\ x - x_e\ \}}$ versus time $\boldsymbol{t}$, was plotted using the data given in Figure 3.4 and is shown in Figure 3.7. (Appendix B4) The resultant straight line curve implies that the rate model is a simple first order absorption process and the rate constant

K = 0.0039 s^{-1}. The data measured for the 800 beads (Figure 3.5) is also plotted in Figure 3.7. It can be seen that this data too follows a straight line, but the rate constant K has a value of -0.002 s^{-1} which is different to that for the large number of beads. This is not that surprising and the next step is the need to investigate the relationship between the number of beads and the rate constant.

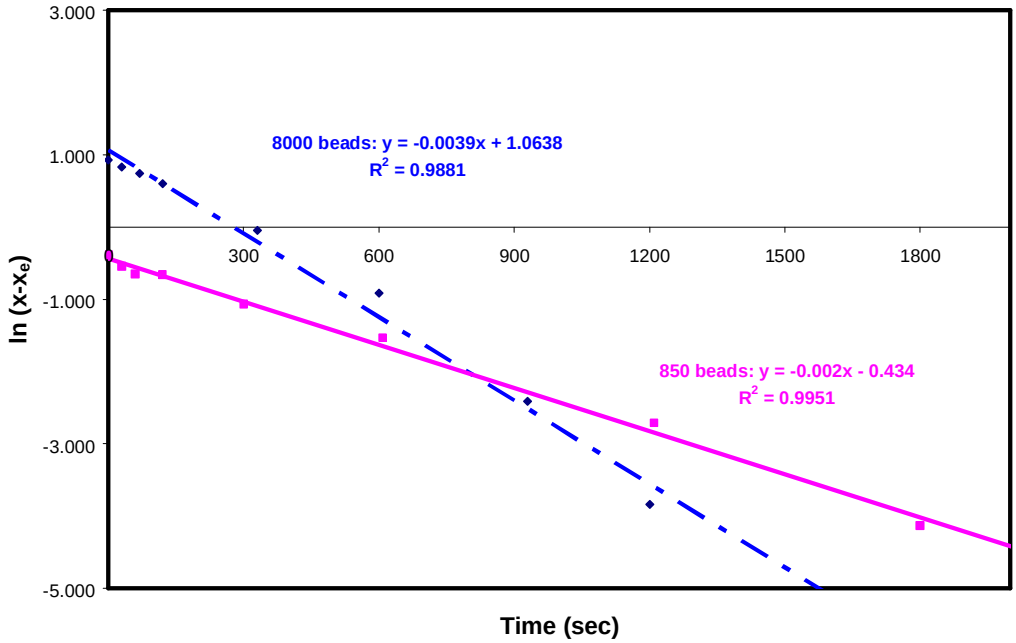

Figure 3.7: Experimental data fitted to a first order absorption process

3.4.3 Relation of absorption rate to the number of beads

In order to design a reactor, it is essential to know the impact that the quantity of the beads would have on the absorption process. It would be useful if a correlation could be obtained between the number of beads and the amount of heparin that can be absorbed by the beads.

Rate experiments in saline solution were performed by varying the number of beads. The previous results together with the new data are presented in Figure 3.8 (Appendix B5).

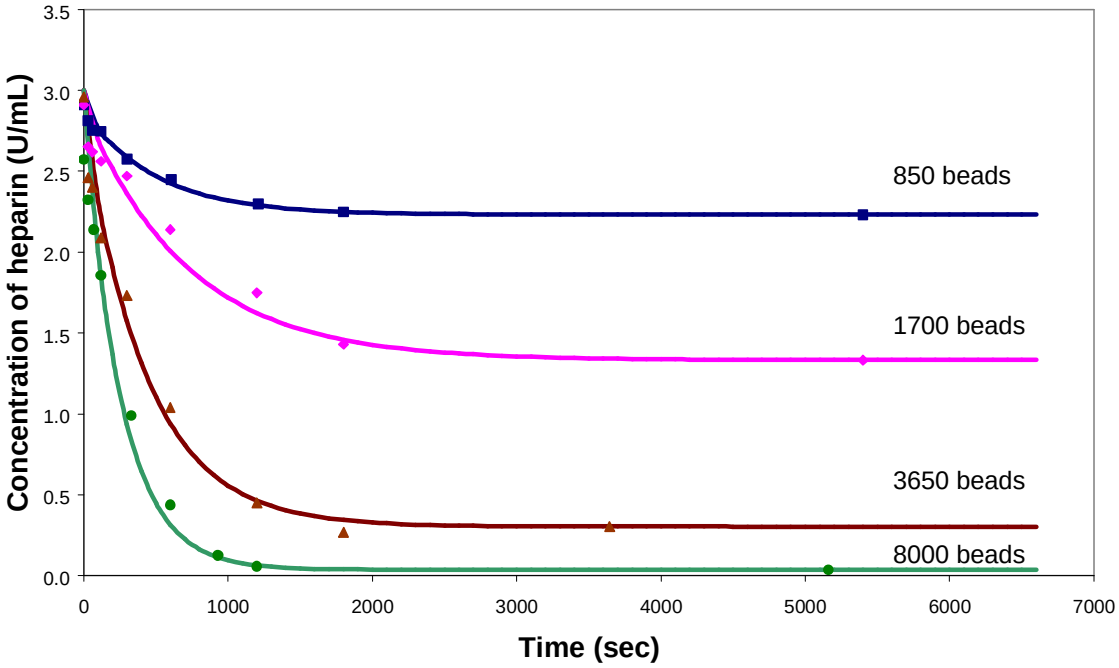

Figure 3.8: Comparison of rate experiments for different number of beads at 25°C.

The final concentration obtained was assumed to be the concentration at the infinite time or the equilibrium concentration since very little change was observed between the last two points. It can be seen that the initial slope depends on the number of beads and hence the rate constant K depends, as observed previously, on the number of beads. The data shown in Figure 3.8 was fitted to equation 3.2 and all the data sets fitted the first order rate model. The rate constants for experiments at 25 ^{O}C and 37 ^{O}C are plotted versus the number of beads in Figure 3.9 (Appendix B6).

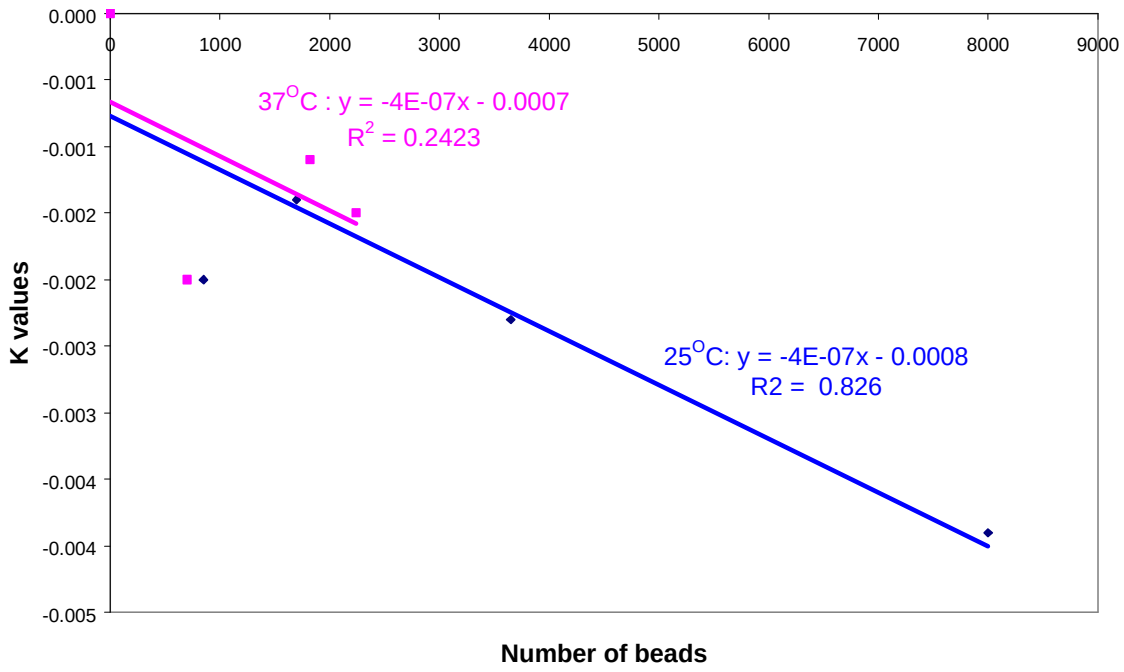

Figure 3.9: Correlation of K-values to the number of beads

It can be seen that the rate of absorption of heparin by the beads is linearly dependent on the number of beads.

3.4.4 Fitting an isotherm to the equilibrium concentration data points

A classical isotherm for a heterogeneous flat surface is the Freundlich Isotherm, which is defined as follows:

$$q_i = k_i x_e^{mi} \qquad \text{.....Equation 3.3}$$

where:

m_i is a constant which is positive and generally not an integer;

k_i is a constant;

x_e is the fluid phase concentration at equilibrium (concentration of heparin in saline solution at infinite time) in U/ml;

q_i is the absorbed phase concentration in U/mL;

q_i = ***Amount of heparin absorbed by the beads***……Equation 3.4

Total volume of the beads

The amount of heparin absorbed by the beads is equal to the initial amount of heparin in saline solution minus the final amount of heparin in the saline solution

The assumptions for these equations are:

- The volume of saline solution removed during the rate experiments is less than 10% of the initial volume of saline solution and is therefore assumed to be negligible. The final volume of the saline solution is thus approximately equal to the initial volume, which is equal to 40 mL
- The membrane thickness of the beads is negligible when compared to the radius of the beads (450 nm) and can therefore be ignored.

Equation 3.3 therefore becomes:

$$q_i = \frac{3\frac{U}{mL}40mL - (x_e 40mL)}{N_b(\frac{4}{3}\pi r^3)} \quad \text{……Equation 3.5}$$

where:

N_b is the number of beads

r is the radius of the beads in m

Equation 3.5 can be rearranged as follows:

$$\ln(q_i) = \ln(k_i) + m_i \ln(x_e) \quad \text{……..Equation 3.6}$$

The data points were used to obtain the values of q_i and the results were plotted on the graph in Figure 3.10 (Appendix B7).

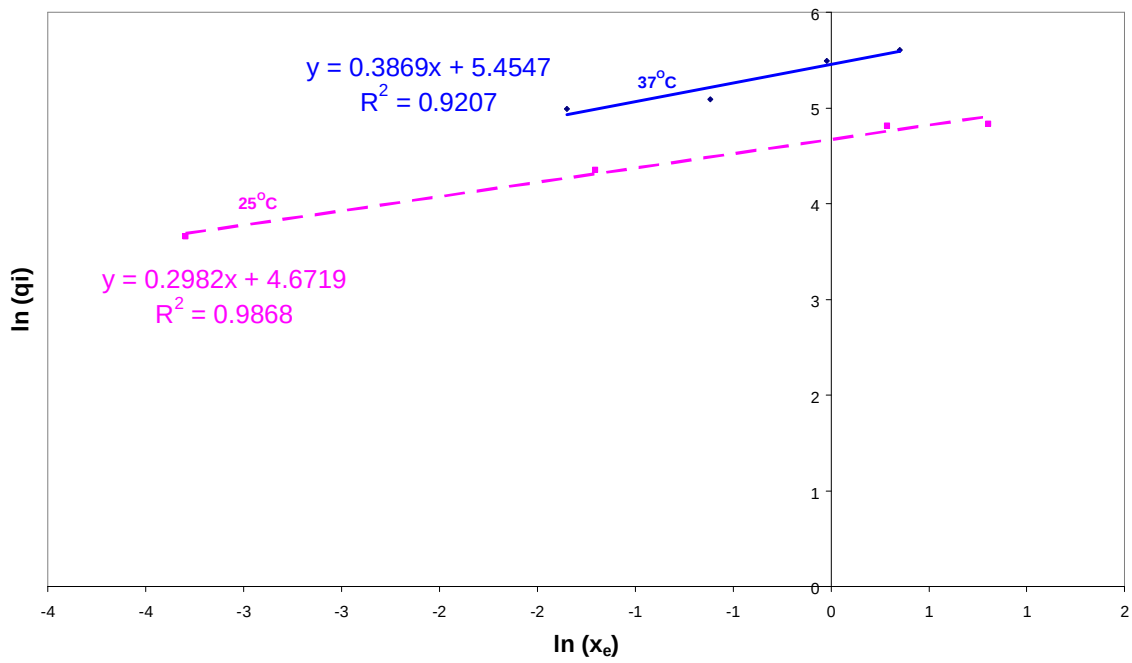

Figure 3.10: Data points obtained from the rate experiments fitted to Freundlich Isotherm

The results indicate that the equilibrium data points obtained from the rate experiments can be modeled using the Freundlich Isotherm. The gradient (m_i) is positive and not an integer; however the values differ for the different temperatures. This difference could be attributed to experimental error. The Freundlich Isotherm can therefore be used to predict the equilibrium parameters that are essential for the reactor design.

3.5. DISCUSSION

The results indicate that heparin can be efficiently removed using poly-L-lysine/alginate beads with no heparinase. Figure 3.5 illustrated that the results obtained from the experiments were consistent and repeatable. The experiment performed in the absence of beads demonstrated that very little of the heparin was absorbed onto the glass surface (Figure 3.6). Results from the incubation experiment indicated that heparin absorbed by the beads was not released back into the solution. If heparin removal were a diffusion process only, heparin would have been released into the saline solution to attain a new equilibrium concentration. Therefore, it was

concluded that the removal was an absorption process. The data obtained from the rate experiments showed an excellent fit to the first order absorption model (Figure 3.7). Increasing the number of beads, as illustrated in Figure 3.8 increased the amount of heparin removed from the saline solution, while the rate constants decreased (Figure 3.9). The results obtained from Figure 3.10 indicated that the Freundlich Isotherm could be used to correlate the data for the equilibrium concentrations.

3.6. CONCLUSION

The present study demonstrates that heparin can be efficiently removed from a saline solution using poly-L-lysine/alginate beads. The removal of heparin from these solutions is a first order absorption process and depends on the number of beads. The rate is fast enough, as the half life is approximately 100 seconds for the initial experiments, thus this method appears to be a feasible method that can be used to remove heparin. The absorption process at a particular temperature can be predicted using the Freundlich Isotherm. These results will ultimately provide some of the required parameters for the bioreactor design using poly-L-lysine/alginate beads. Further research is required to study the effect of these beads on the constituents of blood, the correlation between the volume and size of the beads and the absorption rate.

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CHAPTER 4: INVESTIGATING ABSORPTION IN SALINE, FETAL CALF SERUM AND BLOOD

The following article will be submitted to the Journal: "Artificial Cells, Blood Substitutes and Biotechnology" and is currently being reviewed. This paper investigates the absorption in saline, fetal calf serum and blood. Ways of optimizing the absorption process are explored by performing rate experiments to vary the physical parameters of the beads. All the experiments are designed to obtain the required parameters for the reactor design.

INVESTIGATING ABSORPTION IN SALINE, FETAL CALF SERUM AND BLOOD: TOWARD THE DEVELOPMENT OF A NOVEL REACTOR

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4.1. ABSTRACT

During extracorporeal procedures like hemodialysis, heparin is administered to patients to prevent clotting. Unfractionated heparin has side effects such as excessive bleeding. It would be advantageous if the blood could be deheparinased before it returns to the patient. Previous work has indicated that poly-L-lysine/alginate beads can efficiently remove heparin from saline solutions (Varghese et al[1]., 2006). Heparin is irreversibly absorbed onto the beads. This article explores ways of optimizing the absorption process by performing rate experiments varying the physical parameters of the beads. Fetal calf serum and blood are also used in experiments to investigate the possibility of designing a safe and efficient reactor to absorb heparin. All the experiments are designed to obtain the required parameters for the reactor design. The results indicate that the absorption could be optimized by controlling the membrane thickness of the beads. The beads also showed efficient removal of heparin in blood.

4.2. INTRODUCTION

Heparin has been the basis of initial anticoagulation therapy for more than 80 years (Nielsen[2]., 2003) Heparin reduces clotting during extracorporeal procedures such as hemodialysis. Heparin does not block the platelets from attaching to the foreign objects, but it blocks the cascade of proteins that work with the platelets to form the clot. It does not dissolve the clots but limits or slows clotting by binding and enhancing the activity of a protein called antithrombin III (Patients[3]., 2002). The complex formed with antithrombin III inhibits several activated blood coagulation factors: XIIa, XIa, IXa, Xa and thrombin (Heparin in children[4]., 2002).

Anticoagulation in routine hemodialysis consists of a standard dose of heparin given as a bolus at the start of dialysis treatment with a mid-treatment dose to maintain suitable anticoagulation. Alternatively, heparin modeling can be performed using an initial bolus followed by a constant fixed infusion of heparin, as mentioned above, to maintain an activated clotting time of 200 to 250 seconds (the normal activated clotting time is in the range of 90 to 140 seconds). *Activated clotting time is a*

measure of the anticoagulation effects of heparin in blood. This therapy ensures systematic anticoagulation throughout the dialysis treatment. It is reliable and requires minimal staff intervention after a patient's heparin dose is determined. (Kovalik[5]., 2000)

However, side effects of heparin include excessive bleeding or bruising, blood in urine or stools, abdominal or back pain, heavy menses and skin irritation. (Patients[3].,2002). Heparin is not prescribed to patients with low platelet counts or with bleeding disorders. Patients with some source of bleeding such as recent surgery, injury or ulcer are also in danger of excess bleeding when heparin is administered. Osteoporosis is another serious but uncommon side effect associated with high dose heparin administration over a prolonged period. (Heparin in children[4]., 2002). Various alternative anticoagulation substitutes have been used including extreme hemodilution, low molecular weight heparins, danaparoid, ancrod, r-hirudin, abciximab, tirofiban, argatoban and others. In the presence of heparin-induced thrombocytopenia (HIT) and thrombosis, the use of r-hirudin appears to be an acceptable solution which has been well studied. The main issue with r-hirudin is the difficulty in monitoring its activity (Von Segesser[6]., 2001).

Some of the alternatives for patients with a high risk of bleeding are (Heparin in children[4]., 2002):

- ***Minimum dose heparin***

Minimum dose heparin involves boluses of 500 units of heparin every 30 minutes to keep the activated clotting time greater than 150 seconds but less than 200 seconds. An alternate way is to have a continuous infusion of heparin with frequent activated clotting time monitoring. No additional equipment is required in the dialysis circuit, however a minimal degree of anticoagulation still occurs.

- ***Regional anticoagulation with protamine reversal***

Regional anticoagulation with protamine reversal involves constant infusion of heparin into the dialyser inlet line and simultaneous constant infusion of protamine prior to blood returning to the patient (protamine binds to heparin and eliminates its anticoagulant activity). The infusion pump rates are adjusted to keep the activated clotting time in the dialyser at 250 seconds. The protamine dosage required to neutralise the heparin can be determined by a protamine titration test. Protamine reversal has been largely abandoned due to technical difficulties and due to problems with rebound bleeding two to four hours after the end of dialysis as free heparin is released from the protamine-heparin complex back into the general circulation.

- ***Heparin free hemodialysis***

Heparin free hemodialysis is administered to patients with a high risk of bleeding. Both the dialyser and the blood lines are pre-treated with 2000 to 5000 units of heparin contained in a litre of normal saline prior to the start of the dialysis treatment so that heparin is not administered to the patient. The blood flowrates are in the range of 250 to 500 mL/min and 25 to 30 mL saline flushes are administered every 15 to 30 min into the arterial (pre-dialyser) limb to minimise hemoconcentration and to wash fibrin strands from the kidney. This volume of saline must be removed during dialysis to prevent hypervolemia. Careful monitoring of the arterial and venous pressure alarms is required to detect early clotting. The above method is used in approximately 90% of Intensive Care Unit patients with temporary venous access, with only a 2% clotting rate in the extracorporeal circuit. However, close nursing observation is required, as it may be necessary to convert to minimum dose heparin or stop treatment in 5% of the cases.

- ***Low molecular weight heparin***

The two main types of heparin are unfractionated and fractionated low molecular weight heparin. The molecular weight range of low molecular weight heparin is from 1000 to 10000, while unfractionated heparin is from 3000 to 30000. Low molecular weight heparin is given in a fixed dosage and does not require dose adjustment.

Coagulation assays to monitor its activity are unnecessary. Low molecular weight heparin requires less frequent dosing because of its longer half-life. The risk of osteoporosis is less with prolonged administration of low molecular weight heparin than with unfractionated heparin. However low molecular weight heparin is very expensive.

All of the above alternate modalities have disadvantages and complications. It would therefore be useful to have a system that would eliminate unfractionated heparin after the standard anticoagulation procedure is administered.

It was demonstrated in the earlier article that poly-L-lysine/alginate beads can efficiently remove heparin from saline solutions (Varghese et al[1]., 2006). The absorption process was a first order process and the Freundlich isotherm best fitted the experimental data. This article explores methods of optimizing the conditions to design an efficient reactor.

4.3. APPARATUS AND METHODS

4.3.1 Calibration

Concentration of unfractionated heparin was determined using an Azure II dye assay as described by Ameer et al[7]., 1999a. A cintra 5 UV-VIS double beam spectrometer was used to measure the absorbency of heparin. A standard linear curve for this assay was prepared by using solutions of known heparin concentrations ranging from 0 to 3U/mL.

4.3.2 Formation of poly-L-lysine/alginate beads

The standard encapsulation procedure patented by Chang[8] in 1992 was used to produce poly-L-lysine/alginate beads (Varghese et al[1]., 2006)

Standard poly-L-lysine/alginate beads were formed using a 26-gauge needle of the droplet generator. The beads were then stored in saline solution until they were used in experiments.

4.3.3 Batch rate experiments

Previous work ((Varghese et al[1]., 2006) has indicated that the extraction of heparin from the saline solution by the poly-L-lysine/alginate beads is a irreversible first order absorption process and the equilibrium data can be modeled by using a Freundlich isotherm. For the reactor design, it is essential to determine the absorption capacity of the beads and whether the saline results are representative of the blood results. The aim of this article is to determine whether the absorption process is rate controlling, to determine the rate controlling step, the absorption capacity of the beads and the absorption process in fetal calf serum and in blood.

The batch rate experiments were performed using a saline solution containing a known concentration of heparin was added to a known volume of uniformly sized beads. The contents in the beaker were then stirred to ensure that the beads were in suspension and to maintain uniform exposure of heparin to the beads. Samples of 300μL of the supernatant solution were taken out at time intervals from 0 seconds to 1.5 hours. The samples were centrifuged to separate the fragments of beads from the supernatant solution. The supernatant samples were then analyzed using the spectrometer.

4.4. RESULTS

The first step in the process of determining the absorption capacity of the beads is to determine what characteristics of the beads the absorption process depends on. Experiments were planned to identify whether the absorption process was influenced by the ratio of the volume of the beads to the solution, the surface area of the beads or the membrane volume.

4.4.1 Absorption Dependence on Total Volume Ratio of beads to solution

This experiment was performed to check whether the total volume ratio of beads to solution had an effect on the absorption process. Two batch rate experiments were performed by ensuring that the total volume ratio of the beads to the solution was kept constant. If the absorption process is affected by the total volume ratio of the beads to the solution then the results of both the experiments would be similar to each other.

Poly-L-lysine/alginate beads were placed in a fixed volume of saline solution with a heparin concentration of 3 U/mL, which was stirred continuously at room temperature. The beads of approximate diameters of size 900μm $\pm$ 100μm and 2mm $\pm$ 100μm were used. The number of beads and the volume of the solution used were adjusted such that the total surface volume ratio of beads to solution was kept constant. Aliquots of 300 μL of the heparin solution were pipetted out at different time intervals from zero seconds to one and half-hours. The absorbance of the samples was analyzed at a wavelength of 520 nm using the UV-VIS spectrometer.

Since the membrane thickness was so small when compared to the diameter of the bead, it was neglected:

$$\textit{Volume of one bead} = \frac{4}{3}\pi r^3 \quad \text{.....Equation 4.1}$$

$$\textbf{\textit{Total volume of beads}} = N(\frac{4}{3}\pi r^3) \quad \text{…… Equation 4.2}$$

where:

N is the total number of beads used for the experiment;

r is the radius of the beads;

For the total surface volume ratio of beads to solution to be constant:

$$N_1(\frac{4}{3}\pi r_1^3)V_2 = N_2(\frac{4}{3}\pi r_2^3)V_1 \quad \text{……Equation 4.3}$$

where:

N_1 is the number of beads used in the 900 μm beads experiment = 8000;

r_1 is the radius of beads in the 900 μm beads experiment = 450 μm;

V_1 is the total volume of 3 U/mL heparin solution in mL used for the 900 μm beads experiment;

N_2 is the number of beads used in the 2 mm beads experiment = 848;

r_2 is the radius of beads in the 2 mm beads experiment = 1 mm;

V_2 is the total volume of 3 U/mL heparin solution in mL used for the 2 mm beads experiment.

8000 $\pm$ 1000 beads (diameter 900 μm) were made. A batch rate experiment was performed to obtain the absorption results using 40 mL of saline. Equation 4.3 was then used to obtain the number of 2mm beads and the volume of saline that was required such that the total surface volume ratio remained constant. The obtained values are tabulated in Table 4.1.

Table 4.1 Parameters of beads and solutions used in the total volume ratio experiments.

V_1	N_1	r_1	V_2	N_2	r_2
40 mL	8000	$450*10^{-6}$ m	46 mL	848	$1*10^{-3}$ m

A rate experiment was performed to obtain the absorption results using the 2 mm beads and the obtained results were plotted on the same axes for purposes of comparison in Figure 4.1 (Appendix C1).

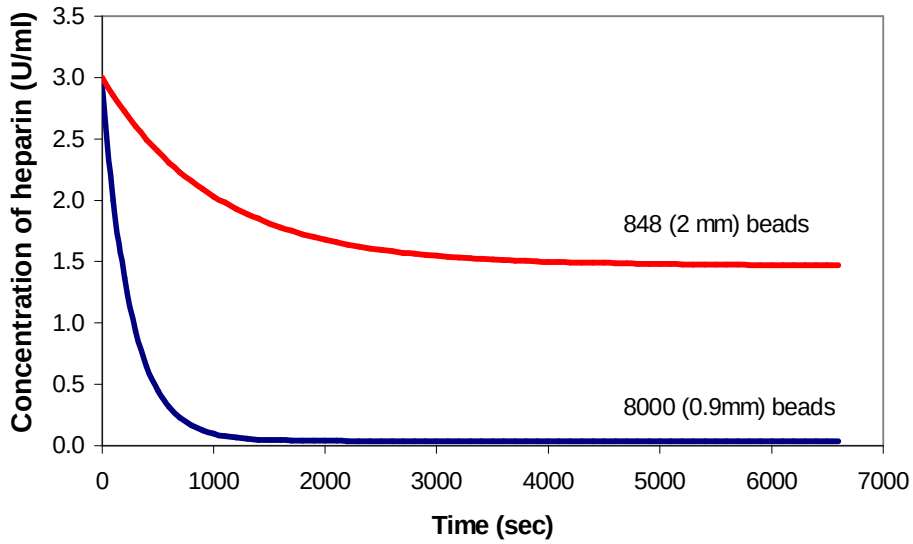

Figure 4.1. Comparison of rate with constant total surface volume ratio of beads to solution

The results clearly indicate that the ratio of the total volume of beads to the solution does not influence the absorption rate.

4.4.2 Absorption Dependence on Total Surface Area of beads

This experiment was performed to check whether the ratio of the total surface area of beads to the volume of solution had an effect on the absorption process. Two batch rate experiments were performed by ensuring that the ratio of the total surface area of beads to the volume of solution was kept constant. If the absorption process is

affected by the ratio of the total surface area of the beads to the volume of the solution then the results of both the experiments would be similar to each other.

The rate experiments were performed for 900 μm beads and 2mm beads. The number of beads and the volume of the solution used were adjusted such that the surface area ratio of the beads to volume the solution was kept constant.

Neglecting the membrane dimension:

Surface area of one bead = $4\pi r^2$ ... Equation 4.4

Total surface area of beads = $N(4\pi r^2)$ ...Equation 4.5

For the surface area ratio to be constant:

$N_1(4\pi r_1^2)V_2 = N_2(4\pi r_2^2)V_1$ …….Equation 4.6

3650 beads of diameter 900 μm were made. A batch rate experiment was performed using 40 mL of saline. Equation 4.6 was then used to obtain the number of 2mm beads and the volume of saline that was required such that the total surface area ratio of beads to the volume of solution remained constant. The obtained values are tabulated in Table 4.2.

Table 4.2 Parameters of beads and solutions used in the surface area ratio experiments.

V_1	N_1	r_1	V_2	N_2	r_2
40 mL	3650	$450*10^{-6}$ m	46 mL	848	$1*10^{-3}$ m

The results obtained from the rate experiments were plotted on the same axes for purposes of comparison (Appendix C2).

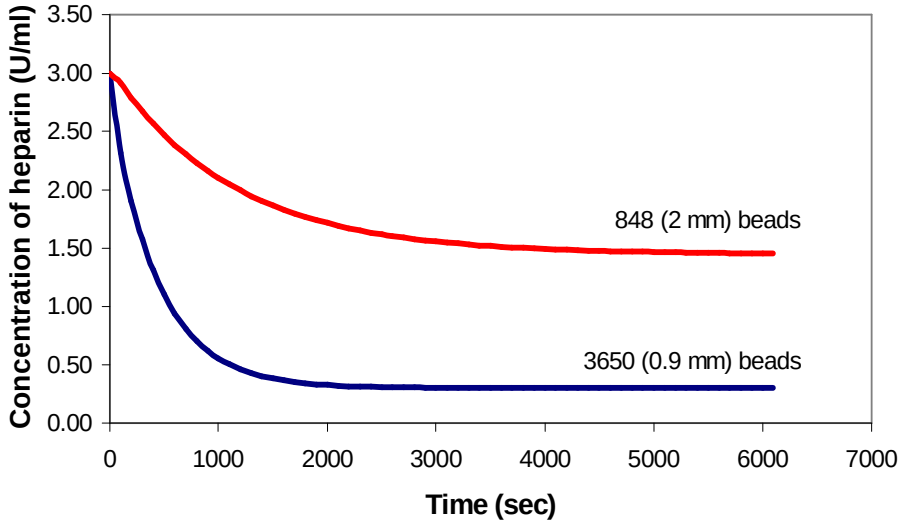

Figure 4.2. Comparison of rate with constant total surface area ratio of beads

The results clearly indicate that the surface area of the beads does not influence the absorption rate.

4.4.3 Effects of the bead membrane on the absorption of heparin

The above experiments have indicated that the absorption process does not depend on the volume or surface area of the beads. Previous research has proved that poly-L-lysine can absorb heparin (Rodger et.al[7]., 1994). During the formation of beads, the poly-L-lysine is contained in the membrane of the beads. The following set of experiments were planned to check if the membrane of the beads had an influence on the absorption process.

It is essential to determine whether if any of the other constituents of the membrane apart from poly-L-lysine affects the absorption process. The following experiment was planned to confirm that the sodium alginate in the membrane had no effect on the absorption process. The sodium alginate used initially to form the beads was expected to have no part in the absorption, as it diffuses out in the final stage of the bead formation (Chang[5]., 1992). A rate experiment involving a variation of the sodium

alginate concentration was performed to investigate the effect of sodium alginate on the absorption process.

Two sets of poly-L-lysine/alginate beads (8000 beads) of size 900 µm were made using the procedure described above. One set of beads was produced using a concentration of sodium alginate of 5%, while the other set was made using 2.5% sodium alginate. Rate experiments were performed for both sets of beads. The results obtained from the experiments are presented in Figure. 4.3 (Appendix C3).

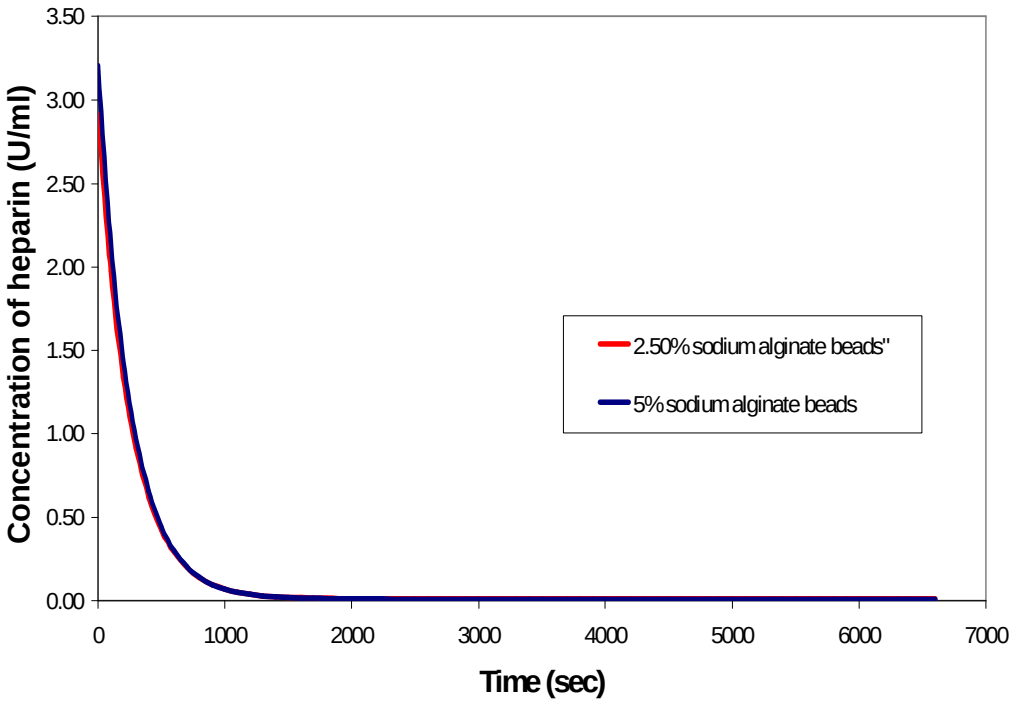

Figure 4.3. Comparison of the absorption of heparin by varying the concentration of sodium alginate

The results clearly indicate that sodium alginate has no effect on the absorption process, as varying the concentration of the sodium alginate does not alter the absorption process.

The following experiment was planned to confirm whether the membrane (poly-L-lysine) was the only factor to consider when determining the absorption capacity of the beads. Beads with no membrane were used in a rate experiment to determine the effect on the absorption process.

Approximately 8000 beads of size 900 µm were produced. Equal amounts of saline and 5% sodium alginate were mixed in a syringe. The droplets were allowed to fall into the calcium chloride solution, thereby forming beads with calcium alginate (gel structure with no membrane). All the other stages were excluded from the formation of the beads. A rate experiment was performed using these beads with no membrane.

The results obtained from the experiment are shown in Figure 4.4 (Appendix C4).

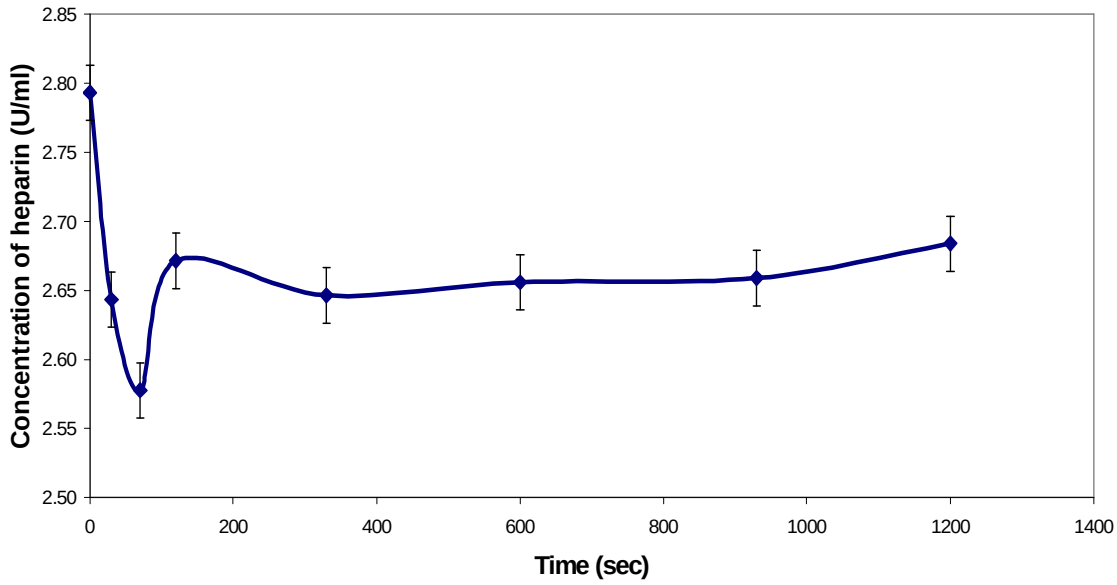

Figure 4.4. Absorption of heparin by beads with no membrane

The results indicate that some of the heparin can be taken by the calcium alginate; however, some of it is released back into the solution to obtain a new equilibrium concentration of 2.66 U/mL, which is much higher than 0.028 U/mL obtained using beads with a membrane. This leads to the conclusion that although calcium alginate

absorbs heparin, the majority of the absorption is due to the poly-L-lysine content in the beads.

As part of the experiments, it was vital to check if the membrane of the smaller beads was different from that of the larger beads and what effects, if any, the difference in membrane thickness would have on the absorption process and ultimately lead to the parameters essential for the reactor design.

Upon microscopic observation, the smaller beads were found to have a thicker membrane than the larger beads (Figure 4.5). The ratio of the dimensions of the membrane thickness was estimated using the programme Scion Image. The thickness of the 2 mm beads was estimated to be 0.006 mm, while the thickness of the 900 μm beads was 0.014 mm.

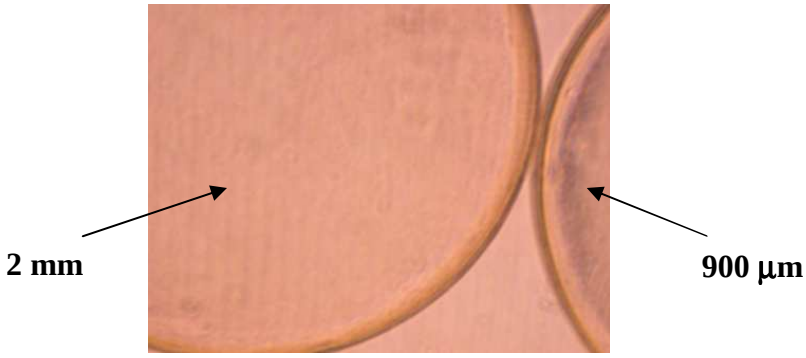

Figure 4.5. Microscopic photo of the 900 μm and 2 mm beads illustrating the different membrane thickness

After observing the noticeable difference in the membrane thickness, batch rate experiments were planned to check if the volume of the membrane of the beads influenced the absorption process.

The ratio of the membrane thickness of the beads is as follows:

$$\frac{\delta_2}{\delta_1} = 0.43 \quad \text{..... Equation 4.7}$$

where:

δ_2 is the membrane thickness of the 2 mm beads in mm;

δ_1 is the membrane thickness of the 900 μm beads in mm.

Volume of membrane of one bead = $4\pi r^2\delta$ … Equation 4.8

Total membrane volume of beads = $N(4\pi r^2\delta)$ Equation 4.9

For the ratio of the total volume of the bead membrane to be constant:

$$N_1(4\pi r_1^2\delta_1) = N_2(4\pi r_2^2\delta_2) \quad \text{.....Equation 4.10}$$

The above equation was used to obtain the following parameters that were used for the rate experiments at 37°C (normal human body temperature), such that the membrane volume ratio of the beads remained the same.

Table 4.3 Parameters used in the bead membrane volume ratio experiments.

N_1	r_1	N_2	r_2	δ_2/δ_1
1800	$450*10^{-6}$ m	850	$1*10^{-3}$ m	0.43

The results obtained from the rate experiment are illustrated in Figure 4.6 (Appendix C5).

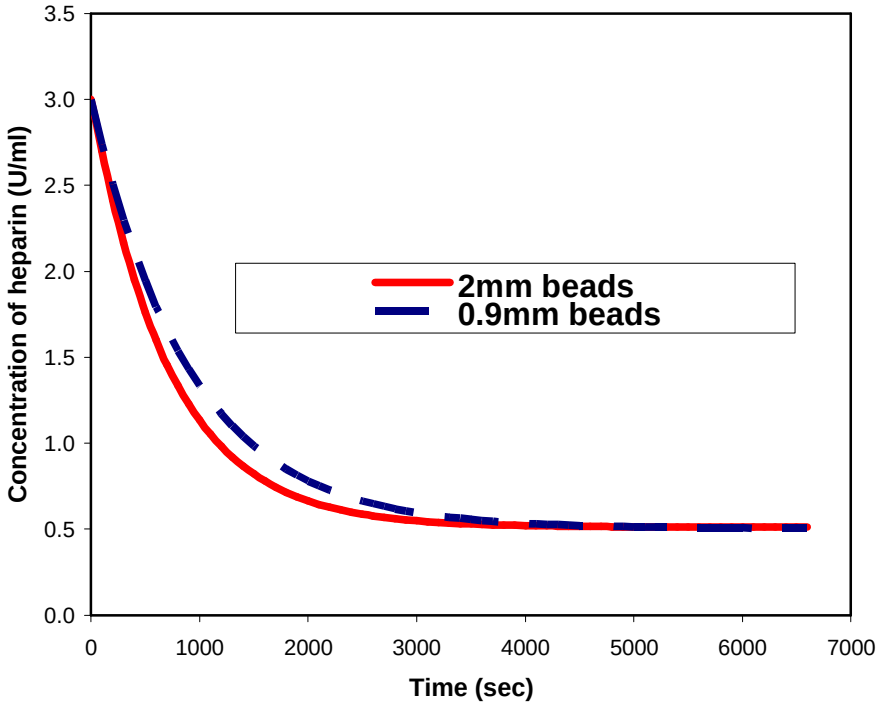

Figure 4.6. Comparison of the rate experiments with constant membrane volume ratio of beads

The above results clearly indicate that the absorption process is dependent on the volume of the membrane of the beads. The absorption capacity of the beads thus depends on the volume of the membrane, which implies that the reactor design must be based on the volume of the membrane of the beads and not on the volume or the surface area of the beads. Previously, the experimental data was fitted to a first order absorption model and the Freundlich Isotherm, however, the volume of the membrane of the beads were neglected. From the above process, it is evident that the membrane of the beads influences the absorption process, thus this parameter cannot be ignored. The effect of the volume of the membrane of the beads needs to be incorporated into both the constants for the First order model and the Freundlich Isotherm. It is essential to check if the models fit the above results in order to obtain a method of determining the constants irrespective of the conditions under which the experiments were performed. This would be beneficial for the reactor design as the value of the constants for a particular number of beads can be easily estimated.

From the previous article (Varghese et al., 2006), the experimental data fitted the following first order absorption model:

$$\frac{dC_{soln}}{dt} = K(C_{soln} - C_{inf}) \quad \text{.....Equation 4.11}$$

where:

C_{soln} is the concentration of heparin in solution in U/mL;

t is the time in seconds;

K is the rate coefficient in seconds^{-1};

C_{inf} is the concentration of heparin U/ml at infinite time (equilibrium concentration)

The curve of $\boldsymbol{ln\ \{C_{soln} - C_{inf}\}}$ versus time $\boldsymbol{t}$, was plotted using the data points obtained from the rate control experiment in saline solution and a straight line was obtained. The gradient of the graph yielded the value of K. The data points from above experiment for both the 900 μm and 2mm beads were used to obtain the values of K. These values were then plotted together with the previous values obtained from the previous article (Varghese et al., 2006) against the volume of the membrane of the beads as shown in Figure 4.7 (Appendix C6):

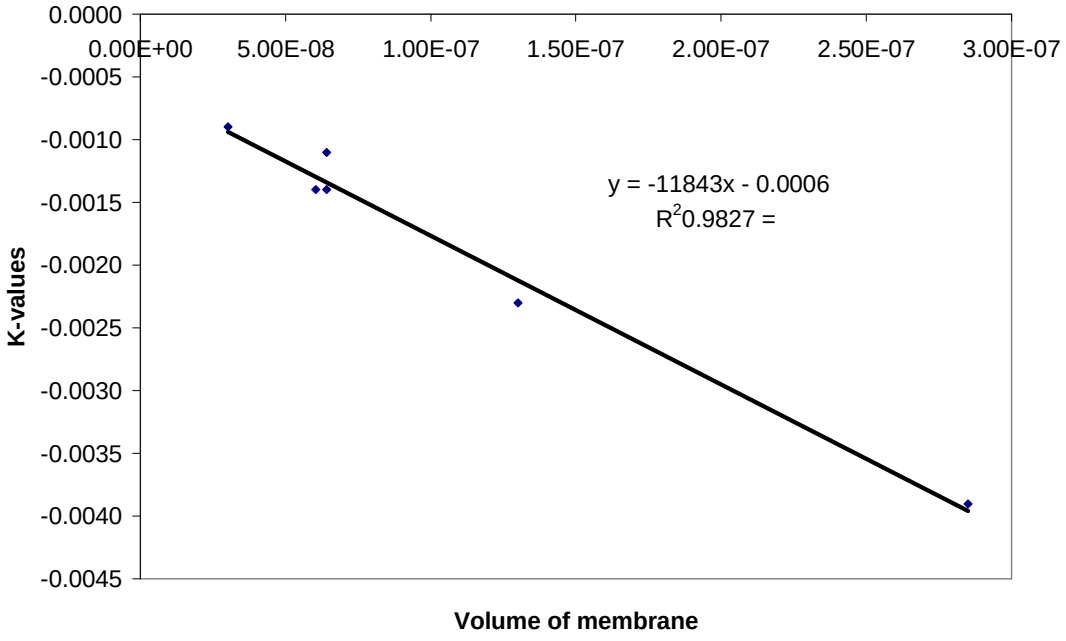

Figure 4.7. K-values for varying volume of membrane of the beads

From Figure 4.7, the K value obtained from the 2mm and the 900μm beads is directly proportional to the volume of the membrane of the beads. Therefore if the volume of the membrane of the beads is known, the value of K can be obtained, irrespective of the size of the beads.

The Freundlich Isotherm in the previous article was obtained using the volume of the beads. The Freundlich isotherm is as follows:

$q_i = k_i C_{eqm}^{mi}$ ……..Equation 4.12

where:

m_i is a constant which is positive and generally not an integer;

K_i is a constant;

C_{eqm} is the fluid phase concentration at equilibrium (concentration of heparin in saline solution at infinite time) in U/ml;

q_i is the absorbed phase concentration in U/mL;

The above equation can be rearranged to:

$$\ln(q_i) = \ln(k_i) + m_i \ln(C_{eqm}) \quad \text{.....Equation 4.13}$$

The absorbed phase concentrations were calculated in the previous article using the volume of the beads. These values are corrected for the volume of the membrane of the beads, and the following graph (Appendix C7) is obtained:

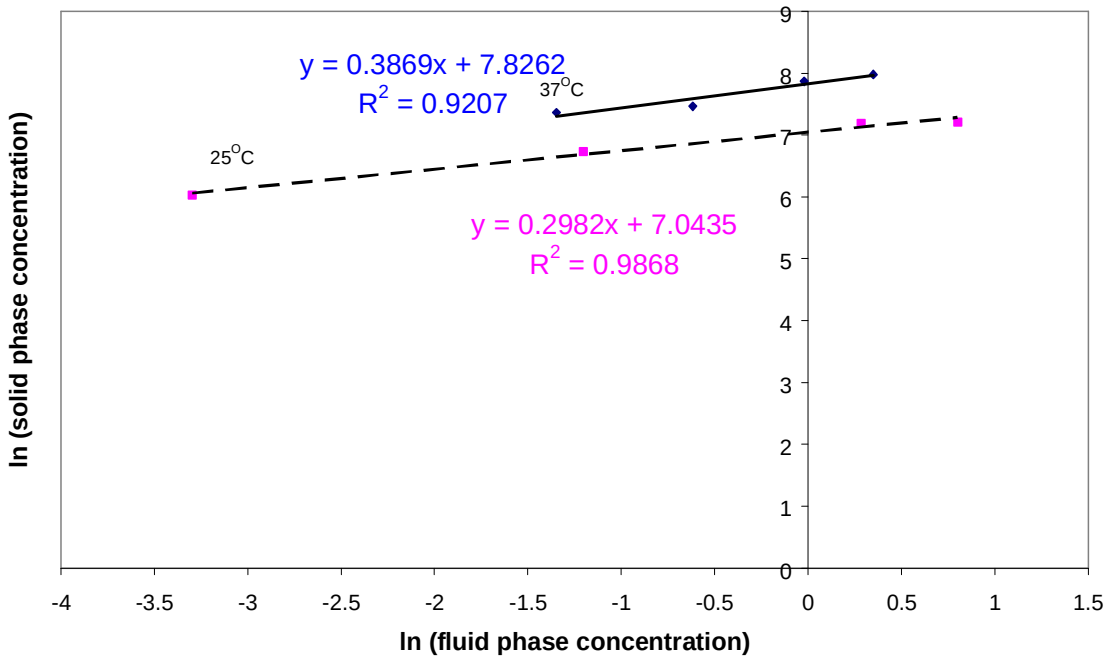

Figure 4.8. Data points fitted to Freundlich Isotherm using the volume of membrane of the beads

The above graph indicates that the data obtained still follows the Freundlich isotherm, and the gradient remains the same as that obtained in Article 1.

4.4.4 Relation of absorption rate to the membrane thickness of the beads

Since poly-L-Lysine is known to absorb heparin (Rodger et.al[18]., 1994) and since the absorption of heparin is directly related to the membrane volume of the beads (Figure 4.6), it would be beneficial if the membrane thickness of the beads could be controlled in order to obtain the optimum absorption conditions. Three sets of 2mm beads were produced, and the membrane thickness was altered by changing the time that the beads were kept in the poly-L-lysine solution. The first set of beads was produced by placing them for the usual 20 minutes in the poly-L-lysine solution. 40 minutes was used for the second set, while 60 minutes was used for the third set. The membrane thickness of the beads was then compared under light microscopy and photographed.

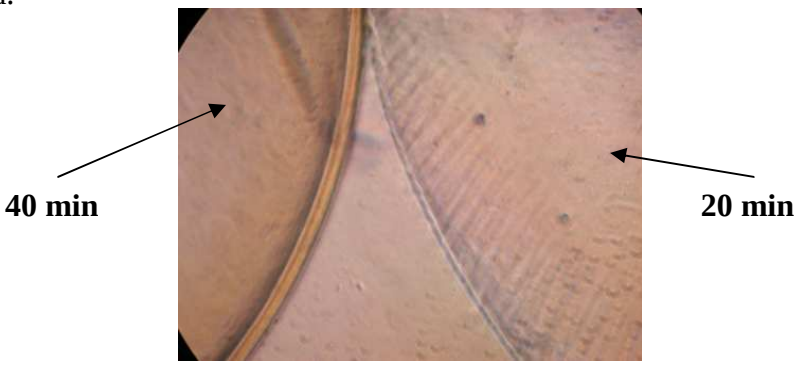

Figure 4.9. Microscopic photo of 2 mm beads placed in poly-L-lysine solution for 20 minutes and 40 minutes

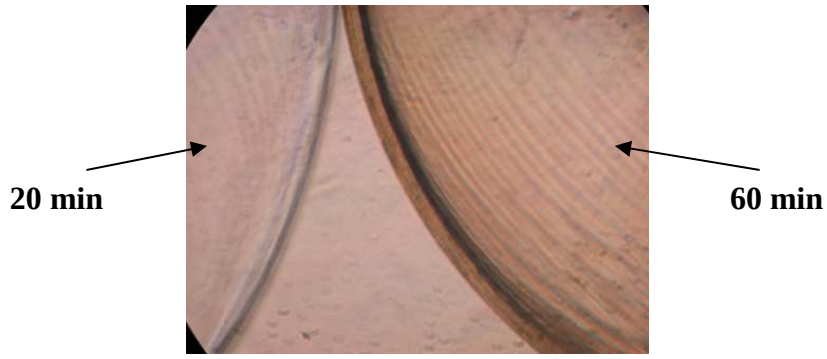

Figure 4.10. Microscopic photo of 2 mm beads placed in poly-L-lysine solution for 20 minutes and 60 minutes

The ratio of the membrane thickness of the beads was determined, using the programme Scion Image, to be approximately

$$\delta_{20} : \delta_{40} : \delta_{60} = 1 : 3 : 5 \qquad \text{..... Equation 4.14}$$

where the subscript of δ represents the time in minutes that the beads were placed in the poly-L-lysine solution.

4.4.5 Rate Experiment for beads with more Poly-L-Lysine content than the standard beads

Since poly-L-lysine is the main constituent that affects the absorption process, it would be beneficial to know if the content of poly-L-lysine were to be increased with the aim of producing solid poly-L-lysine beads, what the effects would be on the absorption process.

850 beads of size 900 μm were produced using Chang's procedure, however the last two steps were omitted in order to produce beads with a poly-L-lysine coating. Three sets of beads were produced. One set was incubated in saline overnight, one set was incubated in poly-L-lysine solution overnight, while the remaining set of beads were used immediately for a rate experiment. Another set of standard beads (containing poly-L-lysine membrane) were made by using the full procedure as described by Chang. The incubated beads were used for rate experiments using 23 mL of saline solution with 3U/mL of heparin. The results (Appendix C8) are as shown in Figure 4.11.

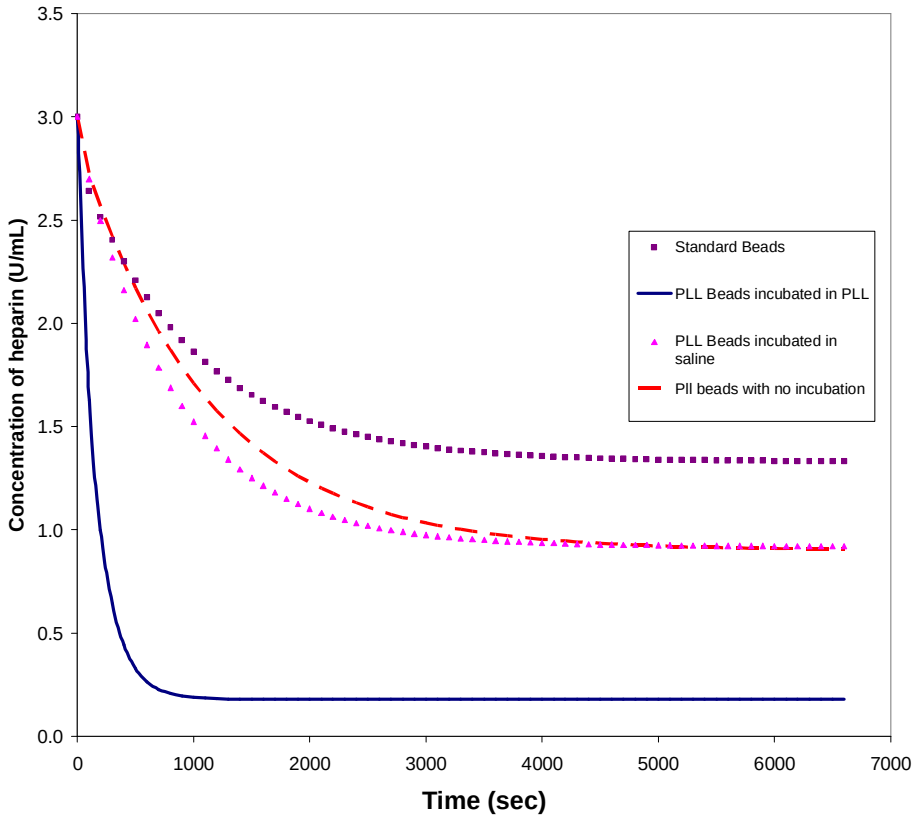

Figure 4.11. Comparison of the rate of heparin using beads with varying poly-L-lysine content

The rate coefficients for the first order model for the above data points are tabulated in Table 4.4.

Table 4.4 Rate coefficients for the beads with varying poly-L-lysine (PLL) content

Beads	**Rate coefficients (K) in sec^{-1}**
Standard beads	-0.001
PLL beads incubated in PLL	-0.0057
PLL beads incubated in saline	-0.0012
PLL beads with no incubation	-0.0009

The results clearly indicate that increasing the poly-L-lysine content of the beads increases the amount of heparin absorbed. This finding creates the possibility of using solid poly-L-lysine beads to improve the efficiency of the reactor.

4.4.6 Heparin absorption in fetal calf serum (FCS) using beads

The above experiments have indicated that the beads can efficiently absorb heparin from saline solutions. However the use of the beads in extracorporeal procedures, where human blood is used, the feasibility of the beads have to tested. As an initial test, fetal calf serum was used. The properties of fetal calf serum are similar to that of blood, thus a rate experiment using the beads in heparinised fetal calf serum would give a clear indication as to whether the beads are feasible or not.

Rate experiments were performed in fetal calf serum to verify the efficiency of the beads at absorbing heparin. Two sets of approximately 1700 beads of size 900 μm were produced. One set was used in a rate experiment using fetal calf serum. The other set was incubated in fetal calf serum for a day, and a rate experiment was performed in the same volume of saline solution. Another experiment was performed with no beads to determine whether any of the heparin was absorbed by FCS. The results (Appendix C9) are presented in Figure 4.12:

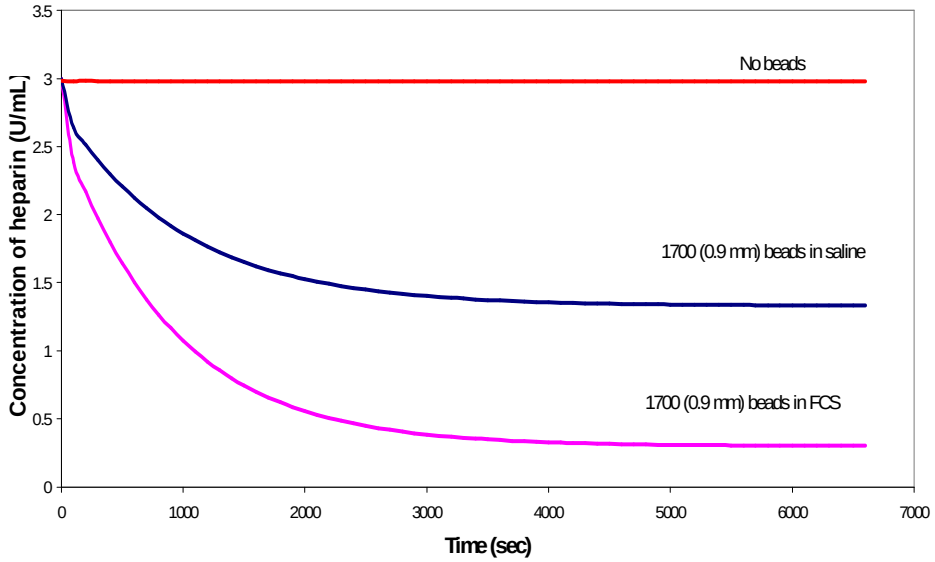

Figure 4.12. Comparison of the rate of heparin in fetal calf serum (FCS)

The rate coefficient for the first order model for the beads in FCS is -0.0011 sec^{-1}.

The results indicate that the beads are more efficient in fetal calf serum.

4.4.7 Heparin absorption in human blood using beads

Since the beads have been tested to be efficient in fetal calf serum, the next step would be to test the feasibility of using the beads in human blood. The efficiency of the beads in blood was tested by performing a rate experiment in blood. Fresh samples of blood (kindly donated by co-authors/supervisors) in citrate tubes were mixed and utilised. The samples, taken at time intervals ranging from 0 to 5400 seconds, were centrifuged for 20 minutes to separate the blood. The plasma was then centrifuged as before to remove the fragments from the samples. The results (Appendix C10) are shown in Figure 4.13:

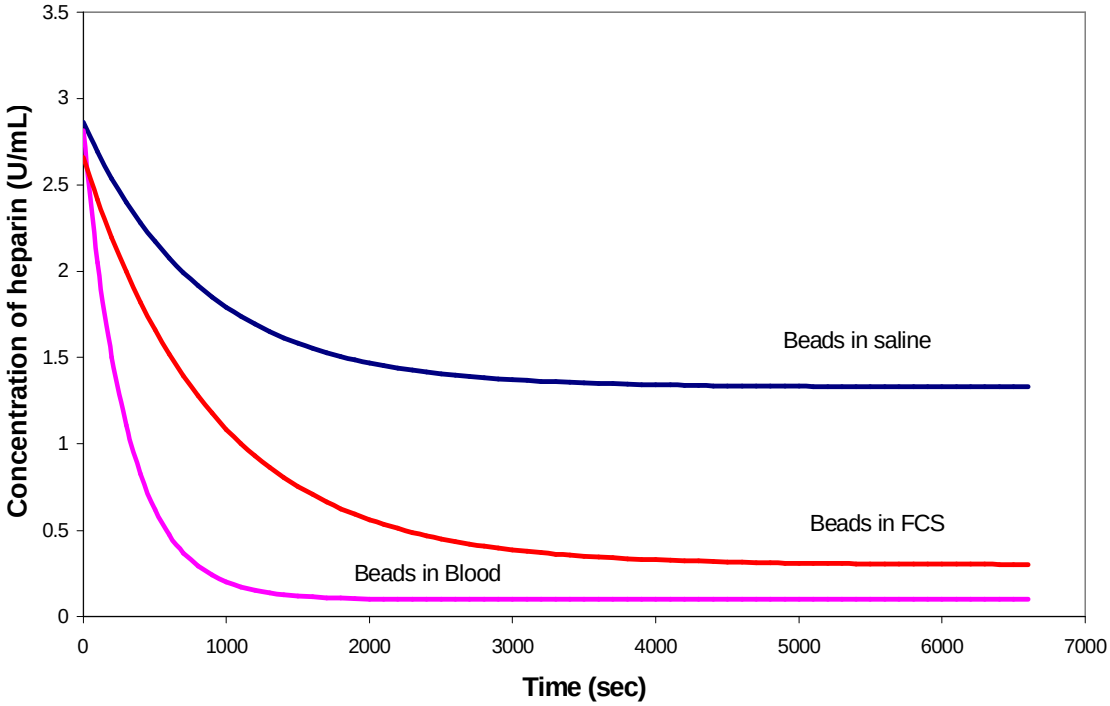

Figure 4.13. Comparison of the rate of heparin absorption in blood, Fetal calf serum and saline

The rate coefficients for the first order model for the above data points are tabulated in Table 4.5.

Table 4.5 Rate coefficients for blood, FCS and saline

Beads	**Rate coefficients (K) in sec^{-1}**
Beads in saline	-0.001
Beads in FCS	-0.0014
Beads in Blood	-0.0027

The results seem to indicate that the beads are more efficient in blood and follow the same trend as the absorption process in saline.

4.4.8 Effect of pH on the absorption process

The above results indicate that the absorption process in blood is more efficient. One of the reasons could be pH. This experiment was performed to obtain the trends, if any, when the pH value is changed.

The effects of pH were observed by performing rate experiments using solutions with varying pH. The volume of the beads and solution were kept constant. The results obtained from the experiments are illustrated in Figure 4.14 (Appendix C11).

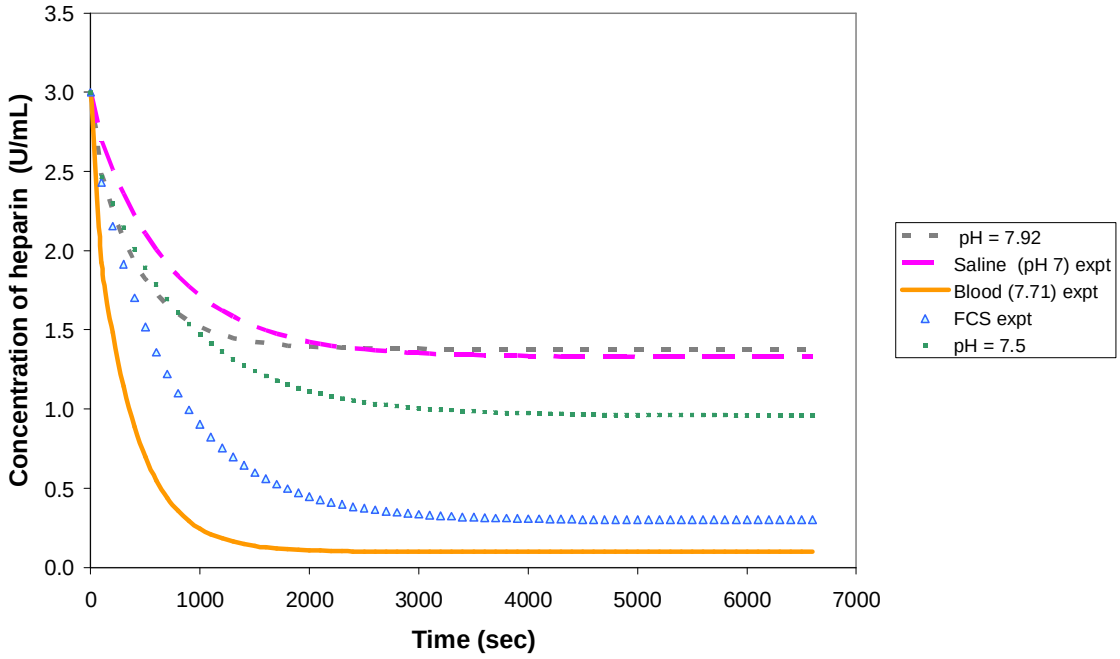

Figure 4.14. Comparison of the rate of heparin using different pH solutions

The rate coefficients for the first order model for the above data points are tabulated in Table 4.6.

Table 4.6 Rate Coefficients for the first order absorption model

Beads	**Rate coefficients (K) in sec^{-1}**
Saline	-0.001
Blood	-0.0027
FCS	-0.0014
pH = 7.5	-0.0012
pH = 7.92	-0.0023

The results indicate that the pH has an effect on the absorption process and cannot be ignored. In the final design of the reactor, one would need to determine the optimum pH for the blood in order to ensure efficient absorption of heparin.

4.4.9 Effect of beads on the normal constituents of blood

To help assess the safety of the beads, the beads were incubated in whole blood for 5 hours. The blood was added to a tube containing a little heparin tp prevent clotting. Measurements of various constituents in blood were taken before and after the incubation, to see if any of the important constituents of the blood were affected by the beads. The results obtained from the measurements are tabulated in Table 4.7.

Table 4.7 Effects of beads on the blood constituents

CONSTITUENTS	READINGS BEFORE EXPT	READINGS AFTER EXPT	% REDUCED
Serum proteins	63	61	3.17%
Serum Albumin	40	38	5.00%
Serum Calcium	2.04	1.89	7.35%
Serum Cholesterol	3.32	3.26	1.80%
Serum Creatine	90	85	5.56%
Serum Urea	5.47	5.26	3.84%
Serum direct bilirubin	2	2	0.00%
Serum iron	16.1	15.7	2.48%
Serum total bilirubin	8	8	0.00%
Serum HDL cholesterol	1.07	1.06	0.93%
Serum magnesium	1.31	1.1	16.00%
Serum triglycerides	0.46	0.44	4.35%
Serum transferin	1.9	1.82	4.21%
Serum uric acid	0.17	0.16	5.88%
CRP	0	0.1	10.0 % increase
Serum phosphate	0.7	0.64	8.57%
Serum ferritin	58	56	3.45%
Serum sodium*	163	155	4.91%
Serum potassium	3.71	3.4	8.36%
Serum chloride	82	82	0.00%
Serum glucose	3.1	2.8	9.68%

* The amount of sodium in the sample is higher than the normal range due to the sodium heparin present in the sample.

The units of the constituents were not listed on the table since only the relative percentage change was considered to determine the effect of the beads on the blood.

The results indicated that there was no significant decrease on any of the important constituents of blood. Although, this experiment proves that the beads have no influence on the constituents of blood, it is essential that all the necessary safety test are performed for the final design of the reactor to ensure that the blood is safe.

4.5. DISCUSSION

The absorption of heparin is not dependent on the total volume (Figure 4.1) or on the total surface area (Figure 4.2) of the beads. Figure 4.1 indicated that fewer larger sized beads absorbed less heparin than the smaller sized beads. The concentration of sodium aliginate did not have an effect on the absorption rate as illustrated in Figure 4.3. Beads with no membrane or poly-L-lysine absorbed heparin initially. However the heparin was released back into the solution until an equilibrium concentration of heparin was attained. The equilibrium concentration of heparin, 2.66 U/mL (Figure 4.4), is much higher than 0.028 U/mL obtained using beads with a membrane.

Large beads have thinner membranes, while the small beads have thicker membranes (Figure 4.5). The results obtained from the rate experiments implied that the absorption of heparin was affected by the membrane thickness (Figure 4.6). The absorption of heparin is solely dependent on the poly-l-Lysine content in the beads.

If the volume of the membrane of the beads is known, the value of the rate coefficient for a first order absorption model can be estimated using Figure 4.7. The Freundlich Isotherm model (Figure 4.8) can also be used to estimate the amount of heparin that can be absorbed from the solution.

The membrane thickness definitely increased as the time of exposure of the beads in poly-L-lysine solution was increased (Figures 4.9 and 4.10). The results obtained from this experiment implied that the thickness of the membrane is directly related to the poly-L-lysine and can therefore be controlled by varying the time in which the beads are kept in the poly-L-lysine solution.

The results from Figure 4.11 indicated that the beads with more poly-L-lysine coating absorbed more heparin than the standard beads. The Poly-L-Lysine coated beads that were incubated in saline and the beads with no incubation absorbed almost the same amount of heparin. The beads that were incubated in poly-L-lysine removed much more heparin, in comparison to the other sets of beads. Controlling the amount of

poly-L-lysine in the beads can optimize the absorption of heparin from the saline solution. Therefore the membrane thickness of the beads can be altered to absorb more heparin.

The beads are efficient at removing heparin from both FCS and human blood (Figures 4.12 and 4.13). More heparin is absorbed in blood when compared to saline using the same number and volume of beads. The absorption rate is affected by pH (Figure 4.14), and there seems to be an optimum pH, however this value has not been identified. Table 4.7 indicates that there is no significant decrease in the important constituents of blood, which would be an important safety consideration.

4.6. CONCLUSION

Poly-L-lysine/alginate beads can efficiently absorb heparin from saline, fetal calf serum and blood. The beads also have minimal effects in blood, as the amount of the constituents in the blood is not significantly affected. The thickness of the membrane of the beads can be adjusted to optimise the absorption process. Heparin absorbs onto the poly-L-lysine in the membrane of the beads, therefore increasing the poly-L-lysine content in the beads will improve the amount of absorption. Smaller sized beads have thicker membranes and therefore absorb more heparin than the larger sized beads. Poly-L-lysine/alginate beads can therefore be used in a reactor to absorb heparin during extracorporeal procedures. The findings of this article will be highly beneficial for the design of the reactor.

4.7. REFERENCES

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CHAPTER 5: INVESTIGATION OF THE USE OF POLY-l-LYSINE/ALGINATE BEADS IN A PACKED BED CONFIGURATION FOR REMOVING HEPARIN.

The following article will be submitted to the Journal: "Artificial Cells, Blood Substitutes and Biotechnology" and is currently being reviewed. This paper investigates the feasibility of using poly-l-lysine/alginate beads in a packed bed reactor configuration using results obtained from previous batch experiments.

INVESTIGATION OF THE USE OF POLY-l-LYSINE/ALGINATE BEADS IN A PACKED BED CONFIGURATION FOR REMOVING HEPARIN

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5.1. ABSTRACT

Bioreactor designs that can eliminate heparin during extracorporeal procedures have remained a challenge. Reactors developed thus far, including an immobilized heparinase I reactor and poly-L-lysine.HBr hollow fiber reactors are not appropriate for clinical purposes. Previous work has proved that poly-l-lysine/alginate beads could efficiently remove heparin from saline and blood. Necessary batch rate experiments were performed in both saline and blood to obtain the information required to design a heparin removal reactor using poly-L-lysine/alginate beads. The results are used to investigate the feasibility of using a packed bed reactor for the absorption process by adjusting the specifications of the reactor and analysing simple flow models.

5.2 INTRODUCTION

Approximately 20 million extracorporeal blood procedures are performed each year, of which between 8 to 30% of bleeding complications arises due to systematic heparinisation (University of Michigan[1]., 2001). Various methods to remove the heparin have been explored including the investigation of using various reactors to treat the blood prior to returning to the patient. The bioreactor designs can be divided into two categories: *membrane-based devices*, such as hollow fiber cartridges, and *porous particle devices* in the form of packed columns or fluidized beds (Ameer et al[2]., 1999a). Several investigators have bound the protein of interest to the walls of a hollow fiber dialyser to enhance the whole blood compatibility of the system, but in many cases the surface area requirements remain impractical (Vallar et al[3]., 1996). Others have suggested the use of porous particles such as agarose or cellulose beads, in either the packed bed or fluidised bed mode as a way to optimize the protein or absorbent loading per volume of device (Gejyo et al[4]., 1993; Langer et al[5]., 1982)

The reactors proposed thus far include the following:

5.2.1 Immobilized heparinase system

A novel approach to enable enzymatic elimination of heparin before the blood returned to the patient was introduced by Langer et al[5]., 1982. This approach consisted of placing a blood filter containing immobilized heparinase at the stream exiting from the extracorporeal device.

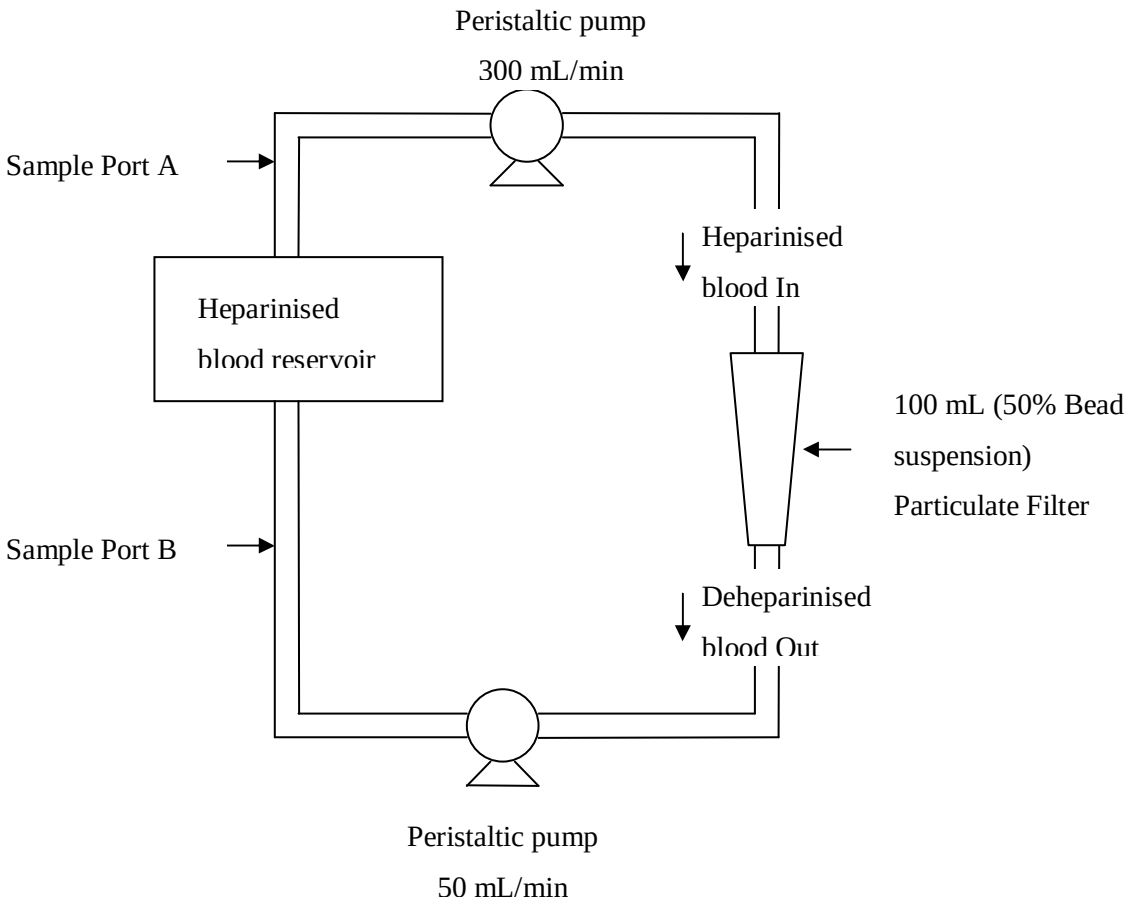


Figure 5.1: Diagram of the immobilized heparinase system
(adapted from Langer et al., 1982[6])

Fresh blood from dogs was heparinised and passed through the filter at a rate of 50 mL/min. The filter was placed between two 1-liter blood transfer packs. After the entire blood volume from one pack had passed through the filter into the other pack (one pass), the packs were disconnected and their positions with respect to the filter

were switched. Six passes of the blood were performed and heparin concentrations were analysed. There was an 80% loss of heparin after the first pass. After two passes 90% loss of heparin occurred. According to the author, the heparin products were observed to be non-toxic; however there was a 30% decrease in the white blood cells count and a 70% decrease in the platelet count. This system therefore required modifications to be utilized in clinical processes because of the damage to the blood cells.

5.2.2 Vortex flow fluidised bed reactor (VFFBR)

Ameer et al., 1999a[2] investigated the use of a whole blood fluidised bed Taylor-Couette flow device for enzymatic heparin neutralization using immobilised heparinase. *Taylor –Couette flow is the resulting fluid flow found in the gap between two infinitely long concentric cylinders, one or both of which are rotating along their common axis.* Heparinase was immobilised using agarose gel beads (200µm average diameter). Immobilization increased the stability of heparinase.

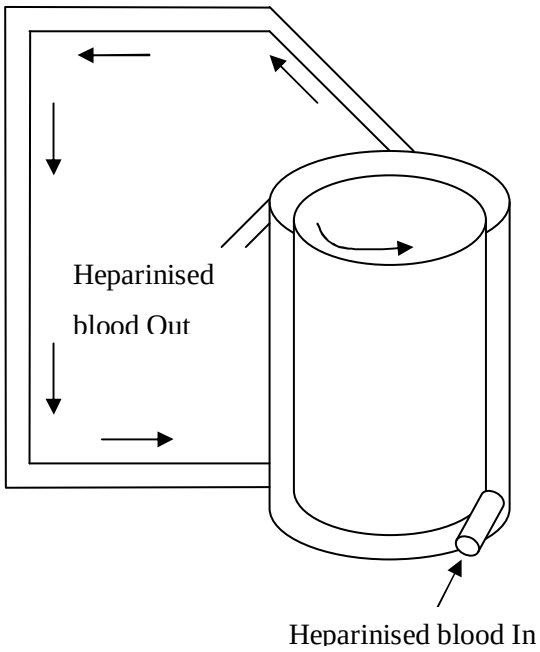


Figure 5.2: Diagram of the vortex flow fluidised bed reactor (VFFBR)
(adapted from Ameer et al.,1999a[2])

Whole blood entered and exited the device while the agarose was continuously recirculated. Taylor-Couette flow together with a "flow induced" recirculation was used as a reactor system to fluidise the agarose immobilized heparinase at high flow rates. The selected tangential location of the recirculation ports allowed continuous flow and fluidisation of the beads without the aid of an external pump. Ninety percent of the heparin was removed within 3 min of operation at a flow rate of 100 mL/min. However extensive damage to the red cells, white cells and platelets was caused by the Taylor vortices. The red cells were ruptured and hemoglobin was released into the plasma. Two steps were taken to reduce the damage. The gap width was increased (design 2) and the rotation rate was reduced to 200 rpm. Eighty percent of the heparin was removed in 3 min and the red cell hemolysis rate was reduced. However for clinical blood flowrates, the rotation rates required to maintain good fluidization was higher than 200 rpm, which lead to extensive damage to the blood cells. The authors felt that the VFFBR required further investigation due to the hemolysis, even though the percentage of heparin degradation was high. Their studies suggested that when Taylor-Couette flow is combined with macroscopic solid particles such as agarose beads, lysis of the red cells becomes an important issue. Further investigations were therefore required to eliminate the hemolysis problem.

5.2.3 Vortex flow plasmapheretic reactor (VFPR)

A device that simultaneously effected plasma separation and enzymatic reaction to minimize blood damage was designed by Ameer et al., 1999[6].

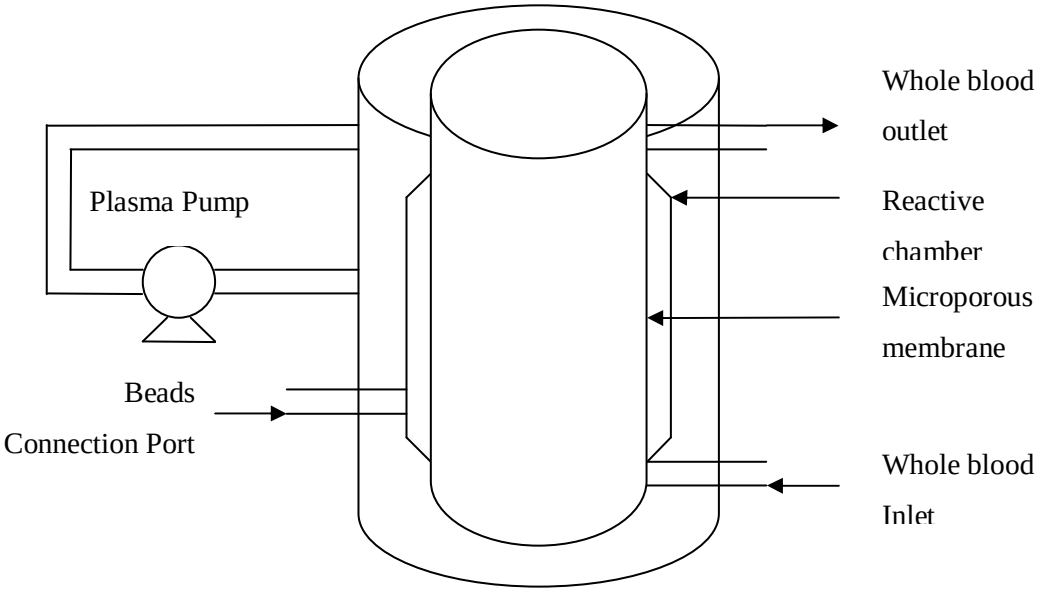


Figure 5.3: Diagram of the vortex-flow Plasmapheretic Reactor (VFPR)
(adapted from Ameer et al.,1999[7])

The reactor consisted of two concentric cylinders and a microporous membrane to separate the blood cells from the agarose immobilized heparinase. The outer stationary cylinder was counter-bored to accommodate an unsupported microporous membrane along the circumference of its inner surface. This set-up created a separate annular compartment (reactive chamber) for the agarose immobilized enzyme. A second, smaller compartment (plasma-collection chamber) was counter-bored into the outer cylinder to accommodate the agarose cylindrical 15-μm-polyester mesh to prevent the agarose beads from escaping the device and to minimize the pressure drop across the beads in the reactive chamber. A plasma pump was used to force convective flow of heparin through the membrane to improve the heparin mass transfer from the main annulus into the reactive chamber. The reacted solution was recombined into the main annulus of the VFPR.

A 500mL volume of the feed solution, heated to 32°C, was recirculated through the circuit at 120 mL/min with the plasma pump adjusted to 60mL/min. The rotation rate

of the inner cylinder was set to 1200 rpm. Approximately 20 mL of the immobilized heparinase was injected into the reactive chamber of the VFPR and was fluidized. The infusion of the heparin pre-reactor was adjusted to achieve clinically relevant heparin-plasma concentrations (5-12μg heparin/mL plasma). Heparin concentrations were measured at the inlet and outlet of the reactor. A mean heparin conversion of 34% in blood was observed. According to the authors, a minimum of 40 to 50% conversion of heparin is required for a reactor to be efficient, therefore the conversion did not fall within the target range; however the data demonstrated the feasibility of using the novel design to achieve regional heparinisation with negligible damage to the blood.

5.2.4 Immobilized Protamine Reactor

A reactor containing immobilized protamine was developed by Yang et al[7] (1991) that provided simultaneous extracorporeal heparin removal and protamine treatment. In vivo studies indicated that the reactor removed about 50% of the heparin in 10 minutes. Hemolysis complements were reduced by approximately 10 to 20% while there was a significant decrease in platelet and white blood cell counts.

5.2.5 Hollow Fiber Reactor

Based on the negative charge density characteristics of heparin, an affinity absorption technique was developed for the removal of heparin using poly-L-lysine.HBr that was immobilized onto a hollow fiber (Xinghang et al[8]., 1992). The activity of heparin decreased by 85% in 25 minutes and no adverse effects on the blood was observed. Clotting was observed to occur within the hollow fibers at low heparin concentrations.

The design criteria required for a reactor to remove heparin are as follows: (Ameer et al., 1999[6])

1. *Efficacy:* The average steady state single pass conversion of heparin must be 40 to 50%.
2. *Safety:* There must be no significant effect on whole blood cells such as hemolysis, rapid cell and platelet-count reduction, or excessive white cell or platelet activation.
3. *Stability:* There must be stable operations at flow rates of up to 300 mL/min.
4. *Simplicity:* A simple and low cost reactor would be preferred.

The reactors described above do not meet all of the four criteria mentioned above. The immobilised heparinase system and the VFFBR had high heparin degradation rates but caused damage to the blood cells at high flowrates. The VFPR met the safety criteria as very little damage to the blood cells was observed. However the VFPR had a low conversion of 34% of heparin. The immobilized protamine reactor had significant damage to the white blood cell counts and the platelets. The hollow fiber reactor had clotting complications. In order to expand all the above studies and based on the batch experiments, it is proposed that the feasibility of using a reactor packed with poly-L-lysine beads is investigated.

Previous work has indicated that poly-L-lysine /alginate beads can efficiently remove heparin from blood (Varghese et al[9]., 2006). Furthermore the spherical beads not only provide a larger surface area to volume ratio for removing heparin, but also provides three-dimensional stability with respect to mechanical stresses (Vally et al[10]., 2005). The beads are also relatively simple to produce at a relatively low cost. A fluidised bed or a packed bed reactor would be ideal for the absorption process, as the beads would have direct contact with the blood. The use of a fluidised bed reactor was investigated for the design of a hybrid liver using the poly-L-lysine/alginate beads (Vally et al[11]., 2005). The fluidized bed reactor was determined not to be feasible as the density of the beads was similar to the density of blood thereby providing

problems to the fluid flow and resulting in inhomogeneous concentrations through the reactor.

This article provides the design of a simple packed bed reactor. Previous work indicated that the absorption was a first order process and fitted the Freundlich Isotherm (Varghese et al[9]., 2006). The absorption process depended on the membrane thickness and the poly-L-lysine content of the beads (Varghese et al[11].,2006). The results are used to investigate the feasibility of using a packed bed reactor for the absorption process by using simple flow models and fundamental rate based models that can be used on both batch and flow systems.

5.3. REACTOR DESIGN MODELS

Various models were used previously (Varghese et. al[9]., 2006 and Varghese et. al[11]., 2006) to fit the batch experimental data. As will be discussed below, these models are not suitable for use in design of processes in flow reactors. Thus more fundamental models are used to analyze the batch data. These models are discussed in Section 5.3.2.

5.3.1 Models used for the Batch Processes

Varghese et. al[9]., 2006 fitted the Freundlich Isotherm to the equilibrium data that was obtained from the batch experiments. The Freundlich Isotherm is of form:

$$q_i = k_i x_e^{\,mi} \quad \text{.....Equation 5.1}$$

where:

m_i is a constant which is positive and generally not an integer;

k_i is a constant;

x_e is the fluid phase concentration at equilibrium (concentration of heparin in saline solution at infinite time) in U/ml;

q_i is the absorbed phase concentration in U/mL;

The Freundlich Isotherm is a commonly used model for analyzing batch data and in particular for relating the equilibrium concentrations x_e to system parameters. However its use for modeling more complex processes, such as occurs in flow systems, is very limited as in these situations the equilibrium concentration x_e is not well defined. In a typical system the solid may be in batch mode while the fluid flows continuously over the solid. In this case the parameter x_e would depend on the history of the solid that the fluid is in contact with and would change with time and position in the reactor.

Varghese et. al[9]., 2006 also analysed the time dependent behaviour by fitting the experimental data to a simple first order process namely:

$$\frac{dx}{dt} = K(x - x_e) \qquad \text{……..Equation 5.2}$$

where:

t is the time in seconds;

K is the rate coefficient in seconds^{-1};

x is the concentration of heparin in the liquid phase in U/mL;

This model was shown to fit the batch data (Varghese et. al[9]., 2006). Although this appears at first glance to be a fairly fundamental model, it is found that different experiments were fitted with different values of the rate coefficient K. It was further shown that K is proportional to the number of beads and to the total volume of the membrane of the beads (Varghese et. al[11]., 2006). This means that this model is again not useful in more complex flow situations as the value of the rate coefficient K as well as the equilibrium concentration x_e is again not well defined in these situations.

5.3.2 Fundamental Models for both Batch and Continuous Absorption Processes

Fundamental rate based absorption models were developed that are applicable in both batch and continuous flow systems. The model used is fundamental and is a simple rate based model which assumes that the absorption process is reversible. The rate of absorption onto the solid is assumed to be proportional to the fluid phase concentration and the number of available sites in the solid. The desorption from the solid to the fluid phase is assumed to be proportional to the concentration of the solid phase. Using this model, one can propose the following rate equation:

$$r = k_1 x \frac{V_y}{V_x}(y^{\Theta} - y) - k_2 \frac{V_y}{V_x} y \quad \text{.....Equation 5.3}$$

where:

r is the rate of reaction or absorption per volume of liquid in U/(mL sec);

k_1 is the rate constant for the process in mL/U sec;

k_2 is the rate constant for the desorption process in 1/sec;

x is the concentration of heparin in the liquid phase in U/mL;

y is the concentration of heparin in the solid phase in U/mL;

y^{Θ} is the capacity of the bead per volume of bead membrane in U/mL;

V_x is the volume of liquid in mL;

V_y is the volume of solid (total volume of membrane of the beads) in mL.

In this model the parameter y^{Θ} is assumed to be fixed for a given solid and hence is the same in both batch and continuous flow systems. The above rate equation has been fitted to a wide range of experimental results in other absorption processes and has given a good fit for both batch and continuous data (Williams, D. F. et. al[15]., 1985). This model is a two-parameter model (k_1 and k_2) and as before the parameters are fitted by using the batch experimental data.

5.3.3 Model simplifications for Batch systems at Equilibrium (Infinite Time)

The Freundlich Isotherm was previously shown (Varghese et al[9]., 2006) to fit the batch equilibrium data. It is therefore necessary that the proposed rate based model simplifies at equilibrium to a form that fits the batch experimental data.

It was experimentally found that when beads loaded with heparin were placed in saline solution; the heparin concentration in the saline solution was negligible, implying that the heparin absorption was fairly irreversible. This implies that the proposed fundamental rate model can be simplified by assuming that $k_2 \approx 0$.

The fundamental rate equation (equation 5.3), with the simplification of $k_2=0$, has two terms that could tend to zero at equilibrium, namely x and (y^{Θ} -y). These two scenarios will be examined separately.

i) *The concentration of heparin in the liquid phase, x, becomes zero*. In this case the equilibrium concentration of heparin in the solid phase is not equal to the capacity of the beads per volume of bead membrane ($y_e \neq y^{\Theta}$), that is the beads have not been fully loaded yet.

ii) *The term (y^{Θ} -y) becomes zero*. In this case the concentration of heparin in the liquid phase, x, is not zero and has attained an equilibrium value ($x = x_e$). The beads are fully loaded in this situation and therefore $y_e = y^{\Theta}$.

If the results obtained from the batch experiments, Figure 3.8 (Varghese et al9., 2006) are observed, it is clear that for the two experiments with the smaller number of beads (that is 848 and 1696 beads), the liquid concentration of heparin, x, at equilibrium is non zero. Thus the liquid concentration is approaching an equilibrium value, xe≠0, and therefore ye can be assumed to be yΘ. As the number of beads increases (the experiments with 3650 and 8000 beads), the liquid concentration of heparin, x,

approaches zero. At equilibrium the beads are not fully loaded and therefore $y_e \neq y^{\Theta}$.

For the smaller number of beads where the beads are fully loaded at equilibrium and $x_e \neq 0$ the fundamental rate equation at equilibrium is:

$$k_1 \frac{V_y}{V_x} x_e (y^{\Theta} - y_e) = 0 \qquad \text{……..…Equation 5.4}$$

which simplifies to:

$$y^{\Theta} = y_e \qquad \text{……..Equation 5.5}$$

The mass balance is:

$$V_x (x_o - x_e) = V_y y \qquad \text{……..Equation 5.6}$$

which simplifies at equilibrium to:

$$y_e = y^{\Theta} = \frac{V_x}{V_y} (x_o - x_e) \qquad \text{……..Equation 5.7}$$

Using the results of the 848 beads and 1696 beads in Equation 5.7 the average value of y^{Θ} is estimated to be 1004 U/mL, (see Table 5.1)

Table 5.1: Estimated values of y^{Θ} from equilibrium data.

	No of beads	Equilibrium concentration x_e U/mL	Estimated y^{Θ} U/mL
Experiment 1	848	2.23	929
Experiment 2	1696	1.33	1079
Average			**1004**

5.3.4 Model predictions of Concentration versus Time for Batch Systems

A more stringent test of the proposed rate based model is to see how well it fits the batch data at times other than equilibrium. In order to do this the rate based model will be fitted to the results obtained previously by Varghese et al[9]., 2006 for batch absorption systems.

Assuming that k_2 is negligible, the fundamental rate equation, r, becomes:

$$r = k_1 x \frac{V_y}{V_x}(y^{\Theta} - y) \qquad \text{……..Equation 5.8}$$

Substituting the mass balance (Equation 5.6) in Equation 5.8 and simplifying:

$$r = k_1 x(a + x) \qquad \text{……..Equation 5.9}$$

Where $a = \frac{V_y}{V_x} y^{\Theta} - x_0$

For the batch absorption process:

$$\frac{dx}{dt} = r = k_1 x(a + x) \qquad \text{……..Equation 5.10}$$

Integrating Equation 5.10 and simplifying, the following equation is obtained:

$$kt = -\frac{1}{a}\ln\left(\frac{(a + x)x_0}{(a + x_0)x}\right) \qquad \text{……..Equation 5.11.}$$

A graph of $-\frac{1}{a}\ln\left(\frac{(a + x)x_0}{(a + x_0)x}\right)$ versus time, t, should yield a straight line passing through the origin with a slope of the rate constant, k_1.

The experimental results obtained previously for the batch absorption experiments (Varghese et al[9]., 2006) are fitted and shown in Figure 5.4. Since the ln term is sensitive at small values of the term in the parenthesises, small experimental errors in the value of the liquid concentration at low rates will cause a large deviation to the value of the ln term. Liquid concentrations of above 70% conversions were thus not used in to fit the value of k_1.

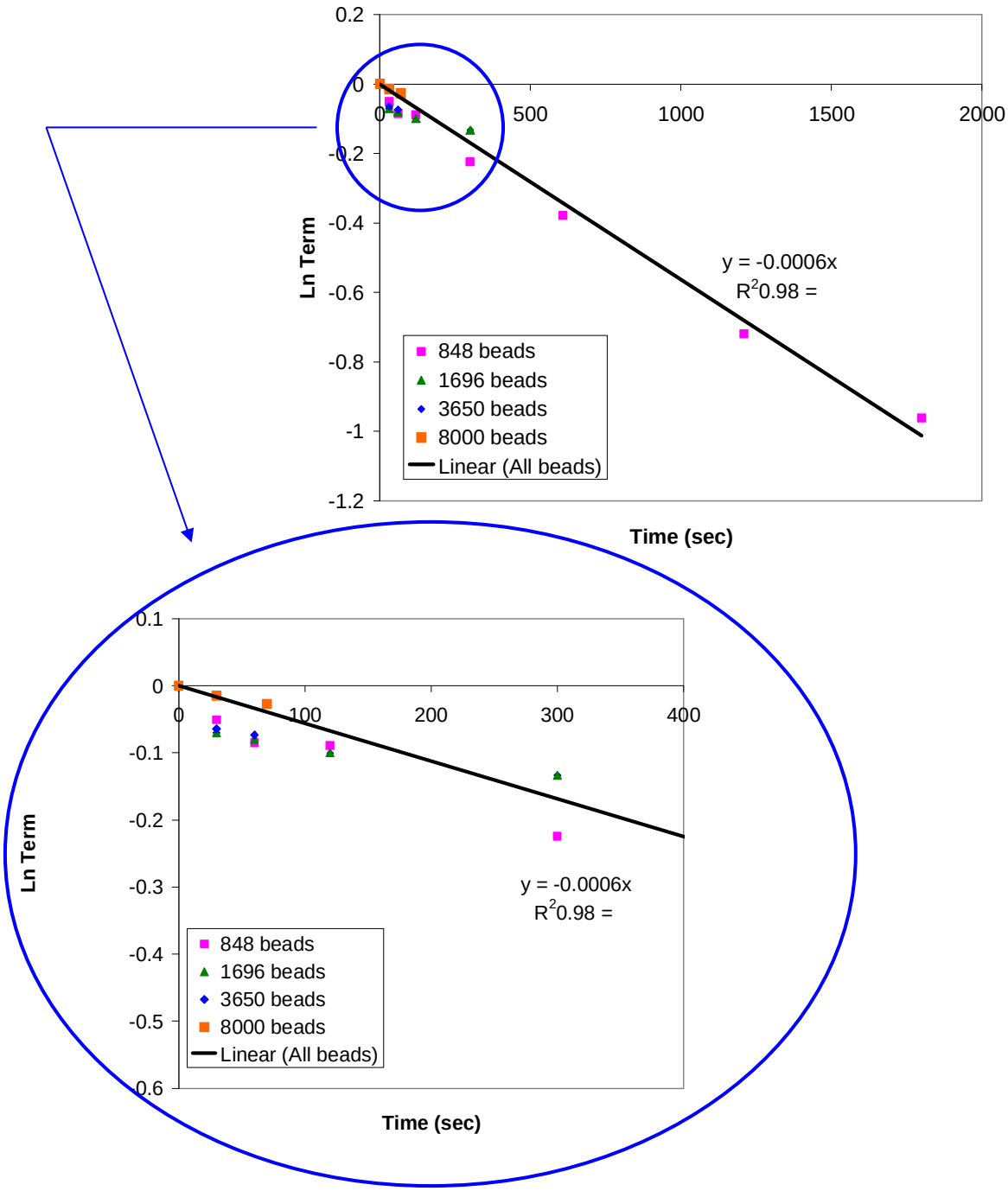


Figure 5.4 Graph to obtain k_1, parameter of the fundamental rate based equation

From Figure 5.4, it can be seen that all four sets of data fit the model all the data falls on a straight line and importantly all the data fits the same slope. One can therefore

have confidence in the model and from the slope one can estimate the rate constant, k_1, to be –0.0006 mL/U sec.

5.3.5 The Effect of Blood and Fetal Calf serum (FCS) on the parameters of the rate equation

The data for the absorption of heparin in FCS and blood can also be analyzed to try to understand how these substances affect the performance of the beads. Assuming that the capacity of the beads, y^{Θ}, is the same value as that obtained for saline (y^{Θ} = 1004 mL/U sec) and using the results obtained from Figure 4.13(Varghese et. al[11]., 2006), the data is fitted for blood and FCS and the results are tabulated in Table xx.

Table 5.2 Comparison of values of k_1 for saline, FCS and blood

	k (mL/Usec)
Saline	-0.0006
FCS	-0.0024
Blood	-0.0039

It should be noted that the data for blood and FCS are limited as only one experiment was performed, however the general effect can be noted from the above results and it is clear that the rate constant value is the greatest for blood. The absorption rate is therefore sensitive to the type of fluid used and the pH as stated previously (Varghese et. al[11]., 2006).

5.3.6 Model Testing

One can compare the experimental data to the model by using the values of the parameters estimated previously in the fundamental rate model. One can compare the measured equilibrium concentration of heparin in the liquid phase with those predicted by the model.

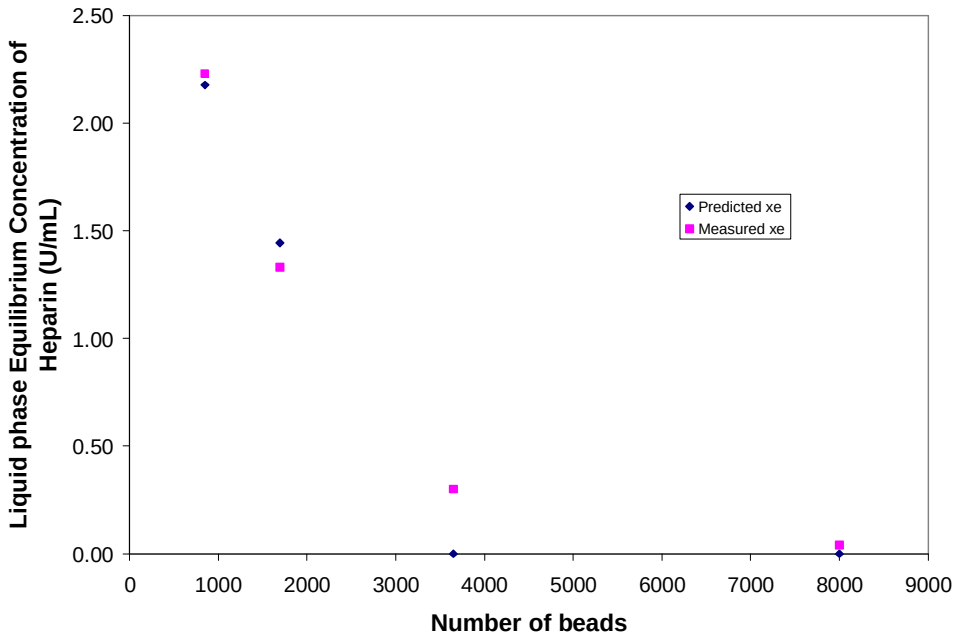


Figure 5.5 Comparison of predicted and measured equilibrium concentration

The results indicate that the fundamental rate model predicts the measured equilibrium concentration data well.

The concentrations of heparin in the liquid phase at a particular time can be predicted by simplifying Equation 5.11 and obtaining the following equation:

$$x = \frac{ax_0}{b} \qquad \text{……Equation 5.12}$$

where

$$b = (a + x_0)\exp(-kta) - x_0 \text{ and } a = \frac{V_y}{V_x} y^{\Theta} - x_0$$

The predicted results are compared with the measured results obtained previously from the batch experiments of saline and is shown in Figure 5.6.

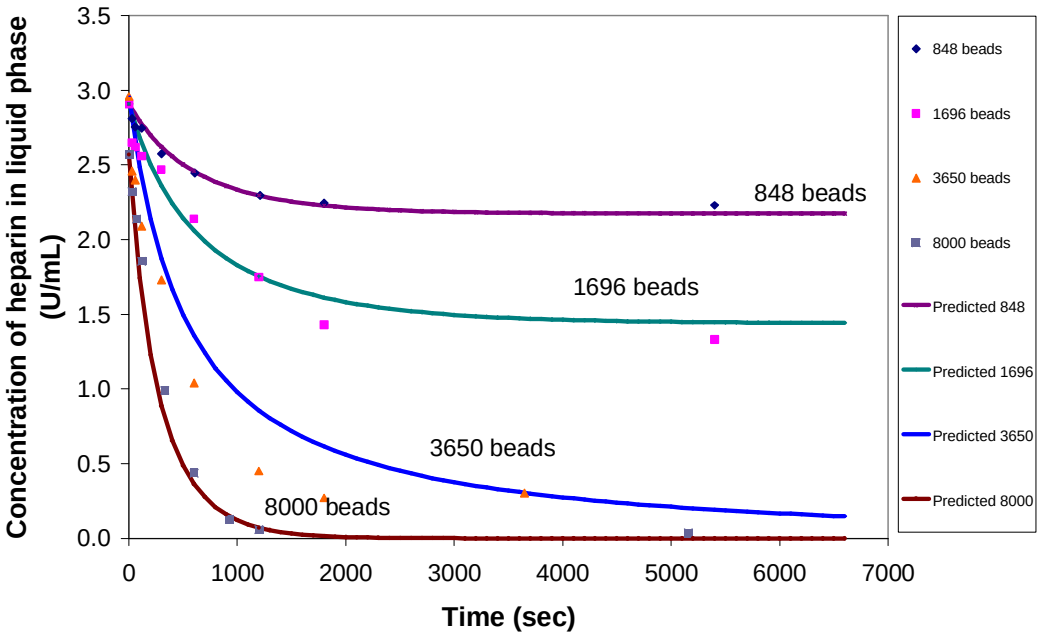


Figure 5.6: Comparison of predicted and measured concentrations of heparin

The results indicate that the fundamental rate model can be used to predict the results for a batch process in saline relatively well, with the exception of the 3650 beads, which may be attributed to experimental error and may require further investigation in future.

5.3.7 Using the Fundamental Rate Based Model to predict the Performance of a Continuous Reactor

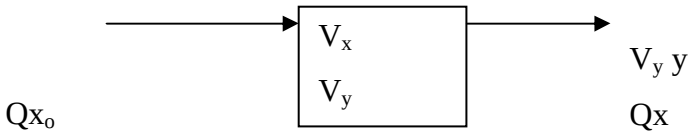
In a batch process, all the reactants are added in the beginning, the reaction is allowed to proceed and samples are withdrawn to obtain the compositions that change with time until the reaction is completed or stopped when the required conversion has been reached. In a flow process, the reactants are fed into the reactor continuously and products are withdrawn continuously. A packed bed reactor with a reasonably low pressure drop would be the ideal choice for a continuous reactor as minimum damage is expected to the blood cells since the shear stresses would be small if pressure drop was low. An added advantage is that there are no mechanical moving parts within the

reactor which also eliminates possible damage to the blood cells. Blood containing heparin can be allowed to flow through a packed bed reactor filled with poly-L-lysine/alginate beads. It is proposed that such a reactor can be designed to achieve the required absorption of heparin within the reactor.

The next step is therefore to predict the performance of a continuous reactor using the information from the batch experiments. Since the aim of this prediction is to check if the option of a reactor packed with beads is feasible to remove heparin in a flow process, one can test this theoretically by using a very simplified reactor model. A model is required for both the liquid and solid phases in this case. One can assume that the change in concentration of heparin in the solid phase is slow in comparison to that in the liquid. As a result of the different time constants of the two phases one can use a pseudo-steady state model in that one can assume that the liquid phase at any time is approximately at steady state, but that the concentration in the liquid phase will change slowly with time.

The simplest model that characterises the major features of a flow reactor is:

- Liquid phase: as the liquid concentration changes slowly with time, it is proposed that a simple CSTR model be used for initial screening. Under this assumption, the liquid concentration in the device is uniform. Further more under this assumption the concentration of heparin in the liquid flowing out of the reactor is the same as that in the reactor. The exit concentration (and hence the concentration in the reactor) will change with time as the beads in the reactor become loaded.
- Solid phase: the solid is in batch mode and it is assumed that the concentration of the solid is uniform in the reactor. Thus the solid concentration is characterised by a single value that is time dependent.



where:

Q is the volumetric flow rate of liquid in mL/min and is constant;

x is the concentration of heparin in liquid in the reactor as well as in the exit stream from the reactor in U/mL. This value will change with time.

x_o is the concentration of heparin in liquid in the feed to the reactor in U/mL and is constant.

V_x is the holdup or volume of liquid within the reactor

y is the concentration of heparin in the solid phase and will change with time.

V_y is the volume of solid (membrane) within the reactor.

The mass balance for heparin in the liquid phase is as follows:

$$Qx - Qx_o = rV_x \qquad \text{……..……..Equation 5.13}$$

One can use the fundamental rate model in the continuous case and very importantly, the values of the parameters k_1 and y^{Θ} that was obtained by fitting batch data can be used in the continuous reactor model. As was stated previously, the process is nearly irreversible and thus it can be assumed that k_1 is much larger than k_2. The fundamental rate model, Equation 5.2, simplifies in this case to:

$$r = k_1 \frac{V_y}{V_x} x\left(y^{\Theta} - y\right) \text{…….....Equation 5.14}$$

Substituting the rate model (Equation 5.14) into the liquid phase mass balance (Equation 5.13) gives:

$$x - x_o = k_1 x \tau_s (y^{\Theta} - y)$$ ……Equation 5.15

where:

τ_S is a residence time like constant for the beads within the reactor and is equal to (V_y/Q) in sec.

The mass balance of heparin in the solid phase is as follows:

$$V_y \frac{dy}{dt} = Qx_o - Qx$$ ……....Equation 5.16

which simplifies to

$$\frac{dy}{dt} = \frac{1}{\tau_s}(x_o - x)$$ ……...…….Equation 5.17

The initial condition from Equation 5.17 is that at t=0, y =0.

Equations 5.15 and 5.17 can be solved simultaneously to obtain the trends of the concentration change of heparin within the reactor. The values of k_1 and y^{Θ} used are those determined for saline from the batch experiments. There is only one parameter that the designer is free to chose, namely τ_s.

The subsequent sections look at the sensitivity of the model to the value of the parameter τ_s and in particular investigates if there is a value of the parameter where the reactor can satisfy the specifications.

5.3.8 Reactor Specifications

In section 5.2.5, the following criteria were given for a reactor to remove heparin:

1. Efficiency: A per pass conversion of 40 to 50 % was specified. This is thus a criterion that can be used in sizing the reactor. Low[15] et al., 1996 states that a 40 to 70 % conversion is required; therefore a range of 40 to 70% conversion will be used to size the reactor.
2. Safety: No significant effect on whole blood cells should be observed, therefore a simple packed bed reactor will be considered to minimize the damage that could be incurred on the cells by additional requirements such as stirring.
3. Stability: Stable operation at flowrates up to 300 ml/min is specified. This flowrate can be used as a design criterion. For kidney dialysis the range of flowrate of blood is between 250mL/min and 500mL/min (Renal Unit, Johannesburg Hospital. Therefore a flowrate of 300mL/min will be considered as a design criterion.
4. Simplicity: The reactor proposed is fairly simple, the most expensive part of it is are the beads. The proposed design is therefore believed to meet this criterion.

From these the following specifications could be made:

- It was assumed that the inlet concentration of heparin in the liquid flowing into the reactor was constant at 1U/mL.

- The reactor must therefore be sized that the outlet concentration of heparin lies between 0.60 U/mL (40% conversion) and 0.40 U/mL (60% conversion). If necessary this criteria could be relaxed as Low[15] et al.,1996 specify that the exit concentration of heparin in blood/saline must be in the range of 0.3U/mL (corresponding to 70 % conversion) to 0.6 U/mL (corresponding to 40% conversion) to avoid clotting within the reactor.

- The volume of blood in the reactor cannot exceed 150 mL as this is a clinical specification to minimize hemodilution of the patient. (Ameer[2] et al., 1999a). Thus the value of V_x must be equal to or less than 150 mL.

- An operating time of more than four hours is preferred for the reactor as the usual dialysis treatment lasts for approximately four hours. This will allow for simplicity of operation in that unit would not need to be changed during the treatment.

5.3.9 Reactor Absorption Behaviour

Equations 5.15 and 5.17 were solved using polymath and a graph of the exit liquid concentration x versus time for various values of the parameter τ_s is given in Figure 5.7. The values of k_1 = -0.0006 mL/U sec and y^{Θ} = 1004 U/mL used are those determined for saline from the batch experiments. The operating range for the reactor to meet specifications is also shown in Figure 5.7. Thus the lines corresponding to 40 and 70 % conversion of heparin are shown. The reactor should operate between these limits. The time constraint of 4 hours is also shown. Thus the curves which lie within this region satisfy the reactor specification. It can be seen that there is no curve which lies within the operating region for a full 4 hours.

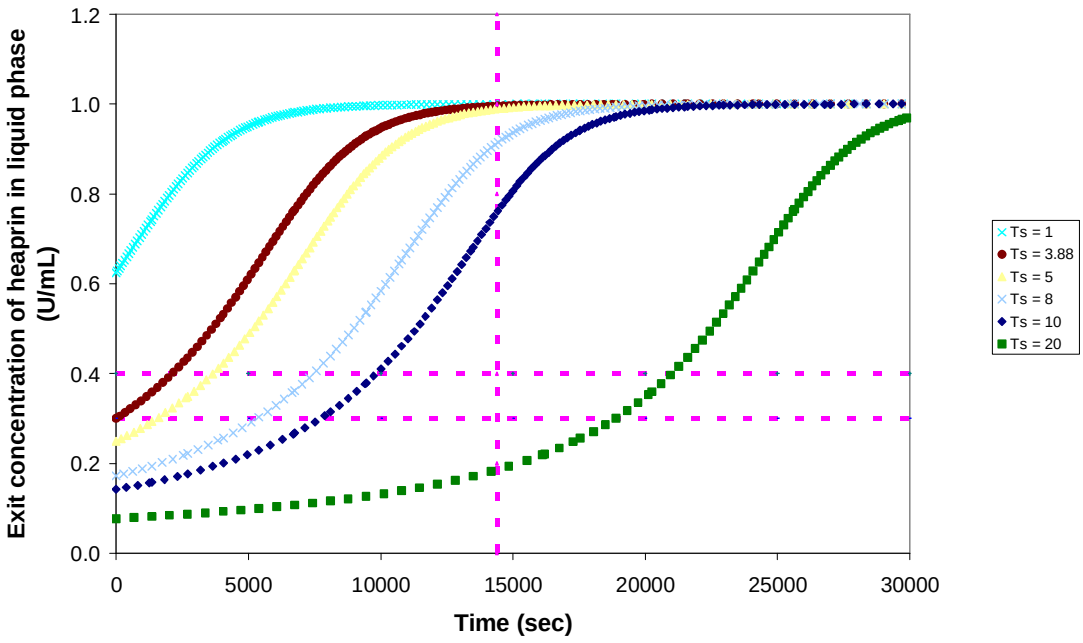


Figure 5.7: Reactor Absorption Behaviour for Saline

It can be seen that Equation 5.15 can be used to estimate the value of τ_s such that the liquid leaving the reactor initially has a 70 % removal of heparin:

$$\tau_s = \frac{(0.3-1)x_o}{0.3x_o k_1 y^{\Theta}} = 3.88\,\text{sec}$$

It can be seen that the curve corresponding to τ_s =3.88 sec meets the upper limit of conversion at 70% and at 4963 sec, corresponding to 1.4 hours, reaches a conversion of 40 % and therefore would no longer meet the design criteria.

One can therefore conclude that a packed bed would meet the design specifications for 1.4 hours and thus if longer operating times where required, the packed bed would need to be replaced every one and a half hours. To see if this is feasible, the following should be considered: sizing the reactor and estimating the number of beads required and more importantly the volume of blood in the reactor.

The volume of membrane V_y required can be estimated using the volumetric flowrate of 300 ml/min and τ_s =3.88 sec from:

$$V_y = \tau_s Q \qquad \text{……...…….Equation 5.18}$$

The total volume of membrane that is required is 19.4 mL.

The number of beads N required can therefore be estimated from:

$$V_y = N\frac{4}{3}\pi(r_{out}^3 - r_{in}^3) \qquad \text{……...…….Equation 5.19}$$

where

r_{out} is the outer radius of the bead and

r_{in} is the inner radius of the bead.

If the outer and inner radius of the beads is used as that of the batch experiments, r_{out} = 0.00045m and r_{in} = 0.000436m it can be seen that 561849 beads are required.

The reactor volume V_r can be estimated for a packed reactor of voidage ε =0.4 from:

$$V_r = \frac{(N\frac{4}{3}\pi r^3)}{(1-\varepsilon)} \qquad \text{……...…….Equation 5.20}$$

The volume of the reactor is thus estimated to be 357 mL. The priming volume of blood is εV_r and is in the range of 143 mL.

According to Ameer[2] et al., 1999a the volume of the reactor cannot exceed 150 mL to minimize hemodilution of the patient. Previous work indicated that as the thickness of the membrane of the beads is increased the amount of heparin absorbed would

increase. The effect of membrane thickness on the volume of the reactor can be investigated and this is shown in Figure 5.8.

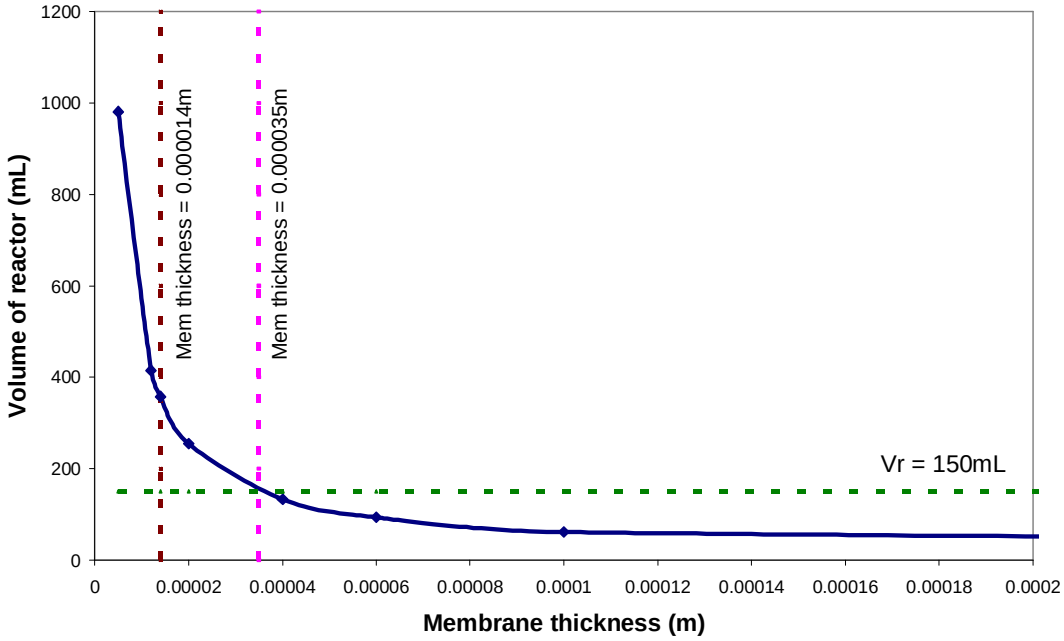


Figure 5.8: Effect of membrane thickness on the reactor volume

In order to satisfy the criterion of minimising hemodilution, from the graph the membrane thickness should be increased from $1.4*10^{-5}$m to $3.5*10^{-5}$m. The specifications for the proposed reactor would therefore be as in Table 5.3.

Table 5.3: Proposed Reactor Specifications for Saline

	Reactor Specifications
Time like variable (τ_s)	3.88 sec
Volume of membrane (V_y)	19.4 mL
Number of beads (N)	235676
Volume of Reactor (V_r)	150 mL
Priming blood volume (V_b)	60 mL
Operating Time	1.4 hours

Thus the reactor meets the specifications given previously. It should be noted that the values of k_1 could change if the membrane of the beads is altered; therefore further experiments are required to determine the exact values.

Using the results obtained from the blood experiment, where the values of k_1 = -0.0039 mL/Usec and y^{Θ} = 1004 U/mL, and solving Equations 5.15 and 5.17, the graph of the exit liquid concentration x versus time for various values of the parameter τ_s is given in Figure 5.9. The results indicate that τ_s must be 0.6 sec and must be replaced every 12 minutes in order to meet the required specifications. Therefore for the duration of the dialysis treatment of 4 hours, at least 20 packed bed reactors/cartridges would be required. The blood results were based on limited data, thus further experiments will be required to confirm the results, however from the experiments the absorption in blood appears to be a lot faster, the reactor should therefore be designed to meet the saline specifications. This can be easily achieved in blood by introducing mass transfer effects to slow down the absorption process. The beads can be coated with an inert substance or the membrane can be made thicker using sodium alginate to reduce the absorption by poly-L-Lysine. In this way the effect of the rate constant will be decreased and the number of cartridge changes could be limited.

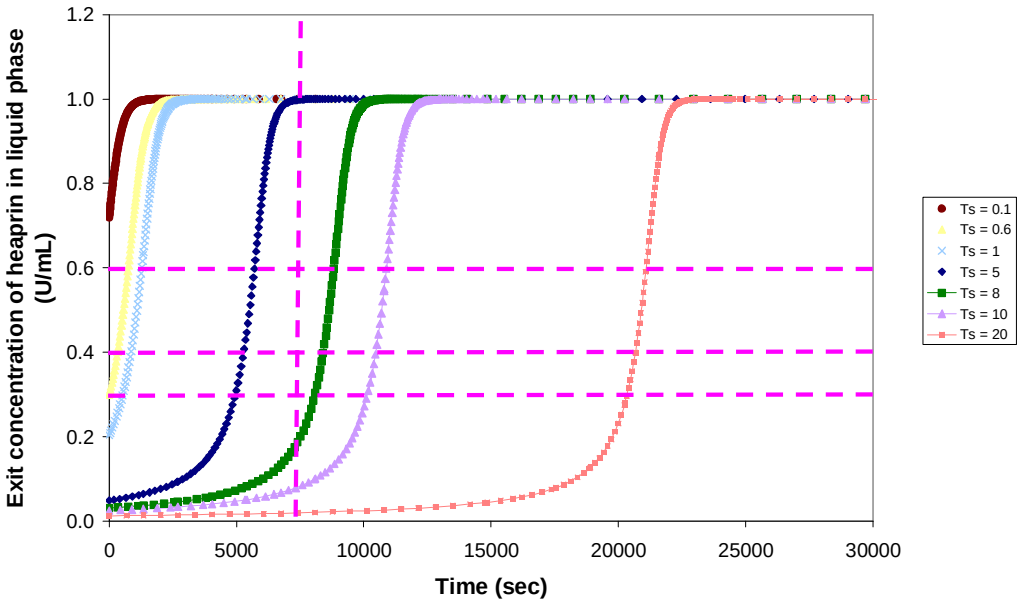


Figure 5.9: Reactor Absorption Behaviour for Blood

The final check that one can do at this stage is to estimate the pressure drop across the reactor.

5.3.10 Pressure drop within the Reactor

Another important consideration of the reactor design is the pressure drop across the reactor. The pressure drop must be as low as possible within the reactor to avoid frictional losses and hence possible damage to the blood.

The pressure drop can be estimated using the Blake –Kozeny equation as follows:

$$\Delta P = \left[\frac{150 \mu L u_o (1-\varepsilon)^2}{D_p^2 \varepsilon^3} \right] \qquad \text{…….…… Equation 5.21}$$

where:

ΔP is the pressure drop in Pa.

μ is the viscosity of blood in kg/ms. The viscosity of blood from literature is 2.72 centipoise.

L is the length of the reactor in m.

u_o is the velocity of the blood through the reactor (based on reactor cross sectional area) in m/s.

ε is the voidage (0.4 for spherical particles).

D_p is the diameter of the beads in m (Range between 800μm and 2000μm).

The velocity of the blood through the reactor is calculated as follows:

$$u_o = \frac{Q}{A} \qquad \text{…….……..Equation 5.22}$$

where:

Q is the flowrate of blood through the reactor in m^3/sec.

A is the area of the reactor in m^2.

It is known from our previous calculations that the volume of the reactor V_r = 150 ml. Furthermore V_r =AL and hence the cross sectional area and length of the reactor are related. Equation 5.21 can thus be rewritten as follows:

$$\Delta P = \left[\frac{150 \mu V_r Q (1-\varepsilon)^2}{A^2 D_p^2 \varepsilon^3} \right] \qquad \text{……..Equation 5.23}$$

One can look at the upper and lower limits on the cross sectional area of the device and compute the pressure drop across the reactor for all reasonable (i.e. between the upper and lower limits) dimensions.

The minimum cross sectional area A_{min} would typically correspond to a device with a diameter equal to 10 particle diameters D_p, i.e.

$$A_{\min} = \pi (5 D_p)^2 \qquad \text{……..Equation 5.24}$$

The maximum cross sectional area A_{max} would roughly correspond to a unit where the diameter D and length were equal. Thus

$$V_r = (\frac{\pi D^2}{4}) D \qquad \text{……..Equation 5.25}$$

And

$$A_{\max} = (\frac{\pi D^2}{4}) \qquad \text{……..Equation 5.26}$$

The Ergun equation can be solved between these limits and the possible range of pressure drops in the device can be estimated. The results for saline and blood are indicated in Table 5.4.

Table 5.4: Pressure Drop Results for Saline and Blood

	Volume of Reactor (mL)	Minimum Area of Reactor (m^2)	Maximum Pressure (kPa)	Maximum Area of Reactor (m^2)	Minimum Pressure (Pa)
Saline	150	$6.36*10^{-5}$	525	0.31	0.022
Blood	150	$6.36*10^{-5}$	193	0.041	0.44

For the estimated Vr = 150 mL, the minimum area of $6.36*10^{-5}$ m^2 yields a pressure drop of 525 kPa, while the maximum area of 1.69 m^2 yields a pressure drop of 0.022 Pa. The typical pressure drop across a haemoperfusion device, such as a dialysis machine is about 13.33 kPa (100mmHg). Therefore the reactor can be designed for a pressure drop of 10 kPa with an area of $4.61*10^{-4}m^2$.

From Equation 5.23, as the diameter of the beads increases, the pressure drop decreases. However increasing diameters may introduce diffusional limitations in the absorption process. From the batch experiments (Varghese et al[11]., 2006) it was shown that beads with the smaller diameter absorbed more heparin than the larger beads. It was also shown that the smaller beads had a thicker poly-L-lysine membrane therefore increasing the quantity of heparin absorbed. However as the bead sizes decrease a wider size distribution is obtained during the production. A wider distribution of sizes will result in an increased pressure drop across the reactor. This trend needs to be taken into consideration when designing the reactor and a balance between pressure drop and capacity will need to be made in deciding on the optimal bead size.

The flow rate of the blood will also affect the pressure drop within the reactor. As the flow rate decreases the pressure drop will decrease. If this device is used in series with existing devices such as the dialysis machine, then the flowrate through the device

will be set by these and thus is not a design variable. The designer however can choose the diameter of the device, and hence the velocity of the liquid phases in the device. A larger diameter of the device will lead to the lower liquid phase velocities and hence lower the pressure drops across the device. Again the designer will need to balance reduced pressure drop against efficient solid- liquid contact when choosing a suitable diameter.

5.4. CONCLUSION

The experiments and estimations have indicated that a reactor with poly-L-lysine beads is a feasible option to absorb heparin from extracorporeal procedures. The reactor should be designed to meet the saline specifications. Using an exit stream for a flowrate of 300 ml/min, a voidage of 0.4 and a reactor volume of 150 mL for saline, 235676 beads are required. The area of the reactor should be 0.31 m^2 in order to limit the pressure drop and should be replaced every one and a half hours.

A packed bed reactor would also be simple, relatively cheap and easy to operate. All the required specifications that are 40 to 70% conversion of heparin, safety (minimum damage to blood cells) stable flowrate of 300mL/min and simplicity are achieved by the proposed reactor configuration. The reactor should be tested experimentally in a flow process to determine the optimum parameter values.

5.5. REFERENCES

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Chapter 6: CONCLUSION

The study indicates that heparin can be efficiently removed from saline, fetal calf serum and blood using poly-L-lysine/alginate beads.

Article 1 indicates that the absorption process is a first order irreversible process and the data fits the Freundlich Isotherm. The amount of heparin absorbed can be increased by increasing the number of beads in a sample solution.

Article 2 illustrates that the beads have minimal effect on the constituents of human blood. The thickness of the membrane can be adjusted to optimize the absorption process. The thicker the membrane, the more heparin is absorbed. The amount of heparin absorbed can also be increased by increasing the poly-L-lysine content of the beads. The smaller beads are more efficient because of their thicker membranes.

Article 3 indicates that a reactor with poly-L-lysine beads is a feasible option to absorb heparin from extracorporeal procedures. The number of beads or reactive sites should be selected to ensure the adequate absorption of heparin to prevent clotting within the reactor but allow clotting once the blood returns back to the patient. The ideal configuration would be to replace a packed bed reactor/cartridge every one and a half hours during the dialysis process.

This study indicates that poly-L-lysine/alginate beads could be used to design a novel reactor. The reactor and bead parameters could be altered to optimize the absorption process. The reactor design provides an efficient, simple and user friendly option of extracting heparin from extracorporeal systems. Further investigation using a flow process for the proposed reactor configuration, more experiments using blood and clinical trials are recommended to optimize the design and to limit all possible problems that may arise during extracorporeal procedures.

APPENDICES

Appendix A Data for Chapter 2: Apparatus and methods

Appendix A1 Calibration

Concentration in U/mL	Absorbance 1	Absorbance 2
0.00	0.0000	0.0000
0.75	0.0253	0.0254
1.50	0.0490	0.0481
3.00	0.0927	0.0947

Appendix A2 Solutions used in the formation of poly-l-Lysine/alginate beads

4% Sodium alginate solution (500 mL)

Sodium alginate	20g
Sodium chloride	2.25g

100 mM Calcium chloride solution (500 mL)

100 mM Calcium chloride	7.35g
10mM HEPES	1.19g
20mM Fructose	1.8g

50mg% Poly-L-Lysine solution (500mL)

Poly-L-Lysine	0.25g
Sodium chloride	4.25g
10 mM HEPES	1.19g
20mM Fructose	1.8g

0.2% Sodium alginate solution (500 mL)

Sodium alginate	1g
Sodium chloride	4.25g
10 mM HEPES	1.19g
20mM Fructose	1.8g

50 mM Sodium citrate solution (500mL)

Sodium citrate	7.35g
Sodium chloride	4.25g
20mM Fructose	1.8g

Appendix A3 Efficiency of the 900 µL pipettes

Measured mass in g	Expected mass in g
0.8935	0.9
0.8937	0.9
0.8947	0.9
0.8945	0.9
0.8957	0.9
0.8956	0.9
0.897	0.9
0.8968	0.9
0.8973	0.9
0.8957	0.9

Appendix A4 Efficiency of the 100 µL pipettes

Measured mass in g	Expected mass in g
0.0976	0.1
0.0974	0.1
0.0976	0.1
0.0973	0.1
0.0981	0.1
0.0979	0.1
0.0979	0.1
0.0979	0.1
0.0979	0.1
0.0979	0.1

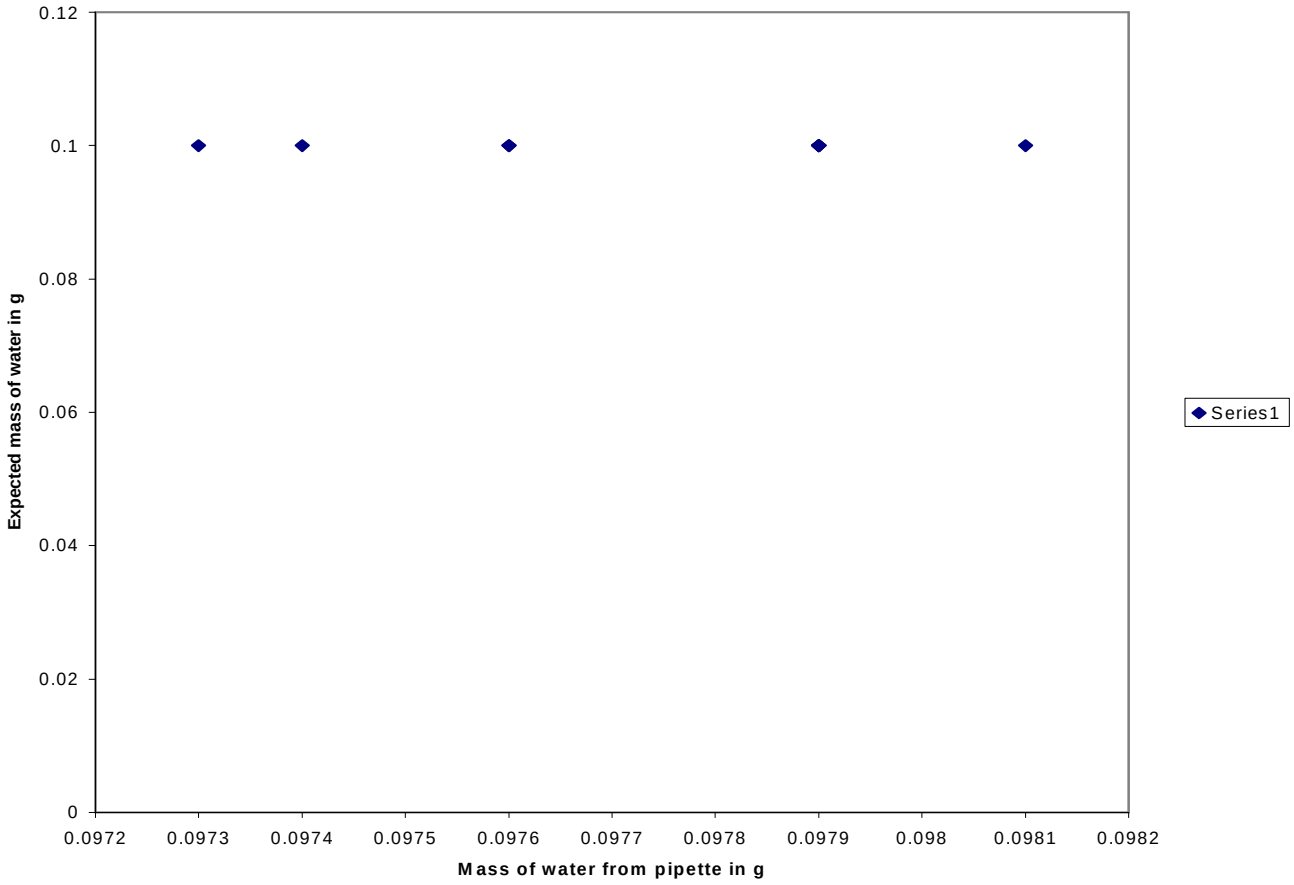

Figure A4.1 Curve of the expected mass of water versus the weighed mass of water for a 100 µL pipette

Appendix B Data for Chapter 3: Efficiency of Polymer Beads in the Removal of Heparin

Appendix B1 Figure 3.4 Results

8000 900 μm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.572	2.572	2.572	0.0000
30	2.320	2.326	2.323	0.0042
70	2.137	2.139	2.138	0.0014
120	1.859	1.851	1.855	0.0057
330	0.991	0.989	0.990	0.0014
600	0.436	0.437	0.437	0.0007
930	0.124	0.128	0.126	0.0028
1200	0.058	0.058	0.058	0.0000
5160	0.035	0.038	0.037	0.0021

Appendix B2 Figure 3.5 Results

Experiment 1				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.914	2.908	2.911	0.0042
30	2.652	2.638	2.645	0.0099
60	2.634	2.629	2.632	0.0035
120	2.568	2.560	2.564	0.0057
300	2.418	2.450	2.434	0.0226
608	2.164	2.163	2.164	0.0007
1210	1.694	1.711	1.703	0.0120
1800	1.339	1.345	1.342	0.0042
5400	1.343	1.326	1.335	0.0120

Experiment 2				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.915	2.907	2.911	0.0057
30	2.653	2.649	2.651	0.0028
60	2.613	2.635	2.624	0.0156
120	2.555	2.558	2.557	0.0021
300	2.468	2.473	2.471	0.0035
608	2.168	2.109	2.139	0.0417
1210	1.748	1.751	1.750	0.0021
1800	1.442	1.426	1.434	0.0113
5400	1.345	1.321	1.333	0.0170

Experiment 3				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.824	2.818	2.821	0.0042
30	2.826	2.814	2.820	0.0085
60	2.741	2.726	2.734	0.0106
120	2.634	2.630	2.632	0.0028
300	2.438	2.449	2.444	0.0078
608	2.025	2.022	2.024	0.0021
1210	1.778	1.769	1.774	0.0064
1800	1.406	1.401	1.404	0.0035
5400	1.345	1.339	1.342	0.0042

Experiment 4				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.823	2.824	2.824	0.0007
30	2.829	2.810	2.820	0.0134
60	2.735	2.731	2.733	0.0028
120	2.637	2.634	2.636	0.0021
300	2.441	2.443	2.442	0.0014
608	2.022	2.026	2.024	0.0028
1210	1.769	1.775	1.772	0.0042
1800	1.406	1.402	1.404	0.0028
5400	1.349	1.343	1.346	0.0042

Appendix B3 Figure 3.6 Results

No Beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	3.000	3.000	3.000	0.000
30	2.893	2.886	2.889	0.005
70	2.925	2.925	2.925	0.000
120	2.950	2.950	2.950	0.000
330	2.943	2.943	2.943	0.000
600	2.950	2.950	2.950	0.000
930	2.943	2.943	2.943	0.000
1200	2.950	2.950	2.950	0.000
5160	2.950	2.950	2.950	0.000

Appendix B4 Figure 3.7 Results

8000 beads		
Time sec	**Con U/mL**	**ln (C-Cinf)**
0	2.57	0.93
30	2.32	0.83
70	2.14	0.74
120	1.86	0.60
330	0.99	-0.05
600	0.44	-0.92
930	0.13	-2.42
1200	0.06	-3.84
5160	0.04	

850 beads		
Time sec	**Conc U/mL**	**ln (C-Cinf)**
0	2.91	-0.39
30	2.81	-0.54
60	2.75	-0.65
120	2.75	-0.66
300	2.58	-1.07
608	2.45	-1.53
1210	2.30	-2.71
1800	2.25	-4.14
5400	2.23	

Appendix B5 Figure 3.8 Results

Number of beads	K-value
848	
1696	-0.0014
3650	-0.0023
8000	-0.0039

Appendix B6 Figure 3.9 Results

Number of beads	K-values
0	0
848	-0.002
1696	-0.0014
3650	-0.0023
8000	-0.0039

Appendix B7 Figure 3.10 Results

Data for 25°C

850 beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.911	2.907	2.909	0.0028
30	2.809	2.813	2.811	0.0028
60	2.753	2.753	2.753	0.0000
120	2.746	2.748	2.747	0.0014
300	2.574	2.576	2.575	0.0014
608	2.447	2.447	2.447	0.0000
1210	2.295	2.300	2.298	0.0035
1800	2.245	2.249	2.247	0.0028
5400	2.237	2.225	2.231	0.0085

1700 beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.911	2.909	2.910	0.0014
30	2.665	2.635	2.650	0.0212
60	2.625	2.614	2.620	0.0078
120	2.561	2.559	2.560	0.0014
300	2.470	2.470	2.470	0.0000
600	2.138	2.141	2.140	0.0021
1200	1.748	1.751	1.750	0.0021
1800	1.432	1.428	1.430	0.0028
5400	1.331	1.333	1.332	0.0014

	3650 beads			
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.961	2.959	2.960	0.0014
30	2.459	2.462	2.461	0.0021
60	2.401	2.399	2.400	0.0014
120	2.090	2.090	2.090	0.0000
300	1.730	1.730	1.730	0.0000
600	1.042	1.038	1.040	0.0028
1200	0.450	0.450	0.450	0.0000
1800	0.270	0.270	0.270	0.0000
3645	0.302	0.304	0.303	0.0014

	8000 beads			
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.572	2.572	2.572	0.0000
30	2.320	2.326	2.323	0.0042
70	2.137	2.139	2.138	0.0014
120	1.859	1.851	1.855	0.0057
330	0.991	0.989	0.990	0.0014
600	0.436	0.437	0.437	0.0007
930	0.124	0.128	0.126	0.0028
1200	0.058	0.058	0.058	0.0000
5160	0.035	0.038	0.037	0.0021

Data for 37°C

700 beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.750	2.758	2.754	0.0052
30	2.743	2.739	2.741	0.0026
60	2.294	2.291	2.293	0.0026
150	2.181	2.181	2.181	0.0000
300	2.032	2.035	2.034	0.0026
600	1.729	1.696	1.713	0.0232
1200	1.711	1.696	1.704	0.0103
1800	1.445	1.441	1.443	0.0026
3615	1.419	1.423	1.421	0.0026

1000 beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.750	2.743	2.747	0.0052
30	2.473	2.477	2.475	0.0026
60	2.287	2.276	2.282	0.0077
150	2.119	2.116	2.118	0.0026
300	1.973	1.966	1.970	0.0052
600	1.849	1.853	1.851	0.0026
1200	1.466	1.470	1.468	0.0026
1800	0.992	0.985	0.989	0.0052
3615	0.956	0.999	0.978	0.0310

	1820 beads			
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.941	2.911	2.926	0.0208
30	2.820	2.859	2.839	0.0278
60	2.816	2.817	2.817	0.0000
150	2.718	2.784	2.751	0.0462
300	2.388	2.404	2.396	0.0116
600	1.852	1.835	1.843	0.0116
1200	1.155	1.174	1.165	0.0139
1800	0.765	0.779	0.772	0.0093
3615	0.507	0.501	0.504	0.0046

	2240 beads			
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.883	2.879	2.881	0.0029
30	2.875	2.883	2.879	0.0057
60	2.791	2.787	2.789	0.0029
150	2.762	2.762	2.762	0.0000
300	2.303	2.299	2.301	0.0029
600	1.061	1.053	1.057	0.0057
1200	0.746	0.754	0.750	0.0057
1800	0.262	0.250	0.256	0.0086
5400	0.262	0.250	0.256	0.0086

Appendix C Data for Chapter 4: Investigating Absorption in Saline, Fetal Calf Serum and Blood

Appendix C1 Figure 4.1 Results

848 2mm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.872	2.912	2.892	0.02818
35	2.842	2.905	2.874	0.04461
60	2.866	2.862	2.864	0.00235
120	2.826	2.816	2.821	0.00704
305	2.653	2.690	2.671	0.02583
600	2.434	2.437	2.436	0.00235
1247	1.986	1.989	1.987	0.00235
1800	1.707	1.703	1.705	0.00235
6120	1.478	1.471	1.474	0.00470

8000 900 µm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.572	2.572	2.572	0.00000
30	2.32	2.326	2.323	0.00420
70	2.137	2.139	2.138	0.00140
120	1.859	1.851	1.855	0.00570
330	0.991	0.989	0.99	0.00140
600	0.436	0.437	0.437	0.00070
930	0.124	0.128	0.126	0.00280
1200	0.058	0.058	0.058	0.00000
5160	0.035	0.038	0.037	0.00210

Appendix C2 Figure 4.2 Results

3650 900 μm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.919	2.957	2.938	0.0271
30	2.488	2.459	2.474	0.0203
60	2.389	2.396	2.393	0.0045
120	2.131	2.086	2.109	0.0316
300	1.723	1.732	1.727	0.0068
600	0.995	1.043	1.019	0.0338
1200	0.424	0.450	0.437	0.0180
1800	0.223	0.271	0.247	0.0338
3645	0.303	0.297	0.300	0.0045

848 2mm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.886	2.902	2.894	0.0117
35	2.876	2.869	2.872	0.0047
60	2.849	2.842	2.846	0.0047
120	2.826	2.806	2.816	0.0141
305	2.700	2.643	2.671	0.0399
600	2.434	2.437	2.436	0.0023
1247	1.979	1.996	1.987	0.0117
1800	1.730	1.677	1.703	0.0376
6120	1.454	1.484	1.469	0.0211

Appendix C3 Figure 4.3 Results

5% Sodium alginate				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	3.001	3.011	3.006	0.007
45	2.771	2.776	2.773	0.003
60	2.519	2.439	2.479	0.057
120	1.807	1.799	1.803	0.006
330	0.996	0.954	0.975	0.029
600	0.343	0.332	0.337	0.008
1200	0.059	0.035	0.047	0.017
1860	0.012	0.012	0.012	0.000

2.5% Sodium alginate				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.566	2.572	2.569	0.004
30	2.295	2.348	2.322	0.037
70	2.132	2.145	2.138	0.009
120	1.846	1.865	1.855	0.013
330	0.985	0.997	0.991	0.009
600	0.437	0.437	0.437	0.000
930	0.145	0.132	0.138	0.009
1200	0.049	0.068	0.058	0.013
5160	0.040	0.043	0.042	0.002

Appendix C4 Figure 4.4 Results

Beads with no membrane				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.784	2.793	2.789	0.0066
30	2.650	2.640	2.645	0.0066
60	2.565	2.587	2.576	0.0155
120	2.659	2.675	2.667	0.0110
300	2.631	2.650	2.640	0.0133
600	2.665	2.656	2.661	0.0066
1200	2.653	2.659	2.656	0.0044
1800	2.668	2.684	2.676	0.0110

Appendix C5 Figure 4.6 Results

1820 900 µm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.941	2.911	2.926	0.0208
30	2.820	2.859	2.839	0.0278
60	2.817	2.817	2.817	0.0000
150	2.718	2.784	2.751	0.0463
300	2.388	2.404	2.396	0.0116
600	1.852	1.835	1.843	0.0116
1200	1.155	1.174	1.165	0.0139
1800	0.765	0.779	0.772	0.0093
3615	0.507	0.501	0.504	0.0046

850 2mm beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.884	2.887	2.885	0.0021
30	2.741	2.798	2.770	0.0399
60	2.718	2.756	2.737	0.0273
150	2.545	2.551	2.548	0.0042
300	2.361	2.337	2.349	0.0168
600	1.823	1.879	1.851	0.0399
1200	0.922	0.892	0.907	0.0210
1800	0.532	0.556	0.544	0.0168
3615	0.485	0.538	0.511	0.0378

Appendix C6 Figure 4.7 Results

Bead Diameter μm	**Number of beads**	**Membrane Thickness mm**	**K**	**Vmem m^3**
900	1800	0.014	-0.0012	6.413E-08
2000	850	0.006	-0.0015	6.409E-08
900	848	0.014	-0.0009	3.021E-08
900	1696	0.014	-0.0014	6.042E-08
900	3650	0.014	-0.0023	1.3E-07
900	8000	0.014	-0.0039	2.85E-07

Appendix C7 Figure 4.8 Results

37°C						
Fluid phase con (ci)	**Absorbed phase con (ni)**	**Volume of beads**	**Volume of membrane**	**Corrected ni**	**ln Ci**	**ln ni**
1.42	272.01	0.27	0.02	2914.43	0.35	7.98
0.98	243.44	0.38	0.04	2608.23	-0.02	7.87
0.54	162.89	0.69	0.06	1745.25	-0.62	7.46
0.26	147.41	0.86	0.08	1579.42	-1.35	7.36

25°C						
Fluid phase con (ci)	**Absorbed phase con (ni)**	**Volume of beads**	**Volume of membrane**	**Corrected ni**	**ln ci**	**ln ni**
2.23	125.96	0.32	0.03	1349.59	0.80	7.21
1.33	123.60	0.65	0.06	1324.24	0.29	7.19
0.3	78.04	1.39	0.13	836.09	-1.20	6.73
0.037	38.84	3.05	0.29	416.16	-3.30	6.03

Appendix C8 Figure 4.11 Results

Beads in saline				
Time Sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.915	2.907	2.911	0.0057
30	2.653	2.649	2.651	0.0028
60	2.613	2.635	2.624	0.0156
120	2.555	2.558	2.557	0.0021
300	2.468	2.473	2.471	0.0035
608	2.168	2.109	2.139	0.0417
1210	1.748	1.751	1.750	0.0021
1800	1.442	1.426	1.434	0.0113
5400	1.345	1.321	1.333	0.0170

Poly-L-lysine Beads incubated in poly-L-lysine						
Time sec	**Con 1 U/mL**	**Con 2 U/mL**	**Con 3 U/mL**	**Con 4 U/mL**	**Average conc U/mL**	**Std dev**
0	2.664	2.668	2.673	2.668	2.668	0.0038
30	2.589	2.593	2.593	2.598	2.593	0.0038
60	2.274	2.279	2.274	2.279	2.277	0.0027
120	1.604	1.609	1.613	1.609	1.609	0.0038
300	1.881	1.885	1.885	1.885	1.884	0.0023
600	0.183	0.183	0.178	0.178	0.181	0.0027
1200	0.234	0.230	0.230	0.225	0.230	0.0038
1800	0.286	0.281	0.272	0.272	0.278	0.0070
5400	0.455	0.455	0.455	0.455	0.455	0.0000

Poly-L-lysine Beads incubated in saline						
Time sec	**Con 1 U/mL**	**Con 2 U/mL**	**Con 3 U/mL**	**Con 4 U/mL**	**Average conc U/mL**	**Std dev**
0	2.91	2.91	2.91	2.91	2.91	0.00
30	2.78	2.78	2.78	2.79	2.78	0.00
60	2.77	2.77	2.77	2.78	2.77	0.00
120	2.68	2.68	2.68	2.68	2.68	0.00
300	2.37	2.37	2.37	2.37	2.37	0.00
600	1.94	1.94	1.94	1.93	1.94	0.00
1200	1.40	1.40	1.40	1.40	1.40	0.00
1800	0.95	0.95	0.95	0.95	0.95	0.00
5400	0.92	0.92	0.92	0.92	0.92	0.00

Poly-L-lysine Beads with no incubation						
Time sec	**Con 1 U/mL**	**Con 2 U/mL**	**Con 3 U/mL**	**Con 4 U/mL**	**Average conc U/mL**	**Std dev**
0	2.879	2.879	2.879	2.879	2.879	0.0000
30	2.836	2.836	2.836	2.840	2.837	0.0024
60	2.719	2.719	2.724	2.724	2.721	0.0028
120	2.651	2.646	2.646	2.651	2.648	0.0028
300	2.471	2.466	2.466	2.466	2.467	0.0024
600	2.150	2.145	2.150	2.150	2.149	0.0024
1200	1.537	1.537	1.537	1.537	1.537	0.0000
1800	0.968	0.963	0.963	0.963	0.964	0.0024
5400	0.905	0.910	0.910	0.910	0.908	0.0024

Appendix C9 Figure 4.12 Results

Beads in saline				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.915	2.907	2.911	0.0057
30	2.653	2.649	2.651	0.0028
60	2.613	2.635	2.624	0.0156
120	2.555	2.558	2.557	0.0021
300	2.468	2.473	2.471	0.0035
608	2.168	2.109	2.139	0.0417
1210	1.748	1.751	1.750	0.0021
1800	1.442	1.426	1.434	0.0113
5400	1.345	1.321	1.333	0.0170

Beads in FCS				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.903	2.928	2.916	0.0174
30	2.627	2.627	2.627	0.0000
60	2.362	2.350	2.356	0.0087
120	2.171	2.171	2.171	0.0000
300	2.030	2.024	2.027	0.0043
608	1.396	1.378	1.387	0.0130
1210	0.935	0.923	0.929	0.0087
1800	0.480	0.474	0.477	0.0043
5400	0.301	0.301	0.301	0.0000

No beads				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.985	2.985	2.985	0.0000
300	2.977	2.985	2.981	0.0054
600	2.985	2.992	2.989	0.0054
1800	2.977	2.962	2.970	0.0108

Appendix C10 Figure 4.13 Results

Beads in saline				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.915	2.907	2.911	0.0057
30	2.653	2.649	2.651	0.0028
60	2.613	2.635	2.624	0.0156
120	2.555	2.558	2.557	0.0021
300	2.468	2.473	2.471	0.0035
608	2.168	2.109	2.139	0.0417
1210	1.748	1.751	1.750	0.0021
1800	1.442	1.426	1.434	0.0113
5400	1.345	1.321	1.333	0.0170

Beads in blood				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.909	2.949	2.929	0.0277
120	1.998	1.940	1.969	0.0416
300	0.999	0.960	0.980	0.0277
600	0.500	0.490	0.495	0.0069
1200	0.225	0.176	0.201	0.0346
1800	0.118	0.078	0.098	0.0277

Beads in FCS				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.903	2.928	2.916	0.0174
30	2.627	2.627	2.627	0.0000
60	2.362	2.350	2.356	0.0087
120	2.171	2.171	2.171	0.0000
300	2.030	2.024	2.027	0.0043
608	1.396	1.378	1.387	0.0130
1210	0.935	0.923	0.929	0.0087
1800	0.480	0.474	0.477	0.0043
5400	0.301	0.301	0.301	0.0000

Appendix C11 Figure 4.14 Results

Beads in saline				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.915	2.907	2.911	0.0057
30	2.653	2.649	2.651	0.0028
60	2.613	2.635	2.624	0.0156
120	2.555	2.558	2.557	0.0021
300	2.468	2.473	2.471	0.0035
608	2.168	2.109	2.139	0.0417
1210	1.748	1.751	1.750	0.0021
1800	1.442	1.426	1.434	0.0113
5400	1.345	1.321	1.333	0.0170

Beads in blood				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.909	2.949	2.929	0.0277
120	1.998	1.940	1.969	0.0416
300	0.999	0.960	0.980	0.0277
600	0.500	0.490	0.495	0.0069
1200	0.225	0.176	0.201	0.0346
1800	0.118	0.078	0.098	0.0277

Beads in FCS				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.903	2.928	2.916	0.0174
30	2.627	2.627	2.627	0.0000
60	2.362	2.350	2.356	0.0087
120	2.171	2.171	2.171	0.0000
300	2.030	2.024	2.027	0.0043
608	1.396	1.378	1.387	0.0130
1210	0.935	0.923	0.929	0.0087
1800	0.480	0.474	0.477	0.0043
5400	0.301	0.301	0.301	0.0000

Beads in pH = 7.92 solution				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.753	2.763	2.758	0.0073
300	2.077	2.056	2.066	0.0148
600	1.411	1.347	1.379	0.0456
1200	1.387	1.381	1.384	0.0042
1800	1.390	1.378	1.384	0.0090
2400	1.387	1.378	1.382	0.0066

Beads in pH = 7.5 solution				
Time sec	**Conc 1 U/mL**	**Conc 2 U/mL**	**Ave Conc U/mL**	**Std Deviation**
0	2.780	2.775	2.778	0.0035
120	2.390	2.388	2.389	0.0014
300	2.010	2.009	2.010	0.0007
600	1.820	1.816	1.818	0.0028
1200	1.340	1.349	1.345	0.0064
1800	0.960	0.958	0.959	0.0014

AppendixD Data for Chapter 5: Feasible Reactor Configuration

Appendix D1 Figure 5.4 Results

	Time (sec)	Ln term
848 beads	0	-0.000297
	30	-0.050950
	60	-0.085041
	120	-0.089082
	300	-0.224361
	608	-0.378268
	1210	-0.719302
	1800	-0.960314
1696 beads	0	0.000000
	30	-0.070334
	60	-0.079887
	120	-0.100092
	300	-0.133507
3650 beads	0	0.000000
	30	-0.063975
	60	-0.073376
8000 beads	0	0.000130
	30	-0.014870
	70	-0.027457

Appendix D2 Figure 5.5 Results

Number of beads	Predicted Eqm Con (U/mL)	Measured Eqm Con (U/mL)
848	2.18	2.23
1696	1.44	1.33
3650	0.00	0.30
8000	0.00	0.04

Appendix D3 Figure 5.6 Results

848 beads

y*	1004.000	U/mL
Vx	40.000	mL
N	848	
Vy	0.02928042	mL
Constant: a	-2.17506138	

mem thickness	0.000014	m
r	0.00045	m
V of mem of one bead	3.45E-05	mL
Inside R	0.000436	

Time Sec	**b**	**Predicted Conc (U/mL)**
0	-2.1751	2.9100
100	-2.2650	2.7945
200	-2.3439	2.7004
300	-2.4132	2.6229
400	-2.4739	2.5584
500	-2.5273	2.5044
600	-2.5741	2.4589
700	-2.6152	2.4202
800	-2.6513	2.3873
900	-2.6829	2.3591
1000	-2.7107	2.3350
1100	-2.7351	2.3142
1200	-2.7565	2.2962
1300	-2.7753	2.2806
1400	-2.7918	2.2672
1500	-2.8062	2.2555
1600	-2.8189	2.2453
1700	-2.8301	2.2365
1800	-2.8398	2.2288
1900	-2.8484	2.2221
2000	-2.8560	2.2162
2100	-2.8626	2.2111
2200	-2.8684	2.2066
2300	-2.8735	2.2027
2400	-2.8779	2.1993
2500	-2.8819	2.1963
2600	-2.8853	2.1937
2700	-2.8883	2.1914
2800	-2.8910	2.1894
2900	-2.8933	2.1876
3000	-2.8953	2.1861
3100	-2.8971	2.1847
3200	-2.8987	2.1835

3300	-2.9001	2.1825
3400	-2.9013	2.1816
3500	-2.9024	2.1808
3600	-2.9033	2.1801
3700	-2.9041	2.1795
3800	-2.9048	2.1789
3900	-2.9055	2.1785
4000	-2.9060	2.1780
4100	-2.9065	2.1777
4200	-2.9069	2.1774
4300	-2.9073	2.1771
4400	-2.9076	2.1768
4500	-2.9079	2.1766
4600	-2.9082	2.1764
4700	-2.9084	2.1763
4800	-2.9086	2.1761
4900	-2.9088	2.1760
5000	-2.9089	2.1759
5100	-2.9091	2.1758
5200	-2.9092	2.1757
5300	-2.9093	2.1756
5400	-2.9094	2.1755
5500	-2.9094	2.1755
5600	-2.9095	2.1754
5700	-2.9096	2.1754
5800	-2.9096	2.1753
5900	-2.9097	2.1753
6000	-2.9097	2.1753
6100	-2.9097	2.1753
6200	-2.9098	2.1752
6300	-2.9098	2.1752
6400	-2.9098	2.1752
6500	-2.9098	2.1752
6600	-2.9099	2.1752

1696 beads

y*	1004.000	U/mL
Vx	40.000	mL
N	1696	
Vy	0.05856085	mL
Constant: a	-1.44012276	

mem thickness	0.000014	m
r	0.00045	m
V of mem of one bead	3.45E-05	mL
Inside R	0.000436	

Time Sec	**b**	**Predicted Conc (U/mL)**
0	-1.4401	2.9100
100	-1.5618	2.6833
200	-1.6734	2.5043
300	-1.7758	2.3600
400	-1.8697	2.2415
500	-1.9558	2.1428
600	-2.0348	2.0596
700	-2.1072	1.9888
800	-2.1737	1.9280
900	-2.2346	1.8754
1000	-2.2905	1.8296
1100	-2.3418	1.7895
1200	-2.3888	1.7543
1300	-2.4320	1.7232
1400	-2.4716	1.6956
1500	-2.5079	1.6711
1600	-2.5411	1.6492
1700	-2.5717	1.6296
1800	-2.5997	1.6120
1900	-2.6254	1.5963
2000	-2.6489	1.5821
2100	-2.6705	1.5693
2200	-2.6904	1.5577
2300	-2.7085	1.5472
2400	-2.7252	1.5378
2500	-2.7405	1.5292
2600	-2.7545	1.5214
2700	-2.7674	1.5143
2800	-2.7792	1.5079
2900	-2.7900	1.5020
3000	-2.8000	1.4967
3100	-2.8091	1.4919
3200	-2.8174	1.4874
3300	-2.8251	1.4834
3400	-2.8321	1.4797
3500	-2.8386	1.4764

3600	-2.8445	1.4733
3700	-2.8499	1.4705
3800	-2.8549	1.4679
3900	-2.8594	1.4656
4000	-2.8636	1.4634
4100	-2.8675	1.4615
4200	-2.8710	1.4597
4300	-2.8742	1.4581
4400	-2.8772	1.4565
4500	-2.8799	1.4552
4600	-2.8824	1.4539
4700	-2.8847	1.4528
4800	-2.8868	1.4517
4900	-2.8887	1.4507
5000	-2.8905	1.4499
5100	-2.8921	1.4490
5200	-2.8936	1.4483
5300	-2.8949	1.4476
5400	-2.8962	1.4470
5500	-2.8973	1.4464
5600	-2.8984	1.4459
5700	-2.8993	1.4454
5800	-2.9002	1.4450
5900	-2.9010	1.4446
6000	-2.9018	1.4442
6100	-2.9024	1.4439
6200	-2.9031	1.4436
6300	-2.9036	1.4433
6400	-2.9042	1.4430
6500	-2.9047	1.4428
6600	-2.9051	1.4426

3650 beads

y*	1004.000	U/mL
Vx	40.000	mL
N	3650	
Vy	0.12603012	mL
Constant: a	0.20335609	

mem thickness	0.000014	m
r	0.00045	m
V of mem of one bead	3.45E-05	mL
Inside R	0.000436	

Time Sec	b	Predicted Conc (U/mL)
0	0.2034	2.9600
100	0.2422	2.4854
200	0.2815	2.1383
300	0.3213	1.8735
400	0.3616	1.6648
500	0.4024	1.4960
600	0.4436	1.3568
700	0.4854	1.2401
800	0.5277	1.1407
900	0.5705	1.0551
1000	0.6139	0.9806
1100	0.6577	0.9152
1200	0.7021	0.8573
1300	0.7471	0.8057
1400	0.7926	0.7594
1500	0.8387	0.7177
1600	0.8853	0.6799
1700	0.9325	0.6455
1800	0.9803	0.6140
1900	1.0287	0.5852
2000	1.0776	0.5586
2100	1.1272	0.5340
2200	1.1774	0.5112
2300	1.2282	0.4901
2400	1.2796	0.4704
2500	1.3316	0.4520
2600	1.3843	0.4348
2700	1.4377	0.4187
2800	1.4916	0.4035
2900	1.5463	0.3893
3000	1.6016	0.3758
3100	1.6576	0.3631
3200	1.7143	0.3511
3300	1.7717	0.3398
3400	1.8298	0.3290

3500	1.8886	0.3187
3600	1.9481	0.3090
3700	2.0083	0.2997
3800	2.0693	0.2909
3900	2.1311	0.2825
4000	2.1936	0.2744
4100	2.2568	0.2667
4200	2.3209	0.2594
4300	2.3857	0.2523
4400	2.4513	0.2456
4500	2.5178	0.2391
4600	2.5850	0.2329
4700	2.6531	0.2269
4800	2.7220	0.2211
4900	2.7917	0.2156
5000	2.8623	0.2103
5100	2.9338	0.2052
5200	3.0062	0.2002
5300	3.0794	0.1955
5400	3.1535	0.1909
5500	3.2286	0.1864
5600	3.3046	0.1822
5700	3.3815	0.1780
5800	3.4593	0.1740
5900	3.5381	0.1701
6000	3.6179	0.1664
6100	3.6987	0.1627
6200	3.7804	0.1592
6300	3.8631	0.1558
6400	3.9469	0.1525
6500	4.0317	0.1493
6600	4.1175	0.1462

8000 beads

y*	1004.000	U/mL
Vx	40.000	mL
N	8000	
Vy	0.276230407	mL
Constant: a	4.363383209	

mem thickness	0.000014	m
r	0.00045	m
V of mem of one bead	3.45E-05	mL
Inside R	0.000436	

Time Sec	**b**	**Predicted Conc (U/mL)**
0.0000	4.3634	2.5700
100.0000	6.4383	1.7417
200.0000	9.1343	1.2277
300.0000	12.6370	0.8874
400.0000	17.1880	0.6524
500.0000	23.1010	0.4854
600.0000	30.7836	0.3643
700.0000	40.7654	0.2751
800.0000	53.7344	0.2087
900.0000	70.5846	0.1589
1000.0000	92.4776	0.1213
1100.0000	120.9225	0.0927
1200.0000	157.8802	0.0710
1300.0000	205.8983	0.0545
1400.0000	268.2867	0.0418
1500.0000	349.3461	0.0321
1600.0000	454.6642	0.0247
1700.0000	591.5010	0.0190
1800.0000	769.2889	0.0146
1900.0000	1000.2835	0.0112
2000.0000	1300.4081	0.0086
2100.0000	1690.3510	0.0066
2200.0000	2196.9924	0.0051
2300.0000	2855.2567	0.0039
2400.0000	3710.5201	0.0030
2500.0000	4821.7386	0.0023
2600.0000	6265.5121	0.0018
2700.0000	8141.3645	0.0014
2800.0000	10578.6042	0.0011
2900.0000	13745.2381	0.0008
3000.0000	17859.5521	0.0006
3100.0000	23205.1593	0.0005
3200.0000	30150.5492	0.0004
3300.0000	39174.4898	0.0003
3400.0000	50899.0301	0.0002

3500.0000	66132.3801	0.0002
3600.0000	85924.6230	0.0001
3700.0000	111640.1015	0.0001
3800.0000	145051.4653	0.0001
3900.0000	188461.8664	0.0001
4000.0000	244863.7222	0.0000
4100.0000	318144.9924	0.0000
4200.0000	413357.1888	0.0000
4300.0000	537063.5917	0.0000
4400.0000	697791.6780	0.0000
4500.0000	906620.9472	0.0000
4600.0000	1177946.6670	0.0000
4700.0000	1530472.1845	0.0000
4800.0000	1988498.2080	0.0000
4900.0000	2583597.9320	0.0000
5000.0000	3356793.4785	0.0000
5100.0000	4361383.6824	0.0000
5200.0000	5666618.1504	0.0000
5300.0000	7362470.8571	0.0000
5400.0000	9565842.3383	0.0000
5500.0000	12428618.0172	0.0000
5600.0000	16148138.1517	0.0000
5700.0000	20980801.1273	0.0000
5800.0000	27259737.8156	0.0000
5900.0000	35417775.3479	0.0000
6000.0000	46017273.2697	0.0000
6100.0000	59788888.7776	0.0000
6200.0000	77681943.2503	0.0000
6300.0000	100929861.9988	0.0000
6400.0000	131135198.2066	0.0000
6500.0000	170380102.0323	0.0000
6600.0000	221369849.8599	0.0000

Appendix D4 Figure 5.7 Results

τs = 1			τs = 3.88			τs = 5		
Time (sec)	**y (U/mL)**	**x (U/mL)**	**Time (sec)**	**y (U/mL)**	**x (U/mL)**	**Time (sec)**	**y (U/mL)**	**x (U/mL)**
0.00	0.00	0.62	0.00	0.00	0.30	0.00	0.00	0.25
49.68	18.55	0.63	146.90	26.39	0.31	184.34	27.57	0.25
107.93	40.01	0.63	336.71	60.18	0.31	417.90	62.21	0.26
171.80	63.21	0.64	533.66	94.83	0.32	661.99	98.07	0.27
247.15	90.11	0.65	735.67	129.94	0.33	913.45	134.60	0.28
323.69	116.91	0.65	941.14	165.18	0.34	1170.94	171.58	0.29
401.25	143.55	0.66	1149.13	200.35	0.35	1350.00	197.02	0.29
479.72	169.95	0.67	1350.00	233.80	0.36	1609.16	233.43	0.30
558.98	196.06	0.67	1556.47	267.66	0.37	1867.84	269.25	0.31
638.94	221.84	0.68	1761.79	300.76	0.38	2125.30	304.37	0.32
719.53	247.25	0.69	1965.57	333.04	0.39	2380.90	338.68	0.33
800.67	272.25	0.70	2167.48	364.43	0.40	2632.39	371.87	0.35
882.31	296.82	0.70	2367.26	394.89	0.41	2700.00	380.69	0.35
964.41	320.93	0.71	2563.07	424.15	0.43	2941.34	411.82	0.36
1046.92	344.57	0.72	2700.00	444.24	0.44	3179.13	441.93	0.37
1129.81	367.71	0.72	2885.85	471.01	0.45	3413.17	470.98	0.39
1213.04	390.36	0.73	3068.99	496.81	0.46	3643.28	498.96	0.40
1296.51	412.47	0.74	3249.38	521.65	0.47	3866.51	525.52	0.41
1350.00	426.32	0.74	3427.02	545.53	0.48	4050.00	546.91	0.42
1432.32	447.17	0.75	3601.93	568.46	0.50	4258.97	570.76	0.44
1514.83	467.49	0.76	3774.14	590.47	0.51	4463.96	593.61	0.45
1597.55	487.29	0.76	3942.11	611.37	0.52	4665.03	615.48	0.46
1680.47	506.58	0.77	4050.00	624.50	0.53	4862.23	636.39	0.48
1763.60	525.34	0.78	4207.58	643.24	0.54	5055.64	656.35	0.49
1846.94	543.60	0.78	4362.88	661.20	0.56	5242.98	675.16	0.50
1930.50	561.35	0.79	4515.98	678.41	0.57	5400.00	690.52	0.52
2014.29	578.59	0.80	4666.98	694.88	0.58	5574.11	707.11	0.53
2098.32	595.34	0.80	4815.97	710.64	0.60	5745.19	722.94	0.54
2182.59	611.60	0.81	4963.04	725.72	0.61	5913.36	738.03	0.56
2267.12	627.38	0.82	5108.28	740.14	0.62	6078.75	752.42	0.57
2351.93	642.68	0.82	5254.14	754.13	0.63	6241.50	766.14	0.59
2437.02	657.52	0.83	5400.00	767.65	0.65	6401.74	779.20	0.60
2522.42	671.89	0.83	5533.28	779.57	0.66	6575.87	792.88	0.61
2611.21	686.30	0.84	5665.33	790.98	0.67	6730.25	804.58	0.63
2700.00	700.17	0.85	5796.23	801.90	0.68	6750.00	806.04	0.63
2783.53	712.75	0.85	5926.07	812.34	0.69	6895.15	816.61	0.64
2867.43	724.93	0.86	6054.93	822.32	0.71	7038.64	826.68	0.66
2951.73	736.73	0.86	6182.90	831.86	0.72	7180.57	836.29	0.67
3036.44	748.14	0.87	6310.06	840.98	0.73	7321.06	845.44	0.68
3121.58	759.18	0.87	6436.48	849.69	0.74	7460.23	854.16	0.69

3207.16	769.85	0.88	6562.26	858.00	0.75	7598.18	862.47	0.70
3293.22	780.17	0.88	6686.63	865.88	0.76	7735.02	870.38	0.72
3379.76	790.13	0.89	6750.00	869.77	0.76	7870.86	877.91	0.73
3466.82	799.75	0.89	6868.58	876.81	0.77	8004.66	885.02	0.74
3554.41	809.03	0.90	6986.92	883.55	0.78	8100.00	889.89	0.75
3642.56	817.99	0.90	7105.09	889.99	0.79	8227.06	896.15	0.76
3731.28	826.62	0.90	7223.15	896.14	0.80	8353.68	902.12	0.77
3820.62	834.94	0.91	7341.16	902.02	0.81	8479.92	907.82	0.78
3910.59	842.96	0.91	7459.20	907.63	0.82	8605.87	913.24	0.79
4000.91	850.65	0.92	7577.32	912.99	0.83	8731.62	918.40	0.80
4050.00	854.69	0.92	7695.59	918.09	0.84	8857.24	923.32	0.81
4138.66	861.73	0.92	7814.08	922.96	0.84	8982.82	928.00	0.82
4228.03	868.51	0.93	7932.85	927.60	0.85	9108.43	932.45	0.83
4318.14	875.04	0.93	8051.34	931.99	0.86	9234.15	936.68	0.84
4409.01	881.32	0.93	8100.00	933.73	0.86	9359.18	940.67	0.84
4500.67	887.36	0.94	8214.71	937.68	0.87	9450.00	943.44	0.85
4593.16	893.17	0.94	8329.99	941.45	0.88	9570.97	946.96	0.86
4686.49	898.75	0.94	8445.89	945.04	0.88	9692.43	950.31	0.87
4780.70	904.11	0.94	8562.47	948.47	0.89	9814.46	953.50	0.87
4875.82	909.26	0.95	8679.79	951.73	0.90	9937.11	956.53	0.88
4971.88	914.19	0.95	8797.92	954.83	0.90	10060.47	959.41	0.89
5068.92	918.92	0.95	8916.90	957.77	0.91	10184.59	962.14	0.89
5166.97	923.46	0.95	9036.82	960.58	0.91	10309.57	964.73	0.90
5266.07	927.80	0.96	9157.73	963.23	0.92	10435.46	967.19	0.91
5365.95	931.94	0.96	9279.71	965.76	0.92	10562.35	969.51	0.91
5400.00	933.30	0.96	9402.25	968.14	0.93	10689.46	971.70	0.92
5498.55	937.10	0.96	9450.00	969.03	0.93	10800.00	973.49	0.92
5598.22	940.73	0.96	9570.30	971.17	0.93	10925.35	975.40	0.93
5699.06	944.21	0.97	9691.88	973.20	0.94	11052.01	977.22	0.93
5801.09	947.53	0.97	9814.81	975.13	0.94	11180.05	978.93	0.94
5904.37	950.71	0.97	9939.17	976.96	0.94	11309.54	980.55	0.94
6008.93	953.74	0.97	10065.02	978.68	0.95	11440.57	982.08	0.94
6114.81	956.63	0.97	10192.44	980.32	0.95	11573.22	983.53	0.95
6222.07	959.39	0.98	10321.51	981.86	0.96	11707.59	984.89	0.95
6330.75	962.01	0.98	10452.31	983.32	0.96	11843.76	986.17	0.95
6440.90	964.52	0.98	10584.92	984.69	0.96	11996.88	987.50	0.96
6552.58	966.90	0.98	10718.74	985.98	0.96	12150.00	988.72	0.96
6665.33	969.15	0.98	10800.00	986.72	0.97	12287.98	989.74	0.96
6750.00	970.74	0.98	10933.35	987.85	0.97	12428.10	990.70	0.97
6862.73	972.75	0.98	11068.76	988.92	0.97	12570.47	991.60	0.97
6977.10	974.66	0.98	11206.33	989.93	0.97	12715.20	992.44	0.97
7093.16	976.47	0.98	11346.14	990.88	0.97	12862.41	993.22	0.97
7210.99	978.19	0.99	11488.32	991.77	0.98	13012.22	993.96	0.98
7330.64	979.82	0.99	11632.96	992.60	0.98	13164.75	994.65	0.98
7452.18	981.36	0.99	11780.20	993.38	0.98	13332.38	995.34	0.98
7575.69	982.82	0.99	11930.15	994.11	0.98	13500.00	995.96	0.98
7701.23	984.19	0.99	12082.18	994.78	0.98	13657.17	996.50	0.98
7828.89	985.49	0.99	12150.00	995.07	0.98	13817.49	997.00	0.99

7964.45	986.77	0.99	12302.45	995.66	0.99	13981.15	997.46	0.99
8100.00	987.95	0.99	12457.91	996.22	0.99	14148.29	997.89	0.99
8230.86	989.00	0.99	12616.52	996.74	0.99	14319.12	998.28	0.99
8364.05	990.00	0.99	12778.45	997.22	0.99	14493.82	998.65	0.99
8499.65	990.93	0.99	12943.86	997.67	0.99	14671.91	998.99	0.99
8637.76	991.80	0.99	13112.92	998.08	0.99	14850.00	999.29	0.99
8778.50	992.62	0.99	13285.85	998.47	0.99	15032.11	999.57	0.99
8921.96	993.39	0.99	13461.99	998.82	0.99	15218.72	999.82	0.99
9068.28	994.11	1.00	13500.00	998.89	0.99	15410.08	1000.06	0.99
9217.58	994.79	1.00	13676.95	999.20	0.99	15606.47	1000.27	0.99
9369.22	995.41	1.00	13858.17	999.49	0.99	15808.18	1000.47	1.00
9450.00	995.72	1.00	14043.88	999.75	0.99	16013.82	1000.65	1.00
9602.98	996.27	1.00	14234.34	999.99	1.00	16200.00	1000.79	1.00
9759.21	996.78	1.00	14429.84	1000.21	1.00	16412.72	1000.93	1.00
9918.84	997.26	1.00	14639.92	1000.42	1.00	16631.75	1001.06	1.00
10082.03	997.70	1.00	14850.00	1000.61	1.00	16857.48	1001.18	1.00
10248.96	998.11	1.00	15056.86	1000.77	1.00	17090.38	1001.29	1.00
10419.81	998.49	1.00	15269.68	1000.92	1.00	17328.64	1001.38	1.00
10609.91	998.86	1.00	15488.85	1001.05	1.00	17550.00	1001.46	1.00
10800.00	999.20	1.00	15714.78	1001.17	1.00	17799.39	1001.53	1.00
10979.41	999.49	1.00	15957.39	1001.28	1.00	18057.56	1001.60	1.00
11163.35	999.75	1.00	16200.00	1001.38	1.00	18325.17	1001.66	1.00
11352.05	999.99	1.00	16442.60	1001.46	1.00	18612.59	1001.71	1.00
11545.79	1000.21	1.00	16693.50	1001.54	1.00	18900.00	1001.76	1.00
11744.85	1000.41	1.00	16953.33	1001.61	1.00	19192.86	1001.80	1.00
11947.43	1000.59	1.00	17251.67	1001.67	1.00	19497.92	1001.83	1.00
12150.00	1000.75	1.00	17550.00	1001.72	1.00	19816.28	1001.86	1.00
12360.72	1000.90	1.00	17834.93	1001.77	1.00	20146.21	1001.89	1.00
12577.72	1001.04	1.00	18131.40	1001.81	1.00	20250.00	1001.89	1.00
12801.39	1001.16	1.00	18440.43	1001.84	1.00	20594.16	1001.91	1.00
13032.20	1001.27	1.00	18760.11	1001.87	1.00	20955.26	1001.93	1.00
13266.10	1001.36	1.00	18900.00	1001.88	1.00	21330.20	1001.94	1.00
13500.00	1001.45	1.00	19234.86	1001.90	1.00	21600.00	1001.95	1.00
13747.65	1001.52	1.00	19585.76	1001.92	1.00	22004.58	1001.96	1.00
14004.01	1001.59	1.00	19949.44	1001.93	1.00	22477.29	1001.97	1.00
14269.74	1001.65	1.00	20250.00	1001.95	1.00	22950.00	1001.98	1.00
14559.87	1001.71	1.00	20643.66	1001.96	1.00	23425.67	1001.98	1.00
14850.00	1001.75	1.00	21059.60	1001.97	1.00	23925.44	1001.99	1.00
15141.13	1001.79	1.00	21495.71	1001.97	1.00	24300.00	1001.99	1.00
15444.35	1001.83	1.00	21600.00	1001.98	1.00	24859.29	1001.99	1.00
15760.75	1001.86	1.00	22062.84	1001.98	1.00	25454.74	1002.00	1.00
16088.56	1001.88	1.00	22548.15	1001.99	1.00	25650.00	1002.00	1.00
16200.00	1001.89	1.00	22950.00	1001.99	1.00	26325.00	1002.00	1.00
16542.30	1001.91	1.00	23494.21	1001.99	1.00	27000.00	1002.00	1.00
16901.37	1001.93	1.00	24072.22	1001.99	1.00	27675.00	1002.00	1.00
17274.10	1001.94	1.00	24300.00	1002.00	1.00	28350.00	1002.00	1.00
17550.00	1001.95	1.00	24975.00	1002.00	1.00	29025.00	1002.00	1.00
17952.47	1001.96	1.00	25650.00	1002.00	1.00	29700.00	1002.00	1.00

18378.26	1001.97	1.00	26325.00	1002.00	1.00	30375.00	1002.00	1.00
18825.49	1001.98	1.00	27000.00	1002.00	1.00	31050.00	1002.00	1.00
18900.00	1001.98	1.00	27675.00	1002.00	1.00	31725.00	1002.00	1.00
19373.24	1001.98	1.00	28350.00	1002.00	1.00	32400.00	1002.00	1.00
19870.28	1001.99	1.00	29025.00	1002.00	1.00	33075.00	1002.00	1.00
20250.00	1001.99	1.00	29700.00	1002.00	1.00	33750.00	1002.00	1.00
20806.46	1001.99	1.00	30375.00	1002.00	1.00	34425.00	1002.00	1.00
21398.63	1002.00	1.00	31050.00	1002.00	1.00	35100.00	1002.00	1.00
21600.00	1002.00	1.00	31725.00	1002.00	1.00	35775.00	1002.00	1.00
22275.00	1002.00	1.00	32400.00	1002.00	1.00	36450.00	1002.00	1.00
22950.00	1002.00	1.00	33075.00	1002.00	1.00	37125.00	1002.00	1.00
23625.00	1002.00	1.00	33750.00	1002.00	1.00	37800.00	1002.00	1.00
24300.00	1002.00	1.00	34425.00	1002.00	1.00	38475.00	1002.00	1.00
24975.00	1002.00	1.00	35100.00	1002.00	1.00	39150.00	1002.00	1.00
25650.00	1002.00	1.00	35775.00	1002.00	1.00	39825.00	1002.00	1.00
26325.00	1002.00	1.00	36450.00	1002.00	1.00	40500.00	1002.00	1.00
27000.00	1002.00	1.00	37125.00	1002.00	1.00	41175.00	1002.00	1.00
27675.00	1002.00	1.00	37800.00	1002.00	1.00	41850.00	1002.00	1.00
28350.00	1002.00	1.00	38475.00	1002.00	1.00	42525.00	1002.00	1.00
29025.00	1002.00	1.00	39150.00	1002.00	1.00	43200.00	1002.00	1.00
29700.00	1002.00	1.00	39825.00	1002.00	1.00	43875.00	1002.00	1.00
30375.00	1002.00	1.00	40500.00	1002.00	1.00	44550.00	1002.00	1.00
31050.00	1002.00	1.00	41175.00	1002.00	1.00	45225.00	1002.00	1.00
31725.00	1002.00	1.00	41850.00	1002.00	1.00	45900.00	1002.00	1.00
32400.00	1002.00	1.00	42525.00	1002.00	1.00	46575.00	1002.00	1.00
33075.00	1002.00	1.00	43200.00	1002.00	1.00	47250.00	1002.00	1.00
33750.00	1002.00	1.00	43875.00	1002.00	1.00	47925.00	1002.00	1.00
34425.00	1002.00	1.00	44550.00	1002.00	1.00	48600.00	1002.00	1.00
35100.00	1002.00	1.00	45225.00	1002.00	1.00	49275.00	1002.00	1.00
35775.00	1002.00	1.00	45900.00	1002.00	1.00	49950.00	1002.00	1.00
36450.00	1002.00	1.00	46575.00	1002.00	1.00	50625.00	1002.00	1.00
37125.00	1002.00	1.00	47250.00	1002.00	1.00	51300.00	1002.00	1.00
37800.00	1002.00	1.00	47925.00	1002.00	1.00	51975.00	1002.00	1.00
38475.00	1002.00	1.00	48600.00	1002.00	1.00	52650.00	1002.00	1.00
39150.00	1002.00	1.00	49275.00	1002.00	1.00	53325.00	1002.00	1.00
39825.00	1002.00	1.00	49950.00	1002.00	1.00	54000.00	1002.00	1.00
40500.00	1002.00	1.00	50625.00	1002.00	1.00			
41175.00	1002.00	1.00	51300.00	1002.00	1.00			
41850.00	1002.00	1.00	51975.00	1002.00	1.00			
42525.00	1002.00	1.00	52650.00	1002.00	1.00			
43200.00	1002.00	1.00	53325.00	1002.00	1.00			
43875.00	1002.00	1.00	54000.00	1002.00	1.00			
44550.00	1002.00	1.00						
45225.00	1002.00	1.00						
45900.00	1002.00	1.00						
46575.00	1002.00	1.00						
47250.00	1002.00	1.00						
47925.00	1002.00	1.00						

48600.00	1002.00	1.00
49275.00	1002.00	1.00
49950.00	1002.00	1.00
50625.00	1002.00	1.00
51300.00	1002.00	1.00
51975.00	1002.00	1.00
52650.00	1002.00	1.00
53325.00	1002.00	1.00
54000.00	1002.00	1.00

Appendix D5 Figure 5.8 Results

Membrane thickness (m)	Reactor Volume (mL)
0.0000050	981
0.0000120	415
0.0000140	357
0.0000200	254
0.0000400	133
0.0000600	93
0.0001000	61
0.0005000	32

Appendix D6 Figure 5.9 Results

τs = 0.1			τs =0.6			τs = 1		
Time (sec)	y (U/mL)	x (U/mL)	Time (sec)	y (U/mL)	x (U/mL)	Time (sec)	y (U/mL)	x (U/mL)
0.00	0.00	0.72	0.00	0.00	0.30	0.00	0.00	0.20
6.03	16.84	0.72	22.45	26.12	0.30	38.95	30.91	0.21
13.13	36.41	0.73	50.96	59.00	0.31	83.07	65.69	0.21
20.95	57.64	0.73	81.11	93.38	0.32	130.23	102.55	0.22
29.31	79.94	0.74	112.30	128.51	0.33	179.22	140.49	0.23
38.83	104.87	0.74	144.11	163.87	0.34	229.26	178.84	0.24
48.56	129.82	0.75	176.24	199.08	0.35	285.29	221.30	0.25
58.46	154.69	0.75	208.47	233.87	0.36	338.53	261.12	0.26
68.52	179.40	0.76	240.62	268.02	0.37	389.10	298.44	0.27
78.72	203.89	0.76	272.56	301.37	0.38	437.15	333.41	0.28
89.05	228.11	0.77	304.18	333.82	0.39	482.79	366.17	0.29
99.49	252.02	0.77	335.41	365.26	0.40	526.15	396.85	0.30
110.03	275.58	0.78	366.17	395.64	0.41	567.34	425.57	0.31
120.66	298.76	0.78	396.44	424.92	0.43	607.65	453.26	0.32
131.38	321.54	0.79	426.16	453.08	0.44	647.22	480.01	0.33
142.18	343.89	0.80	455.33	480.11	0.45	686.03	505.82	0.34
153.06	365.81	0.80	483.92	506.02	0.46	724.09	530.69	0.35
164.00	387.27	0.81	511.94	530.81	0.48	761.37	554.62	0.36
175.01	408.27	0.81	539.39	554.50	0.49	797.88	577.60	0.38
186.09	428.80	0.82	566.26	577.13	0.50	833.61	599.65	0.39
197.22	448.85	0.82	592.59	598.71	0.51	868.57	620.78	0.40
208.42	468.42	0.83	618.36	619.29	0.53	902.77	641.01	0.42
219.67	487.51	0.83	643.62	638.90	0.54	936.23	660.34	0.43
230.97	506.11	0.84	668.36	657.58	0.55	968.94	678.81	0.44
242.33	524.24	0.84	692.62	675.35	0.57	1000.94	696.43	0.46
253.75	541.88	0.85	716.40	692.27	0.58	1032.24	713.23	0.47
265.21	559.05	0.85	739.75	708.37	0.59	1062.87	729.24	0.48
276.74	575.75	0.86	762.66	723.68	0.61	1092.85	744.48	0.50
288.31	591.98	0.86	785.18	738.24	0.62	1122.21	758.98	0.51
299.94	607.74	0.87	807.31	752.09	0.63	1150.97	772.76	0.53
311.63	623.05	0.87	829.09	765.25	0.64	1179.16	785.87	0.54
323.37	637.91	0.88	850.53	777.77	0.66	1206.81	798.31	0.56
335.16	652.32	0.88	871.65	789.67	0.67	1233.95	810.13	0.57
347.02	666.29	0.88	892.47	800.98	0.68	1260.61	821.34	0.59
358.93	679.84	0.89	913.02	811.73	0.69	1286.81	831.98	0.60
370.91	692.96	0.89	934.87	822.72	0.70	1312.58	842.07	0.62
382.94	705.67	0.90	955.63	832.74	0.72	1337.70	851.54	0.63
395.05	717.97	0.90	975.35	841.88	0.73	1350.00	856.04	0.64
407.21	729.87	0.90	994.08	850.23	0.74	1373.97	864.57	0.65
419.45	741.38	0.91	1012.00	857.91	0.75	1397.50	872.62	0.66
431.75	752.51	0.91	1029.88	865.27	0.76	1420.63	880.23	0.68
444.12	763.27	0.91	1047.71	872.33	0.77	1443.39	887.40	0.69
456.57	773.66	0.92	1065.51	879.10	0.78	1465.80	894.18	0.70
469.10	783.69	0.92	1083.28	885.58	0.79	1487.90	900.59	0.72

481.70	793.38	0.92	1101.04	891.78	0.79	1509.72	906.63	0.73
494.38	802.72	0.93	1118.79	897.72	0.80	1531.28	912.35	0.74
507.15	811.73	0.93	1136.55	903.39	0.81	1552.60	917.75	0.75
520.00	820.42	0.93	1154.32	908.82	0.82	1573.72	922.85	0.76
532.94	828.79	0.94	1172.11	914.01	0.83	1594.65	927.67	0.78
545.98	836.86	0.94	1189.94	918.96	0.84	1615.41	932.22	0.79
559.10	844.62	0.94	1207.81	923.69	0.85	1636.03	936.52	0.80
572.33	852.10	0.94	1225.72	928.20	0.85	1656.53	940.59	0.81
585.65	859.29	0.95	1243.70	932.50	0.86	1676.93	944.43	0.82
599.08	866.20	0.95	1261.74	936.60	0.87	1697.24	948.06	0.83
612.61	872.85	0.95	1279.87	940.50	0.87	1717.48	951.48	0.84
626.25	879.23	0.95	1298.08	944.22	0.88	1737.68	954.72	0.84
640.00	885.37	0.96	1316.39	947.75	0.89	1757.84	957.77	0.85
653.88	891.26	0.96	1334.69	951.09	0.89	1778.00	960.65	0.86
667.87	896.90	0.96	1350.00	953.74	0.90	1798.15	963.37	0.87
681.98	902.32	0.96	1368.17	956.73	0.90	1818.32	965.93	0.88
696.22	907.52	0.96	1386.43	959.56	0.91	1838.52	968.35	0.88
710.59	912.49	0.97	1404.78	962.24	0.91	1858.78	970.63	0.89
725.09	917.26	0.97	1423.23	964.78	0.92	1879.10	972.77	0.90
739.74	921.82	0.97	1441.79	967.19	0.92	1899.49	974.79	0.90
754.53	926.18	0.97	1460.48	969.46	0.93	1919.99	976.70	0.91
769.46	930.36	0.97	1479.30	971.61	0.93	1940.59	978.49	0.92
784.54	934.34	0.97	1498.27	973.64	0.94	1961.31	980.17	0.92
799.78	938.15	0.98	1517.39	975.56	0.94	1982.17	981.75	0.93
815.18	941.79	0.98	1536.68	977.37	0.95	2003.19	983.24	0.93
830.75	945.26	0.98	1556.14	979.08	0.95	2024.38	984.63	0.94
846.48	948.57	0.98	1575.79	980.69	0.95	2045.74	985.94	0.94
862.40	951.72	0.98	1595.64	982.21	0.96	2067.31	987.16	0.95
878.49	954.72	0.98	1615.70	983.64	0.96	2089.09	988.31	0.95
894.76	957.58	0.98	1635.98	984.98	0.96	2111.10	989.38	0.95
911.23	960.30	0.98	1656.49	986.24	0.96	2133.35	990.39	0.96
927.89	962.89	0.98	1677.25	987.43	0.97	2155.86	991.32	0.96
944.76	965.34	0.99	1698.26	988.54	0.97	2178.65	992.20	0.96
961.84	967.67	0.99	1719.55	989.58	0.97	2201.74	993.01	0.97
979.13	969.88	0.99	1741.12	990.56	0.97	2226.65	993.82	0.97
997.67	972.10	0.99	1762.98	991.47	0.98	2250.32	994.52	0.97
1015.29	974.06	0.99	1785.16	992.32	0.98	2272.97	995.14	0.97
1032.56	975.86	0.99	1807.67	993.12	0.98	2296.03	995.71	0.98
1050.10	977.57	0.99	1830.51	993.86	0.98	2319.53	996.25	0.98
1067.90	979.20	0.99	1853.71	994.56	0.98	2343.48	996.75	0.98
1085.98	980.74	0.99	1877.29	995.20	0.98	2367.90	997.22	0.98
1104.35	982.20	0.99	1901.25	995.80	0.99	2392.82	997.66	0.98
1123.02	983.58	0.99	1925.62	996.35	0.99	2418.25	998.06	0.98
1141.99	984.88	0.99	1950.42	996.87	0.99	2444.23	998.44	0.99
1161.29	986.12	0.99	1975.67	997.34	0.99	2470.78	998.78	0.99
1180.92	987.28	0.99	2001.38	997.78	0.99	2497.93	999.10	0.99
1200.89	988.38	0.99	2027.58	998.19	0.99	2525.71	999.40	0.99
1221.23	989.41	1.00	2054.30	998.56	0.99	2554.17	999.67	0.99

1241.93	990.38	1.00	2081.55	998.91	0.99	2583.32	999.92	0.99
1263.02	991.30	1.00	2109.36	999.22	0.99	2613.23	1000.14	0.99
1284.51	992.15	1.00	2138.89	999.52	0.99	2643.93	1000.35	0.99
1306.43	992.96	1.00	2166.95	999.78	0.99	2675.22	1000.54	0.99
1328.65	993.71	1.00	2194.86	1000.01	1.00	2700.00	1000.67	0.99
1350.00	994.37	1.00	2223.46	1000.22	1.00	2732.52	1000.83	1.00
1372.82	995.01	1.00	2252.78	1000.41	1.00	2765.86	1000.97	1.00
1396.05	995.62	1.00	2282.87	1000.58	1.00	2800.07	1001.10	1.00
1419.71	996.18	1.00	2313.77	1000.74	1.00	2835.19	1001.22	1.00
1443.81	996.70	1.00	2345.53	1000.89	1.00	2871.28	1001.32	1.00
1468.37	997.18	1.00	2378.19	1001.02	1.00	2908.39	1001.41	1.00
1493.41	997.63	1.00	2411.81	1001.14	1.00	2946.59	1001.49	1.00
1518.94	998.04	1.00	2446.45	1001.25	1.00	2985.95	1001.56	1.00
1545.00	998.43	1.00	2482.19	1001.35	1.00	3026.53	1001.63	1.00
1571.59	998.78	1.00	2519.08	1001.44	1.00	3068.43	1001.68	1.00
1598.75	999.10	1.00	2557.23	1001.51	1.00	3111.74	1001.73	1.00
1626.49	999.40	1.00	2596.71	1001.58	1.00	3158.65	1001.78	1.00
1654.85	999.67	1.00	2637.64	1001.64	1.00	3203.22	1001.81	1.00
1683.84	999.92	1.00	2679.76	1001.70	1.00	3247.42	1001.84	1.00
1713.51	1000.15	1.00	2700.00	1001.72	1.00	3293.37	1001.87	1.00
1743.89	1000.35	1.00	2744.15	1001.77	1.00	3341.21	1001.89	1.00
1775.00	1000.54	1.00	2789.86	1001.80	1.00	3391.08	1001.91	1.00
1806.89	1000.71	1.00	2837.26	1001.84	1.00	3443.20	1001.93	1.00
1839.59	1000.87	1.00	2886.46	1001.87	1.00	3497.75	1001.94	1.00
1873.15	1001.01	1.00	2937.62	1001.89	1.00	3555.00	1001.95	1.00
1907.62	1001.13	1.00	2993.24	1001.91	1.00	3615.22	1001.96	1.00
1943.03	1001.24	1.00	3046.08	1001.93	1.00	3678.76	1001.97	1.00
1980.88	1001.35	1.00	3098.81	1001.94	1.00	3746.00	1001.98	1.00
2016.84	1001.43	1.00	3153.99	1001.95	1.00	3817.42	1001.98	1.00
2052.80	1001.51	1.00	3211.89	1001.96	1.00	3893.59	1001.99	1.00
2089.91	1001.57	1.00	3272.78	1001.97	1.00	3971.80	1001.99	1.00
2128.25	1001.63	1.00	3336.99	1001.98	1.00	4050.00	1001.99	1.00
2167.91	1001.68	1.00	3404.91	1001.98	1.00	4132.13	1002.00	1.00
2208.99	1001.73	1.00	3477.00	1001.99	1.00	4220.24	1002.00	1.00
2251.59	1001.77	1.00	3553.82	1001.99	1.00	4315.26	1002.00	1.00
2295.84	1001.81	1.00	3636.05	1001.99	1.00	4418.37	1002.00	1.00
2341.86	1001.84	1.00	3724.51	1001.99	1.00	4531.09	1002.00	1.00
2389.82	1001.87	1.00	3820.27	1002.00	1.00	4655.38	1002.00	1.00
2439.89	1001.89	1.00	3935.14	1002.00	1.00	4793.90	1002.00	1.00
2492.27	1001.91	1.00	4050.00	1002.00	1.00	4950.33	1002.00	1.00
2547.19	1001.93	1.00	4162.28	1002.00	1.00	5130.01	1002.00	1.00
2604.92	1001.94	1.00	4285.88	1002.00	1.00	5334.74	1002.00	1.00
2665.02	1001.95	1.00	4423.32	1002.00	1.00	5400.00	1002.00	1.00
2700.00	1001.96	1.00	4578.07	1002.00	1.00	5635.46	1002.00	1.00
2767.50	1001.97	1.00	4755.07	1002.00	1.00	5923.64	1002.00	1.00
2831.63	1001.98	1.00	4961.74	1002.00	1.00	6293.79	1002.00	1.00
2896.43	1001.98	1.00	5180.87	1002.00	1.00	6750.00	1002.00	1.00
2964.94	1001.99	1.00	5400.00	1002.00	1.00	7425.00	1002.00	1.00

3037.62	1001.99	1.00	5721.97	1002.00	1.00	8100.00	1002.00	1.00
3114.99	1001.99	1.00	6147.25	1002.00	1.00	8775.00	1002.00	1.00
3197.73	1001.99	1.00	6750.00	1002.00	1.00	9450.00	1002.00	1.00
3286.62	1002.00	1.00	7425.00	1002.00	1.00	10125.00	1002.00	1.00
3382.69	1002.00	1.00	8100.00	1002.00	1.00	10800.00	1002.00	1.00
3487.19	1002.00	1.00	8775.00	1002.00	1.00	11475.00	1002.00	1.00
3601.76	1002.00	1.00	9450.00	1002.00	1.00	12150.00	1002.00	1.00
3728.59	1002.00	1.00	10800.00	1002.00	1.00	13500.00	1002.00	1.00
3870.65	1002.00	1.00	12150.00	1002.00	1.00	14850.00	1002.00	1.00
4028.66	1002.00	1.00	13500.00	1002.00	1.00	16200.00	1002.00	1.00
4050.00	1002.00	1.00	14850.00	1002.00	1.00	17550.00	1002.00	1.00
4215.98	1002.00	1.00	16200.00	1002.00	1.00	18900.00	1002.00	1.00
4407.29	1002.00	1.00	17550.00	1002.00	1.00	20250.00	1002.00	1.00
4632.90	1002.00	1.00	18900.00	1002.00	1.00	21600.00	1002.00	1.00
4907.42	1002.00	1.00	20250.00	1002.00	1.00	22950.00	1002.00	1.00
5241.16	1002.00	1.00	21600.00	1002.00	1.00	24300.00	1002.00	1.00
5400.00	1002.00	1.00	22950.00	1002.00	1.00	25650.00	1002.00	1.00
5877.33	1002.00	1.00	24300.00	1002.00	1.00	27000.00	1002.00	1.00
6575.88	1002.00	1.00	25650.00	1002.00	1.00	28350.00	1002.00	1.00
6750.00	1002.00	1.00	27000.00	1002.00	1.00	29700.00	1002.00	1.00
7425.00	1002.00	1.00	28350.00	1002.00	1.00	31050.00	1002.00	1.00
8100.00	1002.00	1.00	29700.00	1002.00	1.00	32400.00	1002.00	1.00

τs = 5			**τs = 8**			**τs =10**		
Time (sec)	**y (U/mL)**	**x (U/mL)**	**Time (sec)**	**y (U/mL)**	**x (U/mL)**	**Time (sec)**	**y (U/mL)**	**x (U/mL)**
0.00	0.00	0.05	0.00	0.00	0.03	0.00	0.00	0.02
189.87	36.09	0.05	356.81	43.19	0.03	445.63	43.43	0.03
424.01	80.51	0.05	745.03	90.11	0.03	897.82	87.44	0.03
665.41	126.18	0.06	1153.76	139.42	0.04	1350.00	131.40	0.03
910.49	172.41	0.06	1350.00	163.06	0.04	1882.59	183.09	0.03
1154.70	218.34	0.06	1761.71	212.57	0.04	2410.15	234.19	0.03
1350.00	254.94	0.06	2170.12	261.57	0.04	2700.00	262.22	0.03
1581.33	298.15	0.07	2567.32	309.09	0.04	3209.89	311.44	0.04
1808.14	340.34	0.07	2700.00	324.94	0.05	3696.85	358.34	0.04
2029.90	381.41	0.08	3074.99	369.62	0.05	4050.00	392.26	0.04
2246.24	421.27	0.08	3439.50	412.91	0.05	4503.68	435.74	0.04
2473.12	462.84	0.09	3783.35	453.60	0.06	4926.24	476.09	0.05
2674.03	499.42	0.09	4050.00	485.04	0.06	5340.44	515.52	0.05
2700.00	504.13	0.09	4360.00	521.45	0.06	5400.00	521.17	0.05
2875.58	535.87	0.10	4658.43	556.34	0.07	5779.28	557.11	0.05
3045.39	566.36	0.11	4945.38	589.71	0.07	6142.87	591.41	0.06
3209.50	595.62	0.11	5212.31	620.58	0.08	6478.79	622.95	0.06
3368.01	623.66	0.12	5400.00	642.18	0.08	6750.00	648.30	0.07
3521.05	650.49	0.13	5630.13	668.51	0.09	7043.37	675.58	0.07
3668.73	676.14	0.14	5850.59	693.56	0.09	7323.47	701.47	0.08
3811.19	700.63	0.15	6061.72	717.38	0.10	7590.78	726.02	0.09

3944.09	723.21	0.16	6263.86	739.99	0.11	7834.40	748.23	0.09
4050.00	741.01	0.16	6457.34	761.44	0.12	8072.24	769.74	0.10
4152.33	758.02	0.17	6636.22	781.07	0.13	8100.00	772.24	0.10
4251.00	774.23	0.18	6750.00	793.44	0.13	8300.42	790.19	0.11
4346.14	789.66	0.19	6892.51	808.81	0.14	8491.52	807.16	0.12
4437.86	804.33	0.21	7028.96	823.35	0.15	8673.77	823.19	0.13
4526.28	818.27	0.22	7159.62	837.12	0.16	8847.58	838.30	0.14
4611.51	831.48	0.23	7284.75	850.12	0.17	9013.37	852.55	0.15
4693.63	844.00	0.25	7404.58	862.40	0.19	9171.53	865.95	0.16
4772.72	855.83	0.26	7519.36	873.97	0.20	9315.85	878.01	0.17
4848.87	866.99	0.28	7629.26	884.86	0.21	9450.00	889.03	0.18
4922.14	877.49	0.29	7734.48	895.09	0.23	9555.96	897.61	0.20
4992.58	887.35	0.31	7835.16	904.66	0.25	9657.36	905.68	0.21
5060.27	896.58	0.33	7931.44	913.61	0.27	9754.43	913.28	0.22
5125.26	905.20	0.35	8019.93	921.62	0.29	9847.33	920.42	0.24
5187.63	913.23	0.37	8100.00	928.69	0.30	9936.26	927.12	0.26
5247.47	920.70	0.39	8167.50	934.48	0.32	10021.34	933.38	0.27
5304.86	927.61	0.41	8231.63	939.85	0.34	10102.71	939.24	0.29
5358.50	933.85	0.43	8292.54	944.80	0.36	10180.50	944.69	0.31
5400.00	938.52	0.45	8350.42	949.37	0.38	10254.80	949.75	0.33
5449.36	943.87	0.47	8405.40	953.57	0.40	10325.75	954.43	0.35
5496.28	948.75	0.49	8457.63	957.43	0.42	10393.45	958.76	0.37
5540.97	953.21	0.51	8507.24	960.98	0.44	10458.05	962.74	0.40
5583.64	957.28	0.53	8554.38	964.23	0.46	10519.68	966.40	0.42
5624.48	961.00	0.56	8599.16	967.20	0.48	10578.52	969.75	0.44
5663.68	964.40	0.58	8641.71	969.91	0.50	10634.75	972.81	0.47
5701.40	967.51	0.60	8682.12	972.39	0.52	10688.54	975.61	0.49
5737.79	970.36	0.62	8720.51	974.65	0.54	10744.27	978.36	0.52
5772.99	972.98	0.64	8757.26	976.72	0.56	10792.84	980.63	0.55
5807.13	975.38	0.66	8793.04	978.65	0.58	10800.00	980.95	0.55
5840.32	977.58	0.68	8827.95	980.44	0.60	10845.49	982.95	0.57
5872.68	979.61	0.70	8862.07	982.12	0.62	10888.96	984.75	0.60
5904.29	981.47	0.71	8895.50	983.68	0.64	10930.65	986.38	0.62
5935.25	983.19	0.73	8928.30	985.13	0.66	10970.78	987.85	0.64
5965.65	984.77	0.75	8960.56	986.48	0.67	11009.54	989.18	0.67
5995.56	986.22	0.76	8992.35	987.74	0.69	11047.11	990.39	0.69
6025.05	987.57	0.78	9023.74	988.92	0.71	11083.63	991.49	0.71
6054.18	988.80	0.80	9054.78	990.01	0.73	11119.26	992.49	0.73
6083.02	989.94	0.81	9085.54	991.02	0.74	11154.12	993.40	0.75
6111.64	990.99	0.82	9116.08	991.96	0.76	11188.33	994.23	0.77
6140.07	991.96	0.84	9146.44	992.84	0.78	11222.00	994.98	0.79
6170.57	992.92	0.85	9176.69	993.65	0.79	11255.23	995.67	0.80
6199.54	993.75	0.86	9206.87	994.40	0.81	11288.10	996.29	0.82
6227.06	994.49	0.87	9237.03	995.09	0.82	11320.71	996.86	0.83
6253.21	995.13	0.88	9267.22	995.74	0.84	11353.14	997.38	0.85
6278.77	995.71	0.89	9297.49	996.33	0.85	11385.45	997.85	0.86
6304.64	996.25	0.90	9327.87	996.88	0.86	11417.72	998.28	0.87
6330.83	996.76	0.91	9358.43	997.38	0.87	11450.03	998.67	0.89
6357.38	997.23	0.91	9389.21	997.84	0.89	11485.03	999.05	0.90

6384.30	997.67	0.92	9419.86	998.26	0.90	11518.28	999.38	0.91
6411.63	998.08	0.93	9450.00	998.64	0.91	11549.86	999.66	0.92
6439.40	998.45	0.94	9480.89	998.99	0.91	11579.87	999.90	0.92
6467.63	998.80	0.94	9512.03	999.31	0.92	11609.98	1000.11	0.93
6496.35	999.12	0.95	9543.49	999.60	0.93	11640.71	1000.31	0.94
6525.61	999.42	0.95	9575.29	999.86	0.94	11672.09	1000.50	0.94
6555.45	999.69	0.96	9607.51	1000.10	0.94	11704.17	1000.66	0.95
6585.89	999.94	0.96	9640.18	1000.31	0.95	11736.99	1000.82	0.96
6616.99	1000.17	0.97	9673.37	1000.51	0.96	11770.59	1000.96	0.96
6648.79	1000.37	0.97	9707.12	1000.69	0.96	11805.03	1001.08	0.97
6681.34	1000.56	0.97	9741.50	1000.84	0.97	11840.37	1001.20	0.97
6715.67	1000.74	0.98	9776.55	1000.99	0.97	11876.67	1001.30	0.97
6750.00	1000.89	0.98	9812.33	1001.12	0.97	11914.00	1001.39	0.98
6784.49	1001.03	0.98	9848.92	1001.23	0.98	11952.44	1001.48	0.98
6819.80	1001.15	0.98	9886.38	1001.33	0.98	11992.09	1001.55	0.98
6855.97	1001.26	0.99	9924.77	1001.42	0.98	12033.03	1001.62	0.99
6893.07	1001.36	0.99	9968.53	1001.51	0.99	12075.38	1001.67	0.99
6931.16	1001.45	0.99	10010.10	1001.59	0.99	12118.80	1001.72	0.99
6970.32	1001.53	0.99	10049.60	1001.64	0.99	12150.00	1001.76	0.99
7010.61	1001.59	0.99	10088.94	1001.69	0.99	12195.88	1001.79	0.99
7052.13	1001.65	0.99	10129.68	1001.74	0.99	12243.35	1001.83	0.99
7094.96	1001.71	0.99	10171.91	1001.78	0.99	12292.53	1001.86	0.99
7139.20	1001.75	1.00	10215.73	1001.81	0.99	12343.58	1001.88	1.00
7187.24	1001.80	1.00	10261.26	1001.84	1.00	12401.40	1001.91	1.00
7232.88	1001.83	1.00	10308.64	1001.87	1.00	12456.33	1001.93	1.00
7277.76	1001.86	1.00	10358.04	1001.89	1.00	12508.51	1001.94	1.00
7324.45	1001.88	1.00	10409.62	1001.91	1.00	12563.05	1001.95	1.00
7373.09	1001.90	1.00	10463.60	1001.93	1.00	12620.26	1001.96	1.00
7423.86	1001.92	1.00	10520.23	1001.94	1.00	12680.43	1001.97	1.00
7476.94	1001.93	1.00	10579.79	1001.95	1.00	12743.84	1001.98	1.00
7532.56	1001.95	1.00	10642.62	1001.96	1.00	12810.88	1001.98	1.00
7590.97	1001.96	1.00	10709.11	1001.97	1.00	12881.98	1001.99	1.00
7652.47	1001.97	1.00	10778.88	1001.98	1.00	12957.66	1001.99	1.00
7717.42	1001.97	1.00	10800.00	1001.98	1.00	13038.57	1001.99	1.00
7786.24	1001.98	1.00	10867.50	1001.99	1.00	13125.49	1001.99	1.00
7859.42	1001.99	1.00	10938.23	1001.99	1.00	13219.41	1002.00	1.00
7937.59	1001.99	1.00	11013.42	1001.99	1.00	13321.59	1002.00	1.00
8018.80	1001.99	1.00	11093.65	1001.99	1.00	13431.24	1002.00	1.00
8100.00	1001.99	1.00	11179.64	1002.00	1.00	13500.00	1002.00	1.00
8184.84	1002.00	1.00	11272.27	1002.00	1.00	13615.03	1002.00	1.00
8276.08	1002.00	1.00	11372.65	1002.00	1.00	13741.96	1002.00	1.00
8374.75	1002.00	1.00	11482.22	1002.00	1.00	13883.53	1002.00	1.00
8482.17	1002.00	1.00	11602.81	1002.00	1.00	14043.49	1002.00	1.00
8600.04	1002.00	1.00	11736.93	1002.00	1.00	14227.31	1002.00	1.00
8730.60	1002.00	1.00	11888.02	1002.00	1.00	14443.26	1002.00	1.00
8876.93	1002.00	1.00	12055.97	1002.00	1.00	14694.22	1002.00	1.00
9043.35	1002.00	1.00	12150.00	1002.00	1.00	14850.00	1002.00	1.00
9246.68	1002.00	1.00	12341.62	1002.00	1.00	15179.95	1002.00	1.00

9450.00	1002.00	1.00	12567.43	1002.00	1.00	15618.92	1002.00	1.00
9693.32	1002.00	1.00	12841.86	1002.00	1.00	16200.00	1002.00	1.00
9993.14	1002.00	1.00	13170.93	1002.00	1.00	16875.00	1002.00	1.00
10396.57	1002.00	1.00	13500.00	1002.00	1.00	17550.00	1002.00	1.00
10800.00	1002.00	1.00	14052.17	1002.00	1.00	18225.00	1002.00	1.00
11475.00	1002.00	1.00	14850.00	1002.00	1.00	18900.00	1002.00	1.00
12150.00	1002.00	1.00	15525.00	1002.00	1.00	19575.00	1002.00	1.00
12825.00	1002.00	1.00	16200.00	1002.00	1.00	20250.00	1002.00	1.00
13500.00	1002.00	1.00	16875.00	1002.00	1.00	21600.00	1002.00	1.00
14175.00	1002.00	1.00	17550.00	1002.00	1.00	22950.00	1002.00	1.00
14850.00	1002.00	1.00	18225.00	1002.00	1.00	24300.00	1002.00	1.00
15525.00	1002.00	1.00	18900.00	1002.00	1.00	25650.00	1002.00	1.00
16200.00	1002.00	1.00	20250.00	1002.00	1.00	27000.00	1002.00	1.00
16875.00	1002.00	1.00	21600.00	1002.00	1.00	28350.00	1002.00	1.00
17550.00	1002.00	1.00	22950.00	1002.00	1.00	29700.00	1002.00	1.00
18225.00	1002.00	1.00	24300.00	1002.00	1.00	31050.00	1002.00	1.00
18900.00	1002.00	1.00	25650.00	1002.00	1.00	32400.00	1002.00	1.00
19575.00	1002.00	1.00	27000.00	1002.00	1.00	33750.00	1002.00	1.00
20250.00	1002.00	1.00	28350.00	1002.00	1.00	35100.00	1002.00	1.00
20925.00	1002.00	1.00	29700.00	1002.00	1.00	36450.00	1002.00	1.00
21600.00	1002.00	1.00	31050.00	1002.00	1.00	37800.00	1002.00	1.00
22275.00	1002.00	1.00	32400.00	1002.00	1.00	39150.00	1002.00	1.00
22950.00	1002.00	1.00	33750.00	1002.00	1.00	40500.00	1002.00	1.00
23625.00	1002.00	1.00	35100.00	1002.00	1.00	41850.00	1002.00	1.00
24300.00	1002.00	1.00	36450.00	1002.00	1.00	43200.00	1002.00	1.00
24975.00	1002.00	1.00	37800.00	1002.00	1.00	44550.00	1002.00	1.00
25650.00	1002.00	1.00	39150.00	1002.00	1.00	45900.00	1002.00	1.00
26325.00	1002.00	1.00	40500.00	1002.00	1.00	47250.00	1002.00	1.00
27000.00	1002.00	1.00	41850.00	1002.00	1.00	48600.00	1002.00	1.00
27675.00	1002.00	1.00	43200.00	1002.00	1.00	49950.00	1002.00	1.00
28350.00	1002.00	1.00	44550.00	1002.00	1.00	51300.00	1002.00	1.00
29025.00	1002.00	1.00	45900.00	1002.00	1.00	52650.00	1002.00	1.00
29700.00	1002.00	1.00	47250.00	1002.00	1.00	54000.00	1002.00	1.00
30375.00	1002.00	1.00	48600.00	1002.00	1.00			
31050.00	1002.00	1.00	49950.00	1002.00	1.00			
31725.00	1002.00	1.00	51300.00	1002.00	1.00			
32400.00	1002.00	1.00	52650.00	1002.00	1.00			
33075.00	1002.00	1.00	54000.00	1002.00	1.00			
33750.00	1002.00	1.00						
34425.00	1002.00	1.00						
35100.00	1002.00	1.00						
35775.00	1002.00	1.00						
36450.00	1002.00	1.00						
37125.00	1002.00	1.00						

τs = 20		
Time (sec)	**y (U/mL)**	**x (U/mL)**
0.00	0.00	0.01
675.00	33.32	0.01
1350.00	66.62	0.01
2025.00	99.90	0.01
2700.00	133.17	0.01
3375.00	166.42	0.02
4050.00	199.65	0.02
4725.00	232.86	0.02
5400.00	266.04	0.02
6075.00	299.20	0.02
6750.00	332.33	0.02
7425.00	365.43	0.02
8100.00	398.50	0.02
8775.00	431.53	0.02
9450.00	464.51	0.02
10125.00	497.45	0.02
10800.00	530.34	0.03
11475.00	563.17	0.03
12150.00	595.92	0.03
12825.00	628.60	0.03
13500.00	661.17	0.04
14175.00	693.64	0.04
14758.92	721.62	0.04
14850.00	725.97	0.04
15378.65	751.18	0.05
15855.70	773.82	0.05
16200.00	790.08	0.06
16615.46	809.62	0.06
17006.72	827.90	0.07
17360.51	844.32	0.08
17550.00	853.06	0.08
17849.91	866.81	0.09
18132.48	879.66	0.09
18398.77	891.65	0.10
18635.76	902.21	0.11
18865.97	912.35	0.13
18900.00	913.84	0.13
19084.43	921.84	0.14
19258.75	929.30	0.15
19423.60	936.26	0.16
19579.57	942.73	0.18
19727.18	948.74	0.19
19866.90	954.31	0.21
19999.12	959.45	0.23
20117.50	963.94	0.25

20232.60	968.18	0.27
20250.00	968.81	0.28
20328.00	971.59	0.30
20402.48	974.18	0.32
20473.57	976.58	0.34
20541.38	978.79	0.36
20606.05	980.84	0.38
20667.72	982.73	0.40
20726.54	984.46	0.42
20782.69	986.05	0.45
20836.37	987.51	0.47
20887.77	988.84	0.49
20937.09	990.06	0.52
20984.54	991.18	0.54
21030.31	992.20	0.57
21074.59	993.13	0.59
21117.55	993.98	0.62
21159.36	994.76	0.64
21200.17	995.47	0.66
21240.12	996.12	0.69
21279.35	996.72	0.71
21317.99	997.26	0.73
21356.14	997.76	0.75
21393.91	998.21	0.77
21431.41	998.62	0.79
21468.73	998.99	0.81
21505.97	999.33	0.83
21543.22	999.63	0.84
21580.11	999.90	0.86
21600.00	1000.04	0.87
21636.62	1000.27	0.88
21673.35	1000.48	0.89
21710.27	1000.66	0.91
21747.47	1000.83	0.92
21785.06	1000.98	0.93
21823.11	1001.11	0.93
21861.71	1001.23	0.94
21900.95	1001.33	0.95
21940.93	1001.42	0.96
21981.73	1001.50	0.96
22023.46	1001.58	0.97
22069.79	1001.64	0.97
22113.80	1001.70	0.98
22155.61	1001.74	0.98
22197.47	1001.78	0.98
22240.94	1001.82	0.99
22286.12	1001.84	0.99
22333.12	1001.87	0.99

22382.09	1001.89	0.99
22433.20	1001.91	0.99
22486.63	1001.93	0.99
22542.61	1001.94	1.00
22601.40	1001.95	1.00
22663.30	1001.96	1.00
22728.70	1001.97	1.00
22798.03	1001.98	1.00
22874.01	1001.98	1.00
22950.00	1001.99	1.00
23023.52	1001.99	1.00
23101.92	1001.99	1.00
23185.82	1002.00	1.00
23276.05	1002.00	1.00
23373.61	1002.00	1.00
23479.78	1002.00	1.00
23596.22	1002.00	1.00
23725.13	1002.00	1.00
23869.53	1002.00	1.00
24033.66	1002.00	1.00
24218.16	1002.00	1.00
24300.00	1002.00	1.00
24511.42	1002.00	1.00
24764.84	1002.00	1.00
25080.42	1002.00	1.00
25476.20	1002.00	1.00
25650.00	1002.00	1.00
26325.00	1002.00	1.00
27000.00	1002.00	1.00
27675.00	1002.00	1.00
28350.00	1002.00	1.00
29025.00	1002.00	1.00
29700.00	1002.00	1.00
30375.00	1002.00	1.00
31050.00	1002.00	1.00
31725.00	1002.00	1.00
32400.00	1002.00	1.00
33075.00	1002.00	1.00
33750.00	1002.00	1.00
34425.00	1002.00	1.00
35100.00	1002.00	1.00
35775.00	1002.00	1.00
36450.00	1002.00	1.00
37125.00	1002.00	1.00
37800.00	1002.00	1.00
38475.00	1002.00	1.00
39150.00	1002.00	1.00
39825.00	1002.00	1.00

40500.00	1002.00	1.00
41175.00	1002.00	1.00
41850.00	1002.00	1.00
42525.00	1002.00	1.00
43200.00	1002.00	1.00
43875.00	1002.00	1.00
44550.00	1002.00	1.00
45225.00	1002.00	1.00
45900.00	1002.00	1.00
46575.00	1002.00	1.00
47250.00	1002.00	1.00
47925.00	1002.00	1.00
48600.00	1002.00	1.00
49275.00	1002.00	1.00
49950.00	1002.00	1.00
50625.00	1002.00	1.00
51300.00	1002.00	1.00
51975.00	1002.00	1.00
52650.00	1002.00	1.00
53325.00	1002.00	1.00
54000.00	1002.00	1.00
22950.00	1002.00	1.00
24300.00	1002.00	1.00
25650.00	1002.00	1.00
27000.00	1002.00	1.00
28350.00	1002.00	1.00
29700.00	1002.00	1.00
31050.00	1002.00	1.00
32400.00	1002.00	1.00
33750.00	1002.00	1.00
35100.00	1002.00	1.00
36450.00	1002.00	1.00
37800.00	1002.00	1.00
39150.00	1002.00	1.00
40500.00	1002.00	1.00
41850.00	1002.00	1.00
43200.00	1002.00	1.00
44550.00	1002.00	1.00
45900.00	1002.00	1.00
47250.00	1002.00	1.00
48600.00	1002.00	1.00
49950.00	1002.00	1.00
51300.00	1002.00	1.00
52650.00	1002.00	1.00
54000.00	1002.00	1.00

τs = 8			τs = 10			τs = 20		
Time (sec)	**y (U/mL)**	**x (U/mL)**	**Time (sec)**	**y (U/mL)**	**x (U/mL)**	**Time (sec)**	**y (U/mL)**	**x (U/mL)**
0.00	0.00	0.17	0.00	0.00	0.14	0.00	0.00	0.08
282.06	29.11	0.18	382.45	32.71	0.15	675.00	31.12	0.08
628.28	64.64	0.18	806.21	68.77	0.15	1350.00	62.16	0.08
989.14	101.41	0.19	1263.08	107.41	0.16	2025.00	93.12	0.08
1350.00	137.90	0.19	1350.00	114.73	0.16	2700.00	123.99	0.09
1742.01	177.21	0.20	1826.95	154.74	0.16	3375.00	154.77	0.09
2137.40	216.47	0.21	2309.78	194.92	0.17	4050.00	185.45	0.09
2532.09	255.26	0.22	2700.00	227.15	0.18	4725.00	216.02	0.10
2700.00	271.63	0.22	3182.76	266.70	0.18	5400.00	246.47	0.10
3088.43	309.18	0.23	3657.80	305.23	0.19	6075.00	276.81	0.10
3473.76	345.98	0.24	4050.00	336.74	0.20	6750.00	307.01	0.11
3850.65	381.49	0.25	4512.71	373.53	0.21	7425.00	337.08	0.11
4050.00	400.08	0.26	4961.02	408.75	0.22	8100.00	366.99	0.12
4412.29	433.47	0.27	5400.00	442.79	0.23	8775.00	396.74	0.12
4768.62	465.81	0.28	5828.88	475.60	0.24	9450.00	426.32	0.13
5112.55	496.52	0.29	6289.44	510.30	0.25	10125.00	455.70	0.13
5400.00	521.77	0.30	6695.74	540.40	0.27	10800.00	484.88	0.14
5724.08	549.77	0.32	6750.00	544.38	0.27	11475.00	513.83	0.15
6041.04	576.63	0.33	7136.09	572.44	0.28	12150.00	542.53	0.15
6350.72	602.35	0.34	7513.16	599.36	0.29	12825.00	570.95	0.16
6649.00	626.59	0.36	7874.21	624.66	0.31	13500.00	599.08	0.17
6750.00	634.68	0.36	8100.00	640.22	0.32	14175.00	626.88	0.18
7028.89	656.68	0.38	8437.10	663.06	0.33	14850.00	654.30	0.19
7300.78	677.63	0.39	8765.17	684.81	0.34	15525.00	681.31	0.21
7565.72	697.54	0.41	9107.59	706.98	0.36	16113.83	704.50	0.22
7832.86	717.10	0.42	9413.13	726.26	0.38	16200.00	707.86	0.22
8100.00	736.11	0.44	9450.00	728.56	0.38	16756.99	729.38	0.23
8331.73	752.13	0.45	9734.72	746.03	0.39	17283.45	749.36	0.25
8557.71	767.32	0.47	10011.39	762.56	0.41	17550.00	759.33	0.26
8778.16	781.70	0.49	10280.21	778.19	0.43	18041.58	777.44	0.27
8993.29	795.31	0.50	10536.07	792.65	0.44	18503.00	794.09	0.29
9221.65	809.27	0.52	10800.00	807.11	0.46	18900.00	808.12	0.30
9424.58	821.26	0.54	11033.45	819.51	0.48	19324.21	822.78	0.32
9450.00	822.73	0.54	11260.80	831.20	0.49	19733.39	836.57	0.33
9638.94	833.48	0.55	11482.34	842.23	0.51	20120.15	849.26	0.35
9824.10	843.65	0.57	11698.38	852.62	0.53	20250.00	853.44	0.36
10005.69	853.28	0.58	11924.19	863.09	0.55	20606.09	864.68	0.38
10183.93	862.39	0.60	12150.00	873.15	0.56	20949.36	875.20	0.40
10359.03	871.01	0.61	12339.51	881.26	0.58	21271.53	884.77	0.42
10531.22	879.15	0.63	12525.31	888.92	0.60	21600.00	894.21	0.44
10698.86	886.77	0.64	12707.63	896.15	0.61	21890.65	902.26	0.46
10800.00	891.22	0.65	12886.73	902.96	0.63	22171.57	909.78	0.47
10956.67	897.88	0.67	13062.86	909.39	0.64	22443.32	916.78	0.49
11111.49	904.19	0.68	13236.24	915.45	0.66	22700.77	923.17	0.51
11264.61	910.18	0.69	13405.27	921.10	0.67	22950.00	929.11	0.53
11416.19	915.85	0.71	13500.00	924.16	0.68	23183.82	934.45	0.55

11566.38	921.22	0.72	13658.41	929.09	0.70	23411.50	939.44	0.57
11715.34	926.30	0.73	13815.19	933.75	0.71	23633.51	944.10	0.59
11863.19	931.11	0.75	13970.52	938.16	0.72	23850.28	948.44	0.61
12008.50	935.61	0.76	14124.55	942.32	0.74	24075.14	952.73	0.63
12150.00	939.78	0.77	14277.45	946.25	0.75	24300.00	956.79	0.65
12288.24	943.66	0.78	14429.38	949.97	0.76	24491.80	960.08	0.67
12426.13	947.33	0.79	14580.48	953.47	0.77	24680.62	963.16	0.68
12563.78	950.82	0.80	14729.43	956.74	0.79	24866.78	966.05	0.70
12701.28	954.12	0.81	14850.00	959.25	0.80	25050.57	968.74	0.71
12838.76	957.24	0.82	14992.39	962.08	0.81	25232.27	971.26	0.73
12976.31	960.20	0.83	15134.71	964.76	0.82	25412.16	973.62	0.75
13114.04	962.99	0.84	15277.07	967.28	0.83	25588.86	975.79	0.76
13252.06	965.64	0.85	15419.58	969.67	0.84	25650.00	976.52	0.77
13389.36	968.11	0.86	15562.38	971.92	0.85	25817.72	978.42	0.78
13500.00	970.00	0.87	15705.57	974.04	0.86	25984.79	980.21	0.79
13633.45	972.15	0.87	15849.27	976.04	0.87	26151.42	981.88	0.81
13767.68	974.19	0.88	15993.59	977.93	0.87	26317.80	983.45	0.82
13902.77	976.12	0.89	16137.74	979.69	0.88	26484.12	984.91	0.83
14038.82	977.94	0.90	16200.00	980.41	0.89	26650.59	986.28	0.84
14175.93	979.66	0.90	16340.34	981.97	0.89	26825.29	987.62	0.85
14314.19	981.27	0.91	16481.79	983.43	0.90	27000.00	988.86	0.86
14453.70	982.80	0.92	16624.45	984.82	0.91	27160.79	989.91	0.87
14594.57	984.23	0.92	16768.42	986.11	0.91	27322.62	990.90	0.88
14735.90	985.57	0.93	16913.83	987.34	0.92	27485.66	991.82	0.89
14850.00	986.58	0.93	17060.80	988.48	0.92	27650.08	992.68	0.90
14989.97	987.75	0.94	17209.43	989.55	0.93	27816.03	993.48	0.91
15131.70	988.84	0.94	17359.86	990.56	0.94	27983.71	994.23	0.91
15275.29	989.87	0.94	17511.45	991.49	0.94	28166.85	994.98	0.92
15420.85	990.83	0.95	17550.00	991.72	0.94	28350.00	995.66	0.93
15568.50	991.73	0.95	17699.51	992.56	0.95	28517.89	996.22	0.94
15718.36	992.58	0.96	17851.20	993.34	0.95	28688.26	996.75	0.94
15870.56	993.37	0.96	18005.20	994.07	0.95	28861.29	997.24	0.95
16035.28	994.15	0.96	18161.65	994.75	0.96	29037.18	997.69	0.95
16200.00	994.86	0.97	18320.69	995.38	0.96	29216.13	998.11	0.96
16355.24	995.48	0.97	18482.47	995.97	0.97	29398.36	998.50	0.96
16513.28	996.05	0.97	18647.17	996.52	0.97	29582.71	998.85	0.96
16674.28	996.59	0.97	18813.93	997.03	0.97	29700.00	999.06	0.97
16838.39	997.08	0.98	18900.00	997.27	0.97	29885.74	999.36	0.97
17005.78	997.54	0.98	19067.32	997.71	0.97	30075.47	999.64	0.97
17176.65	997.97	0.98	19238.03	998.12	0.98	30269.45	999.89	0.98
17363.33	998.39	0.98	19412.29	998.50	0.98	30467.97	1000.12	0.98
17550.00	998.77	0.98	19590.33	998.85	0.98	30671.31	1000.33	0.98
17727.52	999.09	0.99	19772.36	999.17	0.98	30878.00	1000.52	0.98
17909.15	999.38	0.99	19958.61	999.46	0.99	31050.00	1000.67	0.98
18095.10	999.66	0.99	20148.09	999.73	0.99	31262.15	1000.82	0.99
18285.63	999.91	0.99	20250.00	999.86	0.99	31480.14	1000.97	0.99
18481.02	1000.14	0.99	20442.23	1000.09	0.99	31704.38	1001.09	0.99
18690.51	1000.36	0.99	20639.34	1000.30	0.99	31935.31	1001.21	0.99

18900.00	1000.55	0.99	20841.63	1000.50	0.99	32167.65	1001.31	0.99
19106.09	1000.72	0.99	21049.42	1000.67	0.99	32400.00	1001.40	0.99
19317.99	1000.87	0.99	21263.07	1000.83	0.99	32646.15	1001.48	0.99
19536.06	1001.01	1.00	21481.35	1000.97	0.99	32900.58	1001.56	0.99
19760.72	1001.13	1.00	21600.00	1001.04	0.99	33163.95	1001.62	1.00
20005.36	1001.25	1.00	21823.65	1001.16	1.00	33456.98	1001.68	1.00
20250.00	1001.35	1.00	22054.18	1001.27	1.00	33750.00	1001.73	1.00
20490.94	1001.44	1.00	22292.08	1001.37	1.00	34037.69	1001.78	1.00
20740.01	1001.52	1.00	22537.87	1001.45	1.00	34337.00	1001.81	1.00
20997.80	1001.59	1.00	22789.93	1001.53	1.00	34648.96	1001.84	1.00
21264.99	1001.65	1.00	22950.00	1001.57	1.00	34971.68	1001.87	1.00
21540.36	1001.70	1.00	23211.72	1001.63	1.00	35100.00	1001.88	1.00
21600.00	1001.71	1.00	23483.07	1001.69	1.00	35437.38	1001.90	1.00
21882.59	1001.76	1.00	23764.82	1001.74	1.00	35790.93	1001.92	1.00
22176.48	1001.80	1.00	24054.61	1001.78	1.00	36157.44	1001.94	1.00
22482.67	1001.83	1.00	24300.00	1001.81	1.00	36450.00	1001.95	1.00
22799.19	1001.86	1.00	24607.09	1001.84	1.00	36846.23	1001.96	1.00
22950.00	1001.87	1.00	24927.58	1001.87	1.00	37264.98	1001.97	1.00
23281.90	1001.89	1.00	25288.79	1001.89	1.00	37704.19	1001.98	1.00
23629.52	1001.91	1.00	25650.00	1001.92	1.00	37800.00	1001.98	1.00
23989.57	1001.93	1.00	26010.75	1001.93	1.00	38265.66	1001.98	1.00
24300.00	1001.94	1.00	26390.13	1001.95	1.00	38754.10	1001.99	1.00
24690.07	1001.95	1.00	26785.30	1001.96	1.00	39150.00	1001.99	1.00
25101.99	1001.96	1.00	27000.00	1001.96	1.00	39697.42	1001.99	1.00
25533.56	1001.97	1.00	27424.02	1001.97	1.00	40279.11	1001.99	1.00
25650.00	1001.97	1.00	27887.01	1001.98	1.00	40500.00	1002.00	1.00
26108.56	1001.98	1.00	28350.00	1001.98	1.00	41175.00	1002.00	1.00
26589.04	1001.99	1.00	28848.49	1001.99	1.00	41850.00	1002.00	1.00
27000.00	1001.99	1.00	29374.11	1001.99	1.00	42525.00	1002.00	1.00
27539.15	1001.99	1.00	29700.00	1001.99	1.00	43200.00	1002.00	1.00
28111.32	1001.99	1.00	30286.11	1001.99	1.00	43875.00	1002.00	1.00
28350.00	1002.00	1.00	30912.74	1002.00	1.00	44550.00	1002.00	1.00
29025.00	1002.00	1.00	31050.00	1002.00	1.00	45225.00	1002.00	1.00
29700.00	1002.00	1.00	31725.00	1002.00	1.00	45900.00	1002.00	1.00
30375.00	1002.00	1.00	32400.00	1002.00	1.00	46575.00	1002.00	1.00
31050.00	1002.00	1.00	33075.00	1002.00	1.00	47250.00	1002.00	1.00
31725.00	1002.00	1.00	33750.00	1002.00	1.00	47925.00	1002.00	1.00
32400.00	1002.00	1.00	34425.00	1002.00	1.00	48600.00	1002.00	1.00
33075.00	1002.00	1.00	35100.00	1002.00	1.00	49275.00	1002.00	1.00
33750.00	1002.00	1.00	35775.00	1002.00	1.00	49950.00	1002.00	1.00
34425.00	1002.00	1.00	36450.00	1002.00	1.00	50625.00	1002.00	1.00
35100.00	1002.00	1.00	37125.00	1002.00	1.00	51300.00	1002.00	1.00
35775.00	1002.00	1.00	37800.00	1002.00	1.00	51975.00	1002.00	1.00
36450.00	1002.00	1.00	38475.00	1002.00	1.00	52650.00	1002.00	1.00
37125.00	1002.00	1.00	39150.00	1002.00	1.00	53325.00	1002.00	1.00
37800.00	1002.00	1.00	39825.00	1002.00	1.00	54000.00	1002.00	1.00
38475.00	1002.00	1.00	40500.00	1002.00	1.00			
39150.00	1002.00	1.00	41175.00	1002.00	1.00			

39825.00	1002.00	1.00	41850.00	1002.00	1.00
40500.00	1002.00	1.00	42525.00	1002.00	1.00
41175.00	1002.00	1.00	43200.00	1002.00	1.00
41850.00	1002.00	1.00	43875.00	1002.00	1.00
42525.00	1002.00	1.00	44550.00	1002.00	1.00
43200.00	1002.00	1.00	45225.00	1002.00	1.00
43875.00	1002.00	1.00	45900.00	1002.00	1.00
44550.00	1002.00	1.00	46575.00	1002.00	1.00
45225.00	1002.00	1.00	47250.00	1002.00	1.00
45900.00	1002.00	1.00	47925.00	1002.00	1.00
46575.00	1002.00	1.00	48600.00	1002.00	1.00
47250.00	1002.00	1.00	49275.00	1002.00	1.00
47925.00	1002.00	1.00	49950.00	1002.00	1.00
48600.00	1002.00	1.00	50625.00	1002.00	1.00
49275.00	1002.00	1.00	51300.00	1002.00	1.00
49950.00	1002.00	1.00	51975.00	1002.00	1.00
50625.00	1002.00	1.00	52650.00	1002.00	1.00
51300.00	1002.00	1.00	53325.00	1002.00	1.00
51975.00	1002.00	1.00	54000.00	1002.00	1.00
52650.00	1002.00	1.00			
53325.00	1002.00	1.00			
54000.00	1002.00	1.00			