Analysing the Red Blood Cell Adhesion to the Dialysis Membrane Using the Flow Cell System: Analysis of the Polysulfone and polyethersulfone topography

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The work contained within this document has been submitted

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The research work contained in this thesis was conducted between October 2016 and September 2020. The laboratory experiment was conducted by the researcher assisted by two university research officers at Swansea University. This is an original work utilising university instrumentation; Peak Force Atomic Force Microscope (PF-AFM), Scanning Electron Microscope (SEM), peristaltic pump (for flow cell system), optical microscope, phosphate buffer solution (PBS) and bovine blood samples (from the local abattoir).

It is an original work and due references are made to related research investigations and experiments.

Signature……*Patrick Jolomba*

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Abstract

Background. Red blood cell (RBC) survival in chronic kidney disease (CKD) patients contributes to their anaemia. It has been suggested that the toxic uremic environment accounts for the decreased RBC life span in this group of patients (Vos et al., 2011). These patients are also treated with Haemodialysis (HD), which is argued to contribute to comorbidities such as anemia. The contribution of mechanical damage caused by the extracorporeal devices and the dialysis membranes to the shortened life span of the RBC is still unclear. However, the minimised percentage of the RBC of up to 70% in RBC survival has been reported in CKD patients undergoing Haemodialysis (Vos et al., 2011). To contribute to this field, this study focused on exploring the adhesiveness of the RBC to the dialysis membrane material. This scientific curiosity was triggered by the researcher observing that some dialysis membranes remained pinkish in colour following a dialysis session while others were not, despite rinsing these materials with the same volume of the dialysate solution, or 0.9% of sodium chloride. Currently, there are many different synthetic dialysis membranes in wide use that are made with some of the following polymers: polyethersulfone, polyacrylonitrile, polyamide, polysulfone and their copolymers. It should be noted that whilst these are all generally in use, it has been observed by the researcher that the most popular ones tend to be the polysulfone and the polyethersulfone, hence this study focusing primarily on these two.

Dialysis is a scientific procedure that is based on selective separation by diffusion of molecules across a semi-permeable membrane to separate molecules based on their size and weight. This scientific technique is used for a wide variety of applications such as blood purification, virus purification and water treatment. In blood purification, a buffer solution called the dialysate is placed on the opposite sides of a dialysis membrane which contains pores of a varying size range depending on the molecules to be separated. Molecules that are larger than the pores are retained on the inner side of the membrane, but small molecules pass through the membrane pores, reducing the concentration of those molecules (Hakim, Fearon and Lazarus, 1984).

Methods. The aim of this study is to investigate the adhesiveness of the red blood cells (RBC) to the polysulfone (PSU) and polyethersulfone (PESU) material used in Haemodialysis. A flow cell system that resembles the HD procedure was put together

for the RBC to flow on the PSU, PESU and the glass slide (control) over a period of three hours. At the end of the three-hour period, an optical microscope was used to count and assess the number of RBCs adhering to the surface of these materials. The surface topography of these materials were studied using the Peak Force Atomic Force microscope (PK-AFM), Scanning Electron Microscope (SEM) and the Goniometer, to investigate the surface roughness, similarities and dissimilarities between these membranes and wettability.

Results. The t-test was performed to compare adhesion results of the RBC to these materials. A Mann-Whitney nonparametric test was applied to compare the distributions of unmatched groups. A p-value of less than 0.05 was considered significant. Correlation was calculated with Spearman correlation coefficient and p-value ($P > 0.05$). The AFM and SEM affirmed and quantified that these membranes appeared to be different. They were both confirmed to be hydrophobic, while the glass (control) was hydrophilic. However, there was no obvious significant statistical difference between polysulfone membrane and polyethersulfone membrane adhesion to the RBC.

Conclusion. Despite lack of the significant statistical difference in the RBC adhesion between the PSU and PESU, there was a clear trend that the RBC adhered more to the rougher material (PESU) than the less rough (PSU). Hydrophobicity and hydrophilicity of the material did not seem to have an impact on the RBC adhering to the surface of these materials.

LIST OF ABBREVIATIONS

- 2D……. Two dimensional
- 3D……. Three dimensional
- AFM……. Atomic Force microscope
- ß 2-m……. Beta2 macroglobulin
- BICM…… Bioincompatible
- CRP…… C-reactive protein
- CAMP…… Cyclic Adenosine Monophosphate
- CD……. Cluster of differentiation
- CKD……... Chronic Kidney Disease
- CRP……... C-reactive protein
- CVD……...Cardiovascular disease
- CO…… Carbon monoxide
- ECM……. Extra cellular matrix
- EC……. Endothelial cells
- eGFR…… Estimated Glomeruli Filtration rate
- ESRD…… End stage renal disease
- FE SEM…… Field Emission Scanning Electron Microscope
- GO……. Graphene oxide
- GFR…… Glomeruli Filtration rate
- HA……... Hemadsorption
- HD……. Haemodialysis
- HDF……. Hemodiafiltration
- HF……. Hemofiltration
- Hb……. Haemoglobin (Hb)

- LED…….. Light Emitting Diode
- MHC……. Major histocompatibility complex
- OS……. Oxidative stress
- Pa……...Pascal
- PBS……... Phosphate buffer solution
- PD……. Peritoneal Dialysis
- PSU……. Polysulfone
- PES…… Polyethersulfone
- PESU……. Polyethersulfone
- PF-AFM……. Peak Force Atomic force microscope
- PKA…… Protein kinase
- PSU…… Polysulfone
- PVP…… polyvinylpyrrolidone
- QNM-AFM……. Quantitative Nanoscale Mechanical Atomic Force
- Ra…… Roughness average
- RBC…… Red blood cell
- RRT…….renal replacement therapy
- RMS…… Root mean Square
- RT…… Renal transplant
- SD……. Standard deviation
- SCFS…….. Single Cell Force Spectroscopy
- SC……...Sieving coefficient
- SEM…… Scanning Electron Microspore
- TMP…… Transmembrane pressure
- UF……... Ultrafiltration
- UFR…… Ultrafiltration rate

DEDICATION

I dedicate this research work to my father who with love and effort taught me to keep trying. Rest in peace father. I am still trying.

To my supervisor Ken, who has been my support throughout this difficult process, providing guidance and feedback after feedback throughout this work. Thank you for being a wonderful caring person.

To my wife, to my four girls, Warona, Angela, Alanah and Evie, to my friends, and to all those who believed I can do something worthwhile with my life: love and Unlimited gratitude.

To God Almighty for the strength and wisdom that enabled me to keep trying.

ACKNOWLEDGEMENT

This research is my greatest academic achievement posing to be the greatest academic challenge I have ever faced in my lifetime. My master's degree in this great institution remains the most informative session in my career. It has equipped me with vast insight and wide academic exposure. However, it was not a lonely walk as several people have helped me achieve this.

But my profound gratitude goes to my supervisor, Professor Kenith Meissner whose advice and perseverance encouraged me. Every meeting with him was a problem solved and further insight to more research opportunities. I feel privileged to have worked under his intense supervision and if I am given this same opportunity to work under you, I would gladly accept.

To everyone one else, God bless you all.

CHAPTER 1

1.1 GENERAL INTRODUCTION

The human body is a complex puzzle made up of a complicated series of processes working in coordination with all other bodily systems to maintain a healthy and thriving human being. These processes all happen in a controlled fashion. The kidneys are a component of the bodily system and their function is a key contributor to total solute and fluid removal from the body. They are the major excretory organ for elimination of metabolic wastes from the body. If they fail to function efficiently, dialysis treatment can be started prior to life-threatening complications occurring. Full or partial loss of kidney function results in variable deviations from the series of body processes. Dialysis is needed to continue the process of purification of waste from the body but contact of blood to dialysis membranes can also result in numerous unwanted interactions between the blood elements and the dialysis membrane. Because of this, multiple criteria for biocompatibility need to be understood in the classification of dialysers used in dialysis treatments. Dialysers are manufactured using materials such as polysulfone (PSU) and polyethersulfone (PESU). They are classified as bioincompatible (BICM) or biocompatible (BCM) because they elicit different biological responses when they come into contact with blood. A BCM dialyser material has traditionally been defined as "one that elicits the least amount of inflammatory response in patients exposed to it (Grooteman, et al.,2012). However, a dialysers can be classified as beneficial or deleterious depending on its biological effects on the body system. The properties of a dialyser on biocompatibility level, are said to be related to its microstructural and macrostructural characteristics, like the topography (Mustafa, 2016). Thus, this thesis sought to study the red blood cell (RBC) adherence to common dialyser materials of PSU and PESU in an *in vitro* experiment, to contribute to understanding the interaction(s) of BCM dialysis membranes in chronic kidney disease (CKD) patients.

This chapter will look at the definition of CKD, provide background information and overview on CKD and renal replacement therapy (RRT), including haemodialysis (HD) as a treatment modality. It will analyse the problem statement of this thesis, significance of the study, research questions, justifications of doing the work, and research objectives.

1.2 BACKGOUND

1.2.1 Definition of Chronic kidney disease

CKD is defined as an abnormality of kidney structure or function, present for more than three months, with implications on health (KDIGO, 2013). From this definition, it is clear to note that there are two main criteria for diagnosing CKD and only one is required to be present: abnormality of kidney structure or abnormality of kidney function. Either of the criterion shall be present for a duration of at least three months. Full criteria for diagnosing CKD is outlined in Table 1.1. CKD is a pathological condition with various heterogeneous symptoms reflecting kidney impairment (Kaderjakova, et al., 2012). In CKD, the function of the kidney will usually continue to decline (Lewis et al., 2012) and is classified into 5 stages (stage 1 – stage 5) depending on level of kidney function measured by glomerular filtration rate (GFR). Stage 5 CKD, with GFR equivalent or less than 10 mil/min/1.73 m^2, is also known as end-stage renal failure (ESRD). Because the kidneys cannot clear enough waste material such as urea, creatinine, phosphate or potassium, usually patients at this stage will require renal replacement therapy (RRT). Therefore, the GFR below this rate signifies severely reduced kidney function, and death can occur if the body is not assisted in clearing toxins and excess water. So, at this stage, patients are usually initiated onto RRT (Vos et al., 2011). GFR is therefore, an important parameter to assess kidney function. However as stated above, decrease in kidney function is not the only diagnostic criteria of CKD and therefore GFR is not the only parameter in diagnosing CKD. Abnormality of kidney structure can be identified by the presence of other parameters like albuminuria (the presence of albumin in the urine), electrolyte abnormality and other urine sediments.

Criteria for CKD (either of the following present for >3 months)	
Markers of kidney damage (one or more)	Albuminuria (AER >30 mg/24 hours; ACR >30 mg/g [>3mg/mmol]) Urine sediment abnormalities Electrolyte and other abnormalities due to tubular disorders Abnormalities detected by histology Structural abnormalities detected by imaging History of kidney transplantation
Decreased GFR	GFR <60 ml/min/1.73 m2 (GFR categories G3a–G5)

Table 1.1 Criteria for Chronic Kidney Disease as outlined in KDIGO Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease 2012

The asymptomatic nature of CKD, especially in its early stages, makes diagnosing CKD even more complicated. Many patients are likely to remain undiagnosed, and by the time they are being treated, more physiological damages would have occurred leading to physiological disturbances such as anaemia. CKD treatments such as HD though lifesaving and beneficial to some patients, also have negative contributory factors to the body's anatomy and physiology. This increases the probability of patients succumbing to various associated complications of CKD and its treatments. CKD and HD can cause major physiological dysfunction, especially for those at stage 5.

1.2.2 Overview of chronic kidney disease (CKD) and Red blood cell (RBC)

Responsible for up to two-thirds of the cases, the two main causes of CKD are diabetes and high blood pressure (Hörl and Hörl, 2002). There are three types of RRT; renal transplant (RT), HD and peritoneal dialysis (PD). HD is subdivided into two categories: HD and HDF. The difference between HD and HDF will be outlined in the next chapter. Although the morbidity and mortality of CKD is primarily due to cardiovascular disease (CVD), other factors such as materials used in RRT can also have a negative effect. However, CKD is also considered an independent risk factor for CVD events. Evidence suggests that the risk of developing CVD events increases as GFR decreases, independent of factors like age, sex, treatment modality, treatment

materials, and other risk factors (Tonelli, et al., 2006).Evidence also suggests that a CKD patient is more likely to die of a CVD event rather than from renal failure (Pálsson and Patel, 2015), regales of the treatment modality used. This work will however focus its work on HD related treatments.

HD is a procedure where a dialysis machine and a dialysis filter called a dialyser, are used to remove toxins from the blood. As aforementioned, treatment procedures such as HD, HDF and HF may induce other unforeseen clinical sequela and contribute to or exacerbate the development of certain physiological issues. This is because they use an artificial membrane (dialyser). For example, oxidative stress (OS) and dialyser reactions in patients treated with HD, HDF and HF have been identified as some of the most challenging endeavours in treating CKD patients (Ayli, et al., 2005). This is however not the case when it comes to PD and RT because dialysers are not used in these treatment modalities.

Red blood cells (RBCs) are the most common type of cell found in the blood, with each cubic millimetre of blood containing 4-6 million cells, they have a diameter of about 6 to 8μm (Anselmo, et al., 2013) RBCs are known to have a long *in vivo* survival (approximately 120 days) and a non-random removal from circulation. Therefore, they need to be healthy, with a physiological function unaltered by exposure to the dialysis membranes. The RBC lifespan is an objective index of its clearance that refers to the survival period of RBCs, which enter circulation after having matured in bone marrow (Luo, et al. 2018). RBC lifespan in CKD patients have been estimated by studying their carbon monoxide (CO) release and dividing the total CO release from RBCs by the daily CO release using Levitt formula as indicated below (Luo, et al., 2018):

Equation 1.1 $$RBC_{S_pan} = \frac{4[HB]\times 22400}{0{\cdot}7\times endoPco\times 64400\times 1440} \times \frac{v_b}{v_t}$$

In this equation, the numerator represents the total CO release from haemoglobin (Hb) within the body, and the multiplier of 4 indicates that 1 mol of Hb produces 4 mol of CO upon degradation. '[HB]' is the HB concentration (g/ml), 22,400 is the standardized molar volume (ml), and v_b the blood volume of the body (ml). endoPco is the alveolar

CO concentration and v_t is the volume of resting alveolar ventilation. The denominator represents the daily CO release, where the 0.7 multiplier approximates the ratio of endogenous CO produced by haemoglobin (Luo et al., 2018).

Pertinent to CKD patients on maintenance HD, studies have also suggested that long term HD may reduce the average RBC survival from 120 days because of issues such as compression and twisting of HD extracorporeal circuits and prolonged exposure to filtering membranes (Lequie,r et al., 2013; Stookey, et al., 2013). Stookey, et al. observed the immediate increase in dead RBC debris after dialysis treatment and particularly a notable reduction in the RBC life span post-HD, compared with RBC lifespan at the start of the maintenance of HD treatments (Stookey, et al., 2013). In view of these observations, there is a worrying likelihood that HD procedures may aggravate ESRD-associated reduction of RBCs, anaemia, or simply alter the physiology of the RBCs in patients undergoing HD treatment. Many factors regarding the RBC interaction with the dialysis membranes are still not known and have not been studied.

1.3 PROBLEM STATEMENTS

The HD membrane, and all other components of the extracorporeal circuit and their ability to stimulate biological responses through activation of the complement system (Ronco, et al., 2018), increases the production of inflammatory biomarkers' in HD patients such as cytokines. Cytokines are a targeted inflammatory factor in CKD/ESRD patients on dialysis (Purnell, et al.,.2013). Examples of these cytokines are; tumor necrosis factor-α (TNF-α), interleukin (IL-) 6, and IL-2 as well chemokines such as IL-8 (Lime, et al., 2013). Dysregulation of any component of these inflammatory factors can affect the entire balance, resulting in a wide range of illnesses that may result in variable degrees of inflammation (Foley and Conway, 2016) in CKD patients on HD. Even though anti-inflammatory and modulatory cytokines can also be produced as an attempt to control this process (Han and Boisvert, 2015), it is important for HD patients to use HD membranes that will not hugely contribute to this problem.

1.3.1 Biocompatible dialysers

Several researchers have stated that a biocompatible and efficient dialysis membrane needs to fulfil at least two fundamental necessities. Firstly, the design and structure (defined in terms of the pore size, selectiveness and distribution of the pores on the separating layer of the membrane) must be such that uremic molecules of a defined size and weight range are selectively removed, whilst leaving behind molecules that are essential for the body (Ahrenholz, et al.,2004; Rao, et al., 2004; Asano, et al., 2019). Secondly, and perhaps most importantly, the chemical and physical properties of the plasma and RBCs in contact with parts of any membrane (regardless of the material) must be such that minimal blood-membrane interactions take place. This could either affect the functionality of the dialysis membrane material, cause other clinical issues, contribute to the development of other comorbidities, or lead to an unexplained elevation of inflammatory biomarkers such as C-reactive protein (CRP) and IL-6, or simply cause adverse or anaphylactic reactions for the patient (Tagaya et al., 2017).

The most common cause of inflammation in HD patients is unexplained, and multifactorial in nature (Tattersall, et al. 2013). Impact of stepwise sodium and ultra-filtration profiles and dialysis solution flow rate profile on dialysis adequacy. Iranian journal of nursing and midwifery research, 19(5), 537–541.). Inflammation in CKD patients may directly result in cardiac-related issues. Cachofeiro postulated that the underlying cardiovascular malfunction or complication in these groups of patients may be caused by an inflammatory response (Cachofeiro, 2008). Inflammatory processes in dialysis patients may have an array of different causes: inter alia, vascular access lines, graft or fistula problems such as infections, BICM dialysis membrane, lack of ultra-pure water, and exposure to endotoxins and bacteria (Ramón et al., 2018). Other causes of inflammation in CKD are associated with varied factors such infections, oxidative stress, obesity, and genetic or immunologic factors, HD-related factors mainly dependent on the membrane biocompatibility, water treatment and dialysate endotoxins status (Kerr, et al., 2007). A better understanding of the causes of inflammation in kidney patients and their prevention or treatment will contribute to improving the state of HD patients and, possibly, their quality of lives and mortality.

In this thesis, the researcher focuses his investigation on analysing the adhesion interaction of the RBCs and HD membranes. The researcher has observed that, in some instances, after a dialysis treatment session, some HD/HDF membranes remained more stained with RBCs. This is demonstrated by membranes being pinkish in colour following a rinse with dialysate fluid at the end the treatment Figure 1.1 while others remain whiter than pink Figure 1.2.

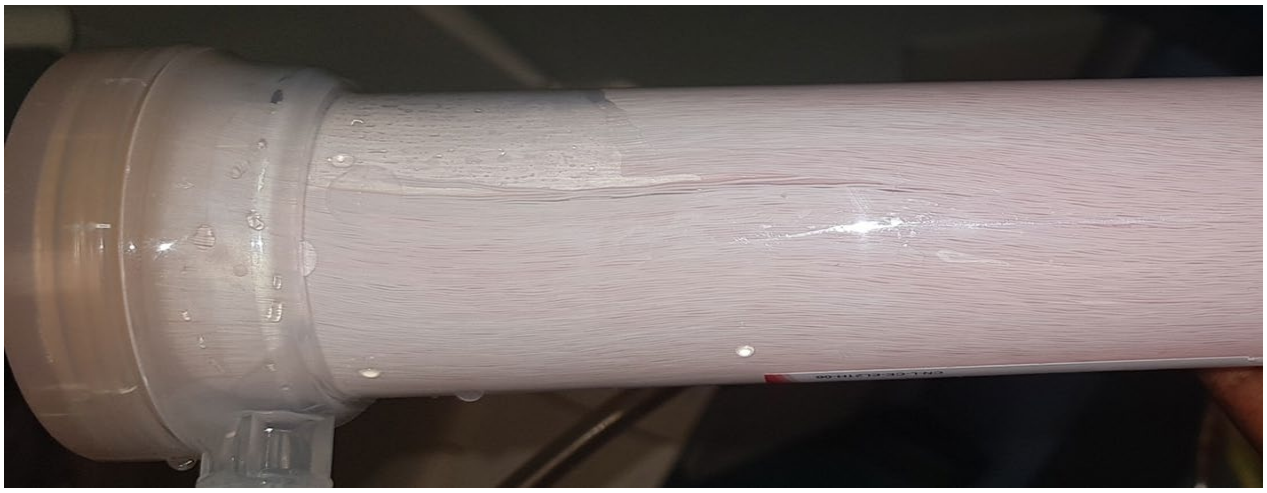

Figure 1.1 Polynephron membrane (second generation high – flux polyethersulfone (PESU)membrane) picture taken following a dialysis session and rinsing with 360mls of dialysate fluid.

Figure 1.2 Polysulfone (PSU) high-flux dialysis membrane, following a dialysis session and a rinse with 360mls of dialysate fluid.

The RBCs remaining in the membranes following a rinse with dialysate are a part of the residual blood volume in the dialysis circuit. However, there are different factors that can potentially influence the level of the residual blood volume in the dialyser and the rest of the extracorporeal circuit used in HD. These factors include the strength of the anticoagulation used, the surface area of the dialyser as well as the rheology, the viscosity, or the haematocrit of the patient's blood. The estimated quantity of total residual blood left in the extracorporeal circuit after rinsing tends to range from 0.01 to 23.9 ml of blood (Otti, et al., 2001, Kalocheretis, et al*.,* 2003). Kalocheretis, et al. have also claimed that there is a link between net ultrafiltration (fluid removal) and residual blood volume (Kalocheretis, et al. 2003). Despite this knowledge, there no studies about RBC adherence to the dialysis membranes.

Based on the observation of these two pictures (Figures 1.1 and 1.2), it could be hypothesised that RBCs attach more to certain dialysis membranes depending on various factors, such as the blood pump speed and the nature of the membrane morphology. Steiner et al. observed in their study that RBCs attach to surfaces and develop tethers if exposed to shear stress above 0.2 Pa (Steiner, et al.,2010). Lima et al. found in their study that PESU was rougher compared to PSU, and they concluded that this property characteristically facilitates RBC adhesion to the test membrane (Lima, et al., 2013). Surface modification of medical polymers can improve biocompatibility and enhance a clinically conducive environment. For example, understanding material surface features such as roughness is key to improving biocompatibility in blood contacting biomaterials. This is supported by Whitehead *et al.* who propose that there is higher adherence of biological cells to solid surfaces with a higher roughness average (Ra) values, showing an increased adhesion to rougher surfaces (Whitehead, et al.,2005). In order to study and understand the above-mentioned membrane discolorations following a dialysis session, an *in vitro* model that allows analysis under controllable conditions such as blood flow and dialysis time as well as eliminating disturbing factors related to flow, was carried out in the research work.

This study will use Peak Force AFM (PK-AFM) to first analyse the membrane materials used in the thesis. Studying various aspects of their topography including (but not limited to) the roughness and the force curve, Similarly, Cavalcanti-Adam et al. conducted a study concerning the effect of roughness on adhesion using AFM. Their

study provided a better understanding of the effect of roughness on adhesion when working on a micro- and nano-scale level (Cavalcanti-Adam, et al., 2008*)*. This is similar to this study, which shows that the interaction between the RBC and the polymeric dialysis membranes will be analysed at a micro/nanoscale level. At this very small scale, the effects of adhesion are significant in understanding the interaction between two surfaces. The RBC and the dialysis membrane's adhesion phenomenon need to be understood better by focusing on nanomolecular behaviour and the forces involved as they interact. The adhesion characteristic of the PSU and PESU was also studied in this thesis to analyse the membrane materials. While using the AFM to analyse this, the total adhesion force created by the contribution of all molecules involved in the process of adhesion can be described by the equation below (Bowen, et al., 1998).

$$F = 2\pi\omega R\left[\frac{R_q}{R + R_q} + \left[\frac{h_c}{h_c + Rq}\right]^2\right]$$

Where: R = tip radius; R_q= RMS of roughness; h_c = distance separating the tip/sample, and 2πωR represents the strength of the AFM system. The total force is normalized by the surface energy so that ω is the work of adhesion force. The adhesion force is associated with increasing surface roughness and increasing radius of the tip used in AFM.

1.4 Study Objective

Driven by the researcher's curiosity of the different colours of the membranes (Pictures 1 and 2), the objective of this study is to explore and analyse the possible adhesion of the RBC to the PSU and PESU dialysis membranes. It should be stated that this study used a flat sheet membrane material of both PSU and PESU. These are slightly different from the cylindrical membranes fibres used in a clinical setting in the sense that (a) Membranes used for dialysis are normally coated with a hydrophilizing agent such as polyvinylpyrrolidone (PVP), and (b) dialysis membranes are manufactured through nano-spinning technology to create hollow fibres. Additionally, in the

manufacturing process, HD dialysis membranes are sterilised through a variety of methods such as ethylene oxide, steam, electron beam and autoclave (Daugirdas and Bernardo, 2012). It should be noted that a few studies have been published in which a method of sterilisation may affect the structure of the membrane topography (Kiaii, et al., 2011; Müller, 1998). This may render membranes slightly different from the flat sheet membrane material used in the thesis.

Many scientific and commercial entities are researching and producing biomaterials for the benefit of patients. On the other hand, sales and marketing departments within these fields aggressively campaign for their products to be recognized as “the products of choice” within the health care industry for commercial gain and clinical benefit (Allard, et al., 2013). This can be overwhelming for clinicians and patients alike to make a choice based on the data presented, regardless of the regulatory compliance and demand put on these commercial companies. Infiltration of information and robust marketing campaigns by manufacturers provide constantly conflicting data about scientific studies and information which further adds a huge cloud of confusion to patients and clinicians. To emphasize this point, Potier, et al. carried out a study that looked at the clearance of the uremic toxins using PSU dialyser. Their study confirmed that effective solute body clearances achieved *in vivo* in HD are significantly lower than instantaneous clearances reported by manufacturers of that dialyser (Potier, et al. 2017*)*. This is a clear example of differing information presented by product owners as opposed to independent academic work. More independent studies on dialyser material and blood interaction may overcome these predicaments in part.

1.4.1 Objective limiting factors

HD membranes are generally decorated or hydrophilized by blending in PVP to enhance their biocompatibility (Hayama, et al., 2004). Studies have shown that in order to design a biocompatible dialysis membrane with a satisfactory capacity to remove uremic toxins and cause minimal invasiveness, hydrophilic agents such as PVP are added in order to suppress plasma protein absorption by an otherwise hydrophobic polymeric membrane such as PESU and PSU (Locatelli, et al., 2009; Liao et al., 2005). Hydrophilizing agents are also affective for inhibiting platelet adhesion

and reducing surface hardness of the membrane (Wolff, 2004; Hayama, et al., 2004; Hoenich, et al., 2000).

The study conducted in this thesis is an *in vitro* study; *in vitro* studies are not always a true representation of *in vivo* conditions. In HD treatment, during the priming of the extracorporeal circuit and the dialyser, the surface of the dialyser may be altered by additives to the priming fluid (heparin in saline and dialysate), and plasma proteins may be adsorbed on the surface of the dialysis membrane as well. These phenomena do not exist in this *in vitro* investigation. Also, Bildyukevich, et al. used scanning electron microscopy (SEM) and the atomic force microscopy AFM to reveal the difference in the structure of the modified and unmodified membranes. Their results showed that the introduction of the PVP led to some changes in membrane water permeability and rejection (Bildyukevich, et al.,2017*).* There is no known documentation on the effects of the PVP on the RBCs. The introduction of the hydrophilizing agents is only a small percentage and does not greatly alter the structure of the PSU and PESU membranes, making this work relevant to clinical dialysis.

1.5 Dialysis as renal replacement therapy (RRT)

Dialysis is a procedure used to remove waste products and excess fluid from the blood when the kidneys lose function. It involves diverting blood via a machine to a dialyser to be cleaned. Figure 1.3 below illustrates how dialysis works, showing different parts of the system, including the dialyser. It can be inferred from Figure 1.1 that dialysis is a technically demanding procedure that requires an extensive array of sophisticated understanding of equipment as well as specifically trained and dedicated staff to perform, monitor, and ensure the integrity and safety of the procedure in these patients (Elliott, 2000).

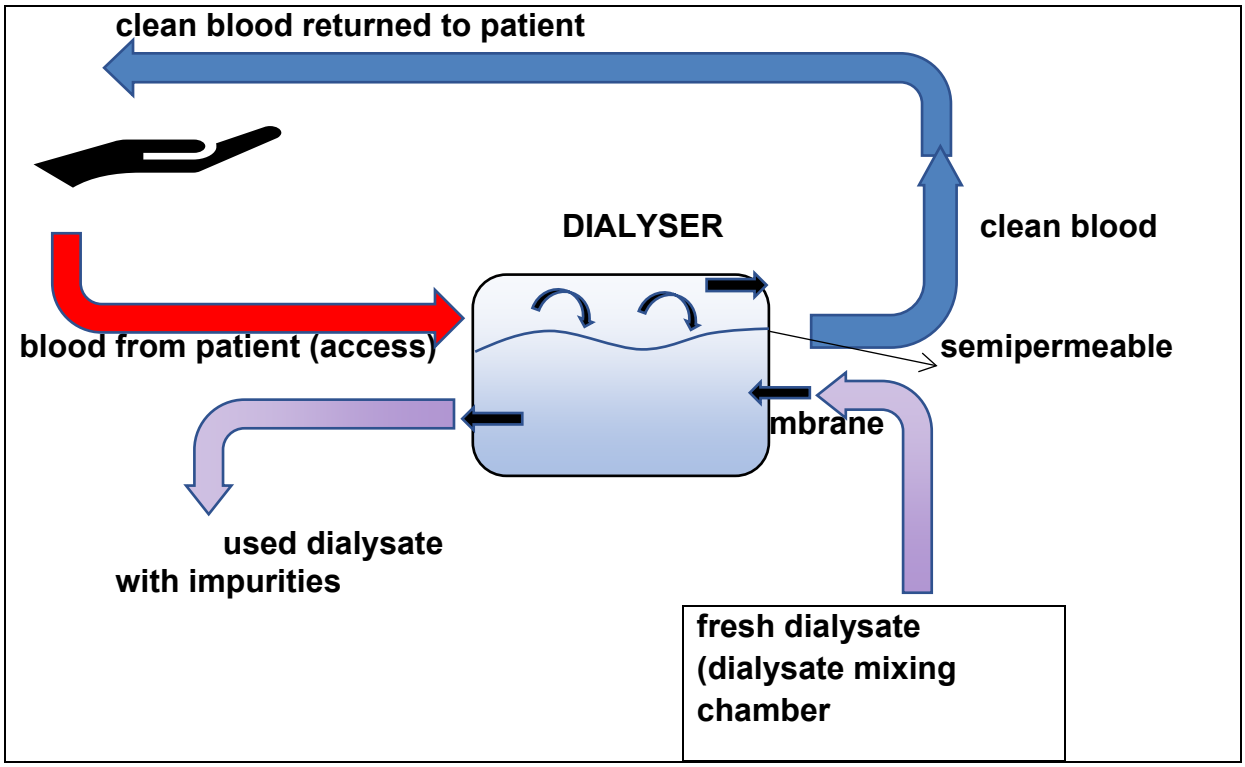

Figure 1.3 Schematics of the dialysis process. The top blue arrow facing left is dialyzed blood returning to the patient. The hand symbol below that is the access point (where blood will be withdrawn and returned) and the red arrow is the blood from the access point to the dialyser/machine. The dialyser is the represented by the square in the middle, with the dialysate fluid entering the dialyser and exiting via the two bottom grey arrows.

A well-functioning access to the bloodstream is a prerequisite in order to have enough blood to be dialyzed in a given treatment time (Elliott, 2000). Good dialysis blood flow must include an easily connectable external HD blood circuit capable of delivering blood flows at a sufficient rate required for a good dialysis session (Sigley, et al., 1979). Blood is taken from a fistula and passed through a peristaltic pump to induce enough pressure (typically 200–400 mm Hg) required to drive water across an exchanger into a fluid called the dialysate. HD machines employ a proportioning system that mixes an acid concentrate with a bicarbonate concentrate and purified water to produce fresh dialysate as in figure 1.3 above. This allows for the generation of a dialysate with a physiologic pH and minimizes the possibility of forming a precipitate between bicarbonate containing alkaline solutions and calcium (Hootkins, 2011). The acid

concentrate contains dextrose and is the source of electrolytes including potassium, calcium, magnesium, and acetic (or citric) acid. The bicarbonate concentrate may contain sodium chloride as well as sodium bicarbonate (36.83) or may contain only sodium bicarbonate (35/45). Dialysate fluid is therefore a nonsterile aqueous electrolyte solution that is similar to the normal levels of electrolytes found in blood with the exception of the buffer bicarbonate and potassium (Pittard, 2017*)*. Dialysate solution is almost an isotonic solution, with the usual osmolality of approximately 300 ± 20 milliosmoles per liter (mOsm/L). To ensure patient safety and prevent RBC destruction, the osmolality of dialysate must be close to the osmolality of plasma (Pittard, 2017).

Anticoagulation medicine is usually administered depending on other co-morbidities the patient may have. The dialysis circuit is also designed as a close circuit without allowing air to get into the system. In modern machines, the bubble catcher is usually an ultrasonic sensor that is connected on the venous (blood returning) as indicated in the above schematic.

1.5.1 Biocompatible dialysers

Dialysers must possess certain requirements, such as bacterial resistance, must be anti-allergenic and non-toxic, have good breathability, and possess the ability to withstand different types of sterilization (Rajendran, et al., 2016). Despite these requirements, unwanted effects such as HD membranes clogging during treatment is not uncommon and that can lead to extracorporeal blood loss (James et al., 2013). So, these devices are not perfect, and more work needs to be done to improve them.

The dialyser is made of material that allows controllable transfer of solutes and water across the semipermeable membrane. In HD the of flows dialysate and blood are separated by a semipermeable membrane (Figure 1.1). Most dialysers have a membrane surface area of about 0.8 to 2.1 m^2 (Chon, et al., 2020). A dialyser has four ports, one inlet and one outlet port each for blood and dialysate. The semipermeable dialysis membrane separates the blood compartment and the dialysate compartment. The transport processes across the membrane are diffusion (dialysis) and convection (ultrafiltration). The composition and the thickness of the membrane varies

considerably and is often more important than the surface area in determining dialyser efficiency (Chon,et al., 2020).

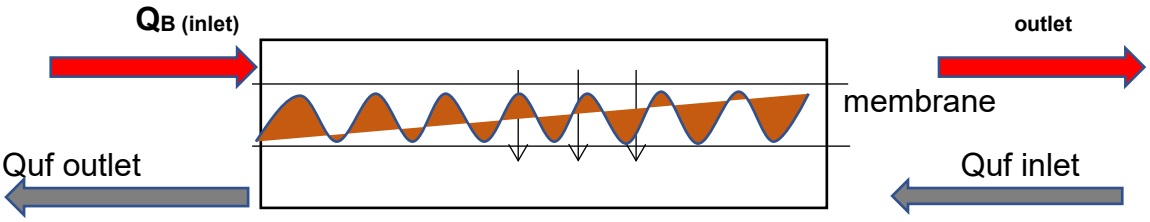

Figure 1.4 Schematic diagram of solute movement in a high-flux membrane. The fresh dialysate (Quf) flows towards the dialyser and the contaminated/used dialysate comes away from the dialyser into the "spent dialysate" section of the dialysate compartment. Qb is the blood flow.

1.5.2 Dialyser and RBC adhesion

A dialyser can be classified based on properties of the chemical composition of its membrane or based on its properties of solute removal and solvent permeability. As blood passes through the dialyser, blood cells may adhere to the membrane's inner surface (Hootikins, 2011). In general, cell adhesion is a complex process regulated by the involvement of the cytoskeleton as well as several surface proteins (Zhu et al., 2016). Adhesion is also regulated by complex extracellular and intracellular signals that may differ from one cell type to another and from a cell to non-biological material (Parsons et al., 2010). However, it is known that lack of nuclear and mitochondrial material on the RBCs make them inactive in general, but they are known to express surface adhesion receptors (Oberleithner, et al., 2015). This is important for this study because these receptors might be involved in the RBC dialyser membrane adhesion. The focus of this thesis is on the observing of the RBCs' adhesion to the dialysis membranes.

1.6 Justification and significance of the study

1.6.1 Justification

It is important to investigate and understand the interaction of RBCs with dialysis membranes since RBC integrity and life span is important for managing anaemia in CKD and ESRD. RBC also plays an important role in the maintenance of systemic and local antioxidant defence system, as they are the first line of defence during contact of the blood with the dialysis membrane. The effect of damage to the RBC appears in many forms, including reduced osmotic resistance of the cellular membrane, susceptibility to disintegration, and this can contribute to a decline in RBC lifespan (Cachofeiro, et al., 2008). However, this is beyond the scope of this work and the researcher will limit his focus to the significance as outlined below.

1.6.2 Significance of the study

ESRD is the final common clinical pathway of several kidney diseases (Thongprayoon, et al., 2015). With HD being the preferred treatment modality in ESRD, RBC interaction with the membrane material used for this treatment therapy is important. It should however be recognised that with renal failure of any aetiology, there are many other physiological derangements, such as, homeostasis of water and minerals (Dessi, et al. 2014). These physiological instabilities also contribute to the overall diseases process, so it is not just the membrane biocompatibility that needs to be scrutinised. Also, toxic end-products of nitrogen metabolism (urea, creatinine, uric acid, among others) that accumulate in blood and tissue can have an impact on how the dialysis membrane functions. Finally, the kidneys are no longer able to function as endocrine organs in the production of RBCs' (Ismail, et al., 2019) which implies that the RBC's physiology is already impaired even before the initiation of dialysis. The ability of a dialysis material to function *in vivo* without eliciting detrimental local or systemic responses in the body is very important. Much of the research into new biomaterials is focused on improving biocompatibility of these materials to assist in avoiding unnecessary complications

Chapter 2 Literature Review

This chapter will provide a detailed description on research background and an assessment of current situation about the research topic. It will cover literature review on RBC interaction with HD membranes, look at and analyse previous studies in this area. It will also have sections on general overview of HD and HDF and subsequently focus on the scientific principles behind these treatment modalities (diffusion, convection and ultrafiltration). Finally, there will also be an exploration of the background of PSU and PESU polymers, dialysers, RBC and adhesion process. Based on this literature review, a research gap is identified and is described, together with the conceptual framework of this research, in the later part of this chapter.

2.1 Red Blood Cell (RBC) interactions with Polymeric Membranes

The interaction of all blood cells with polymeric material and other biomaterial surfaces is an issue crucial for HD. A few studies have shown that adhesion of different blood components to the HD membrane surfaces may lead to inauspicious effects such as clotting and thrombosis (Lipowsky, et al. 1995; Evans, et al. 1980; Bessis, 1973). The reaction of RBCs to the polymeric surfaces is a factor of prime importance in the search for biocompatible blood-contacting HD materials such as PESU and PSU. Voinova, et al. confirmed that the intricate progress involved in RBC material surface contact result in different morphology of RBCs which has been observed microscopically (Voinova, et al., 2019).

2.1.1 Previous work

There are not many studies that have focused on the RBC adhesion to HD membranes. However, one of the latest studies on RBC and HD was by Luo, et al. in 2018 whereby they used CO breath test to investigate HD effects on RBC lifespan in patients with CKD. In their study a cohort of 17 men with CKD undergoing HD via

PSU membrane were subjected to a repeated breath test (Levitt's, CO breath) to compare RBC lifespan before and after HD. They concluded that using PSU membrane did not appear to disrupt RBC nor reduce their lifespan in patients with

CKD (Luo, et al., 2018). However, literature on this topic seems to have a few case studies and a smaller number of higher-level publications. It will be helpful if future polymeric membrane and RBC interaction research would use higher-level research designs, such as randomized controlled trials where possible. Another slightly older study in 2001 by Otti, et al. looked at RBC loss in CKD patients on HD and demonstrated that HD using a PSU membrane had a minimal effect on RBCs survival or anaemia in patients with CKD on HD (Otti, et al., 2001). Their study however did not specifically analyse the RBC HD membranes adhesion, but it demonstrated for the first time that the total RBC loss per HD session was minimal in chronic HD patients (Otti, et al., 2001).

Unlike Otti et al. who only focused on RBC loss per HD session, study by Sato, et al. in 2012 looked at the cause of the reduced RBCs in HD patients in general. Their investigation confirmed that one of the most important complications of renal anaemia is reduced RBC lifespan (Sato, et al. 2012). However, there still has been little research conducted into the causes of and treatments for this anaemia and the role the polymeric HD membranes play in this area. The Sato et al. study measured alveolar CO and then estimated RBC lifespan in patients on HD (Sato, et al. 2012). The RBC interaction with the membrane is not considered in this study, thereby creating a gap in knowledge. The underlying challenges associated with the lack of evidence-based knowledge on HD' materials' interaction with RBC persist in this field, including the accurate comprehension of reaction of RBC morphology and the related clinical outcomes.

Very often HD patients present with anaemia caused by different factors. It is known that erythropoietin (glycoprotein cytokine secreted mainly by the kidney in response to cellular hypoxia) deficiency, decrease in RBC survival, decreased response of marrow precursor cells to erythropoiesis signals, and iron deficiency are among the causes (Brugnara, 2003). A review of literature points mainly to uraemia as a key factor in the short lifespan of RBCs of HD patients, possibly secondary to an increase in osmotic and mechanical fragility of RBCs. However, the interaction of the RBCs with dialysers is still unclear. The fact remains that the cause of this detrimental discount in RBC is largely unknown and therefore studies seeking to understand interaction with polymeric material will add value to the existing knowledge.

An interesting investigation was conducted whereby blood from uremic donors was transfused into healthy recipients. This investigation resulted in normal RBCs survival, implying that the uremic status of patients with CKD is the underlying cause of this phenomenon (Ly, et al.,2004). However, these healthy patients were obviously not on HD and, therefore, their blood was not exposed to dialysis membranes. This study does not rule out the involvement of the dialysers; it merely focuses on uraemia alone and imply that it is the single cause of the problem. It is clear that a more multifaceted study approach is needed. Another point to consider is that stage 5 CKD patients are said to be 16.8 times more likely to develop anaemia as compared to patients with stage 1 and 2 CKD (McClellan, 2004). Stage 5 CKD patients are also mainly treated with HD which uses polymeric membranes, which possibly contributes to this 16.8 increment in RBC depletion.

The behaviour of HD membranes varies greatly, and some are more biocompatible than others (Tagaya et al., 2017). Most research has focused more on the complement activation during dialysis and less on the RBC interaction with the dialysis membrane. This is supported by Hakim, et al., in their work that affirmed that many investigative efforts seem to have centred mainly around leukopenia, complement activation, neutrophil function and peripheral blood mononuclear cells due to their well-known effects on long term clinical issues and contributory factors on the development of other comorbidities (Hakim, et al., 1984*)*. However, Hakim, et al. also confirms that not much is documented on the interaction of dialysis materials with the RBCs (Hakim, et al., 1984). One of the reasons contributing to lack of enough evidence on the interaction of tissues with dialysis membrane is that *in vivo* biocompatibility testing of membranes is at times impractical in daily clinical practice. So, clinicians rely on the manufactures' guide and scientific/clinical papers that are based on *in vitro* work. To date, water flux (the passage of water through a membrane) remains the most viable way to assess the effectiveness of the dialysis membrane during treatment (Jean, et al., 2015). Aoyagi, et al. reiterated this by saying that water flux is a benchmark used to assess the effectiveness of the dialysis membrane during treatment, and it is often evaluated to determine whether membrane fouling has occurred (Aoyagi, et al., 2017).

Other studies looked at a technique for measuring the adhesion between a single RBC attached to an AFM cantilever and a surface coated with purified proteins, and RBC adhesion to other biological cells (Maciaszek et al., 2014; Oberleithner et al., 2015).

In their study, Maciaszek et al. noted some fundamental observations. Firstly, that an increase in the overall cell adhesion measured using Single Cell Force Spectroscopy (SCFS) correlated with an increase in the resultant force measured on 1 μm²-areas of the RBC's membrane. Secondly, the study also demonstrated that SCFS can detect notable changes in the adhesive reaction of the RBCs to the modulation of the cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) pathway (Maciaszek, et al., 2014). From the results of their studies, the RBCs were shown to adhere to biological and non-biological cells (Maciaszek, et al., 2014). Unlike Maciaszek, et al. Oberleithner, et al. study looked at RBC attachment to biological cells and concluded that negative charges on the RBC reduce adhesion between RBC and endothelial cells (EC). Their study further states that, ambient Na+ concentration determines the availability of free negative charges, but Na+ concentrations in the low physiological range (below 140 mM) allow sufficient amounts of vacant negative charges so that adhesion of RBC to the endothelial surface is small (Oberleithner, et al., 2015). So, for patients on regular dialysis therapy, there is regular and constant interaction between the blood cells, the needles, the extracorporeal circuit and the dialysers used. These products are made from different synthetic materials, mainly of polymeric origin (Hoenich, et al., 2010). Though RBCs are in constant contact with these materials, the dialysis membrane has a much bigger surface area, so there is more RBC contact with the membrane than with the other parts of the extracorporeal circuit.

Finally, the authors of a recent study found RBCs from patients on HD were significantly more adhesive than those from healthy controls (Derebail, 2008). They detected clear RBC adhesion to T-cells, platelets and also noted significant RBC adhesion to neutrophils. It is therefore clear that RBC of CKD patients on HD treatment are more adhesive but the degree of adhesion to the dialysis membranes is unclear.

The researcher in this work believes that additional studies should be conducted to assist in this area and investigate different components of blood biocompatibility to HD membranes and its clinical significance.

2.1.2 Reaction of RBCs in Contact with HD membranes

A review of literature also revealed a previous study that looked at RBCs brought in contact with polymeric membrane surfaces of various structure and geometry, in particular, with polymer HD membranes (Grzhibovskis, et al., 2017). Their study

concluded that RBC shape could be different if adsorbed on the inner or outer surfaces of the HD membranes (Grzhibovskis, et al., 2017). Voinova recapped that surface topography and energy of the inner (blood side) and outer (dialysate side) surface can differ due to the variations in the surface density of polymer (Voinova, 2019). The observation of irregularity in surface properties of polymer outer and inner surfaces is confirmed by a recent investigational work where SEM analysis of both surfaces of the HD membranes pores has exhibited different ultrastructure (Hedayat, et al. 2012). This is clearly manifested on the dialysate side of the membrane, as tangles of intricate channels and holes in the spongy area under the skin layer, which are, as concluded, not connected. There is a need to investigate further and gather more insight into this.

Another study by Van Buren, et al. acknowledged that RBC damage is an unavoidable side effect of extracorporeal circulation and membrane contact (Van Bauren, et al., 2016). The effects of RBC damage on patients on HD still need to be explored. One study compared long nocturnal HD with regular 4-hour, three times per week HD treatment. That study concluded that the influence of RBC damage caused by membrane contact and other part of the extracorporeal circuit is minimal compared with biochemical effects (Aoyagi, et al.,.2017). Perhaps this risk can be further reduced by manufactures' quality control and better scientific understanding of the dialysis material. This thesis also highlights that, adverse effects caused by RBC contact with polymeric membranes need to be analysed.

2.2 Haemodialysis (HD) and hemodiafiltration (HDF)

As already mentioned in Chapter 1 of this thesis, there are different treatments for CKD, depending on the stage of the disease. The main categories are RT, PD and HD/HDF. HD and HDF are treatment modalities use a dialyser as a core treatment material. Hence, the reason for this work to focus them. HD is an umbrella term for dialysis but, in practicality, HD is a modality that uses mainly diffusion with minimal convective current involved (Jean, et al., 2015). Whereas, HDF uses both diffusion and convection to achieve treatment goals (Venkataraman, et al., 2003). The effective diffusion coefficient in these treatment modalities are determined by the effective concentration difference across the dialysis membrane (Hu, et al., 2018). The diffusion coefficient is the proportionality between flux and concentration gradient (Baur, 2007).

This principle is derived from Fick's first law of diffusion, which postulates that the flux moves from the region of high concentration to a region of low concentration and diffusion, therefore, becoming the basic principle underpinning these treatments (Perlman, 2019).

HD and HDF modalities use an extracorporeal circuit during the dialysis procedure, this circuit including the dialyser facilitates the passage of blood to and from the patient forming an integral link regarding safety, treatment effectiveness, and molecular clearances (Hoenich, 2007). In the past, most parts of the extracorporeal circuit were subject to problems such as break-up or separation of the material used, causing fragments to be introduced by the blood pump and plasticiser, either from the extracorporeal-circuit material leaching or leaking into the blood. Recently, most of these problems have been largely dealt with by constant scientific improvements on materials used by focusing on the importance of the biocompatibility (Pascual, et al., 1997).

In both HD and HDF, blood is removed from the patient through the extracorporeal circuit consisting of a tubing set connecting the patient's vascular access to the peristaltic machine as in Figure 2.1 Also, in both treatment modalities, a dialyser is connected to the blood lines. However, in HDF, an additional tubing/line segment from the machine is connected to the circuit either before or immediately after the dialyser to allow for the infusion of "replacement fluid" that will cause the convection process within the dialyser. This causes convective removal, a process driven by concentration gradient within the dialyser. This convective removal is based on fluid flow through a membrane and is driven by the pressure difference (known as hydrostatic pressure) between the two sides of the membrane. For example, a higher pressure in the blood compartment or a lower pressure on the dialysate side will force water to squeeze across the membrane from a higher pressure to a lower pressure.

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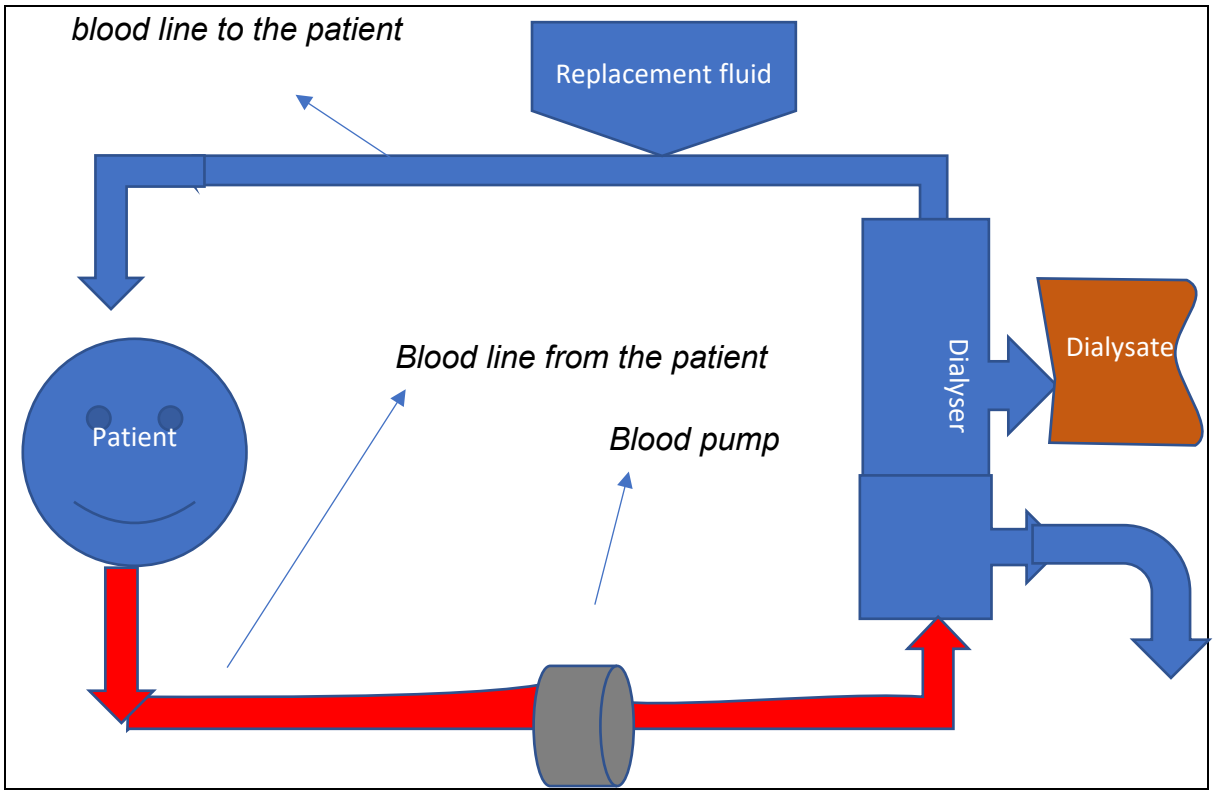

Figure 2.1 Diagram showing replacement fluid for HDF treatment connected after the dialyser. The bottom red part of the diagram is the blood from the patient withdrawn via the peristaltic pump (Blood pump). Blood get into the dialyser (Blue left rectangular shape). The brown part is the dialysate fluid. The blue top part is the blood going back to the patient, and the replacement fluid (applicable for HDF only) will be infused before the blood is returned.

The dialyser is where diffusion and convection will take place. The membrane material in the dialyser is where the blood is physically filtered, and small unwanted molecules/solutes (filtrates) such as urea, creatinine, potassium and middle molecules e.g. Beta 2-microglobulin protein (ß2-m) are lost to the dialysate (Tattersall, et al., 2013). HDF procedure and process is clarified in the Table 2.1 below.

HDF Type	Procedure/process
Post-dilution	Ultrafiltration followed by infusion of replacement fluid
Pre-dilution	Infusion of replacement fluid followed by ultrafiltration
Mid-dilution	Infusion of replacement fluid at the mid-point of ultrafiltration (post-dilution followed by pre-dilution)
Mixed-dilution	Infusion of replacement fluid before and after ultrafiltration (pre-dilution followed by post-dilution)

Table 2.1 HDF treatments modalities (Tattersall, et al., 2013). This table outlines the two different forms of HDF (pre and post dilution) and how they are carried out.

The main short-term and long-term advantages of HDF are supposedly better removal of ß2-m, phosphate, and better haemodynamic stability (Jean *et al.,* 2015). Jean *et al.* also noted that there are minimal elements of convection current in HD created by temperature difference between the blood and the dialysate, and counterflow of the dialysate.

2.2.1 Convective Clearance

Convection in HDF is the movement of molecules/solutes through a semipermeable membrane (within the dialyser) associated with the fluid being removed during ultrafiltration (Swinford, et al., 1997). Convective clearance has also been referred to as the movement of solutes out of the blood compartment of the dialyser, along with the movement of water (Locatelli, et al., 2009). This takes place irrespective of the molecular size, as unwanted materials are dragged along with ultrafiltrate or water across the dialysis membrane at the same rate (Locatelli, et al., 2009).

Convective transport in dialysis consists of solutes passively following a fluid flow of ultrafiltration across a highly permeable membrane commonly referred to as “solvent drag.” TMP is essential for that convection to happen; TMP determines the rate of ultrafiltration (Ledebo, 2010). Treatment modalities involving convection are believed to be more effective in counteracting the middle-size molecules such as ß2-m. Both PSU and PESU are used in HDF and HD, but high flux materials (membrane with bigger pore sizes) are used to allow for excessive fluid movement. The membrane pore size is directly related to sieving coefficient (a measure of equilibration between the concentrations of two mass transfer streams) in HDF. To define sieving coefficient, one might take a literal meaning of the word “sieving” and assume that it is some sort of measure of the degree to which separation occurs, depending on what does the sieving. Sieving coefficient in dialysis is a measure of how easily a substance passes from the blood to the dialysate compartment in a dialysis membrane. A more formal definition is given by Neri, et al. as they said that the sieving coefficient (SC) is the ratio of a specific solute concentration in the ultrafiltrate (removed only by a convective mechanism), divided by the mean plasma concentration in the filter (Neri, et al., 2016). SC is therefore an important factor to be considered in renal dialysis in general, for both HD and HDF as it determines the efficacy of the treatment.

2.2.2 Diffusion Principle in HD and HDF

This section will present the theoretical aspects of diffusion processes and explore the role of the dialyser membranes during diffusion in the dialysis process. The focus will be on the urea movement from the blood side of the membrane to the dialysate fluid via the semipermeable membrane. This is because urea is main molecule that is being removed by diffusion. Diffusion is famously dependent on the radius of the particles, such that molecular mass plays a major role in the diffusional clearance of solutes in the dialyser. This is not the case for convective clearance. There is a size barrier to the HD membrane, but convection is less dependent on molecule size than diffusion.

Sankaran described diffusion as the process by which substances tend to scatter themselves throughout the available space (Sankaran, 2009). The force that drives this process is kinetic energy that is present in all molecules which are in constant motion and at random collision with each other. The general effect of this action is the

movement of the molecules from an area of higher to a lower concentration. Factors such as the size of the colliding molecules and temperature affect the evolved kinetic energy such that the bigger the molecular size and the higher temperature, the faster the rate of diffusion. In terms of the cell-based diffusion, for molecules to passively permeate the plasma membrane, they should either be small enough to get through the pores or they should be fat-soluble (Sørensen, et al., 2018). Molecules can move via simple diffusion if their size permits them, like the small-sized chloride ions, or if they are fat-soluble molecules such as some vitamins, fats, oxygen and carbon dioxide. In HD and HDF, unwanted molecules or toxins should be able to diffuse through the membrane pores because the pores are generally designed to be bigger than these molecules. And pertinent to HDF, slightly bigger molecules will be pushed through the pores by convective force.

2.2.3 Fick's Law of Diffusion in Dialysis Membrane

Fick's law of diffusion describes how particles under random thermal motion tend to move from a region of higher concentration to a region of lower concentration (Seitaridou, 2007). In mathematical terms, three-dimensional diffusion is characterized by Fick's diffusion law (Guenneau and Puvirajesinghe 2013), which states that the diffusion flux is proportional to the concentration gradient:

Equation 2.1 $F = -D\nabla C$

where C is the concentration of the diffusing particles, F is the diffusion flux in particles per square meter per second, and D is the diffusion constant with units of cm^2/s. Therefore, particles tend to flow down a concentration gradient.

To further understand the diffusion principle in the dialysis process, Fick's Law of diffusion explains that the time course of the transfer of a solute between two compartments separated by a thin membrane, is given by:

Equation 2.2 $$\frac{dq}{dt} = DA\frac{dc}{dx}$$

Where, D is the diffusion coefficient, q it's the quantity of solutes, A is the membrane surface area, c is the concentration of the solution or molecules, dx is the membrane thickness and $\frac{dc}{dx}$ is the concentration gradient.

HD procedure relies mainly on three transport mechanisms: diffusion, convection (for HDF) and osmosis (Hörl, 2002). When blood passes through the renal system in the body, essential substances are separated such as toxins and other harmful compounds. Molecules such as urea, creatinine and potassium are small, and they pass through the kidneys' semi permeable membrane to be excreted (Burke, et al., 2010).

In this work, the researcher looks at membrane permeability as a factor which affects the rate of diffusion. Even though the thesis focuses predominantly on the RBC adhesion to the membranes, it also recognises that the pore size and number, thickness, and design of the semi permeable membrane affects the rate of diffusion and efficacy of the dialysis process. The surface area of the semi permeable membrane determines the rate of diffusion (Kim, et al., 1999). Two simple schematic figures showing a diffusion process Figure 2.2 and the one demonstrating diffusion process within a dialyser Figure 2.2 are shown below. Figure 2.2 on the left (initial) shows a solution that is concentrated, and the two arrows indicate movement or area of higher concentration (final) to a lower concentration. In Figure 2.3, blood component (indicated by a red down pointing arrow) is on the left and concentrated with toxins. These toxins diffuse across the semipermeable membrane to the dialysate side (blue upwards pointing arrow) which is less concentrated with these toxins.

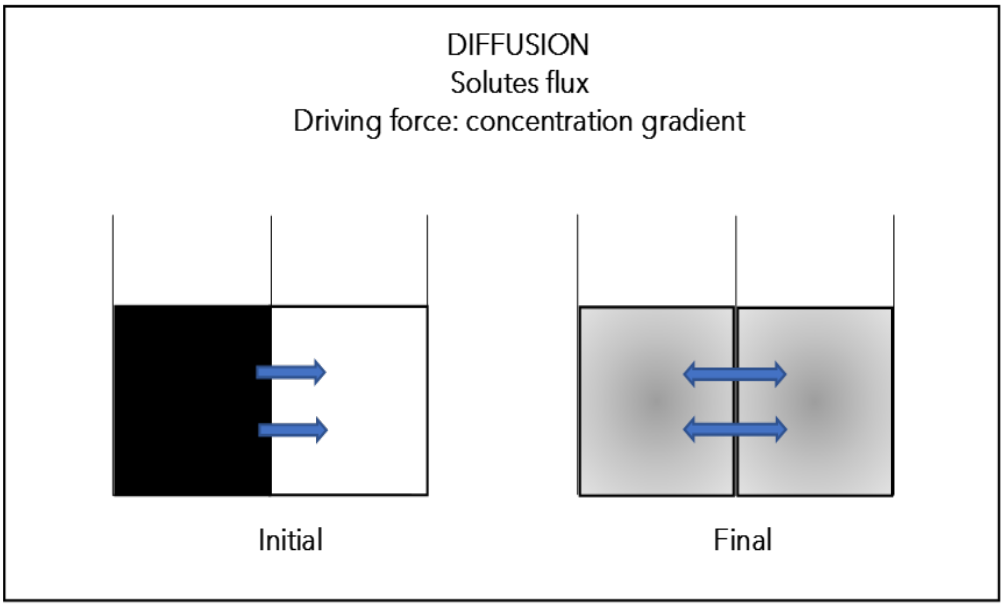

Figure 2.2 Diagram illustrating the diffusion process. The left part shows the concentrated solution and it moves (arrows) to the right and diffusion takes place via semipermeable membrane.

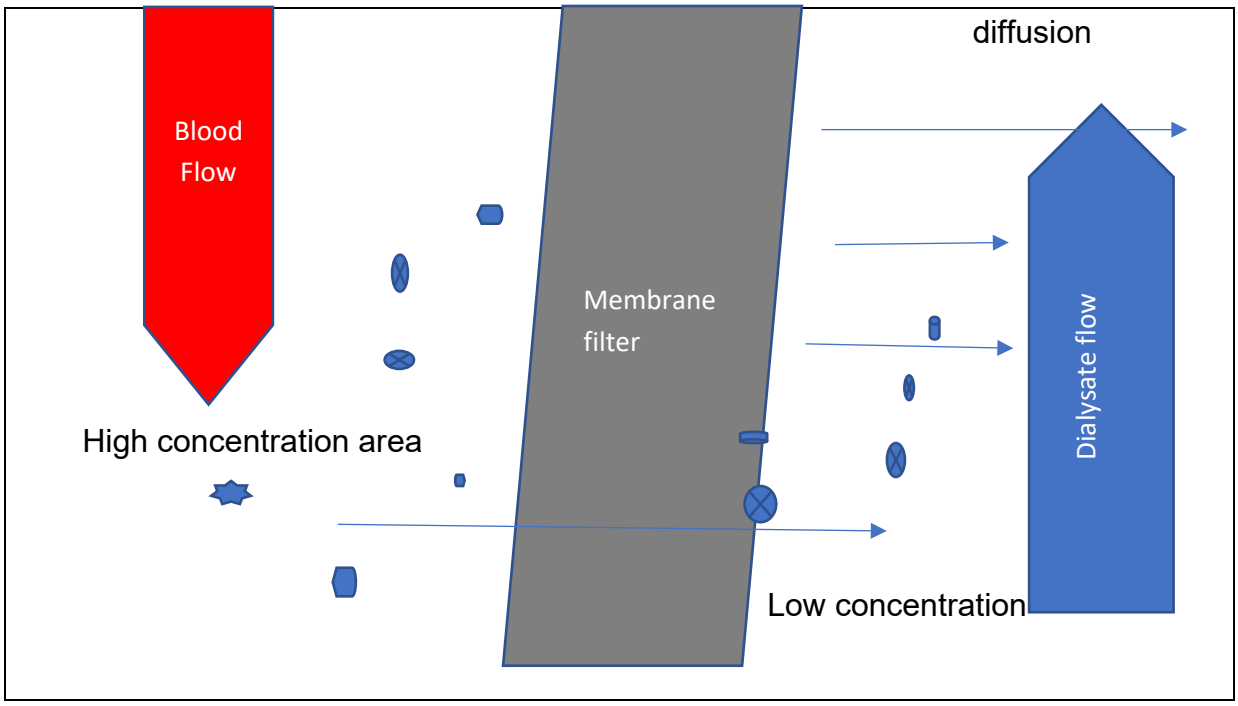

Figure 2.3 Diffusion and counter current flow within a dialyser. The left part of the diagram is the blood section. This is separated by a semipermeable membrane (grey). And the right part represents the dialysate section. Molecules are moving (arrows) from the blood section to the dialysate side.

The dialysing membranes in a dialyser are surrounded by dialysate which is a formulated solution consisting of electrolytes, nutrients and buffers. Gillette, et al. explained that the solute concentration, the control of hydrostatic and osmotic pressure of the dialysate facilitate the dialysis process (Grooteman, et al., 2012). Another factor that affects diffusion is flow geometry. In a dialysis machine blood and dialysate flows in opposite directions. The counter current flow allows fresh dialysate to meet new blood, but it should be emphasised that blood and dialysate will not/should not physically mix. Since the dialysate contains no urea, uric acid or creatinine, a concentration gradient between the blood in the tubes and the surrounding dialysate is established and this facilitates the transfer of urea from the blood to the surrounding dialysate solution.

2.3 Body Water, Ultrafiltration and Dialysis Membrane

Water helps in the transport of various substances within the body and acts as a good solvent. However, excess water needs to be removed from the body otherwise it can cause serious issues in a patient with renal insufficiency, thus dialysis is also responsible for removing excess water via the use of the polymeric membranes.

Removal of water (ultrafiltration/UF) is a type of dialysis membrane filtration in which hydrostatic pressure forces water against a semipermeable membrane (Ficheux, et al., 2011). From the literature reviewed in this work, dialysis membranes differ in their clearance capacities of different solutes, based on thickness and pore sizes. However, increasing the pore size and reducing thickness is almost forcedly linked to water permeability increase (Ficheux, et al., 2011).

When looking at membrane permeability to water, the UF coefficient is extremely important. KUF is defined by the American National Standards Institute (ANSI) as the permeability of a membrane to water, generally expressed in millilitres per hour per millimetre of mercury (Ficheux, et al., 2011). The equation below by Ficheux, et al. depicts how KUF is calculated (Ficheux, et al., 2011).

Equation. 2.3 $KUF=V/T\times P$

Where V is the volume of fluid, T is time and P is pressure. The coefficient of ultrafiltration was first defined by the amount of fluid (V) in mL crossing the dialyser membrane per time (T) in hours and pressure (P) in mmHg.

2.3.1 Dialysis Membrane Flux

The most frequently used dialysis membrane in recent years is a high-flux, non-cellulose membrane with increased permeability (Kerr, et al., 2007). This type of membrane material and design is said to be capable of removing bigger and smaller molecules, including many of the inflammatory proteins such as ß -2m and lipoproteins (Locatelli, et al., 2009). At least two studies have suggested that high-flux membranes improve the removal of small and moderate-sized molecules e.g. lipid profiles or homocysteine (Malik and Raizada, 2015; Johnson, et al., 2012). The use of a high-flux dialysis membrane produced by different commercial companies are designed to eliminate small, medium-sized and larger molecular weight toxins with better biocompatibility. These types of membranes have steadily increased since the discovery of ß -2m amyloidosis in 1985 (Hayama, et al., 2004). Plasma concentration of ß -2m in the case of renal failure is about 20-30x higher than usual, and high-flux membranes are capable of clearing substances of greater molecular weight cross membranes (Hayama, et al., 2004). This is because high flux dialysis membranes have larger pores and allow diffusion of greater amounts of uremic solutes and medium molecules and therefore may decrease the risk of dialysis-related amyloidosis and cardiovascular diseases (Jean, et al., 2015). HD and HDF membranes such as PSU and PESU are, therefore, classified as high-flux or low-flux based on their ability to remove fluid and molecules. It has been suggested that removal of larger solutes across high-flux HD membranes may better mimic normal kidney function and improve clinical outcomes (Maciaszek, et al., 2014, Locatelli, et al., 2009). Removing these larger toxins depend on the size of the pores of the polymeric membrane of the dialyser and other factors such as the temperature of the dialysate solution which is discussed below.

2.3.2 dialysate temperature

During HD treatment, changes in the dialysate temperature can raise or lower body temperature because the blood is returned to the patient in thermal equilibrium with the dialysate (Sherman, et al.,1984). Even a dialysate temperature equal to the patient's body temperature can result in an increase in the patient's body temperature, leading to cutaneous vasodilation and the potential for cardiovascular instability and hypotension (Sherman, et al.,1984). This deleterious cycle of events can be prevented by suitably adjusting the dialysate temperature. Lowering the dialysate temperature from 37°C to 34–35.5°C has improved the cardiovascular stability of many HD patients. Continuous monitoring of blood temperature is very important in HD sessions, and it allows the clinical staff to make proactive changes in dialysate temperature because a small change in body temperature can have enormous cardiovascular implications (Maggiore, et al., 2002; Ayoub and Finlayson 2004; Lackland, et al.,1985). For example, only 0.3°C to 0.8°C separates the thresholds for skin vasodilation from that for shivering (Lackland, et al.,1985). A suggested improvement in the HD procedure is to use devices that allow nonstop monitoring of blood temperatures and adjust the dialysate temperature automatically, keeping the patient, not the dialysate, isothermal (Maggiore, et al. 1981). Hence the use of temperature controller in the work.

2.4 Polymers background

For many years, the use of polymeric membranes has become more popular and have received much attention for the important role they play in clinical and other non-clinical fields. Boer et al. affirmed that, in recent years, there has been a proliferation of PSU and PESU based dialysis membrane introduced on to the market and in clinical settings (Boer, et al., 2017). Almost every dialyser manufacturer, distributor or supplier has been compelled by the state-of-the-art convective (HDF) therapies to include a PSU/PESU based membrane as a major part of their product portfolio (Ji, et al., 2019). This is the main reason this study investigated these two membranes. There are other membranes material used, but they are not as widespread in use as the PSU and PESU. Hence, this work focused on these two.

Polymers have exceptional properties useful in medical products development and application, their ability to be engineered and their abundance in the world have been useful in biomaterials and other medical applications (Jaganathan, et al., 2013). Polymers have replaced many old applications in medicine such as the shift from metal catheters to those made of polyethylene and they have also opened the door for new applications that no other material would permit. Studies shows that costly procedures have now been given new lower cost alternatives (Avery and Prieto, 2018; Okamoto, et al., 2014).

In the renal environment, the polymeric membranes from these polymers allow one to broaden the spectrum of uremic toxins that can be removed depending on its chemical and physical characteristics. Thus, bioengineering advances over the last few years have resulted in the introduction of a wide spectrum of dialysis membranes together with a multitude of different filters that are currently available commercially (Nalesso and Claudio, 2017b). As the bulk properties of a polymer can be directly related to its morphology, the understanding and controls of the morphology are technologically essential. It is also expected that by establishing the relationship between membrane morphology and its sieving behavior the mechanism of mass transport in the membrane can be revealed and understood (Ficheux, et al., 2011). For dialysis treatments, these synthetic polymeric membrane materials should possess properties such as excellent mechanical strength, good anti fouling resistance, high permeability and ability for good UF (Kanani, et al., 2010).

2.4.1 Membrane Technology – Polysulfone (PSU)

PSU is a rigid, strong, tough, high-temperature amorphous thermoplastic (Huang and Yang, 2006) and these desirable industrial properties of PSU makes it versatile in manufacturing of membranes. Apart from HD/HDF, PSU membranes are used in industrial applications such as food and beverage processing, wastewater recovery and gas separation. The chemical structure of PSU is shown in Figure 2.4.

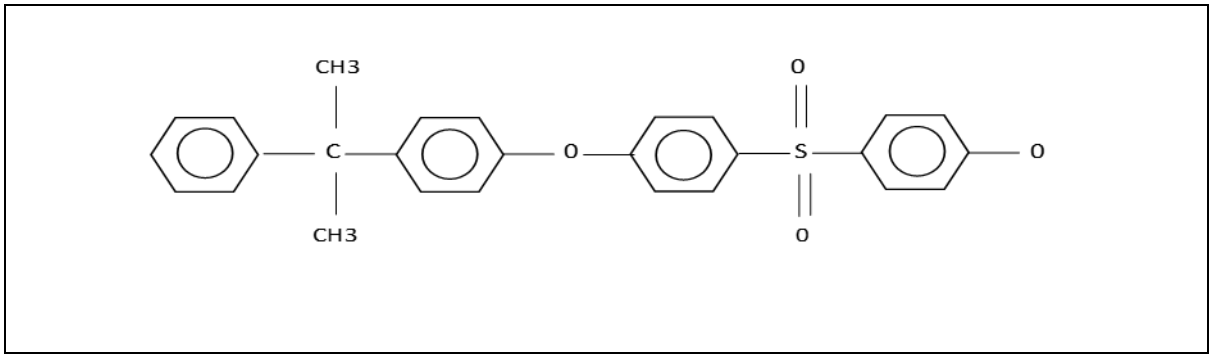

Figure 2.4 Organic structure of PSU (McKeen, 2012), with permission.

Desirable industrial properties of PSU are summarized as high thermal stability, high toughness and strength, good environmental stress crack resistance, inherent fire resistance and transparency.

2.4.2 Polyethersulfone (PESU)

PESU is a thermoplastic with ability to withstand exposure to elevated temperatures in air and water for prolonged periods without compromising on performance (Zhao, et al., 2013). Furthermore, PESU is an amorphous, transparent material, inherently flame retardant as well as resistant to mineral acids and alkalis (Zhao, et al., 2011). The chemical structure of PESU is shown in Figure 2.5.

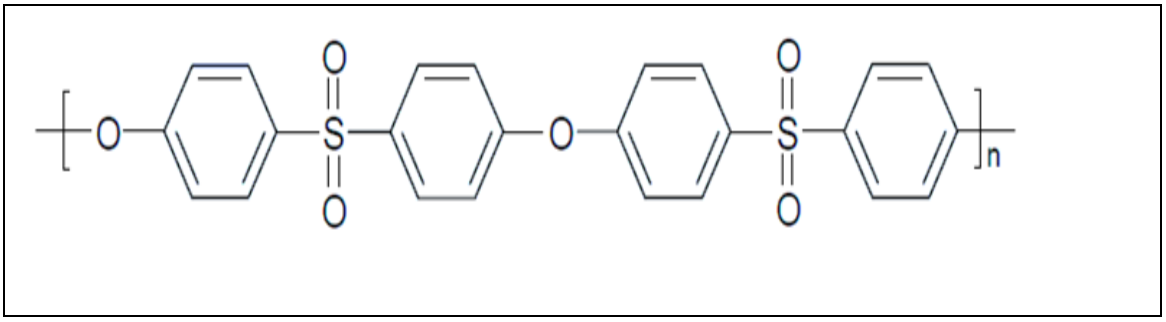

Figure 2.5 Organic structure of PESU *(*McKeen, 2006*) with permission.*

PESU can be prepared by polycondensation of suitable monomers or by ring-opening polymerization of cyclic ethersulfones. One of the most common methods is

nucleophilic substitution of an aromatic chloro- or fluorosulfone by a phenoxide ion (McKeen, 2006). Desirable properties of PESU are the outstanding ability to withstand exposure to elevated temperatures, dimensional stability and Inherently flame retardant.

2.4.3 Manufacturing PESU and PSU

The flat sheet PESU and PSU membranes used in this experiment are processed through the casting of solutions by tape casting method (commonly known as doctor blade)(Huang and Yang, 2006). On the other hand, the hollow fibre polymeric membranes used in dialysis treatment are produced via spinning technology (Mercado-Pagán et al., 2014). It is generally agreed that some orientations, either in plane or out of plane, will be induced during the processes. Hollow fibre polymeric membranes are said to be more popular than flat sheet membranes. The advantages are, firstly, they are less fouling and secondly, they provide larger effective surface area to volume ratios. The low fouling gives them longer life span and the area to volume ratio provides high packing densities. Also, it is effective and easy to use in cross flow mode as compared to flat sheet membranes. Due to these advantages, hollow fibre membranes are playing a prominent role in the development of the membrane field in various sectors including renal dialysis.

Table 2.2 below will summarise the non-manufacturing different properties of the PESU and PSU with reference to density, light transmittance and water absorption equilibrium.

Property	**Units**	**PSU**	**PESU**
Density	g/cc	1.24	1.37
Light transmittance	%	70	70
Water absorption at equilibrium	%	0.5	2

Table 2.2 Comparison of PSU and PESU properties.

As seen highlighted on the Table 2.2 PSU and PESU properties are similar, although PESU exhibits a higher impact strength and better chemical resistance with a slightly higher density compared to PSU. The physical and chemical differences of these two membranes are important for this study and may contribute to RBC adherence to PESU and PSU membranes. Perhaps the most obvious difference between these two materials comes from the fact that PSU is produced via a multi-stage polycondensation reaction (with bisphenol and dichlorosulfonyl sulfone), resulting in a linear amorphous structure (Higuchi, et al., 2002). On the other hand, PESU is created through polysulfonation or in simple terms, PESU come from polyester synthesis (Sokolsky-Papkov, et al., 2011; Zhao, et al., 2004).

2.4.4 PESU and PSU as dialysis membranes

PESU is said to be one of the most important synthetic polymeric materials and is widely used in separation and medical fields (Jin, et al., 2018). PESU based dialysis membranes show better oxidative, hydrolytic and thermal stability as well as good mechanical and film-forming properties. As used in many different application, PESU membranes could endure many kinds of sterilized methods, including, steam sterilization just like PSU. Furthermore, after pores formation and when used as a HD and HDF membrane, PESU and PSU material both show high permeability for low molecular weight proteins when used as a renal replacement membrane (Zhao, et al., 2004). Thus, these membranes are also widely employed not only in biomedical fields such as artificial organs and medical devices used in blood purification but in the plasmapheresis procedures, including plasma collection (Zhao, et al., 2013; Jin et al., 2018; Mercado-Pagán, et al., 2014; Maleka, et al., 2018), and also used widely in other industrial filed and have become very popular in the last few years (Zhao, et al., 2013). In HD clinical practice, dialysis membranes are mainly made from these two materials.

2.5 Biocompatibility of membranes to red blood cells (RBC)

The effectiveness of HD is enhanced by membrane biocompatibility. Biocompatibility simply refers to the ability of a biological material to perform with an appropriate host response in a specific situation (Williams, 1987). It encompasses the behaviour of

biomaterials in various contexts. Biocompatibility involves many physiological changes; therefore, it must be considered when investigating biomaterials of interest. The equivocacy of the term biocompatibility reflects the ongoing development of insight into how biomaterials interact with the human body cells, tissues and organs and eventually how those interactions determine the clinical outcome of a biomedical material (Williams, 1987). It should be noted, that medical devices are often made of more than one material, so it might not always be enough to talk about the biocompatibility of a specific material. Nevertheless, biocompatibility in relation to medical devices is largely determined by the materials used to manufacture the device. Various materials, including cellulose-based and synthetic polymers, are used for dialysis membranes (Sekai, 2000). To enhance biocompatibility, the focus of research in polymeric membranes over recent years has been on membrane materials as well as their surface character and texture, which has evolved over the years. Research and development are directed at altering the chemical and physical properties of membrane surfaces to suppress biological responses that are particularly elicited as a result of blood membrane interaction. To develop membranes with good biocompatibility, membrane materials should be tested on a like-for-like basis under conditions similar to clinical settings. (Kokubo, et al., 2015). Studies have previously focused more on other aspects of the blood interaction and less on RBC interaction or adhesion with the membrane, but to create highly biocompatible dialysis membranes, the overall correlations among biological reactions should be examined by integrating all data on biological responses elicited by blood-membrane interactions or mutual interactions among blood cells (Kokubo, et al., 2015).

In HD, chronic reactions that are not specifically detrimental to patients focus on biocompatibility of dialysis membranes. Some of these chronic reactions are complement activation, contact pathway activation, platelet activation, monocyte activation and neutrophil activation during the HD treatments (Sekai, 2000). Although biocompatibility of the dialysis membrane continues to be studied and investigated, many factors regarding dialysis are yet to be understood.

PSU and PESU in this study are flat sheet material similar in structure to the semipermeable membrane used in HD. It should be noted though that the complexity of dialysis biocompatibility is not limited to the dialysis membrane material alone; all

the system elements of the extracorporeal circuit, and even the design and manufacturing process contribute to biocompatibility (Rao, et al., 2004).

2.6 Red blood cells (RBC) membrane receptors

Widespread adhesion of RBCs in the vasculature would be incompatible with life (Vimal, et al.,2012). But it is known that RBCs can at times adhere to other structures. Therefore, an understanding of the RBC membrane receptors is crucial when dealing with adhesion study. Lodish, et al. looked at cell and extracellular signalling, they stated that RBC membranes are one of the integral membrane proteins in cell behaviour and, will naturally mediate cell signalling via binding extracellular molecules. Specifically, membrane receptors allow communication between the cell and the external environment. Hormones, cytokines, cell adhesion molecules, and immunoproteins are examples of the extracellular molecules (Lodish, et al., 2000). In general terms, cell adhesion molecules interact with membrane receptors of various cells and plays a huge role in the adhesion process (Foley and Conway, 2016).).

2.7 Atomic force microscope (AFM) Background

Light microscopy is historically the first technique to observe objects at micron level where its resolution is limited by the wavelength of visible light. A better imaging performance was achieved when SEM which allows a higher resolution for both biotic and abiotic samples (Chakrabarty, et al., 2008). A drawback of this technique is, however, the need for invasive sample preparation, such as sample coating with a thin conductive layer, and imaging conditions that are often conducted in vacuum: these conditions are accompanied with high level of sample contamination, introducing artifacts and altering sample structures when biological samples, such as cells, are used (Yeow, Tabor and Garnier, 2017). But, despite advances and availability of many types of light microscopy, AFM and SEM remains popular and distinct in their unique ability to examine dimensional topography and distribution of exposed features on a sample (Chakrabarty et al., 2008).

AFM consists of a computer for data processing and visualization, cantilever with or without a sharp tip, photodetector to detect the cantilever deflection, for example, four-

segment photodiode which detects the displacement of a laser beam as it reflects the back of the cantilever, a feedback system which controls the vertical position of the tip on the sample but keeps the cantilever deflection constant and a piezo-electric scanning system. These components of the AFM seen in the below figure 2.6

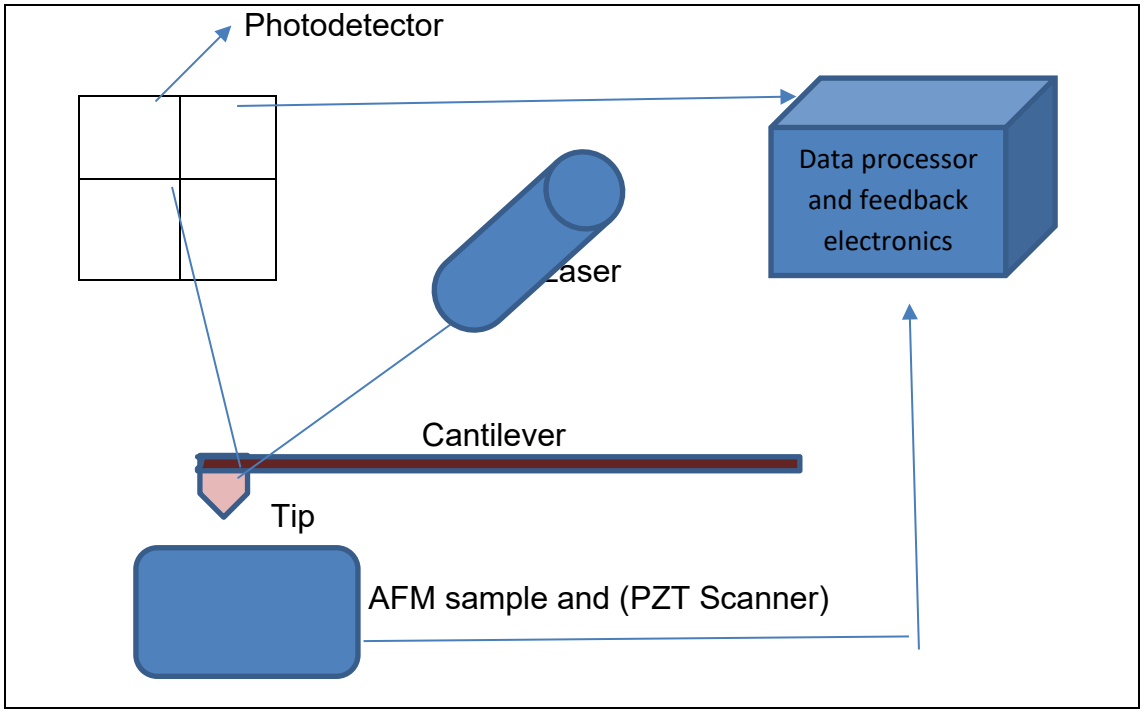

Figure 2.6 Basic AFM schematics demonstrating the principle. The photodetector detects laser from the cantilever. (the laser spot will be centered on the photodetector). The image is generated by scanning the cantilever on the surface of the sample. This information is processed by the photodetector.

The AFM provides a 3D profile on a nanoscale, by measuring forces between a sharp probe (radius less than 10 nm) and surface at very short distance (0.2-10 nm probe-sample separation). The probe is supported on a flexible cantilever and the AFM tip gently touches the surface and records the small force between the probe and the surface (Torrent-Burgués, et al., 2014). This force can be described using Hooke`s law:

Equation. 2.4 $F= -K.x$

Where F is the force, K is the cantilever spring constant, and x is the cantilever deflection. As in seen in the above schematic Figure 1.2, the basic components of an AFM are the tip, the cantilever, the scanner, the laser, a data processor and a photodetector. The cantilever is mounted to a tiny tip which is responsible for scanning the sample and controlling the movement of the cantilever.

PF-AFM will be used in this thesis, and it works simply by the cantilever performing an extremely fast force curve by tapping the sample surface pixel by pixel to create images and analyse the topography. These fast forces between the PF-AFM probe and polymer or sample surfaces have already been reported in literature (Stroh, 2004 and Marrese, et al., 12017). Such forces are said to be nonspecific and can be directly related to the interfacial energy between the AFM probe and the sample (Marrese, et al., 2017).

2.7.1 Scanning Electron Microscope (SEM)

A Field Emission Scanning Electron Microscope (FE-SEM) has a very long history since its first observation done by Max Knoll in 1935, and commercialised 30 years later (Cerqueira et al., 2015). Despite that, it is still used widely in the scientific world and provides valuable information during a study. Modern FE-SEM mechanism of action is different from the AFM, FE-SEM uses an electron beam for imaging (Kremer, et al., 2015). It has a large depth in field for ultra-high-magnification imaging, which enables researchers or investigators to observe an enhanced and detailed image of the sample topography of the surface. This can be observed by 2D scanning of the electron probe over the surface and acquisition of an image from the detected secondary electron (de Haan, et al., 2019). Again, in FE-SEM the beam of electrons is produced using an electron gun and it goes through a vertical path along the microscope, which is placed in a vacuum, hence SEM works in vacuum, while AFM can work in air, liquid and vacuum. However, SEM is said to be the most common methods for examining morphology and roughness of membrane surfaces (Hoek, et al., 2003)

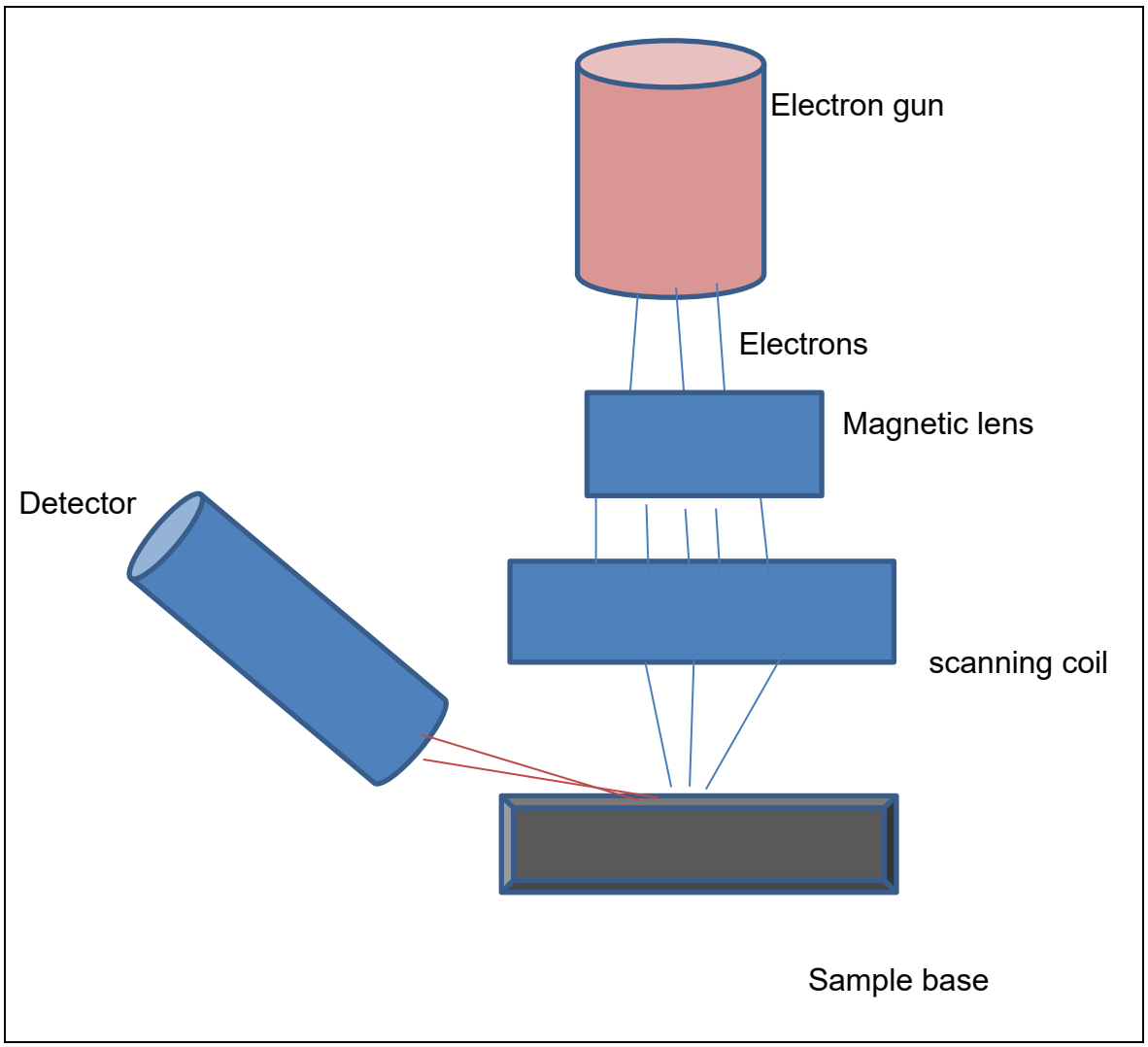

Figure 2.7 Basic schematic diagram of an SEM system with a thermionic electron gun. The electron beam is generated by the electron gun. Electrons emitted by the passes through magnetic lens and scanning coil to the sample base. This image is detected by the detector (left).

SEM uses the electrical and magnetic fields and the lenses helps by focusing the electron beam to the sample. The electron beam will hit on the sample surface and with that impact the electrons and X-rays will be emitted. These emissions are detected and analysed in order to put the material image on the screen. Like the AFM, SEM resolution (can reach 0.4 nm) is in nanometre scale and these similarities makes it easier to compare the SEM images with the AFM images. Another point to consider and perhaps an advantage of the SEM, is that it has more control in the amount of magnification as an electromagnetic system is in use (Yeow, et al., 2017). In SEM,

information on surface topography is mainly contained in the signal coming from secondary electrons Figure 2.7. In AFM, the topography and other physical characteristics of the surface are mapped using lateral and vertical movements of a sharp tip Figure 2.5. Since SEM images are simply 2D projections, some information on surface topography is inevitably lost; therefore, SEM is used mainly for imaging and qualitative comparison. In contrast, AFM produce a genuine 3D topographic image of the surface; thus, various quantitative statistical parameters may be calculated such rms, and ra. Despite these differences, these two useful instrumentations will form the critical part of the material and methods in the work.

2.8 Chapter summary

HD Membrane performance is determined by the effectiveness of solute or waste clearance (Bowman, et al., 2019). However, Haroon and Davenport has reiterated that the biocompatibility of the material is of utmost importance when it comes to blood dialyser interaction (Haroon and Davenport, 2018), and therefore biocompatibility should be considered when looking at dialyser effectiveness. Bioengineering and technical advances in HD and HDF material design, topographic study, and sterilization methods have led to improved performance to the level that HD membranes can now reduce morbidity and mortality. However, more work needs to be done in this area to reduce premature deaths that are still imminent. The literature review in this thesis focused more on the HD membrane because the membrane is the fundamental part of the dialyser, and the dialyser is the essential part of the extracorporeal circuit. It is the author's belief that the difference between the PESU and PSU HD material should be clearly understood including the effects they both have on the RBCs and other blood components. To attain this, the membranes' surface characteristics should also be studied.

CHAPTER 3 Materials, Techniques and Methods

This chapter outlines all the materials, techniques and methods used in this work; It touches on the basic understanding of the instrumentations used, and this covers the AFM, SEM, contact angle, analysis of surface features of the membranes followed by flow cell analysis of the RBCs. The structural difference between PSU and PESU is compared using these tools. The two main areas covered here will be;

a) surface topography analysis employed to investigate and understand the differences in membrane structure. Direct observation of the morphology of the membranes was conducted using PK-AFM, while SEM was also used to study membrane topographical structure, roughness and compare characteristics.
b) the degree of RBC adhesion pertaining to these membranes was evaluated *in vitro* by analysing the number of adherent RBC on the membranes, with the use of bovine blood. A modified flow cell system was designed and built to be used to investigate RBC adhesion to the test membranes. To mimic actual dialysis conditions under controlled temperature, blood was passed over the three membrane samples (PSU, PESU and glass) for three hours over a period of three days. The findings of these investigations are presented on the results section of the next chapter.

3.1 Materials

3.1.1 Bovine blood

This work used bovine blood for this experiment due to its availability and regulatory simplicity. Bovine blood is widely used for pharmaceutical (Aramwit, et al., 2000; Hu et al., 2015) and tissue engineering applications (Novtna, et al., 2016; Zhang et al., 2016). As a by-product of the meat industry, it is relatively inexpensive since large quantities are readily available. It has been reported that human blood groups and that of cattle are very similar in physiological and mechanical terms, even though bovine blood cells are typed by a haemolytic test and human cells by an agglutination test (Lewin, et al., 1994). The differences between human RBC and bovine RBC will be discussed in Chapter 4.

In preparations for the experiment, 500 ml of fresh bovine blood sample Figure 3.1 was collected during the post-slaughter bleed of the animal's carcass at the local abattoir near Swansea. It was mixed with 110 ml of AS-1 solution and stored in a fridge at 4°C or five days before the experiment. The AS-1 solution provides improved preservation of RBCs (Sparrow, 2012). It contains 70 ml of Citrate Phosphate Dextrose Solution with Adenine (CDP) and Gentamicin 0.5 ml as a prophylactic antibiotic. CDP is also required for extracorporeal circulation during HD (Yavari and Becker, 2008; Haroon and Davenport, 2018; Shen et al., 2012). Table 3.1 below shows all the solutions and strength used in the storage of the blood including the doses, molar concentration and mass volume of each solution.

Blood storage solution					
Ingredient	Bovine blood	AS-1	Citrate Phosphate Dextrose Solution with Adenine (CDP)		Gentamicin
Volume [mL]	500	110	70		0.5
AS-1 at pH 5.5-6					
Ingredient	Deionised water	NaCl	Adenine (Adenosine)	Dextrose (Glucose)	Mannitol
Molar concentration [mM]		154	2	111	41
Mass [g] or Volume [mL]	150	1.35	0.0802 2.9997		1.1203

Table 3.1 Bovine RBC and additive solution. Fresh bovine blood sample was collected and mixed with 110 ml of AS-1 solution as per the above Table before storage

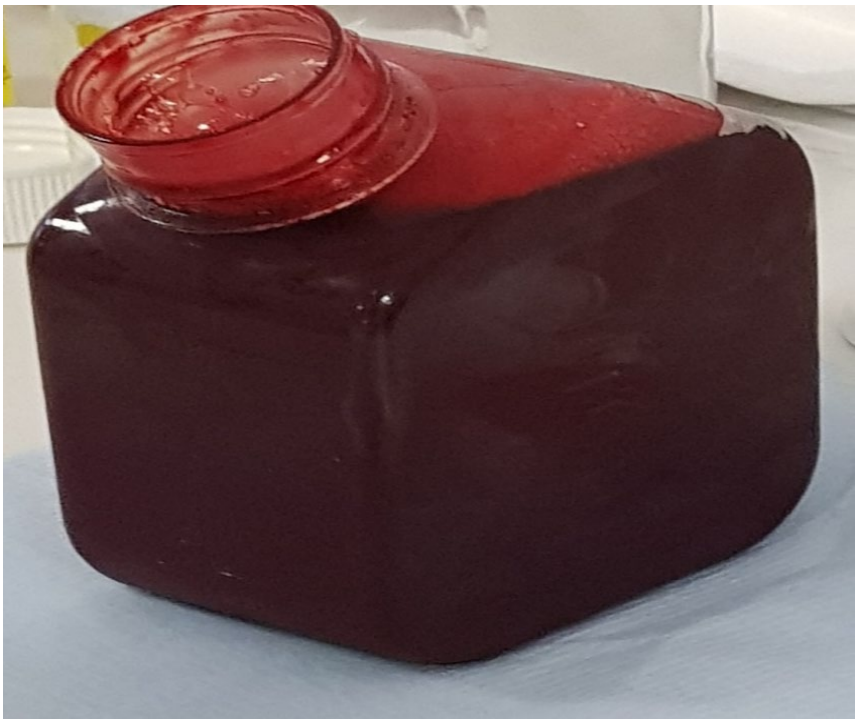

Figure 3.1 Blood sample. Sample containment bovine blood collected and stored in the fridge in the laboratory before the experiments.

3.1.2 Centrifugation and separation of the RBC

The RBCs in this work were then prepared for each experiment from bovine blood. 1 ml of bovine blood was removed using a pipette and injected into 1.5 ml MCF. The tubes were centrifuged (Eppendorf 5424 centrifuge) for 5 min at 3000 rpm (revolution per minutes) for five minutes. Following the 5 min centrifugation process, the RBCs formed a pellet with a clear blood plasma on top, and the white cells and the platelets remained between the plasma and the RBCs. The top layers, approximately 60 percent of the whole volume, were removed with a pipette.

Phosphate buffer solution (PSB) (pH 7.4) was added to the RBC pellet to create a less viscous fluid for the flow cells system. Bubstamante and Meissner predicated this technique in their work dealing with characterization of carrier RBC for biosensing applications (Bustamante and Meissner, 2017).

3.2 Flow Cell System material and techniques

The flow cell system used in this work was custom designed and built by the researcher.

3.2.1 Set up of material samples to the sticky cover slips

The core of the system is the flow cell designed using the pump, tubes, and the Ibidi sticky cover slip (model number 80168 Figure 3.2). The sticky cover slip is the bottomless channel slide used for perfusion applications with a self-adhesive underside to which own substrates can be mounted.

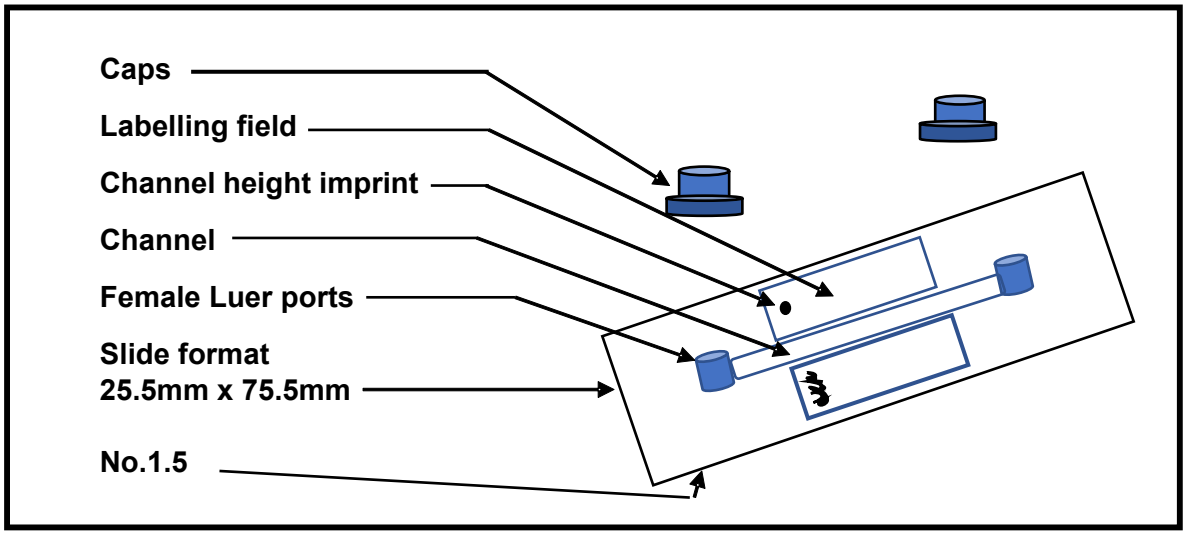

Figure 3.2 Schematics of sticky cover slip with slide luer. This shows the components of the sticky slide and the specifications of these sticky slips are highlighted in Table 3.2 below.

Cover slip components	Dimensions/measurements
Outer dimensions	25.5 x 75.5 mm^2
Channel length	50 mm
Channel width	5 mm
Adapters	Female Luer
Volume per reservoir	60 µl
Growth area	2.5 cm2
Coating area	5.2/5.4/5.6/5.8 cm^2

Table 3.2 Specification of cover slips. This table presents all the specifications of the cover slips including components and dimensions. It highlights the length of the channel and the coating surface area.

The PSE and PSU were cut to fit the sticky glass. Then the cover on the sticky glass was removed for the PESU and PSU samples to be placed underneath the coverslip. These three slides channel in silicone tubing attach directly to the inlet and outlet channel and the tube extending back to the RBC solution jar which is placed in temperature controller/heater, thereby creating a circulatory flow.

3.2.2 The Flow system

The flow cell system, which was originally called “Robbins device” was invented by Jim Robbins to enhance the reproducibility of biofilm formation in a fluid flow and was later modified and named Modified Robbins device (MRD) by McCoy et al. (Azeredo, et al., 2017). This device is now used in various research applications. This work therefore based the basic principle of flow cell design on this system by Jim Robbins. But for this to fit the nature of the investigation in this work, a modified flow cell system was designed, developed and built using existing tools. As challenging as it was for designing and building this flow cell, this was however, appropriate so that the

operating protocol will be tailored to this work. Also, consideration when designing this flow cell operating protocol was based on emulating the dialysis environment. Flow cells system has also been associated with video application to capture the processes of adhesion, and possible attachment of other materials to material such as biofilm (Crusz, et al., 2012). This section of this chapter is dedicated to outline how flow cells system was used in this thesis. This system was efficiently designed to be an optimised system allowing the application of flow of RBCs in a multichannel format running the experiment on all the three slides at the same rate, time and under the same conditions including the temperature control. All the materials used can be seen on the actual picture that was taken during the investigation, below Figure 3.3. below.

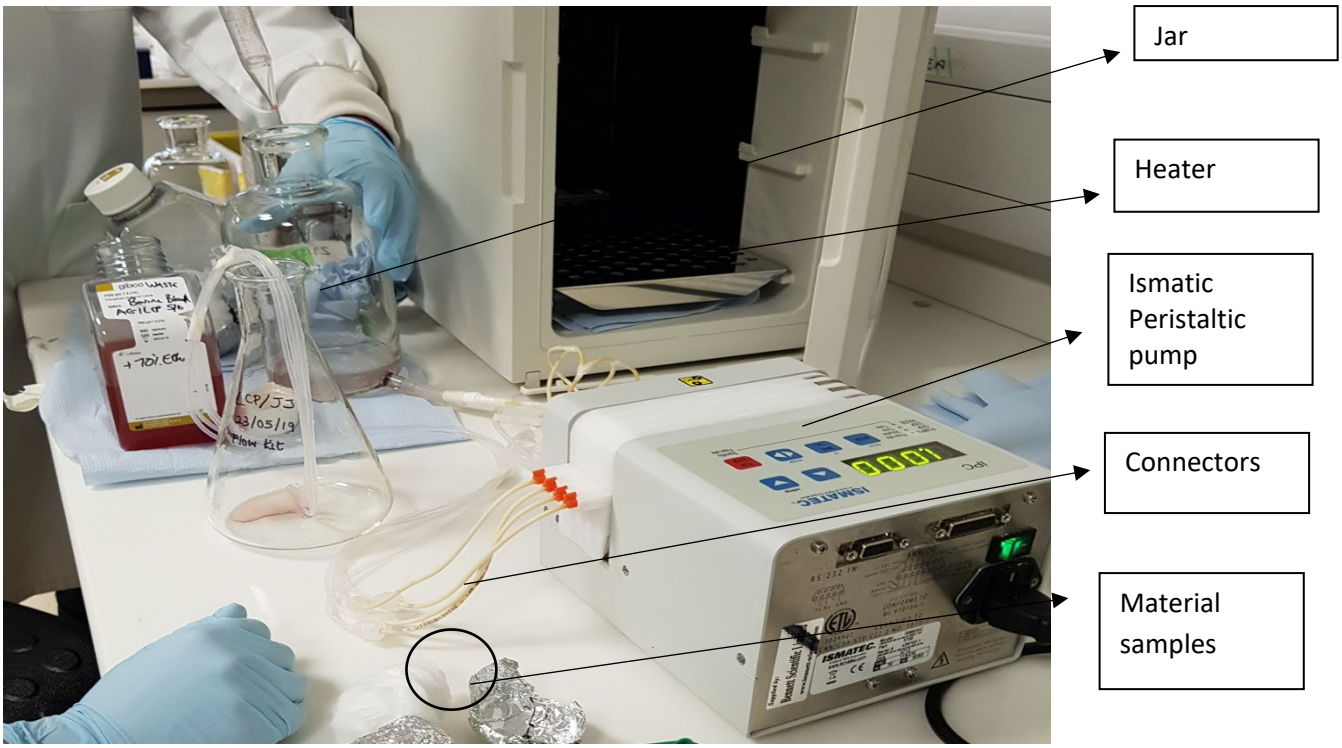

Figure 3.3 Constructed laminar flow cell system used in this thesis. The peristaltic pump (Ismatec) is used to drive the flow of the PBS RBC solution from the jar which was placed inside the heater (thermo scientific temperature control). The peristaltic pump had multi-channel silicone tubing within which the solution will flow and pass by the samples, (PSU, PESU and glass) inlet and an outlet flowing back to the jar, which is also placed inside the heater, thereby creating a continuous flow (recirculation). The tubing was connected to the slides tightly with Leur lock connecters to avoid leaking and air contamination.

To accompany the actual image, the researcher has created a schematic drawing below for the flow system used in this work Figure 3.4

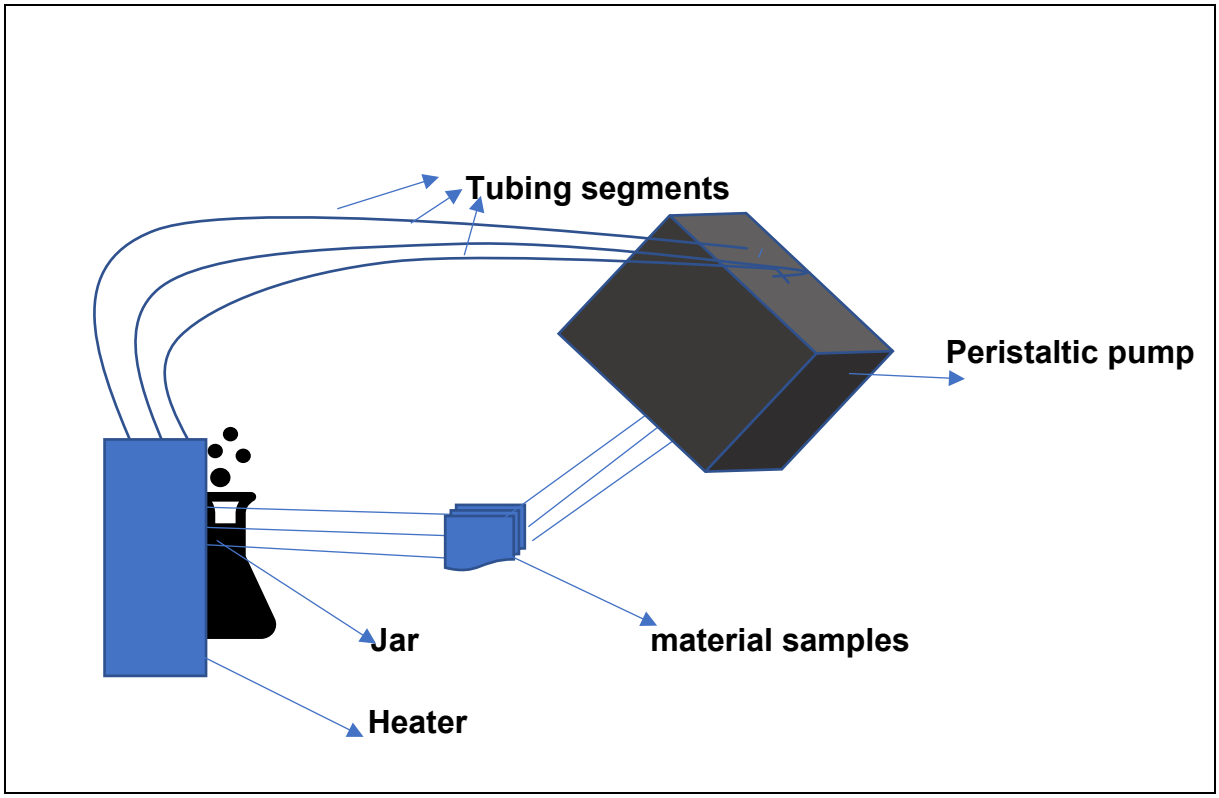

Figure 3.4 An illustrative sketch of the flow system. The flow system showing the heater, RBC sample jar which was placed inside the heater to warm the RBC solution to required temperature, tubing segments and the peristaltic pump.

3.2.3 The peristaltic pump

The peristaltic pump used was a multichannel programmable device featuring planetary gears that drove four stainless steel rollers in a smooth rotation and accurate fluid flow. The machine also had a four-digit light-emitting diodes (LED) display that showed the flow rate when the pump was running. The pump had a feature that allowed for the lines/tubing to be primed, increase and decrease the speed, and to set the pump within the ideal settings/parameters suitable for continuous pumping of the RBC-PBS solution through the slides. The pump had a flow rate of 0.0004 to 44 mL/min (based on the tubing size), catering for sufficient shear stress. This was one of the advantages of using this pump because shear stress is a key factor to be considered when selecting the pump to be used. Shear stress is said to be the

component of stress that acts parallel to a material cross section. The most common source of shear stress occurs when forces are applied directly parallel to a surface like the fluid shear stress that occurs in vascular tissue from flowing blood interacting with the vessel wall (Resnick, et al., 2003). In HD, when blood flows though the dialyser during the treatment session, the membrane surfaces are exposed to shear stress and this may affect the RBC and the surface characteristics of the dialysis membranes. Ramón, et al. confirmed this by suggesting that, the physical and chemical properties of a hollow-fiber dialysis membrane could be changed by shear stress of blood flows in HD (Ramón et al., 2018). Therefore, this system was designed to provide a clinically relevant shear stress, which was equivalent to 1.3 Pa (0.0098nnHg). This is supported by Horobin, et al. as they investigated the shear stress exerted by the uremic blood during the dialysis session and using the AFM, they found out that no changes in the surface structure of the membranes were detected at 1.3Pa (Horobin et al., 2017). The desirable shear stress is achieved within the targeted limits by the pump rate that is controlled by the rotational speed of the inner cylinder within the peristaltic pump.

3.2.4 Controlling the temperature

A key part of the system was the temperature controller (heater) as seen in Figure 3.3 and Figure 3.4 maintained the temperature at 36 °C and kept it steady for three hours so that the experiment is not influenced by the atmospheric temperature and mimics the actual dialysis treatment.

3.2.5 Cleaning the material

Before connection of the RBC-PBS medium jar, every material used was cleaned thoroughly with ethanol, and following each successful 3-hour experiment cycle autoclavable components were sterilised using the autoclave to a temperature of about 121°C. The rest of the equipment used which were not autoclavable were also cleaned using ethanol. This approach has been suggested by Crusz, et al. when they postulated that the non-autoclavable parts of the flow cell system such as the peristaltic pump, and syringes should be disinfected with strong disinfectants such as ethanol (Crusz, et al., 2012). The jar which contained the solution (PBS-RBCs) was

then disconnected from the system by splitting the tubing from the jar (which was in the temperature controller) and connecting to the peristaltic pump.

3.2.6 Modifications of the Flow system to minimise bubble formation and priming of the tubing.

The biggest problem encountered during the process was the air bubble formation especially in the glass slide (control). The detrimental effects of bubble formation in flow cell systems has also been quantified by COMSTAT analysis (Heydorn, et al. 2000). Affirming the existence on this problem, in their work, Crusz, et al. highlighted that researchers are frequently faced with the problem of constant air bubble formation within the flow system (Crusz, et al., 2012). This demonstrates that the constant air-bubbles that we faced on the flow cell, especially on control glass slide, were not unique and is inferred to be caused by a different factor such as the nature of the peristaltic pump, changes in temperature of the fluid. However, measures were taken to minimise or lessen the introduction of the air bubbles in the circuit. Several modifications were made to the traditional flow cell system. Firstly, the slides inlet port was rendered airtight with cellotape around the entry ports. The flow system was placed on a flat work top allowing smooth flow which is influenced by the negative pressure created by the peristaltic pump. This also served to reduce the negative pressure gradient created within the tubing by the pulling action of the pump, which in itself, can lead to air bubbles being drawn out of the solution. This is in keeping with the way dialysis takes place in which the pump (dialysis machine) is on the same level or just slightly above the access point of the patient. Before commencing the flow, the tubing was primed with PBS to get rid of the air in the circuit. For this measure to be effective, the Leur lock on the inlet and outlet of the slides had to be air-tight and there could not be gaps allowing air in. Another action to reduce air bubble formation was to prevent the RBC PBS solution from cooling down by continuously maintaining it at the correct temperature for the experiment which was achieved by placing the solution inside the heater at constant temperature of 36^0C. If the solution is colder than the ambient temperature, air bubbles tend to emerge throughout the system if it is running as the temperature of the solution rises.

3.2.7 Running the experiment and data collection

The Flow cell system techniques will vary depending on the experiment being performed. This experiment was designed to run for three hours. After connecting all the channel slides to the ibidi slides and the pump system the flow commenced. Under flow, the RBCs-PBS solution was continuously pumped through the channel slides. After three hours of flow had elapsed, the peristaltic machine was stopped, the slides disconnected from the inlet and the outlet tubes. The slides were rinsed with 10ml of the PBS to remove all possible nonadherent or unbounded residual cells.

3.2.8 Disassembly and cleaning of flow cell

At the end of each experiment session, the system was emptied and then rinsed with ethanol solution. All tubing was rinsed with PBS and then detached from the pump. The glass coverslip substratum was carefully removed from the flow cell base. Any remaining celloptape was removed from the base and side of the slides and discarded.

3.3 Atomic force microscope (AFM) and Scanning Electron Microscope (SEM)

The AFM and SEM were used to run a surface topography analysis of the three test membranes to investigate the relevant microstructure surface characteristics. The PF-AFM images obtained for all three materials were at different resolutions and the images were analyzed using the nano-scope analysis 1.5 software (Bruker UK). This software was also used to assess the surface roughness. The results of these analysis may provide a better understanding for more efficient dialysis sessions and biocompatibility of the membrane and will be presented in chapter 4 of the work.

3.3.1 Atomic force microscope (AFM) and Scanning Electron Microscope (SEM) background.

To better understand RBC and HD membrane interaction, the physical structure of the material needs to be examined. A microscopic view analysis of the membrane surface topography is usually examined by a scanning electron microscope (SEM) and an atomic force microscope (AFM). Recently the microscope technology has advanced drastically and a field-emission SEM (FE-SEM) with much higher resolutions, is now being utilized widely (Ramón, et al., 2018). This work will combine AFM and SEM analyses of the membranes to provide valuable information regarding the topography of the sample membranes being studied.

AFM as a tool in microscopy is applied in a range of scientific fields especially in the last 15-20years (Yeow, et al., 2017). Kim, et al. reiterated that AFM is not an old system and added that it is now well-developed and, has now become a powerful technology for analyses and characterization of surface of materials down to their atomic scale (Kim, et al., 1999). It can be used to obtain nanoscale chemical, mechanical (stiffness, viscoelastic, roughness), electrical, and magnetic properties of a material. It has indeed become a useful material “friendly” analytical technique which can be used in liquid, vacuum or in air. AFM has contributed to the availability of high-resolution microscopic imaging tools and is continuously increasing in many engineering fields, clinical/medical, and other fields (Kashef and Franz, 2015). In comparison with other microscopy techniques, AFM offers low cost and imaging capability like atomic resolution (Kashef and Franz, 2015). The introduction of AFM by Binnig et al. in 1986 allowed imaging of a wide range of samples, including cells, with a resolution comparable to electron microscopy, but with less invasive sample preparation and the possibility to image samples in different environmental conditions. Nowadays it is a standard approach to scan cells with AFM in the appropriate buffer and temperature, with a high lateral and vertical resolution (Nagornov and Pahomova, 2016). The high signal to the noise of the AFM permits creating images at high resolution making topological reconstruction of the sample more possible (Yeow, et al., 2017).

3.3.2 Peak-Force scanning modality

Although several scanning modes in AFM exist, Peak-Force scanning modality was chosen for this work and samples were analyzed in PBS. Figure 3.5 below explains the operational and scientific principles of Peak-Force AFM. According to Bruker Ltd, the basis of operation for this AFM comes from the “Force against Time display”, colloquially named the “heartbeat.” Point A in Figure 3.5 indicates that very minimal force field is recorded at this stage - but a change occurs (as seen in the graph line) as soon as the tip approaches the surface. The Van der Waal forces (capillary or electrostatic forces) pulls down the cantilever towards the surface and as represented by the negative force (graph line below the horizontal axis). This graph line trend continues until the cantilever stiffness is overcome by the Van der Waal forces pulling the tip down to the surface.

The tip then stays on the surface and the capillary force increases until the Z position of the modulation reaches point at C where the peak force occurs. At point C, the peak force stays constant due to system feedback. The probe then starts to withdraw, and the force decreases until it reaches a minimum at point D. Adhesion is measured by the force at point D. The point where the tip comes off the surface is called the pull-off point; this often coincides with the minimum force. Once the tip has come off the surface, only long-range forces affect the tip, and again, the force is too small or zero when the tip-sample separation is at its maximum (point E). (Web: Bruker. PeakForce QNM Principles of Operation, 2011). Forces acting between tip and sample deflect the cantilever. Adhesion forces is measured by detecting the force interaction during retraction of the tip from the sample surface (Escobar, et la., 2017).

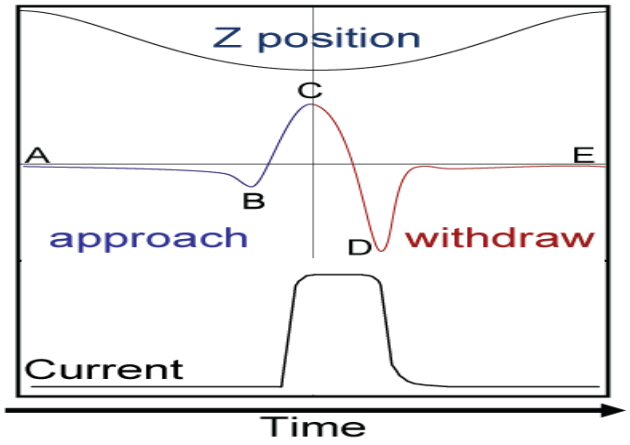

Figure 3.5 Peak force AFM Schematics and Force curve (Web: Bruker. PeakForce QNM Principles of Operation, 2011). The "heartbeat" or Z position is calculated as Force and Current as a function of Time during one Peak Force Tapping cycle. The blue indicates approach while red indicates retract.

To explain this further, the schematic representation of force curves Figure 3.6 below puts this into perspective.

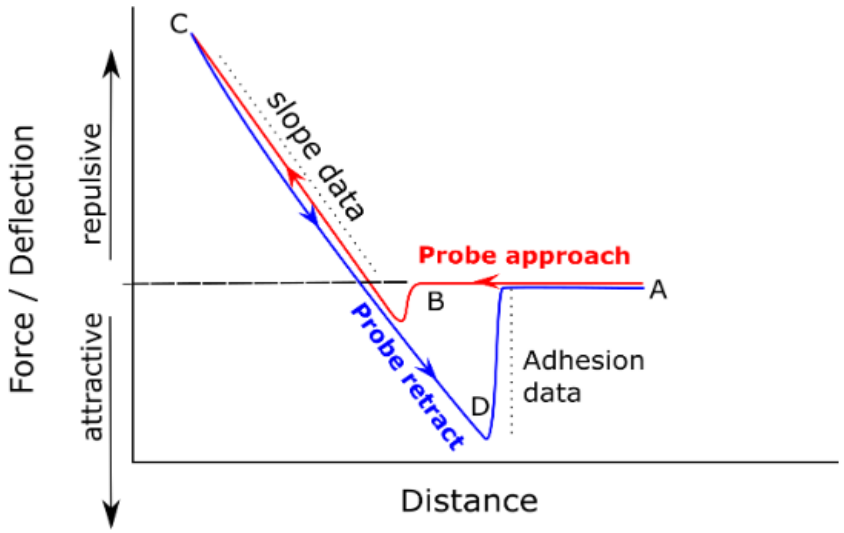

Figure 3.6 Schematic representation of force curves (Hertz model). In adhesion experiments, data recorded during tip retract is used to calculate the adhesion. At point (A) the tip-substrate separation is large with no interaction detected. The red line (B) in this figure represents "snap-in" of the cantilever, while the blue line represents probe

retract, “snap-out”. A complete force curve includes the forces measured as the probe approaches the sample and is retracted. Between points B C, the tip is in contact with the surface whilst the cantilever is bent to a certain degree. From point C, the cantilever withdraws from the surface (blue line). Tip-surface adhesion keeps them in contact (points B–D).

Alongside providing high-resolution 3D information of a wide range of sample, AFM can provide physical and mechanical properties through the quantification of the forces experienced by the cantilever while interacting with the sample. The basic principle of this method is to indent a material (sample) with an AFM tip of selected geometry and measure the applied force from the bending of the AFM cantilever. Fitting the force-indentation curve Figure 3.5 to the Hertz model Figure 3.6 to the corresponding tip geometry gives quantitative measurements of material stiffness (Thomas, et al., 2013). During the retracting cycle, adhesion forces may exist between the tip and the sample, and a pull-off force is required to detach the tip from the surface: this pull-off force is used to measure the adhesion forces between the cantilever tip and the sample and can be linked to wettability and adhesion properties of substrates

The focus of this research with the AFM and SEM was on material/membrane characterisation and testing the adhesive forces on these membranes measured by this Quantitative Nanoscale Mechanical AFM (QNM-AFM). The QNM-AFM distinguishes between nanomechanical properties and deformation and so there is no ambiguity regarding the source of image contrast, as often occurs in other techniques. This information was obtained by scanning over the topography of the PSU and PESU samples, and thus obtaining the roughness, and from the adhesion channel that reflected real time forces with each tip-surface contact. It should be noted that adhesive forces are directly connected to sample wettability properties.

3.4 Set up of the AFM

The glass membrane slide was set up as the control experiment using microscope slides cut edges Ghäasel, srf. hydrophilic b/50. The glass membrane specification for

this study are: Ground edges, Hydrophilic treatment, Corners 45º, Dimensions 26x76 mm, Thickness 1.1 mm.

The data generated from the PK-AFM height images were used to calculate the surface roughness of the material determined by the distance between peaks and valleys within the samples. The roughness of the surface of an object determines the objects interaction with its surrounding environment. Instruments used in this work provide the ability to study adhesion at a very small scale as nanoscale surface roughness strongly affects the adhesion force between surfaces (Hoek, et al. 2003). Numerical values for surface roughness will be obtained from contact angle measurements. Rough surfaces tend to wear down more quickly in addition to having higher friction, though they tend to have better adhesion properties. The surface roughness of a selected area of the flattened sample image was calculated from the height standard deviation using the AFM software by measuring the Rq values and Ra of the height distribution.

3.4.1 Preparation of the samples for imaging

The PK- AFM imaging calibration was done on the glass slide (control) in PBS. Both the PSU and PESU membranes were rinsed with about 4ml of distilled water (dH2O). They were then dried with nitrogen gas (N2) and immobilized on a glass slide using normal double-sided Sellotape. PK-AFM analysis was done in pbs. AFM two-dimensional (2D) and three-dimensional (3D) images present details of all the 3 samples, PESU, PSU and glass surface of 10 μm. Scan Rate 1.00 Hz Areas were scanned in PK-AFM, using Bruker ScanAsyst probe, with an experimentally determined spring constant of 0.92N/m.

During scanning a constant force of 1.5nN, with a rate of 1Hz and an image resolution of 256 x 256 pixels was used. Features of the observed surface images are calculated quantitatively by surface analysis, with high resolution 3D topography, from which is possible to extract surface roughness, defined as the standard deviation of the Z values in a specified area. Surface roughness has been defined as the parameter commonly reported when characterising membrane topography, because of its effect on membrane properties, such as fouling, hydrophobicity and hydrophilicity Fischer,

et al., 2018). However, a surface does not have a single roughness value, Rq or RMS (Root mean square) and Ra (Roughness average) figures of an image are used for judging the roughness of the sample. Rq is the root mean square average of height deviation taken from the mean image data plane, and Ra is the arithmetic average of the absolute values of the surface height deviations measured from the mean plane. Ra provides an overall description of the surfaces height variations and is said to be less sensitive to large peaks and valleys (Kremer, et al., 2015). Equation for calculation of Ra is as below;

Equation 3.1 $$R_a = \frac{1}{n}\sum_{I-1}^{n}|y_i| \cdot$$

where yi is the distance from the average height of a profile (the mean line) for measurement i, and n is the number of measurements. Another parameter considered in the present work is the Rq, which is the same as root mean square (RMS) deviation of the material profile corresponds to the standard deviation of the height distribution, defined on the sampling length. RMS (see equation) provides the same information as Ra.

Equation 3.2 $$R_{Rms} = \sqrt{\frac{1}{n}\sum_{i=1}^{n} y_i^2}$$

where yi is the amplitude of point i, n is the number of sample points, RMS deviation indicates the root mean square along the sampling length (Maciaszek et al., 2014)

3.5 Scanning Electron Microscope (SEM) and Atomic force Microscope (AFM)

The researcher in this thesis did physically do the SEM experiment but dropped off the samples at the SEM laboratory for the SEM investigations to be carried out by SEM experts. Therefore, detailed information on the SEM is limited, but, for clarification of AFM and SEM tools, Table 3.1 below will highlight the most common difference between these two tools; For clarification of AFM and SEM tools, Table 3.3 below will highlight the most common difference between these two tools;

Features	AFM	SEM
Imaging	High Contrast High	Depth of Field
Dimensions	2 and 3 Dimensional	2-Dimensional observation of the topography
Measurements	Physical Properties	Chemical Composition
Environment	Vacuum ., Air, Liquid	Vacuum

Table 3.3 Summary of the differences between AFM and SEM.

SEM is operated in a vacuum as stated above and uses electrons in the imaging process, because of this, a special procedure should be followed in sample preparation, which makes it more complex to operate than the AFM. However, measurements of surface topography forces of the AFM are also strongly affected by the chemistry of the tip and sample surfaces (Kremer, et al., 2015). But, AFM is a suitable technique to evaluate any effect resulting from sample manipulation because it can be applied without any specific treatment unlike the SEM. Kim, et al., carried out a study in 1999 in which AFM was used to investigate the surface of PSU membranes, they confirmed that the AFM method provided useful information on the size and shape of pores and cavities on the surface as well as the roughness of the surface. And, according to their study, the pore sizes obtained from AFM observation were found to be more accurate than those obtained from SEM (Kim et al.,1999), since the potential of altering the pore structure of the membrane during sample preparation was eliminated.

3.6 Surface topography

The data generated from the AFM height images were used to find the surface roughness of the membranes surface using the *nano*-scope analysis 1.5 software (Bruker UK). It is important to study the roughness of the material, because surface topography can alter biological attachment of materials and their morphology (Stroh et al.,2004). And different synthetic topographic features of biomaterial like pillars, ridges, and grooves, are believed to influence the adhesion behaviour of various biological cells. It is believed that topographic features have an influence on the performance of the biomaterial. Hedayat, et al. supported this opinion by stating that surface topography is an important surface property and affects the performance of products at its application fields (Hedayat, et al., 2015). The topography of both the PESU and PSU were therefore analysed to complement the RBC adhesion results of these membranes.

3.6 Wettability of PSU and PESU

In this study, membranes wettability was evaluated using a KRÜSS DSA 25 (Hamburg, Germany) goniometer, for contact angle measurements. Indeed, when in contact with air the silicon nitride cantilevers tip used in this study are known to become hydrophobic due to adsorption of airborne hydrocarbons (Hans-Jürgen, et al., 2005). In liquid environments, a higher adhesion force is expected when the cantilever is brought in contact with surfaces presenting a higher degree of hydrophobicity (Baclayon, et al., 2010). In this way, it is possible to classify surfaces based on the relative degree of hydrophobicity. In particular, it is of interest for this study to quantify the degree of hydrophobicity in the two hydrophobic membranes PSU and PESU, to identify which one is more hydrophobic and to correlate this aspect with RBC-retaining ability of the membranes.

In all membranes used in HD, wettability is very important (Higuchi et al., 2002). The hydrophobicity of both PSU and PESU membranes can be improved and its biocompatibility enhanced by adding polyvinylpyrrolidone (PVP). To address hydrophobicity issues of the PSU and the PESU, solid surface modification methods of these membranes, whether chemical or physical, to reduce membrane surface hydrophobicity, have become the focus of research (Kaur et al., 2018).

3.6.1 Contact angle

To understand how the sample surface responds to water, their contact angles were measured, and that provided information on the interaction energy between the surface and the liquid. The angle at which water droplet interface converges with membrane material surface is the contact angle. This was measured using a contact angle goniometer. Contact angle, θ, is a quantitative measure of the wetting of a solid by a liquid (Baclayon, et al., 2010).

Contact angle is also defined geometrically as the angle formed by a liquid at the three-phase boundary where a water droplet and solid intersect. The well-known Young equation describes the balance at the contact.

Equation 3.3 $Ysv = Ysl + Ylv \circ \cos\theta \mathrm{Y}$

Where γsv, γlv, γsl is the surface tension of solid surface, liquid surface, and solid–liquid interface, respectively. θ is the inherent contact angle on the solid surface. The equation lays the foundation for the study of wettability (Shaoxian and Shizhu, 2019). Shaoxian and Shizhu also point out that this equation also ignores the vertical component of gravity and the surface tension of the liquid, and experiments show that they can be ignored in general (Shaoxian and Shizhu, 2019). It is worthy of note that the Young equation gives the definition of wettability of an absolute smooth surface. However, there is certain surface roughness on the actual surface, and therefore a completely smooth surface does not exist. This applies to both the material studied in this work, they are both rough but at varying degrees.

3.6.2 Sample preparation for contact angle

The wettability of the PESU and PSU, was tested using this tool (KRÜSS DSA 25) as already mentioned in the above section. Measuring the contact angle between 10 µl water drops and the substrates glass, PSU and PESU and the results are shown in Chapter 4 of this work. However, before the contact angle experiment, samples (PSU and PESU) were washed by dipping them in distilled water (dH2O) in a plastic petri

dish, and then dried using nitrogen (N2). The glass was separately washed in dH2O first, dipped in ethanol (EtOH) and lastly dried with N2.

To commence the calculation, 10 µL drop of distilled water was deposited on the substrate. Using the above-mentioned tool (goniometer) a baseline was positioned at the interface substrate-water drop and the angle between the substrate and the drop tangent was calculated.

3.7 Cell Counting

This final section of this chapter will focus on the material and technique used in counting the cells adhesions. RBC in shear flow show a variety of different shapes due to the complex interplay between hydrodynamics and membrane elasticity (Oberleithner, et al.,2015). It should be highlighted here that adhesion of cells to a substrate leads to a reduction in shape variability and to a flipping motion of the non-spherical shapes (Grzhibovskis, et al., 2017). Hence some RBCs seemed deformed and smaller when observed with optical microscope.

In all experiments, the researcher counted all the RBCs on all the slides at the end of each experiment using the optical microscope. It's worth noting that, cell counting is an important routine procedure and can be done in many ways. However, manual cell counting is at times the least expensive method of determining cell numbers. Having said that, Oberleithne, et al. criticized manual counting method saying it is the slowest, time consuming and most tedious. It can also be one of the least reliable due to the possibility for human error, particularly if performing many cell counts sequentially, and performing a large number of cell counts can also cause eye strain (Oberleithner, et al.,2015). Popescu et al. reiterated that counting cells is often a necessary but monotonous step for in-vitro cell culture (Popescu et al.,2008). But consistent cell concentrations ensure experimental reproducibility and accuracy (Dunn and Zicha, 1993). Despite the cell counting being a routine procedure, there is still no comprehensive solution for this it, that is why in some cases it will still be performed manually. If a researcher's need is to count cells in a sporadic or infrequent way, and if a high degree of precision in the cell count is not required, then hemocytometers can be a good choice. For more frequent use, when greater accuracy is required, or in higher-throughput applications, counting chambers fall short. Additionally, human

counters are often poor at discerning between multiple types of cells in a suspension unless the differences in size and / or shape between the various cell types are extreme (Oberleithner, et al.,2015).

All the slides were divided in three equal sections, allowing the microscope imaging field to move, scan and evaluate each section. These sections were then labelled: 1 Top (section on the upper segment of the area scanned). 2. Centre (The section immediately below the top section) 3. Middle/m (the middle section, but below both the top, and the centre). Similar to what is depicted on the haemocytometer below figure 3.6. But unlike on the below haemocytometer, 1, 3 became the top section, 5 was the middle section and 2,4 was the bottom part.

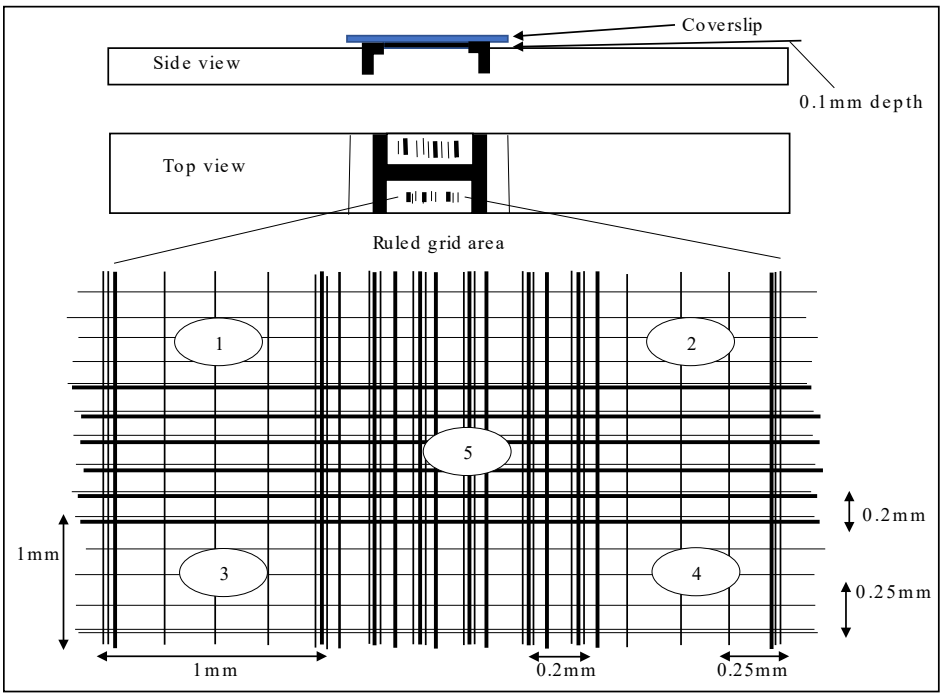

Figure 3.7 Hemocytometer, top, side, and ruled grid area under high magnification (Krediet et al., 2015).

In the Hemocytometer above Figure 3.7, 1 mm^2 areas labelled ①, ②, ③ and ④ have ruling separated by 250µm; while region ⑤ has much tighter ruling of 50µm.

3.8 Statistical evaluation

Results are presented as mean ± standard deviation (SD). All comparisons of results were analysed with Student's t-test. And, all statistics were reported in two-tailed form. Comparisons between means among the study samples were performed using t-Test. A Value of $p < 0.05$ was considered statistically significant. In addition to Student's t-test used for continuous variables, two-tailed Mann-Whitney test was also used.

The t-test is a parametric test of the difference in mean between two groups that assumes that the data are normally distributed, and the groups have equal variances (Whitley and Ball, 2002). t-Tests are widely used by researchers to compare the average values of a numeric outcome between two groups. The availability of software for these statistical tests has simplified the application of these complex statistical analyses and hence facilitates researcher in this work to use them.

A Mann-Whitney nonparametric test was also applied to compare the distributions of unmatched groups. A p value of less than 0.05 was considered significant. Correlation was calculated with Spearman correlation coefficient and p value. Non-parametric methods are referred to as 'distribution-free tests' because generally they don't require any assumptions about underlying population distribution. Nonparametric methods may be applied when the data do not satisfy the distributional requirements of parametric methods (Lee, et al., 2015). There are no limited assumptions in these, and so wider range data are applicable.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Introduction

This study was carried out to investigate the adherence of RBCs to two polymeric membranes (PESU and PSU) usually employed in HD treatments. The study also analysed the topography of the PSU and PESU membranes using the AFM, SEM and goniometer. It is known that cells actively sense and respond to changes in their surroundings; hence this study aimed to understand RBCs adhesive phenomenon to PSU and PESU membranes. Langer at al. reported that cell information and content in the adhesive environment is said to be encoded both in its composition and its organisation on a very tiny scale measured in nanometre to micrometre scales (Langer, et al., 1993). Therefore, the tools used in this study were appropriate to conduct this investigation because of their ability to deal with tiny scale analysis and measurements. For example, they are capable of quantifying surface roughness of samples down to the angstrom-scale.

4.2 Atomic force microscope (AFM) study of the topography of membrane samples.

The AFM and SEM were used to run a surface topography analysis of the three test samples to investigate the relevant microstructure surface characteristics. To better comprehend the various contributions, magnitude to error in the AFM analysis and measurements, and for better evaluation of the PESU, PSU and glass, other quantitative parameters were also obtained such as the projected surface area, Body Y dimension and the image surface area difference.

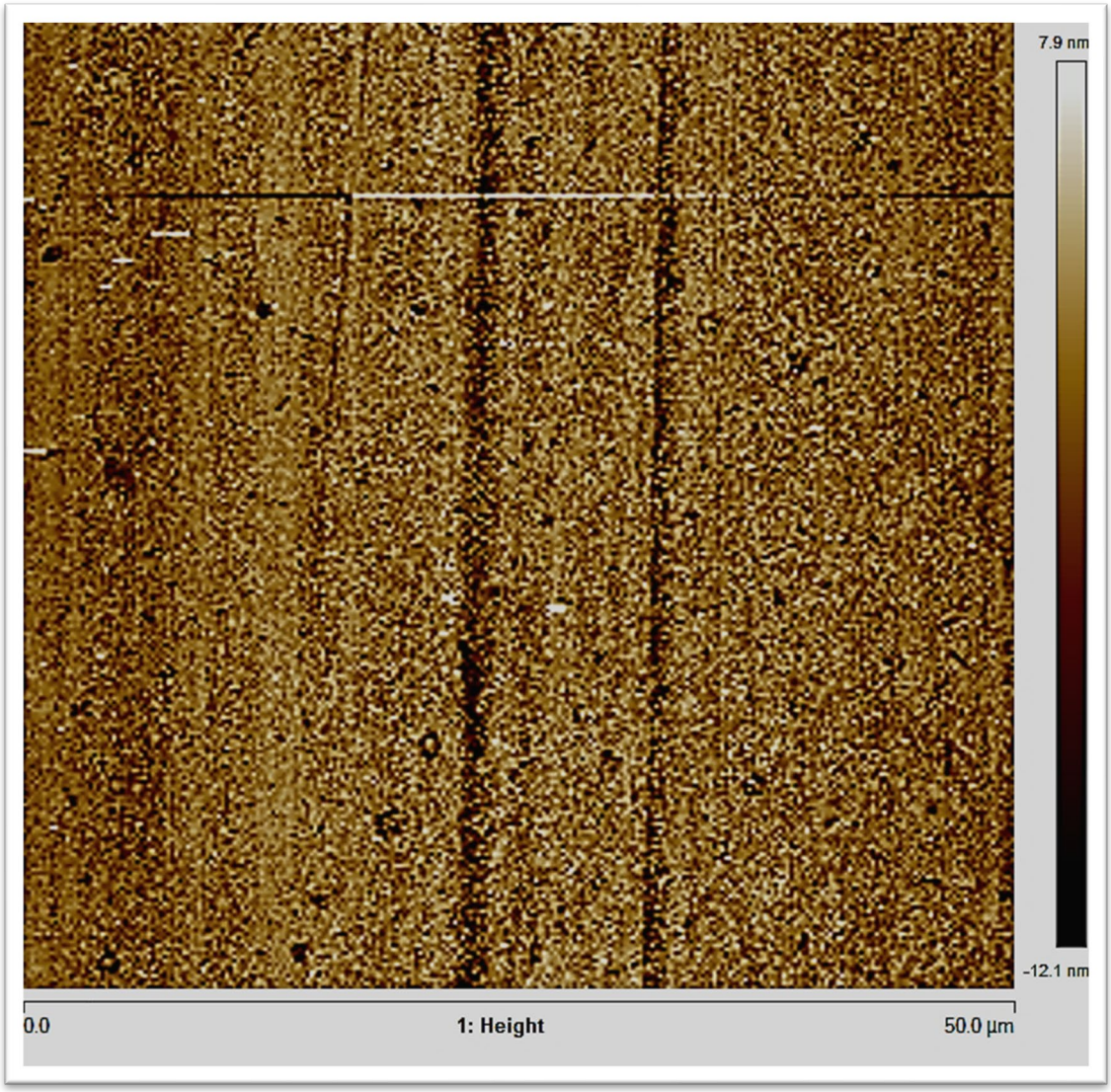

Figure 4.1 Topography of glass surface, 50 µm × 50 µm field of view. 2D-surface topographic image. This shows the morphology on glass material presenting a highly roughness patterns on topography.

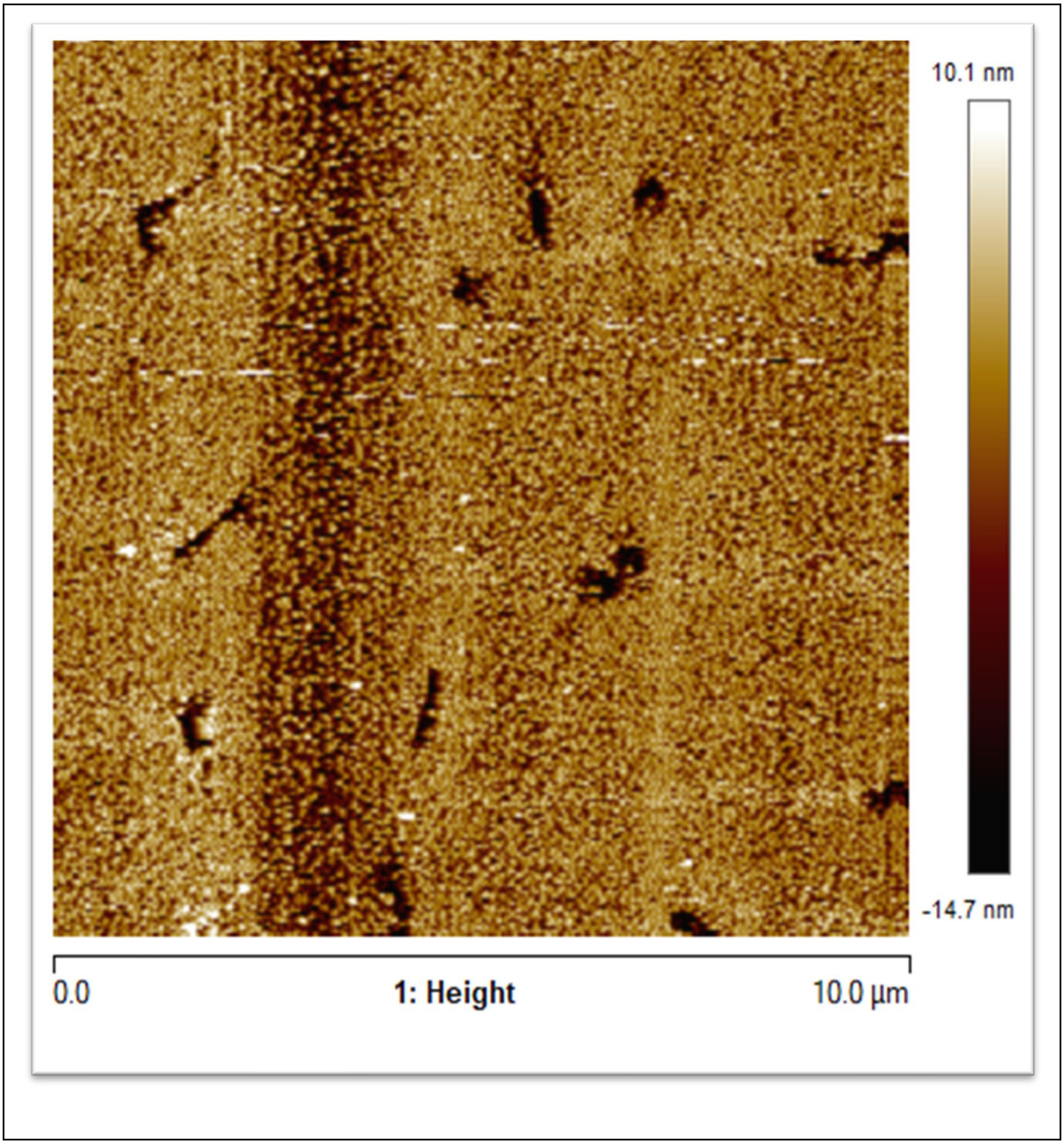

Figure 4.2 Topography of glass surface 10 µm × 10 µm field of view. 2-D image Topography of glass Image. This shows the morphology on glass material presenting an isotropic pattern on topography, similar to Figure 4.1.

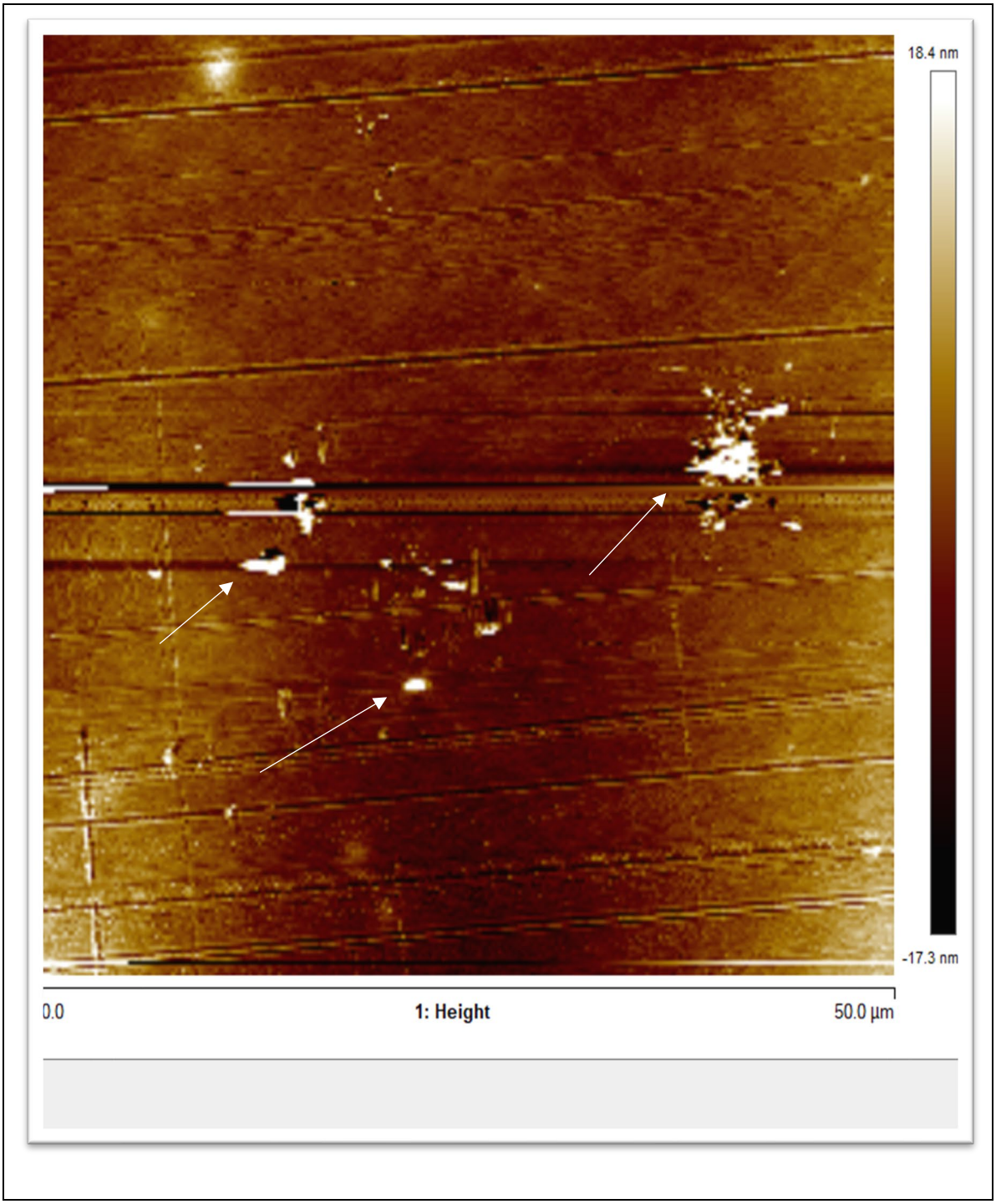

Figure 4.3 Topography of PESU surface, 50 µm × 50 µm field of view. This is the AFM 2-D image and it shows the morphology of the PESU with clear visible horizontal line structures. And some clear artefacts (arrows).

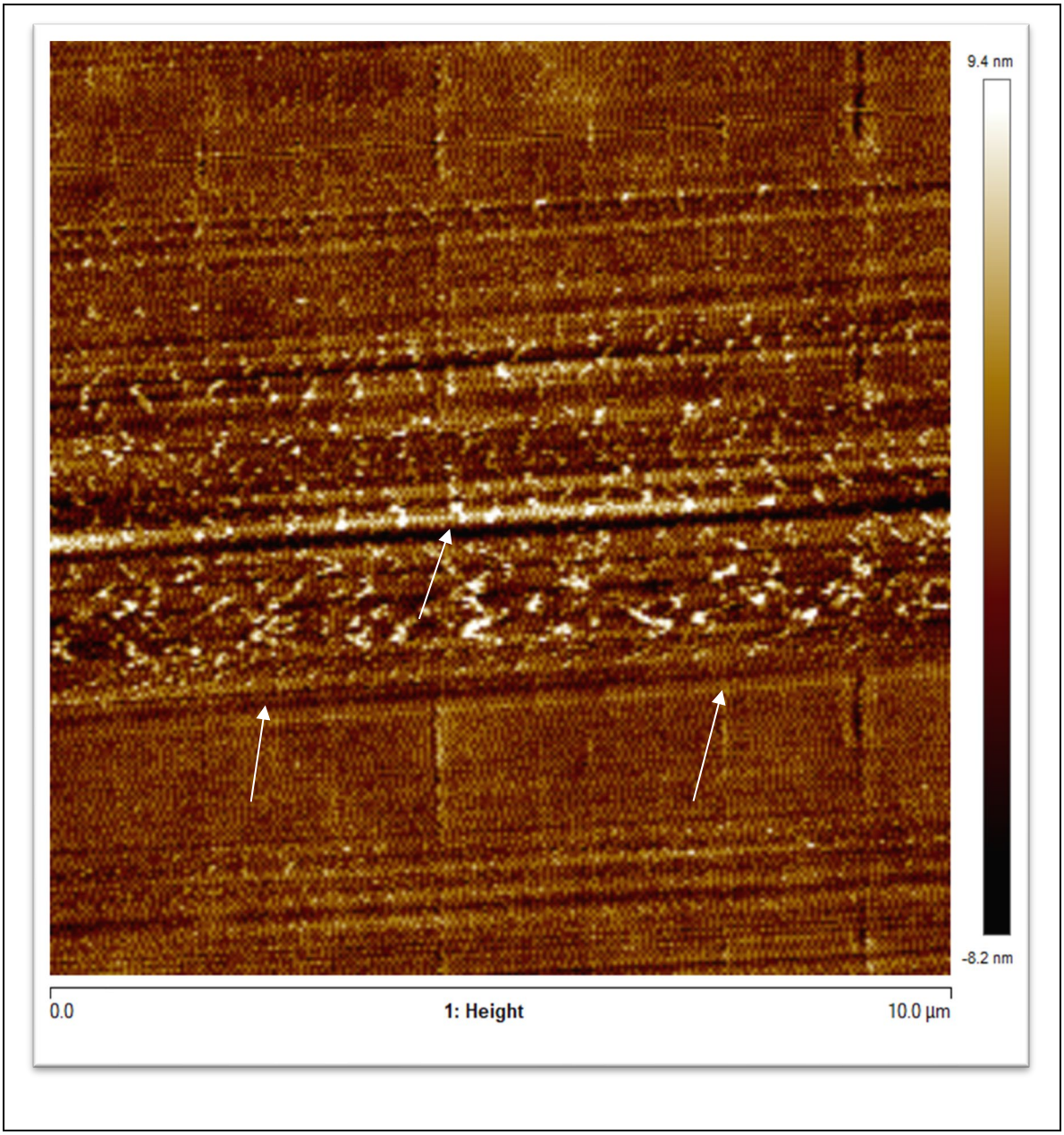

Figure 4.4 Topography of PESU surface, 10 µm × 10 µm field of view measure. This 10µm resolution PESU is clearer view of Figure 4.3. The horizontal lines structures are more visible (arrows).

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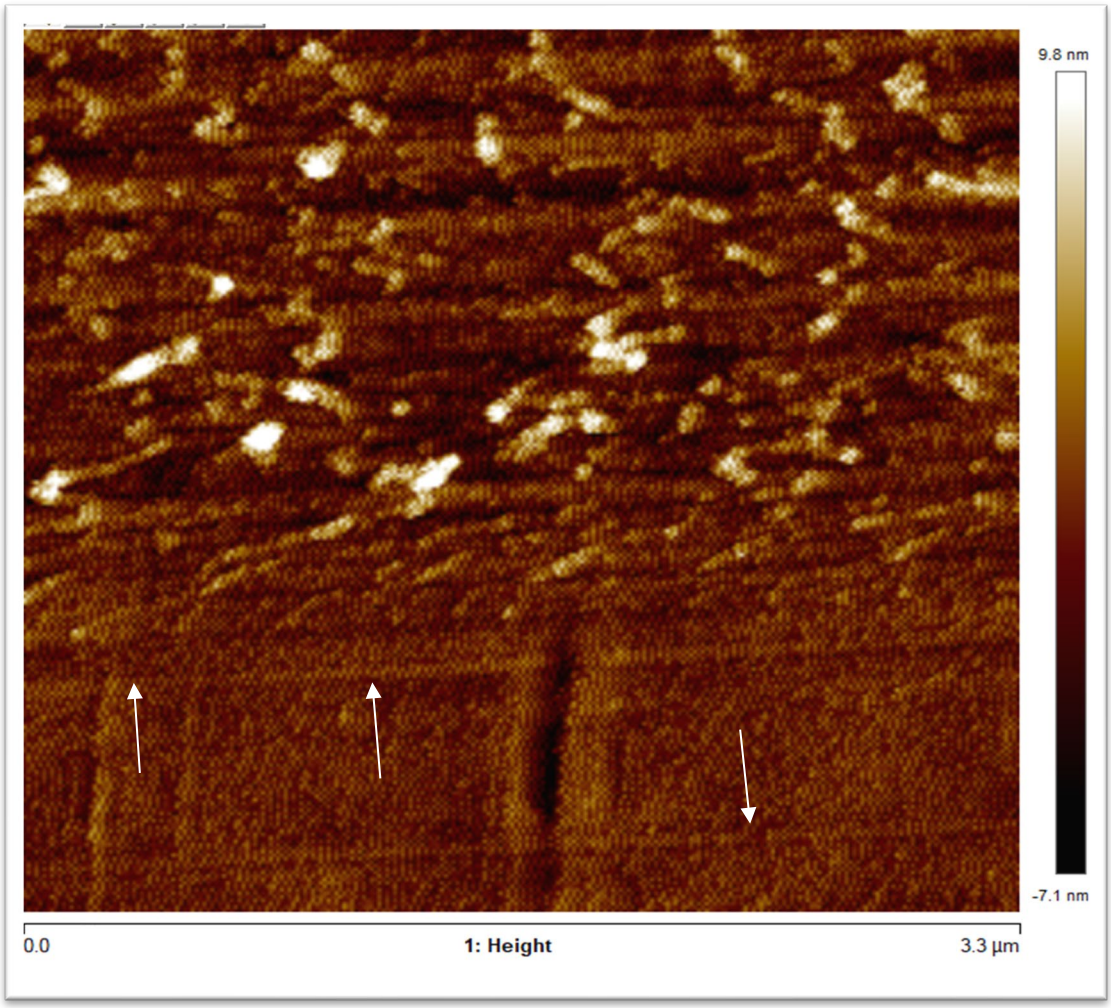

Figure 4.5 Topography of PESU, surface 3 µm × 3 µm field of view. 2D image. This is and AFM image showing visible lines structures (arrows). The upper part of the image seems fuzzy (unclear) possibly due to the AFM noise distortion.

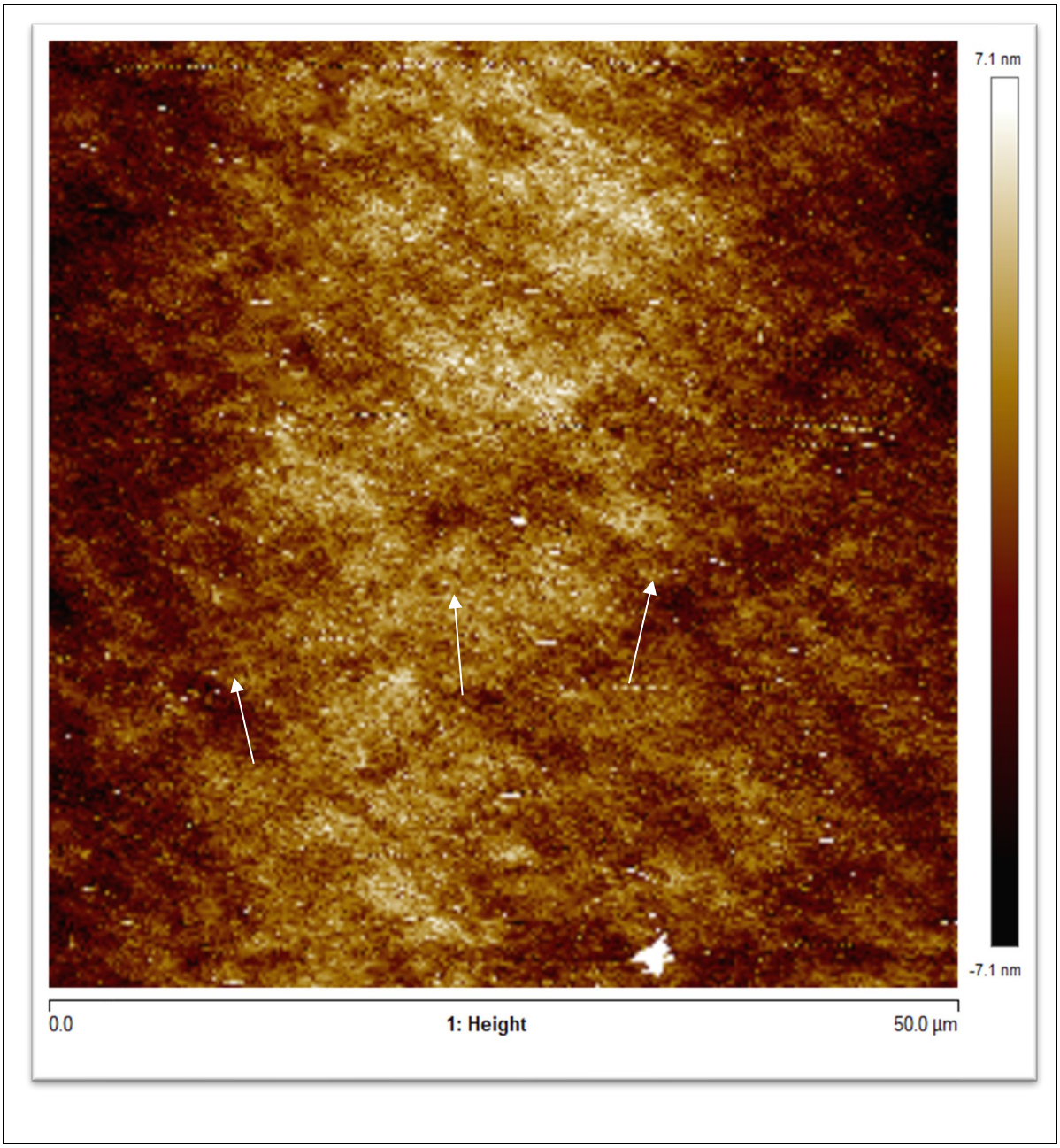

Figure 4.6 2D Topography of PSU surface 50 µm × 50 µm field measure. 2D image. This AFM image exhibits clear spongy structure on the surface with visible bulges or swelling (arrows) that seems to distort a flatter surface.

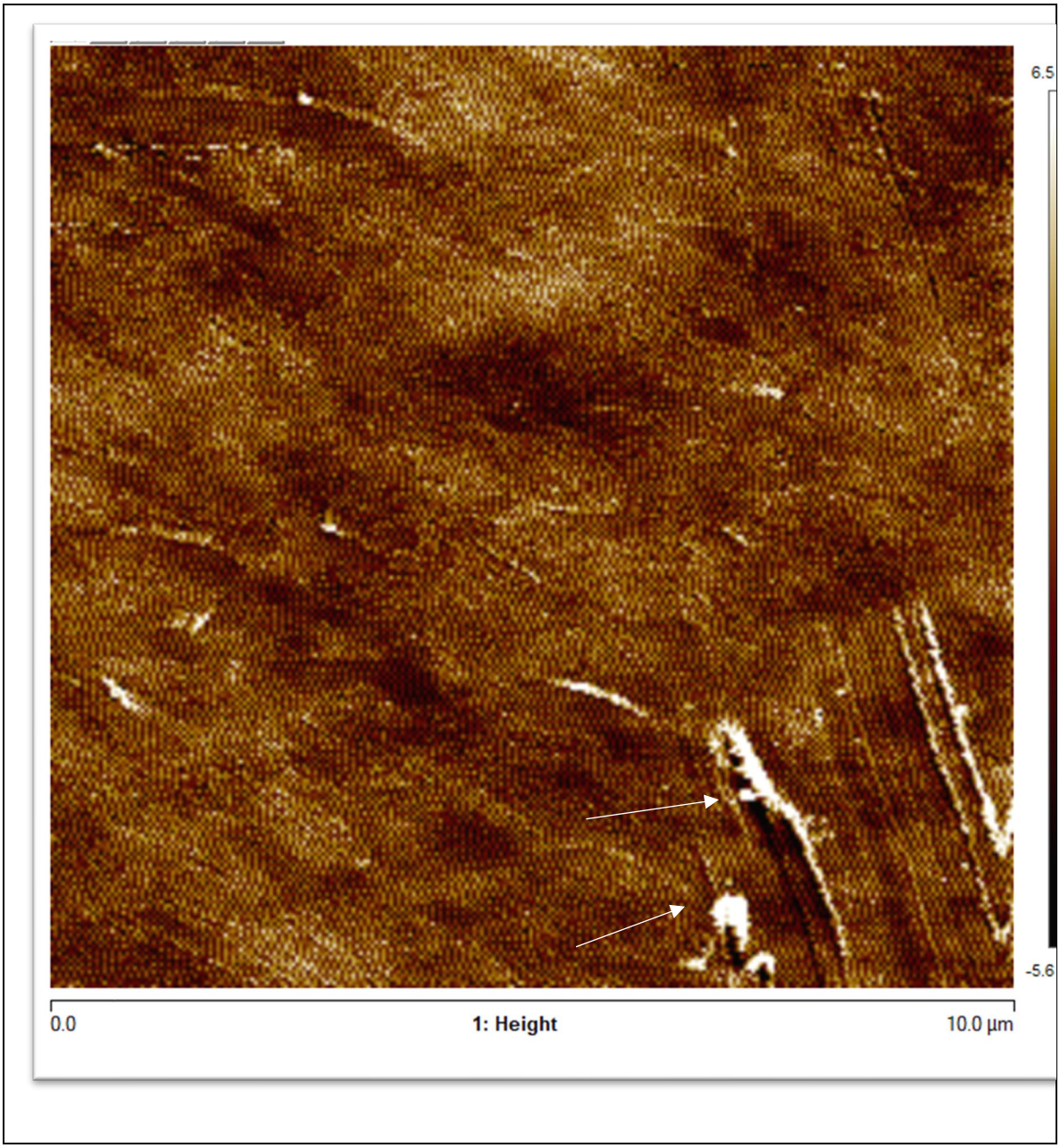

Figure 4.7 Topography of PSU surface 10 µm × 10 µm field measure. 2D image. This AFM image exhibits clear spongy structure on the surface. Visible white patches (arrows) represents image artefacts.

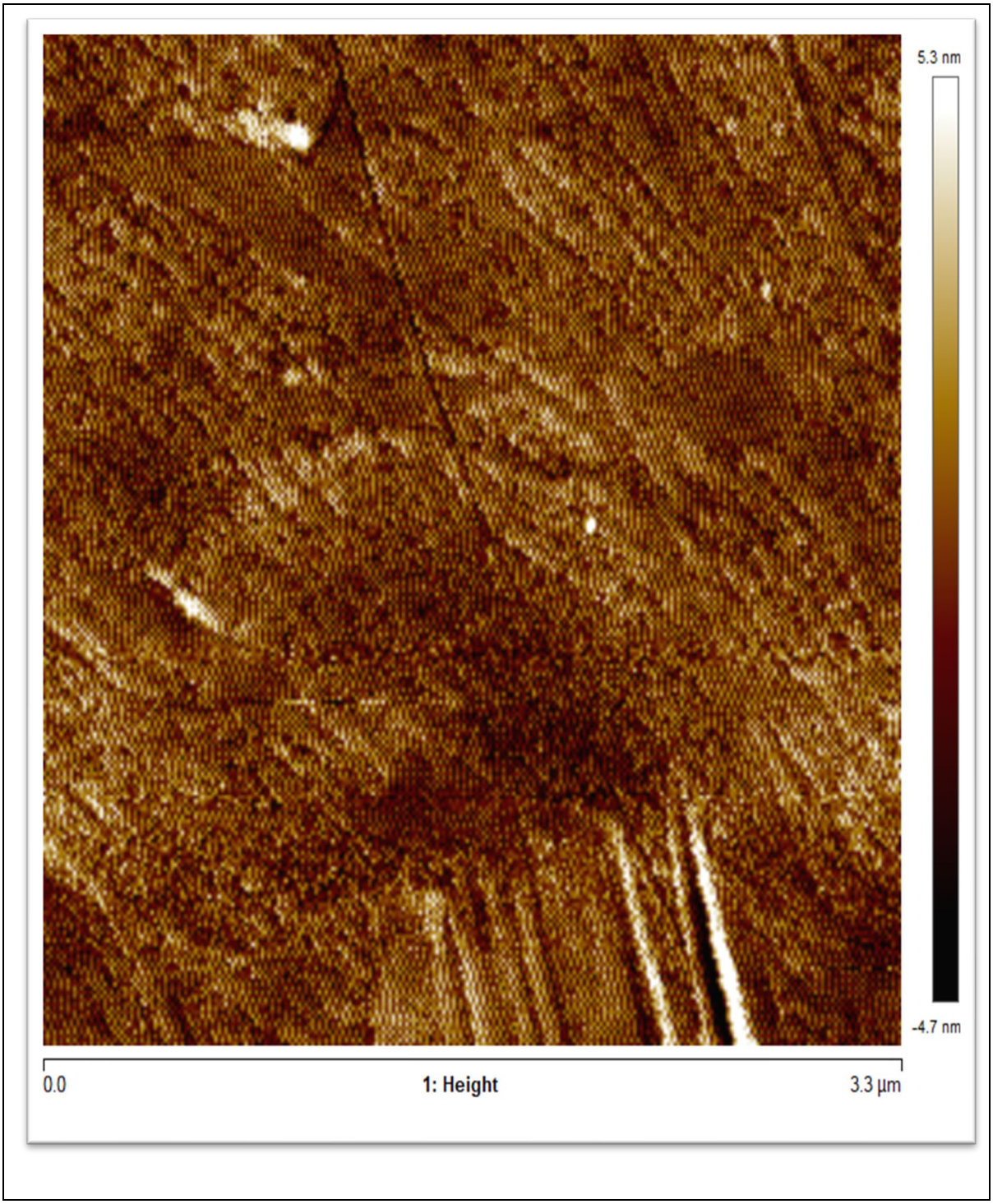

Figure 4.8 Topography of PSU surface 3.3 µm × 3.3 µm field measure. 2D image. This AFM image presents similar morphologic appearance as the above Figure 4.7 and appears to have smoother surface.

Based on the images above, it is notable that all the images look quite different, simply by visual observation. However, it is important to pay attention to the quantitative differences in these membranes to ascertain their differences and/or similarities. These quantitative differences are shown in table 4.1

4.3 Atomic force microscope (AFM) and Scanning electron microscope (SEM) membranes analysis illustrating the differences in topography of PESU and PSU

By using both the AFM and SEM, the researcher exploits the strength of each instrument on the same set of samples analysed.

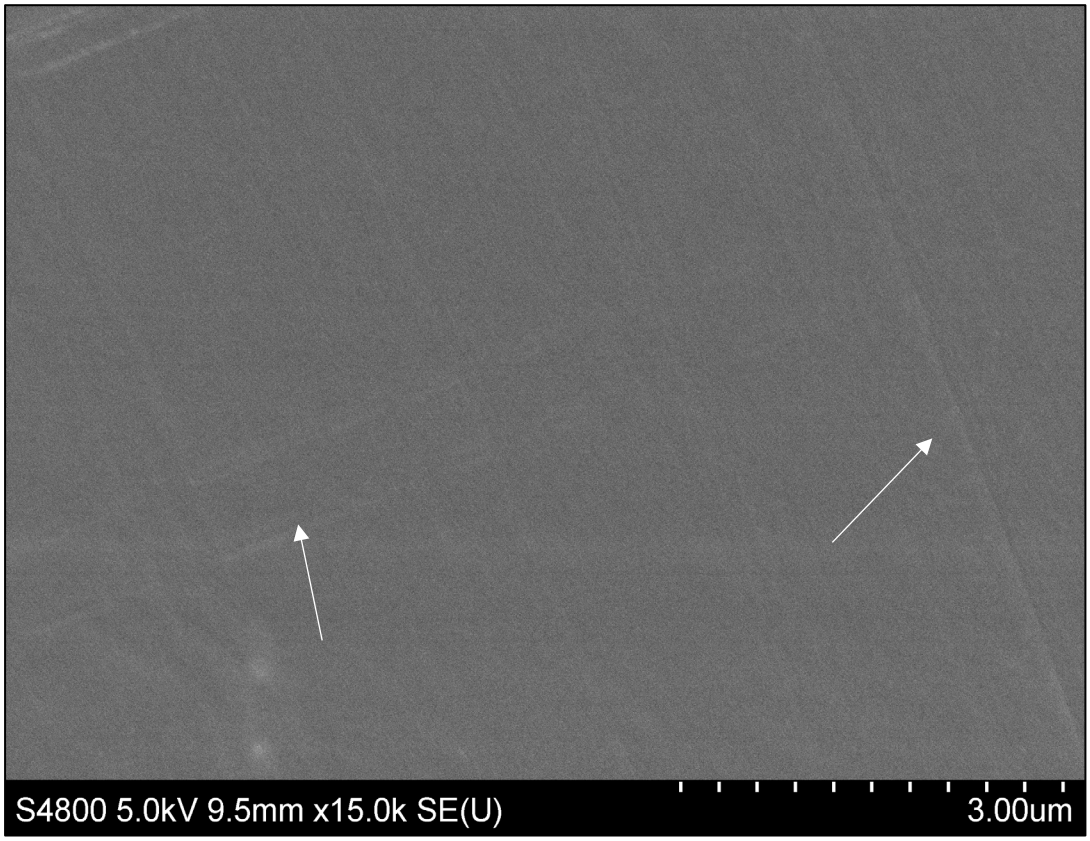

Figure 4.9 PESU image captured using FE-SEM S4800 type imaging at ultra-low voltages of 5.0Kv (voltage of the primary bean at the sample). Visible lines like structures (arrows) can be seen on the morphology of the figure.

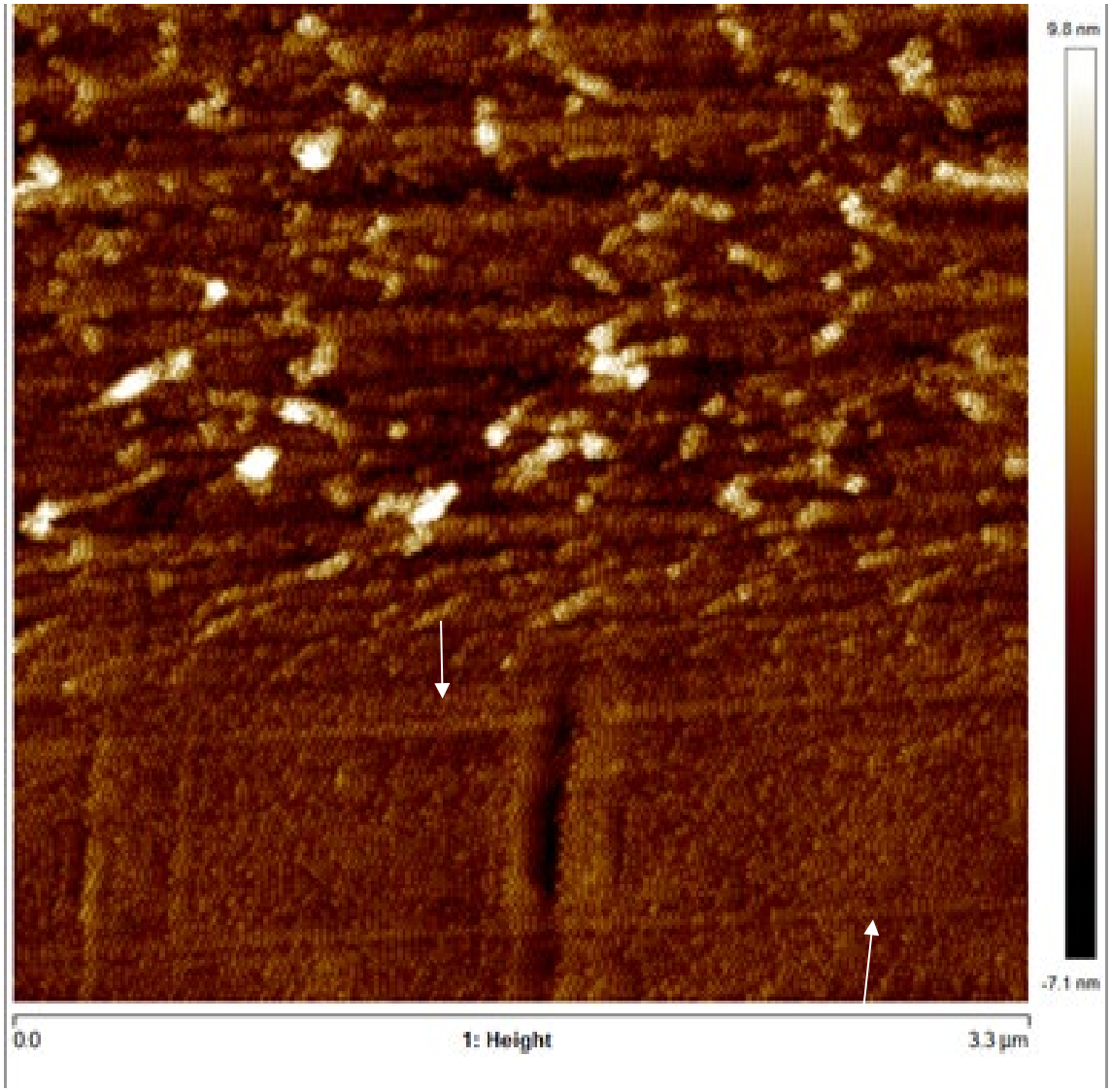

Figure 4.10 PESU AFM image, with 3.3 µm x 3.3 µm field measure on the scanned area. This AFM image showing the lines structures (arrows), but also tiny peaks can be observed with the surface. The line structures seen here are similar to those observed in the SEM Figure 4.9 above.

On the SEM and AFM comparison of the PESU membrane in Figures 4.9 and 4.10 at 3µm field of view, the structural patterns (lines) between the SEM and AFM becomes clear (arrows). These “lines” are however more visible across the PSU membrane than on the PSU membrane Figures 4.7 and 4.8. It is therefore clear that these two membranes’ morphologies are different.

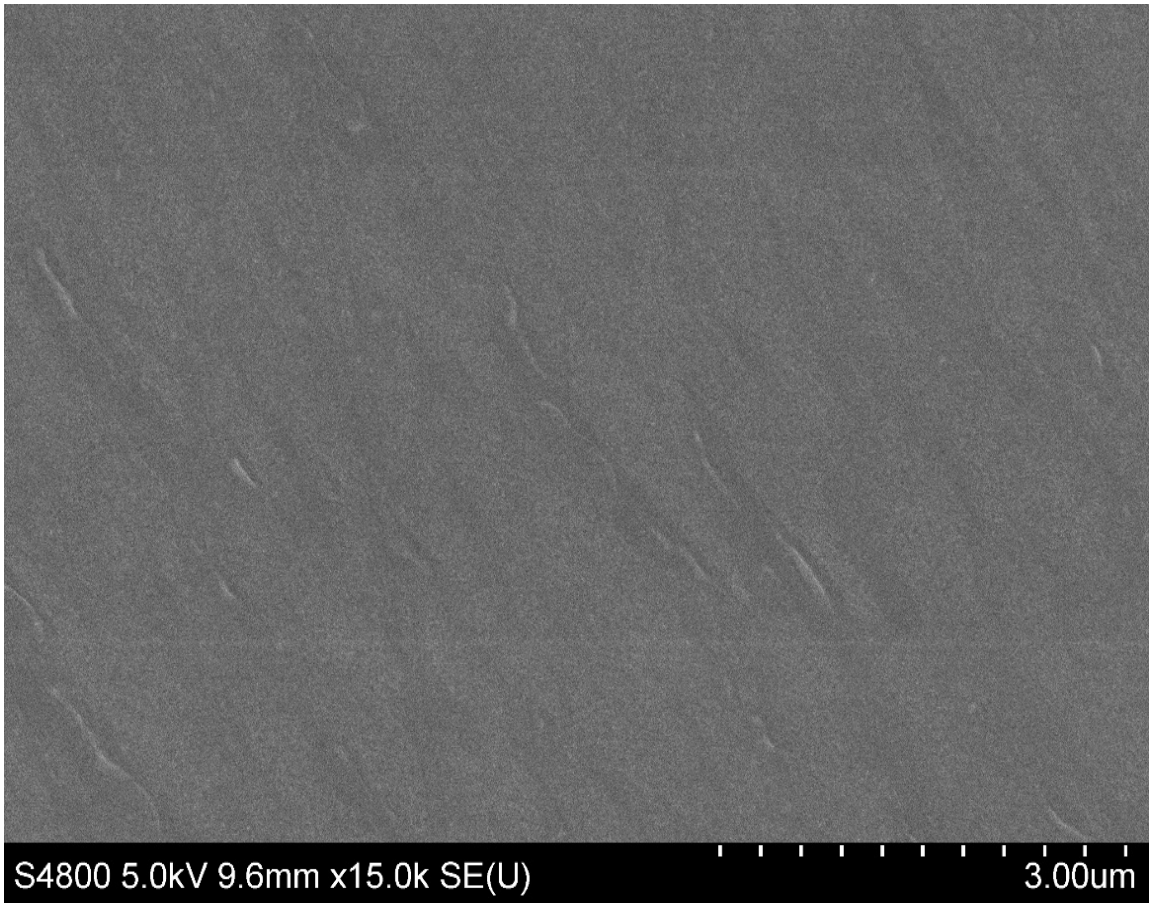

Figure 4.11 Topographic image of PSU captured using FE-SEM S4800 type imaging at ultra-low voltages of 5.0Kv (voltage of the primary bean at the sample). The morphological appearance of this figure resembles that of figure 4.12 below.

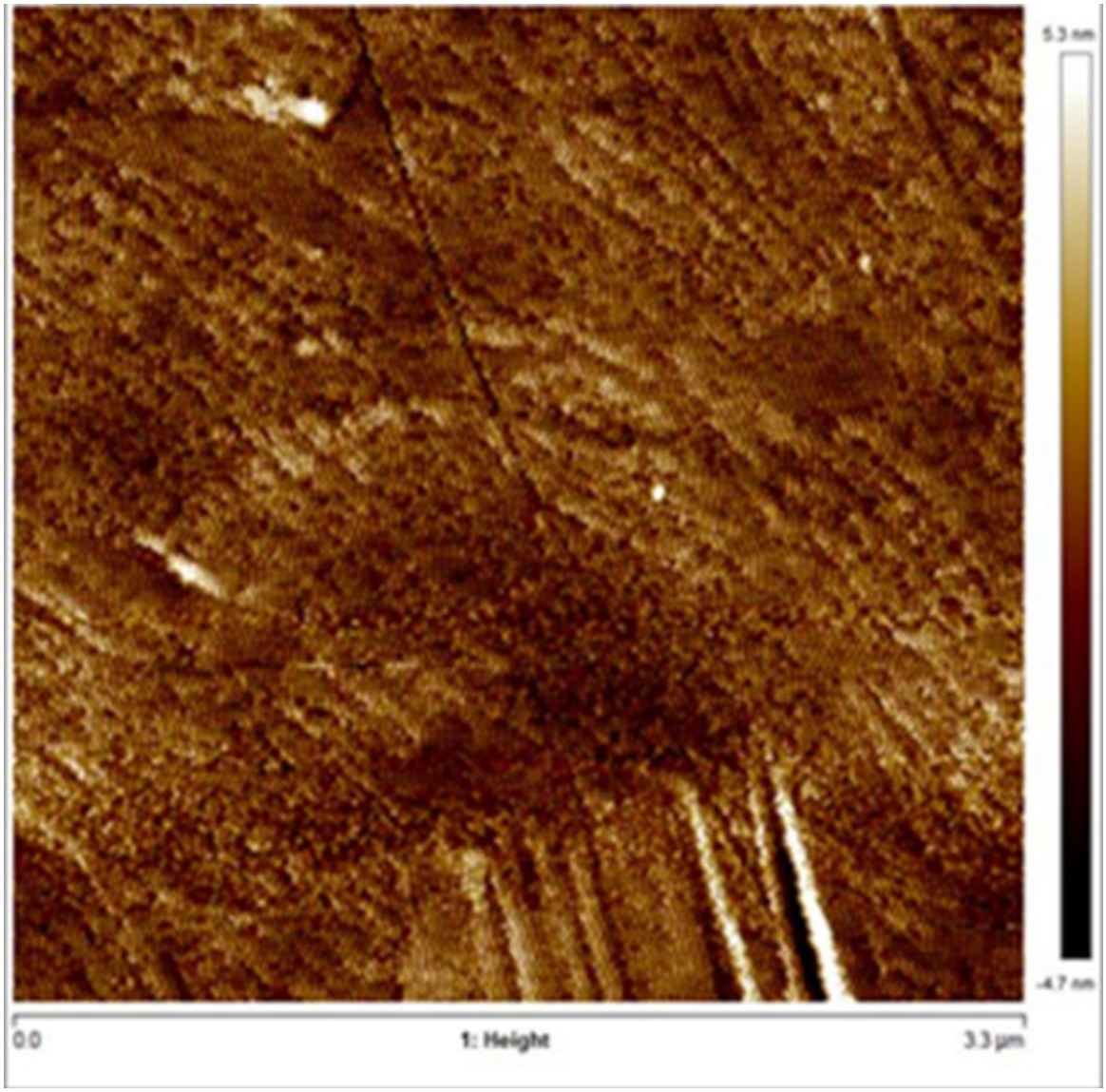

Figure 4.12 AFM topographic image of the PSU. This AFM image is presented to compare AFM and SEM images of the same membranes. They both appear smooth and no visible lines that were seen on the PESU images.

The PSU SEM image in Figure 4.11 shows smoother surfaces and there is obvious membrane integrity and homogeneous morphology compared to the PESU in Figure 4.9 which has lines (arrows) across the sheet. On the AFM images, the surface topography in Figure 4.10 revealed significant differences in surface structure when compared with Figure 4.12. There are no obvious “lines” as seen on the PESU images. The PSU appears more smoother in comparison to the PESU. The white spots seen

on the PSU is a result of artefacts. However, there is no sample that exhibited visible topographic alteration, they all showed uniformly clear surfaces, which means there was no sample damage prior to or during imaging of these membranes.

4.3.1 Summary of the AFM images of the topography of test membranes (Glass, PESU and PSU)

The morphology of PESU, PSU and glass are presented in above mentioned figures and their topographical differences area highlighted. Higher magnification images of the PESU revealed the presence of channels in the material (figure 4.4 and 4.5, arrows). Unlike PESU samples, PSU had spongy structure that formed network of sponges (figure 4.6, arrows). However, these spongy appearances on the surface of the PSU sample were more irregular than the channels seen on the PESU samples. In a similar study, the spongy structure for polymer membranes was a result of the hydrophilic character of these nanomaterials (Wienk, 1996). All the samples depict the numerous nanoscale features within the surface of the materials.

4.4 Assessment of membrane roughness using the AFM.

Surface topography and roughness have a significant impact on cell adhesion to the surface of the material (Kono, et al., 2012). In addition, studies have demonstrated that the existence of increasing roughness on the surface of membranes allows for interaction between cells and the material (Jun, et al., 2018; Cassereau, et al., 2015) results analysis showed that the largest roughness values were obtained for Glass followed by PESU membranes and the smallest was for PSU Figure 4.13. It should be noted here that all parameters describing surface roughness showed similar trends for all samples. The large experimental deviations (determined here as standard deviations) of the Ra parameter denote the magnitude of variations within a given sample type. Thus, a large error of a surface morphology value for a given PESU membrane also indicates a large variability in surface topography. The surface roughness of all the three samples are summarised in Figure 4.13 and Figure 4.14 below. PSU presents lower roughness parameters than glass and PESU. A membrane with lower roughness has less distinct peaks and valleys, but these are

clearly more marked on the glass sample. These valleys are said to provide the path of least resistance (Wienk, 1996); therefore, a majority of permeate are transported through the membrane via these valleys. The box plot results below Figure 4.13 indicates the arrangement surface roughness by plotting data from all three samples and arranging them according to roughness values. Statistical analysis on the ra values shows that glass has ra roughness of 2.6 ± 0.4 nm, PSU of 1.4 ± 0.4 nm and PESU of 2.4 ± 0.6 nm. All values are significantly different (Mann-Whitney p value < 0.05

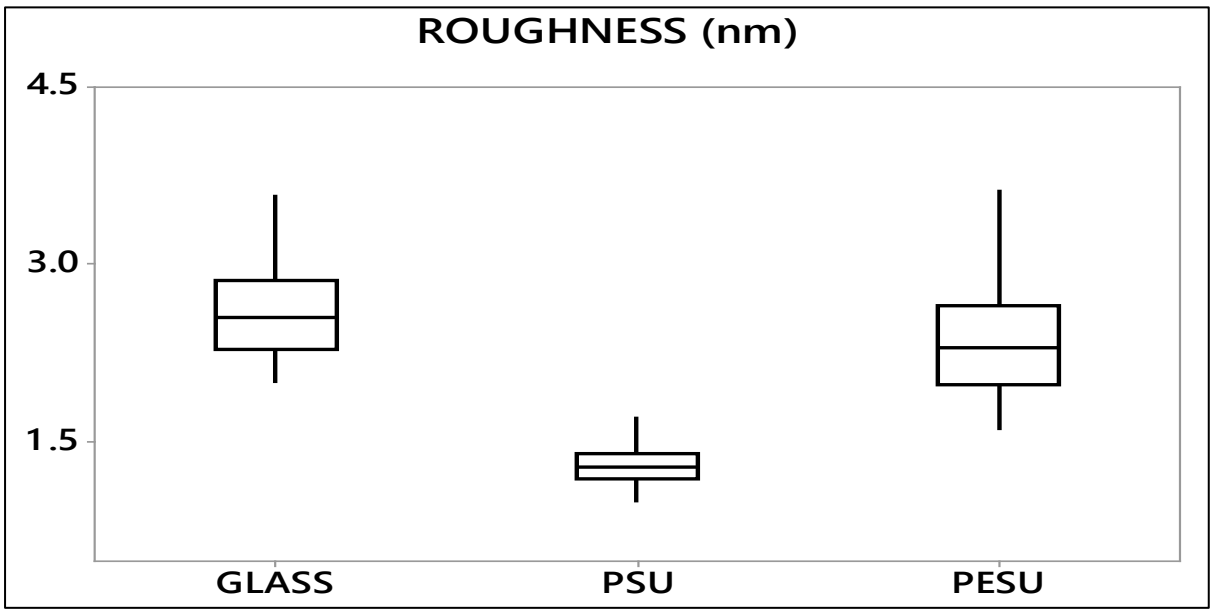

Figure 4.13 Roughness of the test membranes calculated using the AFM (Quantitative Analysis). The data are expressed as mean ± SD measurements; Student's t -test. Statistically significant difference between the PSU and the Glass ($p < 0.05$).

Surface roughness measurements derived from AFM images confirmed that decreasing Rq values were in the order of glass, PESU and PSU being the lowest, as indicated in the below Table 4.1 Different scan areas were used to view and compare surface roughness for all membrane samples. The membrane roughness parameters were achieved by measuring the average roughness (Ra) as indicated in Table. 4.1. The surface roughness is clearly more pronounced on the glass, then on PESU. PSU had the least roughness structures, which is in keeping with the (Ra) and (Rq)

roughness values. The AFM scans also revealed structural characteristics which support the material roughness of the sample surfaces on physical examination. Consistent with rougher material, the glass contained relatively more nano fibre-like structure than the PESU and PSU. The white patches on these images represent artefacts and are not considered to contribute to the measurement and analysis of the membranes.

Membrane parameters	**PSU**	**PESU**	**Glass**
Image Surface Area	11.2 μm²	100 μm²	11.4 μm²
Image Projected Surface Area	11.0 μm²	100 μm²	11.0 μm²
Image Surface Area Difference	1.92 %	0.342 %	3.68%
Image Rq	2.12 nm	2.19 nm	3.04 nm
Image Ra	1.46 nm	2.43 nm	2.60 nm

Table 4.1 AFM analysis results on surface roughness and quantitative analysis.

Based on the image surface area of PESU 11.2 μm², PSU 10.0 μm² and 11.4 μm² for glass, the surface roughness values of PSU and PESU are not too far apart, with PESU (Ra) at 2.43 nm and PSU (Ra) at 1.46 nm. Both surface roughness values are clearly much lower than that of glass which is 2.60 nm

Figure 4.14 shows the Ra and Rq distribution parameters of the samples in correlation with the surface area difference and indicates that glass had the highest value and surface area difference percentage.

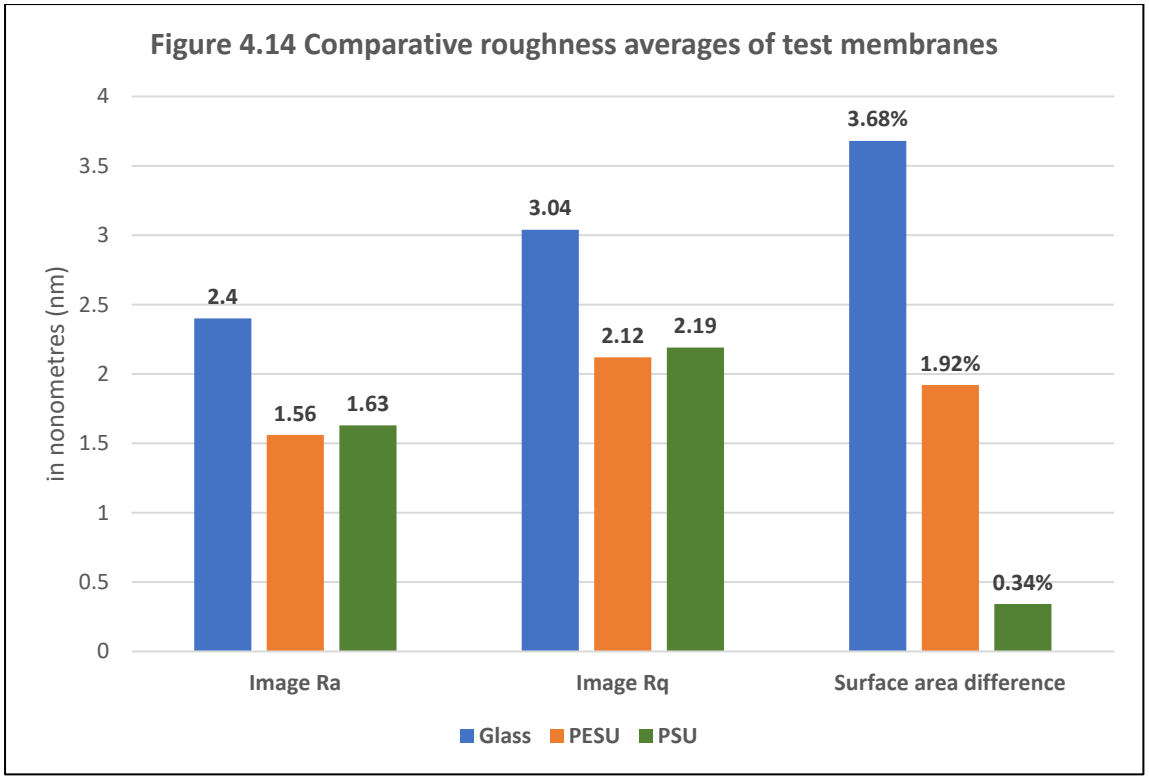

Figure 4.14 captures the roughness average of the membranes in correlation with the surface area difference. This is Ra and Rq values of the Glass (blue), PESU (orange) and PSU (green).

4.4 Hydrophobicity and adhesion force in AFM

As shown in several studies, adhesion forces as detected by AFM can be used to assess the wettability properties of surfaces (Hans-Jürgen, et al., 2005; Spijker, et al., 2003). Findings from the study of Spijker, et al. showed that hydrophobic forces were strongest in aqueous medium (Spijker, et al., 2003). In this study, to measure adhesion forces and hydrophobicity, we considered adhesion events, calculated as percentage of force curves which showed clear adhesion forces to the total curves. For each substrate, a total of 130-150 force curves have been acquired on three different areas at 250 μm^2 each. The total adhesion events for glass amounted to the 11% of the total force curves, for PSU this value increased to 31%, while for PESU all force curves experienced an adhesion force (100% of adhesion events). This strongly suggests that the hydrophobicity increases in the following order: glass, PSU, PESU. Though

literature reports that PSU and PESU are both hydrophobic, from this work it seems that PESU presents a higher hydrophobicity compared to PSU.

4.4.1 Contact angle results

Wettability plays a major role in interaction with cells and biomaterials. It is measured by contact angle. The results of contact angle are shown in figure 4.15. In these results, average contact angles for PSU was lower than PESU membrane. Thus, the topographic material of PSU resulted in a decrease of the water contact angle, the surface wettability of PESU membranes was slightly higher compared to PSU, but lower than for glass. The results indicated that the PESU membranes were more hydrophobic than PSU.

In Figure 4.15 glass has the lowest hydrophobicity, hence highest wettability, as revealed by the very low contact angle between glass and water droplet. Hydrophobicity increased in the case of PSU and PESU as revealed in 10 measurements per substrate type, with PESU presenting the highest contact. The wettability trend seen with AFM force curves is then confirmed by contact angle measurements, further affirming that of the three membranes tested, PESU is the most hydrophobic, followed by PSU and then glass. Hysteresis between receding and advancing contact angle increased with the hydrophilicity of the membrane surface.

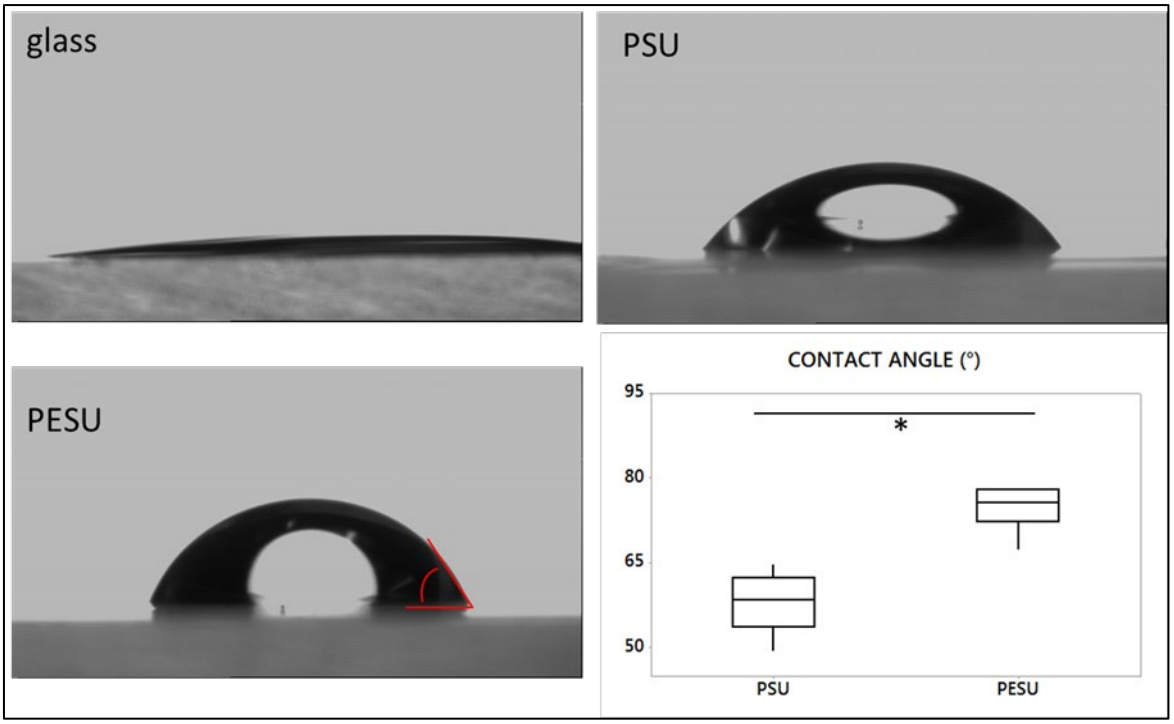

Figure 4.15 Contact angle images of the glass, PESU PSU. The plot on the bottom right hand quantifies the contact angle difference between the PSU and PESU based on these measured contact angle. measurements and hydrophobicity. This shows contact angles for 10µl water droplet on glass (top left), PSU (top right) and PESU (bottom left). To minimize experimental errors, the average value of contact angles was calculated by randomly selecting a few different locations on each sample. In PESU the contact angle measurement is quite visible.

As indicated in the chart (bottom right) PESU presents a contact angle significantly higher than PSU (74.6° ± 3.8° and 57.8° ± 5.2°, respectively. T-test p value <0.05). The glass contact angle was barely measurable using the same program. From this information the low contact angle values indicate that the liquid spreads on the surface while high contact angle values show poor spreading. If the contact angle is less than 90° it is said that the liquid wets the surface, zero contact angle representing complete wetting. If contact angle is greater than 90°, the surface is said to be non-wetting with that liquid. Chieng, et al. explained this by stating that the water droplets on the surface of the hydrophobic material will flow very easily and retain its spherical shape with

contact angle more than 90 degrees (Chieng, et al., 2019), while superhydrophobic materials possess large contact angles above 150 degrees and are difficult to wet. In contrast, for hydrophilic surfaces the water droplets spread out far, and the contact angle is exceedingly small with less than 90 degrees. On these surfaces, the water droplets do not roll but glide. The higher the contact angle, the higher the hydrophobicity as explained by Sangeetha, et al., they stated that contact angle for hydrophilic samples would be < 90° and >90° for hydrophobic samples (Wei, et al., 2020). Therefore, the higher the contact angle, the higher the hydrophobicity.

4.4.2 Force curve

Force-distance curves have been employed for the study of numerous materials properties and for the characterization of different kinds of surface forces (Hans-Jürgen, et al. 2005). To measure the force curve on these samples, the probe of the AFM was supported on a flexible cantilever and the AFM tip gently touches the surface and records the small force between the probe and the surface (Lyubchenko, et al.,2011). However, the AFM does not directly measure force or indentation. Instead, force and indentation (a force curve) are calculated from the deflection and vertical position of the AFM cantilever (Baclayon, et al., 2010). This force can be described using Hooke`s law. Hooke's law can be formulated as;

Equation 4.1 $F = -kx.$

In this equation, F represents the equal and oppositely directed restoring force that causes elastic materials (cantilever) to return to their original dimensions.

The main experiments and outcomes concerning the measurements of such forces on the PSU, PESU and the are highlighted below;

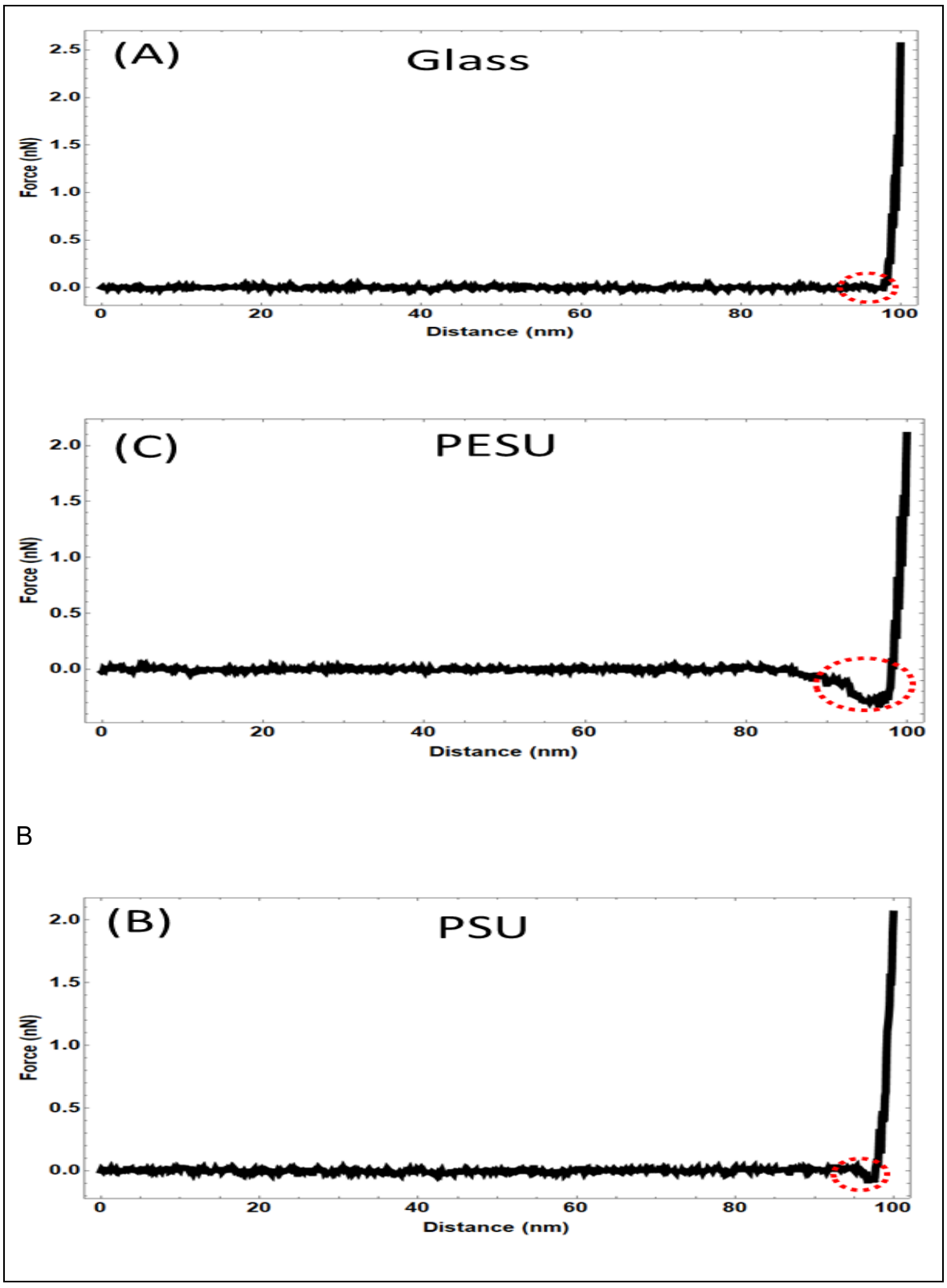

Figure 4.16 Force curve images**.** A typical force curve used to calculate the adhesion force. These are the representatives of retract force curves for glass (A), PESU (B) and PSU (C) as indicated.

The initial contact between the AFM tip and the surface results in the attraction of the tip toward the surface via, for example, van der Waals forces. When the AFM tip makes contact with the sample surface (with a constant force), there is an increase in force resulting in cantilever deflection (due to stiffness of the surface). During the retraction phase, the AFM tip retracts and tries to break contact with the surface. Adhesion forces between the surface and AFM tip attempt to prevent the tip retraction, but the tip eventually overcomes the adhesive forces. The adhesion force measured is ultimately the force required to detach the AFM tip from the surface by quantifying the difference in the approach and retract curves at the point when the 2 surfaces are separated. So, the circled area represents the adhesion force variation between the approach and retraction curves. In glass (A), no clear adhesion event can be identified. Adhesion forces are present in PSU (B) and PESU (C). The difference between PESU and PSU membranes is the roughness as per Ra and Rq values. These differences are perhaps also represented by the outcome of the force curve results in this figure 4.16 Since PSU has less surface roughness, with lower Rq and Ra values than PESU, the RBC adhesion interaction between PSU and RBC might be reduced in comparison to the PESU membrane.

4.5 Flow cell system adhesion results

The degree of RBCs adhesion induced by contact with PESU and PSU membranes was evaluated *in vitro* by analysing the number RBCs' adherent on the membranes, with the use of bovine RBCs. Figures 4.17, 4.18 and 4.19 are representative images showing RBCs' adherent to glass, PSU and PESU samples. The greatest number of RBCs adhered to the glass, and many RBCs also adhered to PESU and PSU membranes respectively. The number of the RBCs remaining adherent to the PESU membrane after washout with the PBS was much higher than that of the PSU.

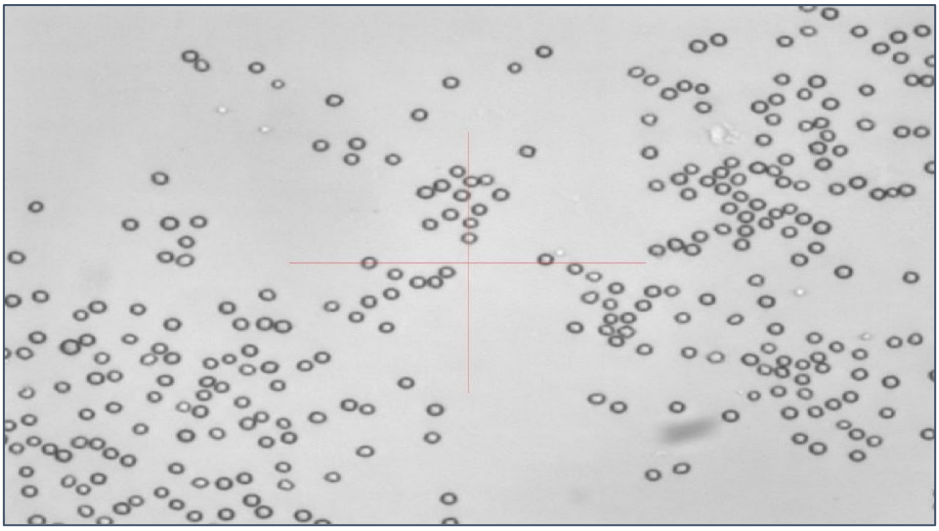

Figure 4.17 Glass sample with adherent RBCs. The RBCs are the round shape, biconcave discs scattered around the slide. The mature RBCs are non-nucleated cells.

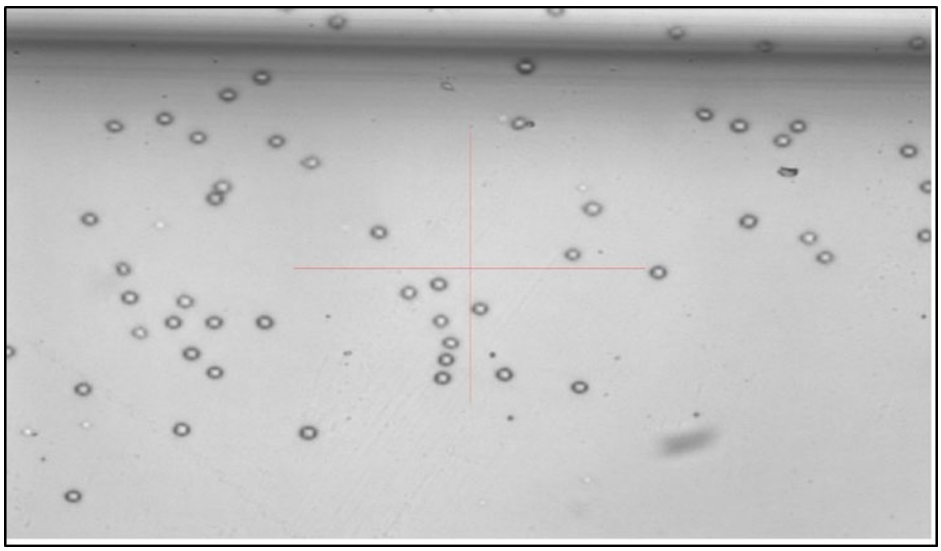

Figure 4.18 PSU sample with adherent RBCs. The RBCs are the round shape, biconcave discs scattered around the slide. The mature RBCs are non-nucleated cells.

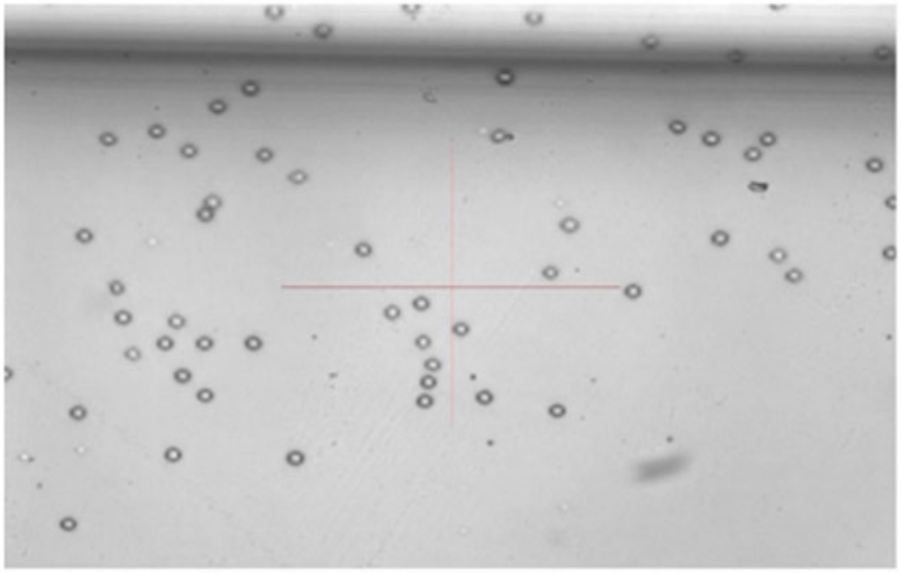

Figure 4.19 PESU sample with adherent RBCs. The RBCs are the round shape, biconcave discs scattered around the slide. The mature RBCs are non-nucleated cells.

Figures 4.17, 4.18 and 4.19 are representative images of RBC's attached to the glass, PESU, PSU. But the total number of sections on all the slides are shown in the below Table 4.2 below.

Polymeric membrane	**PESU**	**PSU**	**Glass**
Number of images	120	120	77

Table 4.2 Total number of RBC membrane section areas.

The experimental plan was to capture 120 membrane sections area for each sample per three experiments for PSU, PESU and Glass. However, the Glass control was a lot more susceptible to air-bubble contamination as indicated earlier. On this Glass samples, out of the 120-glass area sections, 35.8% (43) were completely full of air-bubbles and difficult to analyse. PESU and PSU both had membrane sections of 120 each. Therefore, Glass ended up with 77 membrane sections in total due the presence of these air bubbles Table 4.2. The statistical difference is therefore based on a data set of 77-120 data points, and this demonstrates that the statistical difference in this

analysis was still robust. These results show that the activation of adherent RBCs was very different depending on the membranes used. Among all the tested samples, PSU and PESU induced the lowest and highest activation of RBC, respectively. The total number of RBCs counted on each section is shown in Figure 4.20

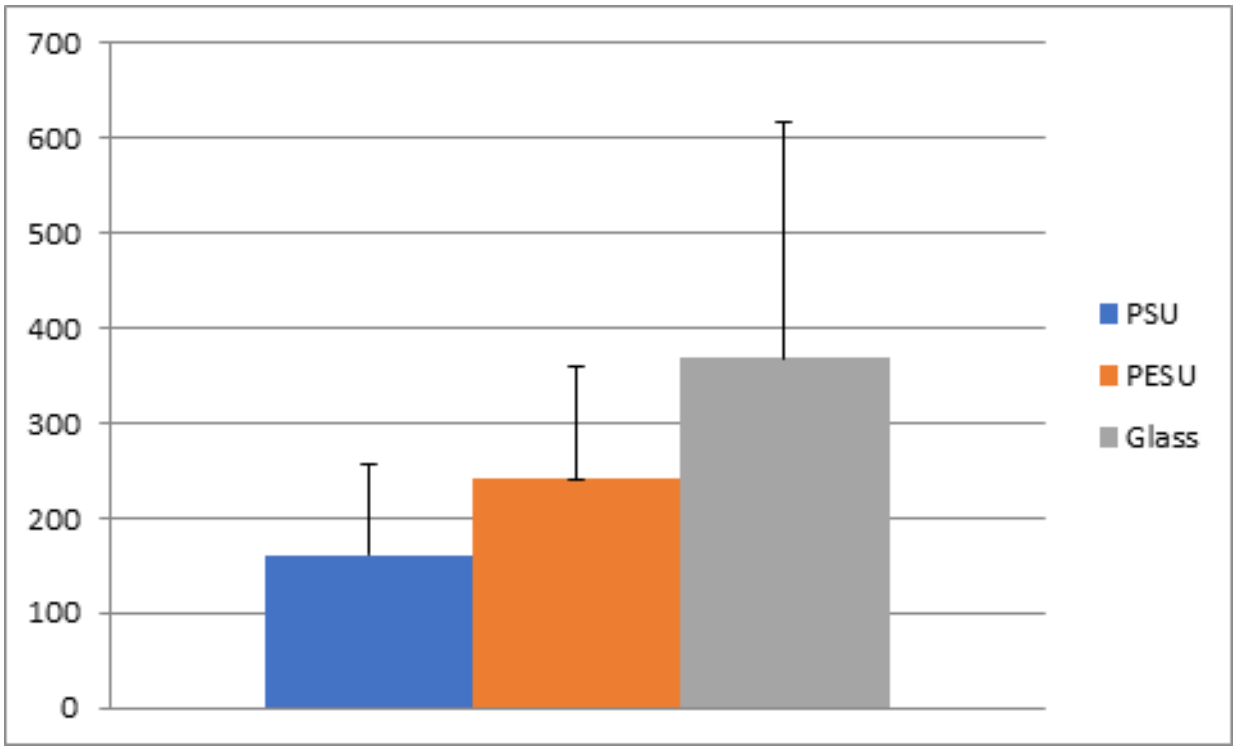

Figure 4.20 The RBC counting on membranes from brightfield images (Y-axis represents the numerical values of the adherent RBCs). This Figure confirms the results captured in Table 4.2. PSU had the lowest (Blue) number of (Grey) RBC attached, while PESU (Orange) came second and glass was by far the highest.

The total flow cell experimental results are therefore represented by the histogram below Figure. 4.20. While Figure 4.21 below breaks down total number attached RBCs on all experiments. Figure 4.20 captures the overall total number of adhered RBCs on all slides captured by experiment - one, two and three. The variables under study are the number of RBCs attached to membranes; PESU, PSU and a glass, post-dialysis (post flow cell investigation) and counted using the microscope.

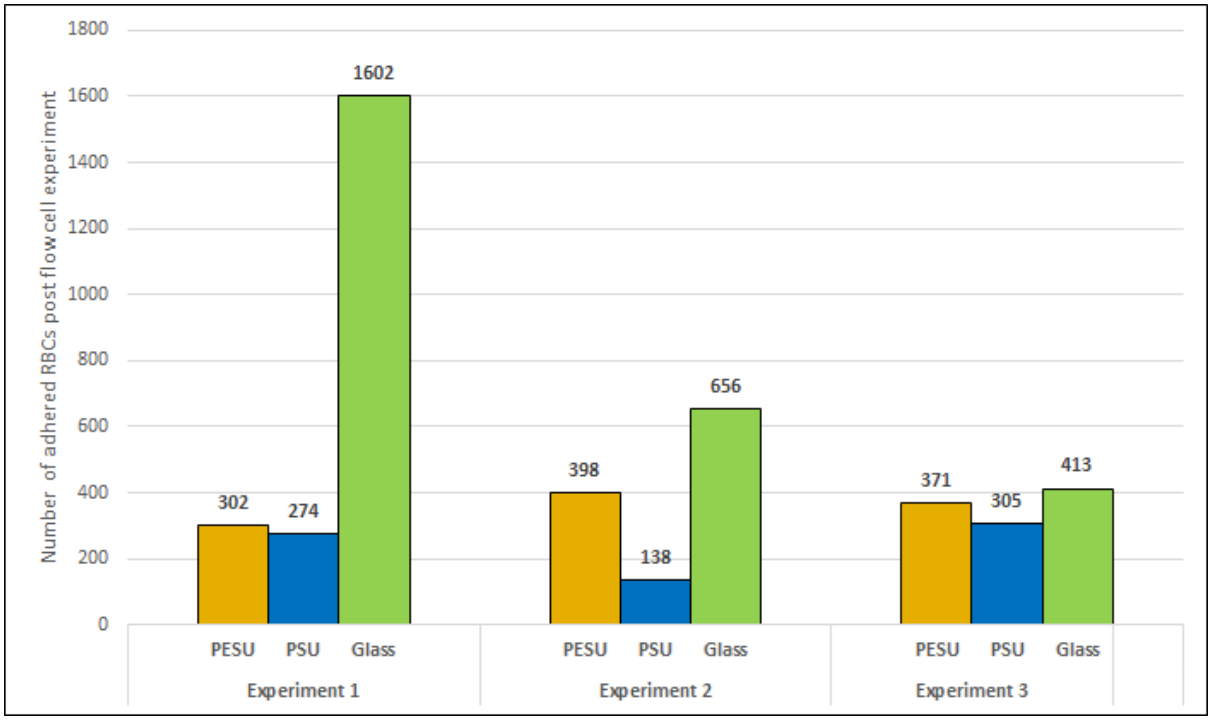

Figure 4.21 Numerical count of cell adhesion to test membrane. The total number of RBC's adhering to the material following the flow cell experiments. This represent all experiments (one to three) and the number of RBC attached to material sample and their results showing PESU (Blue), PSU (yellow) and Glass (green).

To sum-up these results section, this study demonstrated that a membrane with lower roughness topography may reduce RBC adhesion during HD, while wettability may also play a role in this adhesion phenomena. Furthermore, understanding these effects can perhaps decrease the risk of development and progression of various HD-associated complications

4.6 Discussion

The present RBC membrane adhesion analysis of PSU, PESU and glass showed that RBCs adhered more to the Glass, followed by PESU and PSU having the least RBC attachments. There was little difference between PSU and PESU, but a significant difference between PSU and glass. It is imperative to study RBC adhesion, because blood cells incompatibility of dialysis membranes has been reported to be involved in chronic inflammation in CKD patients (Daugirdas and Bernardo, 2011), decreased platelet function (Spijker et al., 2003), and higher mortality and morbidity Hakim et al.,

1996). It is therefore important to investigate blood compatibility with dialysis membranes. For example, Daugirdas and Bernardo proved in their work that significant platelet activation, inflammation and other unwanted reactions can occur in the course of HD and the reasons for that need to be understood (Daugirdas and Bernardo, 2011). In RRT, factors that promote, inflammation, anemia, RBC hemolysis, platelet activation and adhesion on the membrane include hydrophobicity and roughness of membrane surface (Koda, 2000; Kawanishi, 2010), method of sterilization (Kiaii et al., 2011), physicochemical structure (Abe et al., 2011).

It can be inferred from this study that the higher the surface roughness of a membrane, the more hydrophobic it will be and the more the membrane retains RBC. However, the RBCs flowing through the control (glass) were more adherent than for PESU and PSU. In other words, glass retained the most RBC, which highlights the correlation between the characteristics of the glass and the PESU i.e. roughness. The sample material that retains more RBC's was the rougher one and more hydrophilic. The roughness was the common factor for the PESU and glass, but PESU was hydrophobic while glass was hydrophilic. However, their roughness was more pronounced than that of the PSU. Adhesion tests from a different study showed that rougher surfaces have more adhesion to nanosized particles than smooth ones (Lipowsky and Sackmann, 1995). And based on these results, this thesis hypothesis that it is the roughness that mostly affects RBC attachment to the membrane.

An explanation of this finding could be that membrane surface roughness has a more significant role to play in RBC adhesion than hydrophobicity or wettability. This result is probably relevant to observations from a clinical observation in which membrane of PESU origin were pinker in color at the end of the treatment, while PSU membranes were white. But statistically no significant differences were noted between the PESU and PSU membranes, however, it is unclear whether the difference that exists will be clinically important in clinical setting.

4.6.1 Surface topography of the test membranes and roughness

In this study, PSU and PESU were analyzed with SEM and AFM. They were both tested for RBC adhesion using flow cell system. The RBC adhesion in PSU was significantly lower than that of PESU. The rougher membranes are known to influence adhesion than less rougher membranes. In other words, the RBC attachment to HD

membrane was the highest in PESU membrane, which may be explained in the literature that RBC preferentially adhered to rougher surfaces rather than hydrophilic surfaces. Both PSU and PESU are hydrophobic in nature. There was a significant difference in RBC adhesion between PSU membrane and glass in this study. It was presumed that the reason was that PSU was not as rough as glass. The glass was however more hydrophilic than PESU and PSU. Hydrophilicity could presume to have the cause for more RBC adhesion because PESU was more hydrophobic than PSU and yet PESU had more RBC attached.

The AFM surface imaging results of the Glass PSU and PESU membranes showed that glass had the most nano fibre-like structures followed by PESU, in all three microscopic resolutions applied in this study (50x50 µm, 10x10 µm and 3.3x3.3 µm). Surface roughness of test membranes were also calculated as per results in (table 5.1) using roughness average Ra and Rq equations and the Ra value for PESU (1.63 nm) was significantly different ($p > 0.05$) from the Ra value for PSU (1.56 nm).

Sample	**PSU**	**PESU**	**Glass**
A 50x50 µm, 10x10 µm and 3.3x3.3 µm average Image Surface Area Difference	1.92 %	0.342 %	3.68%
Image Rq	2.12 nm	2.19 nm	3.04 nm
Image Ra	1.56 nm	1.63 nm	2.40 nm

Table 4.3 Ra and Rq results showing the Image surface area between the samples.

The surface area difference is the area for the actual 3D object, this shows the exact area used for the measurement of the roughness. In line with other studies, PESU was rougher compared to PSU, and this property characteristically facilitates RBC adhesion to the test membrane (Lima, São José, Andrade, Pires, & Ferreira, 2013). As mentioned in chapter one of this thesis, Whitehead et al. found that there is higher adherence of biological cells to solid surfaces with higher Ra values, indicating an increased adhesion to rougher surface (Whitehead, et al., 2005).

This study found that PSU had the least rough membrane amongst all three samples of interest. Graphical representation of these results showing the roughness of the PSU material as seen in Figures 4.1 and 4.2 mirrors that of other researchers who looked at similar samples including the PSU and PESU topography. Similar rough, spongy-like structures were clearly observed by Razee, et al. n their study of the PSU. The study also used AFM imagery on these materials and presented the 3D surface morphology of the membranes.

In reference to membrane surface roughness to hydrophobicity, the contact angles between water drops and the polymeric membranes were measured. The results from the contact angle test (as seen in Figure 4.16), demonstrated that hydrophobicity increases in the following order: glass, PSU, PESU. Although other research findings have stated that the higher the surface roughness, the more hydrophobic a material would be (Oshihara, 2017 and Bekir Sami Yilbas, 2015). However, in this study, glass had the roughest surface from the AFM analysis and yet the least hydrophobic. There is a need for further investigations to understand this phenomenon. The relationship between roughness and wettability was defined in 1936 by Wenzel, who stated that if contact angle decreases this means that the hydrophilicity increases or the hydrophobic nature of the sample decreases. Roughness would enhance the surface to be more hydrophobic, caused by the chemistry of the surface. For example, if the surface is chemically hydrophobic, it will become even more hydrophobic when surface roughness is added. Wenzel statement can be described with equation below.

Equation 4.2 $\cos \theta m = r \cdot \cos \theta Y$

Where θm is the measured contact angle, θY is the Young contact angle and r is the roughness ratio. The roughness ratio is defined as the ratio between the actual and projected solid surface area (r = 1 for a smooth surface and r > 1 for a rough one). The contact angles calculated from the Wenzel's equation have been found to be good approximations of the most stable contact angles (Decker et al., 1999).

It is however important to note that the Wenzel equation assumes that the liquid penetrates the roughness grooves. It has been stated that if the droplet is larger than the roughness scale by two to three orders of magnitude, the Wenzel equation

applies (BERG, 2002). In relation to this study, the droplet was in fact larger than the roughness scale which means Wenzel's equation will apply.

Finally, roughness is one of the main components that affect interaction of materials with molecules and cells and hence by characterizing it we can interpret the possible difference of membranes in retaining cells and molecules (Torrent-Burgués and Sanz, 2014). There is good evidence in this study to suggest that roughness played a key role in RBC adhesion to sample membranes.

4.6.2 Polyesthesulfone (PESU) vs Polysulfone (PSU)

To some researchers, PESU is a favorable membrane material for HD and HDF membranes. These researchers argue that PESU has better solubility as compared with PSU (Koga et al., 2019; Kremer et al., 2015). This could be related to their characteristics such as their Tg and Tm. This refers to temperatures at which the texture of polymer changes. Most polymers have a certain degree of crystallinity that varies from 5% to 90%, having both amorphous and crystalline regions. In that case, they may have both Tg and Tm (Luis, 2018). These parameters form the basis of the membrane characteristics and surface structure. Topographic properties are very important to membrane applications, yet several polymers that are both suitable for membrane manufacturing and have good quality surface properties are often limited. Thus, modification of membrane surfaces is often used to fulfil diverse requirements (Mercado-Pagán et al., 2014). Bouré and Vanholder stated that the advances in dialysis membrane design, chemical composition, and sterilization methods have led to enhanced performance and versatility to the extent that dialyser choice may reduce morbidity and prolong survival (Bouré and Vanholder, 2004).

4.7 Flow cell system and RBC adhesion

Adhesion of RBCs, proteins, bacteria and other cells to solid surfaces plays an important role in many biological phenomena. In particular, in recent years much work has been devoted to the adhesion process of RBC on various materials (Hammer et al., 1993). In this work, the adhesion of RBCs was analysed and compared for PESU,

PSU and glass. RBC adherence to the PSU membrane was at least 40% lower than its adherence to PESU Figure 4.21, while RBC adhesion to the glass was at least 115% higher than PSU and only 86% higher than PESU membranes Figure 4.21. The results shown in both Figure 4.20 and Figure 4.21 indicate that RBC adhered more to the roughest material (glass).

RBC adhesion results in experiment one and experiment three were very different, an outcome that was hard to comprehend. However, it is known that during storage RBCs undergo a complex and progressive accumulation of physicochemical changes, collectively referred to as the RBC storage lesion (Högman and Meryman 1999). Storage lesions are the adverse effects associated with the storage of blood. Additionally, Glynn identified that older stored RBCs are more strongly implicated in poorer outcomes compared to fresher RBCs (Glynn, 2010). While research effort is being directed to better understand the effects of storage on RBCs (Glynn, 2010), slower progress is being made in finding ways to deter the detrimental effects of the RBC storage RBC lesion. The storage time before the first experiment was ten days, It is therefore a mere speculation that the probably reason for the differences in the RBCs' adhesion of experiment two and three in this study were due to storage-induced damages that accumulate over the shelf life of stored RBCs. It is known that healthy individuals, RBCs' do not appreciably adhere to the vascular endothelium, thus maintaining smooth blood flow. Using an *in vitro* continuous flow model to simulate blood flow, researchers have demonstrated adhesion of stored RBCs to vascular endothelial cells and that the number of adhered RBCs increases with prolonged storage (Luk et al., 2003; Anniss and Sparrow, 2006; Relevy 2008). Pre-storage additives reduced the number of adherent RBCs, but did not eliminate the effect, suggesting that storage-related changes to the RBCs are implicated in the mechanism of adhesion (Anniss and Sparrow, 2006). Perhaps this is the most plausible reason explaining this difference in the work.

PESU dialysis membrane observed by the researcher in a clinical environment, was "pinkish" in colour than the PSU following a dialysis treatment after a rinse with the same amount of the dialysate, usually 300mls. This clinical observation is supported by the findings of this study of a higher adherence of RBC to PES. Future investigations may want to study adhesion of RBCs during HD/HDF sessions under different conditions, such as, viscosity, blood flow rate, hematocrit and anticoagulants.

Future studies should also recognize that HDF and HD are not necessarily similar in the sense that, dialysate or "substitute" fluid is constantly infused in the dialyser on the blood compartment side of the membrane in HDF, whereas in HD the blood is not in direct contact with the dialysate fluid.

In the dialysate compartment, TMP is required to drive internal filtration. TMP is the local pressure gradient across the membrane and the hydraulic and oncotic forces acting along the length of the dialyser on each side of the membrane varies with the length along the whole dialyser according to the following equation:

Equation 4.3 $$TMP(1) = P_b(1) - P_D(1) - \Pi_B(1)$$

where P_B is the hydrostatic pressure in the blood compartment, P_D is the hydrostatic pressure in the dialysate compartment, and π_B is the plasma oncotic pressure.

When TMP is positive, the water flux occurs from the blood compartment to the dialysate compartment. When TMP is negative, back filtration occurs. Thus, removal of middle molecules can be enhanced by raising the positive-pressure differential in the proximal part of the dialyser, thus increasing internal filtration. Adequate net filtration is maintained by the ultrafiltration control system through a parallel increase in the negative-pressure differential in the distal part of the dialyser. This results in greater proximal filtration and distal back filtration without affecting the "net" filtration rate. Given these clear differences between HD and HDF, it seems logical to assume that the condition of dialysis membranes (PSU and PESU) between HD/HDF are different and, therefore, the RBC adherence to the surfaces of the test material in this study are affected by these differences. The study represented HD more than it reflected on HDF because the dialysate flow which will flowing on the outside of the membrane was not introduced in this work. Therefore, more targeted and specific studies need to be conducted to address this issue by modifying the flow system further to cater for the dialysate flow in terms of the HDF.

Another explanation could be that the RBC from the bovine blood samples used in this study could be hydrophilic in nature. To back this up Leffler *et al.* confirmed that RBCs' membranes are rich in glycophorins which give RBC hydrophilic charged coat (Leffler et al., 2017).This is supported by the findings of Tang and Okana, and Mirani et al.

and these studies reported that hydrophobic cells have a stronger adherence to hydrophobic surfaces while hydrophilic cells adhere strongly to hydrophilic surfaces (Tang, and Okano, 2014; Mirani et al., 2018).

Another point to consider is that dialysis membrane can activate inflammatory response in cells, resulting in the production of free radicals and reactive oxidant species (ROS) that can interfere with the normal physiology and function of the RBCs and, ultimately, lead to RBC damage (Dulińska, et al., 2006) and (Borazan, *et al.*, 2004). However, no evidence exists linking inflammatory response of RBCs and their impact on adhesion.

RBCs' membranes are said to be negatively charged which creates a repulsive electric zeta potential between cells. These charges help prevent the interaction between RBC and other cells, (Lopera-Mesa, et al., 2015) and the impact of these charges on dialysis membranes are also unclear. The electrical charges between the RBC and the membrane material (and the glass) is an important factor to consider when studying the RBC's adhesiveness to non-biological materials. Repulsive forces play a role in adhesion of molecules, RBC repulsive force is said to be generated by negative charges on the RBC surface (Fernandes, et al., 2011). Dulińska, et al. conducted a study on the stiffness of normal and pathological RBC using the AFM, they found that the RBCs are negatively charged (Dulińska, et al., 2006). A better understanding of the electrical properties of RBCs and the mechanism of action on adhesion may contribute to the better understanding of the RBCs adhesion to the HD membranes.

This study used the flow cell system to study the adhesion. The flow cell system is an important, widely available and used tool for the *in vitro* cultivation and evaluation of biological material such as bacterial biofilm under hydrodynamic flow condition (Vartoukian, et al., 2016). The flow cell system used in this study has a relatively simple design. The process of its assembly and operation was detailed in Chapter 3. The biggest problem encountered was random bubble formation, particularly in the class sample. However, lessons can be learned from the study. To prevent or at least minimize the air bubble formation in the flow cell system, the design of the system should be correctly conducted as follows: a) All fittings should be checked for leaks before the beginning of the experiment, especially around the glass sample. b) bubble trapping and removal devices should be considered to remove any bubble that may

form in the system. The modified flow cell system developed here can be useful by subsequent researchers as it enables the *in-vitro* analysis of the RBCs observation in flow condition similar to a dialysis procedure and maintaining constant temperature. Based on this flow cell experiment, the Mann-Whitney statistical test has indicted that there is a significant statistical difference between the PSU and the glass when it comes to the RBC adhesion, but not between PESU and the glass. The difference between the PSU and PESU in terms of RBCs' adhesion was not statistically significant, but the trend was clear that RBCs adhered more to PESU material.

This study hypothesizes that PSU can be viewed as more biocompatible with RBCs, particularly when it comes to adhesion. This hypothesis was driven by the researcher's field observations of dialysis membranes after the procedure. Although the adherence differences are quite clear between these materials, differences in biocompatibility between them cannot be explained based on adhesion alone. Therefore, more studies will need to be conducted to scrutinize and validate this hypothesize further. But, based on this work it can be argued that the RBCs compatibility of the PESU membrane is not adequate. A previous study had stated that more injections of anti-coagulants are needed during clinical applications using the PESU as compared to the PSU (Liu, et al., 2009). This supports the researcher's findings that RBCs attachment to the PESU material can lead to more RBC aggregating in the HD membrane leading or contributing to the clogging of the dialyser.

PESU was also seen to be more hydrophobic than PSU material in this study. These potential differences are probably responsible for the differences in RBC adhesion to the membranes in question. It has also been documented that the disadvantage of the PESU is related to the fact that it is relatively more hydrophobic in character. This is in reference to different applications including as filtration devices for separation of viral particles from biologically proteins, for waste applications, as part as several medical drainage devices (Hoek et al., 2003). On that basis, many researchers have also concluded that membrane fouling in PESU is directly related to its hydrophobicity (Van der Bruggen, 2009; Khulbe, et al., 2010). Although, Lan et al. disputed this school of thought by suggesting that both PSU and PESU hollow fibre membranes used in HD/HDF are usually modified by hydrophilic polymers to enhance their hydrophobicity and biocompatibility (Lan, et al.,2013). Based on the experiment done in this work, the researcher shares the view that PSU showed a much higher RBCs attachment

resistance than PESU, perhaps due to the roughness differences and maybe also due to the difference in wettability between these two membranes suggesting more efficient biocompatible characteristics.

Another point to consider in this study is that the average pump speed was maintained at constant rate to give the shear stress of 1.3 Pa for 180 minutes after starting circulation. Shear stress above 1.3 Pa can result in changes in RBC morphology, fragility and haemolysis (Tharmaraj and Kerr, 2017). Horobin et al., highlighted that the supra-physiological shear stress that blood is exposed to while traversing mechanical circulatory assist devices such as dialysis affects the physical properties of RBCs, deforms them, and may induce hemolysis (Horobin et al., 2017). From these results, it was considered that RBCs are likely to adhere to both dialysers but at different rates perhaps. The membrane surfaces were significantly different when studied by the AFM and SEM as highlighted in chapter four. As both membranes were generally hydrophobic in nature, this will further enhance the RBC adhesiveness to the membrane as indicated. Togo, et al. shared the same view by suggesting that, blood components, that is, proteins and blood cells such as RBCs, platelets tend to adhere to the membrane surfaces during a dialysis procedure in general (Togo et al., 2008). Therefore, the possibility of biological reaction such as thrombus formation as a result of, platelets adhering to the PSU and PESU membrane is higher than that of the membrane made of hydrophilic material. Since the roughness increases in the order PSU, PESU and glass, it may be inferred that membrane surface roughness is a contributing factor for RBC adhesion. To support this hypothesis, Hoek et al. concluded in their study that colloidal particles may preferentially deposit in the valleys of a rougher membrane, as the energy well predicted by DLVO theory becomes deeper compared to a smooth surface (Hoek, et al., 2003).

Although membranes with improved permeability and biocompatibility have been developed over the past few decades and the blood contact surface of some membranes have been modified, previous investigations had not considered the adhesion of the RBCs to either the PSU or the PESU. HD membranes commercialised in recent years still use the materials that have been in use for many years and studies employing the use of polymeric membranes, such as PSU and PESU, have not been fully explored to understand how they affect RBC adhesion

Chapter 5 Research Summary

5.1 Summary

This study focused on the flow cell system experiment to understand RBC adhesion to polymeric membranes under conditions similar to those experienced during dialysis. The experiment can now be viewed as relatively simple to conduct and the researcher hopes it will enhance the rate and quality of studies in many dialysis studies examining the blood flow and the interaction of the various blood component with different dialysis membrane currently in clinical use. The overall study type would be compatible to many studies involving blood flow analyses and examination of cell interaction with the extracorporeal circuit of the renal replacement therapy.

In addition, this work also investigated the topographic characteristics of polymeric membrane materials (PESU and PSU). These membranes are mainly used in dialysis treatments and are also seen to be excellent for use in various medical devices such as plasma separation filters (plasmapheresis) and devices used in the removal of LDL cholesterol (LDL-C). The investigation started with background study and literature review. The researcher analysed the AFM and SEM as tools used in the study of the membrane topography. This investigation progressed to flow cells equipment gathering and assembling and continued with assessing RBC adhesion to the membrane using the flow cell system. More RBCs adhered to the PESU sample than the PSU. Adhesion was explored in relation to wettability, biocompatibility and membrane roughness. Surface topography analysis showed that order of highest surface roughness was glass, followed by PESU and then PSU.

The number of studies of dialysis membrane biocompatibility have increased tremendously in the last few years. Many studies have been dedicated to developing the understanding of biological responses, involving humoral and cellular pathway induced by HD membranes. However, the number of studies focused on RBC adhesion to HD membrane is limited. The functional properties of RBCs, haemolysis and any alteration of the RBC caused by the HD membrane can affect biological systems of CKD patients. The interaction between RBC and HD membranes make HD membranes an attractive material of interest for both academia and industry. It has

already been demonstrated in previous chapters that efficacy of HD procedure is determined by untreated blood and dialysate flow passing through the dialyser as well as dialyser characteristics.

Even though the experimental system used in this work represents a rather simple *in-vitro* model, it could be argued that the results of this work also suggest that adhesiveness of RBCs to a membrane could play a significant role for in *vivo* dialyser clogging. Dialyser clogging during a four-hour treatment is a common phenomenon sometimes regardless of the anticoagulation used. Clotting of the dialysis membrane can lead to extracorporeal blood loss, a reduction in dialysis efficiency, and procedural interruptions (James et al., 2013). The adhesion to the membrane might promote close cell-cell contacts and thereby allow more-specific adhesive mechanisms perhaps leading to clogging.

To develop membrane material with better blood membranes interaction especially RBCs compatibility, the focus of future research should consider more studies on the interaction of the RBCs and the dialysis membranes, over and above the RBCs' adhesion ability to the membranes already identified from thesis. Careful consideration should also be given to studying other material which are used in HD and HDF treatments in order to understand the clinical impact of RBC interaction with all dialysis membranes in general.

This thesis has already identified that there is a significant difference between the membrane's roughness and the RBC adhesion. What is not clear or has not been investigated is the translation of that adhesion phenomena in physiological terms. In the absence of membrane material with clear superior characteristics in all respects, immediate and future investigation should look at RBCs interaction with existing material. However, the biocompatibility issues and understanding for PSU, PESU and other membrane materials should be regarded as a priority research area since there is still great disparity among authors, clinician and commercial companies in terms of the ultimate superiority of either PSU or PESU and their derivatives studied to date.

In conclusion, the contribution of mechanical damage caused by HD membranes to the shortened life span and other RBC related anomalies in CKD still is unclear. But it has been well documented that reductions up to 70% in RBC survival have been reported in CKD patients. One of the reasons why the researcher in this work was

interested in this topic is because, to date, no accurate well-controlled RBC survival data exist in CKD patients treated using PSU and PESU dialysis membranes (Vos et al., 2011). The focus of this study was based on the PSU and PESU membranes. This study has shown that whereas these two membranes have similarities, the AFM and SEM have also highlighted topographical and characteristically differences between them. These notable physical disparities may induce changes that result in clinical difference, most notably in the way that the RBCs' adheres to the membrane during a typical dialysis session. The differences observed during this study between these two polymeric membranes were pronounced and obvious and therefore it can be argued that these membranes cannot be considered equivalent.

As future work, deeper insights into the relationship between RBCs' adhesion and surface structure of HD membranes could be gained by focusing on the overall physiology of the RBC in CKD patients on HD and studying if there is a link between this adhesion and anaemia or other blood related abnormalities in CKD (Voinova et al. 2019). The thesis concludes by reinforcing that surface adhesion of RBC remains a highly undesirable process that needs more detailed *in vivo* and *in vitro* investigations. Clinical and academic researchers ought to understand that valuable information on why RBC adhesion occurs, and they occur more on PESU than PESU, the short-term and long-term effect it has on HD patients need to be studied.

5.2 Study limitations

The result of this study indicates that there are some differences between the test membranes (PESU and PSU) and further investigations are needed to explore these differences and better understand how best to improve membrane technology for HD applications.

One of the limitations of this work was that the membranes analysed and tested here were not coated with either PVP or any hydrophilic material to resemble the ones used in a clinical setting. This is a shortfall because it is clearly known that such coating increase hydrophilicity of dialysis membranes (Oberleithner, et al., 2015). All dialysis membranes used in clinical settings are therefore coated with some form of hydrophilic material (David F. Williams, 2017). It was difficult to get these materials because the

researcher had moved jobs and no longer had access to such materials. Commercial companies are also reluctant to share these materials for fear of falling into competitors' hands.

Another limitation of the study was the use of bovine RBCs in this *in vitro* investigation, not of human origin nor a HD patient. However, there are similarities between the human RBC and bovine blood, and so it is assumed that dialysis membranes would exhibit similar characteristics and behavior if the same investigation is performed using blood from HD patients. However, as in all species, a certain amount of physiological variability is observed in hematologic profiles of cattle (Gordon et al., 2007). Variables that contribute to the thresholds and width of reference intervals include age, sex, stress, diet, body condition, reproductive status, recent activity, hydration, ambient temperature, and altitude Bovine RBCs have an average diameter of 5–6 μm in cattle (Boudreaux et al., 2011), which is small compared to other species . While Human RBCs have an avarage diameter of 6-8 μm (Eric, 2013).

Other limitations of this study are as follows:

a) CKD patients tend to have comorbidities that can also affect RBCs. Togo *et al.* supported this by mentioning that the blood cells of CKD patients on dialysis are more vulnerable than those of healthy subjects (Kim et al., 2014).

b) During normal clinical dialysis session, the amount of fluid removal (ultrafiltration) varies among patients. The effect of this on the RBC adhesion to the dialysis membranes is not known, and the role the ultrafiltrate will play in blood cells adhesion has not been studied either. The blood within the hollow fibre of the dialysis can become more concentrated based on various factors including the amount of the fluid being removed, and there is the possibility that blood cells will more easily adhere to the dialysis membrane. It has been documented by researchers that some sterilisation techniques induce structural modifications of the dialysers components such as gamma radiation (Nalesso and Claudio, 2017; Allard *et al.*, 2013). On the other hand, Allard et al. argued that beta radiation causes fewer modifications than gamma radiation; these structural modifications can release cytotoxic components and certainly affect the biological reaction in the surface of the dialyser. It is the researcher's experience that steam sterilization is the most commonly adopted method of sterilising the dialysis membranes by commercial

companies. Steam is a reference in terms of safety, security for thermostable materials with an added benefit for steam process, and it allows the removal of waste generated during the dialyser's manufacturing as well (Fumagall et al., 2013)

c). Sterilization method of both the PSU and PESU dialysis membrane varies, some are by autoclave and gamma-ray while others are steam sterilised. We have learnt from Togo et al. that the sterilization method impacts some reactions such as anaphylactic reaction (Togo et al., 2018). Kessler et al. also said that it has been possible to blame sterilizing agents (ethylene oxide, formaldehyde) and in a few cases of reaction related to membranes in HD (Kessler et al. 1984). The sterilization method was not considered in this study and can have an impact on the membranes surface.

Despite the limitations, the research reported the thesis has several advantages over some of the existing data on RBC adhesion to PESU and PSU membranes, such as the ability to run the Flow system for more than 3 hours simulating the actual *in vivo* dialysis time. The ability to compare two most used materials. The ability to study the topographical structure of the material prior to running the flow cell system. The findings of this work may also present opportunities for more studies aiming understanding the clinical effect of the difference between the PSU and PESU. Future, larger studies, *in vivo* analysis will need to be conducted to determine the actual problem caused by RBC adhesion to the artificial membranes. This would allow for a more effective management of HD patients and potentially improved material biocompatibility of HD membranes. Additionally, more scrutiny in this area will improve the study's applicability in the clinical setting.

Finally, this work will demonstrate that whereas these two membranes (PSU and PESU) are similar to a certain extent and are both used in clinical dialysis, the AFM and SEM clearly highlighted topographical and characteristically differences. These notable physical disparities may be hypothesised to induce changes that attain clinical difference, most notably in the way that the RBCs' adheres to the membrane during a typical dialysis session. The differences observed during this study of these polymeric membranes were pronounced and obvious and therefore it can be argued that these membranes cannot be considered equivalent.

References

1. Abe, T., Kato, K., Fujioka, T., Akizawa, T. 'The blood compatibilities of blood purification membranes and other materials developed in Japan'. *International journal of biomaterials.* **2011** 375390 (2011).

2. Allard, B. et al. 'Dialyser biocompatibility and efficiency of sterilization method choice'. *Le Pharmacien Hospitalie*r *et Clinicien,* **48**(4) (2013).

3. Aramwit, P. 'Introduction to biomaterials for wound healing'. Wound Healing Biomaterials', Woodhead Publishing. **2** 3-38, (2016).

4. Ahrenholz, P.G., Winkler, R.E., Michelsen, A., Lang D.A., Bowry S.K. 'Dialysis membrane-dependent removal of middle molecules during hemodiafiltration'. the beta2-microglobulin/albumin relationship. *Clin Nephrol*. **62**(1):21-28. (2004).

5. Anniss, A., Sparrow R.L. 'Storage duration and leukocyte content of red cell products increases adhesion of stored red blood cells to endothelium under flow conditions'. *Transfusion*. **46**:1561–7. (2006).

6. Anselmo, A. C., Gupta, V., Zern, B. J., Pan, D., Zakrewsky, M., Muzykantov, V., Delivering nanoparticles to lungs while avoiding liver and spleen through adsorption on red blood cells. *ACS Nano* **7**, 11129–11137 (2013).

7. Arijit, S., Allison, R. Linehan, P. M., Iovine, 'Impact of starch content on protein adsorption characteristics in amphiphilic hybrid graft copolymers'. International, *Journal of Biological Macromolecules*, **82**, 2016, 256-263,(2016).

8. Aoyagi, S., Abe, K., Yamagishi, T., Iwai, H., Yamaguchi, S., Sunohara T., 'Evaluation of blood adsorption onto dialysis membranes by time-of-flight secondary ion mass spectrometry and near-field infrared microscopy'. *Anal Bioanal Chem.* **27** 6387-6396 (2017).

9. Asano, M., Ishii, T., Hirayama ,A., et al., 'Differences in peritoneal solute transport rates in peritoneal dialysis'. *Clin Exp Nephrol*.**23**(1):122-134. (2019).

10. Aquino, R.S., Pereira, M.S., Vairo, B.C, Cinelli, L.P., Santos, G.R., Fonseca, R.J., Mourão, P.A., 'Heparins from porcine and bovine intestinal mucosa. Are they similar drugs? *Thromb Haemost'*. **2010**; 103:1005–1015. (2010).

11. Avery, M., Prieto, J., Okamoto, I., et al. 'Reuse of intermittent catheters: a qualitative study of IC users' perspectives. *BMJ Open* **2018**;8:e021554. (2018)

12. Ayoub, A., Finlayson, M., 'Effect of cool temperature dialysate on the quality and patients' perception of haemodialysis'. *Nephrol Dial Transplant*. **2004**; 19 190-194 (2004).

13. Ayli, M., Ayli, D., Azak, A., Yüksel, C., Atilgan, G., Dede, F., Akalin, A., Abayli, E & M Çamlibel, M., The Effect of High-Flux Hemodialysis on Dialysis-Associated Amyloidosis, *Renal Failure*, **27**:1, 31-34 (2005).

14. Azeredo, J., Azevedo, N. F., Briandet, R., Cerce, N., Coynye, T., Costa, A et al 'Critical review on biofilm methods', *Critical Reviews in Microbiology*, **43**:3, 313-351 (2017).

15. Baclayon, M., Roos, W. H., & Wuite, G. J. 'Sampling protein form and function with the atomic force microscope'. Molecular & cellular proteomics: *MCP*, **9**(8), 1678–1688. (2010).

16. Bowman, B., Mitchell, H., Rosner., 'Acute Complications of Hemodialysis, Chronic Kidney Disease', *Dialysis, and Transplantation,* **4** 411-426.e6 (2019).

17. Brugnara C. 'Iron deficiency and erythropoiesis: new diagnostic approaches. *Clin Chem*. **49**(10):1573-1578. (2003).

18. Bessis, M. '*Living Blood Cells and Their Ultrastructure'*. Springer; New York, NY, USA: (1973).

19. Bildyukevich, A.V., Plisko, T.V., Usosky, V.V. 'Hydrophobization of Polysulfone Hollow Fiber Membranes. *Pet. Chem*. **58**, 279–288 (2018).

20. BERG, JC. Chapter 1 - Semi-empirical strategies for predicting adhesion, Editor(s): D.A. Dillard, A.V. Pocius, M. Chaudhury, Adhesion Science and Engineering, *Elsevier Science* B.V., **2002**, 1-73, (2002)

21. Brugnara, C. 'Iron deficiency and erythropoiesis: new diagnostic approaches. Clin Chem. **49**(10):1573-1578(2003).

22. Borazan, A., Ustün, H., Ustundag, Y. 'The effects of peritoneal dialysis and hemodialysis on serum tumor necrosis factor-alpha, interleukin-6, interleukin-10 and C-reactive-protein levels'. *Mediators Inflamm*. **2004;**13(3):201-204(2004)

23. Boudreaux, M.K., Spangler, E.A., Welles, E.G. 'Hemostasis. *In*: Duncan and Prasse's veterinary laboratory medicine: clinical pathology, ed. Latimer, KS 5th ed., 107–144. Wiley, Chichester, UK. (2011).

24. Bowen, W.R, Hilal, N, Lovitt, R.W, Wright, C.J.. Direct measurement of interactions between adsorbed protein layers using an atomic force microscope. *J Colloid Interface* Sci **197**:348-352.(1998).

25. Burke, J. M., & Ivory, C. F. 'Influence of the semi-permeable membrane on the performance of dynamic field gradient focusing'. *Electrophoresis,* **31**(5), 893–901 (2010).

26. Baur, J., '19 - Diffusion Coefficients'. Handbook of Electrochemistry, Elsevier, 829-848, -*Caspin J Intern Med*. 2013 Spring; **4**(2): 627-635 829-848 (2007).

27. Boer, W.H, Liem Y, de Beus E, Abrahams AC. 'Acute reactions to polysulfone/polyethersulfone dialysers'. *literature review and management. Neth J Med*. **2017**;75(1):4-13 (2017)

28. Bustamante Lopez and Meissner, *Journal of Biomedical Optic*s **22**(9), 091510 (2017).

29. Cachofeiro, V., Goicochea M., de Vinuesa, S.G, Oubiña P, Lahera V, Luño J. 'Oxidative stress and inflammation, a link between chronic kidney disease and cardiovascular disease'. *Kidney Int Suppl*. **2008**; 111:S4-S9(2008).

30. Cassereau, L., Miroshnikova, Y. A., Ou, G., Lakins, J., & Weaver, V. M. A 3D tension bioreactor platform to study the interplay between ECM stiffness and tumor phenotype. *Journal of biotechnology,* **193**, 66–69. (2015).

31. Cavalcanti-Adam, E. A., Aydin, D., Hirschfeld-Warneken, V. C., & Spatz, J. P. 'Cell adhesion and response to synthetic nanopatterned environments by steering receptor clustering and spatial location. *HFSP journal,* **2**(5), 276– (2008).

32. Cerqueira, B., Arenas-Lago, D., Andrade, M. L., & Vega, F. A. Validation of TOF-SIMS and FE-SEM/EDS Techniques Combined with Sorption and Desorption Experiments to Check Competitive and Individual Pb2+ and Cd2+ Association with Components of B Soil Horizons. PloS one, **10**(4)(2015).

33. Chakrabarty, B et al., 'Preparation, characterization and performance studies of polysulfone membranes using PVP as an additive' · *Journal of Membrane Science* **315**(1):36- (2008).

34. Chang, Y. R., et al. Automated AFM force curve analysis for determining elastic modulus of biomaterials and biological samples. *Journal of the mechanical behaviour of biomedical materials*, **37**, 209–218(2014).

35. Chieng, W., Ibrahim N.A, Daud A, Abidin Talib A.Z., 'Synthesis, Technology and Applications of Carbon Nanomaterials', Chapter 8 Functionalization of Graphene Oxide via Gamma-Ray Irradiation for Hydrophobic Materials . *Micro and Nano Technologies* 177-203.(2019).

36. Chakrabarty, B., Ghoshal, A.K., Purkait, M.K. 'Ultrafiltration of stable oil-in-water emulsion by polysulfone membrane'. J*. Memb. Sci*. **325**. 427-437 (2008)

37. Chon, M.Y., Yeun, E.J, Jung ,K.H, Jo YI, Lee K.R. 'Perceptions of resilience in patients undergoing peritoneal dialysis'. *A Q-methodology study. Nurs Health Sci*. **22**(1):108-117(2020)

38. Crusz, S. A., Popat, R., Rybtke, M. T., Cámara, M., Givskov, M., Tolker-Nielsen, T., Diggle, S. P., & Williams, P. 'Bursting the bubble on bacterial biofilms: a flow cell methodology'. *Biofouling,* **28**(8), 835–842. (2012).

39. Daugirdas, J.T., Bernardo, A.A., 'Hemodialysis effect on platelet count and function and hemodialysis-associated thrombocytopenia'. *Kidney Int* **2012**;82:147(2012).

40. Davenport, A. 'Optimization of heparin anticoagulation for hemodialysis'. *Hemodial Int*. **2011**;15(S1): S43–S48. (2011)

41. Decker, E.L, Frank, B., Suo, Y., Garoff ,S. (1999) 'Physics of contact angle measurement'. *Colloids Surf A*. **1999**; 156:177–189.(1999).

42. de Haan, K., Ballard, Z. S., Rivenson, Y., Wu, Y., & Ozcan, A. (2019). Resolution enhancement in scanning electron microscopy using deep learning. *Scientific reports*, **9**(1), 12050. (2020).

43. Derebail, V.K., McGregor, J.G., Colindres, R.E., Singh, H.K., Kshirsagar AV. 'The Case: Acute kidney injury in a patient with P. carinii pneumonia'. *Kidney Int.***75**(8):865-6 (2008).

44. Dessì, M., Noce, A., Bertucci, P., Noce, G., Rizza, S., De Stefano, A., Manca di Villahermosa, S., Bernardini, S., De Lorenzo, A., & Di Daniele, N. 'Plasma and erythrocyte membrane phospholipids and fatty acids in Italian general population and hemodialysis patients'. *Lipids in health and disease*, **13**, 54. (2014).

45. Ducker, W.A., Senden, T.J., Pashley, R.M. 'Measurement of forces in liquids using a force microscope'. *Langmuir* **8:**1831–1836 (1992).

46. Dunn GA, Zicha D. 'Long-term chemotaxis of neutrophils in stable gradients: preliminary evidence of periodic behavior. *Blood Cells*. **1993**;19(1):25-41. (1993).

47. Dulińska,I., Targosz, M., Lekka, M., Czbu, P 'Stiffness of normal and pathological erythrocytes studied by means of atomic force microscopy, *Journal of Biochemical and Biophysical Methods*. **661–3**, 20061-11, (2006).

48. Escobar, J.V., Garza, C., Castillo, R. 'Measuring adhesion on rough surfaces using atomic force. microscopy with a liquid probe'. *Beilstein J. Nanotechnol*. **2017**, 8, 813–825. (2017).

49. Elliott. D.A., 'Hemodialysis. (2000) '*Clin Tech Small Anim Pract*.;**15**(3):136-148. (2000).

50. Evans, E., Skalak, R. 'Mechanics and Thermodynamics of Biomembranes'. CRC Press; Boca Raton, FL, USA: (1980).

51. Faia-Torres, A. B, Charnley, M., Goren, T., Guimond-Lischer, S., Rottmar, M., Maniura-*Weber Acta Biomater*. **28** 64(2015).

52. Ficheux, A., Kerr, P.G., Brunet, P., Argilés A.'The ultrafiltration coefficient of a dialyser (KUF) is not a fixed value, and it follows a parabolic function: the new concept of KUF max' *Nephrology Dialysis Transplantation*, **26**(2) 636-640 (2011).

53. Foley, J. H., Conway, E. M. 'Cross talk pathways between coagulation and inflammation'. *Circulation Research.* **2016**;118(9):1392–1408. (2016).

54. Fumagall, G. and Panichi, V 'Chapter 151- Biocompatibility of the dialysis system', in Ranco, C. et al. (eds). Philadelphia: Content repository.918-922.(2017).

55. Gordon, J. E., Gagnon, Z., & Chang, H. C. 'Dielectrophoretic discrimination of bovine red blood cell starvation age by buffer selection and membrane cross-linking. *Biomicrofluidics,* **1**(4), 44102. (2007).

56. Grooteman, M. P., van den Dorpel, M. A., Bots, M. L., Penne, E. L., van der Weerd, N. C., Mazairac, A. H., den Hoedt, C. H., van der Tweel, I., Lévesque, R., Nubé, M. J., ter Wee, P. M., Blankestijn, P. J., & CONTRAST Investigatoret. 'Effect of online hemodiafiltration on all-cause mortality and cardiovascular outcomes. *Journal of the American Society of Nephrology: JASN*, **23**(6), 1087–1096 (2012).

57. Guenneau, S., & Puvirajesinghe, T. M.'Fick's second law transformed: one path to cloaking in mass diffusion. *Journal of the Royal Society, Interface*, **10**(83), 20130106. (2013).

58. Grzhibovskis R, Krämer E, Bernhardt I, Kemper B, Zanden C, Repin N.V, Tkachuk B.V, Voinova M.V. 'Shape of red blood cells in contact with artificial surfaces'*. Eur Biophys J.* **46**(2):141-148.(2017).

59. Guillard R.R.L, Sieracki, M.S.'Counting cells in cultures with the light microscope In: Andersen R, editor. Algal culturing techniques. Academic Press; 2005. 239–267 (2005).

60. Glynn SA. 'The red cell storage lesion: a method to the madness'. *Transfusio*n. **2010**; 50:1164–9.(2010).

61. Hakim, R.M.; Breillatt, J.; Lazarus, J.M.; Port, F.K. 'Complement activation and hypersensitivity reactions to dialysis membranes. *N. Engl. J. Med*. **1984,** 311, 878–882 (1984).

62. Hakim RM, Held PJ, Stannard DC et al 'Effect of the dialysis membrane on mortality of chronic hemodialysis patients'. *Kidney Int* 1**996** 50:566–70 (1996).

63. Hess JR. Red cell changes during storage. *Transfus Apher Sci*. **2010**;43:51–(2010).

64. Heydorn, A., Nielsen, A.T., Hentze,r M, 'Quantification of biofilm structures by the novel computer program COMSTAT'. *Microbiology (Reading).* **2000**;146(200).

65. Hammer DA, Tempelman LA, Apte SM. ''Statistics of cell adhesion under hydrodynamic flow: simulation and experiment. Blood Cells. **1993**;19(2):261-277.(1993)

66. Hans-Jürgen, Butt, Cappella, B., Michael K. 'Force measurements with the atomic force microscope'.Technique, interpretation and applications, *Surface Science Reports* **59,** 1–6, 2005, 1-152 (2005).

67. Han X., Boisvert W. A. Interleukin-10 protects against atherosclerosis by modulating multiple atherogenic macrophage function. Thrombosis and Haemostasis. **2015**;113(3):505–512. 9 (2015).

68. Hedayat ,A., Szpunar, J., Kumar, N.A., Peace, R., Elmoselhi H, Shoker ,A. 'Morphological Characterization of the Polyflux 210H Hemodialysis Filter *Pores'. Int J Nephrol.* (2012).

69. Higuchi A, Shirano K, Harashima M, Yoon OB, Hara M, Hattori M, Imamura K. 'Chemically modifi ed polysulfone hollow fibers with vinylpyrrolidone having improved blood compatibility. *Biomaterials* **2002;** 23:2659–2666 (2002).

70. Hoenich, A,N., Stamp, S., Roberts, J.S., 'A microdomain-structured synthetic high-fl ux hollow-fiber membrane for renal replacement therapy.*' ASAIO* J **2000**; 46:70–75(2000).

71. Hoenich ,N.A,. Levin, R., Ronco, C. 'Water for haemodialysis and related therapies: recent standards and emerging issues'. *Blood Purif*. **2010**;29(2):81-85. (2010).

72. Horobin J.T, Sabapathy, S., Simmonds, M.J.' Supra-Physiological Shear Stress Impairs Red Blood Cell Deformability and Induces Hemolysis'. *Artif Organs*. **2017**;41(11):1017-1025. (2017).

73. Hoek, E.M, Bhattacharjee S, Elimelech M. Langmuir, 'Effect of Membrane Surface Roughness on Colloid−Membrane DLVO Interactions' **19**:4836–4847 (2003).

74. Hörl MP, Hörl WH. Hemodialysis-associated hypertension: pathophysiology and therapy. *Am J Kidney Dis*. **2002**;39(2):227-244. (2002).

75. Hu, X., Sang, Y., Yang, M., Chen, X., & Tang, W. ‘Prevalence of chronic kidney disease-associated pruritus among adult dialysis patients’. A meta-analysis of cross-sectional studies. *Medicine,* **97**(21), (2018).

76. James, M.T, Samuel, S.M, Manning, M.A., ‘Contrast-induced acute kidney injury and risk of adverse clinical outcomes after coronary angiography: a systematic review and meta-analysis. *Circ Cardiovasc Interv*. **2013**;6(1):37-43. (2003).

77. Fischer, H, Bansiwal, A., Hippargi, G., Pophali ,G.R. ‘Effect of hydrophobicity of pharmaceuticals and personal care products for adsorption on activated carbon’*. Environ Sci Pollut Res Int.* **2018**;25(21):20473-20485. (2018).

78. Foley, J. H., Conway E. M. ’Cross talk pathways between coagulation and inflammation’. Circulation Research. **2016**;118(9):1392–1408. (2016).

79. Hayama M., Yamamoto, K., Kohori, F., Sakai, K.‘ How do polysulfone dialysis membranes containing polyvinylpyrrolidone achieve excellent biocompatibility?’ *J Membr Sci* **2004**;243: 389–399 (2004).

80. Guenneau, S., & Puvirajesinghe, T. M. ‘Fick's second law transformed: one path to cloaking in mass diffusion. *Journal of the Royal Society, Interface*, **10**(83), (2013).

81. Hedayat, A, Szpunar J., Kumar N.A, Peace, R, Elmoselhi, H, Shoker, A. (2012) ‘Morphological Characterization of the Polyflux 210H Hemodialysis Filter Pores’. Int J Nephrol**. 2012**;2012:304135. (2012).

82. Han X., Boisvert W. A. ‘Interleukin-10 protects against atherosclerosis by modulating multiple atherogenic macrophage function’. *Thrombosis and Haemostasis***. 2015**;113(3):505–512. (2015).

83. Hakim, R.M, Held P.J, Stannard DC. ‘Effect of the dialysis membrane on mortality of chronic hemodialysis patients’. *Kidney Int* **1996;**50:566–70 (1996).

84. Hakim, R.M.; Breillatt, J.; Lazarus, J.M.; Port, F.K. ‘Complement activation and hypersensitivity reactions to dialysis membranes. *N. Engl. J. Med*. **1984**, 311, 878–882. (1984).

85. Haroon, S., Davenport, A., ‘Haemodialysis at home: review of current dialysis machines*. Expert Rev Med Devices*. **2018**;15(5):337-347(2018).

86. Hess JR. Red cell changes during storage. *Transfus Apher Sci*. **2010**;43:51–9(2010).

87. Heydorn, A, Nielsen, A.T, Hentzer M, et al. (2000) ‘Quantification of biofilm structures by the novel computer program COMSTAT’*. Microbiology (Reading)*. **(2000).**

88. Higuchi, A., Shirano, K., Harashima, M., Yoon, O.B,, Hara, M., Hattor,i M., Imamura, K. ‘Chemically modifi ed polysulfone hollow fibers with vinylpyrrolidone having improved blood compatibility’. *Biomaterials* **2002**;23:2659–2666 (2002).

89. Hoenich NA, Levin R, Ronco C. ‘Water for haemodialysis and related therapies: recent standards and emerging issues’. *Blood Purif*. **2010**;29(2):81-85. (2010).

90. Horobin JT, Sabapathy S, Simmonds MJ. Repetitive.‘ Supra-Physiological Shear Stress Impairs Red Blood Cell Deformability and Induces Hemolysis’. Artif Organs. **2017**;41(11):1017-1025 (2017).

91. Högman, C.F, Meryman ,H.T. ‘Storage parameters affecting red blood cell survival and function after transfusion’. *Transfus Med Rev*. **1999**;13:275–96. (1999).

92. Hörl MP, Hörl WH. Hemodialysis-associated hypertension: pathophysiology and therapy. *Am J Kidney Dis*. **2002**;39(2):227-244. (2002).

93. Hu, D., Zhang, F., Zhou, J., (2015) 'The clearance effect of bovine anti-Helicobacter pylori antibody-containing milk in O blood group Helicobacter pylori-infected patients: a randomized double-blind clinical trial. *J Transl Med*. **2015;**13:205. (2015).

94. Ismail, H., Abdul Manaf M.R., Abdul Gafor, A.H., 'Economic burden of ESRD to the Malaysian health care system'. *Kidney Int Rep*. **2019**;4:1261-1270. Kidney Int Rep. 2019;4(12):1770. (2019).

95. James, M.T, Samuel S.M, Manning, M.A, 'Contrast-induced acute kidney injury and risk of adverse clinical outcomes after coronary angiography: a systematic review and meta-analysis. *Circ Cardiovasc Interv*. **2013**;6(1):37-43.(2013).

96. Jaganathan, Saravana Kumar et al. 'Biomaterials in cardiovascular research: applications and clinical implications." *BioMed research international* **2014** (2014).

97. Jean, G., Hurot, J., Deleaval, P. 'Online-haemodiafiltration vs. conventional haemodialysis: a cross-over study'. *BMC Nephrol* **16**, 70 (2015).

98. Jin, S., Yu, J., Zheng, Y., Wang, W. Y., Xin, B., & Kan, C. W. 'Preparation and Characterization of Electrospun PAN/PSA Carbonized Nanofibers: Experiment and Simulation Study. *Nanomaterial*s (Basel, Switzerland), **8(**10), 821 (2018).

99. Johnson, David W et al. 'Effect of timing of dialysis commencement on clinical outcomes of patients with planned initiation of peritoneal dialysis in the IDEAL trial." Peritoneal dialysis international*: journal of the International Society for Peritoneal Dialysis'*. **32**,6 595-604. (2012).

100. Jung, S., Bohner, L., Hanisch, M., Kleinheinz, J., & Sielker, S. 'Influence of Implant Material and Surface on Differentiation and Proliferation of Human Adipose-Derived Stromal Cells. *International journal of molecular sciences*, **19**(12), 4033 (2018).

101. Kaderjakova, Z et al. ‘Effects of chronic kidney disease on blood cells membrane properties’, *Bioelectrochemist,* **87**. 226-229 (2012).

102. Kalocheretis, P., Zerbala S, Zorbas S et al. ‘The post-dialysis potassium rebound phenomenon and its relationship to hormonal and metabolic factors’. *Nephrol Dial Transplant 2003;* **18** [Suppl 4]: 562(2003).

103. Kanani, D. M., Fissell, W. H., Roy, S., Dubnisheva, A., Fleischman, A., & Zydney, A. L. ‘Permeability - Selectivity Analysis for Ultrafiltration: Effect of Pore Geometry. *Journal of membrane science*, **349**(1-2), 405 (2010).

104. Kashef J, Franz CM. ‘Quantitative methods for analyzing cell-cell adhesion in development’. *Dev Biol*. 2015;401(1):165-174 (2015).

105. Kim, Hyung Wook et al. “Comparison of the impact of high-flux dialysis on mortality in hemodialysis patients with and without residual renal function.” PloS one vol. 9,6 e97184. (2014).

106. Kim. Lee and Km, ‘Experimental study on the Effects of the Performance of Polymer Electrolyte Membrane Fuel cell, materials Science Forum. 544-545 (1999)

107. KDIGO ‘Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. Kidney International Supplements 3(1): (2013).

108. Kawanishi H. ‘Reaction by Contact with Dialysis Membrane.’ Biocompatibility of Dialytic Membrane. Tokyo: Igakusha, 2010 in Japanese.(2010).

109. Kerr, P. G et al. ‘The impact of Standard High-flux polysulfone vs novel high-flux polysulfone dialysis membranes on inflammatory markers: A

randomized, single-blinded, controlled clinical trial'. *American journal of kidney disease,* **49**(4), 533-539 (2007).

110. Kiaii M, Djurdjev O, Farah M, Levin A, Jung B, MacRae J.' Use of electron-beam sterilized hemodialysis membranes and risk of thrombocytopenia'*. JAMA 2011*; **306**:1679–87.(2011).

111. Krimer, PM 'Generating and interpreting test results: test validity, quality control, reference values, and basic epidemiology'. In: Duncan and Prasse's veterinary laboratory medicine: clinical pathology, ed. Latimer, KS , 5th ed. 365–382. Wiley, Chichester, UK (2011).

112. Krediet, C. J., DeNofrio, J. C., Caruso, C., Burriesci, M. S., Cella, K., & Pringle, J. R., 'Rapid, Precise, and Accurate Counts of Symbiodinium Cells Using the Guava Flow Cytometer, and a Comparison to Other Methods. PloS one, 10(8), e0135725. (2015).

113. Kremer, A.., Lippens, S. Goossens, A. 'Developing 3D SEM in a broad biological context'. *Journal of microscopy*, **259**(2), 80–96. (2015).

114. Koda Y. (200) 'HDF and Biocompatibility'. HDF Handbook. Tokyo: Nankodo Co., Ltd., 2000 (in Japanese). (2002).

115. Koga, Y., Meguro, H., Fuji, H. 'A new hydrophilic polysulfone hemodialysis membrane can prevent platelet-neutrophil interactions and successive neutrophil activation'. *The International journal of artificial organs*, **42**(4), 175–181. (2019).

116. Kokubo K, Kurihara Y, Kobayashi K, Tsukao H, Kobayashi H. Evaluation of the Biocompatibility of Dialysis Membranes. Blood Purif. 2015;40(4):293-297. (2015).

117. Kono, M., Mori, R., & Uchio, Y.'Bone screws have advantages in repair of experimental osteochondral fragments. *Clinical orthopaedics and related research*, **470**(7), 2043–2050. (2012).

118. Lan, M. Y., Liu, C. P., Huang, H. H., & Lee, S. W. ‘ Both enhanced biocompatibility and antibacterial activity in Ag-decorated TiO2 nanotubes. PloS one, 8(10), e75364.(2013).

119. Langer, R., Vacanti, J.P. ‘Tissue engineering. Science. 1993;260(5110):920-926.(1993).

120. Lan, M. Y., Liu, C. P., Huang, H. H., & Lee, S. W. ‘Both enhanced biocompatibility and antibacterial activity in Ag-decorated TiO2 nanotubes. PloS one, 8(10), e75364.(2013).

121. Lackland D, Israelachvilli JN, Schmitt FJ, Knoll W. ‘Temperature and vascular stability in hemodialysis. *Nephron.* **1985**; **39**: 130-133 (1985).

122. Lee HY, Kim YJ, Ahn T,‘ A Randomized, Multicenter, Double-blind, Placebo-controlled, 3 × 3 Factorial Design, Phase II Study to Evaluate the Efficacy and Safety of the Combination of Fimasartan/Amlodipine in Patients With Essential Hypertension. *Clin Ther*. **2015**;37(11):2581-2596.(2015).

123. Ledebo I, Blankestijn PJ. ‘ Haemodiafiltration-optimal efficiency and safety’. NDT Plus. 2010 Feb;3(1):8-16. (2010).

124. Lequier L, Horton SB, McMullan DM, Bartlett RH. ‘Extracorporeal membrane oxygenation circuitry’. Pediatr Crit Care Med. 2013;14(5 Suppl 1):S7-S12. (2013)

125. Lewis S, Raj D, Guzman NJ. 'Renal failure: implications of chronic kidney disease in the management of the diabetic foot'.Semin Vasc Surg. 2012;25(2):82-88. (2012).

126. Lima S. M. d., Otoni A., Sabino Ade P., et al. 'Inflammation, neoangiogenesis and fibrosis in peritoneal dialysis'. *Clinica Chimica Acta*. **2013**;421: 46–50. (2013).

127. Liao, Y. C., Hargrove, W., & Weeks, B. L. (2013). Effect of humidity and hydrophobicity on the tribological properties of self-assembled monolayers. TheScientificWorldJournal, (2013)

128. Lipowsky R., Sackmann E. 'Structure and Dynamics of Membranes: From Cells to Vesicles'. Elsevier; Amsterdam, The Netherlands: (1995).

129. Lisa M. Ryno, et al. (2014) ' Biomacromolecules 2014 15 (8), 2944-2951 (2014).

130. Lewin, A J E Beever, Y Da, H C Hines, D B Faulkner, ('The bovine B and C blood group systems are not likely to be the orthologues of human RH: an interesting twist in the comparative map, Animal Genetic. S1, 13-18 (1994)

131. Liao Z, Klein E, Poh CK, Huang Z, Lu J, Hardy PA, Gao D. 'Measurement of hollow fi ber membrane transport properties in hemodialyzers'. *J Membr Sci* **2005**;256:176–183 (2005).

132. Locatelli, F., Martin-Malo, A., Hannedouche, T., Loureiro, A., Papadimitriou, M., Wizemann, V., Jacobson, S. H., Czekalski, S., Ronco, C., Vanholder, R., & Membrane Permeability Outcome (MPO) Study Group. Effect of membrane permeability on survival of hemodialysis patients. *Journal of the American Society of Nephrology*: (**2009).**

133. Lopera-Mesa, T. M. et al ‘Effect of red blood cell variants on childhood malaria in Mali: a prospective cohort study’. The Lancet. Haematology, 2(4), e140–e149. (2015).

134. Luis, P. (2018). Chapter 1 – Introduction ‘Fundamental Modelling of Membrane System’, Elsevier, 1-23,(2018).

135. Luk CS, Gray-Statchuk LA, Cepinkas G, Chin-Yee IH. ‘WBC reduction reduces storage-associated RBC adhesion to human vascular endothelial cells under conditions of continuous flow in vitro. *Transfusion.* **2003**; 43:151–56.(2003).

136. Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 1.6, Molecular Cell Biology: An Integrated View of Cells at Work. Available from: (2000).

137. Luo H, Gauthier M, Tan X, ‘Sodium Transporters Are Involved in Lithium Influx in Brain Endothelial Cells’. *Mol Pharm*. **2018**;15(7):2528-2538. (2018).

138. Ly J, Marticorena R, Donnelly S. ‘Red blood cell survival in chronic renal failure. Am J Kidney Dis. 2004;44:715–719. doi: 10.1016/S0272-6386(04)00951-5. (2004).

139. Lyubchenko, Y. L., Shlyakhtenko, L. S., & Ando, T. ‘Imaging of nucleic acids with atomic force microscopy. Methods (San Diego, Calif.), 54(2), 274–283.(2011).

140. Maciaszek JL, Partola K, Zhang J, Andemariam B, Lykotrafitis G. ‘Single-cell force spectroscopy as a technique to quantify human red blood cell adhesion to subendothelial laminin’. *J Biomech*. **2014**;47(16):3855-3861(2014).

141. McKeen, L. W ‘Fluorinated Coatings and Finishes HandbookThe Definitive User's Guide and Databook A volume in Plastics Design Library Book • Hardcover ISBN: (2006).

142. Mota A. P., Vilaça S. S., das Mercês F. L‘Cytokines signatures in short and long-term stable renal transplanted patients’. *Cytokine***. 2013**;62(2):302–309(2013).

143. Maggiore Q. Pizzarelli F. Santoro A. ‘The effects of control of thermal balance on vascular stability in hemodialysis patients’.Am J *Kidney Dis*. **2002**; 40: 280-290 (2002).

144. Maggiore Q, Pizzarelli F, Zoccali C, Sisca S, Nicolò F, Parlongo S.(1981) ‘ Effect of extracorporeal blood cooling on dialytic arterial hypotension’. (1981).

145. Maleka P. Hashmi, Trever M. Koester, ‘Application of synthetically produced materials in clinical medicine’. in Reference Module in Materials Science and Materials Engineering, (2018)

146. Malik, U., & Raizada, V. ‘Some Aspects of the Renin-Angiotensin-System in Hemodialysis Patients. Kidney & blood pressure research, 40(6), 614–622 (2015).

147. Marrese M, Guarino V, Ambrosio L. ‘Atomic Force Microscopy: A Powerful Tool to Address Scaffold Design in Tissue Engineering’*. Journal of Functional Biomaterials.* (**2017)**.

148. McClellan W, Aronoff SL, Bolton WK, Hood S, Lorber DL, Tang KL, Tse TF, Wasserman B, Leiserowitz ‘ The prevalence of anemia in patients with chronic kidney disease’. Curr Med Res Opin. 2004;20:1501–1510. (2004).

149. McKeen, L. ‘Fluorinated Coatings and Finishes Handbook 2nd EditionThe Definitive User's Guide Hardcover ISBN: 9780323371261 eBook ISBN: 9780323374675 (2006).

150. Mercado-Pagán ÁE, Ker DF, Yang Y. 'Hemocompatibility evaluation of small elastomeric hollow fiber membranes as vascular substitutes. *J Biomater Appl*. **2014**;29(4):557-565 (2014).

151. Mirani, Z. A., et al. 'Relationship of cell surface hydrophobicity with biofilm formation and growth rate'. A study on Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli. *Iranian journal of basic medical sciences,* **21(**7), 760–769. 2018).

152. Müller TF, Seitz M, Eckle I, Lange H, Kolb G. Biocompatibility differences with respect to the dialyzer sterilization method. Nephron. 1998;78(2):139-142. doi:10.1159/000044901

153. Nalesso, F. and Clausio, R. (2017) 'Chapter 17- selecting a Dialyser: Techenical and Clinical consideration', in Nissenson, A. R and Fine, R.N.B.T, H. of D.T. (Fith E. (eds). Elsevier, pp.227-238.e4.doi:

154. Mustafa RA, Bdair F, Akl EA, et al. (2016) 'Effect of Lowering the Dialysate Temperature in Chronic Hemodialysis: A Systematic Review and Meta-Analysis. Clin J Am Soc Nephrol. 2016;11(3):442-457. doi:10.2215/CJN.04580415

155. Nagornov, Y.S., Pahomova, R.A. Atomic force microscopy of the erythrocyte membrane in obstructive jaundice. BIOPHYSICS 61, 405–412 (2016). https://doi.org/10.1134/S0006350916030106

156. Novotna, K., et al. 'Adhesion and Growth of Vascular Smooth Muscle Cells on Nanostructured and Biofunctionalized Polyethylene. Materials (Basel, Switzerland), 6(5), 1632–1655 (2013).

157. Oberleithner H, Wälte M, Kusche-Vihrog K. (2015), 'Sodium renders endothelial cells sticky for red blood cells'. Front Physiol. 2015;6:188.(2015).

158. Neri, Mauro et al. "Nomenclature for renal replacement therapy in acute kidney injury: basic principles." *Critical care (London, England)* vol. 20,1 318. 10 Oct. 2016, doi:10.1186/s13054-016- London, England), 20(1), 318. (2016).

159. Oberleithner H, Wälte M, Kusche-Vihrog K. ‘Sodium renders endothelial cells sticky for red blood cells’. Front Physiol. 2015;6:188. Published 2015 Jun 30. (2015).

160. Oshihara W, Ueno Y, Fujieda H. A ‘New Polysulfone Membrane Dialyzer, NV, with Low-Fouling and Antithrombotic Properties. Contrib Nephrol. 2017;189:222-229. (2017).

161. Otti T, Khajehdehi P, Fawzy A, Gellens M, Bastani B ‘Comparison of blood loss with different high-flux and high-efficiency hemodialysis membranes’. Am J Nephrol. 2001;21(1):16-19. (2001).

162. Pálsson, R., & Patel, U. D. (2015). Cardiovascular complications of diabetic kidney disease. Advances in chronic kidney disease, 21(3), 273–280. (2015).

163. Parsons, J. T., Horwitz, A. R., & Schwartz, M. A.’Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nature reviews. Molecular cell biology*, **11**(9), 633–643. (2010).

164. Pascual M, Swinford RD, Tolkoff-Rubin N. ‘Acute renal failure: role of dialysis membrane biocompatibility’. *Annu Rev Med*. **1997**; 48:467-476. (1997).

165. Perlman RL, Zhao J, Fuller DS, et al.’ International Anemia Prevalence and Management in Peritoneal Dialysis Patients’. Perit Dial Int. 2019;39(6):539-546. (2019).

166. Pittard, J. D Chapter 13 - Safety Monitors in Hemodialysis, Editor(s): Allen R. Nissenson, Richard N. Fine, Handbook of Dialysis Therapy (Fifth Edition), Elsevier,2017, Pages 162-190. (2017).

167. Popescu, G et al, (2008) ‘Imaging red blood cel dynamics by quantitative phase microscope’, Blood cells, Molecules, and Diseases, 41 (1), pp. 10-16 doiHttps://doi.or/1016/j.bcmd.2008

168. Potier J, Bowry S, Canaud B. Clinical Performance Assessment of CorDiax Filters in Hemodialysis and Hemodiafiltration. Contrib Nephrol. 2017; 189:237-245. doi:10.1159/000450810

169. Purnell, T. S., Auguste, P., Crews, D. C., Lamprea-Montealegre, J., Olufade, T., Greer, R., Ephraim, P., Sheu, J., Kostecki, D., Powe, N. R., Rabb, H., Jaar, B., & Boulware, L. E. (2013). Comparison of life participation activities among adults treated by hemodialysis, peritoneal dialysis, and kidney transplantation: a systematic review. American journal of kidney diseases: the official journal of the National Kidney Foundation, 62(5), 953–973. https://doi.org/10.1053/j.ajkd.2013.03.022

170. Ramón et al (2018) ‘Bedtime hypertension treatment improves cardiovascular risk reduction: the Hygia Chronotherapy Trial’ European Heart Journal, ehz754, nhttps://doi.org/10.1093/eurheartj/ehz754

171. Ramón M, A Martínez Miguel P, Bohorquez L, et al. (2017) ‘Asymmetric cellulose triacetate is a safe and effective alternative for online haemodiafiltration’. 2018;38(3):315-320. doi:10.1016/j.nefro..11.015

172. Rao, M., Guo, D., Bertrand J. ‘Dialyzer membrane type and reuse practice influence polymorphonuclear leukocyte function in hemodialysis patients Kidney International Volume 65, Issue 2, 682-691 (2004).

173. Resnick N, Yahav H, Shay-Salit A, et al. Fluid shear stress and the vascular endothelium: for better and for worse. Prog Biophys Mol Biol. 2003;81(3):177-199. (2003).

174. Ronco C, Levin N, Brendolan A, et al. Flow distribution analysis by helical scanning in polysulfone hemodialyzers: effect of fibre structure and design on the flow patterns and solute clearances*. Hemodial Int.* **2006**; 10:380–388. (2006).

175. Ronco, et al. 'Expanded haemodialysis: from operational mechanism to clinical results, *Nephrology Dialysis Transplantation* **33**, 3, October 2018, iii41–iii47, (2018).

176. Relevy H, Koshkaryev A, Manny N, et al. Blood banking-induced alteration of red blood cell flow properties. *Transfusion.* **2008;** 48:136–46. (2008).

177. Reisz, J. A., Nemkov, T., Dzieciatkowska, M., Culp-Hill, R., Stefanoni, D., Hill, R. C., Yoshida, T., Dunham, A., Kanias, T., Dumont, L. J., Busch, M., Eisenmesser, E. Z., Zimring, J. C., Hansen, K. C., & D'Alessandro, A. (2018). Methylation of protein aspartates and deamidated asparagines as a function of blood bank storage and oxidative stress in human red blood cells. *Transfusion*, **58**(12), 2978–2991. (2018)

178. Reisz, Julie A et al. "Methylation of protein aspartates and deamidated asparagines as a function of blood bank storage and oxidative stress in human red blood cells." *Transfusion* 58,12: 2978-2991 (2018).

179. Sankaran, J., Manna, M., Guo, L., Kraut, R., & Wohland, T. 'Diffusion, transport, and cell membrane organization investigated by imaging fluorescence cross-correlation spectroscopy'. *Biophysical journal,* **97**(9), 2630–2639. (2009).

180. Sato, Y., Miziguchui, T., Shigenaga, S Yoshikawa, E. 'Shortened Red Blood Cell Lifespan Is Related to the Dose of Erythropoiesis-Stimulating Agents Requirement in Patients on Hemodialysis'. Therapeutic Apheresis and Dialysis. *International Society for Apheresis*. **16**(6) 522-528 (2012).

181. Seitaridou, E., Inamdar, M. M., Phillips, R., Ghosh, K., & Dill, K. 'Measuring flux distributions for diffusion in the small-numbers limit'. *The journal of physical chemistry.* B, **111**(9), 2288–2292 (2007).

182. Shaoxian and Shizhu, 'Vapor-condensed gas lubrication of face seals. Gas Thermohydrodynamic Lubrication and Seals, Bessis M. Living Blood Cells and Their Ultrastructure. Springer; New York, NY, USA: 1973. (2019).

183. Sigley RD, May KJ Jr, Mack RM. Scribner shunt conversion to arteriovenous fistula. Am J Surg. 1979;137(3):423-424. doi:10.1016/0002-9610(79)90081-3

184. Steiner, M.et et al. 'Addressing the question of the effect of RBC storage on clinical outcomes: The Red Cell Storage Duration Study (RECESS) (Section 7), *Transfusion and Apheresis Science*, **43**(1), 107-116 (2010).

185. Sakai K. 'Dialysis membranes for blood purification. Front *Med Biol Eng.* **2000**;10(2):117-129. (2000).

186. Stroh, Cordula M., 'Simultaneous topography and recognition imaging using force microscopy." *Biophysical journal*, 87,3 (2004)

187. Strohm, E. M., Berndl, E. S., & Kolios, M. C. 'Probing red blood cell morphology using high-frequency photoacoustics'. *Biophysical journal*, **105**(1), 59–67 (2013).

188. Shen, J.I, Winkelmayer WC. Use and safety of unfractionated heparin for anticoagulation during maintenance hemodialysis. *Am J Kidney Dis.* 2012;60:473–486(2012).

189. Sokolsky-Papkov, M., Langer, R., & Domb, A. J. 'Synthesis of aliphatic polyesters by polycondensation using inorganic acid as catalyst'. *Polymers for advanced technologie*s, **22**(5), 502–511 (2011).

190. Sørensen, O.E, Borregaard ,N. ' Neutrophil extracellular traps - the dark side of neutrophils'. *J Clin Invest*. **2016**;126(5):1612-1620. (2016).

191. Spijker, H.T., Graaff, R., Boonstra, P.W, Busscher, H.J, van Oeveren W. 'On the influence of flow conditions and wettability on blood material interactions'. Biomaterials 24 (26) 4717-4727 (2003).

192. Stookey, J. D., Klein, A., Hamer, J., Chi, C., Higa, A., Ng, V., Arieff, A., Kuypers, F. A., Larkin, S., Perrier, E., & Lang, F. 'RBC deformability and amino acid concentrations after hypo-osmotic challenge may reflect chronic cell hydration status in healthy young men. *Physiological reports*, **1**(5), e00117 (2013).

193. Tagaya, M., Nagoshi, S., Takashi, S., Okana, S. "Hemodialysis membrane coated with a polymer having a hydrophilic blood-contacting layer can enhance diffusional performance." *The International journal of artificial organs* **40,**12 (2017).

194. Tang, Z., & Okano, T. 'Recent development of temperature-responsive surfaces and their application for cell sheet engineering'. *Regenerative biomaterials*, **1**(1), 91–102. (2014).

195. Tattersall , J., Ward, R., et al. 'Online haemodiafiltration: definition, dose quantification and safety revisited'. Nephrology Dialysis Transplantation, Volume 28, Issue 3, March 2013, Pages 542–550 (2013).

196. Tonelli, M, Wiebe N, Culleton B, House A, Rabbat C, Fok M, McAlister F, Garg AX. 'Chronic kidney disease and mortality risk: a systematic review'. *J Am Soc Nephrol*. **2006**; 17:2034–2047(2006).

197. Sherman R. Faustino E. Bernholc A. Eisinger R. 'Effect of variations in dialysate temperature on blood pressure during hemodialysis**.** *Am J Kidney Dis.* 1984; 4: 66-68 (1984).

198. Togo, K., Yamamoto, M., Imai, M. 'Comparison of biocompatibility in cellulose triacetate dialysis membranes with homogeneous and asymmetric structures'. Ren Replace Ther 4, 29 (2018).

199. Torrent-Burgués J, Sanz F. AFM in mode Peak Force applied to the study of un-worn contact lenses. *Colloids Surf B Biointerfaces*. **2014**; 121:388-39(2014).

200. Thomas, G., Burnham, N. A., Camesano, T. A., & Wen, Q. 'Measuring the mechanical properties of living cells using atomic force microscopy. *Journal of visualized experiments: JoVE*, (**76)**, 50497(2013).

201. Tharmaraj, D., & Kerr, P.G. 'Haemolysis in haemodialysis. Nephrology, 22(11), 838-847. doi:10. 1111/neph, 13119(2017).

202. Thongprayoon, C., Cheungpasitporn, W., Phatharacharukul, P., Edmonds, P. J., Kaewpoowat, Q., Mahaparn, P., Bruminhent, J., & Erickson, S. B. 'Chronic kidney disease and end-stage renal disease are risk factors for poor outcomes of Clostridium difficile infection: a systematic review and meta-analysis. *International journal of clinical practice*, **69**(9), 998–1006 (2006).

203. Tyler Van Buren, Gilad Arwatz, Alexander J. Smits, A simple method to monitor hemolysis in real time, *Scientific Reports*, **10**.1038/s41598-020-62041-8, 10, 1, (2020).

204. Van Buren, P.N, Inrig, J.K. 'Mechanisms and Treatment of Intradialytic Hypertension'. Blood Purif. 2016;41(1-3):188-193. (2016).

205. Van der Bruggen (2009) 'Chemical modification of polyethersulfone nanofiltration membranes'. Department of Chemical Engineering, *Laboratory for Applied Physical Chemistry and Environmental Technology* **(2009).**

206. Venkataraman, R., Subramanian, S., & Kellum, J. A. 'Clinical review: extracorporeal blood purification in severe sepsis'. *Critical care (London, England),* **7**(2), 139–145 (2003).

207. Vartoukian, S. R., Adamowska, A., Lawlor, M., Moazzez, R., Dewhirst, F. E., & Wade, W. G. ‘In Vitro Cultivation of 'Unculturable' Oral Bacteria’, Facilitated by Community Culture and Media Supplementation with *Siderophores. PloS one,* **11**(1), (2016).

208. Voinova, M., Repin, N., Sokol, E., Tkachuk, B., Gorelik, L. ‘Physical Processes in Polymeric Filters Used for Dialysis’. Polymers (Basel). 2019;11(3):389. (2019)

209. Voinova, V., Bonartseva, G., & Bonartsev, A. ‘Effect of poly(3-hydroxyalkanoates) as natural polymers on mesenchymal stem cells’. World journal of stem cells, 11(10), 764–786.(2019).

210. Vos, F.E., Schollum J.B., Coulter C.V, Doyle T.C., Duffull ,S.B., Walker, R.J. ‘Red blood cell survival in long-term dialysis patients’. *Am J Kidney Dis.* **2011**;58(4):591-598. (2011).

211. Wei, D . Haiying Wei, Alec C. Gauthier, Junlong Song, Yongcan Jin, Huining Xiaoet al. ‘Superhydrophobic modification of cellulose and cotton textiles’. Methodologies and applications, *Journal of Bioresources and Bioproducts*, **5**, 1, 2020, 1-15, (2020)

212. Whitehead KA, Colligon J, Verran J. (2005) ‘Retention of microbial cells in substratum surface features of micrometer and sub-micrometer dimensions’. Colloids Surf B Biointerfaces. 2005;41(2-3):129-138. doi:10.1016/j.colsurfb.2004.11.010

213. Wienk,I R.M. Boom, M.A.M. Beerlage, A.M.W. Bulte, C.A. Smolders, H. Strathmann, ‘Recent advances in the formation of phase inversion membranes made from amorphous or semi-crystalline polymers, *Journal of Membrane Science*, **113**,(2) 361-371 (1996).

214. Williams, D . (2017) ‘ A Systems Engineering Approach to Restoring Kidney Structure and Function,Editor(s): Transplantation, Bioengineering and Regeneration,Academic Press, chapter 55 769-784,ISBN 9780128017340,

215. Williams D.F. ‘Progress in Biomedical Engineering’. Elsevier; Amsterdam, The Netherlands: *Definitions in Biomaterials*; **49–59** (1987).

216. Whitley E, Ball J. ‘Statistics review 6: Nonparametric methods’. *Crit Care*. **2002**;6(6):509-513. (2002).

217. Yavari M, Becker RC. ‘Anticoagulant therapy during cardiopulmonary bypass. J Thromb Thrombolysis’. 2008; 26:218–228 (2008).

218. Yeow, N, Tabor, R.F., Garnier G. Mapping the distribution of specific antibody interaction forces on individual red blood cells’. *Sci Rep.* 2017; 7:41956. (2017).

219. Yilbas, B. S., Ali, H., Khaled, M. M., Al-Aqeeli, N., Abu-Dheir, N., & Varanasi, K. K. ‘Influence of dust and mud on the optical, chemical, and mechanical properties of a pv protective glass’. *Scientific reports* 15-83 (2015).

220. Zhao, W.., Huang, J., Fang, B., Nie ,S., Yi N., Su, B., Li ,H., Zha,o C. ‘Modification of polyethersulfone membrane by blending semi-interpenetrating network polymeric nanoparticles.’ J*. Membr. Sci*. **2011**;369:258–266. (2011).

221. Zhao, C., Xu X, Chen J, Yang F. Effect of graphene oxide concentration on the morphologies and antifouling properties of PVDF ultrafiltration membranes. *J Environ Chem Eng***. 2013**; 1:349–54. (2013).

222. Zhang LX, Wang F, Wang L, Wang WK, Liu BC, ‘Prevalence of chronic kidney disease in China: a cross-sectional survey’. *The Lancet* **379**: 815–822. (2012).

223. Zhu, X., Siamantouras ,E., Liu, K.K., Liu ,X. ‘Determination of work of adhesion of biological cell under AFM bead indentation. *J Mech Behav Biomed Mater*. **2016**; 56:77-86 (2016).