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Red Blood Cell Passage through Narrow Capillaries: Sensitivity to Stiffness and Shape

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The University of Melbourne

Abstract

Red blood cells (RBCs) squeeze through narrow capillaries as they transport oxygen to tissues and carbon dioxide to the lungs. The deformability of RBCs has been shown to depend on the viscoelasticity of the cell membrane and cytoplasm as well as the surface area to volume ratio (SA:V ratio) of the cell. In certain pathological diseases such as malaria, RBCs undergo restructuring of the membrane structure and modifications to the cell shape, which significantly reduce their deformability. Nonetheless, it is still unclear which factor has the greatest impact on the passage of RBCs through small capillaries. Here, we present a systematic analysis designed to identify the individual contributions of cell stiffness and SA:V ratio to the ability of RBCs to traverse narrow capillaries in a microfluidic device. We modified cellular rigidity using glutaraldehyde fixation, changed SA:V ratio by altering the buffer osmolarity and probed RBCs passage through microchannels. Our results showed that dramatic stiffening (~8 fold) had little effect (~6% retardation) on the ability of RBCs of the same geometry to traverse the channels. On the other hand, a moderate decrease (~13%) in the SA:V ratio affected the traversal of RBCs of similar stiffness more markedly (~19% decrease). We further studied RBCs infected by two different species of malaria parasites known to affect humans, *Plasmodium falciparum* and *knowlesi*. We found that *P. falciparum* rigidified the host RBC, but infected RBCs penetrated into microchannels with similar efficiency to uninfected RBCs. By contrast, *P. knowlesi* reduced the SA:V ratio of the host RBC resulting in restricted passage. We found that the earliest stage immature RBCs (reticulocytes) exhibited a similar SA:V ratio to mature RBCs and, despite being 30% larger, travelled into microchannels as efficiently as mature cells. Our finite element (FE) model provides a coherent rationale for our experimental observations, indicating that cell stiffness changes do not significantly affect RBC traversal in small capillaries due to the highly nonlinear mechanical behaviour of the cell membrane. Our numerical simulations predict that RBCs with low SA:V ratios are more prone to trapping in small capillaries (within the physiological size range) than RBCs with high membrane stiffness. Therefore, therapies targetting surface area to volume ratio of RBCs may be more effective than those that target cell stiffness.

Declaration

This is to certify that:

1. The thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the Preface.
2. Due acknowledgement has been made in the text to all other material used.
3. The thesis is fewer than 100,000 words in length exclusive of tables, bibliographies and appendices.

Arman Namvar

July 2020

Preface

This thesis has received contributions from others:

- (a) Figure 1-2, Figure 1-3, Figure 1-4 and Figure 1-7 are adopted from (Hochmuth, 2000), (Mills et al., 2004), (Gifford et al., 2003) and (Malleret et al., 2013).
- (b) The scanning electron microscopy images presented in Figure 1-5, Figure 1-6, Figure 2-3 were acquired by Dr Adam Blanch (the University of Melbourne).
- (c) The transmission electron microscopy images presented in Figure 1-5 and Figure 1-6 were acquired by Dr Boyin Liu (the University of Melbourne). This work is published (Liu et al., 2019).
- (d) Parts of the ektacytometry analysis were kindly performed by Dr Adam Blanch (The University of Melbourne).
- (e) The fluorescent labelling of RBCs and preparation of media were kindly performed by Dr Adam Blanch (The University of Melbourne).
- (f) Culture of malaria parasites was kindly performed by Ms Olivia Carmo, Mr Dean Andrew, Dr Snigdha Tiash and Dr Oliver Looker (The University of Melbourne).
- (g) Purified reticulocyte samples from cord blood were prepared by the group of Dr Waihong Tham (Walter and Eliza Hall Institute of Medical Research).
- (h) Mutant mice with defects in RBC proteins were generated by Dr Gaetan Burgio (The Australian National University).

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Publications

- **Surface-to-volume ratio, not cellular rigidity, determines red blood cell traversal through small capillaries**

Arman Namvar, Adam J. Blanch, Matthew W. Dixon, Sebastien Menant, Olivia Carmo, Boyin Liu, Snigdha Tiash, Oliver Looker, Dean Andrew, Peter V. S. Lee, Wai-Hong Tham, Vijay Rajagopal*, Leann Tilley*

* Equal author contribution.

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- **Multimodal analysis of Plasmodium knowlesi-infected erythrocytes reveals large invaginations, swelling of the host cell, and rheological defects**

Boyin Liu, Adam J. Blanch*, Arman Namvar*, Olivia Carmo*, Snigdha Tiash, Dean Andrew, Eric Hanssen, Vijay Rajagopal, Matthew W.A. Dixon[#] and Leann Tilley[#]

* Equal second author contribution.

[#] Equal author contribution.

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- **Erythrocyte β spectrin can be genetically targeted to protect mice from malaria**

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Conference Presentations

Oral Presentations

2019 10th Australia and New Zealand Nano and Microfluidics Symposium

Wollongong, NSW, Australia

2017 ANFF & AMMRF Research Showcase

Sydney, NSW, Australia

Poster Presentations

2018 8th World Congress of Biomechanics

Dublin, Ireland

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Chapter 1

1 Introduction to the red blood cell

1.1 Motivation

Red blood cells (RBCs) circulate the body to deliver oxygen to tissues and return carbon dioxide to the lungs. Throughout its journey, a healthy RBC needs to squeeze through small capillaries with diameters well below its own diameter to reach various organs and tissues. RBCs undergo large mechanical deformations when passing through these small constrictions and then, recover their initial shape when released. The remarkable deformability of RBCs is an intrinsic mechanical property primarily derived from (i) the viscoelastic properties of the cell and (ii) its biconcave disk shape (Cooke et al., 2001; Nash et al., 1989). The disk shape provides a high surface area to volume ratio that maximises oxygen exchange but also facilitates large deformations. However, RBC deformability can be significantly impaired under certain pathophysiological conditions, which, in turn, can initiate severe consequences like capillary occlusions.

For example, malaria is one of the most life-threatening diseases in the world, causing 405,000 deaths in 2018 (Organization, 2019). The malaria parasite invades RBCs in the bloodstream to proliferate and hide from the host immune system. Intraerythrocytic growth of a malaria parasite is associated with altered cell stiffness and morphology (Burns et al., 2019; Dixon et al., 2012). These changes compromise the infected RBC deformability (Safeukui et al., 2013; Shelby et al., 2003; Suresh et al., 2005). Some studies have reported impaired microcirculatory blood flow and organs failure in severe malaria, which is postulated to be consequences of poor

deformability of malaria parasite-infected RBCs. Thus, a thorough examination of RBC deformability can enable a better understanding of deadly RBC-related diseases like malaria.

A variety of experimental techniques and numerical models have been developed to examine the roles of cell stiffness and shape on RBC deformability (Aingaran et al., 2012; Glenister et al., 2002; Mills et al., 2004; Shelby et al., 2003; Zhang et al., 2015b). These studies have been conducted on different stages of the RBC lifespan, disease-affected RBCs and chemically-treated RBCs. Several studies reported that cell stiffness (membrane and cytoplasm) changes substantially affect cellular deformability (Deplaine et al., 2011; Park et al., 2008; Suresh et al., 2005), while a few studies demonstrated the important role of cell shape on its deformability (Diez-Silva et al., 2010; Herricks et al., 2009; Li et al., 2018; Pivkin et al., 2016). However, a key limitation of all these studies is that the individual impacts of cell stiffness and cell shape were not clearly differentiated in these studies. There is still uncertainty about which factor has the greatest impact on the circulation of RBCs through small constrictions like capillaries in the brain and splenic inter-endothelial slits.

The specific objective of the work described in this thesis is to investigate the biomechanics of normal RBCs and physiologically or pathologically altered RBCs, including stiffened, swollen, immature, malaria parasite-infected and mutant RBCs. A numerical model was used to seek an explanation of the biophysical basis for the impaired deformability of RBCs that is associated with certain pathological conditions. This work focuses on the relative contributions of cell stiffness and cell shape to RBC passage through small constrictions and provides insights that could be used to develop new therapies that target cell deformability to halt life-threatening diseases like malaria.

This chapter provides a brief background on RBC structure and then, a review of the literature on varying experimental observations and numerical simulations focused on cellular deformability of RBCs in healthy and disease conditions. This is followed by an outline of the thesis research aims.

1.2 The molecular structure of red blood cells

A mature RBC comprises a composite membrane and an enclosed cytoplasm. The cytoplasm is a viscous incompressible fluid, mainly consisting of a concentrated solution of haemoglobin. The RBC membrane consists of two layers: a lipid bilayer and an underlying membrane skeleton. The lipid bilayer is an incompressible layer comprising phospholipids, cholesterol and transmembrane proteins and is strongly resistant to surface area dilation (Peng et al., 2010; Skalak et al., 1973; Yoon and You, 2015). The membrane skeleton is a hexagonal array of flexible proteins, called spectrin tetramers, which are linked to junctional complexes comprising actin filaments and accessory proteins. Each spectrin tetramer consists of two spectrin heterodimers i.e. intertwined α -spectrin and β -spectrin filaments. The membrane skeleton is tethered to the membrane through transmembrane proteins, namely band-3 and glycophorin (Klöppel and Wall, 2011; Li and Lykotrafitis, 2012; Mankelov et al., 2012). Figure 1-1 illustrates the molecular structure of a human RBC.

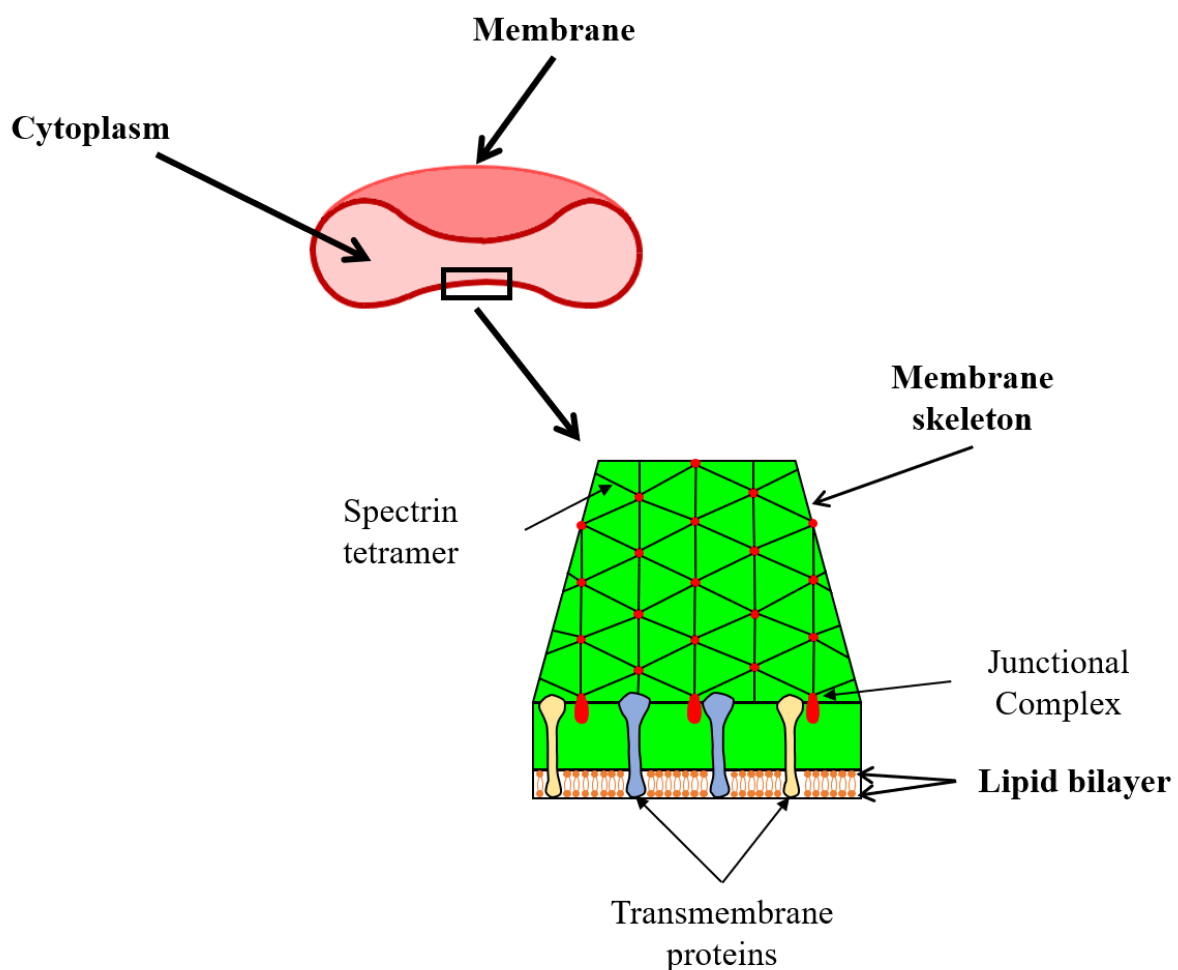


Figure 1-1 Morphology of an RBC and microstructure of the membrane. The membrane skeleton is comprised of spectrin tetramers and junctional complexes and is connected to the lipid bilayer through vertical links to the transmembrane proteins.

The mechanical properties of an RBC can be attributed to the microstructure of the cell membrane and cell cytoplasm. The strength and flexibility of the membrane are mainly derived from the network of spectrins in the membrane skeleton, while the lipid bilayer cannot resist in-plane shear stresses due to its fluid-like character (Hansen et al., 1996; Mohandas and Evans, 1994). However, the lipid bilayer does exhibit resistance to bending deformation (Bassereau et al., 2014; Evans, 1983). The cell membrane and cytoplasm are also reported to be a source of viscosity dissipation during deformation recovery (Evans and Hochmuth, 1976; Mills et al., 2004). The cytoplasmatic viscosity is determined by the concentration of haemoglobin, while the membrane viscosity is mainly derived from mechanical properties of the membrane skeleton (Fischer, 2016; Mohandas and Gallagher, 2008).

1.3 Red Blood Cell deformability

Cellular deformability is an essential feature needed for RBCs to fulfil their functions in the body. The disc-shaped RBCs circulate in the microvasculature about a million times within their 120 days lifespan (Allison, 1960), squeezing through small capillaries as narrow as 3 μm and rapidly recovering their disc shape on exiting capillaries. The deformability of an RBC is broadly dependent on two major factors: the overall cellular stiffness and the surface area to volume ratio (SA:V ratio) (Chien, 1987; Cooke et al., 2001; Huang et al., 2014; Nash et al., 1989). The shear stiffness and viscosity of the membrane skeleton, bending stiffness of the lipid bilayer and cytoplasm viscosity each make contributions to the overall cell stiffness of an RBC. The biconcave disk shape of an RBC provides a high SA:V ratio (compared with a sphere of the same volume), facilitating large deformations. Changes to these two properties of RBCs are expected to compromise RBC deformability and impair their function. Over the past few decades, a variety of experimental techniques have been developed to examine the deformability of healthy and diseased RBCs. A brief review of conventional measurement techniques is presented next, followed by a review of studies focused on changes to the deformability of malaria parasite-infected, immature, mutant cells and chemically-treated RBCs.

1.3.1 Measurement techniques

1.3.1.1 Micropipette aspiration

Micropipette aspiration technique has been widely used to study the mechanical properties of the RBC membrane (Evans, 1983; Hochmuth, 2000; Rand and Burton, 1964). Micropipette aspiration uses a small glass pipette, with a typical inner diameter of 1-3 μm , to aspirate the

membrane of an RBC into the pipette by applying a negative pressure. The membrane shear modulus can be estimated by measuring the aspirated length of the RBC (Kim et al., 2012). Figure 1-2 shows a normal RBC and a swollen RBC aspirated into a micropipette. Micropipette aspiration can also be used to measure the surface area and volume of RBCs (Evans, 1989); however, this technique is time-consuming for studying a large number of cells. Micropipette aspiration technique is normally carried out on a portion of the cell membrane; therefore, this technique primarily measures the visco-elasticity of the cell membrane rather than cellular stiffness (Mohandas et al., 1980).

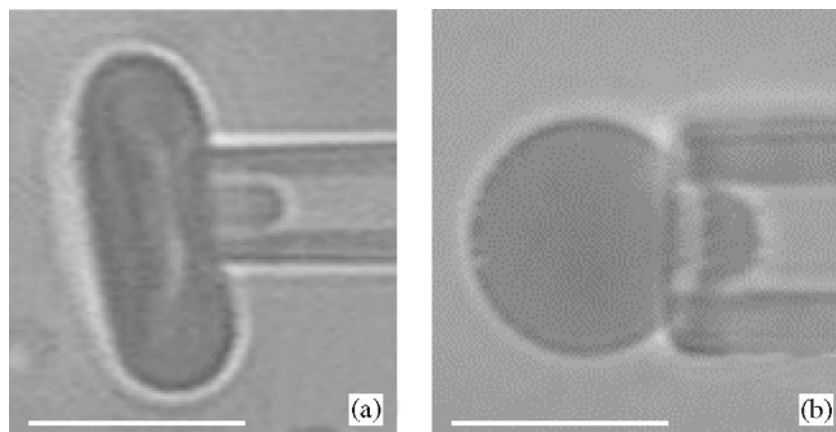


Figure 1-2 Aspiration of (a) a normal RBC and (b) a swollen RBC into a micropipette **adapted from** (Hochmuth, 2000). The scale bars represent 5 μm .

1.3.1.2 Atomic force microscope

Atomic force microscope (AFM) is another method to study cell mechanics. AFM employs of a rectangular or V-shaped cantilever with a sharp tip (probe) at its end. The probe applies small forces, on the nanonewton (nN) scale, to the cell membrane, and the response of the RBC can be measured using high-resolution imaging (Kim et al., 2012; Kirmizis and Logothetidis, 2010). This technique has been used to measure Young's modulus and the Poisson ratio of RBCs in health and disease (Dulińska et al., 2006).

1.3.1.3 Optical tweezers

Optical tweezers are another device to measure the mechanical properties of cells. Highly focused laser beams use light refraction to generate forces, on the order of piconewton (pN), on microscopic objects. There are two methods to stretch an RBC in optical tweezers. In the first method, the stretching force is applied to two silica microbeads attached at opposite ends of the RBC (Figure 2-3). In the second method, the RBC is stretched using two diverging laser beams in opposite directions. The second method is also called optical stretcher (Kim et al.,

2015). The advantage of this technique is that the stretching force and deformed diameters of cells can be measured. Shear moduli of RBCs in health and disease have been measured using optical tweezers (Mills et al., 2004; Suresh et al., 2005).

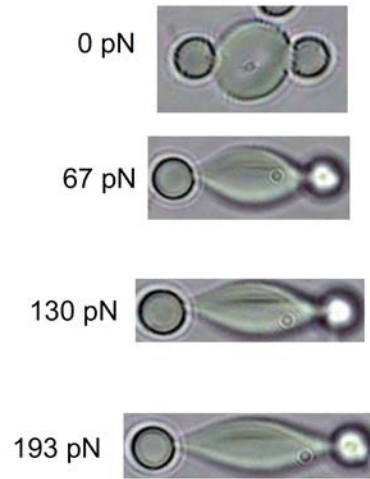


Figure 1-3 An stretched RBC in an optical tweezers experiment using silica beads **adapted from** (Mills et al., 2004)

1.3.1.4 Microfluidic devices

Microfluidic technologies offer a high-throughput approach to study mechanical and physiological properties of RBCs. Microchannels can be manufactured with dimensions in the order of a few microns to mimic tiny capillaries in the human body (Shelby et al., 2003; Shevkoplyas et al., 2006). Microfluidic devices are mostly made from polydimethylsiloxane (PDMS). Being inexpensive, easy to fabricate, transparent and biocompatible makes PDMS the best option for microfluidic devices. PDMS chips are fabricated through casting over a silicon mould which is manufactured through soft lithography (Lake et al., 2015; Madou, 2002).

Using microfluidic devices, mechanical properties of RBCs have been studied by introducing cells to small microchannels with a few micrometres, mimicking small physiological blood vessels (Santoso et al., 2015; Shevkoplyas et al., 2006). Microfluidic devices enable measuring various parameters such as entry time, transit time and velocity, and deformed length of the RBC through microchannels (Hou et al., 2011; Shelby et al., 2003).

The mechanical properties of RBCs have been studied using microfluidic devices, (Santoso et al., 2015; Shevkoplyas et al., 2006), providing data on parameters such as entry time, transit time and velocity, and deformed length of the RBC (Hou et al., 2011; Shelby et al., 2003).

Microfluidics have been also used to estimate SA:V ratio of RBCs. The Human Erythrocyte Microchannel Analyser (HEMA) is a microfluidic device that was designed to measure the surface area and the volume of RBCs (Gifford et al., 2003). This device consists of thousands of channels, some of which are wedge-shaped to trap RBCs (Figure 2-4). The surface area and volume of a trapped RBC can be calculated by knowing the geometry of microchannels and delineating the cell position. The HEMA microchip is a high-throughput technique which has been used to measure the surface area and volume of large numbers of RBC (Gifford et al., 2006; Herricks et al., 2009). This device is modified and used in this thesis to examine SA:V ratio of RBCs. Further details of the modified version of the HEMA microchip are presented in the methods section (Chapter 2).

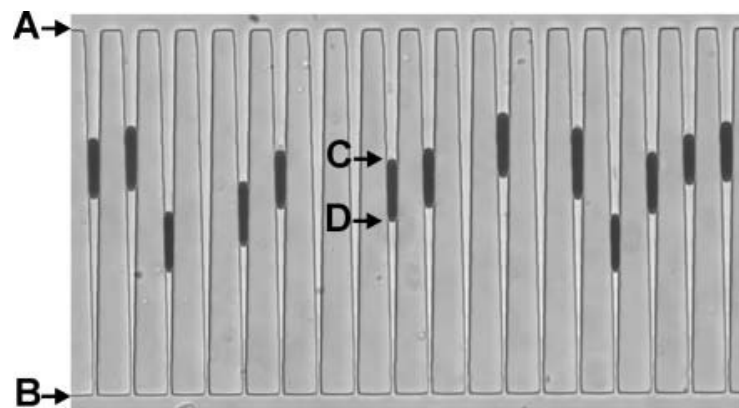


Figure 1-4 Arrested RBCs in wedge-shaped microchannels in the HEMA device **adapted from** (Gifford et al., 2003). The arrows A and B show the entry and exit of the microchannels, and the arrows C and D show the top and bottom of an arrested RBC.

1.3.1.5 Microbeads filtration

Microbeads filtration consists of a mixture of metal microbeads with diameters of 5 to 25 μm , providing interstices that mimic the geometry of inter-endothelial slits in the human spleen. A surrogate measure of deformability of RBCs is their rate of retention during in the microbeads filtration. Rigidified RBCs are not able to passage through the microbeads (Deplaine et al., 2011) (Lelliott et al., 2017).

1.3.1.6 Ektacytometry

Ektacytometry measures elongation index (EI) of RBCs in response to increasing shear stress from 0-20 Pa. This device passes a laser beam through the cell suspension to form a diffraction pattern. The RBC's ability to elongate can be measured from the diffraction pattern (Bessis et al., 1980).

Ektacytometry is an effective technique to measure the average ability of a population of RBCs to elongate. This technique is less effective when the target cells of interest represent only a minority of the population (Huang, 2014). Ektacytometry has been widely used to measure the elongation index of healthy and diseased RBCs (Barber et al., 2018b; Clark et al., 1983; Dearnley et al., 2012; Renoux et al., 2019). Further details on how ektacytometry works are presented in the methods chapter (Chapter 2).

1.3.1.7 Limitations

The review of experimental techniques highlights that each of them measures different physical parameters and different techniques may be more sensitive to different biomechanical changes. As discussed above, membrane viscoelasticity, cytoplasmic viscosity and SA:V ratio each make contributions to RBC deformability. Some techniques such as micropipette aspiration and ektacytometry exhibit more sensitivity to changes in membrane shear modulus rather than SA:V ratio changes. Hence, such techniques may not provide a complete understanding of cellular deformability changes in pathophysiological conditions. Therefore, the results of different experimental techniques need to be interpreted with caution, and where possible different techniques should be employed to interrogate changes to RBC properties.

1.3.2 Cellular deformability changes in pathophysiological conditions

Different pathological conditions alter the properties of RBCs and in turn influence circulation through the bloodstream. Therefore, it is important to study the separate contributions of cell stiffness and cell shape to RBC deformability, as this may provide insights into the underpinning mechanisms of different pathological diseases and lead to appropriate, targeted treatments. An overview of studies focused on the deformability of malaria parasite-infected, mutant and immature RBCs is presented next.

1.3.2.1 Infection of RBCs with malaria parasites

Malaria is one of the most life-threatening diseases in the world. It is caused by protozoan parasites of the genus *Plasmodium* that can be transmitted to humans through bites of infected mosquitos. Malaria parasites hijack red blood cells. Host cell invasion by malaria parasite is followed by restructuring the RBC membrane structure and modifying the cell morphology, compromising host cell deformability (Aingaran et al., 2012; Burns et al., 2019; Dearnley et al., 2016; Dixon et al., 2012; Wu and Feng, 2013). Some studies have reported the impaired microcirculatory blood flow in severe malaria, and have attributed these pathological

consequences to the poor deformability of malaria-parasite infected RBCs (Dondorp et al., 2000; MacPherson et al., 1985; Miller et al., 1971). An overview of two species of malaria, *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium knowlesi* (*P. knowlesi*), which are known to affect humans, is presented here.

1.3.2.1.1 *P. falciparum*-infected RBCs

Within the *Plasmodium* genus, *P. falciparum* causes the most severe disease. Upon the RBC invasion, the parasite undergoes an asexual development cycle of about 48 hrs, progressing through the ring, trophozoite and schizont stages (Figure 1-5a) (Haldar and Mohandas, 2007) (Maier et al., 2009). During asexual development, the intracellular *P. falciparum* parasite exports proteins to the RBC membrane skeleton, stiffening the host cell membrane through composite strengthening, strain hardening, and vertical coupling effects (Zhang et al., 2015b). Formation of adhesive knobs at the outer surface of the RBC is another notable change occurring during the asexual development (Figure 1-5b,c), facilitating the sequestration of rigid infected RBCs in the microvasculature (Barnwell, 1989) (Rug et al., 2006). The altered cellular properties of *P. falciparum*-infected RBCs result in trapping and sequestration of infected RBCs in the microvascular network, especially in cerebral capillaries (Dondorp et al., 2000) (MacPherson et al., 1985).

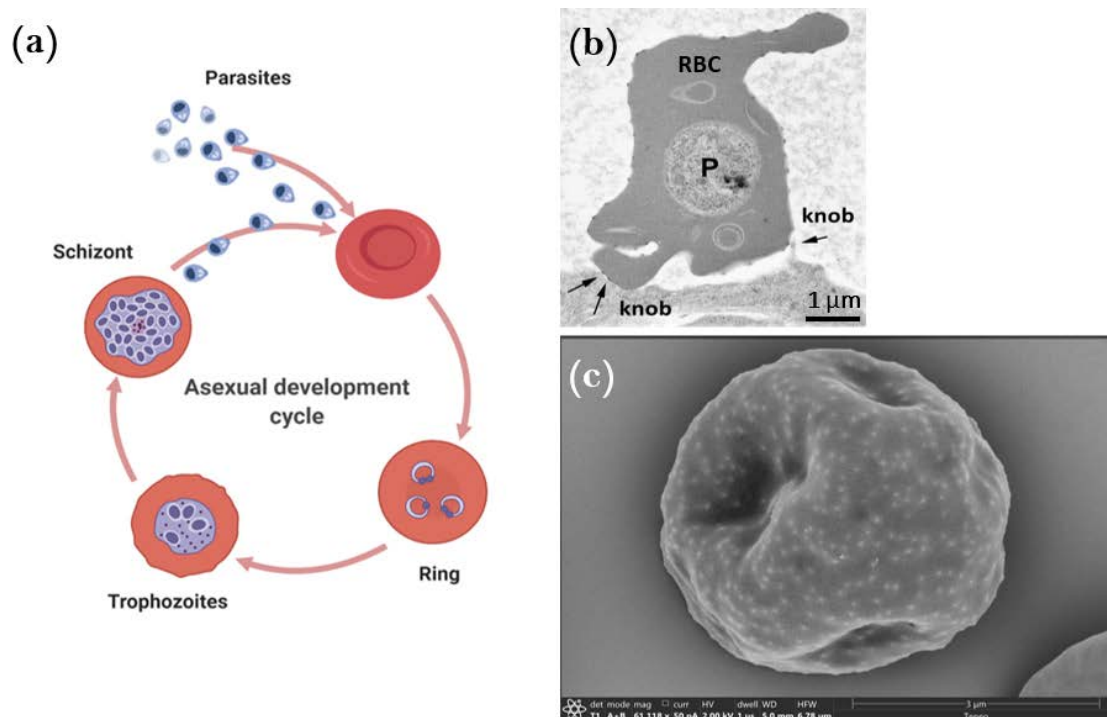


Figure 1-5 (a) Development of *P. falciparum* in human RBCs. *P. falciparum* parasites invade healthy RBCs and undergo an asexual development cycle of 48 hrs through the ring (0-24 hrs), trophozoite (24-36 hrs) and schizont (30-38 hrs) stages. (b) Transmission electron microscopy (TEM) image, showing the ultrastructure of a *P. falciparum*-infected RBC. Adhesive knobs

form on the outer surface of the infected RBC (image courtesy of Dr Boyin Liu). (c) Scanning electron microscopy (SEM) image of a *P. falciparum*-infected RBC (image courtesy of Dr Adam Blanch).

Overall, it remains unclear whether increased rigidity of *P. falciparum*-infected RBCs is the main determinant of capillary obstructions in the body or whether changes to the SA:V ratio play a more prominent role in compromising the circulation of infected cells in narrow capillaries.

1.3.2.1.2 *P. knowlesi*-infected RBCs

P. knowlesi is a parasite of long-tailed macaques that causes infection in humans and is potentially fatal (Singh and Daneshvar, 2013). While infections are relatively few, this species of the genus *Plasmodium* is reported to cause severe malaria in Malaysia with a higher risk of severity than *P. falciparum* (Barber et al., 2012). The asexual development cycle for culture-adapted *P. knowlesi* is 32 hrs (Liu et al., 2019). The ultrastructure and morphology of a *P. knowlesi*-infected RBC are shown in Figure 1-6. Similar to *P. falciparum*, a previous study has reported that severe *knowlesi* malaria is associated with impairment of microcirculatory blood flow (Miller et al., 1971), despite the lack of adhesive knobs on the outer surface of infected RBCs (Barber et al., 2018a). Further, splenic rupture has been observed in *P. knowlesi*, suggesting capillary blockages (Chang et al., 2018). It is anticipated that the mechanism of microvascular obstructions in *P. knowlesi* likely differs from that for *P. falciparum*. Using micropipette aspiration, Barber et al. measured an increased membrane shear modulus of 47 $\mu\text{N/m}$ in human RBCs infected with severe *P. knowlesi*, which is less than the one measured for *P. falciparum* (Barber et al., 2018b). No attempt was made to examine changes in volume and surface area of infected RBCs during asexual development of *P. knowlesi*. Therefore, it is still unclear why infected RBCs with *P. knowlesi* exhibit passage restriction through small capillaries.

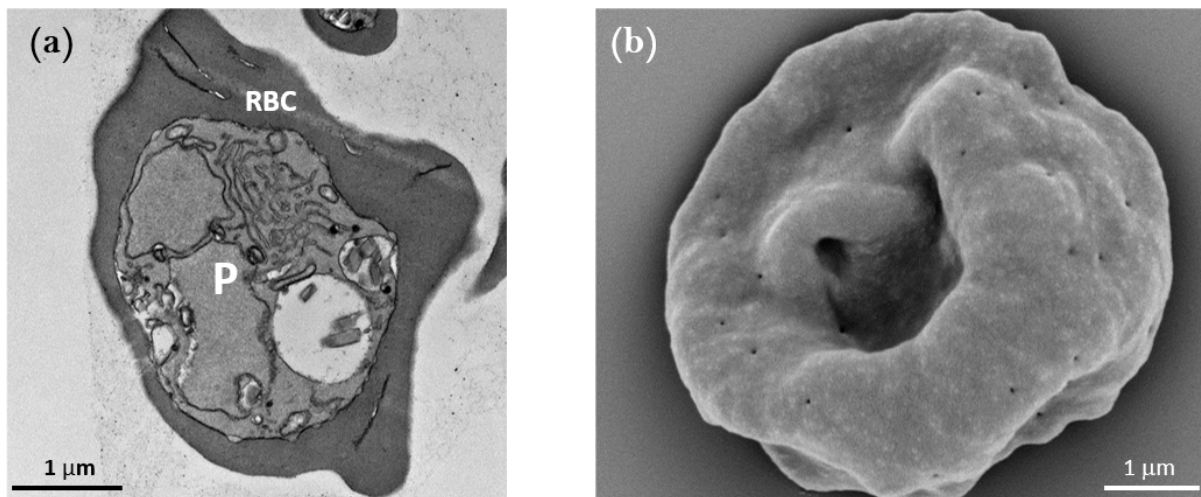


Figure 1-6 Ultrastructure and morphology of a *P. knowlesi*-infected RBC. (a) Transmission electron microscopy (TEM) image . (b) Scanning electron microscopy (SEM) image (image courtesy of Dr Adam Blanch).

Taken together, RBCs infected with these two *Plasmodium* species undergo ultrastructural changes, which affect their cellular deformability. Much uncertainty still exists about the consequences of changes to cell stiffness and morphology of malaria parasite-infected RBCs on their circulation in the body.

1.3.2.2 Mutant RBCs

Defects in some of the protein components of the membrane skeleton can result in abnormal RBCs that are susceptible to splenic clearance (An and Mohandas, 2008). Interestingly, some RBC abnormalities exhibit resistance against malaria parasite invasion and can provide protection against malaria (Schulman et al., 1990) (Chishti et al., 1996) (Rank et al., 2009) (Greth et al., 2012) (Leffler et al., 2017). These mutations in the membrane skeleton proteins are associated with physical changes to the properties of the RBC. Membrane cohesion derives from vertical interactions between proteins embedded in the lipid bilayer and the proteins of the membrane skeleton. Mechanical stability also requires stable lateral linkages between proteins of the membrane skeleton (An and Mohandas, 2008). Mutations can decrease membrane cohesion or mechanical stability, leading to membrane surface area loss, reduced RBC lifespan and anaemia, as observed in RBC disorders, such as hereditary spherocytosis and hereditary elliptocytosis (An and Mohandas, 2008).

Hereditary spherocytosis (HS) is a common inherited RBC disorder where the affected RBCs transform from discocyte to spherocyte, and is associated with haemolysis, leading to anaemia and splenomegaly, depending on its severity. Mutations or truncations of spectrins account for 20% of HS; α -spectrin defects typically lead to severe HS in patients, while β -spectrin defects

cause mild to moderately severe HS (Eber and Lux, 2004). Hereditary elliptocytosis (HE) is another inherited RBC disorder where RBCs are characterised with an elliptical shape and mechanically unstable membrane. HE can be caused by defects in any of the protein components involved in lateral linkages in the membrane skeleton, such as α -spectrin and β -spectrin (An and Mohandas, 2008).

Mutations in β -spectrin (*Sptb*) can result in both HS and HE; however, the effect of *Sptb*-mutations on the biomechanics of mutant RBCs have not been well studied. No previous study has investigated whether *Sptb*-mutant RBCs are more likely to obstruct narrow constrictions compared to normal cells.

1.3.2.3 Immature RBCs

Mature RBCs have a lifespan of about 120 days, and their journey begins with the generation of immature RBCs (reticulocytes) in the bone marrow and their release into the peripheral blood. When reticulocytes start circulating in the peripheral blood, they undergo some significant changes in the structure and membrane properties as they transform into mature RBCs within 1 to 2 days. Reticulocyte maturation is accompanied by a decrease in membrane stiffness, loss of surface area and elimination of residual cytosolic organelles. Early reticulocytes are reported to lose about 20% of the membrane surface area by vesiculation to acquire the biconcave shape of the mature RBCs, taking with it some markers such as the transferrin receptor (CD71) (Blanc and Vidal, 2010) (Vaughn et al., 1997) (Liu et al., 2010). Remnant RNA is also lost during maturation (Ney, 2011). Figure 2-2 shows the morphological changes of a reticulocyte as the reticulocyte loses the CD71 in the process of maturation.

Using a microfluidic device, it was previously reported that the surface area decreases from $170 \mu\text{m}^2$ in RNA positive reticulocytes to $147 \mu\text{m}^2$ in mature RBCs, and similarly, the volume reduces from 115 fL to 99 fL. The SA:V ratio, however, remained unchanged during reticulocyte maturation (Gifford et al., 2006). Micropipette aspiration experiments have shown that membrane shear modulus of reticulocytes ($11.4 \text{ pN}/\mu\text{m}$) is almost twice that for mature RBCs ($6.1 \text{ pN}/\mu\text{m}$) (Malleret et al., 2013).

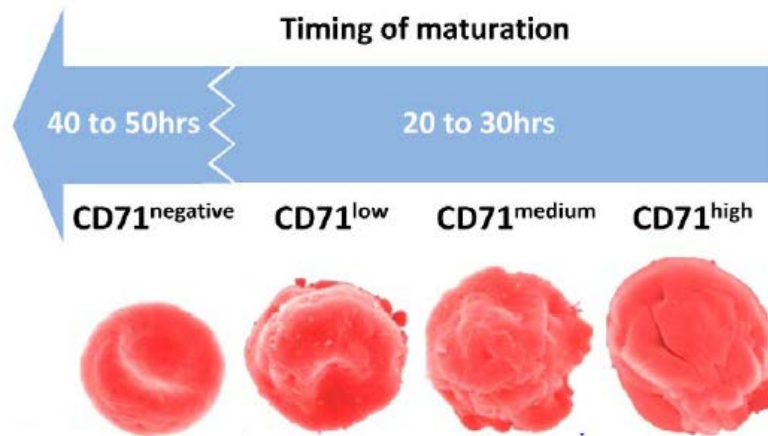


Figure 1-7 Morphological changes of a reticulocyte as it matures in the maturation process **adopted from** (Malleret et al., 2013).

There has been no detailed investigation of the ability of early-stage reticulocytes to circulate through small constrictions. Thus, it is unclear whether the sub-optimal mechanical properties of early-stage reticulocytes restrict their passage through the smallest capillaries in the body compared with mature RBCs. This comparison would provide a better understanding of the roles of cell membrane stiffness and cell shape in RBCs traversal through narrow blood vessels.

1.3.2.4 Insights from computational modelling

In addition to experimental studies, many numerical simulations have been performed mainly using continuum modelling and particle-based methods to investigate the mechanical characteristics of the RBC under large deformations. However, few numerical studies have focused on cell stiffness and SA:V ratio changes and their individual impacts on cellular deformability. Finite element analyses on different stages of *P. falciparum* gametocytes (sexual stages of parasites) predicted that both cell stiffness and cell shape significantly impact the passage of gametocytes in small capillaries and inter-endothelial slits (Aingaran et al., 2012). Using a particle-based model, Wu and Feng studied the effects of membrane stiffness, loss of surface area and parasite presence on the passage of *P. falciparum*-infected RBCs through a constricting channel. Their numerical simulations indicated that all three factors contribute to the channel occlusion in infected RBCs (Wu and Feng, 2013).

Imai et al. used a particle-based model to study the deformation of *P. falciparum*-infected RBCs in optical tweezers, shear flow and narrow channels. Their simulations predicted that changes in both sphericity and rigidity of *P. falciparum* trophozoites make these cells poorly deformable in blood flow (Imai et al., 2010). Using a dissipative particle dynamics (DPD)

model, Pivkin et al. indicated that the passage of *P. falciparum*-infected RBCs through inter-endothelial slits is more impacted by surface area changes rather than membrane stiffness changes (Pivkin et al., 2016). However, some previous studies have measured no significant changes in SA:V ratio of *P. falciparum* trophozoites compared to uninfected cells (Herricks et al., 2009) (Liu et al., 2019), indicating that morphological changes might not be the reason for the restricted passage of infected cells through inter-endothelial slits. A recent study also analysed the traversal of two typical hereditary RBC disorders through splenic inter-endothelial slits using a coarse-grained molecular dynamics (CGMD) model and predicted that both hereditary spherocytosis and elliptocytosis RBCs would not escape the inter-endothelial slits due to their lower SA:V ratios (Li et al., 2018).

Taken together, pathological and physiological changes to cells stiffness and SA:V ratio are known to impact RBC deformability, but no systematic study has been done to understand the individual contributions of these two factors to RBC traversal through small capillaries. It remains unclear which factor has the greatest impact.

1.3.3 Cellular deformability changes in chemically treated RBCs

In addition to studies on pathophysiological changes to RBCs, some other studies have focused on cellular deformability changes of RBCs by chemically manipulating either cell stiffness or SA:V ratio of cells. The following sections review the findings of these studies.

1.3.3.1 Altering cell stiffness

Cell stiffness has long been understood to an important contributor to RBC deformability. The deformability of chemically rigidified RBCs has been investigated using different experimental techniques (Shevkoplyas et al., 2006) (Sosa et al., 2014) (Safeukui et al., 2018) (Renoux et al., 2019). Sosa et al. stiffened RBCs using glutaraldehyde and diamide and showed that elongation index of RBCs measured by ektacytometry was highly sensitive to both glutaraldehyde and diamide treatment, while the ability of stiff RBCs to perfuse through an artificial microvascular network remained unaffected by the treatment of RBCs with diamide but decreased linearly with increasing concentration of glutaraldehyde. Similarly, the ability of stiff cells to pass through micropore filters was not affected by the diamide treatment, but high concentrations of glutaraldehyde (>0.04%) impact the RBCs passage through micropore filters (Sosa et al., 2014). Safeukui et al. also assessed the retention of diamide-treated RBCs in a perfused human spleen and microsphilters. The splenic retention was found to be strongly correlated with the diamide concentration, while the treated RBCs were retained to a lesser extent in the

microsphilters (Safeukui et al., 2018). These results showed that treated RBCs with glutaraldehyde and diamide behaved differently depending on experimental techniques, and it is unclear whether changes to SA:V ratio also contributed to poor deformability of treated RBCs.

These studies showed that changes to cell stiffness impact the response of RBCs under large deformations. However, the sensitivity of the circulation of an RBC through narrow capillaries to its cell stiffness has not been well studied, and this indicates a need to quantify the separate contribution of cell stiffness and compare with SA:V ratio's contribution.

1.3.3.2 Altering surface area to volume ratio

SA:V ratio is also known to be an important determinant of RBC deformability (Cooke et al., 2001; Diez-Silva et al., 2010; Murdock et al., 2000). Murdock et al. treated mouse RBCs to reduce their surface area and consequently, reduce their SA:V ratio. A decrease was observed in the number of circulating cells when the sphericity was greater than 20% above the normal (Murdock et al., 2000). Gifford et al. designed a novel microfluidic device to trap RBCs in wedge-shaped microchannels in order to measure surface area and volume (Gifford et al., 2003). Their results showed that cells with a higher SA:V ratio can squeeze further into the wedge-shaped microchannels. In another study, treated RBCs with reduced SA:V ratio (~27%) were completely entrapped in an *ex vivo* human spleen (Safeukui et al., 2012). In recent research, Renoux et al. measured the elongation index (EI) of chemically treated RBCs using an osmotic gradient ektacytometry (Renoux et al., 2019). Lysolecithin (LPC), diamide and nystatin (OSMO) were used to alter SA:V ratio and cell stiffness of RBCs. They concluded that a measured decrease in the EI at shear stresses lower than 3 Pa is due to an increase in membrane stiffness, while a decrease in the EI at shear stresses above 3 Pa could be due to either a reduction in SA:V ratio or an increase in membrane stiffness (Renoux 2019). This work was not able to completely differentiate the relative contributions of cell stiffness and SA:V ratio to elongation of RBCs under shear stress.

These studies showed that a high SA:V ratio is crucial for the maintenance of RBCs in the circulation. However, the sensitivity of RBCs passage through narrow capillaries to SA:V ratio has not been studied in sufficient detail and not been compared with the separate impact of cell stiffness. Therefore, a systematic analysis of relative impacts of these two factors will deepen our understanding of the circulation of RBCs affected by diseases and shed light on mechanisms of the impaired microvascular network in RBC-related diseases.

1.4 Research aims

The main objective of the work present in this thesis was to examine the passage of RBCs through small constrictions and determine the relative contributions of cell stiffness and cell shape to RBC traversal. This study examined different types of RBCs, including chemically treated, malaria parasite-infected and genetically modified RBCs. The specific research aims are:

1. To investigate the independent impacts of cell stiffness and the SA:V ratio on traversal of chemically-treated RBCs into small channels (Chapter 3).
2. To examine the consequences of altered cellular properties in immature, malaria parasite-infected and mutant RBCs on their ability to circulate through small channels (Chapter 4).
3. To use a finite element model to explain the experimental observations and predict critical bounds for membrane stiffness and SA:V ratio which allow RBCs to passage through capillaries with dimension similar to cerebral capillaries (Chapter 5).

1.4.1 Thesis outline

Chapter 2 presents the experimental framework used in this thesis. Two microfluidic-based devices (i) ektacytometry and (ii) a Human Erythrocyte Microchannel Analyser (HEMA) were used to measure cellular properties of RBCs and monitor their passage through small microchannels. Cell stiffness was assessed under increasing shear stress using ektacytometry, while volume and surface area were measured in the HEMA microchip. The experimental setups are explained in detail in this chapter.

Chapter 3 focuses on independently analysing the effects of cell stiffness and SA:V ratio on RBCs passage through narrow microchannels. Two fundamental experiments were carried out in this chapter: (i) increasing the cell stiffness while maintaining the SA:V ratio unchanged and (ii) tuning the SA:V ratio of cells while minimising the cell stiffness changes. This study is the first detailed investigation of the independent impacts of cell stiffness and SA:V ratio on RBC passage through microchannels.

In Chapter 4, case studies on immature, malaria parasite-infected and mutant RBCs were undertaken to examine whether altered cellular properties of RBCs relevant to pathophysiological conditions allow passage through narrow microchannels or not.

Our computational model is described in Chapter 5, where parametric studies were performed independently on membrane stiffness and morphology of the RBC. We identified the impacts of these two physical properties of the RBC on stress and strain distributions within the cell. Our finite element model was also used to predict critical bounds for cell stiffness and SA:V ratio which allow cells to pass through small channels comparable to narrow cerebral capillaries in the brain.

Finally, Chapter 6 recaps the findings in this thesis and suggests future research.

Chapter 2

2 Methods

2.1 Introduction

In the work described in this thesis, two microfluidic-based techniques, (i) ektacytometry and (ii) a microfluidic chip called a Human Erythrocyte Microchannel Analyser (HEMA) (Gifford et al., 2003), were used to measure rigidity and SA:V ratio of RBCs and examine their passage through small microchannels. This chapter details these experimental techniques and explains how measurements and calculations were performed to assess the morphology and deformability of RBCs.

2.2 Measuring cell stiffness

All measurements of stiffness of RBCs were conducted using ektacytometry. To conduct an ektacytometry experiment, blood samples are diluted to approximately 0.2% haematocrit in 6% 360 kDa polyvinylpyrrolidone (PVP) solution and inserted into the sample chamber of a slit-flow ektacytometer (see Figure 2-1a). Once the ektacytometer is switched on, a high pressure is introduced, and the RBCs are sucked into the ektacytometer slit. A laser diode emits a laser beam to traverse through the suspension, which generates a diffraction pattern on the projection screen (Figure 2-1a). The diffraction pattern of normal RBCs is circular at low shear stress and gradually approaches ellipsoidal shape as shear stress increases in the chamber (Figure 2-1b). The elongation index (EI) is determined by measuring the diameters of the diffraction pattern under given shear stress:

$$EI = \frac{A - B}{A + B} \quad (2 - 1)$$

where A and B are the major and minor diameters of the diffraction pattern (Figure 2-1b). Reduction in the EI of a blood sample compared with that for normal RBCs indicates an increase in cell stiffness of the examined RBCs.

In our studies, 10 μL of packed cells were dispersed in 500 μL PVP solution, and the EI was measured in a RheoScan ektacytometer (Shin et al., 2005). Each measurement was performed in triplicate, and the averaged data is presented. An example of elongation index plot for normal RBCs is presented in (Figure 2-1c).

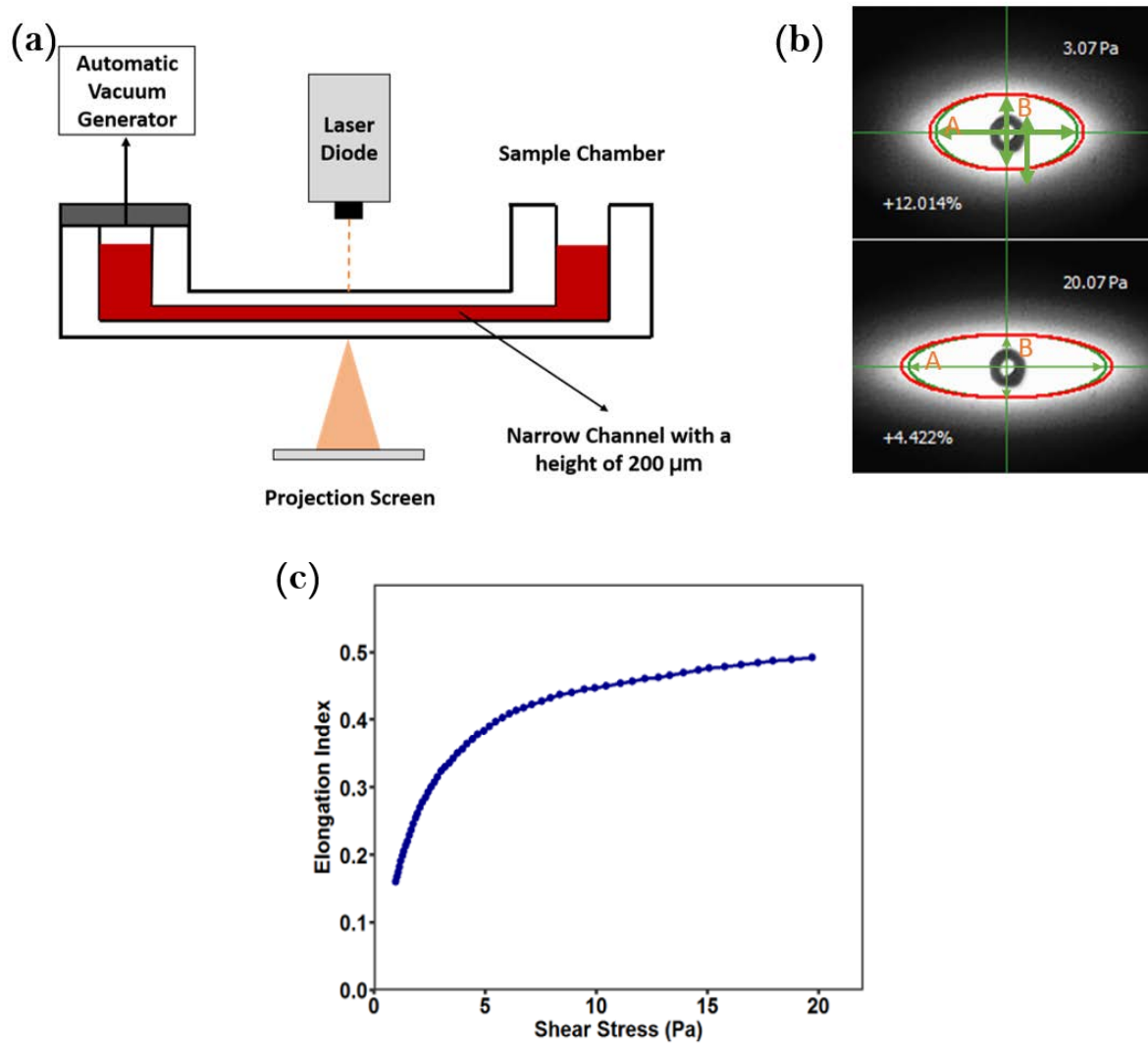


Figure 2-1 (a) Schematic diagram of a slit-flow ektacytometer, and (b) diffraction patterns of normal RBCs at shear stresses of 3 and 20 Pa. Major diameter (A) and minor diameter (B) of the diffraction patterns are shown. (c) An example of elongation index plot for normal RBCs subjected to shear stress.

2.3 Measuring the surface area to volume ratio and the ability of RBCs to traverse through microchannels

A modified version of the microfluidic device called a Human Erythrocyte Microchannel Analyser (HEMA) (Gifford et al., 2003) was used to entrap RBCs and measure their ability to squeeze into wedge-shaped microchannels. Conforming RBCs into the wedge-shaped microchannels also allowed estimation of the cell surface area and volume.

2.3.1 Fabrication of the HEMA microchip

The HEMA device consists of a 7×8 mm array of rows of parallel microchannels. Two types of microchannels were fabricated in the HEMA microchip: (i) straight microchannels which allow passage of cells and (ii) wedge-shaped microchannels which are designed to trap RBCs. The cell surface area and volume were estimated from the final position of the arrested RBC and the known dimensions of the channel in the trapped region.

The microchips were fabricated using standard soft lithography at the Melbourne Centre for Nanofabrication (MCN) in the Victorian Node of the Australian National Fabrication Facility (ANFF) (Lake et al., 2015; Madou, 2002). SU-8 2005 photoresist was spin coated on the surface of silicon wafer for 2 sec at 500 rpm and 30 sec at 5000 rpm. Then, the wafer was soft-baked at 95°C for 2 min. The photomask was designed using AutoCAD 2017 (Autodesk, Inc., USA), and the inverse of the microchip pattern was printed on the silicon wafer by UV light exposure using a UV mask aligner. The inverse pattern was etched into the wafer using deep reactive-ion etching. The etched silicon wafer served as a master mould to fabricate PDMS microchips with good accuracy. The master moulds were kindly fabricated by Mr Dan Smith.

PDMS is a transparent and biocompatible material widely used in the fabrication of microfluidic chips. The PDMS was mixed in a 1:10 ratio of curing agent and base elastomer. Then, the PDMS was poured onto the master mould and fully degassed in a vacuum desiccator for 1 hr to remove air bubbles before curing in an oven at 80°C for 1 hr. After curing, the PDMS microchip was easily peeled off from the mould, and the inlet and outlet were made into the PDMS using a biopsy punch for connection of tubing to a fluid reservoir and pressure pump. Finally, the PDMS microchip was bonded to a coverslip using a Harricks Plasma cleaner to activate the surfaces before the attachment. For bonding, the feature side of the PDMS microchip and the coverslip were cleaned carefully from residues using a scotch tape before placing them in the plasma cleaner. The PDMS microchip and the coverslip were treated with

air plasma for 2 min at a pressure of 300 mTorr. Then, the microchip was placed on the coverslip, and gentle pressure was applied for 1 min uniformly over the microchip surface. The microchip was baked at 90°C for 10 min to complete the bonding.

The HEMA pattern was firstly designed using the same dimensions that were proposed for microchannels by Gifford et al. (2003). Using the original version of the HEMA microchip, some RBCs were not able to squeeze into the wedge-shaped microchannels, and many cells were found stuck at the entrance of the microchannels. Therefore, we redesigned the microchannels by extending their length from 80 μm to 125 μm and increasing their entrance width from 3 μm to 5 μm to allow for a broader range of cells with different sizes to be studied using the microchip. Figure 2-2 shows the schematic design of a small part of the array of microchannels in the HEMA microchip.

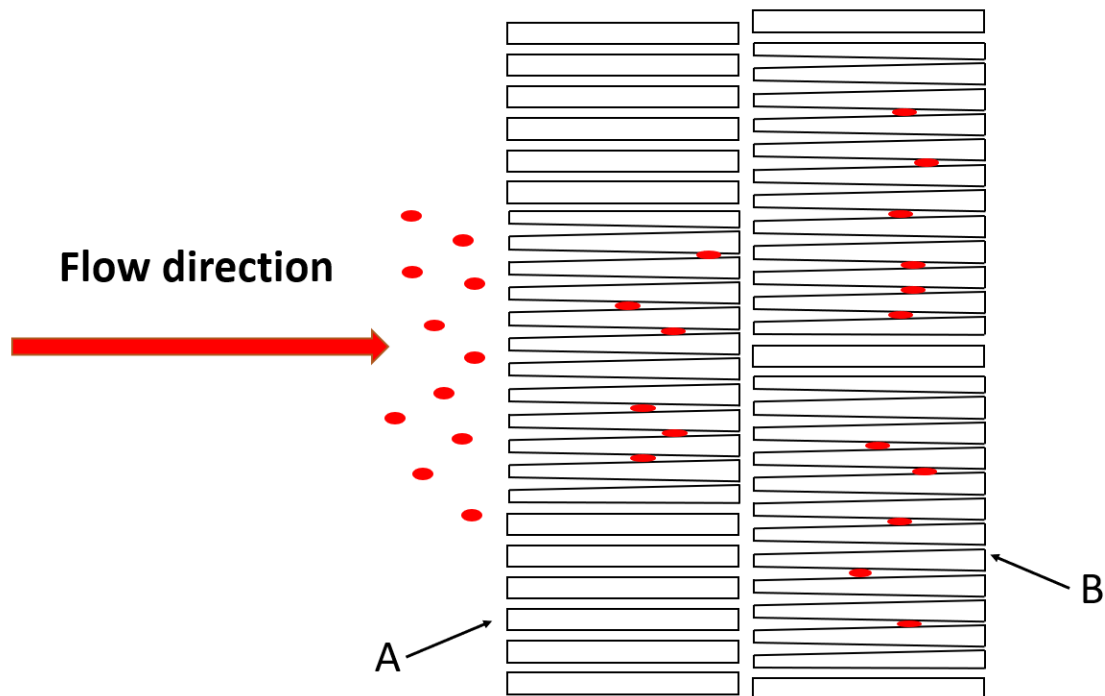


Figure 2-2 A schematic diagram of the array of microchannels in the modified version of HEMA microchip with arrested RBCs. Only a small portion of the array is shown. Most of the channels were designed straight with a width of 6 μm to allow the passage of RBCs with small deformation (A), while a small number of microchannels in each row was designed in a wedge-shaped to entrap RBCs (B).

Actual dimensions of wedge-shaped microchannels were measured via a scanning electron microscope (SEM). The SEM images were kindly collected by Dr Adam Blanch using FEI Teneo, 2kV beam voltage and 6.5 mm working distance after coating a thin layer of gold (<1nm) on PDMS microchips. The microchannels were $125.2 \pm 3 \mu\text{m}$ long and $3.23 \pm 0.4 \mu\text{m}$

deep. The width of wedge-shaped microchannels was $1.44 \pm 0.4 \mu\text{m}$ at the entrance and $4.98 \pm 0.4 \mu\text{m}$ at the exit. SEM images of the PDMS chip are presented in Figure 2-3.

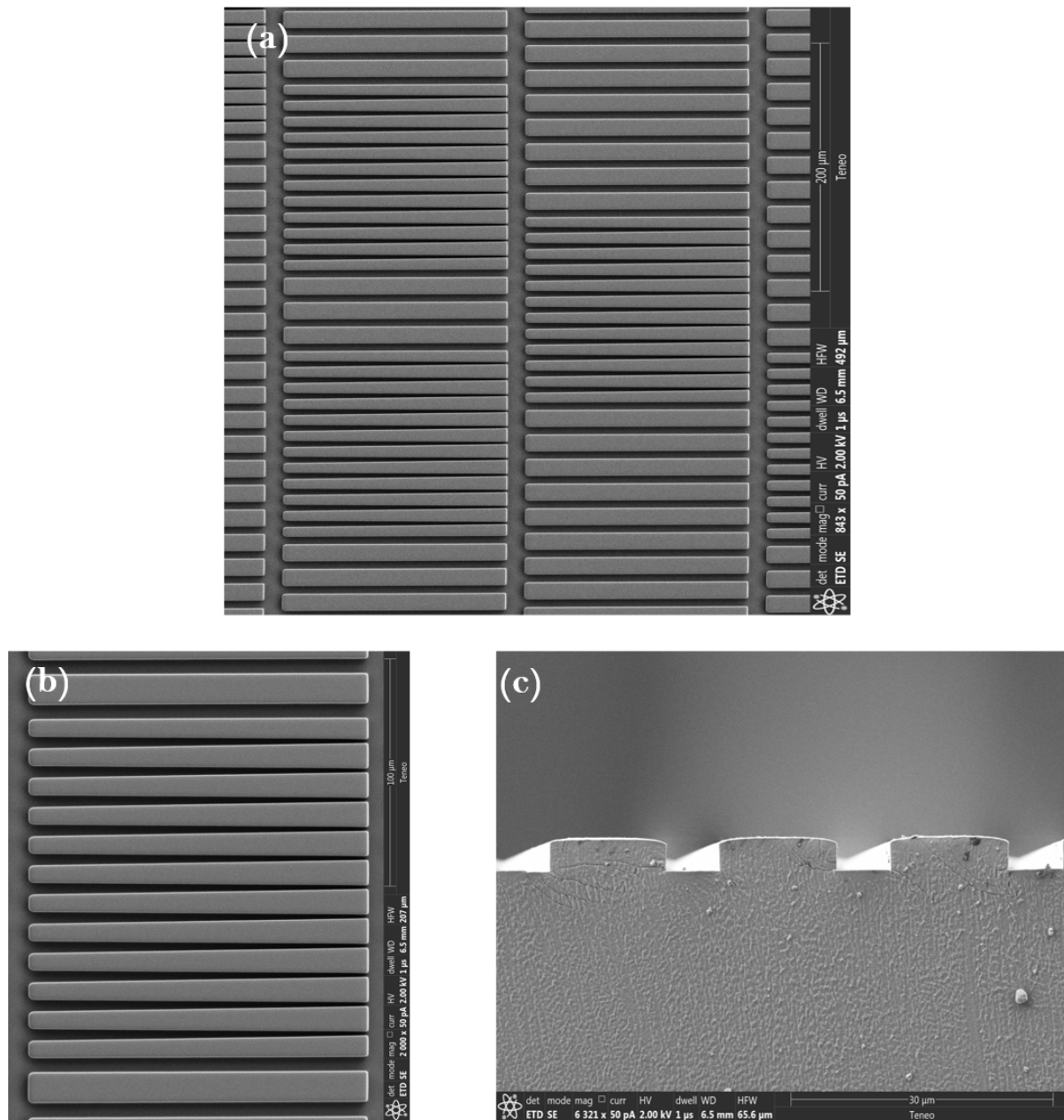


Figure 2-3 Scanning electron microscopic images of the HEMA PDMS chip. The $125.2 \pm 0.06 \mu\text{m}$ long and $3.23 \pm 0.05 \mu\text{m}$ deep as measured via SEM. The width of wedge-shaped microchannels was $1.44 \pm 0.04 \mu\text{m}$ at the entrance and $4.98 \pm 0.07 \mu\text{m}$ at the exit. These images illustrate that the PDMS cast is an excellent replica of the designed pattern.

2.3.2 Experimental setup

The HEMA chip was purged with 80% ethanol at a flow rate of $2 \mu\text{L}/\text{min}$ for 30 minutes. Afterwards, it was flushed with phosphate-buffered saline (PBS) or complete culture media for 45 minutes. Purging steps were completed using a syringe pump. For more precise control of pressure, diluted washed blood (at 0.2-0.5% haematocrit) was driven into the microchannels at

a flow pressure of 180-250 mbar using an Elveflow AF1 pressure pump, except for the glutaraldehyde study, where a syringe pump was used to drive cells to the microchip at a flow rate of 1 $\mu\text{L}/\text{min}$. Figure 2-4 displays a schematic diagram of the experimental setup for HEMA experiments. Since small chip to chip variation was evident in early experiments focussed on glutaraldehyde treated and mutant mouse cells, later experiments were completed using a single microchip that was washed between samples for reliable comparison between samples. Microfluidic chips were mounted in a Deltavision Elite (GE Healthcare) inverted microscope and imaged using a 40x air objective at 37 °C for malaria-infected cells and at room temperature for other experiments.

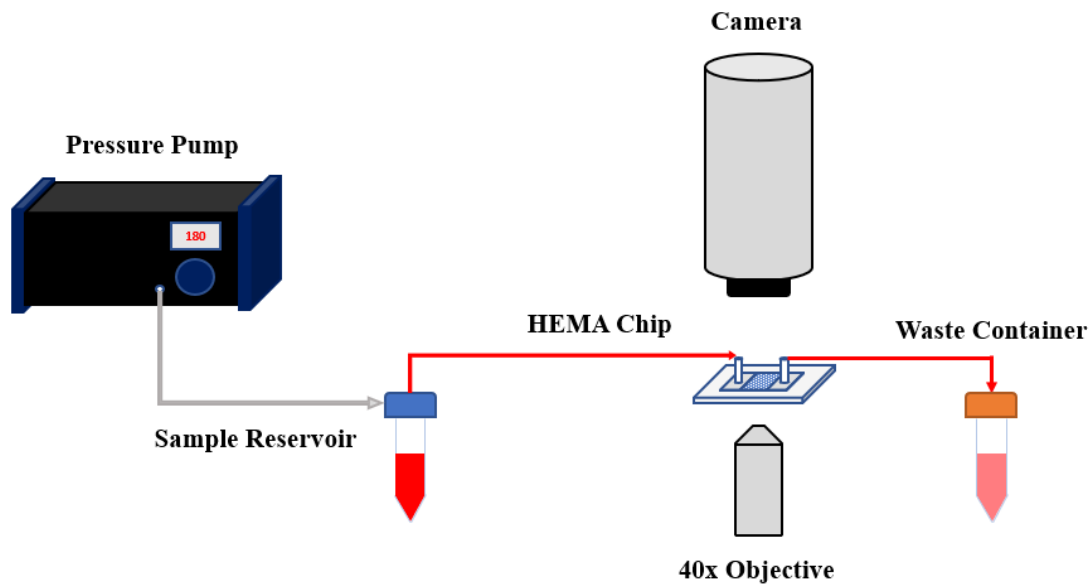


Figure 2-4 A schematic diagram of the experimental setup for HEMA experiments. The diluted blood sample was driven into the HEMA chip using a pressure pump, and the HEMA chip was mounted in a Deltavision Elite inverted microscope and imaged using a 40x air objective.

2.3.3 Measuring volume, surface area and SA:V ratio

To examine the deformed shape of normal RBCs trapped in narrowing microchannels, cells were labelled with PKH67 dye before introduction into the HEMA chip. 20 μL of packed RBCs were washed in PBS and redispersed in 125 μL DilC. An additional 125 μL of DilC containing 1 μL of PKH67 was added to the mixture. After ~ 1.5 min, 250 μL of 2% BSA in DilC was also added to this mixture and inverted to mix and halt the labelling process. After ~ 1 min, the cells were pelleted and redispersed in PBS and washed further three times before introduction to the HEMA chip.

Z stacks of trapped cells were acquired using the green channel (475 nm excitation; 523 nm emission) of the Deltavision Elite fluorescence microscope using a 100x oil objective. No creases or folds in the membrane of any cell were observed in 55 Z-planes with the nominal spacing of 0.2 μm . Fluorescent microscopic images of Z-stacks of a trapped normal RBC in microchannels are shown in Figure 2-5.

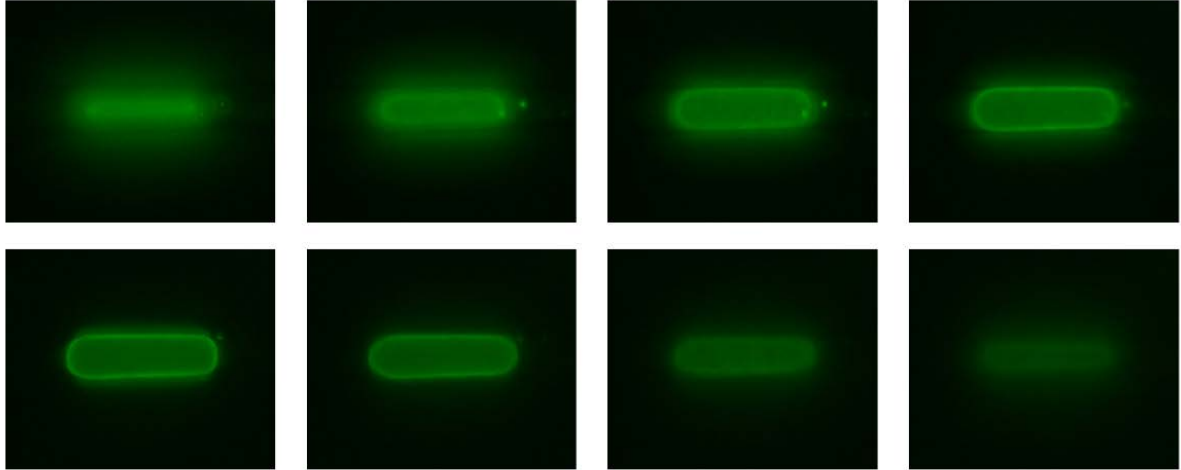


Figure 2-5 Fluorescent microscopic images in a Z-stack of a trapped normal RBC in the microchannels, acquired using the Deltavision Elite fluorescence microscope, indicating unfolded membrane of the trapped cell.

The geometry of an arrested RBC is comprised of the main body and two spherical end caps (Figure 2-6a). To calculate the volume and surface area of each arrested RBC, four points were accurately delineated on each cell: (A) the left point of each cell, (B) the point where the cell touches the wall, (C) the point where the cell is detached from the wall, and (D) the right point of the cell (Figure 2-6b). These points were automatically located in acquired images using a written macro plugin developed in ImageJ. The automated RBCs identifier is described in section 2.3.5. By measuring the distances of each of these four points from the narrow end of microchannels, the mid-point position (d_{mid}), the height of the end caps (h_{lc} and h_{rc}) and the length of the main body (L_b) of each arrested cell can be calculated as follows:

$$d_{\text{mid}} = \frac{d_A + d_D}{2} \quad (2 - 1)$$

$$L_b = d_B - d_C \quad (2 - 2)$$

$$h_{\text{lc}} = (d_A - d_B), \quad h_{\text{rc}} = (d_C - d_D) \quad (2 - 3)$$

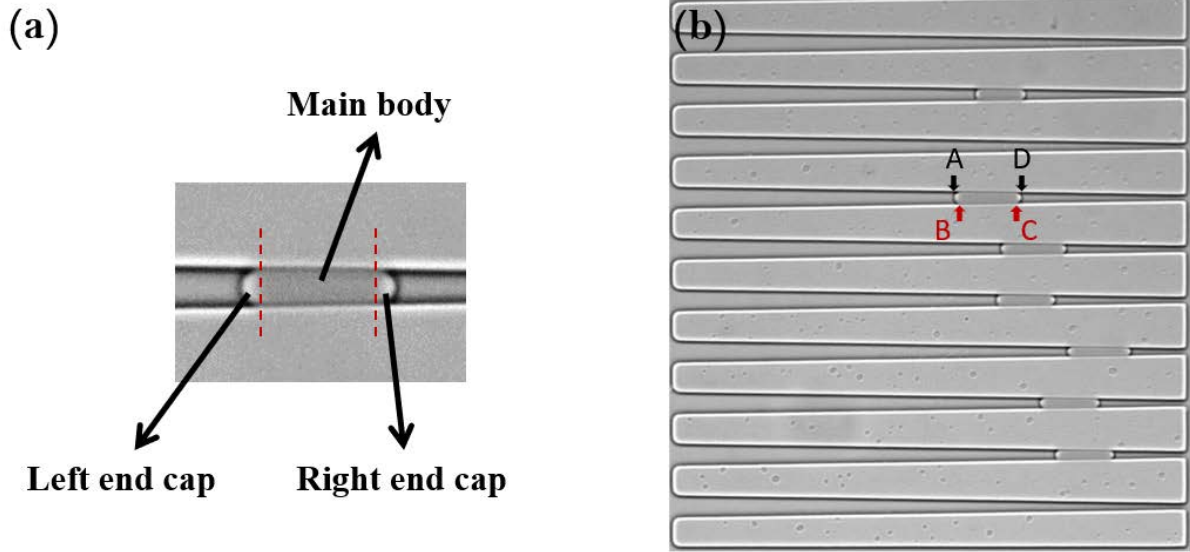


Figure 2-6 Arrested RBCs in wedge-shaped microchannels. (a) each arrested RBC comprised a main body and two spherical end caps. (b) Delineating four points for each arrested RBC to calculate the volume and surface area of individual cells. (A) shows the left point of the cell, (B) represents the point where the cell is attached to the wall, (C) shows the point where the cell is detached from the wall, and (D) represents the right point of the cell.

It was previously reported (Gifford et al., 2003) that arrested cells did not fully fill the rectangular cross-section of the wedge-shaped channels, and some space between the arrested cells and the corners of the microchannels were left unfilled. As a schematic diagram of an arrested RBC in microchannels is shown in Figure 2-7, R_c represents an effective radius of the cell cross-section, simplifying the two caps of the arrested RBC as spherical caps smoothly fitted on the main body of the cell, and R_E is the radius of rounded corners of the cell cross-section.

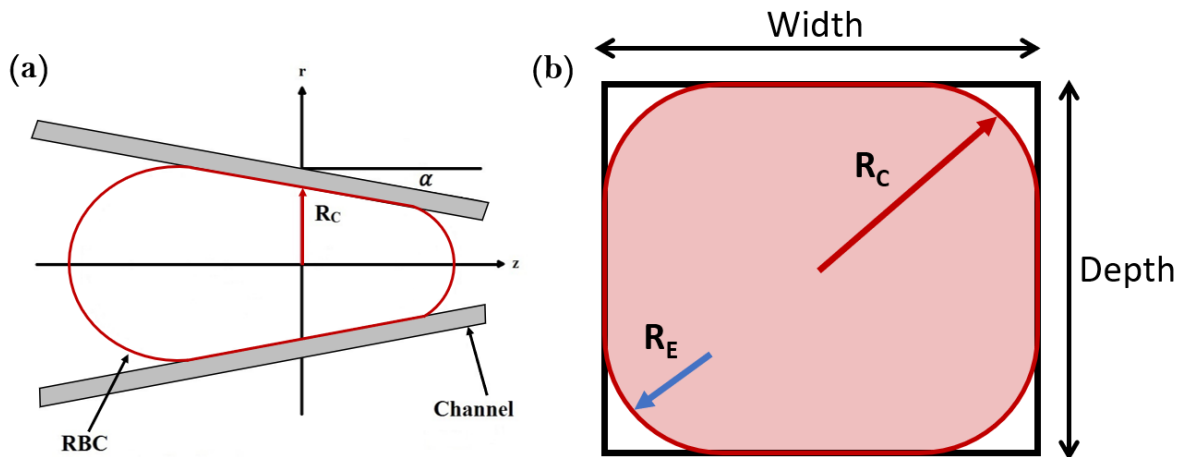


Figure 2-7 (a) The schematic diagram of an arrested RBC in wedge-shaped microchannels. R_c represents an effective radius of the cell cross-section, which is assumed constant all along the cell, and α is the slope of wedge-shaped microchannels. (b) Cross-section of an arrested RBC in microchannels, indicating that the cell does not fully fill the cross-section of microchannels. R_E shows the radius of rounded corners of the cell cross-section.

An empirical relationship between R_E and the cell position in the wedge-shaped channels was previously established (Gifford et al., 2003):

$$R_E = 0.385 \mu\text{m} + 0.008 \times d_{\text{mid}} \quad (2-4)$$

By calculating R_E and the geometry of wedge-shaped microchannels ($\alpha = 0.82^\circ$), R_C can also be calculated using Eq. (2-5)

$$R_C = \sqrt{\left(\frac{\text{Depth}}{2} - R_E\right)^2 + \left(\frac{\text{Width}}{2} - R_E\right)^2} + R_E \quad (2-5)$$

For simplicity, R_C is assumed constant all along the arrested RBC. Then, the area (Area_s) and circumference (Circ_s) of the cell cross-section can be calculated as follows:

$$\text{Area}_s = \text{Width} \times \text{Depth} - 4 \times \left(1 - \frac{\pi}{4}\right) R_E^2 \quad (2-6)$$

$$\text{Circ}_s = 4 \times \left(\frac{2\pi R_E}{4}\right) + 2 \times (\text{Width} - 2R_E) + 2 \times (\text{Depth} - 2R_E) \quad (2-7)$$

Finally, we calculated the volume and surface area of each arrested RBC using Eq. (2-8) and Eq. (2-9).

$$\text{Volume} = \underbrace{(L_b \times \text{Area}_s)}_{\text{Main body}} + \underbrace{\frac{\pi}{6}(3R_C^2 + h_{lc}^2)L_{lc}}_{\text{Left end cap}} + \underbrace{\frac{\pi}{6}(3R_C^2 + h_{rc}^2)L_{rc}}_{\text{Right end cap}} \quad (2-8)$$

$$\text{Surface Area} = \underbrace{(L_b \times \text{Circ}_s)}_{\text{Main body}} + \underbrace{\pi(R_C^2 + h_{lc}^2)}_{\text{Left end cap}} + \underbrace{\pi(R_C^2 + h_{rc}^2)}_{\text{Right end cap}} \quad (2-9)$$

2.3.4 Minimum equivalent diameter

The distance that each RBC travelled into wedge-shaped microchannels before fully stopping is indicative of the ability of RBCs to squeeze into the microchannels. By measuring the position where the cells became lodged, we calculated the minimum equivalent diameter, which represents the smallest diameter of an equivalent circular tube where an RBC can pass through. The minimum equivalent diameter was calculated using Eq. (2-10).

$$\text{Minimum Equivalent Diameter} = 2 \times \sqrt{\frac{\text{Area}_s}{\pi}} \quad (2-10)$$

Where $Area_s$ is the section area of the cell calculated from Eq. (2-6).

2.3.5 Automated RBCs identifier

An automated RBCs identifier was developed to facilitate the analysis of hundreds of cells per experiment and to minimise random measurement errors. Our automated RBCs identifier was written in a custom macro plugin in ImageJ, and the idea was to find suitable threshold values to identify the arrested cells and accurately delineate them in the channels. Images were collected in brightfield along with the blue fluorescence channel (390/18 nm excitation and 435/48 nm collection filter sets and long (2-3 sec) exposure time) to facilitate automated RBCs identification using the shadow of RBC haemoglobin in the fluorescence images. Figure 2-8 displays microscopic images collected from an experiment on malaria-parasite infected RBCs and uninfected cells, indicating the ability of our automated RBCs identifier to detect all types of RBCs, even infected cells with malaria parasites. After delineating the four required points on each arrested RBC, all the calculations for estimating the volume, surface area and minimum equivalent diameter were carried out by importing the measurements into R software. The code script in R and the macro plugin in ImageJ are attached in the Appendix (see Appendix1).

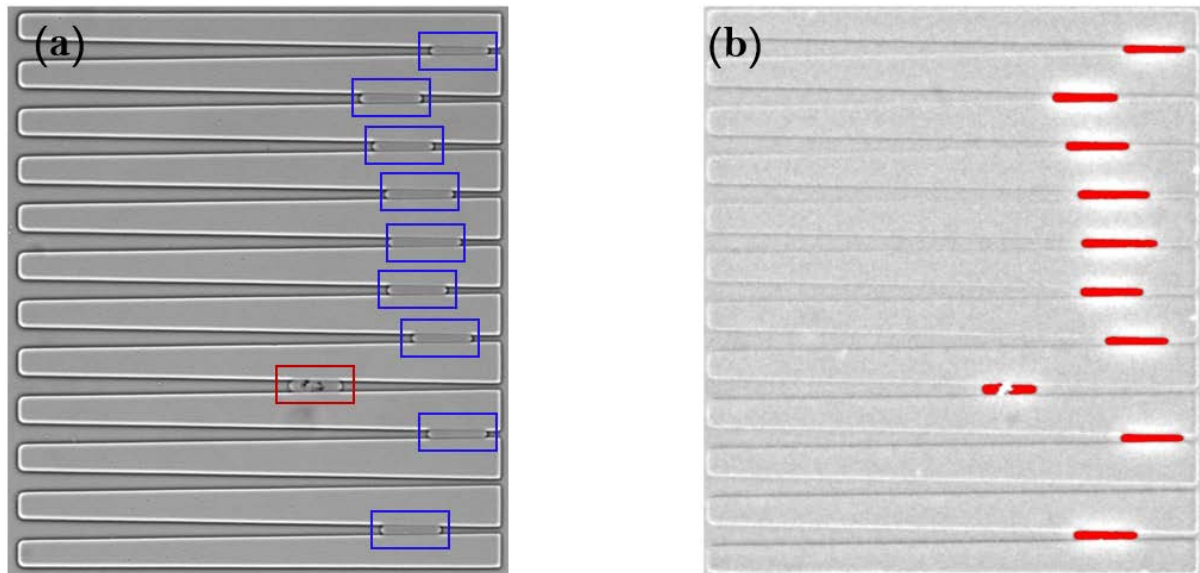


Figure 2-8 Cells identification in microchannels using the automated RBCs identifier. (a) Brightfield microscopy image. uninfected RBCs and malaria-parasite infected cells are marked in blue and red respectively. (b) Fluorescent image, where cells are identified using the shadow of RBC haemoglobin in the blue channel.

To validate our automated RBCs identifier, we compared the automated identification of ~300 normal RBCs with the measurements done manually to locate the cells. The comparison showed that the automated measurements overestimated the volume and surface area of cells

by 3-4% compared with the manual measurements (Figure 2-9a, b). In total, less than 2% differences was measured in SA:V ratio and minimum equivalent diameter of cells between the manual and automated measurements (Figure 2-9c, d). This comparison indicates that our automated RBCs identifier has a high accuracy while significantly saving time by eliminating manual measurements.

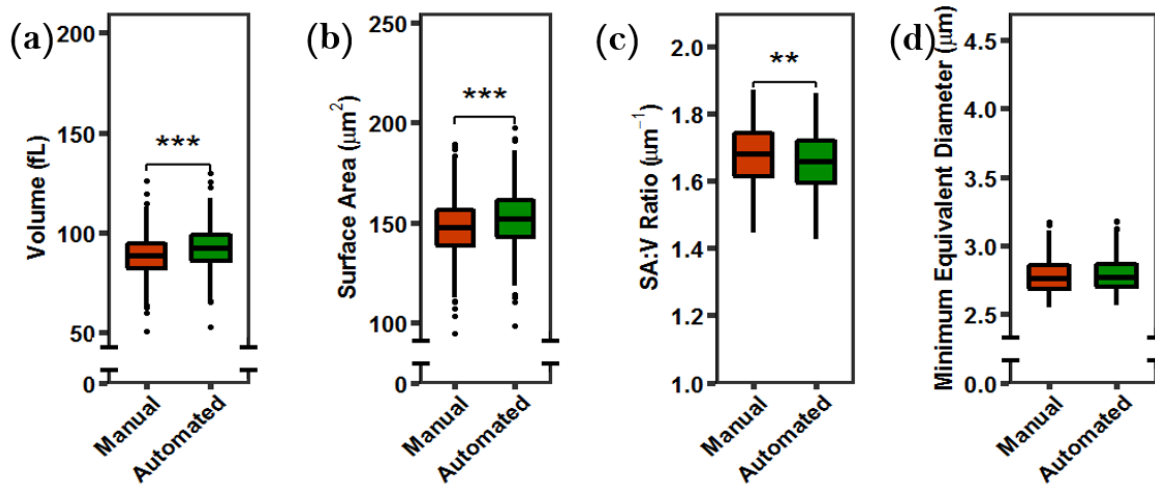


Figure 2-9 Comparison of manual and automated measurements: (a) volume, (b) surface area, (c) SA:V ratio, and (d) minimum equivalent diameter of cells by locating manually (orange) and using our automated RBCs identifier (green).

2.1 Summary

Cellular deformability of RBCs is known as an effective indicator of certain pathological conditions like malaria. We used two microfluidic-based devices to study the rigidity, SA:V ratio and ability of RBCs to traverse narrow microchannels. The ektacytometry analysis was used to examine the cell stiffness of RBCs by measuring their elongation index under shear stress, and a modified version of the human erythrocyte microchannels analyser (HEMA) was used to estimate the volume and surface area of RBCs and evaluate their traversal through narrow microchannels.

Chapter 3

3 Impacts of cell stiffness and surface area to volume ratio on RBC passage through narrow channels

3.1 Introduction

The remarkable deformability of RBCs is primarily derived from cell stiffness and surface area to volume ratio (SA:V ratio) (Chien, 1987; Cooke et al., 2001; Huang et al., 2014; Nash et al., 1989). However, the independent effects of SA:V ratio and cell stiffness on RBC traversal in small constrictions have not been investigated experimentally in sufficient detail. It remains unclear which factor has the greatest impact on RBC passage through small capillaries.

In this chapter, we studied the passage of RBCs through an array of microchannels on a size scale commensurate with the smallest physiological blood vessels and probed the individual contributions of cell stiffness and SA:V ratio to RBC passage. Two series of experiments were conducted where: (i) cell stiffness was modified by chemical treatment, but the cell SA:V ratio was minimally perturbed, and (ii) SA:V ratio was modified by modulating the osmolarity of the media over a range of conditions where cell stiffness changes were low. Our experiments indicate that the SA:V ratio plays a dominant role in passage of RBCs through narrow microchannels.

3.2 Impact of cell stiffness

RBCs were treated with increasing glutaraldehyde concentrations (0.001%-0.005%) for one hour to increase the cell stiffness. Glutaraldehyde has been widely used for chemical fixation

due to its rapid effect (Morel et al., 1971). Glutaraldehyde cross-links amine groups on proteins in the RBC membrane and consequently stiffens the cell membrane and also increases intracellular viscosity by cross-linking haemoglobin in the cytosol. We refer to these changes collectively as an increase in cell stiffness.

3.2.1 Glutaraldehyde treatment

Aliquots of a stock glutaraldehyde solution (2.5% in PBS) were added to 1 mL PBS to obtain the desired concentrations of 0.001, 0.002, 0.003, 0.004 and 0.005%. O-positive (O⁺) human RBCs obtained from the Australian Red Cross Blood Service were gently dispersed into the diluted glutaraldehyde solutions at 2% haematocrit and incubated at room temperature for 1 hr. The cells were subsequently centrifuged at 2500 rev/min for 2 minutes, and the packed cells were resuspended in 1 mL PBS. The suspension of treated RBCs was washed three times. The treated RBCs were then mixed at a 1:1 ratio with untreated RBCs from the same aliquot before introduction to the microfluidic device. The untreated RBCs were kept in PBS during the treatment period and serve as an internal control. Treated cells were identified by their autofluorescence in long-exposure images taken with the red (632 nm excitation; 676 nm emission) filter set of the microscope. An image of the microchannels with arrested cells is shown in Figure 3-1.

3.2.2 Experimental setup

As described in Chapter 2, we used ektacytometry as a surrogate measure of stiffness and used the HEMA chip to measure the SA:V ratio and extent of passage inside microchannels. The HEMA chip was purged with 80% ethanol at a flow rate of 2 μ L/min for 30 minutes. Afterwards, it was flushed with PBS for 45 minutes. The diluted washed blood (at 0.2-0.5% haematocrit), including glutaraldehyde-treated and untreated RBCs, was driven into the HEMA chip at a flow rate of 1 μ L/min using a syringe pump. New microfluidic chips were used for each glutaraldehyde concentration. This introduced a small amount of measurement variability between samples due to the small variation in dimensions from chip to chip. Images were collected in brightfield along with the red fluorescence channel (632 nm excitation; 676 nm emission) at room temperature. The number of analysed cells in each glutaraldehyde experiments using the HEMA device is given in Table 3-1.

Table 3-1 Number of analysed cells in each glutaraldehyde experiments using the HEMA chip

Cell type Glutaraldehyde Concentration	Number of fixed cells	Number of unfixed cells
0.001%	154	320
0.002%	81	314
0.003%	80	386
0.004%	76	279
0.005%	104	257

3.2.3 Results

Ektacytometry analyses showed a gradual decrease in the elongation index (EI) for fixed cells as the glutaraldehyde concentration increased from 0.001% to 0.005% (Figure 3-1a). At the shear stress of 3 Pa, the EI reduced by more than eight times in the cells treated with the highest concentration of glutaraldehyde (0.005%), demonstrating a dramatic increase in the cell stiffness.

In the HEMA experiment, fixed cells and unfixed cells were mixed in phosphate-buffered saline (PBS) at a ratio of 1:1 and introduced into the microfluidic chip. An image of arrested cells in the microchannels is shown in Figure 3-1b, where the cells treated with 0.005% glutaraldehyde were detected in the red fluorescence channel while untreated cells were unlabelled. No significant change in volume or surface area was observed between fixed and unfixed cells at glutaraldehyde concentrations of 0.001- 0.004% (Figure 3-1c, d). However, the surface area of the cells treated with 0.005% glutaraldehyde was ~4% lower than the surface area in unfixed cells (Figure 3-1d). This slight difference is likely due to the formation of creases in the membrane (0.005% glutaraldehyde) of fixed cells when they became trapped in microchannels (see Figure 3-1b). Thus, the surface area and SA:V ratio were likely underestimated for this sample (Figure 3-1d, e). No creases were observed in the membrane of cells fixed with lower glutaraldehyde concentrations or for unfixed cells. In general, the SA:V ratio of cells remained unchanged during fixation by glutaraldehyde (Figure 3-1e).

Our results showed that fixed cells treated by glutaraldehyde (concentrations from 0.001%-0.004%) were able to pass into microchannels to the same equivalent diameter as unfixed cells. Only cells fixed at 0.005% glutaraldehyde (with ~8-fold less EI) were trapped earlier, being trapped at a ~6% greater equivalent diameter compared to unfixed cells (p value < 0.0001, Welch two sample t-test) (Fig 1d). The mean value and standard deviation of measured parameters are provided in Table 3-2. We repeated the fixation experiment and found results consistent in all three repetitions (see Appendix2).

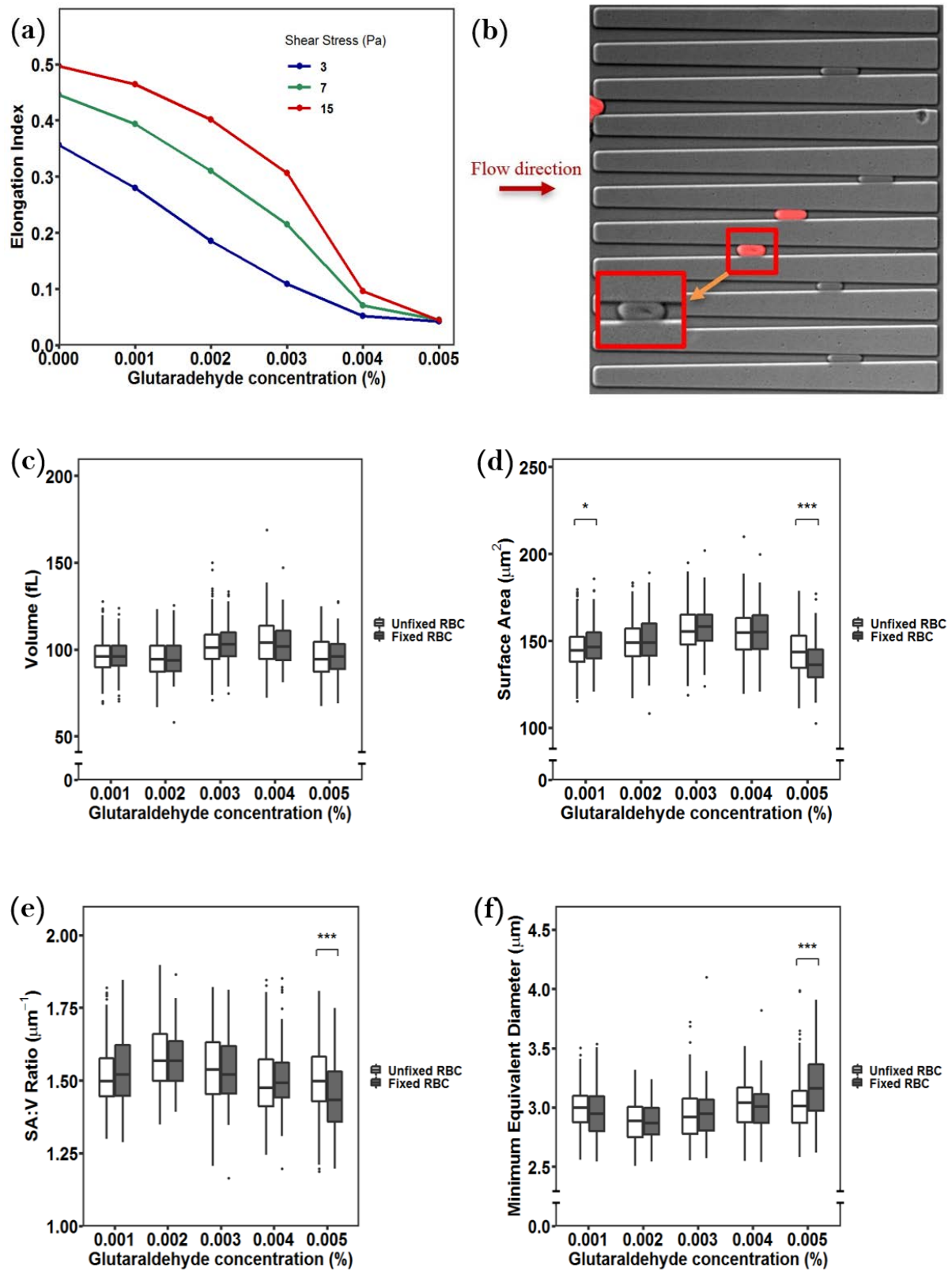


Figure 3-1 Rigidity and physical dimensions of unfixed cells and fixed cells treated with glutaraldehyde. (a) Comparison of the elongation index (EI) for the cells treated by various glutaraldehyde concentrations (0.001%-0.005%) at shear stresses of 3, 7 and 15 Pa. (b) An image of the wedge-shaped microchannels in the HEMA chip with arrested unfixed cells (no stain) and fixed cells treated by 0.005% glutaraldehyde (red stain) with one cell showing a crease (inset). (c-f) Mean values of volume, surface area, SA:V ratio and minimum equivalent diameter for unfixed cells (white) and fixed cells (grey).

Table 3-2 Mean values and standard deviation (SD) of volume, surface area, SA:V ratio and minimum equivalent diameter for fixed and unfixed RBCs.

Glutaraldehyde concentration Parameter (Mean±SD)	0.0001% (Control)	0.0002% (Control)	0.0003% (Control)	0.0004% (Control)	0.0005% (Control)
Volume	97±10 (96±10)	95±13 (95±11)	103±12 (101±11)	103±13 (104±13)	96±12 (95±11)
Surface area	148±13 (145±12)	150±16 (149±12)	157±14 (156±13)	155±14 (155±14)	136±14 (143±14)
SA:V ratio	1.54±0.11 (1.52±0.10)	1.58±0.12 (1.58±0.11)	1.53±0.11 (1.54±0.12)	1.51±0.13 (1.50±0.12)	1.44±0.13 (1.51±0.11)
Minimum equivalent diameter	2.96±0.20 (2.98±0.18)	2.88±0.16 (2.88±0.17)	2.95±0.21 (2.93±0.20)	3.00±0.23 (3.02±0.21)	3.19±0.31 (3.02±0.22)

3.3 Impact of SA:V ratio

We investigated the impact of SA:V ratio on the ability of RBCs to enter small channels by altering the osmolarity of the buffer medium from 139-484 mOsm/L and changing the cell volume. The RBC volume changes in various osmolarity solutions due to diffusion of water across the cell membrane in response to an imbalance of solutes inside the cell versus outside the cell.

3.3.1 Altering medium osmolarity

The PBS buffer osmolarity was tuned over the range 139-484 mOsm/L by adjusting the NaCl content of the medium while keeping the Na₂HPO₄, KH₂PO₄ and KCl concentrations constant at 10, 1.8 and 2.7 mM respectively. The pH was adjusted to 7.35 with small volumes of 2M HCl for individual buffer solutions before measurement of osmolarity using an Advanced Instruments 3320 freezing-point osmometer. This work was kindly performed by Dr Adam Blanch. RBCs were washed in 1X PBS once before being redispersed in osmolarity-modified PBS at 2% haematocrit. RBC suspensions were then further diluted with modified PBS before introduction to the microfluidic device.

3.3.2 Experimental setup

Similar to the glutaraldehyde experiments, ektacytometry and the HEMA chip were used to study behaviour of RBCs in various osmolarity solutions. The HEMA chip was flushed with PBS with the buffer osmolarity of 239 mOsm/L; then, the diluted washed blood (at 0.2-0.5% haematocrit) was driven into the HEMA chip at a flow pressure of 250 mbar using the pressure pump. The same microchip was used in all experiments (239–350 mOsm/L) for a reliable comparison between samples. After each experiment, the chip was flushed with the next osmolarity medium for 30 min before introducing the cells. Images were collected in brightfield along with the blue fluorescence channel at room temperature. Approximately 400 cells were measured for each osmolarity-modified buffer.

3.3.3 Results

The EI as a function of buffer osmolarity is shown in Figure 3-2a. The EI decreased as the buffer osmolarity was adjusted away from the physiological range (~ 275 - 295 mOsm/L). The EI was significantly lower for both hypotonic and hypertonic solutions, in good agreement with previous research (Clark et al., 1983). To study the impact of SA:V ratio on RBCs passage into microchannels, we used the HEMA device over the range of osmolarity from 239–350 mOsm/L where differences in cellular stiffness are minimal according to the ektacytometry results (Figure 3-2a). To avoid errors introduced by small variations in geometry from device to device all five experiments for this range of medium osmolarity were conducted in one single HEMA chip, starting from the medium osmolarity of 239 mOsm/L.

Our results showed a gradual increase in cell volume as the buffer osmolarity decreased (Figure 3-2d), in good agreement with previous reports (Park et al., 2011; Reinhart et al., 2015). A slight decrease in cell surface area was also observed with reduction in the buffer osmolarity (Figure 3-2e). A consequence of these changes is a moderate decrease in SA:V ratio (Figure 3-2f). A steady decline in minimum equivalent diameter was observed as the osmolarity increased (Figure 3-2g). For instance, a $\sim 13\%$ decrease in SA:V ratio reduced RBCs traversal in the microchannels by $\sim 14\%$. The mean value and standard deviation of measured parameters are provided in Table 3-3. Data from a repeat HEMA experiment over a broader range 215-387 mOsm/L are presented in the Appendix (see Appendix3). The results are in good agreement with the data shown here.

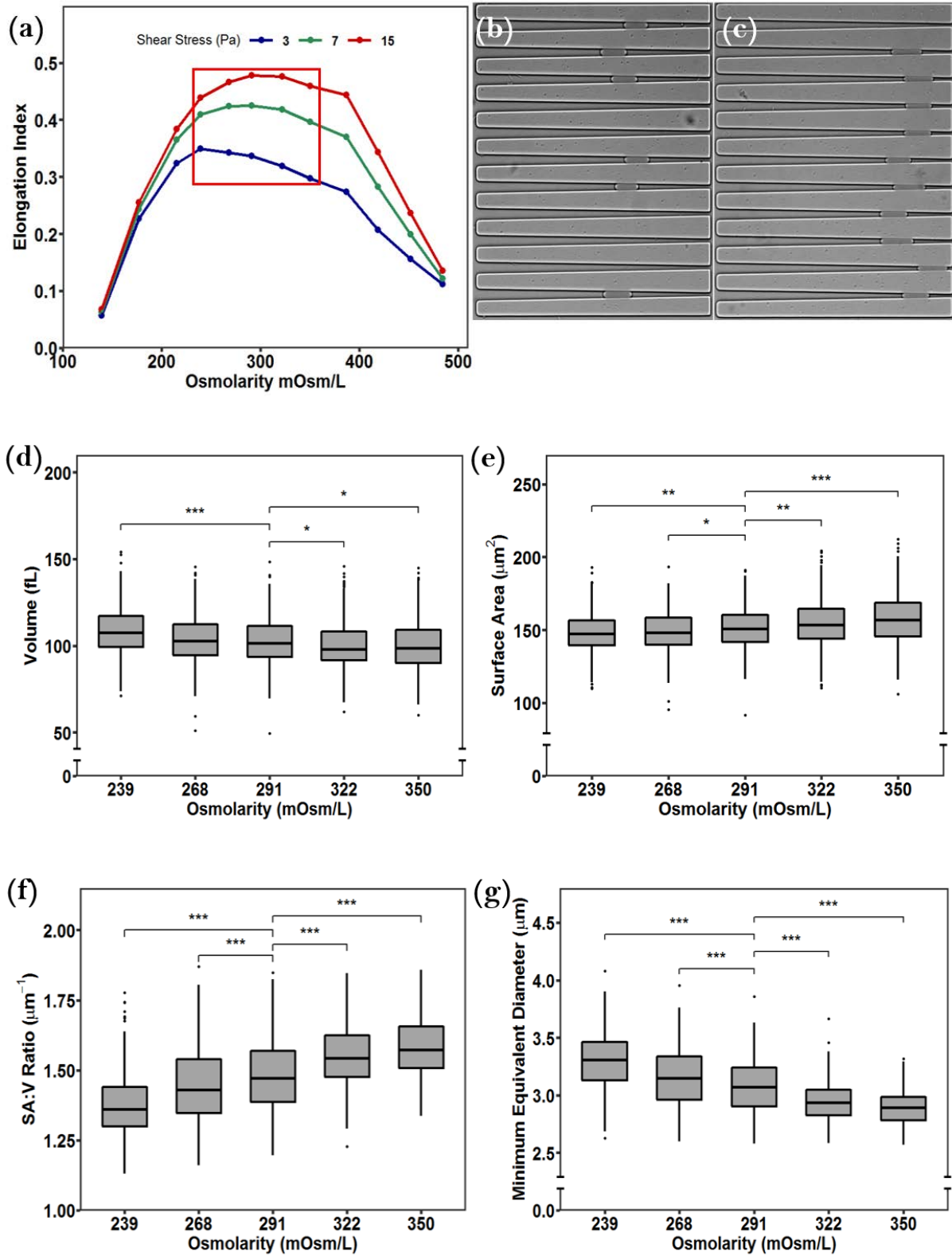


Figure 3-2 Rigidity and physical dimensions of RBCs subjected to different buffer osmolarities. (a) Elongation index as a function of the medium osmolarity at shear stresses of 3, 7 and 15 Pa. (b-c) Images of the wedge-shaped microchannels with arrested RBCs in the media with osmolarities of 239 mOsm/L (b) and 350 mOsm/L (c). (d-g) Mean values of volume, surface area, SA:V ratio and minimum equivalent diameter for cells subjected to different buffer osmolarities.

Table 3-3 Mean values and standard deviation (SD) of volume, surface area, SA:V ratio and minimum equivalent diameter for RBCs subjected to different buffer osmolarities.

Buffer Osmolarity Parameter (Mean±SD)	239 mOsm/L	268 mOsm/L	294 mOsm/L	322 mOsm/L	350 mOsm/L
Volume	108±14	104±14	103±14	100±14	100±14
Surface area	148±14	149±14	151±15	154±17	158±17
SA:V ratio	1.38±0.11	1.45±0.13	1.48±0.12	1.55±0.10	1.58±0.10
Minimum equivalent diameter	3.29±0.24	3.15±0.24	3.07±0.22	2.94±0.16	2.89±0.15

We also studied the rigidity and physical characteristics of normal RBCs in different carrier media (PBS, RPMI 1640 medium, and complete culture media (CCM)) and found that RBCs exhibit different SA:V ratios depending on the dispersing media (see Appendix4). Our results show that even though the ability of RBCs to elongate in fluid flow remained unchanged in these media, the SA:V ratio of RBCs in RPMI and CCM increased in comparison with PBS. Therefore, RBCs in RPMI and CCM were able to traverse into smaller minimum equivalent diameters in the HEMA chip. These results also confirm that the ability of RBCs to passage small capillaries is highly dependent on the RBC SA:V ratio.

3.4 Discussion

We used two microfluidic-based techniques to study the effects of SA:V ratio and cell stiffness. Ektacytometry was used to measure the elongation index of cells, an effective surrogate measure of average cell stiffness in a large population of RBCs. We used the HEMA device to entrap RBCs and measure their ability to squeeze into wedge-shaped microchannels. This study is the first detailed investigation of the independent impacts of cell stiffness and SA:V ratio on RBC passage through microchannels.

The ektacytometry analyses showed that treating RBCs with 0.005% glutaraldehyde significantly increased their stiffness (~800%). Surprisingly, the RBCs treated with 0.005% glutaraldehyde exhibited to traverse an equivalent diameter that was only 6% larger than untreated cells, while fixed cells treated with lower concentrations (0.001%-0.004%) reached

the same equivalent diameter as untreated cells. A slight variation was observed in the results across glutaraldehyde experiments, which emanated from the small variation in dimensions from chip to chip. Nonetheless, the important purpose of this study was to compare the characteristics of glutaraldehyde treated RBCs with untreated cells. Our results show that treated glutaraldehyde-treated RBCs and untreated cells traversed into minimum equivalent diameters depending more on their SA:V ratio rather than cell stiffness. These results are consistent with a previous study that reported that the ability of treated RBCs to pass through micropore filtration assay was only affected at high concentrations of glutaraldehyde (>0.04%) (Sosa et al., 2014). However, it was reported that the ability of treated RBCs to perfuse to an artificial microvascular network decreased moderately with increasing concentration of glutaraldehyde from 0.02% to 0.08% (Shevkoplyas et al., 2006; Sosa et al., 2014). But, in these studies, RBCs were treated with higher glutaraldehyde concentrations than those we used to fix RBCs in our experiments.

Previous studies have using experimental techniques like optical tweezers and micropipette aspiration to study RBC physical properties, and showed that cell stiffness is an important determinant of the behaviour of RBCs as measured by these techniques (Nash et al., 1989; Park et al., 2008; Suresh et al., 2005). By contrast, our results indicate that changes to the overall cell stiffness have little effect on RBCs traversal into the microchannels, consistent with a previous study that demonstrated that increased membrane rigidity is not the main cause of splenic clearance of diamide-treated RBCs (Safeukui et al., 2018). We explain in chapter 5 why increasing the membrane shear modulus would have little effect on RBC traversal in narrow capillaries.

In all glutaraldehyde experiments, we found a smaller number of fixed cells trapped in microchannels, although the fixed and unfixed cells were mixed at a ratio of 1:1, and evidence for lysed cell membranes was observed in the collected fluorescence images. This suggests that glutaraldehyde-treated cells are more fragile and more prone to rupture upon deformation in the wedge-shaped microchannels.

Next, we altered the SA:V ratio of RBCs by modifying the buffer osmolarity. Our experiments revealed that the increase in SA:V ratio of RBCs was associated with a decrease in the minimum equivalent diameter from $\sim 3.3 \mu\text{m}$ to $\sim 2.9 \mu\text{m}$, while the cellular stiffness changed only minimally. Overall, it was observed that RBCs reached smaller minimum equivalent diameters as the buffer osmolarity increased, due to the increases in their SA:V ratio. By

contrast, ektacytometry analysis showed that the ability of RBCs to elongate in fluid flow was not greatly affected in the range (~ 239 - 350 mOsm/L) but decreased as the buffer osmolarity moved away from that range.

A previous study showed that deviations in osmolarity (± 100 mOsm/L) minimally changed the perfusion rate of RBCs in an artificial microvascular network with the minimum diameter of $5\text{ }\mu\text{m}$ (Reinhart et al., 2015). However, based on our experiments, capillaries with diameter of $5\text{ }\mu\text{m}$ are large enough even for swollen RBCs in low osmolarity solutions (e.g. 239 mOsm/L) to squeeze through efficiently. Nonetheless, comparing to the impact of cell stiffness, it can be concluded from this experiment that the ability of RBCs to conform into microchannels is highly sensitive to their SA:V ratio. Our findings are consistent with previous reports suggesting that the high SA:V ratio of RBCs facilitates undergoing large deformations (Antia et al., 2008; Gifford et al., 2003; Herricks et al., 2009; Reinhart and Chien, 1985; Safeukui et al., 2012).

We also studied characteristics of normal RBCs trapped using different carrier media (PBS, RPMI 1640, and CCM), and the comparison revealed that RBCs exhibited slightly different behaviours depending on the nature of the dispersing medium (see Appendix4). Indeed, RBCs in RPMI or CCM reached smaller equivalent diameter ($\sim 11\%$) in wedge-shaped channels than those in PBS, due to a $\sim 12\%$ higher SA:V ratio, while the EI of RBCs remained unchanged in these media. This finding indicates that a component or a combination of components comprising RPMI acts to decrease the volume of RBCs slightly and also, confirms that RBC's ability to squeeze into microchannels is sensitively dependent on the cell SA:V ratio.

Taken together, these experimental findings imply that the SA:V ratio is by far the dominant factor affecting RBC traversal in microchannels, consistent with some previous studies (Herricks et al., 2009; Murdock et al., 2000; Safeukui et al., 2012; Safeukui et al., 2013).

3.5 Conclusion

RBCs need to undergo large deformations when they squeeze through small constrictions like capillaries in the body. It has been thought that the RBC passage in small capillaries and inter-endothelial slits depends on both cellular stiffness and SA:V ratio of the cell. In this chapter, two fundamental studies were conducted using microfluidic-based techniques to identify the independent impacts of cell stiffness and SA:V ratio on RBCs ability to traverse microchannels: (i) cell stiffness was varied using glutaraldehyde treatment, and (ii) SA:V ratio was varied by

altering the osmolarity of external media. Our experimental results show that the RBC passage into narrow microchannels is highly sensitive to the SA:V ratio of the cell. On the contrary, a dramatic increase in cell stiffness has comparatively little effect on RBCs passage through microchannels. In following chapter, we investigate the implications of our findings in different physiological and pathological conditions.

Chapter 4

4 Impacts of SA:V ratio and cell stiffness in RBC pathology

4.1 Introduction

In the previous chapter, I described the application of chemical treatments with known impacts on SA:V and cell stiffness to investigate the impact of these physical properties on RBC traversal through small capillaries. In this chapter, I describe work designed to probe the impact of changes to SA:V and cell stiffness that occur in a variety of physiological and pathological conditions.

RBCs undergo changes in deformability, morphology and mechanical properties in a variety of contexts including RBC maturation, malaria infection and genetic mutations. For example, immature RBCs, called reticulocytes are released from the bone marrow into the bloodstream with a higher membrane stiffness and ~20% higher surface area than mature RBCs (Liu et al., 2010; Malleret et al., 2015; Malleret et al., 2013). RBCs adopt the characteristic biconcave shape after about one day of circulation through the vascular system, following a process of maturation. It remains unclear whether the sub-optimal mechanical properties of reticulocytes affect passage through small capillaries.

RBCs are also known to undergo changes to their morphology and deformability in certain disease states. Pathologies such as malaria are associated with changes to RBC molecular organisation and ultrastructure, which affect cellular deformability. A pathological consequence of two different species of malaria parasite, *Plasmodium falciparum* (*P. falciparum*) and *Plasmodium knowlesi* (*P. knowlesi*) is the occlusion of small blood vessels

especially within cerebral capillaries containing trapped or sequestered infected RBCs (iRBCs) (Dondorp et al., 2000; Miller et al., 1971). Both the increased rigidity of iRBCs and the presence of adhesive knobs on the outer surface of iRBCs are thought to contribute to the impaired microcirculatory blood flow (Dondorp et al., 2000; MacPherson et al., 1985; Miller et al., 1971) (Barnwell, 1989; Rug et al., 2006). The individual contributions of altered cell stiffness and morphology of iRBCs to the occlusion of small capillaries have not been well studied.

Some RBC abnormalities result in decreased efficiency of malaria parasite invasion, which can protect against infection (Chishti et al., 1996; Leffler et al., 2017; Rank et al., 2009; Schulman et al., 1990). Mutations in β spectrin (*Sptb*) are thought to enhance the clearance of mutant mouse RBCs in the spleen by compromising their ability to survive splenic filtration (Lelliott et al., 2017). It is poorly understood whether *Sptb* mutation affects SA:V ratio of RBCs and if this would restrict passage into small channels.

We provided evidence, detailed in the previous chapter, that SA:V ratio is the more important determinant of RBCs traversal in microchannels. In this chapter, we continue our investigation of the relative contributions of cell stiffness and SA:V ratio to RBC traversal in narrow microchannels by studying (i) reticulocytes, (ii) RBCs infected with two human *Plasmodium* species, *P. falciparum* and *P. knowlesi*, and (iii) mutant mouse RBCs. We employed ektacytometric analysis and HEMA microchip analysis, as described in Chapter 2. We probed whether the stiffness changes that have been reported previously are sufficient to limit RBCs passage into small constrictions and found that SA:V ratio is still the critical factor that determines their traversal through narrow channels. Our experimental analyses showed that reticulocytes exhibit similar ability to traverse through narrow microchannels to mature RBC, despite having more rigid membranes. *P. falciparum* trophozoite-infected RBCs traversed into a similar equivalent diameter as uninfected RBC, whereas, the passage of *P. knowlesi* trophozoite-infected RBCs was compromised due to their lower SA:V ratio.

4.2 Cellular deformability of RBCs during reticulocyte maturation

During maturation of reticulocytes, about 20% of the plasma membrane is lost by a process of vesiculation, taking with it some markers such as the transferrin receptor (CD71) (Blanc and Vidal, 2010). The reticulocyte membrane is known to be more rigid than that of mature RBCs, with a measured shear modulus almost twice that for mature cells (Malleret et al., 2013). In this study, we studied two stages of reticulocytes (i) CD71⁺ (immature) reticulocytes and (ii)

CD71⁻ (older) reticulocytes and examined whether the higher membrane stiffness of reticulocytes restricts their passage into narrow microchannels.

4.2.1 Reticulocyte purification

Reticulocytes from cord blood were purified by centrifugation on a Percoll cushion and compared directly to mature cells from the pellet. A population of mature RBCs (i.e. no longer containing RNA) remained in the reticulocyte fraction at a percentage which varies with preparation, as quantified via flow cytometry with an RNA stain (Thiazole Orange). The reticulocyte preparation work was all performed in the laboratory of Dr Waihong Tham.

4.2.2 Experimental setup

Immature reticulocytes (CD71⁺) were labelled using anti-CD71, which recognises the transferrin receptor (TfR), while mature RBCs were labelled using an antiserum recognising complement receptor (CR1). A mixture of CD71⁺ (green fluorescence) and CD71⁻ (no label) reticulocytes was mixed with mature RBCs (red fluorescence) before introduction into the HEMA device, in PBS at a flow rate of 1 μ L/min. Images were collected in brightfield mode at room temperature along with the green fluorescence channel (475 nm excitation; 523 nm emission) and red fluorescence channel (632 nm excitation; 676 nm emission) to detect immature reticulocytes CD71⁺ and mature RBCs. The blue fluorescence channel was used to facilitate automated RBCs identification. Ektacytometry experiments were kindly conducted by Dr Adam Blanch.

4.2.3 Results

The EI of immature CD71⁺ reticulocytes-enriched blood (49-80% RNA positive) was ~13% lower than that of mature RBCs at shear stress of 3 Pa (Figure 4-1a), confirming that the reticulocytes are more rigid than mature RBCs. These data are in good agreement with that reported previously, based on micropipette aspiration (Malleret et al., 2013).

The HEMA analysis showed that the volume and surface area of reticulocytes decreased during the process of maturation. The mean volume of immature CD71⁺-reticulocytes was 130 fL compared to 115 fL for the older CD71⁻ reticulocytes and 102 fL for mature RBCs. The surface area also decreased by 12% and 21% as immature reticulocytes matured to older reticulocytes and mature RBCs (Figure 4-1c, d). Importantly, no significant difference was observed in SA:V ratio between reticulocytes and mature erythrocytes. Accordingly, both reticulocytes (CD71⁺ and CD71⁻) and mature RBC passaged into the HEMA chip to reach the same equivalent diameter, 3.1 μ m (p value =0.79, 0.44) (Figure 4-1e, f). The mean value and standard deviation

of measured parameters are provided in Table 4-1. This result was consistent across three repeat experiments (see Appendix5).

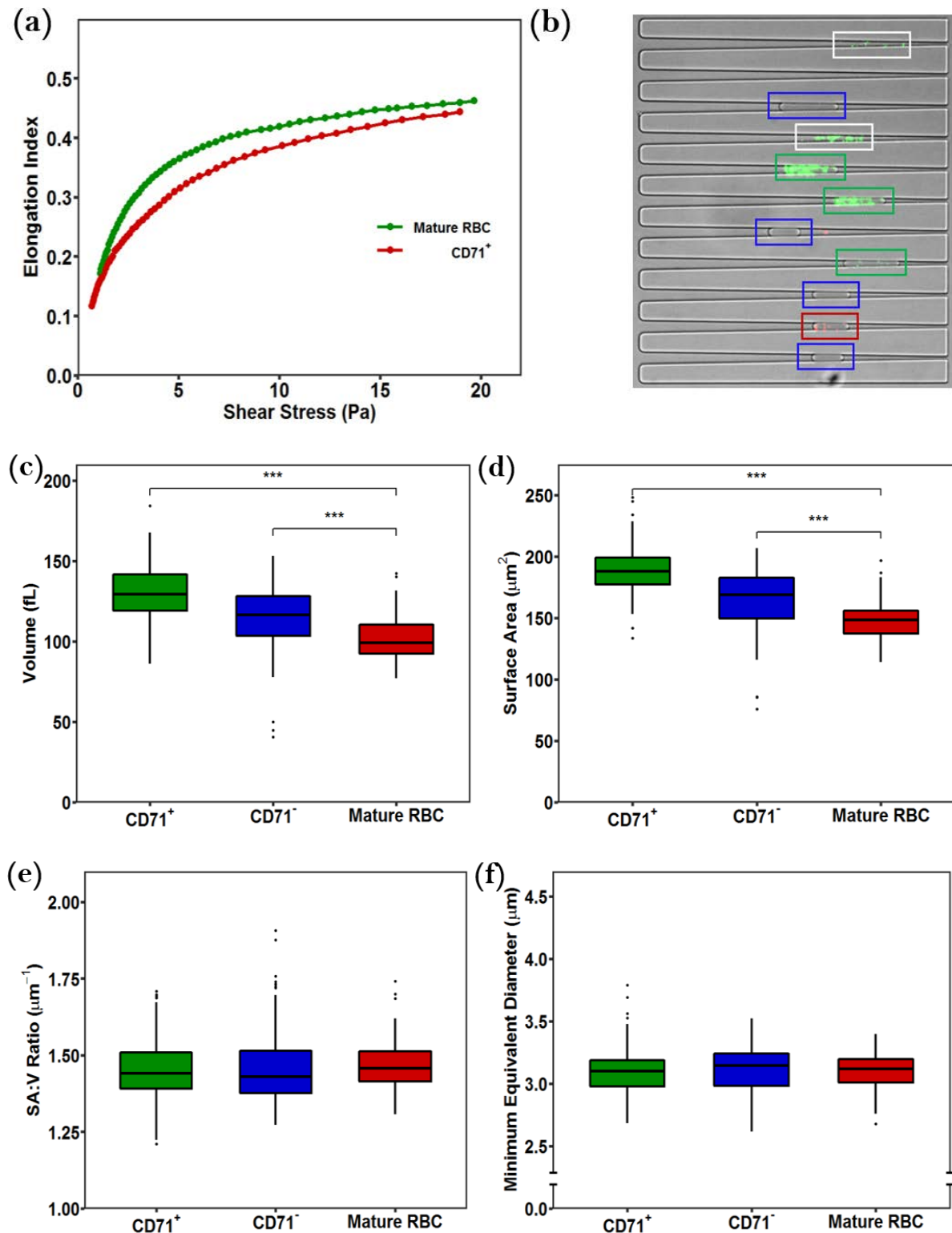


Figure 4-1 Rigidity, SA:V ratio and cellular rigidity of reticulocytes. (a) Ektacytometry analysis (0-20 Pa shear stress) for mature RBCs (red line) and purified cord blood reticulocytes (80% RNA+ve, 59% TfR +ve; green line). (b) Mature RBCs were labelled for CR1 (647, marked in red) and immature reticulocytes CD71⁺ (488, marked in green). Unlabelled cells are assumed to be older reticulocytes CD71⁻ (marked in blue) but may also include any unlabelled cells from the mature pellet. Some lysed cell membranes are visible in the channels (marked in white). Trapping in the HEMA device allows comparison

of mean values of volume (c), surface area (d), SA:V ratio (e) and minimum equivalent diameter (f) for mature (69 cells, red), older reticulocytes CD71⁻ (TfR⁻, 168 cells, blue) and immature reticulocytes CD71⁺ (TfR⁺, 155 cells, green) cells.

Table 4-1 Mean values and standard deviation (SD) of volume, surface area, SA:V ratio and minimum equivalent diameter for immature reticulocytes CD71⁺, older reticulocytes CD71⁻ and mature RBCs.

Cell type Parameter (Mean±SD)	Reticulocyte CD71 ⁺	Reticulocyte CD71 ⁻	Mature RBC
Volume	130±16	115±19	102±13
Surface area	189±19	166±23	149±17
SA:V ratio	1.45±0.10	1.45±0.12	1.47±0.09
Minimum equivalent diameter	3.09±0.18	3.12±0.20	3.10±0.15

4.3 Traversal of malaria parasite-infected RBCs into microchannels

Both *P. falciparum* and *P. knowlesi* cause disease in humans, and are associated with rheo-pathological consequences, including increased stiffness of the infected RBC membranes (Park et al., 2008) (Suresh et al., 2005) (Glenister et al., 2002). We measured the ability of RBSc infected with these two *Plasmodium* species to traverse into HEMA chip microchannels and measured surface area, volume and the SA:V ratio to determine whether changes in these parameters or in cell stiffness affect their passage.

4.3.1 Culture of *P. falciparum* and *P. knowlesi*

Parasites were cultured in O+ human RBCs (5% haematocrit) in CCM containing AlbuMAX™ II (5%), 10 mM D - glucose (Sigma), pooled human serum (5%), 200 µM hypoxanthine and 20 µg/ml gentamicin (Sigma). Parasitemia was kept below 5%. Magnetic separation was used to enrich mature stage infected RBCs from culture. This work was kindly performed by Ms Olivia Carmo, Mr Dean Andrew, Dr Snigdha Tiash and Dr Oliver Looker.

Preliminary experiments showed that the presence of bovine serum albumin (BSA) in the carrier medium does not alter the results of this experiment by, for example, changing the mechanics of the cells' interaction with the microchannel walls (see Appendix4).

4.3.2 Experimental setup

RBCs infected with *P. falciparum* (3D7-line) trophozoites (32-36 h) or *P. knowlesi* trophozoites (22-26 h) were magnet purified and washed in complete culture media (parasitemia > 80%). After flushing the HEMA chip with CCM, the diluted blood was pumped into the channels at a flow pressure of 180 mbar and a temperature of 37 °C. Images were collected in brightfield along with the blue fluorescence channel.

For ektacytometry analysis, infected samples at the same trophozoite stage and at a parasitemia > 97% were thoroughly mixed with 6% polyvinylpyrrolidone (PVP) in PBS. This work was kindly performed by Dr Adam Blanch.

4.3.3 Results

Ektacytometry analysis (Figure 4-2a, Figure 4-3a) showed that both malaria parasite species substantially compromise the ability of their host RBCs to extend in conditions of fluid flow. The EI decreased four-fold for *P. falciparum* and three-fold for *P. knowlesi* iRBCs compared to uninfected RBCs (uRBCs) at a shear stress of 3 Pa. Thus, *P. knowlesi* had a more moderate effect than *P. falciparum* on the ability of the host RBC to elongate.

Infected samples at a high initial parasitemia (> 80%) were introduced into the HEMA microchip. In all experiments, however, a smaller number of infected RBCs was found in the microchannels in comparison with the number of uninfected RBCs. Using the HEMA microfluidic chip, we observed no difference in the mean values of volume and surface area of *P. falciparum* trophozoite-infected RBCs as compared to uRBCs (Figure 4-2c-d), with the SA:V ratio remaining unchanged. Despite the extremely high cell stiffness of *P. falciparum* trophozoite-infected RBCs, which has a contribution from the presence of a rigid parasite inside the host cell, they were able to traverse to the same minimum equivalent diameter as uninfected RBCs (p value = 0.18) (Figure 4-2e-f). The mean values and standard deviation of measured parameters for infected and uninfected RBCs are provided in Table 4-2.

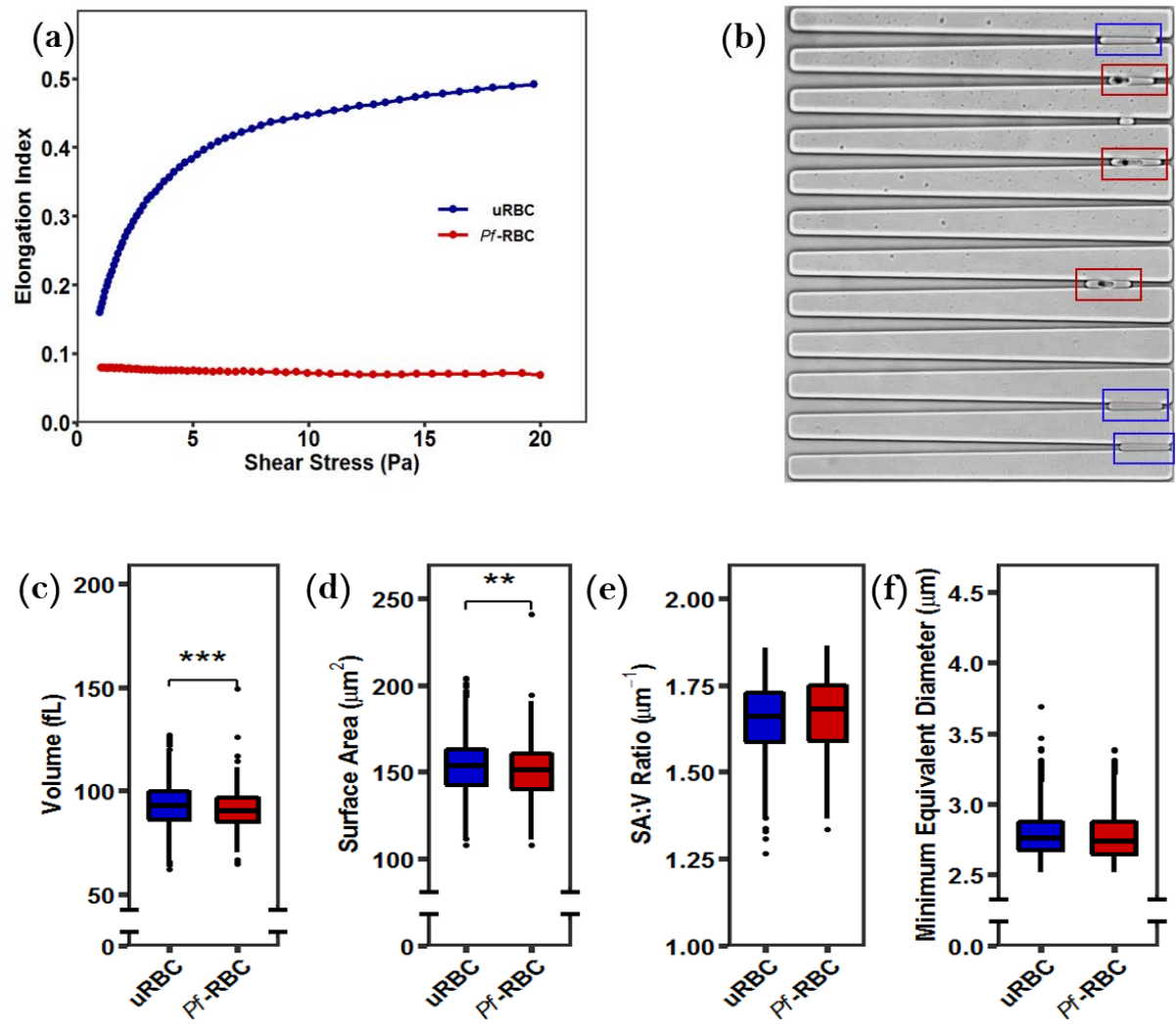


Figure 4-2 Physical parameters for *P. falciparum* 3D7 iRBCs (32-36 h). (a) Ektacytometry analysis (0-20 Pa shear stress) for uRBCs (blue line) and *P. falciparum* iRBCs (red line). (b) An image of trapped uninfected RBCs and *P. falciparum* trophozoite-infected RBCs in the wedge-shaped microchannels, uninfected RBCs and infected RBCs are marked in blue and red, respectively. (c-f) Comparison of mean values of volume, surface area, SA:V ratio and minimum equivalent diameter in uninfected RBCs (1038 cells, blue) and iRBCs (529 cells, red).

Table 4-2 Mean values and standard deviation (SD) of volume, surface area, SA:V ratio and minimum equivalent diameter for *P. falciparum*-infected and uninfected RBCs.

Cell type Parameter (Mean±SD)	<i>P. falciparum</i> - infected RBC	Uninfected RBC
Volume	91±9	93±10
Surface area	151±16	153±15
SA:V ratio	1.66±0.11	1.65±0.10
Minimum equivalent diameter	2.78±0.17	2.79±0.15

We also studied another strain of *P. falciparum* (CS2) and found results similar to those for the 3D7 strain. That is, RBCs infected with trophozoites of *P. falciparum* CS2 (30-40 h) maintained the same SA:V ratio and reached the same equivalent diameter as uninfected RBCs (see Appendix6).

In contrast to *P. falciparum*, RBCs infected with *P. knowlesi* trophozoites exhibited increased volume and surface area increased (~23% and ~12%, respectively) as compared to uninfected RBCs (Figure 4-3c-d). The SA:V ratio also was increased by ~9% which resulted in *P. knowlesi*-infected RBCs being arrested earlier in the microchannels than uninfected cells (~8% increase in the minimum equivalent diameter) (p value < 0.0001) as shown in Figure 4-3e-f. The mean value and standard deviation of measured parameters for *P. knowlesi*-infected and uninfected RBCs are provided in Table 4-3. Data from a repeat of this study on mature stage *P. knowlesi*-infected RBCs (22-32 h) was published (Liu et al., 2019).

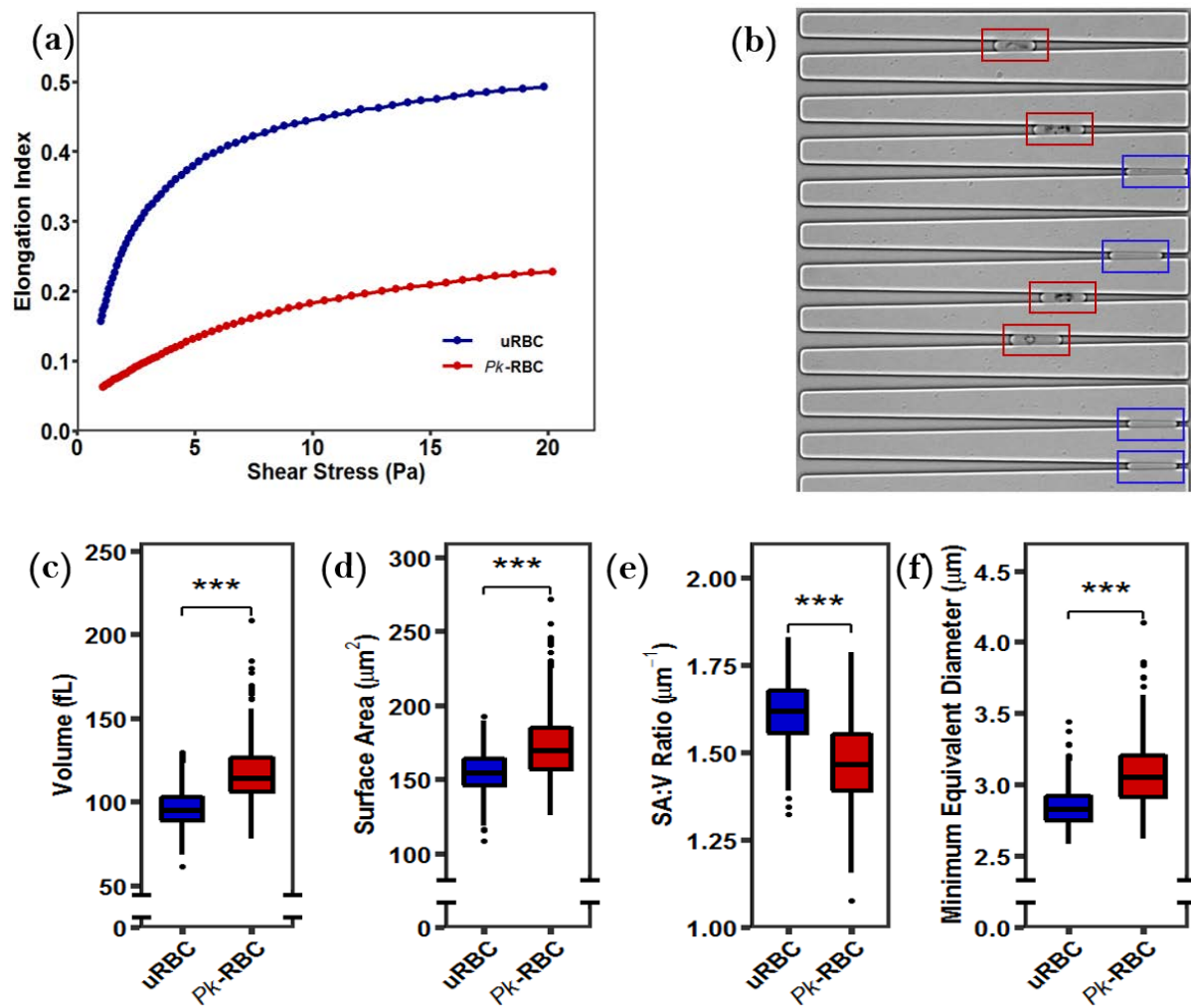


Figure 4-3 Physical properties of *P. knowlesi* iRBCs (22-26 h). (a) Ektacytometry analysis (0-20 Pa shear stress) for uRBCs (blue line) and *P. knowlesi* iRBCs (red line). (b) An image of trapped uninfected RBCs and *P. knowlesi* trophozoite-infected RBC in the wedge-shaped microchannels. Uninfected RBCs and infected RBCs are marked in blue and red, respectively. (c-f) Comparison of mean values of volume, surface area, SA:V ratio and minimum equivalent diameter in uRBCs (490 cells, blue) and iRBCs (406 cells, red).

Table 4-3 Mean values and standard deviation (SD) of volume, surface area, SA:V ratio and minimum equivalent diameter for *P. knowlesi*-infected and uninfected RBCs.

Cell type Parameter (Mean±SD)	<i>P. knowlesi</i> - infected RBC	Uninfected RBC
Volume	118±17	96±11
Surface area	172±24	154±14
SA:V ratio	1.47±0.11	1.61±0.09
Minimum equivalent diameter	3.08±0.23	2.84±0.13

4.4 Measuring the SA:V ratio in mutant mice cells

Mutations in different RBC proteins can result in abnormal physical properties that render them resistant to infection by malaria parasites, either by preventing invasion, compromising parasite development or disrupting the ability of infected RBCs to escape spleen filtration (Chishti et al., 1996; Leffler et al., 2017; Rank et al., 2009; Schulman et al., 1990).

Dr Gaetan Burgio (ANU) generated mutant mice with RBC defects using the mutagen, ENU (Lelliott et al., 2017). One of the mice lines generated, *Sptb* (MRI26194), harbours a single-point mutation in the gene for the RBC membrane skeleton protein, beta spectrin (*Sptb*). The MRI26194 mutation disrupts splicing of intron 7 and results in translation of a truncated (188 amino acid residue) beta spectrin protein. The defect is associated with microcytosis (volume reduction). Here we studied the physical properties of this mutant.

Our collaborators showed that mutant MRI26194 RBCs exhibit a reduced ability to elongate in fluid flow (lower EI) compared with RBCs from a wild-type (WT) mouse (Lelliott et al., 2017). We measured the SA:V ratio change due to *Sptb* mutation and examined the traversal of *Sptb*-mutant RBCs in the HEMA microchannels.

4.4.1 *Sptb* mutation

Generation of *Sptb*^{MRI26194} mice was performed as previously described (Lelliott et al., 2017). Mutant RBCs were kindly supplied by Dr Gaetan Burgio (ANU).

4.4.2 Experimental setup

In this experiment, we used HEMA chips with a depth 3.75 μm , as described in the original publication (Gifford et al., 2003), to measure the volume, surface area and minimum equivalent diameter of mouse cells. See Methods in Chapter 2. The microchip was purged with ethanol at a flow rate of 2 $\mu\text{L}/\text{min}$ for 10 minutes, followed by flushing with mouse-tonicity phosphate-buffered saline (MT-PBS, 150 mM NaCl, 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , pH 7.3) for an additional 30 minutes before introduction of blood cells. Mouse blood was washed twice in MT-PBS and resuspended at a concentration of 1 μL packed cells per mL. Diluted blood samples were driven into the microchannels at a flow rate of 1 $\mu\text{L}/\text{min}$. WT samples were taken from 3 mice, and mutant samples from 2 mice. New microfluidic chips were used for each mouse blood sample.

4.4.3 Results

We examined the volume and surface area of *Sptb*^{MR126194} RBCs using the HEMA chip. The average volume of the mouse RBCs was ~ 50 fl, which is much smaller than that of human RBCs (~ 90 fl, see Chapter 3). We found that volume and surface area of mutant cells decreased by 12% and 16%, respectively as compared to WT cells (Figure 4-4a, b). However, in spite of the microcytosis of mutant cells, only a subtle decrease was measured in their SA:V ratio of mutant RBCs (Figure 4-4c), consistent with the observation that the mutant RBCs maintain their biconcave shape. Mutant mouse RBCs were trapped at a slightly larger minimum equivalent diameter ($\sim 4\%$) than WT cells (p value < 0.0001) (Figure 4-4d). The mean value and standard deviation of measured parameters for mutant and WT RBCs are provided in Table 4-4.

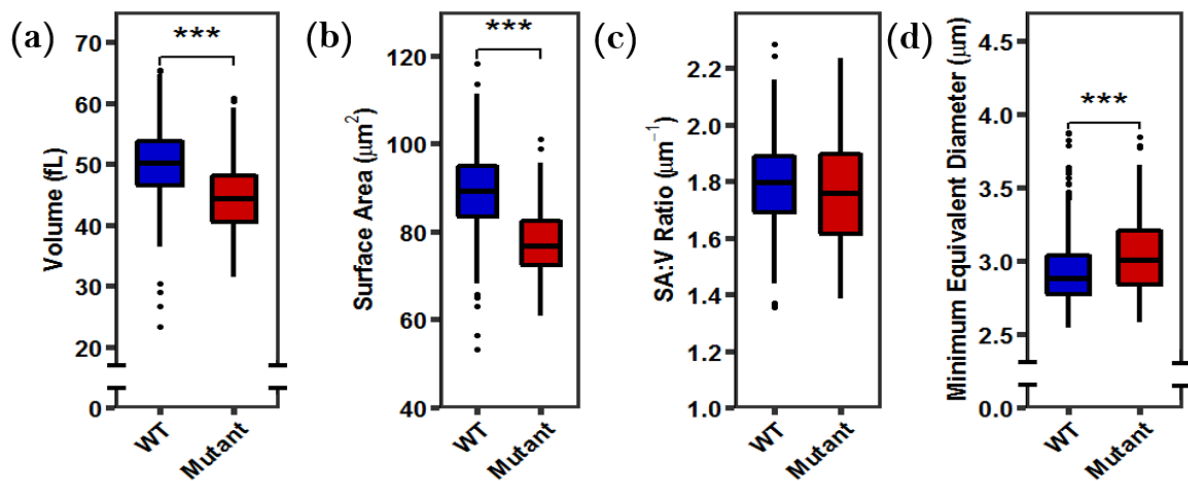


Figure 4-4 Mean values for (a) volume, (b) surface area, (c) SA:V ratio and (d) minimum equivalent diameter of cell populations for WT (3 mice, 642 cells) and MRI2694 mutant (2 mice, 233 cells) RBCs.

Table 4-4 Mean values and standard deviation (SD) of volume, surface area, SA:V ratio and minimum equivalent diameter for mutant and WT mouse RBCs.

Cell type	Mutant RBC	WT RBC
Parameter (Mean $\pm$ SD)		
Volume	44 $\pm$ 6	50 $\pm$ 6
Surface area	78 $\pm$ 8	89 $\pm$ 9
SA:V ratio	1.77 $\pm$ 0.18	1.79 $\pm$ 0.15
Minimum equivalent diameter	3.04 $\pm$ 0.27	2.92 $\pm$ 0.21

4.5 Discussion

In this chapter, the physical characteristics of immature, malaria parasite-infected, and mutant RBCs were examined in two microfluidic-based devices, ektacytometry and HEMA analysis to evaluate the contributions of cell stiffness and SA:V ratio on traversal of these different RBC types in small blood vessels.

Reticulocytes undergo significant changes in the structure and membrane properties as they transform into mature RBCs. Reticulocyte maturation is associated with the loss of remnant RNA and surface receptors like CD71, is accompanied by the acquisition of a biconcave shape. We used CD71 labelling to distinguish the earliest (least mature) stage reticulocytes and found that RBCs lose ~20% of both their volume and surface area in the transition to mature RBCs. This surface area loss is slightly larger than that found in previous studies (Gifford et al., 2006; Waugh et al., 1997), which used RNA staining to detect reticulocytes. Our data indicate that the volumes of cells just released into the peripheral blood may be higher than earlier work suggests. Despite being more rigid and being larger in size, both reticulocyte populations (CD71⁺ and CD71⁻) reached a similar minimum equivalent diameter to mature RBCs in the microchannels, due to exhibiting a similar SA:V ratio to mature RBCs, in line with a previous report (Gifford et al., 2006).

P. falciparum trophozoite-infected RBCs exhibited a four-fold lower ability to elongate in fluid flow than uninfected RBCs, likely due to increased membrane stiffness of the host RBC (Park et al., 2008; Suresh et al., 2005) and the presence of a rigid intracellular parasite, which occupies ~50% of the host cell volume (Hanssen et al., 2012). Park et al. measured the membrane shear modulus of infected RBCs at the trophozoite ($G=29\text{ }\mu\text{N/m}$) and schizont stage ($G=71\text{ }\mu\text{N/m}$) (Park et al., 2008). By contrast, the in-plane membrane shear modulus is reported to be in the range of 5-10 $\mu\text{N/m}$ for the healthy RBC (Evans et al., 1984; Hochmuth and Waugh, 1987). In spite of these rigidity changes, our experimental measurements show that trophozoite-infected RBCs preserve the host cell SA:V ratio and were able to traverse into microchannels to the same extent as uninfected RBCs. Some previous studies reported that the SA:V ratio of the host RBC decreases in later (schizont) stage of *P. falciparum*-infected RBCs (Hanssen et al., 2012; Herricks et al., 2009), and it is possible that these swollen iRBCs would obstruct microcapillaries. Our results are in agreement with a previous study (Herricks et al., 2009), who found that RBCs infected with the trophozoite stages of two other strains of *P. falciparum* were able to pass through similar equivalent diameters to uninfected RBCs.

Some previous studies used a microbeads assay to mimic the geometry of splenic inter-endothelial slits. It was reported that the retention rate of RBCs infected with *P. falciparum* in microbeads filtration increased with parasite development (Deplaine et al., 2011). For instance, Safeukui et al. reported a ~10% loss of surface area in ring-stage infected RBCs and found a 50% rate of retention. They observed complete retention of trophozoite-infected cells (Safeukui et al., 2013), as has also been reported in previous studies from the Tilley laboratory (Dearnley et al., 2016).

It is important to note that these different methods, HEMA and microbead filtration, measure different physical parameters, and each method exhibits sensitivity to the changes in cell stiffness and RBC SA:V ratio to different extents. Further work is needed to dissect the different contributions and to determine which of these assays provides a better surrogate measure of the likely survival of physically altered RBCs in the human circulation.

Our findings support an adhesive sequestration-based mechanism being the main cause of microvascular obstructions in the case of *P. falciparum*, with a more minor contribution for rigidity changes. This is consistent with a previous finding that knob-less infected RBCs are observed in the peripheral circulation (Langreth and Peterson, 1985), despite remaining highly rigidified (Glenister et al., 2002).

Our findings have important implications for the pathology *P. knowlesi* is reported to cause severe malaria in Malaysia with risk of acute kidney injury (Barber et al., 2018a). In this study, we found that the EI of *P. knowlesi*-infected RBCs decreased by three-fold compared to uninfected RBCs, which is less dramatic than the defect observed for *P. falciparum*-infected RBCs. However, *P. knowlesi*-infected RBCs exhibited restricted traversal (8% less) in microchannels, as a consequence of a ~9% reduction in the SA:V ratio of the host RBCs. A previous study showed increased membrane stiffness of RBCs infected with *P. knowlesi* and suggested that rigidity changes may contribute to microvascular accumulation (Barber et al., 2018b). However, we anticipate that the decrease in SA:V ratio of *P. knowlesi*-infected RBCs likely underpins *P. knowlesi*'s ability to sequester within the vasculature despite the absence of adhesive knobs in this species (Knisely et al., 1964; Menezes et al., 2012; Miller et al., 1971).

Our study mainly focused on the trophozoite stages of *P. falciparum* (32-36 h) and *P. knowlesi* (22-26 h), and we compared the physical parameters of RBCs infected with these two

Plasmodium species. However, any further comparison should be drawn with caution, as *P. falciparum* and *P. knowlesi* have life cycles of different length.

In all of malaria parasite-infected RBC studies, we found a smaller number of infected RBCs than uninfected RBCs trapped in the microchannels, despite the high initial parasitemia (> 80%). We observed many intracellular parasites without the host cell membrane in wedge-shaped microchannels. The rupture rate of RBCs infected with *P. falciparum* was higher than that for RBCs infected with *P. knowlesi*. The mismatch between the parasitemia and the number of intact infected cells trapped in channels suggests that malaria parasite-infected RBCs are more fragile than healthy RBCs, likely because of their altered membrane properties, such as deposition of knobs and cross linking of the membrane skeleton (Zhang et al., 2015a).

We also examined the impact of truncation of β spectrin (*Sptb*) on traversal of mouse RBCs in microchannels and found that *Sptb* mutation reduced the size of mutant RBCs. However, only a slight decrease was observed in the SA:V ratio, consistent with the maintenance of the biconcave disc shape observed in SEM images (Lelliott et al., 2017). Mutant RBCs were found to be trapped at 4% larger equivalent diameter than the one for WT cells, consistent with the greater retention of mutant RBCs in the spleen bead filtration assay (Lelliott et al., 2017). However, the 4% difference in minimum equivalent diameter between mutant and WT cells was more than expected, as the SA:V ratio was only reduced by 1% for mutant cells. This result may be due to the small volume of mouse RBCs (~50 fL) which may amplify the effects of any further loss in volume. Another possible explanation is that we used new microfluidic chips for each sample of WT and mutant cells, which might have introduced a small amount of experimental measurement error due to a slight variability in dimensions from chip to chip.

4.6 Conclusion

Pathological and physiological changes to RBC membrane properties can affect the cell's ability to circulate through small blood vessels and impair its functionality. As examples, we probed the behaviour of immature, malaria parasite-infected and mutant RBCs using microfluidic devices. Our experiments confirmed that newly released reticulocytes adopt a similar SA:V ratio to mature RBCs and travelled into narrow channels efficiently despite being larger and more rigid. RBCs infected with *P. falciparum* showed an extremely compromised ability to deform under fluid flow but preserved the same SA:V ratio as uninfected RBCs and penetrated into small capillaries to the same extent. By contrast, the less rigid *P. knowlesi* infected RBCs exhibited a reduced SA:V ratio and penetrated less into small capillaries

compared with uninfected RBCs. These findings confirm that passage of RBCs through small constrictions is more dependent on SA:V ratio than cell stiffness and illustrate some potential pathological sequelae of this physical parameter.

Chapter 5

5 Finite element analysis of RBC behaviour in microchannels and small capillaries

5.1 Introduction

Our results presented in previous chapters showed that a decrease in SA:V ratio significantly compromises the RBC's ability to traverse into narrow microchannels, while increases in cell stiffness have little effect on RBC traversal. In this chapter, I describe the development of a whole-cell computational model using the finite element (FE) method to enable a complete understanding of the relative contributions of SA:V ratio and membrane stiffness to the ability of cells to traverse into narrow microchannels as well as small capillaries such as cerebral capillaries.

We used our computational model to perform parametric studies of alterations to the shear modulus of the membrane and the SA:V ratio of the cell. Consistent with our experimental data, the simulations indicate that a 15-fold increase in the shear modulus of the membrane only slightly reduces the ability of the cell to traverse the microchannels (by ~6%). On the other hand, a 20% decrease in the SA:V ratio of the cell (from 1.42 to 1.15) compromises RBC passage through the microchannels by ~9%. Finally, we estimated the minimum required pressure for RBCs with different SA:V ratio and membrane stiffness to pass through vessels with dimensions equivalent to those of cerebral capillaries in the cortex. These results provide a mechanistic rationale for the relatively high impact of SA:V ratio as compared to membrane stiffness on RBC traversal of narrow blood vessels of physiological sizes, such as cerebral capillaries in the brain.

5.2 3D computational model

We developed a nonlinear 3D Finite Element (FE) model for numerical simulations using the commercial Abaqus software (SIMULIA, Providence, RI, USA) following previous studies (Aingaran et al., 2012; Mills et al., 2004). The RBC has a biconcave shape with a high SA:V ratio, facilitating passage through small constrictions such as small capillaries and the spleen inter-endothelial slits. We used a previously standardised RBC geometry (i.e. SA:V ratio of 1.42) for the simulations, generated according to the formula published in a previous study (Evans and Fung, 1972). The thickness distribution of a normal RBC is given in Eq. 5-1:

$$T(r) = \sqrt{1 - \left(\frac{r}{R_0}\right)^2} \left[C_0 + C_2 \left(\frac{r}{R_0}\right)^2 + C_4 \left(\frac{r}{R_0}\right)^4 \right] \quad (5 - 1)$$

where R_0 is the initial radius of the cell, and C_0 , C_2 and C_4 are shape coefficients.

Mechanical properties of the cell microstructure determine the deformation-related behaviour of the RBC. The RBC cellular microstructure comprises a lipid bilayer, a membrane skeleton, and the cytoplasm. The network of proteins in the membrane skeleton dominates contributions to the shear modulus of the membrane, while the lipid bilayer is resistant to bending deformation (Hansen et al., 1996; Mohandas and Evans, 1994). These two layers of the membrane lead to the nonlinear elastic behaviour of the RBC. In addition to the elasticity of the RBC, it was observed that the RBC exhibits viscous behaviour, being resistant to strain rate (Evans and Hochmuth, 1976; Fischer, 2016; Lim et al., 2004; Tomaiuolo et al., 2011). Some studies have considered the cell membrane as the major source of viscous dissipation during recovery (Evans and Hochmuth, 1976; Hochmuth and Waugh, 1987; Yoon and You, 2015). Others have argued that the cytoplasm viscosity plays an important role when the RBC is subjected to large deformation, and then, the force of deformation is suddenly removed (Chee et al., 2008). Viscosity plays an important role when the RBC is subjected to large deformation, and then, the force of deformation is suddenly removed. During the unloading, the time required for the RBC to recover its original shape is determined by its membrane viscosity. In this study, we mainly focused on the static state of RBCs trapped in microchannels; thus, we neglected the viscous dissipation of the membrane and cytoplasm in our FE model and ran all simulation using a quasi-static analysis, similar to some previous studies (Aingaran et al., 2012; Jiao et al., 2013). Moreover, it was previously reported (Mills et al., 2004) that neglecting the viscous dissipation introduces a negligibly small error during mechanical loading.

We regarded the lipid bilayer and the membrane skeleton layers as one homogenous layer in our whole-cell model. Given the large deformation of the RBC, as it passes through small capillaries, we reasoned that a hyper-elastic model would be an appropriate choice to define the constitutive equations for the cell membrane. Two hyper-elastic models, the Neo-Hookean model, which is an extension of Hook's law, and the Yeoh model, which is a third-order model, are commonly employed when modelling RBC mechanics. Considering the incompressibility of the membrane, the strain energy potential functions in the Neo-Hookean and the Yeoh model are expressed as follows (Mills et al., 2004):

$$U_{Neo-Hookean} = \frac{G_0}{2}(\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3) \quad (5 - 2)$$

$$U_{Yeoh} = \frac{G_0}{2}(\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3) + C_3(\lambda_1^2 + \lambda_2^2 + \lambda_3^2 - 3)^3 \quad (5 - 3)$$

$$\lambda_1\lambda_2\lambda_3 = 1 \quad (\text{compressibility})$$

where G_0 is the initial value of bulk shear modulus, C_3 is a model parameter ($C_3=G_0/30$ in this study), and λ_i ($i=1-3$) is the principal stretch ratio in the i -th direction. Since the membrane is quite thin, the in-plate shear modulus was used instead of the bulk shear modulus (G_0) (Jacobs et al., 2012). If the initial thickness is h_0 , the initial in-plane shear modulus is represented by $\mu_0=G_0 h_0$.

Bending stiffness is another important mechanical feature of the membrane, which represents resistance to bending and buckling. Bending stiffness in thin shells is related to the bulk shear modulus and its thickness by Eq. 5-4 (Yoon and You, 2015):

$$B = \frac{2}{3}G_0h_0^3 \quad (5 - 4)$$

Bending stiffness was reported to be in the range of 1.7×10^{-19} - 7×10^{-19} J in a previous study (Evans, 1983). In this study, the bending stiffness was assumed to be 2×10^{-19} J, resulting in a greater thickness value than the actual membrane thickness, according to Eq. 6-4.

The cytoplasm of the RBC mainly comprises a concentrated solution of haemoglobin. It is an incompressible fluid that keeps the cell volume constant under the deformation. Thus, we regarded the cytoplasm as a constant volume constraint using a fluid cavity model to enforce volume conservation.

5.2.1 Verification of the continuum model using an optical tweezer experiment

To verify our model, we reproduced simulations of optical tweezer experiments from (Mills et al., 2004) by employing 3D shell elements in ABAQUS. In the optical tweezers experiments, two silica microbeads, each with a diameter of 1-2 μm are attached to the RBC at diametrically opposite points, and forces are applied to these microbeads to gradually stretch the cell (Figure 5-1a). Mills et al. conducted an optical tweezer experiment on a normal RBC by increasing the applied force to 193 pN and measuring the axial and transverse diameters at different forces (Mills et al., 2004). We ran numerical simulations using our FE model and compared the results with experimental data. Because of symmetry, it sufficed to only model one-eighth of the RBC with the proper symmetrical boundary conditions, as shown in Figure 5-1b. In this simulation, the microbeads were considered to be attached to the cell membrane over a small circular area with a diameter of 1.5 μm , and the stretching force (surface traction) was directly applied to the contact area on the cell membrane (Figure 5-1b). The cell membrane was discretised using 4,100 four-node linear shell elements (Figure 5-1c), and the first -order Neo-Hookean model and the third-order Yeoh hyper-elastic model were used to represent the stress-strain relationships in the membrane. The undeformed and deformed shapes of a standard RBC undergoing the optical tweezer simulation are shown in Figure 5-1.

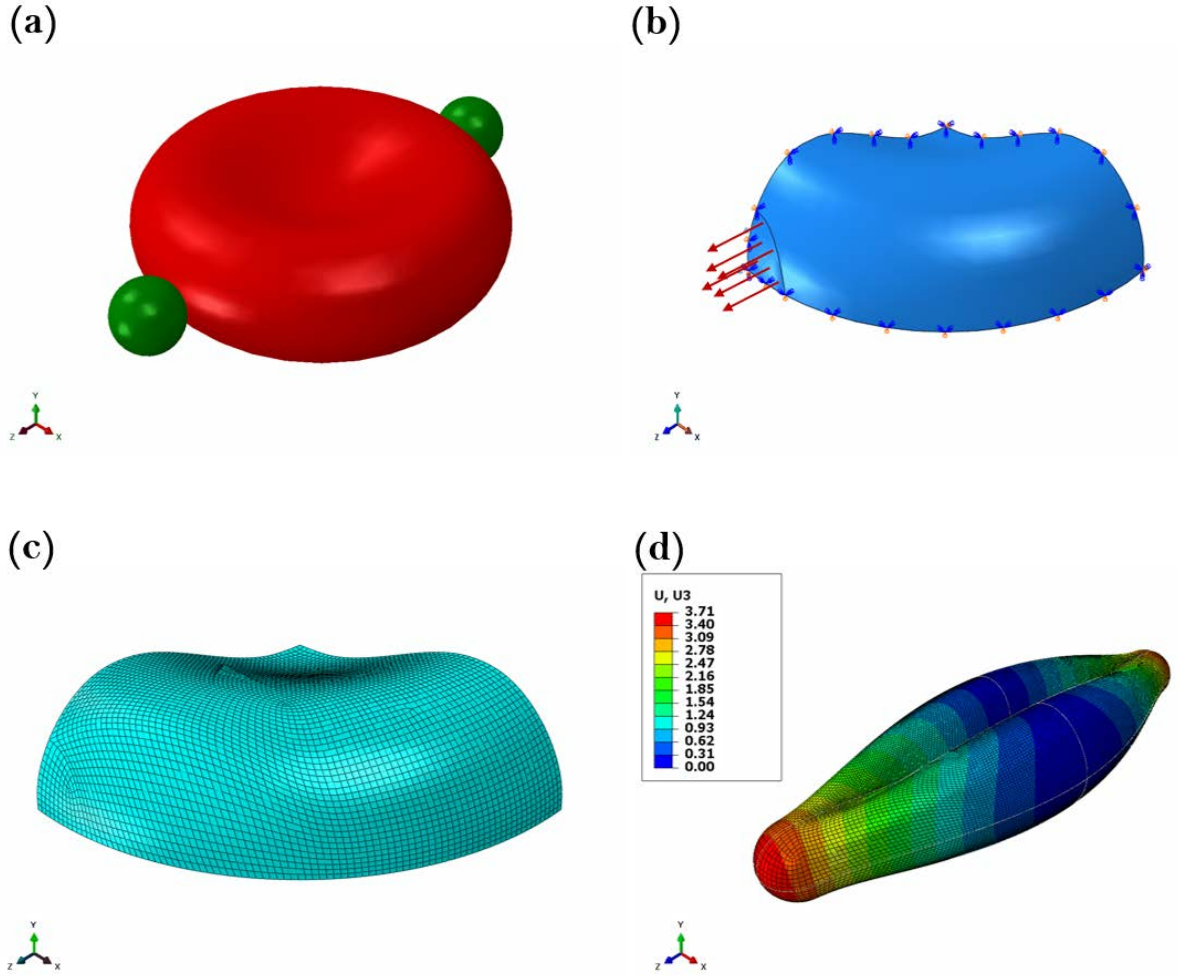


Figure 5-1 FE model setup of the optical tweezers experiment. (a) The undeformed shape of an RBC in the optical tweezer experiment. The stretching forces were applied to small microbeads attached to the cell. (b) Only one-eighth of the RBC was modelled because of symmetry. Stretching force was applied to the contact area with a diameter of 1.5 μm . (c) The membrane was discretised using 4,100 four-node linear shell elements. (d) Deformed shape of the RBC after applying 193 pN force. The displacement distribution (μm) is shown for a standard RBC modelled with the Yeoh model and a shear modulus of $\mu_0=7.3 \mu\text{N/m}$.

Our simulation results using the Neo-Hookean and Yeoh models are compared with the experimental data (Mills et al., 2004) in Figure 5-2. Figure 5-2 shows the variation of axial and transverse diameters of the RBC relative to the magnitude of the stretching force. Using the first order Neo-Hookean model, an initial shear modulus of $\mu_0=7.3 \mu\text{N/m}$ appeared to match the average values of the experimental data over the range of 0-70 pN load, while the Neo-Hookean model was not able to capture the experimental trends at higher stretching forces (Figure 5-2a). The main reason is that the Neo-Hookean is an extension of Hook's law for linear elastic deformation and is not able to encompass large deformation. On the other hand, Figure 5-2b demonstrates that using the third-order Yeoh model with an initial shear modulus of $\mu_0=7.3 \mu\text{N/m}$ matched well with the experimental data over the complete range of 0-193 pN load, as the Yeoh model benefits from a third-order strain energy potential function. In

summary, these simulations showed that the Yeoh hyper-elastic model with an initial shear modulus of $\mu_0=7.3 \mu\text{N/m}$ resulted in a better fit to the data than lower-order hyper-elastic models like the Neo-Hookean model, in agreement with previous studies (Chee et al., 2008; Mills et al., 2004; Yoon and You, 2015).

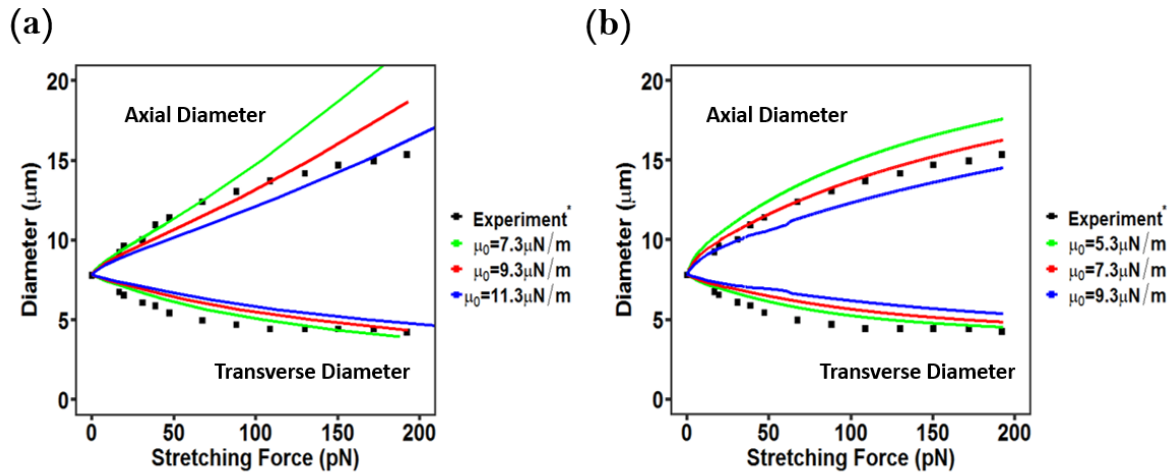


Figure 5-2 Comparison of our numerical results using (a) the Neo-Hookean model and (b) the Yeoh hyper-elastic model with the experimental results from optical tweezers experiment. *Experimental data was taken from (Mills et al., 2004).

5.3 Numerical simulation of the HEMA experiment

In order to gain a mechanistic understanding of the higher importance of SA:V ratio compared with cell stiffness on passage into microchannels, we used our numerical model to simulate RBCs traversal in the HEMA device and performed stress-strain analysis within the cell membrane.

Considering that the membrane surface area is constant due to incompressibility (Peng et al., 2010; Skalak et al., 1973; Yoon and You, 2015), the whole-cell membrane was discretised using 2600 shell elements with a constant thickness ($\lambda_3 = 1$), and the third-order Yeoh hyper-elastic model was used to represent the stress-strain relationships in the membrane (Figure 5-3a). We modelled the wedge-shape microchannels as rigid parts in the simulation of the HEMA experiment (Figure 5-3b) because the shear modulus of PDMS is four orders of magnitude higher than the membrane stiffness of the RBC (Nunes, 2010).

In our experimental work, the microfluidic chip was purged and flushed with ethanol 80% and PBS/CCM (> 1 hr) before introducing the cells to minimise the friction between the microchannels and cells. Accordingly, the contact between the cell and the microchannel/coverslip was assumed to be low-friction. We incorporated a friction coefficient of 0.01 in the tangential direction and a hard contact in the normal direction. Figure 5-3 shows

the FE model setup for RBC passage into the narrowing microchannels in the HEMA device. In the simulations, the cell was introduced into the microchannels using surface traction along the flow direction.

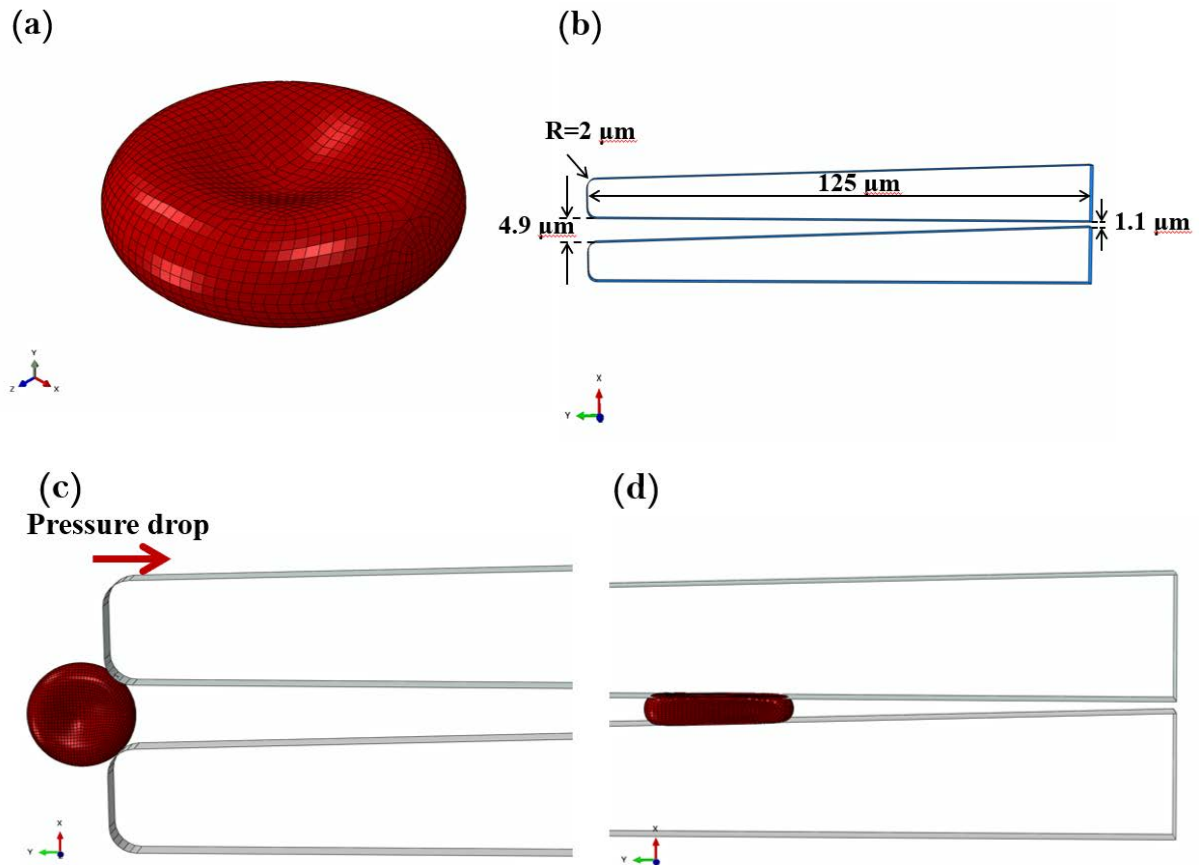


Figure 5-3 The FE model of a normal RBC passing through the wedge-shaped channels in the HEMA device. (a) 3D geometry of an RBC with a SA:V ratio of 1.42, discretised using 2600 shell elements for the cell membrane. (b) The wedge-shaped channels were modelled as rigid parts, and their dimensions are given in the figure. (c) The FE model setup of the RBC passage into the narrowing microchannels before applying the pressure drop. (b) The deformed shape of the RBC being stretched in the microchannels under a pressure drop of 2.2 pa.

The HEMA chip readout does not provide a direct measurement of the forces acting on an RBC squeezing into the microchannels. Indeed, estimation of the force acting on the RBC in the HEMA chip is difficult because of the many channels through which cells can traverse the microchip. Thus, we resorted to estimating the load that an RBC would experience as it flows through the microchannels using our FE model. The RBC is subjected to a net force along the flow direction when it is introduced to the microchip due to the pressure difference (drop) across the cell surface. The pressure drop per cell increases as the cell squeezes into the narrowing microchannels, and it reaches a maximum once the cell is fully trapped. To estimate the pressure drop per cell in the wedge-shaped channels, we applied a pressure drop on a normal RBC (with the typical SA:V ratio of 1.42 and a shear modulus of 7.3 μN/m) and increased the

pressure drop gradually to 4.4 Pa. The minimum equivalent diameter and the length of the elongated cell were calculated, as the cell squeezed into the microchannel (Figure 5-4). Figure 5-4 shows the changes in the minimum equivalent diameter and the length of the deformed cell as the pressure drop increases. We collected the volume and surface area data for 33 RBCs from the HEMA experiments and found that applying a pressure drop of 2.2 Pa to the cell is in good agreement with the experimental data (green zone in Figure 5-4). The estimated pressure drop value is the same magnitude of that reported in previous studies (Aingaran et al., 2012; Guo et al., 2012; Pivkin et al., 2016). This value of pressure drop per cell was used in the following simulations, assuming that cells with different SA:V ratios and different membrane stiffness experience the same level of pressure drop in microchannels.

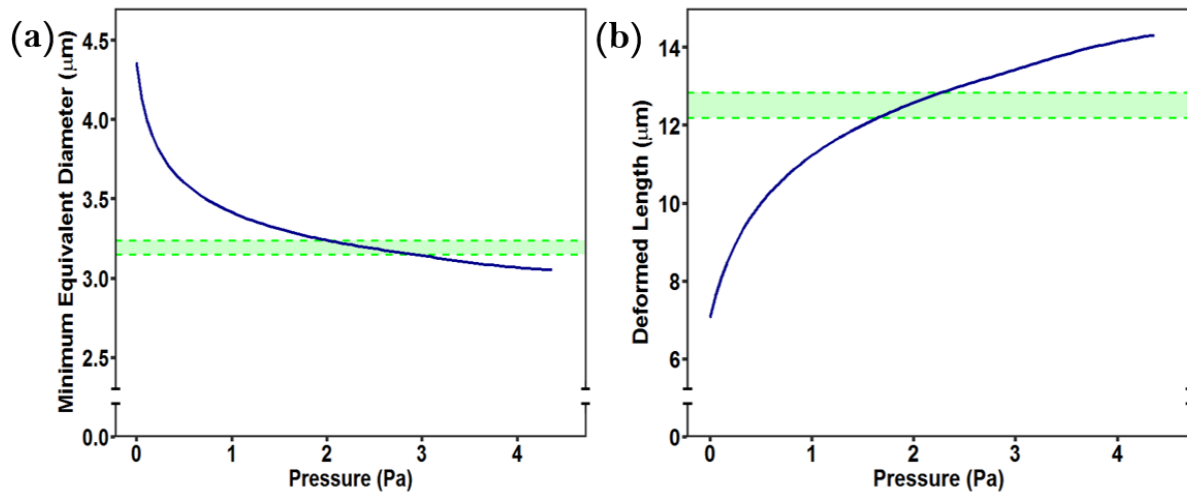


Figure 5-4 Pressure analysis on a normal RBC with a SA:V ratio of 1.42. (a) The minimum equivalent diameter that a normal RBC traversed as a function of the applied pressure drop, (b) the elongated length of the cell as a function of the applied pressure drop. The green zone shows the experimental data for 33 cells with similar volume and surface area.

5.4 Parametric studies

Next, parametric studies were conducted to study the impacts of membrane stiffness and SA:V ratio. We carried out two numerical analyses of RBCs within the HEMA device considering (i) only membrane stiffness variation, and (ii) only SA:V ratio variation. Finally, we used our FE model to predict critical bounds for membrane stiffness and SA:V ratio which allow cells to pass through small channels (3-4 micron in diameter), comparable to the minimum diameter cerebral capillaries in the brain.

5.4.1 Membrane stiffness change

To study the impact of membrane stiffness, we performed simulations using the standard RBC geometry for a variety of membrane shear moduli. The in-plane membrane shear modulus is reported to be in the range of 5-10 $\mu\text{N/m}$ for a normal RBC (Evans et al., 1984; Hochmuth and

Waugh, 1987), while according to previous measurements on malaria-parasite infected RBCs (Barber et al., 2018b; Park et al., 2008; Suresh et al., 2005), the in-plane shear modulus can increase to 100 $\mu\text{N/m}$ in *P. falciparum* schizont-infected RBCs. Thus, the in-plane membrane shear modulus was varied from 7.3 to 109.5 $\mu\text{N/m}$ in these simulations.

Figure 5-5a shows the minimum equivalent diameter as a function of the membrane shear modulus that RBCs with the same SA:V ratio reach before being trapped. Our simulations show that cells with shear moduli less than 51 $\mu\text{N/m}$ are trapped in the microchannels at an equivalent diameter of 3.2 μm which is within the experimental range for normal cells with similar SA:V ratio. Importantly, increasing the shear modulus higher than 51 $\mu\text{N/m}$ had only a moderate effect on cell passage through the microchannels. For instance, an RBC with a shear modulus of 109.5 $\mu\text{N/m}$ exhibited an equivalent minimum diameter that is only ~6% higher than that for a normal rigidity RBC. This was accompanied by the formation of creases in the membrane of the cell at $\mu_0=109.5 \mu\text{N/m}$.

We observed that creases are formed in the RBC membrane in the simulations when the membrane shear modulus was higher than 87.6 $\mu\text{N/m}$ (Figure 5-5b,c) similar to those seen in cells treated with 0.005% glutaraldehyde. Formation of creases in the membrane prevents the cell from travelling into regions of smaller equivalent minimum diameter, as the strain and stress in the membrane are significantly increased in the vicinity of creases. We calculated the maximum in-plane principal logarithmic strain and von Mises stress at the integration point of all membrane shell elements. Ranges of strain and stress were calculated at the position of 60 μm inside the microchannels for membrane stiffness ranging from 7.3-109.5 $\mu\text{N/m}$ (Figure 5-5d, e). Our simulations indicate that cells with shear moduli less than 73 $\mu\text{N/m}$ experience a similar strain range; however, both strain and stress increase substantially as the shear modulus increases to more than 87.6 $\mu\text{N/m}$. Formation of creases within the membrane is the leading cause of the substantial increase in strain and stress within the elements in the vicinity of creases in such rigid RBCs, and subsequently, such cells were trapped at larger equivalent diameter, i.e. earlier within the microchannels.

These results suggest that a large increase in the membrane shear modulus will have little effect on RBC traversal in the microchannels. The reason is that the cell membrane has extremely nonlinear mechanical behaviour due to the membrane skeleton layer, which means that the cell can be extensively stretched, even in RBCs with high membrane rigidity, such as malaria-parasite infected cells. Nominal stress-strain curves for all shear moduli are displayed in Figure

5-5f. The nominal stress-strain plot shows that according to the Yeoh-model model of mechanical behaviour, RBCs would exhibit a highly nonlinear mechanical behaviour across a wide range of shear moduli. This highly nonlinear behaviour makes the RBC stress insensitive to large strains ranging from 0 to 60% as shown by the long plateaus in Figure 6-5f, similar to the strains that cells experience in the wedge-shaped microchannels (Figure 5-5d).

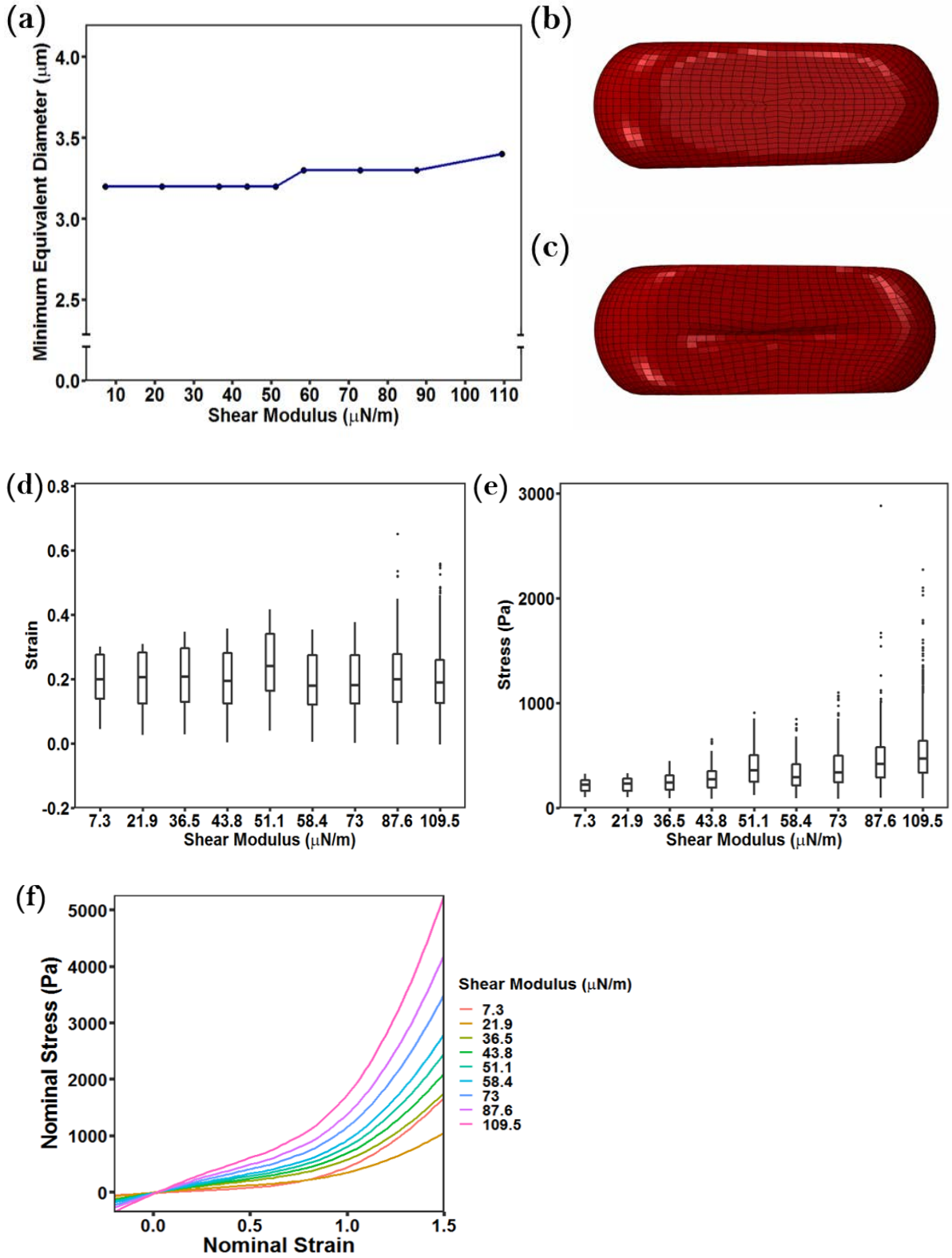


Figure 5-5 Numerical simulations of RBC passage through narrow microchannels as the membrane shear modulus changes from 7.3-109.5 $\mu\text{N/m}$. (a) The minimum equivalent diameter that cells traverse in the microchannels as the membrane shear modulus increases. Deformed shapes of RBCs with shear moduli of (b) $\mu_0 = 7.3 \mu\text{N/m}$ and (c) $\mu_0 = 87.6 \mu\text{N/m}$ passing through the microchannels, showing strain around creases in the membrane of the RBC with a shear modulus of 87.6 $\mu\text{N/m}$. (d,e) Variation of maximum in-plane principal logarithmic strain and von Mises stress in the cell membrane calculated at the position of 60 μm inside the microchannels for different shear moduli. (f) Nominal stress-strain curves for different shear moduli.

5.4.2 SA:V ratio change

The impact of SA:V ratio was studied through simulations performed on five RBC geometries with different SA:V ratios ranging from 1.15-1.76. These RBC models were generated by altering the geometric parameters in Eq 5-1. The volume of generated cells was reduced from 117 to 78 fL while keeping surface area constant at $\sim 134 \mu\text{m}^2$. The geometric parameters for various SA:V ratios are given in Table 5-1.

Table 5-1 Geometric parameters for various SA:V ratios

SA:V Ratio(μm^{-1})	R_0 (μm)	C_0 (μm)	C_2 (μm)	C_4 (μm)
1.15	3.80	2.10	7.58	-5.59
1.33	3.85	1.20	7.60	-4.50
1.42	3.91	0.81	7.83	-4.39
1.54	4.02	0.60	8.05	-5.59
1.76	4.23	0.20	8.50	-6.70

In these simulations, the membrane shear modulus was fixed as $\mu_0 = 7.3 \mu\text{N/m}$. Figure 5-6a shows the minimum equivalent diameter that cells with various SA:V ratio reach within the microchannels. Our simulations demonstrate that the passage of RBCs through the microchannels is highly dependent on the cell SA:V ratio. As an illustration, decreasing the SA:V ratio by 20% (from 1.42 to 1.15) compromises the ability of the RBC to squeeze in the microchannels by $\sim 9\%$.

As observed in our experiments, the numerical results also indicate that the SA:V ratio plays a much more important role than cell membrane stiffness in RBC passage through the microchannels. Our results illustrate that as the RBC morphology approaches a spherical shape (i.e. as its SA:V ratio decreases) a higher deformation is imposed on the cell as it squeezes into the narrow channels. The range of stress and strain in the membrane for the various SA:V ratio is shown in Figure 5-6b, c. The maximum von Mises stress in the membrane is doubled to 900 Pa when the SA:V ratio of the RBC is decreased from 1.33 to 1.15.

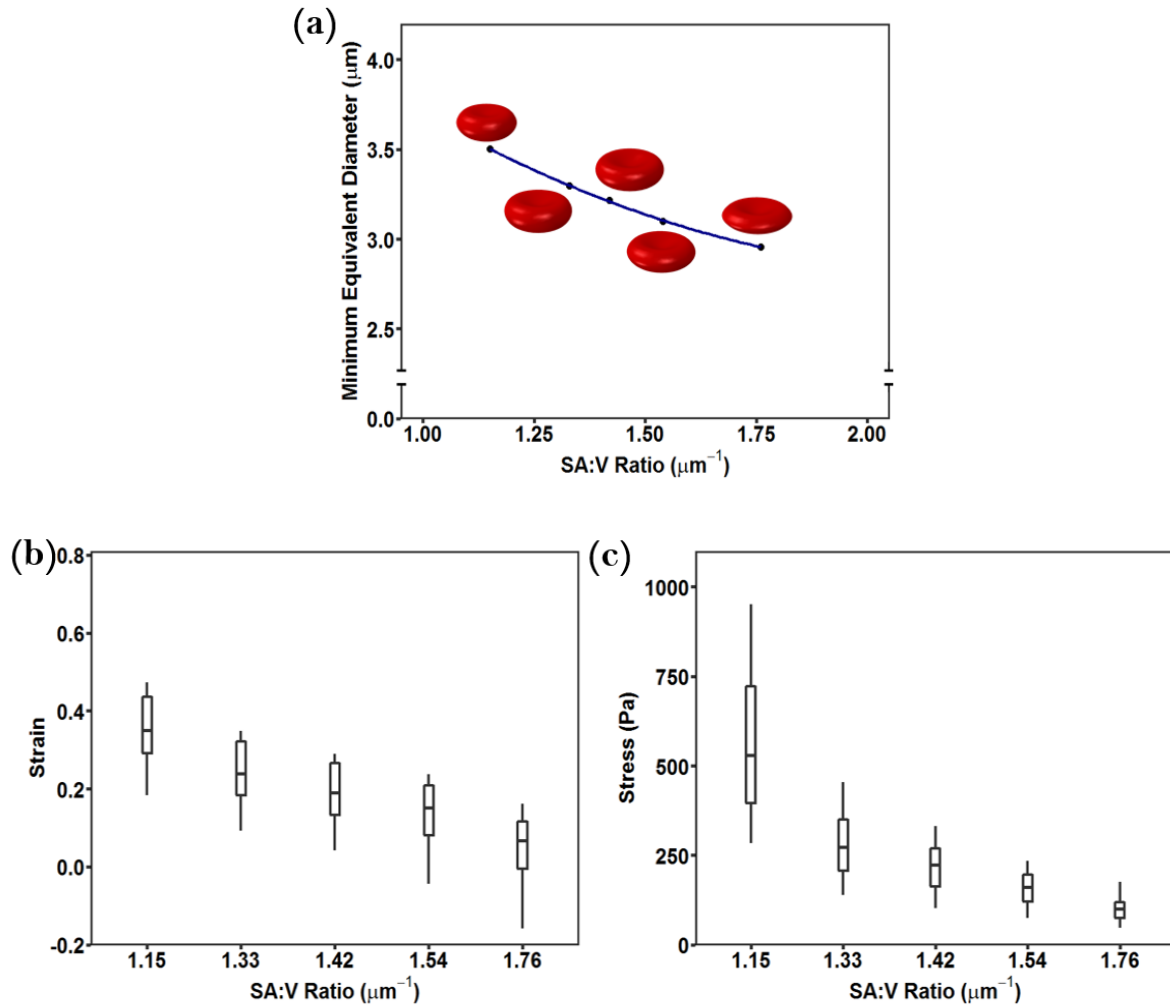


Figure 5-6 Numerical simulations for RBCs passing through narrow microchannels as the SA:V ratio changes from 1.15-1.76. (a) The minimum equivalent diameter reached as cells traverse into microchannels as a function of the SA:V ratio while keeping the surface area unchanged. (b-c) Variation of maximum in-plane principal logarithmic strain and von Mises stress in the cell membrane for different SA:V ratios.

5.4.3 Prediction of RBC Passage in Cerebral Capillaries

We investigated the passage of RBCs through the smallest capillaries of physiological size using our whole-cell FE model to predict whether high cell membrane stiffness or low SA:V ratio would restrict RBC passage through small cerebral capillaries. We performed simulations to evaluate the minimum required pressure for RBCs to squeeze into small capillaries with three diameters of 3, 3.5 and 4 μm (Figure 5-7). We ignored deformation of the capillary wall and regarded the capillaries as rigid parts in the FE model, as the blood vessel wall is two to three orders of magnitude stiffer than the RBC (Lu et al., 2003; Xie et al., 1995). A contact interaction similar to the simulation of the HEMA experiment was considered between the cell and the capillary wall (Figure 5-7a, b).

These simulations predict that stiffening RBCs does not significantly influence the minimum required pressure for cells to pass through small capillaries (Figure 5-7c). All stiffened cells except the one with the highest shear modulus (109.5 $\mu\text{N/m}$) were able to pass the minimum diameter capillary (3 μm) upon application of a pressure drop of 4 Pa. In contrast, the minimum required pressure steeply increased as the SA:V ratio decreases, most strikingly for the 3 μm capillary. RBCs with a SA:V ratio of less than 1.4 are far more prone to be trapped in the capillary (Figure 5-7d). Figure 5-7d shows a five-fold increase in the minimum required pressure to traverse the capillary with a diameter of 3 μm , as the SA:V ratio reduced from 1.33 to 1.15. The minimum required pressures for RBCs to pass through the 3 μm capillary significantly differ from those calculated for the 3.5 and 4 μm capillaries. This difference is due to the relatively higher amount of strains that RBCs would experience during the passage to the 3 μm capillary. For example, the maximum in-plane principal strains that a normal RBC with a SA:V ratio of the 1.42 undergoes during the passage through the 4, 3.5 and 3 μm capillaries are 0.17, 0.34 and 0.57, respectively.

Our simulations confirm that RBCs with low SA:V ratios are more prone to trapping in small cerebral capillaries than cells with high cell rigidity, and the minimum required pressure is much more sensitive to the SA:V ratio of the RBC. On the other hand, changing membrane stiffness up to 87.6 $\mu\text{N/m}$ would subtly affect the required pressure for the cell to traverse into small capillaries.

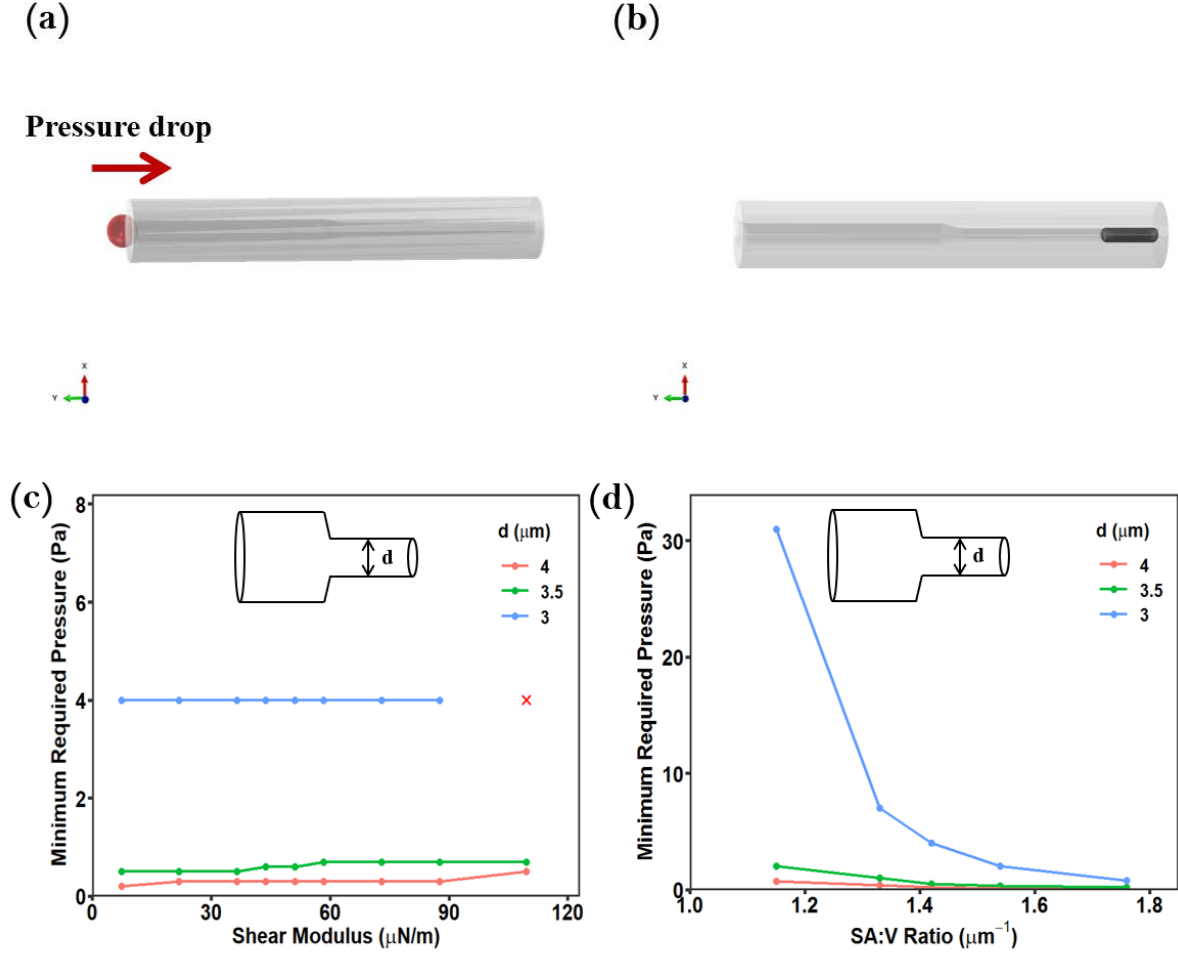


Figure 5-7 Numerical simulations of RBCs traversal in small capillaries of physiological size. The capillary diameter was varied from 4 to 3 μm . (a) The FE model setup of an RBC passing through a small capillary with a diameter of 3 μm , using 2,600 shell elements for the cell membrane and considering the capillary as a rigid part. (b) The deformed shape of the RBC being stretched inside the capillary. The minimum required pressure was predicted for RBCs with (c) different membrane shear moduli and (d) various SA:V ratios.

5.5 Discussion

In this chapter, we provide a coherent rational for our experimental observations using a 3D nonlinear finite element model and predict critical bounds for membranes stiffness and SA:V ratio which allow RBCs to passage through small capillaries with diameters similar to cerebral capillaries.

Our FE model was developed using the third-order Yeoh hyper-elastic model (Yeoh, 1990) combined with a cell volume conservation and is designed to represent the mechanical response of a healthy RBC during the loading stage. This model does not account for the viscosity and time-dependent behaviours of the RBC; however, as we mainly focused on a static state of the RBCs, this model provides valuable insights into understanding RBC passage through small channels and capillaries. The pressure drop per cell was difficult to measure experimentally.

Therefore, in the simulations, the pressure drop per cell was estimated from a pressure sensitivity analysis and assumed consistent for all cells analysed in this computational study. In reality, RBCs may experience slightly different pressure drops in the experiments based on their position in the microchannels.

We utilised our FE model to further study the impacts of membrane stiffness and SA:V ratio on RBC passage through the microchannels of the HEMA device. Our studies show that RBCs with shear moduli less than 51 $\mu\text{N/m}$ are trapped in the microchannels at an equivalent diameter of 3.2 μm . Importantly, our simulation suggests that increasing the shear modulus of the membrane (~ 15 -fold) has little effect on RBC passage into the narrow microchannels ($\sim 6\%$ smaller equivalent diameter). The reason is that the cell membrane has extremely nonlinear mechanical behaviour due to the membrane skeleton layer, which means that the cell can be extensively stretched even in RBCs with high membrane rigidity such as malaria-parasite infected cells. This is consistent with our observation in the glutaraldehyde experiments that increasing cell stiffness by ~ 8 times only slightly affected the RBC's ability to passage into the microchannels (by $\sim 6\%$). Glutaraldehyde cross-links amine groups on proteins in the cell membrane. It also cross-links haemoglobin in the cytoplasm, increasing the cytoplasm viscosity as well. However, here we ignored the viscous dissipation within the cell, which mainly contributes to dynamic behaviours during the unloading stage and the recovery process.

Our simulations revealed the high sensitivity of the RBC traversal to SA:V ratio, which is in good agreement with our experimental results from the osmolarity and malaria-parasite infection studies. As the SA:V ratio of the RBC decreased, higher strains and stresses were observed within its membrane, which prevented the RBC from squeezing into smaller equivalent diameters. It is important to note that the present computational model is limited by the lack of information on the threshold stress and strain for RBC membrane rupture. A further study could assess the maximum stress and strain that the RBC membrane can withstand in healthy and disease states before rupturing.

Finally, we used our FE model to investigate the passage of RBCs through the smallest capillaries of physiological size in an effort to predict whether high shear stiffness or low SA:V ratio would restrict cell passage through small capillaries with diameters of 3-4 μm . Our simulations suggest that RBCs with SA:V ratios lower than 1.3 and RBCs with membrane stiffness higher than 90 $\mu\text{N/m}$ are more prone to trapping in small cerebral capillaries.

Overall, the SA:V ratio appears to play a much more important role than membrane stiffness in RBC passage through both narrow microchannels and small capillaries. However, this work does not address biophysical limits for RBCs to traverse through splenic inter-endothelial slits. Further work is needed to probe the impacts of cell stiffness and SA:V ratio on the splenic clearance of RBCs. Nonetheless, our findings are in agreement with previous computational studies (Li et al., 2018; Pivkin et al., 2016), which have shown that the surface area loss plays a more important role on the passage of malaria parasite-infected RBCs and two typical hereditary RBC disorders through splenic inter-endothelial slits than stiffness changes.

Similarly, we observed in our experimental study on malaria-parasite infected cells that even though the cell stiffness in *P. falciparum*-infected RBCs was significantly higher than that for *P. knowlesi*-infected RBCs, *P. falciparum*-infected RBCs showed a similar ability to uninfected cells to traverse into the microchannels. This is consistent with their preservation of the host cell SA:V ratio. By contrast, the lower SA:V ratio in *P. knowlesi*-infected RBCs led to trapping at larger minimum equivalent diameter (~8%) in the microchannels than uninfected cells. Therefore, we anticipate that *P. knowlesi*-infected RBCs with SA:V ratios less than 1.3 may occlude small cerebral capillaries. The ability of circulating *P. falciparum*-infected RBCs to block small capillaries is a moot point, as mature stage *P. falciparum*-infected RBCs adhere to the blood vessel walls, particularly in post-capillary venules (Pasloske and Howard, 1994). They may nonetheless decrease flow through the microcirculation by inhibiting the flow of uninfected RBCs.

5.6 Conclusion

Two important computational studies were performed to gain a more detailed understanding of the impacts of membrane stiffness and SA:V ratio on RBC traversal through:

- (i) the narrow microchannels of the HEMA device
- (ii) the smallest capillaries of physiological size

RBCs have a nonlinear elastic membrane that enables them to undergo high deformations while passing through spleen slits and small capillaries. Our results show that increasing the membrane shear modulus has little effect on RBC traversal in narrow microchannels and capillaries. By contrast, the SA:V of the RBC is an important determinant of the ability of RBCs to undergo passage through both microchannels and small cerebral capillaries. Our

computational model predicts that RBCs with low SA:V ratios are more prone to trapping in small cerebral capillaries than RBCs with high membrane stiffness.

Chapter 6

6 Conclusions and future work

6.1 A systematic study of the ability of RBCs to traverse into small capillaries

A variety of experimental techniques have been used to elucidate mechanical properties changes in RBCs under pathophysiological conditions. However, independent impacts of changes to cell stiffness and SA:V ratio on the ability of RBCs to circulate through small capillaries are poorly understood. In this work, a systematic study is presented to identify the impacts of cell stiffness and SA:V ratio on the ability of RBCs to penetrate narrow capillaries in a microfluidic device. Furthermore, a finite element model was developed to provide a mechanistic rationale for the different contributions.

6.2 Independent effects of cell stiffness and SA:V ratio

In this study, a microfluidic chip was used to monitor RBCs passage into an array of narrow microchannels, with diameters commensurate with the smallest physiological vessels. This allowed analysis of the impacts of independently manipulating cell stiffness and SA:V ratio (Chapter 3). Modifying cell stiffness of RBCs using glutaraldehyde cross-linking indicated that an extreme increase in cell stiffness (~800%) had little effect on RBCs passage through microchannels, being trapped at a ~6% greater equivalent diameter (section 3.2). By contrast, tuning SA:V ratio of RBCs by altering the osmolarity showed that a ~13% decrease in SA:V ratio of RBCs substantially restricted the passage of cells in microchannels (~14% retardation) (section 3.3). Overall, RBCs with low SA:V ratios exhibit more restriction than RBCs with high cell stiffness.

6.3 The major determinant of the passage of immature, malaria parasite-infected and mutant RBCs into microchannels

Having demonstrated that SA:V ratio is the more important determinant of RBCs traversal in microchannels, we investigated RBCs characteristics subject to different physiological and pathological conditions to determine whether changes to cellular properties of such RBCs are sufficient to impact passage into small blood vessels and whether they might be expected to occlude small capillary-sized apertures.

Our experimental results showed that newly released reticulocytes adopted a similar SA:V ratio to mature RBCs and exhibited equivalent passage through microchannels despite being larger and more rigid. RBCs infected with trophozoite stage *P. falciparum* showed extremely high rigidity under shear stress in ektacytometry but maintained the same SA:V ratio as the host RBCs and travelled into small microchannels to the same extent as uninfected cells. By contrast, the RBCs infected with *P. knowlesi* were less rigid but exhibited reduced SA:V ratio and obstructed microchannels of greater equivalent diameters than of uninfected RBCs. These findings suggest that an adhesive sequestration-based mechanism is likely the main cause of decreased cerebral blood flow in severe *falciparum* malaria while a decrease in SA:V ratio of *P. knowlesi*-infected RBCs may compromise their circulation through small capillaries, potentially leading to capillary obstructions and increased host cell fragility in severe *P. knowlesi* malaria.

The impact of mutations in β spectrin (*Sptb*) on traversal of mouse RBCs in microchannels was also examined. Our results showed that *Sptb* mutation reduced the size of mutant RBCs but slightly decreased SA:V ratio of cells, and mutant RBCs were found to be trapped at 4% larger equivalent diameter than wildtype RBCs.

Taken together, our experiments indicate that RBCs passage through small constrictions is mainly determined by their SA:V ratio rather than their cellular stiffness.

6.1 Parametric studies on the impacts of cell membrane and SA:V ratio on RBCs passage through microchannels using finite element analysis

We developed a nonlinear 3D finite element (FE) model to enable a complete understanding of our experimental results. Two important parametric studies were conducted to study the relative impacts of cell membrane stiffness and SA:V ratio on the ability of RBCs to squeeze into microchannels. Our FE model explains that membrane stiffness changes have little effect

on RBCs passage because that the cell membrane has extremely nonlinear mechanical behaviour, which means that the cell can be extensively stretched, even in RBCs with high membrane rigidity, such as malaria-parasite infected cells. By contrast, our numerical simulations showed that as the RBC SA:V ratio decreases, it becomes more spherical in shape, and subsequently the RBC experiences a higher range of strain and stress during the passage through small constrictions. This is consistent with our observation in the study on *P. knowlesi*-infected RBCs, where infected cells obstructed larger equivalent diameters because of a decrease in SA:V ratio of the host RBCs.

6.2 Prediction of RBC passage in cerebral capillaries

It is essential for healthy RBCs to pass through cerebral capillaries as narrow as 3 to 7 μm to deliver oxygen and nutrition to all over the human cerebral cortex. In this work, we performed simulations to evaluate the minimum required pressure for RBCs to squeeze into small capillaries with three diameters of 3, 3.5 and 4 μm . Our computational model predicts that RBCs with SA:V ratios lower than 1.3 and RBCs with membrane stiffness higher than 90 $\mu\text{N/m}$ are more prone to trapping in 3 μm diameter cerebral capillaries. Therefore, *P. knowlesi*-infected RBCs with low SA:V ratios are likely to obstruct small capillaries like cerebral capillaries. By contrast, in the absence of an adhesive phenotype, *P. falciparum*-infected RBCs (cell membrane stiffness $\leq 90 \mu\text{N/m}$) would efficiently circulate through the same capillaries.

6.1 Future Research

6.1.1 Impacts of cell stiffness and SA:V ratio on RBC dynamic behaviours in small capillaries

The presented study did not consider dynamic behaviours of RBCs, such as the rate of flow in microchannels and time constant (shape recovery rate). Dynamic behaviours of RBCs are of great importance for normal blood flow circulation in the microvasculature. It was observed in some of our experiments that some malaria parasite-infected RBCs traversed slower than other RBCs in microchannels. Therefore, a further study could assess the effects of cell stiffness and SA:V ratio on RBCs dynamic behaviours in small capillaries and examine whether RBCs with high cell stiffness or low SA:V ratios would disrupt the blood flow in small capillaries.

6.1.2 Impacts of cell rigidity on cell membrane fragility in RBCs

In all malaria parasite-infected RBC studies, we found a smaller number of infected RBCs than uninfected RBCs arrested in microchannels, despite the high initial parasitemia ($> 80\%$).

Furthermore, many intracellular parasites without the host cell membrane were observed in wedge-shaped microchannels. This mismatch suggests that infected RBCs are more fragile than uninfected cells, likely because of their altered membrane properties, such as cross-linking of the membrane skeleton and adhesive knobs formation on the outer surface of infected cells (Rug et al., 2006; Zhang et al., 2015b). Therefore, more detailed research is needed to determine what increases host membrane fragility of malaria parasite-infected RBCs.

6.1.3 RBCs behaviour in different carrier media

Our experiments showed that RBCs exhibited different behaviour when we changed the media from PBS to RPMI 1640 medium or complete culture medium (CCM). The results revealed a 12% increase in SA:V ratio of RBCs in RPMI and CCM as compared to PBS, as a result of volume reduction of RBCs in RPMI and CCM. It appears that a component or a combination of components comprising RPMI moderately reduces the volume of RBCs, subsequently increasing the SA:V ratio. Therefore, a greater focus on different components of RPMI could determine what is responsible for the volume reduction of RBCs in RPMI as compared to PBS.

6.1.4 Traversal of different stages of malaria parasite-infected RBCs

In this study, we mainly focused on malaria parasite-infected RBCs with trophozoite stages of two human *Plasmodium* species, *P. falciparum* and *P. knowlesi*. Our experimental results showed that *P. falciparum* trophozoites maintained the SA:V ratio of host RBCs; however, some previous studies reported decreases in the SA:V ratio of the earlier and later stages of *P. falciparum*-infected RBCs (Hanssen et al., 2012; Herricks et al., 2009). Therefore, different stages of malaria parasite-infected RBCs might exhibit different physical properties and thus different behaviours in the microvasculature network. A further experimental study on other stages of these two *Plasmodium* species could precisely probe the rigidity and physical changes to host RBCs during the parasite development and determine the possibility of microvascular obstructions in different stages of malaria parasite life cycle.

6.2 Takeaway

This study is the first detailed investigation of the independent impacts of cell stiffness and SA:V ratio on RBC passage through microchannels. All our experimental findings as well as computational simulations imply that circulation of RBCs through small capillaries is substantially impacted by changes to SA:V ratio rather than cell stiffness change. Our study has implications for pathophysiological conditions, such as senescent RBCs, malaria parasite-

infected RBCs and inherited RBC disorders like hereditary spherocytosis, where RBC shape changes. Our work also suggests that the measurements from techniques like ektacytometry and micropipette aspiration need to be interpreted with caution, as they exhibit more sensitivity to rigidity changes rather than SA:V ratio changes. Therefore, a combination of different techniques would provide a better measure of cellular deformability changes in RBCs affected by certain pathological diseases.

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Appendices

Appendix1. Code scripts for automated RBCs identifier

A macro plugin was written in ImageJ to locate RBCs in the wedge-shaped microchannels.

```
// Change the directory
dirc=getDirectory("C:\Arman\PhD\Experiment\Microfluidics\HEMA\test");
f=0;
for (q=1;q<=100;q++){
    if (q <= 9) {
        open(dirc+"Image-0"+q+"_R3D.dv.tif");
    }
    if (q >=10) {
        open(dirc+"Image-"+q+"_R3D.dv.tif");
    }
    f = f+1;
}

// Convert to black & white image using threshold
setSlice(2);
setAutoThreshold("Default");

// Ask use to select a point on the edge of wedge-shaped channels
setSlice(1);
setTool("point");
waitForUser("Select a point on the edge of wedge-shaped channels");
getSelectionCoordinates(x, y);
getPixelSize(unit, pw, ph);

// Split the channels and merge three of them
run("Split Channels");

// Convert to black & white image using threshold
if (q <= 9) {
    selectWindow("C2-Image-0"+q+"_R3D.dv.tif");
}
if (q >=10) {
    selectWindow("C2-Image-"+q+"_R3D.dv.tif");
}
setAutoThreshold("Default");
```

```

run("Threshold...");
waitForUser("Adjust the threshold");
// Set measurements
run("Set Measurements...", "area centroid bounding redirect=None decimal=3");
// Geometry parameters for 3.2 um depth microchip
teta = 3.14159/180*0.822;
a0 = 0.714;
pi = 3.14159;
// Run ROI Manager
run("ROI Manager...");
if (f > 1) {
    roiManager("Deselect");
roiManager("Delete");
}
// Ask user to enter number of intact RBCs trapped in microchannels
nn = getNumber("Number of cells in this image?",nn);
if (nn == 0) {
    f = 0;
}
for (j=1;j<=nn;j++) {
    setTool("wand");
    waitForUser("Choose cells one by one and click on Ok");
    roiManager("Add");
}
roiManager("Measure");
s= newArray(nn);
xm = newArray(nn);
w = newArray(nn);
for (j=0;j<nn;j++) {
    s[j] = getResult("Area", j);
    BX = getResult("BX", j);
    w[j] = getResult("Width", j);
    xm[j] = BX + w[j]/2;
}
//IJ.renameResults("ROI");
run("Clear Results");

```

```

n = nResults;
for (j=1;j<=nn;j++) {
    // Ask user to determine if the RBC is infected or not
    label = getNumber("Is the cell #"+j+" labelled? ('0'No,'1'Yes)",nn);
    dm = (x[0]*pw - xm[j-1]);
    area = s[j-1];
    width = w[j-1];
    d1 = (2*dm - 2*a0 + width)/(2*(tan(teta) + 1));
    d2 = -(2*a0 + 2*dm - width)/(2*(tan(teta) - 1));
    R2 = d2*tan(teta) + a0;
    R1 = d1*tan(teta) + a0;
    setResult("length", n, d1+R1);
    setResult("image_num", n, q);
    setResult("label", n++, label);
    setResult("length", n, d1);
    setResult("image_num", n, q);
    setResult("label", n++, label);
    setResult("length", n, d2);
    setResult("image_num", n, q);
    setResult("label", n++, label);
    setResult("length", n, d2-R2);
    setResult("image_num", n, q);
    setResult("label", n++, label);
}
saveAs("Results",dirc+"measurement"+q+".csv");
close();
if (q <= 9) {
    selectWindow("C1-Image-0"+q+"_R3D.dv.tif");
}
if (q >=10) {
    selectWindow("C1-Image-"+q+"_R3D.dv.tif");
}
close();
}

```

After locating RBCs in microchannels, the measurements were imported into R for performing calculations of volume, surface area and minimum equivalent diameter. Below is the code script written in R:

```
setwd("C:/Arman/PhD/Experiment/Microfluidics/HEMA/Infected RBCs/HEMA2_3.2_Pk-22_26h_21-6-2018")
rm(list=ls())
library("ggplot2")
# Load the data
data <- read.csv("measurement__pk_22-26h_olivia_auto_21-6-2018.CSV",header=TRUE,sep=",")
# Organising the dataset
# variable "att"
names(data)[1] <- "measure"
data$att [data$measure %% 4 == 1] <- 1
data$att [data$measure %% 4 == 2] <- 2
data$att [data$measure %% 4 == 3] <- 3
data$att [data$measure %% 4 == 0] <- 4
for (i in 1:nrow(data))
{
  if (i %% 4 == 0)
  {
    data$measure[i] <- i/4
  }
  if (i %% 4 == 1)
  {
    data$measure[i] <- (i+3)/4
  }
  if (i %% 4 == 2)
  {
    data$measure[i] <- (i+2)/4
  }
  if (i %% 4 == 3)
  {
    data$measure[i] <- (i+1)/4
  }
}
# Change the name of "measure" variable
```

```

names(data) [names(data) == "measure"] <- "cell_num"

# Reshaping to wide format
data <- reshape(data, timevar = "att", idvar = c("cell_num", "image_num", "label"), direction = "wide")

# Surface area and volume calculation
d.mean=(data$length.1+data$length.4)/2
data$d_mean = d.mean
del.d=data$length.2-data$length.3
del.d.1=data$length.1-data$length.2
del.d.2=data$length.3-data$length.4
re=0.385+0.008*d.mean
L=(d.mean*tan(3.14159/180*0.822)+0.714)*2
depth=3.23
area=L*depth-4*re^2*(1-3.14159/4)
circ=4*(2*re*3.14159/4)+2*(L+depth-4*re)
rc=sqrt((L/2-re)^2+(depth/2-re)^2)+re
data$volume <- 3.14159/6*del.d.1*(3*rc^2+del.d.1^2) + 3.14159/6*del.d.2*(3*rc^2+del.d.2^2) + del.d*area
data$surf_area <- circ*del.d + 3.14159*(rc^2 + del.d.1^2) + 3.14159*(rc^2 + del.d.2^2)
data$SA_V <- data$surf_area/data$volume
data$eq_dia <- 2*sqrt(area/3.14159)

# Label cells
data$label <- factor(data$label, levels = c(0,1), labels = c("NO", "GREEN"))

# Number of cells
n0 <- sum(data$label == "NO", na.rm = F)
n1 <- sum(data$label == "GREEN", na.rm = F)

# Calculating mean and sd values
data$d_mean_mean [data$label == "NO"] <- mean(data$d_mean [data$label == "NO"], na.rm = F)
data$d_mean_mean [data$label == "GREEN"] <- mean(data$d_mean [data$label == "GREEN"], na.rm = F)
data$d_mean_sd [data$label == "NO"] <- sd(data$d_mean [data$label == "NO"], na.rm = F)
data$d_mean_sd [data$label == "GREEN"] <- sd(data$d_mean [data$label == "GREEN"], na.rm = F)
data$vol_mean [data$label == "NO"] <- mean(data$volume [data$label == "NO"], na.rm = F)
data$vol_mean [data$label == "GREEN"] <- mean(data$volume [data$label == "GREEN"], na.rm = F)
data$vol_sd [data$label == "NO"] <- sd(data$volume [data$label == "NO"], na.rm = F)
data$vol_sd [data$label == "GREEN"] <- sd(data$volume [data$label == "GREEN"], na.rm = F)
data$surf_mean [data$label == "NO"] <- mean(data$surf_area [data$label == "NO"], na.rm = F)
data$surf_mean [data$label == "GREEN"] <- mean(data$surf_area [data$label == "GREEN"], na.rm = F)
data$surf_sd [data$label == "NO"] <- sd(data$surf_area [data$label == "NO"], na.rm = F)

```

```

data$surf_sd [data$label == "GREEN"] <- sd(data$surf_area [data$label == "GREEN"], na.rm = F)
data$SA_V_mean [data$label == "NO"] <- mean(data$SA_V [data$label == "NO"], na.rm = F)
data$SA_V_mean [data$label == "GREEN"] <- mean(data$SA_V [data$label == "GREEN"], na.rm = F)
data$SA_V_sd [data$label == "NO"] <- sd(data$SA_V [data$label == "NO"], na.rm = F)
data$SA_V_sd [data$label == "GREEN"] <- sd(data$SA_V [data$label == "GREEN"], na.rm = F)
data$eq_dia_mean [data$label == "NO"] <- mean(data$eq_dia [data$label == "NO"], na.rm = F)
data$eq_dia_mean [data$label == "GREEN"] <- mean(data$eq_dia [data$label == "GREEN"], na.rm = F)
data$eq_dia_sd [data$label == "NO"] <- sd(data$eq_dia [data$label == "NO"], na.rm = F)
data$eq_dia_sd [data$label == "GREEN"] <- sd(data$eq_dia [data$label == "GREEN"], na.rm = F)

# Relabel label
data$label <- factor(data$label, labels = c("Uninfected", "Infected"))

# plot volume
ggplot(data=data, aes(label, volume)) + theme_bw() + geom_boxplot() + labs(x="Cell type", y="Volume (fL)") +
  theme(text = element_text(size=20))

# plot surface area
ggplot(data=data, aes(label, surf_area)) + theme_bw() + geom_boxplot() + labs(x="Cell type",
y=expression(paste('Surface Area (', mu, m^2, ')'))) + theme(text = element_text(size=20))

#plot surface area/volume ratio
ggplot(data=data, aes(label, SA_V)) + theme_bw() + geom_boxplot() + labs(x="Cell type",
y=expression(paste('SA/V ratio (', mu*m^-1, ')'))) + theme(text = element_text(size=20))

#Plot equivalent diameter
ggplot(data=data, aes(label, eq_dia)) + theme_bw() + geom_boxplot() + labs(x="Cell type",
y=expression(paste('Equivalent Diameter (', mu*m, ')'))) + theme(text = element_text(size=20))

#export as csv
write.csv(data, file="Output-HEMA3.2_pk_22-26h_olivia_21-6-2018.csv")

```

Appendix2.Characteristics of RBCs treated with glutaraldehyde

Data from a repeat of study on passage of RBCs treated with glutaraldehyde confirmed that 0.005% glutaraldehyde concentration significantly rigidified RBCs (~800%) but maintained the SA:V ratio of cells (Figure S. 1c). Our results only showed a ~5% retardation in traversing microchannels for fixed RBCs compared to unfixed cells (Figure S. 1d).

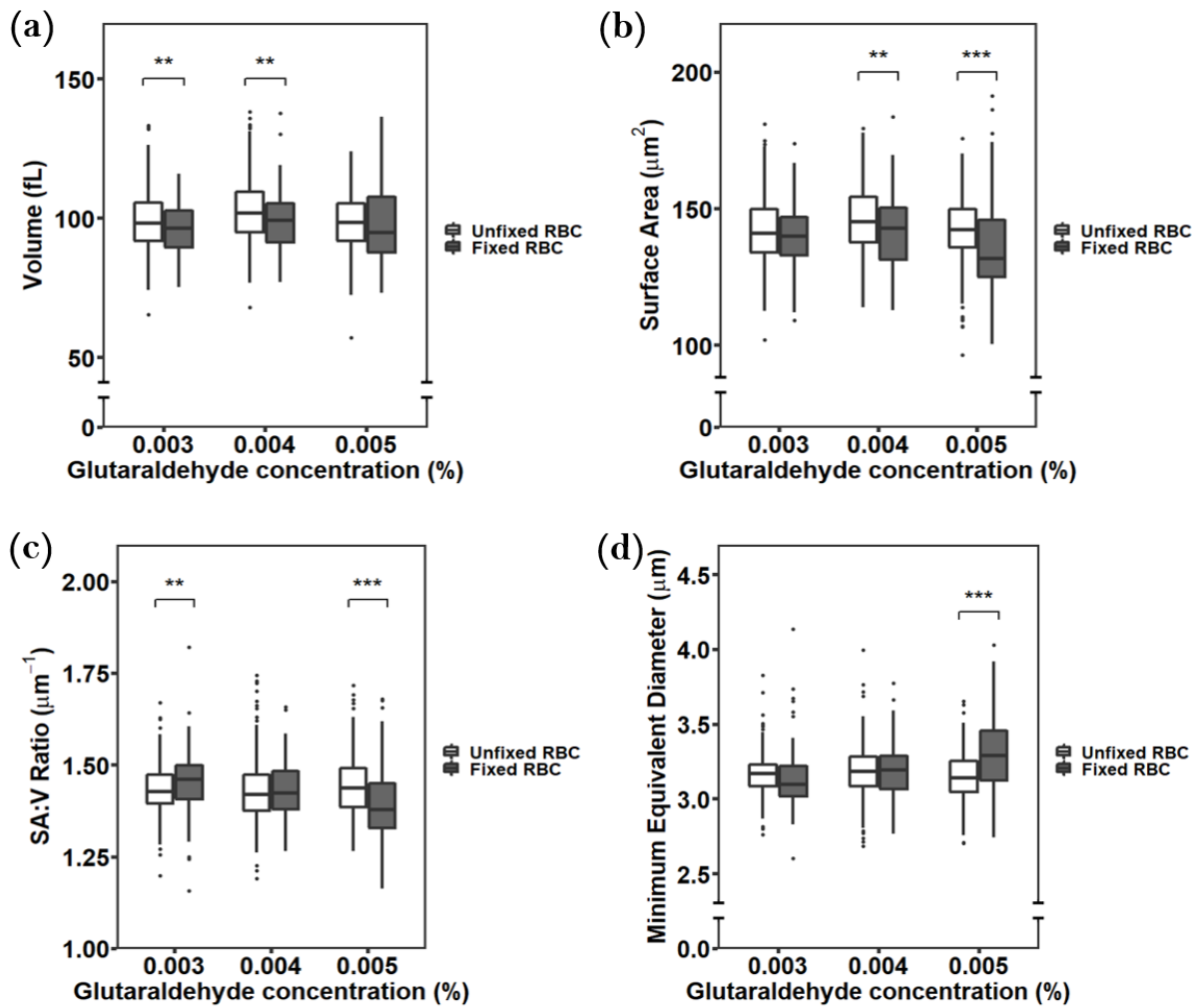


Figure S. 1 Characteristics of unfixed cells and fixed cells treated with glutaraldehyde. (a-d) Mean values of volume, surface area, SA:V ratio and minimum equivalent diameter for unfixed cells (white) and fixed cells (grey).

Appendix3.Characteristics of RBCs as the buffer osmolarity altered over 215-387 mOsm/L

The osmolarity study was repeated over a broader range of buffer osmolarity 215-387 mOsm/L. The characteristics of RBCs in various buffer osmolarities are shown in Figure S. 3. Our results showed a smooth decrease of volume of RBCs as the osmolarity increases, and subsequently, a moderate increase was observed in SA:V ratio of cells (Figure S. 3a-c). Overall, RBCs exhibited more efficient passage with increase in their SA:V ratio (Figure S. 3d).

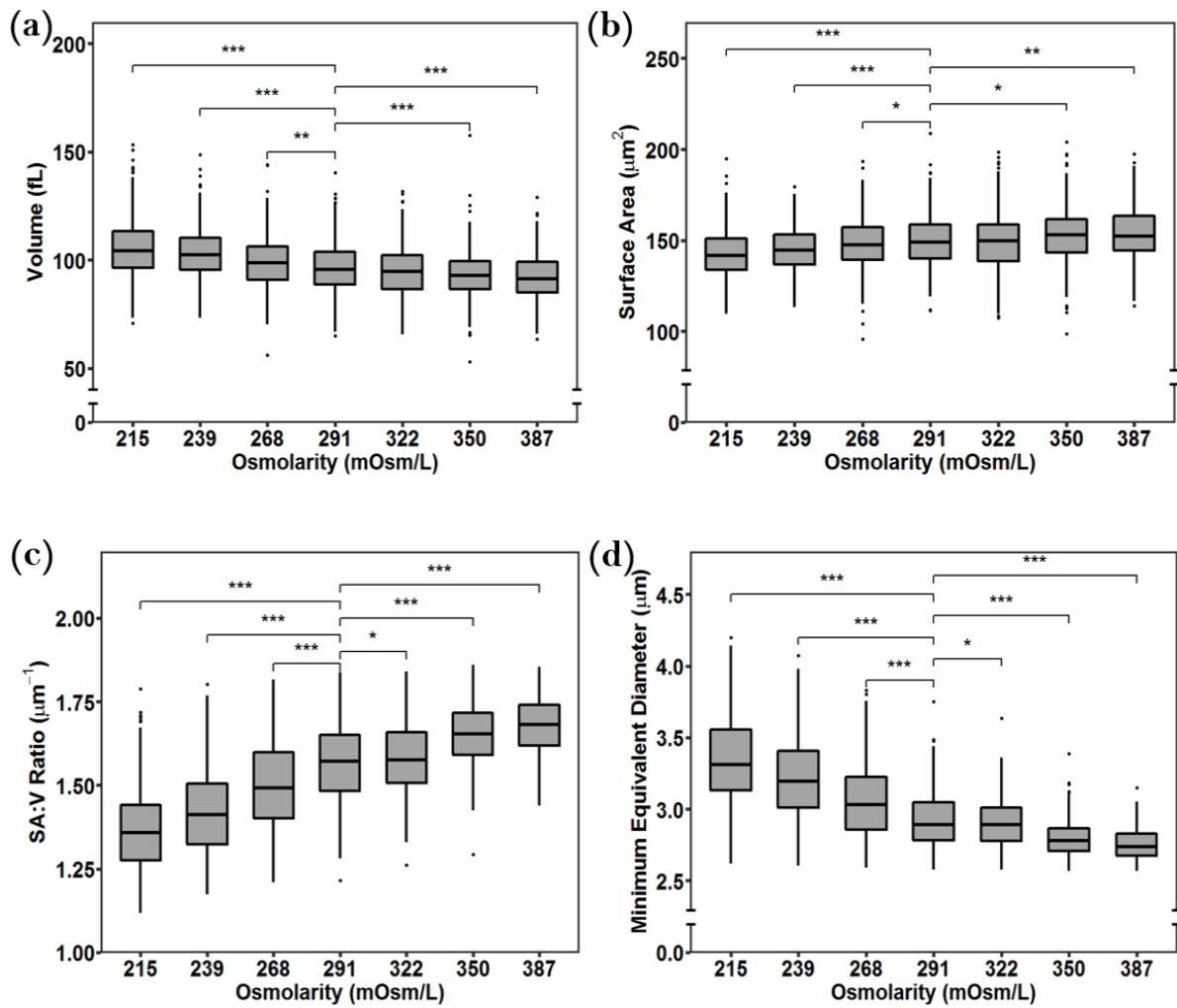


Figure S. 2 Characteristics of RBCs subjected to different buffer osmolarities. (a-d) Mean values of volume, surface area, SA:V ratio and minimum equivalent diameter for cells subjected to different buffer osmolarity.

Appendix4.Characteristics of normal RBCs in various media

We observed during our experiments that RBCs exhibited different behaviour when we changed the media from PBS to RPMI 1640 medium or complete culture medium (CCM). RPMI and CCM are widely used for cell culture applications, and both contain a variety of amino acids, vitamins and other components more than phosphate-buffered saline (PBS). In order to directly compare the impact of various media on cell characteristics, we subjected normal RBCs to four different media solutions: PBS, PBS + AlbuMAX (1%), RPMI 1640 and complete culture media (CCM) having similar osmolarity (284-292 mOsm/kg) and pH (7.3-7.5) before trapping cells in the HEMA device.

Experimental setup

The diluted blood was pumped into the microchip at a flow pressure of 180 mbar. All four experiments were conducted in the same microchip at room temperature, and after each

experiment, the microchip was flushed back with the next medium for 45 min at a flow rate of $2\ \mu\text{L}/\text{min}$ before the introduction of the next sample.

Results

Although the ektacytometry analyses showed no difference in the elongation index of cells in these media (Figure S. 3a), the microchip results showed that the volume of cells in RPMI and CCM reduced by $\sim 10\%$ as compared to PBS and PBS + AlbuMAX, and the volume reduction increased the SA:V ratio of cells by $\sim 12\%$ in RPMI and CCM (Figure S. 3b-d). As a result, cells in RPMI and CCM traversed into smaller minimum equivalent diameter ($\sim 10\%$) (Figure S. 3e). Clearly, a component or a combination of components comprising RPMI acts to decrease the volume of RBCs slightly, subsequently increasing the SA:V ratio. The presence of 1% AlbuMAX (lipid-rich BSA) did not alter the results from standard PBS.

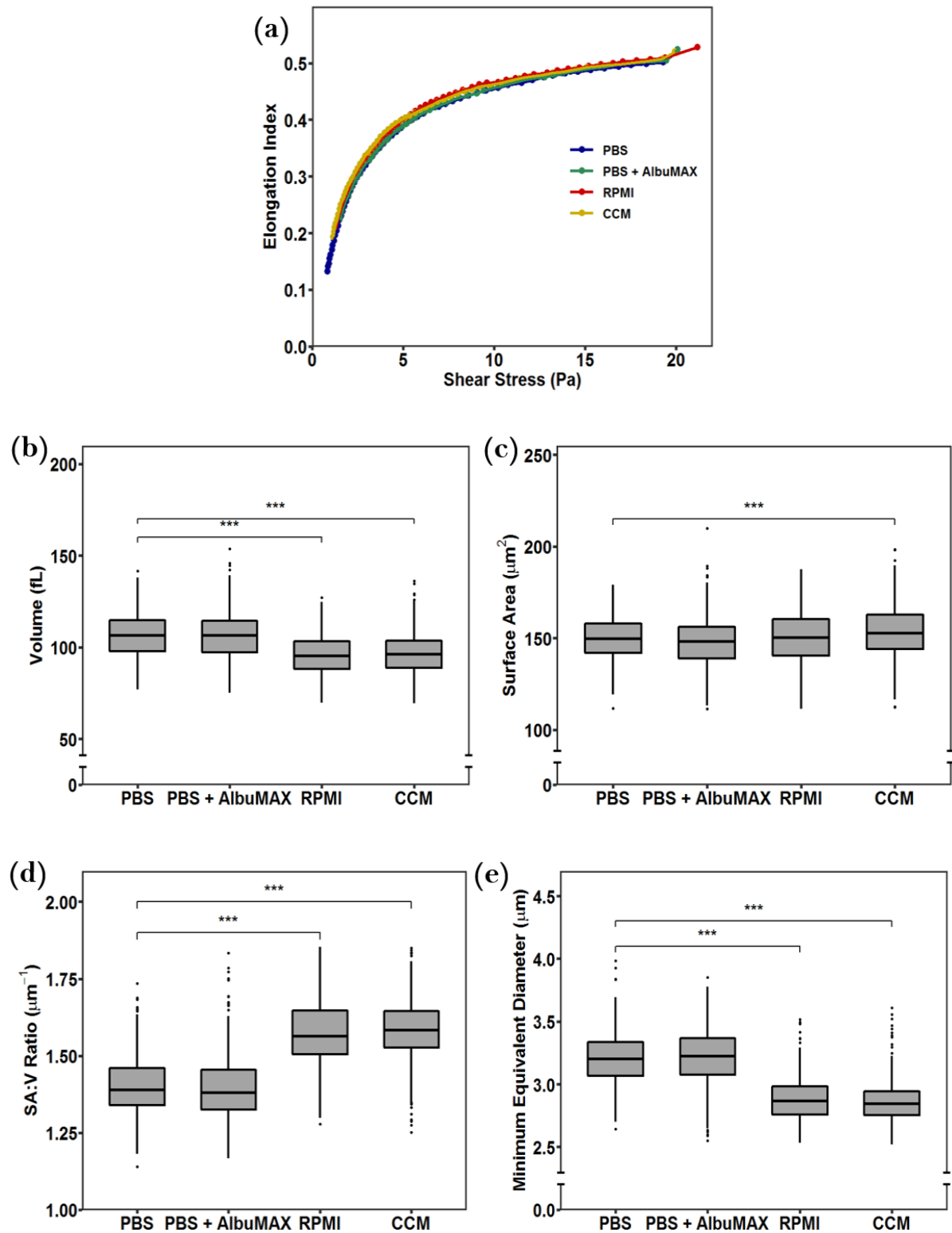


Figure S. 3 Rigidity and characteristics of RBCs subjected to various media: PBS, PBS + AlbuMAX, RPMI and CCM. (a) Ektacytometry analysis for normal RBCs in the four media. (b-e) Mean values of volume, surface area, SA:V ratio and minimum equivalent diameter for cells in the four media.

Next, we conducted a further experiment to figure out what components of RPMI are potentially responsible for the morphology alteration in cells. This time we looked further into

the impacts of glucose and inorganic compounds like calcium nitrate and magnesium sulphate with the formulas $Ca(NO_3)_2$ and $MgSO_4$. Our results showed that glucose made no changes to the cell morphology or its deformability while adding calcium nitrate and magnesium sulphate to PBS increased the SA:V ratio of cells slightly by ~4% (Figure S. 4c). However, adding calcium nitrate and magnesium nitrate to PBS did not fully account for the ~12% higher SA:V ratio of cells in RPMI in comparison with PBS. Thus, calcium nitrate and magnesium nitrate slightly facilitated the cells traversal to a smaller equivalent diameter (~3%) as compared to PBS (Figure S. 4d).

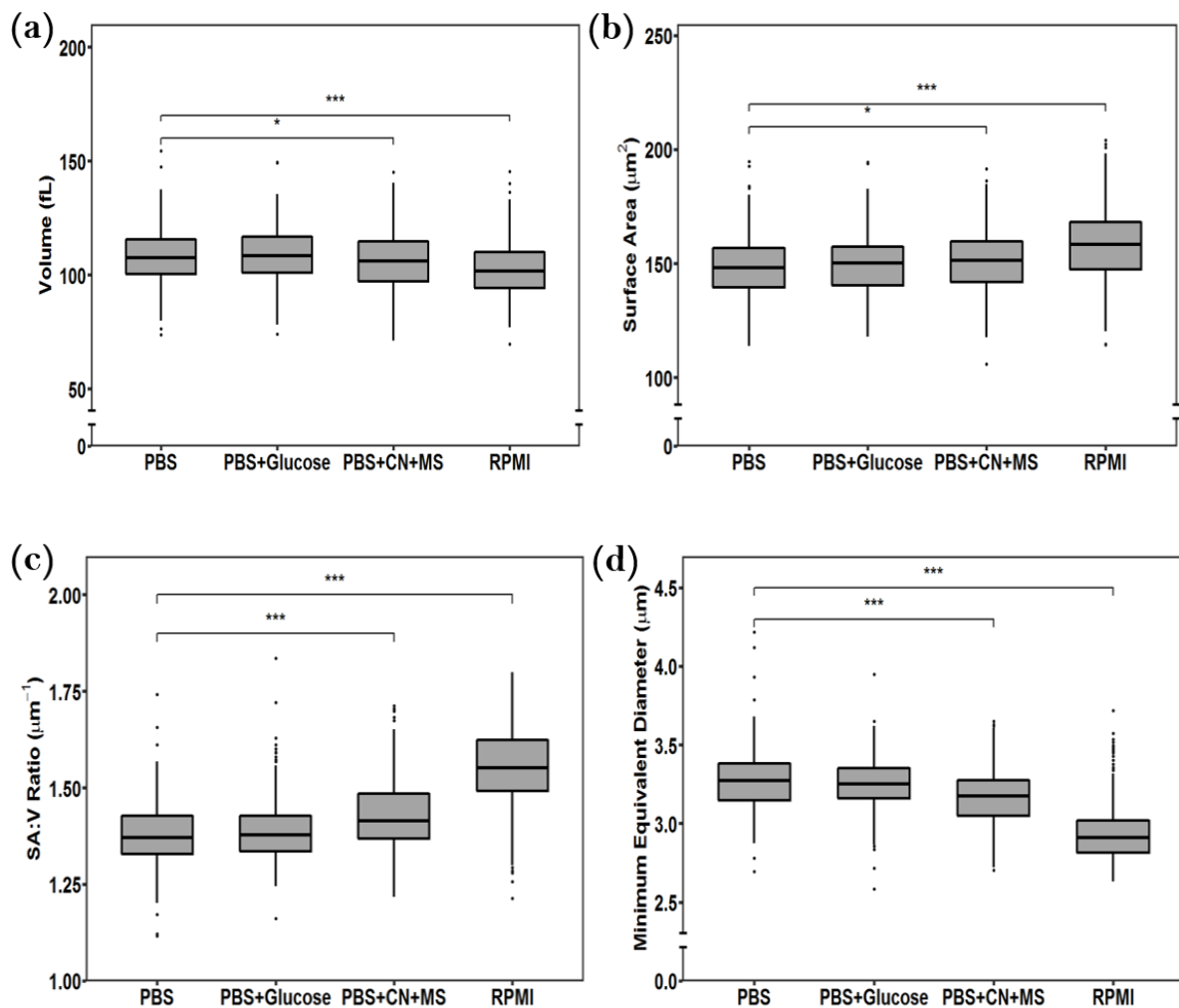


Figure S. 4 Impacts of compounds, glucose and calcium nitrate (CN) and magnesium sulphate (MS) on mean values of (a) volume, (b) surface area, (c) SA:V ratio and (d) minimum equivalent diameter compared with PBS and RPMI.

Appendix5.Characteristics of immature RBCs (reticulocytes)

Data from a repeat of examining characteristics of reticulocytes confirmed that both volume and surface area of reticulocytes decreased during the maturation, but reticulocytes exhibited a

similar SA:V ratio to mature RBCs (Figure S. 5a-c). Therefore, despite being larger, reticulocytes penetrated into microchannels efficiently like mature RBCs (Figure S. 5d).

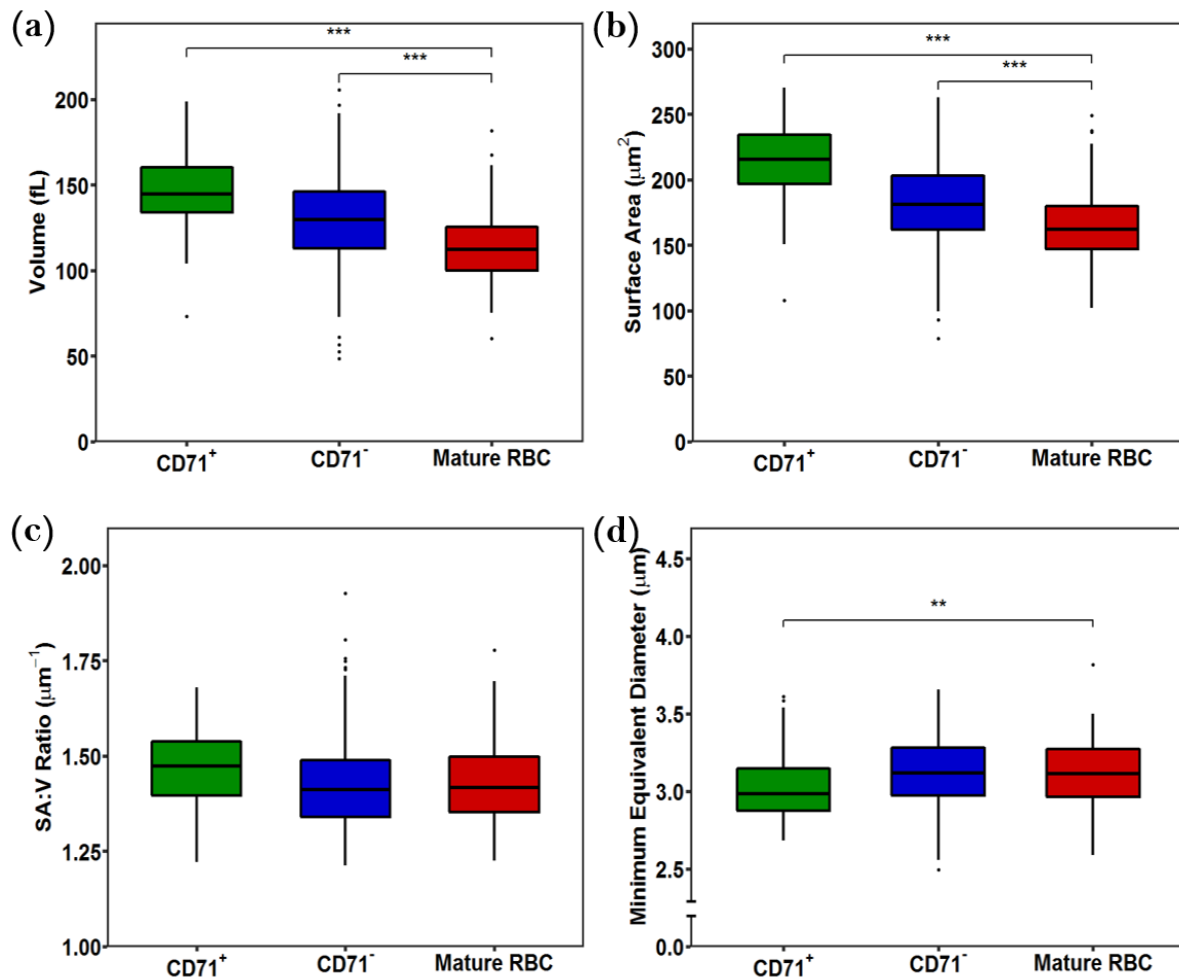


Figure S. 5 Characteristics of reticulocytes. Trapping in the HEMA device allows comparison of mean values of volume (a), surface area (b), SA:V ratio (c) and minimum equivalent diameter (d) for mature (red), older reticulocytes CD71⁻ (TfR⁻, blue) and immature reticulocytes CD71⁺ (TfR⁺, green) cells.

Appendix6.Characteristics of infected RBCs with *P. falciparum* CS2

Our results showed that the elongation index of *P. falciparum*-CS2 trophozoites (30-40 h) was substantively decreased compared with uRBCs (Figure S. 6a). While CS2 trophozoites preserved the host cell SA:V ratio likewise 3D7 strain, subsequently reaching into the same minimum equivalent diameter as did uninfected cells from the same culture (Figure S. 6d, e).

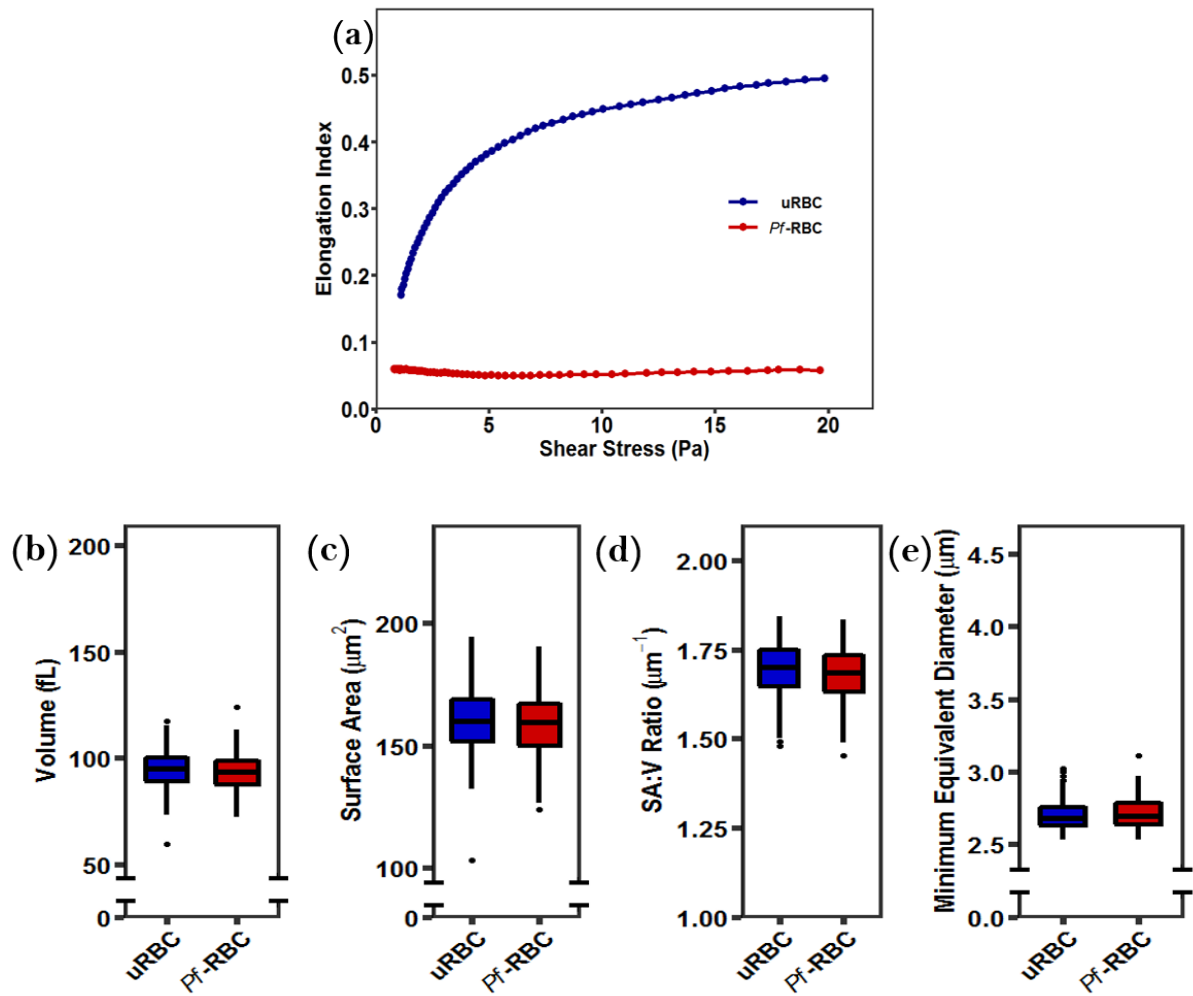


Figure S. 6 Physical parameters of *P. falciparum* CS2 iRBCs (30-40 h). (a) Ektacytometry analysis (0-20 Pa shear stress) for uRBCs (blue line) and *P. falciparum* iRBCs (red line). (b-e) Comparison of mean values of volume, surface area, SA:V ratio and minimum equivalent diameters in uninfected RBCs (blue) and iRBCs (red).