The development of a low-powered and portable erythrocyte aggregometer for point-of-care use

Juan M. Arrieta

BExSc

A thesis submitted in partial fulfilment of the requirements for the award of the degree of Master of Medical Research

Griffith Health
School of Allied Health Sciences
Gold Coast, Australia
July 7th 2017
Author Information

Juan Manuel Arrieta

Email: juanmanuel.arrieterreros@griffithuni.edu.au

School of Allied Health Sciences

Menzies Health Institute Queensland

Gold Coast Campus Griffith University Queensland, 4222

AUSTRALIA
Declaration and copyright statement

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Juan M. Arrieta

The new portable RBC capillary tube aggregometer (PCA)
Acknowledgements

“Trust in the Lord with all thine heart; and lean not unto thine own understanding. In all thy ways acknowledge him, and he shall direct thy paths.” Proverbs 3:5-6

“The fear of the Lord is the beginning of knowledge: but fools despise wisdom and instruction.” Proverbs 1:7

“Delight thyself also in the Lord; and he shall give thee the desires of thine heart.” Psalm 37:4

First and foremost, I would like to thank my Heavenly Father for making me the curious man that I am. Thank you God for everything you have given me in my life, for all the love that you have filled in my heart that unfailingly encourages me to be a better human every day. Without you my Lord, I am less than nothing, but with You by my side I am inspired to produce meaningful achievements; the completion of this project is just another example of how wonderful life is when you direct my path.

Dr Michael Simmonds: I would like to thank you for the infinite support you have always brought me since you allowed me to be part of your elite research team. I was knocking on many doors seeking for an opportunity to exploit my curiosity by blending different knowledge disciplines and you were the only one who saw the potential that resides within me. With your guidance, knowledge, commitment, and compassion I was inspired to achieve the very best version of myself every day. I cannot easily express how significant this journey has been not only for my academic and professional life, but for my personal growth. I thank God for the honour of having you as my mentor, as part of my Australian family.

Dr Surendran Sabapathy: I would like to thank you for your support and advice, especially for your continued interest on this project.
Mr Guillermo Jacuinde: I am deeply grateful for all your technological advice. Your kind support and perspective helped me find novel solutions to overcome technical numerous obstacles throughout the development of this study.

This study was also made possible due to the expertise, direction and support by my blood rheology labmates. I would like to thank my friend, Mr Jarod Horobin, for the selfless assistance and patience you have, that allowed me to get back on track during times of frustration. To Mr Antony McNamee, thank you for your friendship and continuous encouragement since we started exploring this research world as undergraduate students. To my friend, Mr Kieran Richardson, thank you for your valuable contribution in the participants’ recruitment process. To the staff of the School of Allied Health Sciences, thank you for your continued interest and consistent encouragement.

To Mum and Dad, I can still feel your love in my thoughts and my prayers even though you are literally on the other side of the world. Thanks for teaching me the truly important things in life, for raising me in a family full of love, joy, warmth and faith. The man who I am is the result of the Heavenly inspiration my Lord gave you to be the wonderful parents you are. I love you with my all my soul, God bless you.
**Abstract**

It is well known that the cardiopulmonary system is crucial for the delivery of oxygen to various bodily tissues as well as for the removal of metabolic waste. In addition, the cardiopulmonary system has a vital role in regulating bodily temperature, as well as assisting in the transport of various hormones and nutrients to bodily tissues. Whole blood is a two-phase suspension, consisting of plasma (liquid phase) and numerous cellular components (solid phase); given that erythrocytes are the primary cellular component, the unique mechanical properties of blood can be explained by the characteristics of red blood cells (RBC). RBC tend to form three-dimensional microstructures (*rouleaux*) when under low shear conditions. The rate and magnitude of RBC aggregation can be quantified using photometric methods, which measures the amount of light passing through a blood sample for a discrete time period. The quantification of specific aspects of RBC aggregation, such as the extent of the aggregation, the time course of aggregation, and the magnitude of the aggregating forces, has been shown to be important from a clinical perspective. Analysis of the RBC aggregation process provides valuable information, which can be utilised to determine the presence of various adverse health conditions (e.g., sepsis, diabetes mellitus and myocardial ischemia). Therefore, RBC aggregation analysis – as a possible health indicator – may play a crucial role in the clinical management in several patient groups.

The Laser-Assisted Optical Rotational Cell Analyser (LORCA®) and the Myrenne® aggregometer are photometric instruments commercially available for quantifying RBC aggregation. Disadvantages of these systems include elevated cost and lack of portability (i.e., size, weight, power consumption). The Myrenne aggregometer is possibly the most extensively adopted hemorheological analyser; however, the absence of a temperature control and the inability to provide information about the time course of RBC aggregation, represents a significant disadvantage in regards to experimental research. In this context, a new instrument which is designed to overcome the major limitations of current commercial aggregometers may have an
important impact in the health care domain.

Low cost, portability, low-power consumption, computer independency, and built-in graphic interface are the cardinal features of a newly-developed instrument described in the present thesis. Furthermore, the portable capillary tube RBC aggregometer (PCA) is able to analyse the aggregation time course and provide relevant parameters. The design of the PCA facilitates an intuitive way to control its operation through the various phases of the aggregation process. Moreover, the PCA’s integrated graphic interface allows the user to visualise the aggregation curve in real time during the data collection process. The engineering process of the present device was conceived as the result of the latent need to bring low-cost specialised equipment to remote regions where budget, transportation facilities and/or power supply restrictions are major limitations for use of current technologies.

Blood samples from 43 individuals were analysed to compare the results yielded by the new newly-developed aggregometer, those produced by a commercial device, and the measurements obtained using the erythrocyte sedimentation rate (ESR) technique. The results obtained with the new PCA showed an enhanced signal quality evidenced by a superior signal-to-noise ratio when compared to that yielded by the Myrenne aggregometer. In addition, the precision assessed for the PCA from the aggregation index at 120 seconds (AI$_{120}$) and aggregation half-time (T $\frac{1}{2}$) measurements reflected a good reliability of the instrument. Furthermore, a strong correlation between PCA and the Myrenne aggregometer for the AI$_{120}$ parameter was found.

An unexpected finding allowed this study to hypothesise that the PCA may be able to predict ESR measurements due to the sedimentation phenomenon observed on the blood sample contained in the capillary tube. Unusual aggregation curves were obtained as a result of the RBC reorganisation being detected by the PCA’s infrared sensor. Based on these results, it was possible to obtain the linear equations to predict the ESR in a fraction of the time required for the traditional practice (i.e., Westergren method). Moreover, the possibility to predict ESR by using a small blood sample (~50
µl) at a fraction of the current required time (i.e., 5 min) will expand the PCA’s applicability in a wide range of scenarios.

The significance of this study is represented by the overall performance of the PCA as a modern medical tool. Given that the newly constructed PCA accurately determines various RBC aggregation parameters, it may be suitable for use as a regular screening tool, and assist in the early detection of particular diseases. Importantly, utilising the newly constructed PCA device at point-of-care (i.e., health care facilities) would promote the use of preventative medicine.
Contents

Author Information ........................................................................................................................................... 2
Declaration and copyright statement .................................................................................................................. 3
Abstract ............................................................................................................................................................. 6
Contents ............................................................................................................................................................ 9
List of Abbreviations ......................................................................................................................................... 12
List of Figures and Tables .................................................................................................................................. 13
Chapter 1 – Introduction and literature review ............................................................................................ 15
  1.1 Introduction ............................................................................................................................................... 15
    1.1.1 Importance of blood flow for mammalian life .................................................................................. 15
    1.1.2 Determinants of blood flow ............................................................................................................ 15
    1.1.3 Determinants of blood viscosity ..................................................................................................... 17
    1.1.4 Significance of Haematocrit and Exercise effect on blood rheology .............................................. 18
    1.1.5 The aggregation phenomenon ......................................................................................................... 19
    1.1.6 Measurement of Red Blood Cell Aggregation ............................................................................... 23
    1.1.7 Photometric methods ....................................................................................................................... 24
    1.1.8 Commercial available devices for RBC aggregation measurement .............................................. 25
    1.1.9 Limitations of current instrumentation ............................................................................................ 28
  1.2 Introducing PCA, a newly designed aggregometer .................................................................................. 28
Chapter 2 – Experimental design .................................................................................................................. 30
  2.1 Subjects and sampling ............................................................................................................................ 30
  2.2 Engineering of the capillary-tube RBC aggregometer .......................................................................... 31
  2.3 Engineered aggregometer: main components ....................................................................................... 33
    2.3.1 Arduino MEGA microcontroller board ............................................................................................ 33
    2.3.2 Infrared light emitter and receiver .................................................................................................. 34
    2.3.3 Motor shield ..................................................................................................................................... 34
    2.3.4 Heater element and temperature sensor ........................................................................................ 35
2.3.5 Disaggregation mechanism ................................................................. 35
2.3.6 Touch shield/screen ........................................................................... 36
2.4 Software component ............................................................................. 36
  2.4.1 Disaggregation subroutine ............................................................... 37
  2.4.2 Heating subroutine ........................................................................... 38
  2.4.3 Rescaling subroutine ....................................................................... 39
  2.4.4 Analog processing subroutine ........................................................ 40
  2.4.5 Aggregation parameters calculations subroutine .......................... 44
2.5 Engineered aggregometer .................................................................... 46
  2.5.1 Sample processing ........................................................................... 46
  2.5.2 Discrete phases explained ................................................................ 47
  2.5.3 Analysis of sensor performance ...................................................... 49
2.6 Statistical analysis .................................................................................. 49

Chapter 3 – Results .................................................................................. 51
3.1 Pre-experimental results – Design considerations ..................................... 51
  3.1.1 Battery power supply ...................................................................... 51
  3.1.2 Disaggregation mechanism ............................................................... 52
3.2 Experimental Results ............................................................................ 54
  3.2.1 Performance of the newly developed device: Signal-to-noise ratio ...... 54
  3.2.2 Obtained parameters and instrument precision .................................... 55
  3.2.3 Repeatability assessment on the new device ....................................... 56
  3.2.4 Agreement between results analysis for the new instrument ............. 58
  3.2.5 Comparison of aggregation parameters between the new PCA, the Myrenne device and the ESR results ......................................................... 58
  3.2.6 Additional readings obtained by the new PCA ................................... 60
  3.2.7 Light refraction analysis ................................................................. 62

Chapter 4 – Discussion ............................................................................. 63
4.1 Signal-to-noise ratio (SNR) .................................................................... 64
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>Obtained parameters and instrument precision</td>
<td>68</td>
</tr>
<tr>
<td>4.3</td>
<td>Repeatability assessment</td>
<td>70</td>
</tr>
<tr>
<td>4.4</td>
<td>Level of agreement between results</td>
<td>71</td>
</tr>
<tr>
<td>4.5</td>
<td>Comparison between PCA, the Myrenne device and ESR results</td>
<td>72</td>
</tr>
<tr>
<td>4.6</td>
<td>Limitations</td>
<td>76</td>
</tr>
<tr>
<td>4.7</td>
<td>Future directions</td>
<td>77</td>
</tr>
<tr>
<td>4.7.1</td>
<td>Modifications to the analysis chamber</td>
<td>77</td>
</tr>
<tr>
<td>4.7.2</td>
<td>Signal digitisation enhancement</td>
<td>78</td>
</tr>
<tr>
<td>4.7.3</td>
<td>Technical matching and testing protocols</td>
<td>78</td>
</tr>
<tr>
<td>4.7.4</td>
<td>Commercialisation</td>
<td>79</td>
</tr>
<tr>
<td>4.8</td>
<td>Conclusion</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>83</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>AAC</td>
<td>Area above the curve</td>
<td></td>
</tr>
<tr>
<td>ADC</td>
<td>Analogue to digital converter</td>
<td></td>
</tr>
<tr>
<td>AI</td>
<td>Aggregation index</td>
<td></td>
</tr>
<tr>
<td>AI&lt;sub&gt;120&lt;/sub&gt;</td>
<td>Aggregation index at 120 seconds</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>Aggregation amplitude</td>
<td></td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
<td></td>
</tr>
<tr>
<td>CEA</td>
<td>Capillary tube erythrocyte aggregometer</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
<td></td>
</tr>
<tr>
<td>IDE</td>
<td>Integrated Development Environment</td>
<td></td>
</tr>
<tr>
<td>KVL</td>
<td>Kirchhoff voltage law</td>
<td></td>
</tr>
<tr>
<td>LED</td>
<td>Light-emitting Diode</td>
<td></td>
</tr>
<tr>
<td>LORCA</td>
<td>Laser-Assisted Optical Rotational Cell Analyser</td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td></td>
</tr>
<tr>
<td>PCA</td>
<td>Portable RBC capillary tube aggregometer</td>
<td></td>
</tr>
<tr>
<td>PEN</td>
<td>Public electrical network</td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cells</td>
<td></td>
</tr>
<tr>
<td>SEE</td>
<td>Standard error of estimate</td>
<td></td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-noise ratio</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;½&lt;/sub&gt;</td>
<td>Aggregation half-time</td>
<td></td>
</tr>
</tbody>
</table>
### List of Figures and Tables

#### Tables

1. Power consumption for the PCA’s main components ........................................51
2. Battery voltage measurements in different operation modes ..........................52
3. Testing frequencies for the stepwise disaggregation protocol ..........................53
4. Coefficient of variation (CV; %) for the different parameters measured with the new PCA .................................................................................................................. 56
5. Standard error of estimate (SEE) for the different parameters measured with the new PCA .................................................................................................................. 57
6. Results of t-test and Descriptive Statistics for Aggregation Index scores and T ½ values for two consecutive trials for the same sample ................................................57
7. Spearman’s rho coefficient correlations for relations between the different parameters measured by the new engineered aggregometer and those obtained by other methods ........................................................................................................ 59
8. Diffracted angles calculations for soda-lime glass capillary tube .........................62

#### Figures

1. The effect of shear rate on whole blood viscosity .................................................16
2. RBC aggregation parameters calculated from the collected syllectogram ..........25
3. Light transmittance measurement to determine RBC aggregation .....................31
4. Block diagram of the capillary tube RBC aggregometer (PCA) .........................33
5. Signals collected during disaggregation processes for PCA and Myrenne ..........54
6. SNR calculated for unloaded and measurement conditions for the PCA and Myrenne aggregometer ........................................................................................................ 55
Relationship between the data acquired using the PCA for the same measurement on different trials

Differences between aggregation index and aggregation half time scores plotted against the average corresponding scores for two consecutive trials for the same participant.

Syllectograms obtained using PCA and Myrenne device for the same sample in consecutive trials.

Sigmoidal syllectograms observed during RBC aggregation measurements.

Configuration setup for the phototransistor.

Light intensity versus time for a blood sample with stepwise increase and sudden cessation of vibration.

Syllectograms obtained using PCA (downscaled) and Myrenne device for the same sample.

The diffracting effect produced by the capillary tube walls on infrared light.
Chapter 1 – Introduction and literature review

1.1 Introduction

1.1.1 Importance of blood flow for mammalian life

It is well established that the adequate delivery of oxygen and the removal of waste products from body tissues is a critical objective of the cardiopulmonary system. In addition, the transportation of substances (hormones, nutrients, etc.) and regulation of body temperature are also important functions of the circulatory system specifically associated with body maintenance and the response of the immune system. Blood is composed by plasma (~55%), leukocytes and platelets (<1%) and erythrocytes (~45%). Plasma consists of 90% water and 10% of dissolved solutes including electrolytes and important proteins such as albumins, globulins and fibrinogen. Variations in the proportion of the different blood components can modify their physical properties and thus its flow in the circulatory system. Optimal blood flow is, therefore, essential for the preservation of mammalian life (Tran-Son-Tay & Shyy, 2006). Impaired blood flow may precipitate different disease conditions secondary to poor perfusion which can lead to decreased delivery of oxygen for target tissues and organs, as can be observed in myocardial ischemia and cardiac infarct.

1.1.2 Determinants of blood flow

The flow of blood in the circulatory system has been of scientific interest for many years, and it has been shown through simplified models that blood flow is dependent upon the geometry (i.e., length and diameter) of the tube (e.g., vein), and the velocity and viscosity of the fluid (i.e., blood). Much is known regarding the regulation of blood vessel geometry, and its resultant effects on blood flow, given that the diameter of the vessel through which blood flows is perhaps the primary determinant of flow (Chien, 1984). In brief, vessel geometry is regulated by neuroendocrine control, where the sympathetic branch of the autonomic nervous system, for example, maintains vasomotor tone in
response to homeostatic requirements (e.g., maintaining blood pressure etc.). The velocity of blood flow for a given vessel diameter is governed by the pressure difference across the length of the vessel; the highest pressures exist in the central circulation where the heart generates large pressures during contraction, while the peripheral circulations tend to have much lower pressures, facilitating flow away from the heart and towards the metabolically active tissues (e.g., skeletal muscle). Accordingly, the direct relationship between blood pressure, cardiac output and peripheral resistance allows the physiological adjustment of intrinsic (i.e., stroke volume and heart rate) and extrinsic cardiac factors (i.e., neurological and endocrine control) to preserve the required pressure of the blood in the circulatory system for the human body’s adequate functioning.

An often overlooked determinant of blood flow is the physical properties of blood itself. Of particular importance is the viscosity of blood, which due to its two-component nature, is a non-Newtonian and shear-thinning fluid. Figure 1.

![Viscosity vs Shear Rate Graph](image)

Fig. 1. The effect of shear rate on whole blood viscosity. Decrease in the viscosity of blood (centipoise) at increasing shear rates (1/s). The shear rate refers to the velocity gradient between adjacent layers of blood and is directionally related to the rate of flow. The units for viscosity are centipoise (cp) or millipascal seconds.
As the main determinants of blood viscosity, plasma and red blood cells (RBC) can influence directly the flow properties of blood by any change in their own flow properties. RBC physical attributes including deformability have a determinant effect on blood flow. Erythrocytes have an average diameter of 8 μm, with a mean volume of 90 fl (Bull & Breton-Gorius, 1995). The cell membrane flexibility and the biconcave shape of RBC, allows the erythrocytes to enter capillaries with a diameter between 2 and 5 μm (Wiedeman, 1963). Accordingly, the proportion of the cellular component to the whole blood volume (i.e., haematocrit) plays also a crucial role in determining the blood viscosity within a wide range of applied shear rates.

Under high shear stress, RBC experiment a change in structure to ellipsoidal configurations due to their exceptional flexibility when compared with other cells (Chien, 1987). This unique ability to deform fosters the alignment of erythrocytes to flow stream lines in blood vessels, which results in a significant decrease in blood viscosity. This alteration in form and orientation is enhanced under high shear rates conditions (Baskurt & Meiselman, 2003). Conversely, at low-shear conditions or stasis erythrocytes recover their original biconcave-discoid shape and tend to clump together into larger particles. As a result of the aggregation phenomena, the formed structures also known as aggregates contribute to the increase of the frictional resistance between flow-stream lines which results in elevated viscosity under low-shear stress (Baskurt & Meiselman, 2003).

Blood flow is determined, in part, by RBC deformability, orientation and arrangement, and in particular RBC aggregation (collectively the study of these properties is referred to as “blood rheology”). Blood rheological properties govern the capacity of erythrocytes to flow into capillaries or pass through tissues. Detrimental alterations in blood rheology may result due to various diseases (cardiovascular, peripheral vascular, diabetes, etc.) and may be routinely and easily identified. Increased levels of RBC aggregation have been detected in different diseases in which vascular inflammation occurs (Justo et al., 2003; Samocha-Bonet et al., 2003; Schechner et al., 2003).
addition, a positive correlation between inflammatory markers and the observed aggregates was established, suggesting that RBC aggregation can be enhanced by the inflammatory processes (Crawford, Chacko, Kevil, & Patel, 2004). Accumulating evidence indicates that the degree of elevated RBC aggregation is fundamental to the aetiology and progression of various disorders, including type 2 diabetes, where it is reported that the resultant hyperviscosity of blood may contribute to ischaemic necrosis (Simpson, 1988). Moreover, some studies have reported that blood viscosity can be approximately 20% higher in diabetes patients showing also significant increments in fibrinogen and other large plasma proteins such as globulins (Skovborg, Nielsen, Schlichtkrull, & Ditzel, 1966).

1.1.4 Significance of Haematocrit and Exercise effect on blood rheology

The functional properties of RBC determine the capacity of the blood to perform its basic functions. Since RBCs are the most numerous cells in the blood, the flow properties of blood are strongly influenced by its volume fraction in a suspension, termed the haematocrit, which can be expressed as either a fraction (litre/litre, l/l) or as volume percent (%) (Wells & Merrill, 1962). In healthy humans, the average haematocrit for men and women is 42 and 38%, respectively (Brown, Hopper, Hodges, Bradley, Wennesland, & Yamauchi, 1962; Wennesland, Brown, Hopper, Hodges, Guttentag, Scott, Tucker, & Bradley, 1959). The significance of haematocrit as a determinant of blood viscosity is evidenced in the disproportionate (exponential) increase in blood viscosity for a given unit increase in haematocrit (Tran-Son-Tay & Shyy, 2006).

Correspondingly, available evidence suggests that exercise and physical training produce several effects that improve blood rheology in normal healthy subjects and in patients (El-Sayed, Sale, Jones & Chester, 2000). Moreover, significant changes in blood rheology have been reported as a result of heavy exercise performance (Brun et al., 1998; Neuhaus & Gaehtgens, 1994; Szygula, 1990). Specifically exercise type, intensity, work period and individual's athletic capacity have demonstrated to be determinant factors in the domain of the effects of exercise on blood rheology.
(Yalcin, Erman, Muratli et al., 2003). For example, athletes and subjects who train on a regular basis have altered blood properties such as increased blood fluidity and decreased plasma viscosity when compared to sedentary individuals (Fendler & Matrai, 1985; Letcher, Pickering, Chien, & Laragh, 1981). This alteration constitutes a rheological advantage, since reduced blood and plasma viscosity will improve the delivery of oxygen throughout the microcirculation during physical exertion. (El-Sayed, 1998; Letcher et al., 1981). In addition, there is consensus that endurance exercise can not only promote important modifications in blood properties in healthy subjects but in coronary heart disease patients (Brun, Miccallef, & Orsetti, 1994; Ernst, Daburger, & Saradeth, 1991; Galea & Davidson, 1985; Letcher et al., 1981; Martin, Ferguson, Wigutoff, Gawne, & Schoomaker, 1985).

1.1.5 The aggregation phenomenon

Interestingly, when the blood of healthy humans is analysed at stasis or at very low rates of flow, red blood cells (RBC) are observed to cluster or aggregate. In human blood, the clumping of RBC (i.e., aggregation process) tends to form loose linear and three-dimensional clusters with a characteristic face-to-face morphology, in a structure that resembles a stack of coins (i.e., rouleaux formations; Chien & Jan, 1973; Chien, Sung, Kim, & Usami, 1977). It should be noted that RBC aggregation is a reversible phenomenon, with adequate shear forces able to disperse the rouleaux; at stasis or low flow, aggregate reformation occurs. While rouleaux may be formed in the venous circulation, it has generally been accepted that high shear forces in arterial vessels prevent the aggregate formation (Baskurt & Meiselman, 2008). However, the phenomenon of aggregation cannot be observed if RBCs are suspended in isotonic salt solutions, and experimental studies support that the extent and rate of erythrocytes aggregation strongly depend on the concentration and type of the macromolecules present in the suspending medium (Rampling, 1988). Aggregation is directly affected by the number of RBC per unit volume (i.e., haematocrit); haematocrit is one of the most important determinants of RBC aggregation (Agosti et al. 1988), given that elevated cell-
cell contact facilitates the formation of aggregates.

Consequently, the aggregation phenomenon starts when the arbitrary movement of erythrocytes allows them to be in close proximity (Kim et al., 2005; Kim et al., 2007). Moreover, the required time for the RBC to aggregate is reduced as the haematocrit is increased (Baskurt et al. 1998; Hardeman et al. 2001; Shin et al. 2009).

RBC aggregation has also been associated to non-continuum behaviour of blood in the proximity of the vessel wall, where a significant decrease in the apparent viscosity was evidenced. This response can be explained by the formation of a cell-free or cell-depleted layer at the wall of the vessel facilitated by the aggregation process. The aggregates formation leads to an axial migration of erythrocytes to the centre of the tube promoting the development of an outer lubricant layer. In the same way, evidence from different studies where RBC aggregation was analysed in tube flow has demonstrated a complex reciprocity between the augmented local viscosity of an aggregate and the likelihood of that aggregation process to thicken the cell-free layer (Reinke et al. 1987). In spite of the fact that the aggregation process can affect the velocity profile by increasing the apparent viscosity, it also promotes a greater axial migration, which results in a thicker cell-free layer at the wall. Therefore, while some aggregation usually increases apparent viscosity, enhanced levels of aggregation can produce the opposite effect (Goldsmith et al. 1999). The degree of RBC aggregation is influenced by both intrinsic and extrinsic factors (Baskurt & Meiselman, 2012). Fibrous plasma proteins, such as fibrinogen, are proaggregating and represent one of the primary extrinsic factors that determine the rate and magnitude of RBC aggregation (Rampling, 1988).

Fibrinogen is a soluble glycoprotein that is widely accepted to be the most determinant plasma protein in regards to RBC aggregation induction. Fibrinogen is an acute phase protein, and as such when fibrinogen levels are elevated (for example, in several diseases), an inflammatory response is triggered. As a result, higher content of fibrinogen in plasma can lead to altered conditions in the formation of RBC aggregation with particular changes affecting the size of the aggregate, the blood

The term “aggregability” has been defined as the intrinsic tendency of erythrocytes to form aggregates independent of plasma factors (such as fibrinogen), in order to differentiate this process from the effects of the extrinsic aggregation factors. The aggregation behaviour of RBCs can be understood as the resultant equilibrium between aggregating and disaggregating forces including fluid shear stress, the potential mechanical energy of the cell membrane and the electrostatic repulsive forces among cells (Chien & Sung, 1987; Meiselman, 1993; Kobuchi & Ogiwara, 1988). Shear forces may be produced by mechanical means such as blood flowing through the vasculature, and these forces prevent rouleaux formation and disperse previously formed aggregates; shear forces are thus the primary disaggregating factor in vivo. Another disaggregation factor is the electrostatic force created by the net negative charge of RBC, which promotes repulsion between adjacent blood cells and thus opposes aggregate formation (Baskurt, Neu, & Meiselman, 2011). There is also important evidence that supports the fact that the biconcave-discoid shape of erythrocytes plays a crucial role for aggregation, showing that any divergence from this typical shape can impair the normal aggregation behaviour (i.e., oval RBC of camelids with no aggregation) (Windberger & Baskurt 2007; Meiselman 2009).

Two different models have been proposed to explain the process leading to RBC aggregation formation which are mutually exclusive due to the way both statements resort to the repulsive and attractive forces to support their own proposals: The “Bridging Model” states that bridging forces produced by adsorbed macromolecules on adjacent cell membranes surpass the forces associated to disaggregation such as the tension on the membrane and the electrostatic repulsive force (Brooks, 1973, 1988; Chien, 1975; Chien and Jan, 1973; Chien and Lang, 1987); whereas the “Depletion Model” refers to an osmotic gradient produced by a selective exclusion of macromolecules from the erythrocyte surface which results in a diminished cell-solvent affinity due to the movement of fluid away from the gaps between RBCs (Van Oss, Arnold, & Coakley, 1990; Evans, Berk, Leung, &
Mohandas, 1991; Baskurt, Tugral, Neu, & Meiselman, 2002; Neu & Meiselman, 2002). It is evident that these two models have different predictions in regards to the effects on RBC aggregation when the concentration of macromolecules increases in the proximity of the cell surface. Furthermore, a recent study has analysed the electrostatic repulsion forces and also the magnitude of forces produced by depletion providing conceptual support for the depletion model (Neu & Meiselman, 2002).

It is known that RBC aggregation increases due to various diseases such as sepsis (Baskurt, Temiz, & Meiselman, 1997), diabetes mellitus (Bauersachs, Shaw, Zeidler, & Meiselman, 1987), and myocardial ischemia (Dormandy, Ernst, Matrai, & Flute, 1982). The systemic inflammatory response associated with these diseases is thought to be a major cause of elevated RBC aggregation (Stuart, 1991). This elevation in RBC aggregation is associated with an increase in plasma concentrations of acute phase reactants, since these plasma proteins play a crucial role in the aggregation process (Simmonds, Christy, Marshall-Gradisnik, Meiselman, & Baskurt, 2011).

Evidence supports that structural and chemical alterations are exhibited in RBC due to sepsis or when RBC are subjected to biochemical factors associated with this condition (Todd & Mollitt, 1995; Todd, Poulos, Davidson, & Mollitt, 1993). Additional studies performing a cell electrophoresis procedure under experimental sepsis conditions, evidenced changes in RBC surface properties including the electric charge, which results in an enhanced aggregation due to the significant reduction in electrostatic repulsion forces (Baskurt et al. 2002).

With regards to diabetes mellitus, it was found that blood viscosity is inversely related to flow and therefore may contribute to flow-related insulin resistance (Tamariz, Young, Pankow, Yeh, Schmidt, Astor, & Brancati, 2008). Prior studies have also found a relationship between elevated blood viscosity, insulin resistance, and type 2 diabetes (Høiegen, Fossum, Moan, Enger, & Kjeldsen, 1998; Caimi, Sinagra, Scarpitta, & Lo, 2001; Capoğlu, Ünüvar, Bektaş, Yiğmaz, & Kaya, 2002).
1.1.6 Measurement of Red Blood Cell Aggregation

For the purpose of measuring RBC aggregation, different methods and devices have been developed. A direct method for the measurement of RBC aggregation has not been regularly used in routine clinical laboratories. However, measurement of the erythrocyte sedimentation rate (ESR), in which the sedimentation of RBCs is observed in a vertical glass tube, has been extensively adopted in clinical pathology laboratories to monitor disease for decades (Lynch & Raphael, 1983). The ESR is widely recognised as a nonspecific inflammation indicator. This laboratory test is often prescribed as a screening method. The outcome of this clinical test is interpreted with reference to the presence and level of inflammation or acute phase reaction, but is infrequently associated with the erythrocyte aggregation process by most medical practitioners.

The measurement of ESR has several limitations, and is not the preferred method to assess RBC aggregation. The rationale behind this is due to several factors including the amount of time required for the test (i.e., one hour), the lack of sensitivity to identify differences in sedimentation from healthy individuals (Rampling et al., 1989), and its strong dependence on haematocrit level which is not standardised prior to measurement. Nevertheless, ESR has been proven as an indirect predictor of the RBC aggregation process (Mayer, Pospisil & Litzman, 1991; Oka, 1984); significant correlations between ESR and RBC aggregation indexes determined using photometric methods have been reported (Baskurt, Uyuklu, Ulker, Cengiz, Nemeth, Alexy, Shin, Hardeman, & Meiselman, 2009b; Hardeman, Levitus, Pelliccia, & Bouman, 2010; Potron, Jolly, Nguyen, Mailliot, & Pignon, 1994; Rampling & Martin, 1989).
1.1.7 Photometric methods

Photometric methods may be used to determine RBC aggregation, and is based upon the interaction between light and RBC, where light may be reflected (i.e., backscattered) or absorbed by the cell. Light transmission may be used to measure RBC aggregation dynamics by detecting the level of light that passes through a blood suspension: when RBC are free of aggregates, the level of light transmission is diminished due to the dispersed cells, whereas once RBC start to form aggregates, gaps between clusters of cells facilitates light passage through the suspension which may be detected by the photomultiplier (Figure 1). On these grounds, under proper conditions that foster RBC aggregation (i.e., low shear forces), rouleaux formation begin to form leaving fluid gaps between them (Kaliviotis & Yianneskis 2008). As a result, light transmittance is a function of the amount of light that passes through these gaps, therefore representing the degree of aggregation. The time course of the intensity of transmitted or reflected light during RBC aggregation was named syllectogram by Zijlstra et al. (1958). The examination of a syllectogram provides two different types of information about the aggregation process which starts from the high shear rate for the disaggregation stage to the low shear rate or stasis: the extent of the aggregation and the time course of the aggregation process. In order to understand the aggregation response of the blood samples, a certain number of parameters can be calculated through the mathematical analysis of syllectograms. This analysis is performed using the aggregation phase of the curves beginning when the shape recovery process finishes. At this specific instant following the disaggregation period, it can be observed a sharp decline in light transmittance reaching minimum in fractions of second. This minimum represents the beginning of the aggregation process. Based on the information represented by the syllectogram, it is possible to calculate RBC aggregation parameters including the surface areas above (AAC) and under (AUC) the curve (syllectogram), the aggregation index (AI), the amplitude (AMP), aggregation half-time ($T_{\frac{1}{2}}$) and the time constants $T_{\text{fast}}$ and $T_{\text{slow}}$ from the double exponential function which best fits the data collected. Figure 2.
The photometric method is used by different instruments which are able to measure the amount of light transmitted or reflected through or from a blood sample to monitor the aggregation process under specific shear stress conditions.

1.1.8 Commercial available devices for RBC aggregation measurement

The Laser-Assisted Optical Rotational Cell Analyser (LORCA®) and the Myrenne® aggregometer are photometric instruments commercially available for quantitating RBC aggregation. The LORCA device combines an ektacytometer designed for measuring erythrocytes deformability and an aggregometer based on the photometric principle. For this device to operate it is required a 1 ml of sample, with the shearing stress produced by a special arrangement of concentric cylinders driven by a stepper motor which is controlled by an external computer. The LORCA system utilises a 670-nm laser beam which is directed through the sample. The backscattered light is subsequently recorded by a photodiode sensor. An important feature of this instrument is the temperature control which allows the measurements to be performed in a range from ambient temperature to 37°C. Specialized software on an external computer controls all the optoelectronic and mechanical
components of the LORCA to perform the RBC aggregation measurement. The first phase of the measurement corresponds to the disaggregation of the erythrocytes (i.e., aggregates) contained in the sample during 10 seconds. The syllectogram is then recorded for the following 120 seconds. Based on the syllectogram information gathered during the data collection period, the LORCA system’s software calculates the RBC aggregation parameters previously explained. In addition, the LORCA is able to variate the shear rate during disaggregation, which represents a significant advantage during the analysis of samples with particular strong aggregation tendency (e.g., blood samples from multiple melanoma sufferers) (Zhao et al. 1999); a shear rate of approximately 500 s\(^{-1}\) is required to achieve complete disaggregation for such samples. This powerful system can fully analyse a sample in around five minutes, however, some disadvantages of the LORCA system are represented by the high cost of this instrument, lack of portability (i.e., size, weight, power consumption), computer dependence, and the considerable amount of blood required (1 ml) to process a sample.

The Myrenne aggregometer is possibly the most extensively adopted hemorheological analyser. The small amount of blood required for this instrument is approximately 50 µL, which is around 20 times smaller than that used by the LORCA system and thus represents a significant advantage. The shearing mechanism of this device consists of a cone that is rotated by a motor and a plate situated on top of it. The Myrenne aggregometer uses an infrared Light-emitting Diode (LED) as a light source and the light beam is directed through the blood sample located between the cone and the plate at which the gap is ~50 µm (Kiesewetter et al., 1982). The initial stage of the measurement process starts with applying shearing stress to the blood sample mediated by the rotation of the cone for 10 seconds. Once the cone has stopped or reduced its speed of rotation, the light transmission signal is integrated resulting in a numerical value which corresponds to the aggregation index parameter as discussed previously. The Myrenne instrument is not temperature controlled, which makes this device only suitable to perform ambient temperature measurements. Furthermore, the Myrenne aggregometer does not provide information about the time course of the aggregation
program, which represents a significant limitation for experimental research. Although the Myrenne aggregometer has been recognised as the Gold Standard in regards to RBC aggregation measurements, major limitations include the cost of the system, lack of portability (i.e., power consumption), computer dependence in order to acquire the syllectogram information for further parameters calculations, and the absence of a temperature control module.

Alternatively, experimental and custom-built instruments have been used in some studies (Baskurt, Meiselman, & Kayar, 1998; Baskurt, Uyuklu, Ozdem, & Meiselman, 2011). It should be noted that these instruments are primarily designed for use in research laboratories (Baskurt, Cokelet, Connes, Cooke, Forconi, Liao, Hardeman, Jung, Meiselman, Nash, Nemeth, Neu, Sandhagen, Shin, Thurston, & Wautier, 2009a), and thus, a major limitation of current devices is a lack of portability and availability.

The capillary tube erythrocyte aggregometer developed by Baskurt and colleagues in 2011, utilises a photometric method by monitoring the light transmittance through a blood sample contained in a disposable glass capillary tube during the RBC aggregation process. The disaggregation mechanism consists of a small piston and cylinder setup driven by an electrical actuator which connects to the capillary tube through a plastic adapter. The main disadvantage of this disaggregation system is the blood contamination, which is very likely to occur when the piston accidentally pulls blood inside the cylinder or the plastic adapter from the capillary tube. Once the blood sample has gone through the disaggregation procedure, an infrared light beam (LED; wavelength = 910 nm) is directed through the sample and the transmitted light is sensed by a phototransistor (BP101, Siemens, Berlin, Germany). The control of the system, data collection and analysis is performed by specialised software LabVIEW 9.0 (National Instruments, Austin, Texas, USA) running on an external computer linked to the aggregometer via an analog-digital (AD/DA) interface (USB-6009, National Instruments, Austin, Texas, USA). In accordance with the previous description, it is evident that the lack of portability and also computer dependency represent a big limitation especially when field
tests would be required.

1.1.9 Limitations of current instrumentation

Taking into account the fact that most of the devices previously described here are designed for research studies purposes, significant limitations are evidenced as follows:

Size and power consumption

The equipment described in the previous section have considerable sizes and weights. In addition, they all depend on the public electrical network to operate at 240 Volts which represents a clear disadvantage when portability is an assessment factor.

Cost

The range of prices for the commercial available aggregometers goes from several tens of thousands of dollars to hundreds of thousands of dollars, which in most cases doesn’t fit tight budgets of medical institutions around the world.

External computer dependency.

The available commercial aggregometers described in this document, require an external computer for the multiple process control and also for data visualisation analysis. This is a significant limitation in regards to portability and over costs, since expensive software licences may be required.

1.2 Introducing PCA, a newly designed aggregometer

Based on the design of the capillary tube aggregometer by Baskurt and colleagues (Baskurt et al., 2011), a portable and low-power consumption version of this device described herein will not only preserve its most important features, such as easy application and rapid provision of results using a very small amount of blood, but will be designed to overcome the major limitations of the currently
and mostly used available aggregometers. The newly optimised instrument is aimed at quantifying the RBC aggregation process, while incorporating the following innovative designs requirements: i. low-cost of manufacture; ii. portability – thus light weight, battery powered; iii. low power consumption; iv. independence from a personal computer, and; v. incorporate a built-in graphical user interface. The new engineered portable capillary tube RBC aggregometer (PCA) was able to operate with a USB power supply from either a portable or desktop computer USB connection port or a portable battery pack. Moreover, the new portable design had a weight reduction of about 20 fold when compared to the capillary tube aggregometer. A powerful ATMEL® microcontroller regulated the entire measurement process with accurate control phases. Through the use of a touch screen, the device was provided with an intuitive way to control its operation through the different phases of the aggregation parameters measurement. At the same time, the touch shield performed as an integrated graphic interface in which the information was visualized in real time during the data collection process. In addition, at the end of the measurement procedure, the outcome of the calculations associated with the aggregation parameters measurement appeared available on the screen. The engineering process of the present device was conceived as the result of the latent need to bring low-cost specialised equipment to remote regions where budget, transportation facilities and/or power supply restrictions are major limitations for use of current technologies.
Chapter 2 – Experimental design

2.1 Subjects and sampling

Blood samples were obtained via venepuncture from recruited volunteers from the Griffith University located at the Gold Coast campus. Male individuals aged between 20 and 50 years old took part of this blood collection process. No further exclusion criteria were considered for this study. Blood samples were drawn into heparinised vacutainer tubes and aliquots were subject to specific experimental assessments of several types of aggregometers. One ml of blood was transferred to a glass Westergren tube in order to measure the ESR after one hour. Then, 1 mL of blood was required to run the aggregation test on the Lorca system. Additional 0.5 mL of blood was used in the Myrenne aggregometer in order to obtain repeated measurements (i.e., 10) for the same subject. Finally, 0.5 mL was used for repeated measurements using the new device proposed in this study. The analysis samples were transferred to non-heparinised soda lime glass capillary tubes (approx. 50 µL) and subsequently studied for RBC aggregation using the presently described and newly engineered RBC aggregometer. The resultant information obtained from the RBC aggregometer was available for visual inspection in real time and also, stored in a comma-separated-values file for further review and analysis. All the separate outcomes obtained by the different methods were analysed using statistical methods in order to find possible correlations among them (e.g. Section 2.6 Page 50). All participants in the present study provided informed consent, and the experimental design and protocols were reviewed and approved by the Griffith University Human Research Ethics Committee (GU Ethics Reference # 2016/533).
2.2 Engineering of the capillary-tube RBC aggregometer

The measurement principle of the PCA in the present study is based upon light transmission through a suspension of RBC in plasma (i.e., blood) that is contained in a disposable, glass capillary tube. The intensity of light transmission through the blood sample is dependent upon two primary determinants: i. the concentration of cells in the suspension, and; ii. the degree of aggregation of the formed cellular elements. Given RBC are the primary cell-type in blood (~1000:1), the intensity of light transmission therefore represents the aggregation of RBC at a given haematocrit. The range of light transmission intensity through the blood sample reflects two extremes of RBC aggregation tendency (Figure 3): the lowest level of light transmission corresponds to the highest voltage level on the phototransistor output for a given blood sample. This condition reflects a disaggregated state of RBC (Region ii; Figure 3), whereas the highest level of light transmission corresponds to the lowest voltage level on the phototransistor output which is achieved following total aggregation of RBC (Regions i & iii; Figure 3).

Fig. 3. Light transmittance measurement to determine RBC aggregation. Three distinct regions are illustrated in the figure which reflect the underlying behaviour of RBC: Region i is the light intensity of blood that is already aggregated in the rested state; Region ii reflects the change in light intensity following the application of a disaggregating shear, and; Region iii describes the time-course change in light intensity following the cessation of the disaggregating shear.
The following parameters of RBC aggregation will be computed based on the light transmittance-time curves as previously described and presented by Hardeman et al., 2001:

1. Area under the curve (AUC), area above the curve (AAC), and an aggregation index (AI) calculated as the ratio of AUC to the addition of the AUC and the AAC (i.e. $AUC/(AUC+AAC)$) following 120 seconds of the sample being at stasis;

2. Aggregation half-time ($T_{1/2}$), the period of time for light transmittance to increase by one half of maximal amplitude (AMP), following total disaggregation.

To address the limitation in currently available RBC aggregometers, a newly-engineered device was designed and developed that uses the light-transmission principle described above, but requires only a 5-V power source (e.g., Lithium Ion battery). The general design of the PCA was based on a specific microcontroller: the Arduino MEGA system. The new instrument requires five primary components and secondary shields. The shields are basically boards that can be plugged on top of the Arduino board extending its capabilities. The system’s main components are related to: i. the generation and detection of light transmission; ii. heating of blood samples to a desired temperature (i.e., 37°C); iii. a disaggregation motorised mechanism to disperse the RBC in the blood samples for a discrete time period; iv. a touch shield that will provide the control panel for the instrument, the storage device controller and also, the visualisation in real time of the light transmittance signal across the sample and the calculated aggregation parameters; v. and the software required to control and coordinate all the electronic, optoelectronic, electro-mechanic and electro-thermic components and their operation correspondingly.

The block diagram of the PCA is shown in Figure 4. The system is governed by an Arduino microcontroller (AT91SAM3X8E, ATMEL, San Jose, California, USA) which controls the heating panel (10 x 15 cm Electric Heating Pad, Adafruit, New York, USA) and its temperature sensor (MCP9808, Adafruit, New York, USA), the disaggregation mechanism, the light-emitting diode (LED; wavelength=910 nm), the transmitted light sensor phototransistor (PT100MF0MP1, Sharp,
Osaka, Japan), the motor shield (Motor Shield v2.3, Adafruit, New York, USA), and the touch shield (STMPE610, Adafruit, New York, USA).

![Block diagram of the capillary tube RBC aggregometer](image)

Fig. 4. Block diagram of the capillary tube RBC aggregometer (PCA). Temperature increase and control of the heating pad was mediated by the temperature sensor’s feedback. Disaggregation process was initiated by the electromagnetic component activation during 10 seconds once 37°C of temperature is achieved. Analogue data from the phototransistor represented the light transmittance intensity variations caused by the disaggregation and aggregation processes.

2.3 Engineered aggregometer: main components

2.3.1 Arduino MEGA microcontroller board

The brain power of the proposed instrument relies on an open-source and widely supported platform – the Arduino development system. This prototyping platform was designed to be easy-to-use hardware and software. Arduino provides also an open-source programming tool, the Arduino IDE (Integrated Development Environment) for writing code and uploading to the microcontroller system. The main reason why this open-source platform was selected among many other options, was that this system enjoys a large user base where developers from around the world share...
knowledge and thus support is easy to provide/receive. In addition, the low cost of this microcontroller system and the required peripheral components was a primary determinant. Important features of the Arduino MEGA board include 54 digital input/output pins, 16 analogue inputs, 4 hardware serial ports, microprocessor speed (16MHz) and its compatibility with shields (i.e., touch screen, motor shield) that are of vital importance for the present project.

2.3.2 Infrared light emitter and receiver

The sensor system of the instrument operates according to the light transmittance principle. A 910-nm infrared LED was selected, because non-visible light at this wavelength provides the ideal balance between absorbance and reflectance when infrared light is directed through a sample of human tissue. The light source produces the light beam which passes through the blood sample contained on a capillary tube. Accordingly, the light transmittance was sensed by an infrared phototransistor operating at the same wavelength as the emitter. This sensor setup allowed the system to transduce the amount of light which effectively passed through the sample into a readable voltage analogue signal that is processed and visualised in real time. In addition, the data collected from the sensor represents the basic input to the microcontroller to calculate the RBC aggregation.

2.3.3 Motor shield

The motor shield is a fully stackable and compatible board for the Arduino platform, designed to drive DC and Stepper motors. However, due to its capacity to drive currents up to 1.2 Amperes, the motor shield was used to isolate the power phase (i.e., heater component and disaggregation mechanism) from the microcontroller. In this way, the delicate electronics from the Arduino board (i.e., microprocessor, flash memory, digital ports, etc.) were isolated from the increased current values typically driven in the motor shield during the activation of the heating and disaggregation mechanisms.
2.3.4 **Heater element and temperature sensor**

A flexible, light fabric with stainless steel fibres which only requires 5 volts to operate is used in this project to warm up the blood sample and therefore, preserve the in-vivo conditions. The amount of current required for this component to operate is approximately 600mA, which is significantly larger when compared to the maximal current driven by the Arduino MEGA board (~80 mA). For this reason, the use of a power shield (i.e., motor shield) is compulsory in order to prevent an eventual overcurrent drawn by the high power consumption components which may cause irreversible damage to the Arduino board. The temperature sensor helps the microcontroller to maintain the desired temperature by a feed-back system which will modulate the power delivered to the heating component.

2.3.5 **Disaggregation mechanism**

The mechanism designed to achieve a full disaggregation state of the RBCs contained in the blood sample before starting the aggregation measurement consists of an electromagnetic actuator, a mechanical to pneumatic transducer, and connecting tubing. During the electric activation phase, the electromagnetic component produces the forth movement of its core, which is necessary to create a deformation of a silicon membrane. Accordingly, the pressure on the air chamber increases causing a quick and small displacement of the blood sample contained in the capillary tube (~ 1 mm) previously attached to the disaggregation mechanism by a miniature silicon hose. On the other hand, during the electric deactivation phase, the electromagnet’s metallic core returns to its resting position, releasing the pressure on the silicon membrane. Consequently, the blood sample moves backwards to its initial position inside the capillary tube due to the membrane’s elastic recoil effect. In this manner, with a proper electric control signalling provided by the microcontroller, the disaggregation mechanism produces the shear stress as a small-scale agitation, necessary to achieve
the complete disaggregation state of the erythrocytes which represents the desired starting point of
the RBC aggregation measurement. Based on experimental testing performed during the prototype
stage of this project, and also supported in evidence from another important study (Shin, Jang, Park,
Ku, & Suh, 2005), a stepwise disaggregation routine including two different frequencies was
designed to achieve the highest disaggregated state before starting the RBC aggregation
measurement. It is important to highlight that the electromagnet requires a current of approximately
500mA to activate, reason why this component is also energised via the motor shield as the heating
element is, based on the rationale previously explained.

2.3.6 Touch shield/screen

The touch shield/screen is also a fully stackable and compatible board for the Arduino platform,
designed to provide an interactive visual interface and storage capabilities for the instrument.
Specifically, the touch screen implements the control panel for this new aggregometer. In other
words, different options to operate the new device appear available on the screen for the operator to
activate by using touch technology. Moreover, as a visual interface, the touch shield allows the
operator to observe the data represented as a syllectogram in real time. Furthermore, at the end of
the two minutes period when the data collection and visualisation occurs, all the information
produced as a result of the aggregation parameters calculations are also available on the screen for
inspection. Finally, the touch shield provides a micro SD card slot, available to be programmed to
save the syllectogram’s data points and also the parameters calculations into a standard comma-
separated values file, which represents a significant feature for further analysis and revision.

2.4 Software component

For the engineered capillary tube aggregometer to perform as a medical instrument, different
software routines, functions and procedures were developed in C++ programming language.
Arduino is a powerful development platform whose technical capabilities can be enhanced by
connecting different shields or elements. In order to produce an efficient functioning system, which maximises processing power, sensor and actuator configuration as well as collaborating interfaces, novel software was developed. Following subsections will describe the development of the software utilised in the current thesis.

2.4.1 Disaggregation subroutine

Controlling the power and speed of the electromagnetic component of the disaggregation mechanism was a crucial mission for the new engineered instrument. The final power and speed were obtained after many experimental trials during the development of this project. Different aspects were taken into consideration being the most relevant ones the adequate sample agitation into the capillary tube in order to avoid any blood contamination to the system and also the desired degree of RBC disaggregation. Optimally, the frequencies that were experimentally found to achieve a complete disaggregation state preceding the measurement of the blood aggregation were 9.3Hz and 8.1Hz for a non-interrupted sequence of 5 seconds duration for each. Also, the blood contamination protection was achieved by controlling the power delivered to the solenoid at 70% of its maximal capacity. The source code is presented below.

```c
void disaggregation() {
  uint16_t i;
  //Serial.println("Disaggregation process...");
  mySolenoid->run(FORWARD);
  //myHeater->setSpeed(120);
  //Step-wise disaggregation process cycle
  for (i = 0; i < 40; i++) {  //fast cycle
    mySolenoid->setSpeed(180);
    delay(54);
    mySolenoid->setSpeed(0);
    delay(54);
  }
  for (i = 0; i < 50; i++) {  //slow cycle
    //
  }
}```
mySolenoid->setSpeed(180);
delay(62);
mySolenoid->setSpeed(0);
delay(62);
}

mySolenoid->setSpeed(0);
mySolenoid->run(RELEASE);
disaggregated = true;
}

## 2.4.2 Heating subroutine

Activating the heating element at different intensities and also deactivating it allows the new instrument to achieve in-vivo conditions during the test by an accurate temperature control. The source code is presented below.

```c
void heating(int indicator) {
    switch (indicator) {
        case 0: // turning on the heater
            myHeater->setSpeed(255);
            myHeater->run(FORWARD);
            break;
        case 1: // setting steady temperature
            myHeater->setSpeed(130);
            myHeater->run(FORWARD);
            break;
        case 2: // turning off the heater
            myHeater->setSpeed(0);
            myHeater->run(RELEASE);
            break;
    }
}```
### 2.4.3 Rescaling subroutine

In order to obtain an optimal visualisation scale of the syllectogram depicted in real time, a rescaling subroutine was created. Particularly, when the syllectogram data points reach the minimal visualisation value on the screen, this mighty subroutine starts to rescale the already acquired data up to that specific moment. In addition, this function resets the visual data grid and repaints the scaled syllectogram at high speed to create the auto scale effect on the screen for the operator. This function receives as parameters the sequence pointer (i.e., the coordinate of the pixel in the x axis) at which the rescaling is required and also the new scale to be applied to the already collected data at that specific sequence. The source code is presented below.

```c
void rescaling (int sequence, uint16_t scale) {
    uint16_t k;
    //String myScaledDataBuffer = String();
    //float myInitialScaledPlot = 0.0;
    float myScaledPlot = 0.0;
    //Cleaning the screen and drawing the grid
    dataGrid();

    //Serial.print("new Scale: "); Serial.println(scale);
    myInitialData = (myAnalogData[0][1] * scale);
    //Serial.print("new InitialData: "); Serial.println(myInitialData);

    for (k = 0; k <= sequence; k++) {
        myScaledPlot = (myAnalogData[k][1] * scale) - myInitialData;
        //tft.drawPixel(k, myScaledPlot, ILI9341_WHITE);
        if (k > 0) {
            tft.drawLine(k - 1, myAnalogData[k - 1][1] * scale, k, myScaledPlot, ILI9341_WHITE);
        } else {
            tft.drawPixel(k, myScaledPlot, ILI9341_WHITE);
        }
    }
}
```
2.4.4 Analog processing subroutine

This complex subroutine was created to perform different tasks and also to coordinate other subroutines. Important processes included in this routine are the initialisation of the micro SD card for further file storage; the creation of the CSV file where the syllectogram and the aggregation parameters data will be stored; the data acquisition of the infrared sensor data via the Arduino’s in-built analogue to digital converter (ADC); the real-time visualisation of the syllectogram and the invocations to the rescaling subroutine when required; and the call to the subroutine responsible for the aggregation parameters calculations which will be describe next. At the processing data level, this is the most important routine developed for the system. The source code is presented below.

```c
void analogProcessing() {
    File myPhoenixFile;
    myPhoenixFileName = ("File1.csv");
    String myHeaderFileName = ("File");
    String myDataBuffer = String();
    boolean myRescaled = false;
    float valueADC = 0;
    float bufferVoltage = 0;
    float myAnalogToVoltage = 0;
    float myScale = 10;
    uint16_t myInitialAnalogData = 0;
    int h = tft.height();
```
int w = tft.width();
//Serial.print(" -> h: "); Serial.print(h);
Serial.print(" w: "); Serial.println(w);
dataGrid();
// read the input on analog pin 7:
uint16_t i = 0;
uint16_t sensorValue = 0;
//Storing the plots into a file
uint32_t j = 0;
Serial.print("Initializing SD card...");
if (!SD.begin(SD_CS)) {
    Serial.println("failed!");
} else {
    Serial.println("OK!");
    while (SD.exists(myPhoenixFileName)) {
        myPhoenixFileName = myHeaderFileName;
        myPhoenixFileName += j++;
        myPhoenixFileName += ".csv";
    }
    Serial.print("File to use: ");
    Serial.println(myPhoenixFileName);
    myPhoenixFile = SD.open(myPhoenixFileName, FILE_WRITE);
    Serial.println("File opened!");

while (i < 320) {
    sensorValue = analogRead(A7);
    //Serial.print(" -> Voltage on receptor: ");
    Serial.print(i); Serial.print(" : ");
    Serial.println(sensorValue);
    if (i == 0) {
        myInitialAnalogData = sensorValue;
    }
    myAnalogData[i][0] = i;
myAnalogData[i][1] = abs(myInitialAnalogData - sensorValue);
myPhoenixFile.print(i);
myPhoenixFile.print(",");
myPhoenixFile.println(myAnalogData[i][1]);
if (i == 0) {
//myInitialData = ((5 - (sensorValue * (5 / 1023.0))) - 2) * (myScale);
myInitialData = abs(myInitialAnalogData - sensorValue) * myScale;
} else if (myRescaled) {
myInitialData = myAnalogData[0][1] * myScale;
myRescaled = false;
}
//Convert the analog reading (which goes from 0 - 1023)
//to a range (0 - 5000):
//myAnalogToVoltage = myAnalogData[i][1] * (5 / 1023.0);
valueADC = (myAnalogData[i][1] * myScale) - myInitialData;
//voltage difference from no transmittance
Serial.print(" -> Value on ADC: "); Serial.print(i);
Serial.print(" "); Serial.println(valueADC);
if (valueADC >= 240) {
//Adjusting the scale
if (myScale > 2) {
    myScale = myScale - 1;
    myRescaled = true;
} else {
    myScale = myScale * 0.8;
    myRescaled = true;
}
rescaling(i, myScale);
//Serial.print(" -> Scale "); Serial.println(myScale);
} else {
//tft.drawLine(i - 1, myAnalogData[i - 1][1] * myScale,
i, valueADC, ILI9341_WHITE);

} else {
    tft.drawPixel(i, valueADC, ILI9341_WHITE);
}

//Serial.print(" -> Pixel: "); Serial.print(i);
Serial.print(" "); Serial.println(valueADC);
delay(375);    // delay in between reads for stability
}
i++;

aggregationIndex();

//Including the calculated aggregation parameters into the file
myPhoenixFile.print("AI:");
myPhoenixFile.print(",");
myPhoenixFile.println(myAI);
myPhoenixFile.print("AAC:");
myPhoenixFile.print(",");
myPhoenixFile.println(myAAC);
myPhoenixFile.print("AUC:");
myPhoenixFile.print(",");
myPhoenixFile.println(myAUC);
myPhoenixFile.print("T1/2:");
myPhoenixFile.print(",");
myPhoenixFile.println(myTHalf);
Serial.println("File filled!");
myPhoenixFile.close();
Serial.println("File created!");

//curveFittingProcess();
//plotting the curve filtered data
filteredSignalDisplay(myScale);
//toDo create a routine for plotting that can be used from different calls
}
2.4.5 Aggregation parameters calculations subroutine

Based on the information collected during the RBC aggregation measurement, this subroutine performs simple calculations to determine the aggregation parameters of interest (i.e., AAC, AUC, AI\textsubscript{120}, and T\textsubscript{1/2}). Additional auxiliary functions are invoked by this subroutine to find the maximum and minimum values from the data collection as part of the aggregation parameters calculations.

The source code is presented below.

```c
void aggregationIndex() {
    //Calculation of the aggregation index based on the areas of the curve
    float myMaxYValue = maxValue(myAnalogData, 1);
    float myMinYValue = minValue(myAnalogData, 1);
    Serial.print("myMaxYValue: "); Serial.println(myMaxYValue);
    Serial.print("myMinYValue: "); Serial.println(myMinYValue);
    float myAmpHalf = ((myMaxYValue - myMinYValue) / 2) + myMinYValue;
    Serial.print("myAmpHalf: "); Serial.println(myAmpHalf);
    //Area above and under the curve
    for (uint16_t p = 0; p < 320; p++) {
        //Subtract the y value from the y max value for AAC
        myAAC += myMaxYValue - myAnalogData[p][1];
        //Subtract the min value from the y value for AUC
        myAUC += myAnalogData[p][1] - myMinYValue;
        if ((myAnalogData[p][1] >= myAmpHalf) && (myTHalf == 0)) {
            Serial.print("Analog data for T1/2: ");Serial.println(myAnalogData[p][1]);
            myTHalf = myAnalogData[p][0];
            myTHalf = myTHalf * 3 / 8;  //Conversion from pixels to seconds
        }
    }
    Serial.print("myAAC: "); Serial.println(myAAC);
    Serial.print("myAUC: "); Serial.println(myAUC);
}
```
Serial.print("myTHalf: "); Serial.println(myTHalf);

//Verification of operators to calculate AI
if (myAAC > 0 && myAUC > 0) {
    myAI = (myAUC / (myAAC + myAUC)) * 100;
} else {
    myAI = 0;
}
Serial.print("myAI: "); Serial.println(myAI);

//Function returning the max value from an array
//@param double array: The array to be examined
//@param int: Indicates the specific position in the array to be examined
float maxValue (double array[][2], int n) {
    float myMaxValue = 0;
    for (uint16_t q = 0; q < 320; q++) {
        //Subtract the y value from the y max value
        if (myMaxValue < myAnalogData[q][n]) {
            myMaxValue = myAnalogData[q][n];
        }
    }
    return myMaxValue;
}

//Function returning the min value from an array
//@param double array: The array to be examined
//@param int: Indicates the specific position in the array to be examined
float minValue (double array[][2], int n) {
    float myMinValue = 0;
    for (uint16_t q = 0; q < 320; q++) {
        //Subtract the y value from the y max value
        if (myMinValue > myAnalogData[q][n]) {
            myMinValue = myAnalogData[q][n];
        }
    }
    return myMinValue;
}
//Serial.print("Stored data: ");
Serial.println(myAnalogData[q][n]);
if (myMinValue > myAnalogData[q][n]) {
    myMinValue = myAnalogData[q][n];
}
return myMinValue;

2.5 Engineered aggregometer

2.5.1 Sample processing

To initiate analysis of RBC aggregation, approximately 50 microliters of the blood sample previously collected into a heparinised vacutainer are transferred into a disposable soda lime glass capillary tube. The capillary tube is approximately 75% filled with respect to its total length (75 mm). The capillary tube is subsequently placed on a thermoconductive polymer block attached to a heating panel, and one end will then be connected to the aggregometer via a miniature silicon hose, which is the final end of the disaggregation motorised mechanism. At this stage, the capillary tube is in position between the light transmitter and receiver arrangement, such that light is directed through the blood sample. The instrument is then activated through an option at the control panel. The control panel implemented through the touch shield allows the system to activate different components of the instrument at non sequential mode. Accordingly, the heater component, the disaggregation mechanism or the full process can be triggered by touching the corresponding option on the screen. By selecting the “Heater” option, the heating component is activated by the microcontroller at its maximal power. This activation may be appropriate for a pre-heating stage when the instrument is required to be ready for testing after some hours of general deactivation. Another available option on the screen is “Disaggregation” which triggers the disaggregation
mechanism during ten seconds at two different frequencies to achieve a full disaggregation state in a stepwise procedure. The disaggregation routine may be used as a preliminary way to determine the proper function of this electro-mechanical stage as well as to verify the connection between the capillary tube containing the blood sample and the disaggregation mechanism. The complete procedure can be triggered by “Full Proc” option on the screen. This routine starts with the activation of the heating element until the desired temperature (37°C) is achieved. Immediately after the temperature has risen up to in-vivo conditions, the disaggregation mechanism starts to agitate the blood sample for ten seconds. The disaggregation routine was designed to be stepwise, performing two different sample agitation frequencies in order to get a full disaggregation state. After the ten seconds timeframe, the touch screen changes from control panel to a graphic interphase where the real-time light transmittance signal appears on the display. During this two minutes period of data collection, the syllectogram depicts the photometric outcome of the infrared light passing through the blood sample during the RBC aggregation process. Moreover, at the end of the data collection period, the calculations performed by the microcontroller in regards to the RBC aggregation parameters also appear on the screen on top of the syllectogram.

2.5.2 Discrete phases explained

The operation of the new instrument, can be explained through three discrete phases:

Phase I: The heating panel activation, whereby a current is passing through a very fine (270 µm) conductive yarn, which transfers heat to a textile and therefore creates a heat source for the blood sample. The temperature of the heating pad is measured in real-time via the temperature sensor at 1 Hz. This Phase is activated dynamically to regulate the blood temperature at 37°C.

Phase II: Once the blood sample reaches 37°C, the disaggregation mechanism is activated to induce the mechanical shear forces that result in RBC dispersion. The disaggregation mechanism contains a small electric solenoid (CB0830, Takaha Kiko Co Ltd, Fukuoka, Japan) that intermittently creates a differential tension on a silicon membrane by pushing it to induce air flow into the connected
glass capillary tube and thereby displaces the blood column by approximately 1 mm. During the magnetic deactivation of the solenoid (i.e., “off” period), the blood column returns to its original position via suction produced by the elastic recoil of the membrane. For the present design, an estimate of 3 mm strain compression on the silicon membrane produces approximately 1 mm of back and forth movement of the blood in the capillary tube. This movement is the result of alternating push and pull, resulting in small pneumatic forces caused by the compression action of the solenoid over the silicone membrane and its elastic recoil. This activation and deactivation of the solenoid occurs for a period of 10 seconds by two different frequencies (9.3 Hz and 8.1 Hz), after which the disaggregation motorised mechanism is deactivated for the remaining two minutes.

Phase III: Concurrently during Phase I and II, the transmittance of light throughout the blood sample in the capillary tube is then registered during a 120 seconds sampling period by the analogue-digital converter (ADC) interface built in the Arduino microcontroller board. The region of interest in order to determine the RBC aggregation is the voltage-time curve that is obtained following the cessation of Phase II.

Phase IV: During the data collection period, the syllectogram information is visualised and also stored into a standard CSV file at the same time. Once the data is finally collected, the data processing subroutines calculate the aggregation parameters of interest to be included in the previously created file and also to be available on the screen for review. The data acquisition, visualisation, the data processing and all the processes of the engineered RBC aggregometer are governed and coordinated by custom-written software developed for the Arduino (Arduino LLC, Instruments, Austin, Texas, USA) platform.
2.5.3 **Analysis of sensor performance**

The performance assessment for the selected phototransistor as the light-transmittance detector of the PCA, was performed by the analysis of their corresponding signal-to-noise ratio (SNR). In addition, the same evaluation was performed on the Myrenne aggregometer in favour of the comparison between instruments performance. Accordingly, two different tests were designed under static and dynamic conditions respectively. The first method was performed in the absence of a blood sample to evaluate the performance of the sensor once activated by the LED infrared light. During 23 minutes, the signal produced by the sensors was digitized and recorded to compare its mean value against the noise represented by the standard deviation. In this manner, the intrinsic SNR of both sensors (i.e., PCA and Myrenne) were measured. For the SNR calculations under dynamic conditions, representative syllectograms obtained from each device during experimental trials for RBC aggregation were selected. The second method for the SNR analysis was performed by examining the plot values from the syllectograms in the interest of determining the intensities of the signal and the associated noise.

2.6 **Statistical analysis**

SNR for the instruments (i.e., PCA and Myrenne) was calculated based on the raw signals acquired from the infrared sensors; the calculations were performed by using the mean signal intensity $S$ divided by the standard deviation SD caused by the electrical noise under unloaded conditions: $\text{SNR} = S / \text{SD}$. Coefficient of variation (CV) was used for each sample to determine the device precision for various parameters calculated from the information provided by the engineered capillary tube aggregometer. Data for each sampling method was then compared using paired t-tests to determine whether significant differences in the means exist. Linear regression analysis and Spearman’s rho correlation coefficients were used to analyse the relationship between the parameters calculated
from the aggregation measurements yielded by the PCA, the Gold Standard device and the ESR method. The standard error of estimate (SEE) was calculated based on the linear regression analysis. The regression line follows to minimize the sum of the squared errors of prediction. The square root of the average squared error of prediction is used as a measure of the accuracy of prediction. The formula for the standard error of the estimate is:

$$\sigma_{est} = \sqrt{\frac{\sum(Y-Y')^2}{N}}$$  \hspace{1cm} (1.0)

Where

- $Y =$ Actual score
- $Y' =$ Predicted score
- $N =$ number of pairs of (X, Y) points

Bland and Altman analysis will be used to investigate the agreement between sampling methods for key parameters of RBC aggregation (Bland and Altman, 1986).
Chapter 3 – Results

3.1 Pre-experimental results – Design considerations

In this section, the results of some pre-experimental tests are presented. These specific tests were executed in order to provide the best technical solutions to the PCA’s design and performance in regards to the RBC aggregation measurement.

3.1.1 Battery power supply

As a result of voltage fluctuations early detected on the signal delivered by the infrared sensor, different electrical measurements were specifically done. Several considerations were taken into account for the power supply final design, especially the power demand of the PCA’s components during the different functioning phases. Electrical measurements were performed using a precision multimeter (FLUKE 115, FLUKE, Everett, Washington, USA). The power consumption discriminated by elements can be observed in Table 1.

<table>
<thead>
<tr>
<th>Table 1. Power consumption for the PCA’s main components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Element</td>
</tr>
<tr>
<td>I (mA)</td>
</tr>
<tr>
<td>V (V)</td>
</tr>
<tr>
<td>P(W)</td>
</tr>
</tbody>
</table>

I = current expressed in milliamperes (mA); V = voltage expressed in Volts (V); P = power expressed in Watts (W)

The amount of power required by the instrument during the beginning of the aggregation
measurements (i.e., disaggregation mechanism and heating pad activation) raises from ~3W (standby state) to ~9.5W which represents the PCA’s peak power demand. The power consumption required for the solenoid and the heating pad during operation represents the 68.4% of the peak power demand. Consequently, the measured voltages on the battery before and during the peak power demand are presented in Table 2. As it can be observed, a total voltage drop of 651 mV was registered which represents 12.82% of the battery’s maximal voltage capacity.

<table>
<thead>
<tr>
<th>Table 2. Battery voltage measurements in different operation modes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standby</td>
</tr>
<tr>
<td>operation</td>
</tr>
<tr>
<td>V (V)</td>
</tr>
</tbody>
</table>

V = voltage in Volts (V)

3.1.2 Disaggregation mechanism

In order to achieve the most adequate RBC disaggregated state, several tests to determine the speed and type of acceleration for the erythrocytes dispersion mechanism were performed.

Various protocols were used to determine the most effective frequencies for the operation of the solenoid to produce the highest RBC disaggregation. See Table 3.
Protocol 4 allowed the system to achieve the maximal disaggregation state as it can be observed in Figure 5. In Figure 5a, the two phases disaggregation process can be clearly identified; the beginning of the cells dispersion starts at ~2 seconds reflected by a sudden drop in the light transmittance. At the end of the first stage at ~7 seconds, the change in frequency of the blood sample agitation takes place for additional RBC disaggregation. It is important to notice that at the end of the second phase (~12 seconds), when the cells start reshaping, there is no significant difference in light transmittance (i.e., voltage) during this transition which indicates that the maximal possible disaggregation has been reached. In contrast, in Figure 5b can be observed the light transmittance signal with micro fluctuations as an indication of the disaggregation forces acting on the sample using the Myrenne device. Following the end of the disaggregation process, the cells reshaping phase initiates as it is represented as the large voltage change presented at ~7 seconds. In this case, this remarkable transition possibly indicates that the disaggregation process was not efficient enough to produce complete dispersion of RBC within the sample.
Fig. 5. Signals collected during disaggregation processes for PCA (a) and Myrenne (b).

3.2 Experimental Results

3.2.1 Performance of the newly developed device: Signal-to-noise ratio

The graphical analysis performed in order to calculate the SNR is presented in Figure 6. Two conditions are represented in the figure for each instrument (i.e., PCA and Myrenne) to provide measures of intrinsic noise (using the device with no blood sample), while the SNR calculated during routine measurements provided an index of dynamic performance of the device (i.e., during a measurement using blood). In the case of the PCA, the electrical noise (represented by the standard deviation of the raw light transmission date) was 500 µV (0.0005 V), which represents 0.011% of the mean signal (mean = 4.75 V). The representative aggregation curve (Figure 6b) indicated that the intensity of the noise relative to the signal represents 1.3% of the amplitude of the signal which can be expressed as SNR =78. In regard to the Myrenne aggregometer, the electrical noise under unloaded conditions (i.e., no blood sample) (Figure 6c) was 1.2 mV (0.0012 V), representing the 0.07% of the mean signal (mean = 1.69 V). Based on these calculations, it can be observed that the intrinsic noise of the Myrenne is approximately six times greater than that from the PCA. With respect to the dynamic noise analysis, the representative syllectogram produced by the Myrenne aggregometer (Figure 6d) revealed that the intensity of the noise relative to the signal...
was 5% of the amplitude of the signal (SNR = 20).

Fig. 6. (a) SNR calculated for unloaded conditions for the PCA: mean = 4.75, SD = .0005, SNR = 9248.16 (b). SNR calculated for the acquired RBC aggregation signal for the PCA: N = 0.002, S = 0.156, SNR = 78 (c) SNR calculated for unloaded conditions for the Myrenne device: mean = 1.69, SD = .0012, SNR = 1460.90 (d). SNR calculated for the acquired RBC aggregation signal for the Myrenne device: N = 0.003, S = 0.060, SNR = 20. * indicates signal interference caused by electrical noise.

3.2.2 Obtained parameters and instrument precision

The aggregation parameters calculated using the areas analysis of the syllectograms are presented in Table 4. Measurements on 86 separate samples collected from healthy volunteers were performed; all subjects (n=43) were within the normal range of body mass index (20.0-24.9 kg·m⁻²). The biological variability depicted by the natural differences between subjects, is expressed in our analyses as coefficient of variation (CV_{BIO}) for all subjects. The related intra-assay variation
(CV\textsubscript{INTRA}) provides an index of measurement precision and data resulting from parameters observations by the PCA device showed that CV\textsubscript{INTRA} values for AI\textsubscript{120} and T\textsubscript{1/2} were 0.99\% and 4.22\% respectively. These values reflect a very high precision score for the new instrument.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SE</th>
<th>Biological variation</th>
<th>Intraassay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI\textsubscript{120} (%)</td>
<td>78.73 ± 0.82</td>
<td>6.83</td>
<td>0.99</td>
</tr>
<tr>
<td>T \textsubscript{1/2} (s)</td>
<td>9.76 ± 0.66</td>
<td>44.6</td>
<td>4.22</td>
</tr>
</tbody>
</table>

### 3.2.3 Repeatability assessment on the new device

The degree of repeatability for the PCA is illustrated in Figure 7. The relationship between two (2) successive measurements of the parameters AI and T\textsubscript{1/2} revealed a high level of repeatability for the instrument. The linear regression analysis yielded goodness of fit values for AI and T\textsubscript{1/2} equal to $r = 0.94$ and $0.89$, respectively, for repeated trials.

![Fig. 7. Relationship between the data acquired using the PCA for the same measurement on different trials. The information was obtained for two consecutive trials on the same sample for the Aggregation Index (Panel A) and T\textsubscript{1/2} (Panel B).](image-url)
In addition, the SEE was calculated to measure the accuracy of the linear regression equations obtained for the aggregation parameters; the resultant SEE values are presented in Table 5.

Table 5. Standard error of estimate (SEE) for the different parameters measured with the new PCA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SEE ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI$_{120}$ (%)</td>
<td>1.36 ± 0.39</td>
</tr>
<tr>
<td>T $\frac{1}{2}$ (s)</td>
<td>1.38 ± 1.10</td>
</tr>
</tbody>
</table>

AI$_{120}$: AI calculated using curves recorded in 120 seconds expressed in arbitrary units (au); T $\frac{1}{2}$: expressed in seconds (s).

In order to determine significant differences between the measurements performed during consecutive trials, a paired t-test was performed. The results are shown in Table 6.

Table 6. Results of t-test and Descriptive Statistics for Aggregation Index scores and T $\frac{1}{2}$ values for two consecutive trials for the same sample

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>n</th>
<th>t</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI$_{120}$</td>
<td>78.76 5.38</td>
<td>78.71 5.46</td>
<td>43</td>
<td>.24</td>
<td>42</td>
<td>.813</td>
</tr>
<tr>
<td>T $\frac{1}{2}$</td>
<td>9.75 4.58</td>
<td>9.78 4.25</td>
<td>43</td>
<td>.11</td>
<td>42</td>
<td>.914</td>
</tr>
</tbody>
</table>

Results show no statistically significant difference (p>0.05) in AI$_{120}$ for the scores obtained during two consecutive trials. The same observation can be made for the T $\frac{1}{2}$ values registered under the same conditions.
3.2.4 Agreement between results analysis for the new instrument

Bland and Altman plots were used to analyse the agreement between results for consecutive trials for the aggregation parameters described above (Fig. 8). AI$_{120}$ and T $\frac{1}{2}$ determined using consecutive trials demonstrated strong agreement (Fig. 8); the bias scores for both AI$_{120}$ and T $\frac{1}{2}$ measurements were -0.02 and -0.05 respectively.

![Bland and Altman plots](image)

Fig. 8. (a) Difference between aggregation index scores plotted against the average scores for two consecutive trials for the same participant. (b) Difference between T $\frac{1}{2}$ plotted against the average values for two consecutive trials for the same participant. Dotted line: mean difference (bias) between trials. Dashed lines: 95% limits of agreement.

3.2.5 Comparison of aggregation parameters between the new PCA, the Myrenne device and the ESR results

The new PCA test results were compared with those obtained by the Myrenne device and ESR procedure previously described. In Table 7, Spearman’s rho correlation coefficients were calculated to determine significant correlations between the parameters and measurements performed during this study. AI parameter from PCA was significantly correlated with that from Myrenne. There was a significant negative correlation between PCA and Myrenne for the T1/2 parameter. Aggregation
parameters (AI and T\(_{1/2}\)) produced with PCA did not exhibit significant correlations with ESR values.

Table 7. Spearman’s rho coefficient correlations for relations between the different parameters measured by the new engineered aggregometer and those obtained by other methods.

<table>
<thead>
<tr>
<th></th>
<th>AI My</th>
<th>AI PCA</th>
<th>T (1/2) My</th>
<th>T (1/2) P PCA</th>
<th>ESR</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI My</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AI PCA</td>
<td>.587**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T (1/2) My</td>
<td>-.842**</td>
<td>-.724**</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T (1/2) PCA</td>
<td>-.482**</td>
<td>-.782**</td>
<td>-.692**</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ESR</td>
<td>.637**</td>
<td>.076</td>
<td>-.305*</td>
<td>-.015</td>
<td>1</td>
</tr>
</tbody>
</table>

AI: Aggregation index calculated using curves recorded in 120 seconds; T\(1/2\): Aggregation half time; ESR: Erythrocyte sedimentation rate measured at 1 hr; Suffixes My and PCA identify whether Myrenne or PCA were used in order to determine any specific parameter. Significant correlations are marked with *: p < 0.05; **: p < 0.01, and also printed in bold; n = 43.

In addition, a comparison of syllectograms shape between the PCA and the Myrenne device is shown in Figure 9. The curves shown in panel (a) represent the syllectograms obtained using the PCA and the Myrenne device for the same sample; the T\(1/2\) values collected for PCA and Myrenne are 9.3 s and 13.4 s respectively. In a similar way, for a different subject, in panel (b) the reported values for PCA and Myrenne are 9.9 s and 15.5 s respectively. It can also be observed that the Myrenne syllectograms reflect a faster initial rise when compared to those produced by the PCA.
(a) Syllectograms obtained using PCA and Myrenne device for the same sample; reported values for aggregation half time (T ½) are 9.3 s and 13.4 s respectively. (b) Syllectograms obtained for a different individual using the same sample; reported values for aggregation half time (T ½) are 9.9 s for PCA and 15.5 s for Myrenne device. Syllectograms are identified by trace colour: PCA (grey) and Myrenne (black). Relationships between half amplitude (½ AMP) and half aggregation time (T ½) are identified by dotted lines for the Myrenne device and dashed lines for the PCA.

### 3.2.6 Additional readings obtained by the new PCA

During the assessment of the RBC aggregation parameters, the new PCA produced unexpected syllectograms with sigmoidal shapes for particular samples. These results may be associated to ESR results and are presented in Figure 10. In Figure 10a, a typical trial for aggregation measurement was obtained at 140 seconds; the associated ESR was 13 mm/hr and the slope of the projection line calculated for the second half of the sigmoid signal \( m = 4.12 \times 10^{-3} \text{ V} \cdot \text{s}^{-1} \). Figure 10b shows the experimental trial performed over a 1400 seconds period; the associated ESR was 9 mm/hr and the slope of the projection line calculated for the second half of the sigmoid signal \( m = 2.3 \times 10^{-3} \text{ V} \cdot \text{s}^{-1} \).
Fig. 10. Sigmoidal syllectograms observed during RBC aggregation measurements. (a) Aggregation curve obtained during 140 seconds. ESR 1 hr = 13 mm. (b) Aggregation curve obtained during 1400 seconds. ESR 1 hr= 9 mm / hr. Dashed lines: projection of the slope calculated on the second half of the sigmoid syllectograms.

The dashed line shown in Figure 10b depicts a constant linear trend of the light transmittance across the sample which was used to determine a relationship with ESR as follows:

With the slope value and the result of ESR, equivalence is established (Eq. 2.0)

\[ 2.3 \, \frac{mV}{s} \Leftrightarrow 9 \, \frac{mm}{h} \]  \hspace{1cm} (2.0)

Then the equation is rewritten converting time units from seconds to minutes (Eq. 2.1)

\[ 138 \, \frac{mV}{m} \Leftrightarrow 9 \, \frac{mm}{h} \]  \hspace{1cm} (2.1)

Finally, the expression of equivalence is simplified (Eq. 2.2)

\[ 15.3 \, \frac{mV}{m} \Leftrightarrow 1 \, \frac{mm}{h} \]  \hspace{1cm} (2.2)

Equation 1.2 indicated that a change of 15.3 millivolts per minute in the amplitude of the signal following erythrocyte aggregation will produce one mm of RBC sedimentation per hour.
3.2.7 Light refraction analysis

Due to the special design of the PCA’s analysis chamber, a diffraction analysis was performed for a better understanding of the infrared beam trajectory alterations from the light source, passing through the capillary tube to finally reach the sensor; the Snell’s law (Eq. 3.0) and the refractive index for soda-lime glass ($\eta = 1.52$) were used to calculate the diffracted angles presented in Table 8.

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{\eta_2}{\eta_1} \quad (3.0)$$

Where

$\theta_1 =$ Incident angle

$\theta_2 =$ Diffracted angle

$\eta_1 =$ Refractive index medium 1

$\eta_2 =$ Refractive index medium 2

<table>
<thead>
<tr>
<th>$\theta_1$ wall 1</th>
<th>$\theta_2$ wall 1</th>
<th>$\theta_2$ wall 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.5</td>
<td>4.9</td>
<td>3.2</td>
</tr>
<tr>
<td>15</td>
<td>9.8</td>
<td>6.4</td>
</tr>
<tr>
<td>30</td>
<td>19.2</td>
<td>12.5</td>
</tr>
<tr>
<td>45</td>
<td>27.7</td>
<td>17.8</td>
</tr>
</tbody>
</table>

$\theta_1$ Incidence angle on the first wall of the capillary tube. $\theta_2$ Refracted angle at walls 1 and 2. All the angles are expressed in degrees with respect to the line perpendicular to the main axis of the capillary tube.
Chapter 4 – Discussion

The purpose of the present study was to develop a new aggregometer in order to achieve important advancements in the sector that highlight low-cost, size reduction, portability, low-maintenance and user/operator safety. Special attention was given to the reliability of RBC aggregation measurements yielded by this new aggregometer. Moreover, important aspects were taken into account during the planning of this project such as results accuracy and precision. Accordingly, blood samples from healthy young males were analysed with different devices and methods. As a result, RBC aggregation parameters (i.e., aggregation index (AI) and aggregation half time \( T^{1/2} \)) data were collected to assess the operation and general performance of the proposed instrument.

The results obtained with the new PCA showed an enhanced signal quality evidenced by a superior signal-to-noise ratio when compared to that yielded by the Myrenne aggregometer. In addition, the precision assessed for the PCA from the AI and \( T^{1/2} \) measurements reflected a good reliability of the instrument. Furthermore, a strong correlation between PCA and the Myrenne aggregometer for the \( AI_{120} \) parameter was found.

The significance of the improvements achieved during this study was focused on the attainment of positive impact not only in medical research and clinical applications but also in social development. As the specialised equipment used for diagnosis and disease management is evolving towards expensive devices using high-tech engineering, communities worldwide with limited economic resources are disadvantaged due to poor access to healthcare; however, the proposed study and instrument implementation present advantages that may improve accessibility to medical tests and general healthcare treatments, ultimately leading to an important enhancement in the quality of life for disadvantaged populations. Research and medical testing may also be expanded fostering improved healthcare opportunities to people from the most remote and poor regions of the world.
4.1 Signal-to-noise ratio (SNR)

The signal-to-noise ratio of the newly developed aggregometer was excellent. This finding is fully supported on the results section Figure 5, where the data indicated that the noise for the static and dynamic conditions reached levels as low as 0.011% and 1.3% respectively. The reference values with which the SNR of the PCA were compared came from the Myrenne aggregometer experimental trials. Based on that comparison, it was found that for baseline measurements (i.e., without a blood sample) the PCA intrinsic noise was six times less than that from Myrenne. Moreover, under dynamic conditions (i.e., blood sample analysis), it was found that the SNR for PCA was four times superior than that for the Myrenne device. Unfortunately, there are no existing studies that have investigated similar data in similar instruments. That is, to date, there are no reported values for the measurement of SNR for photometric methods similar to the light transmittance principle used by the new PCA. Thus, the present study provided the first report of such data for the Gold Standard device (Myrenne) and also provided a comparison between the new PCA and Myrenne aggregometer under different conditions (i.e., static and dynamic). Interestingly, as can be observed in Figure 5d, it was found that electrical interference within the signal was detected that likely reflects internal switching transients of the Myrenne device. However, this undesirable effect is not observed for the PCA at any stage (i.e., static or dynamic) of measurement.

Several measures were taken during the development of the PCA to improve the quality of the signal. Firstly, the analysis chamber of the new device was luminously isolated from undesired light sources; thus, the noise caused by external sources rather than the infrared light source was diminished. Secondly, the battery powered feature of the PCA represents a significant advantage in regards to noise reduction in comparison with all the commercially available aggregometers – which rely on the public electrical network (PEN) for operation. While the PEN supplies a continuous source of power, the primary disadvantage of a system reliant the PEN is observed through decreased SNR due to numerous extraneous sources of noise (Zimmermann & Dostert,
Three main interfering sources of noise present within the PEN are: i) the coloured background noise caused by summation of numerous noise sources of low power, ii) periodic impulsive synchronous noise to the main frequency caused by many electrical appliances, and iii) the asynchronous impulsive noise caused by switching transients in the power network (Zimmermann & Dostert, 2002). In fact, noise associated to the PEN is often a threat to sensitive and delicate electronic instruments; therefore, complex filtering systems must be implemented to maintain the quality of the diagnostic signal. Finally, a double independent battery arrangement for the PCA power supply was designed with the purpose of limiting fluctuations in the power supply which may affect the performance of the sensor element (i.e., the phototransistor). This unwanted effect is mostly due to the current demand produced by the power elements of the system (i.e., solenoid, heating pad) which can produce a voltage drop in the battery. Specifically, before the implementation of the double independent battery arrangement, unwanted fluctuations were observed at the output signal of the sensor. This abnormality was evidenced during the activation of the solenoid and heating pad elements affecting the quality of the signal. In the course of the power phase activation (i.e., disaggregation and heating processes). As it can be observed in tables 1 and 2, it was determined that the PCA increases the current demand from approximately 500 mA to 1900 mA causing a notable voltage drop on the battery of 12.82% of its maximal voltage capacity), which is coherent with the findings of Ng et al. (2009), which demonstrated typical drops in voltage for Li-ION batteries are substantial and dependant on the applied working load and its associated current demand. Clear implications on the sensor’s performance derived from voltage fluctuations are evidenced using circuits theory analysis at the output of the sensor’s configuration (Figure 11A) as follows:
To calculate and determine the effect of the voltage fluctuations on the sensor, the Kirchhoff voltage law (KVL) must be considered:

$$\sum_{k=1}^{n} V_k = 0$$  \hspace{1cm} (4.0)

Where \( V_k \) = voltage of the \( k \) element of the loop

Applying KVL to the loop at the output sensor circuit, the voltage’s equation can be expressed as follows:

$$V_{CC} + V_{RC} + V_{CE} = 0$$  \hspace{1cm} (4.1)

Where \( V_{CC} \) = Collector supply voltage  
\( V_{RC} \) = Collector resistance voltage  
\( V_{CE} \) = Collector-Emitter voltage

Using the Ohm’s law, the Eq. 4.1 can be rewritten as follows:

$$V_{CC} + I_c \cdot R_c + V_{CE} = 0$$  \hspace{1cm} (4.2)

Where \( I_c \) = Collector supply voltage
\[ R_C = \text{Collector resistance} \]

To simplify the analysis, assumption of complete saturation on the phototransistor \( (V_{CE} = 0) \) is made (maximal light transmittance crossing the sample). Therefore, rearranging Eq. 4.2 can be expressed as follows:

\[
I_C = \frac{-V_{CC}}{R_C}
\]  

(4.3)

The Eq. 4.3 illustrates the direct relationship between the collector supply voltage and the collector current which means that any voltage drop in the battery will cause a drop in the collector current at the same proportion (i.e., 12.82\%). This alteration is reflected on decreased amplitude of the output which is detrimental to the quality (i.e., decreased SNR) and coherence of the signal produced by the sensor component.

Based on the previous analysis, it is opportune to highlight the benefits of the battery operated feature of the PCA compared to the PEN dependent devices: i) the amount of noise present at the output may dramatically be reduced (i.e., superior SNR) ii) the quality of the signal is enhanced since the internal electrical interference may be eliminated. iii) medical equipment is better protected from sudden electrical fluctuations (i.e., switching transients in the power network) when battery systems provide the required power supply for adequate functioning.

With respect to the Li-ION batteries typical voltage drops when exposed to high current demands, the final design of the PCA’s power supply (i.e., dual battery system) prevents undesirable electrical behaviour on the sensor’s output (i.e., signal amplitude variations) which can affect the quality of the signal produced by the device. Moreover, the double independent battery arrangement designed for the PCA provides a strong advantage when considering the reliability of a clean steady signal crucial for the analysis of the aggregation parameters. This special design allowed the first battery to support all the power phases (i.e., disaggregation and heating) whereas the second one provides the required electrical energy for the microcontroller, peripherals and the sensor arrangement.
The PCA’s enhanced quality of the signal as a result of a superior SNR when compared to the Myrenne device represents an important technical feature with respect to RBC aggregation parameters analysis. This improvement as a medical instrument was essentially achieved by the fine tuning of the setup experimentally determined for the phototransistor (PT100MF0MP1, Sharp, Osaka, Japan), the analysis chamber’s light insulation, and the double independent battery arrangement of the PCA. As a result, the sensor component of the PCA performs at a superior level when compared with that of the Gold Standard device in regards to quality of the signal (i.e., SNR).

4.2 Obtained parameters and instrument precision

The precision calculated for the PCA from the AI and T ½ measurements reflected a good reliability of the instrument.

The intraassay variation, as presented in Table 4, indicated that the precision of the instrument was very high with differences between measurements for the AI and T ½ parameters of 0.99% and 4.22% respectively. These precision values are comparable to those previously reported by Baskurt et al. (2011), who developed a capillary tube erythrocyte aggregometer (CEA); the reported values by Baskurt and colleagues for the AI and T ½ parameters were 0.70% and 8.11% respectively. As it can be observed, for the AI parameter, both instruments (i.e. PCA and CEA) reported very similar intraassay variation values whereas for the T ½ measurement the variation reported for the CEA doubled the PCA reported value. In other words, this study found that the PCA is more precise than the CEA when the variation between consecutive trials for both instruments was assessed. The reason behind this finding may be associated to significant differences in the operation of the disaggregation mechanisms for both devices. Namely, the PCA’s disaggregation mechanism implements a stepwise protocol whereas the CEA doesn’t; stepwise disaggregation protocols have demonstrated higher level of RBC dispersion when compared to single step protocols (Shin et al.,
As presented by Shin et al. (2005), following the development of a novel aggregometer, an interesting stepwise approach to disaggregate the RBC using mechanical vibrations was introduced. Essentially, different intensities were used by consecutive stages to achieve a maximal RBC disaggregation state; those gradual intensity increments of the signal took place as long as a cells dispersion plateau was reached (Figure 12).

Based on the stepwise approach previously described, this project adopted and adapted that new concept to enhance the disaggregation mechanism for the proposed PCA. Experimentally, different frequencies were tested for the solenoid activation to produce different agitation speeds to disaggregate the blood sample contained in the capillary tube. In addition, increasing and decreasing agitation speeds were tested to find the most adequate frequencies combination protocol to achieve maximal disaggregation (Table 3).

At the end of the experimental phase to find the most efficient disaggregation protocol, it was concluded that the two staged protocol with gradual decrease in frequency (Protocol 4) appeared to
be the most efficient as it is shown in Figure 5.

Moreover, the final frequencies were adjusted according to the cells dispersion (i.e., amount of disaggregation observed) and the speed of the plateau achievement per stage. In fact, the final protocol was determined due to non-significant observed differences in additional RBC disaggregation between the second and third stage.

The final design of the stepwise disaggregation mechanism for the PCA operates at two different five seconds duration phases; during phase one the pneumatic forces are applied to the blood sample at 9.3 Hz to produce a quick dispersion of the RBC among the sample. However, the second phase is characterised by a reduced agitation frequency (8.1 Hz) which fosters the shape recovery stage producing maximal RBC disaggregation. Subsequently, achieving a maximal disaggregation state for every trial is crucial to set a precise starting point to yield high-precision measurements. Thus, this finding indicates that stepwise disaggregation may foster more precise results for RBC aggregation measurements.

4.3 Repeatability assessment

The degree of repeatability assessed for the PCA was very high. The repeatability, as shown in Figure 6, reported high goodness of fit values \( r \geq 0.89 \) for the aggregation parameters of interest (i.e., AI and \( T_{1/2} \)) as a result of the linear regression analysis performed for this validation. Furthermore, the t-test results (Table 2) and the standard error of estimate calculations (Table 5) provided additional evidence supporting the finding reported in this section. Notwithstanding, similar instruments developed by Baskurt et al. (2011) and Shin and colleagues (2005) did not include goodness of fit values as reported results which prevent this study to have a useful comparison among these devices.
However, the results evidenced a technical advantage for this instrument in comparison with similar devices; the PCA’s final design including the stepwise disaggregation mechanism combining a fine tuned sensor provided a superior level of repeatability. As a consequence the PCA in regards to the aggregation parameters measurements (i.e., Al_{120} and T \frac{1}{2}) was proven to be a reproducible and precise instrument.

4.4 Level of agreement between results

The level of agreement between measurements for the PCA was very strong. The level of agreement, as reported in Figure 8, indicated that not only the vast majority of the results were limited by the 95% of the upper and lower LOA but the mean difference between trials (bias) was almost zero (0.05 and -0.02). Moreover, taking into account the LOA are narrow and the bias values were very small (0.05 and -0.02), it can be concluded that the two measurements obtained for consecutive trials may be treated as equivalent. In contrast, the studies from Baskurt et al. (2011), and Shien et al. (2005), did not present analysis related with the level of agreement between measurements for their instruments. The high level of agreement observed for the PCA may be due to several design features, including: i) the light insulation of the analysis chamber which significantly contributed to the noise reduction factor, ii) the independent double battery arrangement which maintained the measurement signal free from electrical fluctuations and iii) the stepwise disaggregation mechanism which facilitated a precise RBC dispersion as a starting point for the erythrocyte aggregation measurement.
4.5 Comparison between PCA, the Myrenne device and ESR results

The comparison of the RBC aggregation parameters measured by the new PCA and the Myrenne aggregometer indicate further development of the PCA may be necessary. The relations between the measurements, as listed in Table 7, indicated only a strong correlation between PCA and Myrenne for the AI$_{120}$ parameter. Unexpectedly, the half time aggregation parameter was inversely correlated between those two instruments.

Considering the shapes of the syllectograms obtained from the PCA and the Myrenne instruments (Figure 9), it can be observed that the flatter syllectograms (Myrenne) indicate a slower initial rate of signal increase (i.e., light transmission) when compared to the more “rounded” signal of the PCA. This difference can be explained by the time constants (fast and slow) which represent the earlier and later phases of change in light intensity during the syllectogram data collection (Hardeman et al., 2001). While the early stage of the syllectogram reflects the primary rouleaux formation (i.e., aggregates formation in two dimensions), the late stage reflects the secondary rouleaux formation when the aggregates become tri-dimensional structures (Hardeman et al., 2001). Accordingly, it is valid to hypothesize that a better RBC dispersion initial state may increase the duration of the aggregation early stage during the syllectogram collection; therefore, the more “rounded” signals produced by the PCA may be an indication of superior disaggregation mechanism efficiency with reference to the Myrenne device.

Unfortunately, this study did not consider an experimental assessment of the PCA’s disaggregation mechanism, thus a valid statistical comparison with that of the Myrenne device is not feasible. Nevertheless, by the analysis of the syllectogram differences and the PCA’s superior disaggregation mechanism hypothesis, the discrepancies for the half aggregation time parameter can be explained. As it can be observed in Figure 9a, the difference in shape of the two presented syllectograms relates a discrepancy greater than 4 s in the reported values for T$_{1/2}$. Similarly, in Figure 9b a difference greater than 5 s for the same parameter is reported between instruments. In both cases,
the fast initial rise observed in the early stage of the aggregation phenomenon appears to be higher for the PCA when compared to Myrenne, possibly explaining why the $T\frac{1}{2}$ reported values which are directly affected by the time constants (i.e., fast and slow) are larger for the Gold Standard device. Additional questions have arisen in the direction of the need for standardization (i.e., disaggregation level) towards an adequate outcome comparison of two different medical instruments. Figure 13 presents a comparison of the two syllectograms previously shown in Figure 8B where the PCA trace has been down scaled to match their amplitude; it can be observed that the early stage for the Myrenne trace rises at a lower rate with respect to that from PCA. This comparison exercise also supports the disaggregation hypothesis previously formulated.

![Figure 13](image_url)

Fig. 13. Syllectograms obtained using PCA and Myrenne device. Syllectograms are identified by trace colour: PCA down scaled (grey) and Myrenne (black).

The relationship between ESR and the yielded PCA measurements was not significant. This finding contrasted previous studies where strong correlations have been reported between ESR and RBC aggregation quantitated using various instruments (Baskurt et al., 2009b; Ramplin & Martin, 1989). Different arguments and theories can be used in order to explain this disagreement. Firstly, some studies suggest that the geometry of the measurement chamber has a direct influence on the syllectograms using the light transmittance principle (Gaspar-Rosas & Thurston, 1987). Although Baskurt’s erythrocyte capillary tube aggregometer (Baskurt et al., 2011) demonstrated strong correlations between some measured parameters (i.e., AUC and $T\frac{1}{2}$) and the ESR scores, the PCA,
which have similar chamber geometry and use the same photometric principle, did not. In fact both instruments process the blood sample in a capillary tube, but the distance between the light source and the sensor are different. The increased separation between the emitter and the receiver of the PCA’s analysis chamber may cause undesirable light refraction and reflection through the cylindrical symmetry of the glass capillary tube causing a distortion on the final measurement. As it can be seen in Figure 14, the diffraction caused by the walls of the soda-lime-glass capillary tube (diffraction index = 1.52) prevents a significant proportion of infrared light coming from the LED reaching the phototransistor. In fact, according to the calculations presented in Table 8, just 25% of the light emitted by the source is able to enter the capillary tube in an incidence angle lesser than 7.5 degrees required reaching the sensor according the current analysis chamber configuration.

Secondly, the temperature at which the sample is analysed for the two different instruments is different. The Myrenne aggregometer lacks from a temperature control system for the sample whereas the PCA maintains 37 °C during the complete measurement procedure. Moreover, the accurate temperature control of the PCA can maintain the regulation of this parameter with a
resolution of 0.25°C without causing any alteration to the power supply due to the double independent battery arrangement finally implemented. Thus, the differences in RBC aggregation parameters were also affected by the measurement temperature (Neumann, Schmid-Schönbein, & Ohlenbusch, 1987).

Nonetheless, by the analysis of sigmoidal syllectograms yielded by the PCA, some clues pointing to ESR prediction were found. In the first half of Figure 10A, it can be observed a typical aggregation curve, reaching a minimum at the end of the disaggregation process and starting to rise exponentially as a consequence of the variation in light transmittance due to the aggregates formation. Interestingly, this typical behaviour is disrupted at ~75 seconds where an inflection point is reached. From that point forward, a rising trend is observed which cannot be related with RBC aggregation since it was demonstrated that in ~120 seconds for most samples, complete aggregation is observed (Baskurt et al., 2011). Subsequently, the sudden increase observed in Figure 10A at ~75 seconds reflects variations in light transmittance perceived by the PCA’s infrared sensor which can only be produced by a different RBC reorganization within the blood sample. For that reason, and based on the linear trend observed in figures 10A and 10B following aggregation, a new hypothesis is formulated in which the unexpected rise may be a direct consequence of the RBC sedimentation inside the capillary tube containing the blood sample. The slope of the projection line for the second section of the sigmoid curve (Figure 10A and 10B) may be directly associated to the RBC sedimentation rate. The second inflection point of the sigmoid signal indicates a new alteration in the light transmittance acquired by the PCA, which can be explained with the sedimentation of cells reaching a level where the infrared light crosses more plasma than RBC. Particularly, the infrared light increasingly finds less resistance crossing the sample due to the separation of the RBC from the blood plasma. From Eq. 1.2 can be inferred the potential that the PCA has to eventually predict ESR in a fraction of the time required for the traditional practice (i.e., Westergren method). Additional calculations can be derived from this observation, considering known information such as the internal diameter of the capillary tube (1 mm), the elapsed time, and the light transmittance.
gradient; however further experiments are required to find the most accurate correlation between the sigmoid signals and the ESR to further increase statistical power. Nevertheless, the possibility to predict ESR by using a small blood sample (~50 µl) and a fraction of the current required time (i.e., 5 min) will expand the PCA’s applicability in a wide range of scenarios.

4.6 Limitations

The major limitation of the present study was the fact that the testing protocols were always performed in parallel with the device development. In other words, different tests were performed at particular stages of the instrument, which is not ideal for results analysis consistency. This situation was not deliberate and was the result of continual prototype development that was guided by discrete technical and experimental issues detected from state-to-stage. Specifically the noise reduction was the biggest limitation this project faced from its beginning; several strategies and techniques were applied during all stages of the project including the fine tuning of the sensor’s configuration, the light insulation of the analysis chamber and the independent double battery power supply.

Another obstacle this project had to confront was the implementation of the on-board analysis module. This module depended on the microcontroller’s analogue-to-digital converter (ADC) power to digitise the raw signal produced by the sensor of the system. It is important to highlight that the resolution of the Arduino’s ADC was inferior when compared to that from the National Instruments board that was used to assess the quality of the signal and to acquire the syllectogram during most stages of this development. This technical disadvantage coupled to the symmetry of the analysis chamber may be responsible for the lack of correlation between the aggregation parameters produced by the PCA and those yielded by the Myrenne device. During the last phase of testing, measurements were forced to bypass the on-board processing module to use a National Instruments
ADC and LabVIEW routine as an independent signal processing unit.

The results obtained during this last phase of testing allowed this study to find a strong correlation for the $A_{I120}$ parameter between devices (i.e., PCA and Myrenne). In addition, the proven precision and the high SNR score represented an important achievement for the development of the new instrument.

In conclusion, the need for continuous improvement coupled with unforeseen challenges (i.e., analysis chamber symmetry and ADC resolution) as a result of this new device development process did not allow this study to advance as smooth as expected. However, significant technical advantages for a medical instrument were achieved; more important, the PCA is no longer a prototype, it is a functional and precise diagnostic device which will continue improving beyond this study. Future directions and suggestions are discussed in the following section which can represent important advances for the developed instrument.

4.7 Future directions

In order to achieve an enhanced version of the PCA, some directions are suggested in this section.

4.7.1 Modifications to the analysis chamber

It is required to design an improved analysis chamber for the PCA. The new design of this element will allow the sensor to be situated at a minimum distance ($< 1$ mm) from the capillary tube channel to enhance the light reception across the sample. Another required modification will consist of a small compartment between the capillary tube channel and the light source, able to allocate a biconcave lens. This optical element will focus the infrared light beam across the blood sample in order to decrease the undesirable effect of light reflection and diffraction through the capillary tube.

As a result of the described modifications to the analysis chamber, an enhanced amplitude and...
quality of the signal (i.e., increased SNR) may be expected, which can improve the correlation between the aggregation parameters yielded by the PCA and those from other devices.

4.7.2 Signal digitisation enhancement

With the purpose of increase the quality of the syllectograms produced by the PCA, the use of an external analogue-to-digital converter (ADC) will enhance the digitisation process for the on-board analysis module of the proposed instrument. Currently, the Arduino board includes a 10-bit resolution ADC which is less powerful when compared to the 12-bit resolution feature of the National Instruments converter. Particularly, digitisation refers to the conversion of an analogue signal (i.e., the output voltage at the sensor) to a digital value (i.e., a number as a series of ones and zeroes). In this context, resolution represents the number of small steps in which the analogue signal may be represented. For example, 10-bit resolution means that there are $2^{10}$ (1024) possible digital values that each sample can be converted to whereas 12-bit resolution relates $2^{12}$ (4096) possible digital values. Based on the results provided by the PCA, a typical analogue signal at the sensor has amplitude of 150mV; using a 10-bit converter means that the amplitude of the signal can be divided by $2^{10}$ digital values which is represented in a resolution of 146.5 µV / bit. In contrast, a 12-bit converter will provide a resolution of 36.7 µV / bit. In sum, a better resolution ADC will allow the PCA to detect smaller variations of the analogue signal which can be digitised more precisely in order to calculate more accurate aggregation parameters. Ideally, this upgrade should not be implemented without the modifications to the analysis chamber in the pursuance of a substantial signal analysis improvement.

4.7.3 Technical matching and testing protocols

It is highly recommended to adjust the testing protocols according to technical features in order to achieve a more reliable comparison between instruments. Specifically, temperature measurement
and disaggregation levels should be matched to make a proper validation between the PCA and Myrenne aggregometer; the effect of those specifications on the RBC aggregation parameters is significant and well supported by different studies and cannot be neglected in the interest to achieve a solid validation of the new device based on the Gold Standard results under almost identical conditions.

### 4.7.4 Commercialisation

The new PCA is intended to be commercialised in order to bring all its capabilities as a medical instrument to different clinical settings and research laboratories. The improved features achieved for the new aggregometer, represent a great opportunity to introduce the RBC aggregation as an inexpensive and reliable screening test. Accordingly, the serial production of the PCA may facilitate the presence of a new era of aggregometers at many point-of-care institutions worldwide, promoting preventive medicine and early detection of diseases.
4.8 Conclusion

The physiological phenomenon of erythrocytes aggregation has been accepted as an important determinant in the normal regulation of blood rheology (Baskurt et al., 2011). RBC aggregation may help regulating fluctuations in blood pressure at microcirculation level during exercise and increased perfusion to the active tissue by the adjustment of venous resistance (i.e., reduced viscosity) according to blood flow (Bishop et al., 2001). Accordingly, athletic capacity understood as maximal oxygen consumption, is a clear indication of more efficient systems for improving tissue metabolism and perfusion when required; this is characterised by higher RBC aggregation (Popel et al., 1994). RBC characteristics may be altered by different pathologies including the extent and time course of aggregation and the aggregates cohesion (i.e., the force required to disperse the aggregates) (Baskurt et al., 2011). In fact, these alterations may have influence on flow dynamics; increased aggregates cohesion is expected to impair the RBC disaggregation in microcirculation increasing flow resistance (Baskurt et al., 2011); similarly, an affected aggregation time course may alter the aggregation process in postcapillary micro vessels influencing venous vascular resistance (Baskurt et al, 2011). Moreover, the degree of erythrocytes aggregation plays a crucial role determining whether tissue perfusion is fostered or impaired (Mchedlishvili 1998; Yalcin et al. 2004). In addition, enhanced RBC aggregation would produce a more effective axial migration which leads to lower haematocrit, and hence a lower viscosity which is reflected in decreased flow resistance (Baskurt et al., 2011). Accordingly, impairment in the RBC aggregation due to pathological conditions may alter tissue perfusion whereas aggregation process within physiological limits may improve tissue perfusion (Baskurt et al., 2011). In contrast, pathological enhanced RBC aggregation leads to increase vascular resistance as a result of the negative feedback acting on nitric oxide (NO) production mechanisms; the downregulation of these mechanisms is proven to augment degeneration in the vessel wall hence promoting atherosclerosis (Lloyd-Jones & Bloch 1996; Nerem et al. 1998). Thus, chronically enhanced erythrocyte aggregation may be
associated with atherosclerotic damage. In sum, the role that RBC aggregation process plays in the preservation of normal hemodynamic conditions is very important, with specific emphasis on the effects on tissue perfusion in reference to the degree and type of aggregation. Consequently, the assessment of different parameters of RBC aggregation has been proven to be significant from a clinical perspective; as a result several methods and devices have been developed to determine erythrocyte aggregation. However, the measurement of RBC aggregation has been relegated to specific research fields, despite its physiological and clinical importance. One of the primary limitations to its common utilisation is that the required equipment to perform this measurement is expensive; many clinical institutions around the world are limited in purchasing specialised devices (Peters et al., 2008). Another limitation in its widespread adoption is that the determinants of RBC aggregation, and its effects on disease, is not widely understood; indeed, most university textbooks neglect to inform medical doctors and clinical scientists about this important aspect of blood fluidity in any detail (Baskurt et al., 2011). Moreover, despite the limitations in existing clinical methods (i.e., Westergren ESR determination) such as extended analysis time (i.e., one hour) and blood sample size (i.e., 1 mL) which traditionally can provide a much more simplistic evaluation of related processes, the benefits of commonly utilised methods outweigh any disadvantage by far.

With the development of the new PCA, this study provides an alternative to some of the limitations in adoption of RBC aggregation in routine clinical assessment. The new instrument possesses numerous advantages, including: affordability, portability and low power consumption. Moreover, a small blood sample (50 µL) is required to execute the aggregation analysis procedure and no special skills or expertise are needed to operate the PCA.

One of the main purposes of this study is to create awareness at clinical level of how important blood rheology is, and to make the red blood cell the main player for diagnostic performances. The amount of information that can be extracted from a single sample of erythrocytes is underestimated; the way certain conditions directly affects blood rheology (i.e., viscosity, aggregation,
deformability) represents a noteworthy indication of disease. Furthermore, the influence that different conditions have on RBC mechanical properties is an enormous opportunity to expand the power of diagnostic tools by knowing precisely how haemorheology works and most importantly, how it is altered by different health conditions.

With the introduction of a new diagnostic instrument, able to provide specific RBC aggregation parameters, this study attempts to bring haemorheology at a relevant position at clinical level; this mission can be accomplished by using novel ideas, inexpensive technology and alternative improved methods.

Increased levels of RBC aggregation are not only associated to health conditions. Healthy active individuals may enhance their erythrocytes aggregation in response to exercise and regular physical activity which is in agreement with Wood et al. (1991) who reported that enhanced aggregation may be a part of the physiological adaptation response during long-term exercise protocols.

This study is also interested in transform the information of the RBC aggregation parameters in a regular screening tool that may help in the detection of silent conditions or the early detection of particular diseases such as inflammatory processes, prediabetes or heart failure.

Having the new PCA performing at the clinical field, ideally in many point-of-care institutions in different countries, this study would promote the preventive medicine at a new whole level. The new instrument will provide valuable diagnostic information which may improve the early detection of diseases and general diagnostic outcomes; with the help of God and this study, the new small device developed at the blood rheology laboratory of Griffith University will have the potential to improve the quality of life of many individuals, having an unprecedented impact on health care systems around the world.
References


sepsis. *Journal of Laboratory and Clinical Medicine, 130*(2), 183-190.


Szygula Z. (1990) Erythrocytic system under the influence of physical exercise and training. *Sports Medicine, 10*, 181-197


