A NUMERICAL STUDY OF MORPHOLOGIES AND MORPHOLOGICAL TRANSFORMATIONS OF HUMAN ERYTHROCYTE BASED ON MEMBRANE MECHANICS

by

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A NUMERICAL STUDY OF MORPHOLOGIES AND MORPHOLOGICAL TRANSFORMATIONS OF HUMAN ERYTHROCYTE BASED ON MEMBRANE MECHANICS

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Abstract

Certain chemical agents cause normal human erythrocytes to undergo morphological transformations in which they assume cup (stomatocytic), biconcave discoid (discocytic), or spiculated (echinocytic) shapes. We use continuum mechanics to give the first solid theoretical explanation of these morphologies and morphological transformations.

We argue that erythrocyte morphologies are governed by erythrocyte membrane mechanical properties, namely, the plasma membrane’s resistance to bending deformations plus the area and volume constraints imposed by the plasma membrane’s resistance to area dilation and the requirement of osmotic balance and, in addition, the membrane skeleton’s resistance to local shear and area deformations. These properties result in an overall free energy $F$. Stable morphologies minimise $F$.

The plasma membrane is described using the area-difference-elasticity model. The membrane skeleton is treated as an isotropic hyperelastic surface. The resultant $F$ contains two important parameters, $\bar{m}_0$ and $V_{\text{rest}}$, the reduced effective preferred area difference between the neutral surfaces of the plasma membrane leaflets and the volume of the unstressed shape of the membrane skeleton, respectively. $\bar{m}_0$ is the parameter that responds to inducing agents: echinocytogenic agents increase $\bar{m}_0$, whereas stomatocytogenic agent decrease $\bar{m}_0$.

$F$ is minimised numerically at given values of $\bar{m}_0$ and $V_{\text{rest}}$ using a Monte Carlo technique. This gives predicted locally stable morphologies and morphological transformations as a function of $\bar{m}_0$ and $V_{\text{rest}}$. We group these morphologies into 16 classes. At each value of $\bar{m}_0$ and $V_{\text{rest}}$, one or several morphologies are locally stable. We are, thus, able to construct a stability diagram describing the morphological transformations. We deduce that $V_{\text{rest}}$ needs to be approximately 95% of the volume of a sphere with the same area as an erythrocyte for there to be qualitative agreement with observed morphological transformations. We predict the existence of hysteresis in some morphological transformations. Lastly, we are able to predict the membrane skeletal strains as $V_{\text{rest}}$ and $\bar{m}_0$ are varied.

Our calculations clarify, extend, and validate the so-called bilayer couple hypothesis of Sheetz and Singer (1974).
Dedicated to the late
Professor Marcel Bessis (1917–1994).
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Chapter 1

Introduction

The normal human red blood cell (RBC) or erythrocyte is formed of a thin, semi-permeable outer membrane surrounding an inner solution of hæmoglobin and salts. When suspended in a medium with physiological osmolarity (~ 290 mosmol/L) and pH (~ 7.4) at room temperature (20–25 °C), it generally assumes a biconcave discoid shape with an approximate diameter of 8 μm and an intracellular hæmoglobin concentration in the range 0.27–0.37 g/mL (based on whole blood measurements [144]). The volume $V$ of its liquid content depends on the osmolarity of the suspending medium. At the aforementioned physiological osmolarity, a RBC with area $A$ has a volume $V$ that is less than the maximum value, which corresponds to the volume of a spherically swollen RBC with the same area. In other words, $A$ is in excess of the minimum required to hold $V$ under physiological conditions. This excess area is commonly gauged by the quantity $A/V$, the RBC surface area-to-volume ratio$^1$. Like the intracellular hæmoglobin concentration, $A$ and $V$ have a range of natural variability, in that their values vary within the RBC population of an individual as well as from individual to individual. The values of $A$ and $V$ have been measured extensively because of their medical importance. Table 1.1 shows a compilation of some measurements of $A$ and $V$ under physiological conditions. The average of the mean values of $A$ is $(135 \pm 14) \, \mu m^2$ and the average of the mean values of $V$ is $(97 \pm 14) \, \mu m^3$, respectively.

The membrane of the RBC strongly resists area dilation and, thus, effectively imposes

---

$^1$Although $A/V$ is not dimensionless, it is nevertheless widely used in hæmatology as a measure of the RBC’s geometry [141].
CHAPTER 1. INTRODUCTION

Table 1.1: A chronologically-ordered compilation of some previous measurements of \( A \) and \( V \) for unfractionated adult blood samples in isotonic solutions at room temperature (20–25 °C). In the fourth column, the sample size is given in the form: number of subjects from whom blood was drawn / combined total number of cells. Each entry in columns 7 and 8 is given in the form: mean (±) standard error of mean (±) population standard deviation.

<table>
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<tr>
<th>Ref.</th>
<th>Note</th>
<th>Year</th>
<th>Sample Size</th>
<th>Osmolarity/Osmolality</th>
<th>pH</th>
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<th>( V (\mu m^3) )</th>
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<td>SEM</td>
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<tr>
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<td>136.9</td>
<td>0.5</td>
</tr>
<tr>
<td>104</td>
<td>c, e</td>
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<td>310 ± 2 mOsm</td>
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<td>133.4</td>
<td>0.5</td>
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<tr>
<td>68</td>
<td>b</td>
<td>1981</td>
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<td>298–307 mOsm</td>
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<td>129.95</td>
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<td>127</td>
<td>f</td>
<td>1982</td>
<td>5 / 200</td>
<td>297 mosmol/kg</td>
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<td>134.1</td>
<td>13.8</td>
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<tr>
<td>154</td>
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<td>295–300 mosmol/kg</td>
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<td>198</td>
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<td>295 mosmol/kg</td>
<td>7.4</td>
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<td>9</td>
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\( a \) Manual tracing of diametrical cross-sections of cells hanging vertically from underside of microscope coverslips photographed edge-on.

\( b \) Determination of geometries of cells by interference holography.

\( c \) Digital tracing of diametrical cross-sections of cells hanging vertically from underside of microscope coverslips photographed edge-on.

\( d \) In Ringer solution without albumin.

\( e \) In Ringer solution with albumin.

\( f \) Video recordings of geometries of micropipette-aspirated cells.

\( g \) From text of Ref. 68; values given in the abstract of Ref. 68 are different (1.03 \( \mu m^2 \) for \( A \) and 1.06 \( \mu m^3 \) for \( V \)).

\( h \) Digitized images of diametrical cross-sections of cells hanging vertically from underside of microscope coverslips viewed edge-on.

an area constraint on its deformation. Within this geometric constraint, however, the RBC still exhibits a remarkable deformability because of the excess area and the weak bending and shear elasticities of its membrane [38, 61, 144, 145, 151]. The excess area imparts the necessary geometrical freedom for it to deform, while the weak bending and shear elasticities offer weak resistance to large bending and shear strains. This deformability, in turn, allows the RBC in vitro to assume numerous distinctive steady-state shapes, among
**CHAPTER 1. INTRODUCTION**

1.1 RBC shapes and shape transformations

A pictorial classification of these steady-state shapes is shown in Figure 1.1. In this context, the normal, disc-shaped RBC is known as a discocyte. By *in vitro* treatment with a large variety of so-called *echinocytogenic* or *stomatocytogenic* agents [14, 232], some typical examples of which are listed in Table 1.2, discocytes may be transformed into either *echinocytes*\(^2\) or *stomatocytes* [14, 33, 46, 57, 66, 69, 116, 152, 153, 171–173, 188, 199].

---

\(^2\)Also known as *spur* cells, especially in older medical literature.
CHAPTER 1. INTRODUCTION

<table>
<thead>
<tr>
<th>Stomatocytogenic</th>
<th>Echinocytogenic</th>
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<tbody>
<tr>
<td>cationic amphipathic drugs</td>
<td>anionic amphipathic drugs</td>
</tr>
<tr>
<td>cholesterol depletion</td>
<td>cholesterol addition</td>
</tr>
<tr>
<td>low salt (hypotonic saline)</td>
<td>high salt (hypertonic saline)</td>
</tr>
<tr>
<td>low pH</td>
<td>high pH</td>
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<tr>
<td></td>
<td>ATP depletion</td>
</tr>
<tr>
<td></td>
<td>proximity to glass</td>
</tr>
</tbody>
</table>

Table 1.2: Typical examples of stomatocytogenic and echinocytogenic agents.

These terms allude to the former’s surface crenations and the latter’s cup shapes, as illustrated by representative images of echinocytes and stomatocytes in Figure 1.2.

Each of these broad classes consists of four stages of shape evolution, differentiated by their finer morphological characteristics. In order of increasing strength of an echinocytogenic agent, the four observed stages of echinocytosis are:

**Echinocyte I** A disc with several undulations on its rim.

**Echinocyte II** A roughly elliptically oblate body with distinct spicules distributed quite evenly over its surface.

**Echinocyte III** An ovoid or sphere with regularly spaced spicules that are sharper and more numerous, numbering about 30–50.

**Sphero-echinocyte** A sphere with spicules that have shortened and sharpened to needle-like projections.

Representative photographs of these shapes are shown in Figure 1.2 (e, f and g), which show clearly the RBC membrane’s increasing tendency towards *exterior budding*.

For stomatocytosis, on the other hand, the four observed stages in order of increasing strength of a stomatocytogenic agent are:

**Stomatocyte I** A cup shape with a shallow, circular invagination.

**Stomatocyte II** A cup shape with a deep invagination.

**Stomatocyte III** A cup shape with a deep invagination that elongates into a mouth-like slit, possibly accompanied by other pit-like membrane indentations.

**Sphero-stomatocyte** A sphere with minute intracellular sacs attached to the membrane and small vesicles in the cytoplasm.
Figure 1.2: Stomatocytes III, II and I (a, b and c), discocyte (d) and echinocytes I, II and III (e, f and g). Images a, b and f are reprinted from Fig. 119 of Ref. 12, Plate I(a) of Ref. 29 and Fig. 1 of Ref. 106, respectively, with permission of Elsevier. Images c and d are reprinted from Fig. 2A of Ref. 104 with permission of the Biophysical Society. Images e and g are reprinted from Figs. 28-20 and 28-22, respectively, of Ref. 112.
In contrast to echinocytosis, stomatocytosis is marked by the RBC membrane’s increasing tendency towards interior budding, as shown in Figure 1.2 (a, b and c).

In both echinocytosis and stomatocytosis, shape reversal is not possible once a terminal stage is reached, as the precursor to the formation of a spherocytosis or spherostomatocytosis is the shedding of excess membrane area by exocytotic or endocytotic vesiculation. Rapid haemolysis (loss of RBC membrane integrity) occurs on further loading of the terminal stages. The observations leading to the pictorial classification above seem to be based entirely on the behaviour of populations of cells. Not surprisingly, with the natural variation of cellular properties in a population, there is a spread of cell morphologies across several stages in a population [66, 69, 89, 171, 172, 188] at a given strength of the inducing agent; but, all can be driven to the terminal stages. Qualitative observations of RBC shape transformations suggest that every normal RBC can be made to cycle through each of the non-terminal stages in a reversible fashion, with the shape reversal being accomplished by removing the inducing agent or adding the agent’s antagonist (adding a stomatocytogenic agent after an echinocytogenic agent has been applied or vice versa). This sequence of shape transitions, depicted in Figure 1.1, is commonly known in hæmatology as the stomatocyte-discocyte-echinocyte (SDE) shape transformations.

In addition to shapes belonging to the dominant SDE transformations, other minor shapes can occur naturally or by design. Three of these are of particular interest to us as a consequence of their being found among the catalogue of shapes successfully predicted in this work. These three shapes (Figure 1.3), with the last two occurring naturally, are:

Asymmetric discocyte A discocytic RBC with an uneven rim thickness, produced by treating an osmotically swollen, nearly spherical RBC with diamide and then osmotically shrinking it back to the normal volume [67].

Triangular stomatocyte A cup shape with a deep triangular invagination [29]. Stomatocyte II sometimes transforms to this shape instead of stomatocyte III [13].

Knizocyte A triconcave RBC [14], found predominantly in healthy newborn infants [179].

It can also be observed in certain hæmolytic anæmias, such as hereditary spherocytosis [13].

The history of RBC shape observation dates back to the seventeenth century. The detection of individual human RBCs was made possible by the invention of the light microscope by Anton van Leeuwenhoek of Holland (1632–1723). Not surprisingly, he was also the first
CHAPTER 1. INTRODUCTION

Figure 1.3: Light (a) and scanning electron (b and c) microscope images of (a) an asymmetric discocyte, (b) a triangular stomatocyte and (c) a knizocyte. (a) is reprinted from Fig. 5C of Ref. 67 with permission of the Biophysical Society; (b) is reprinted from Plate I(b) of Ref. 29 with permission of Elsevier; and (c) is reprinted from Fig. 28-16 of Ref. 112.

person to observe individual human RBCs in the year 1674, using his invention. Much later, in 1895, Hamburger [79] made the first observation of reversible RBC morphological transformation induced by changes of solution tonicity. Ponder [166] performed comprehensive investigations in the 1930’s to identify all shape classes of the discocyte-echinocyte transformation accompanied or unaccompanied by volume changes. His discoveries constitute the echinocytic half of the SDE transformations. Subsequent experiments in the 1960’s, particularly the work of Deuticke [46], firmly established the discocyte-stomatocyte transformation. Bessis [14] combined the morphological observations of both the discocyte-echinocyte transformation and the discocyte-stomatocyte transformation into an overall pictorial classification of RBC morphologies, namely, the SDE transformations illustrated in Figure 1.1, which has become the standard description in use today.

In spite of the wealth of knowledge about the RBC accumulated over more than three centuries, a convincing explanation for the shapes and shape transformations of the normal human RBC had remained elusive. As recently as 2001, the RBC physiologist J.F. Hoffman [92] stated this problem as the first of his “… own most perplexing and cherished red cell problems…” in a commentary entitled ‘Questions for Red Blood Cell Physiologists to Ponder in This Millenium.’ We believe our work, a brief report of which has been published
in 2002 [125], is a good answer for Dr. Hoffman.

1.2 Structural organisation of the RBC membrane

Unlike most other cells, the RBC lacks internal stress-bearing structure; therefore, its shape can only be governed by its membrane. The schematic structural organisation of the RBC membrane is shown in Figure 1.4. The thin, quasi-two-dimensional RBC membrane is a composite structure, composed of a fluid outer layer and an inner layer of polymerised proteins [146]. These are referred to as the plasma membrane and membrane skeleton,
respectively. These two layers are coupled together mainly by protein anchors at discrete
locations. The plasma membrane is organised into a fluid bilayer structure with a highly
heterogeneous distribution of phospholipids, cholesterol, and membrane-bound proteins,
including the transmembrane proteins band 3 and glycophorin C that are involved in cou-
pling the plasma membrane to the membrane skeleton. The membrane skeleton is a quasi-
hexagonal multi-protein network composed mainly of actin, band 4.1, ankyrin, and spectrin.
Spectrin in the RBC exists mainly in the tetrameric form; the tetramer arises from the head-
to-head association of two spectrin heterodimers. Each junction of the protein network is
a protein complex formed by the band 4.1-assisted binding of approximately six spectrin
tetramers to one actin, with one band 4.1 molecule for every spectrin tetramer at the spectrin
binding site on actin [10]. Each junction complex is coupled to the plasma membrane by the
binding of each of its approximately six band 4.1 molecules to a glycophorin C molecule
of the plasma membrane [233]. In addition, one ankyrin molecule binds to a site near the
midpoint of each spectrin tetramer of the membrane skeleton and to a band 3 tetramer of
the plasma membrane [221]. Of these two protein linkages, the latter is known qualitatively
to be much stronger. A review of the structural organisation of the RBC membrane is given
in Appendix A.

1.3 RBC membrane mechanical properties governing
      RBC shapes and shape transformations

When an agent interacts with the RBC membrane and causes shape changes, one infers
that the agent must be changing certain properties of the RBC membrane which govern
the RBC shape. We argue that the RBC shape is governed by mechanical properties of
the RBC membrane (what these properties are will be explained shortly). The fact that
the various shape transformations induced by chemically diverse agents all belong to the
same sequence of SDE transformations suggests that the agents drive shape transformations
predominantly through one parameter of the RBC membrane mechanical properties (what
this parameter is will be explained shortly).

We shall argue that the mechanical properties governing the RBC shape are:

1. The plasma membrane’s resistance to (a) dilation of the overall area \( A \), (b) change in
CHAPTER 1. INTRODUCTION

volume $V$ away from the volume set by the osmolarity of the suspending medium, and (c) bending deformations which change the local mean curvature away from its preferred value.

2. The resistance of the membrane skeleton to local shear and area deformations.

A stable, steady-state shape of the RBC is assumed to be a shape that minimises the net mechanical energy corresponding to these elasticities.\(^3\)

As will be shown in Section 2.3, the characteristic energies required to change $A$ and $V$ are much larger than the other energies. Therefore, $A$ and $V$ are effectively constrained. For example, a prototypical discocytic RBC under physiological conditions is subjected to the constraints

$$A \approx A_{RBC},$$
$$V \approx V_{RBC},$$

where $A_{RBC}$ and $V_{RBC}$ are, respectively, the nominal average area and volume of the normal RBC, as shown in Table 1.1.

The relative size of $A$ and $V$ plays an important role in RBC morphology. $A$ is set by the amount of material in the plasma membrane. $V$ is determined by the osmolarity of the suspending medium and can be varied by changing that osmolarity. It is well appreciated that osmotically swelling the RBC reduces the geometrical freedom available for the RBC to deform. In particular, when it is fully swollen in a hypotonic solution, the RBC can only assume a spherical shape, regardless of the presence of any inducing agent. When fully swollen to a sphere, the RBC develops an elevated internal pressure and is said to be turgid. A turgid RBC cannot be deformed easily (the effects of an inducing agent are insufficient) because of its complete loss of excess area. When the osmolarity of the suspending medium is such that $V$ is sub-maximal, the RBC can undergo deformation at fixed $A$ with

\(^3\)Strictly speaking, the predicted locally stable shapes correspond to observed equilibrium shapes at temperature $T = 0$ only. Nevertheless, we can generally neglect thermally excited deviations from these locally stable shapes at room temperature. The mean-square amplitude of an eigenmode of these thermal shape fluctuations is roughly $k_B T/\epsilon_k$ [236], where $k_B$ is Boltzmann's constant and $\epsilon_k$ is the energy of the eigenmode. The usual situation is $\epsilon_k \gg k_B T$, which means thermal effects are negligible. In exceptional cases where a low-lying eigenmode becomes "soft" (the corresponding $\epsilon_k$ becomes comparable to or smaller than $k_B T$) in the vicinity of a mechanical instability, thermal fluctuations will, of course, play an important role.
little energy cost and is said to be flaccid. We deal exclusively with shape transformations of a flaccid RBC, where the changes in $A$ and $V$ from $A_{RBC}$ and $V_{RBC}$, respectively, are negligible.

In Chapter 2, we shall write down the free energy functional that governs the shapes available to the flaccid RBC. This functional contains several parameters; however, only one is crucial in driving the universal shape changes referred to above. This key parameter, denoted $\overline{m}_0$ in our theory, is known as the reduced effective preferred area difference between the neutral surfaces of the leaflets of the plasma membrane. $\overline{m}_0$ is a dimensionless parameter that measures the "bending tendency" of the plasma membrane, i.e., its tendency to bulge outward, as in echinocytes, or inward, as in stomatocytes. The effect of an echinocytogenic agent is to increase $\overline{m}_0$, whereas the effect of a stomatocytogenic agent is to decrease $\overline{m}_0$. $\overline{m}_0$ encapsulates the net effect of the two mechanisms by which an inducing agent drives shape transformations, namely, changing the area of one leaflet of the plasma membrane relative to the other and preferentially changing the average molecular shape of molecules in one leaflet of the plasma membrane. The qualitative notion of the first mechanism was proposed initially by Sheetz and Singer [189]. In the modern interpretation of their so-called bilayer couple hypothesis, a relative expansion of the outer leaflet (or relative contraction of the inner one), such as a preferential intercalation of amphipathic molecules into the outer leaflet, produces a tendency to form convex structures on the cell surface to accommodate the extra area, thereby inducing echinocytosis. Conversely, a relative contraction of the outer leaflet (or relative expansion of the inner one) favours concavities, thus inducing stomatocytosis. The second mechanism by which inducing agents drive shape transformations is especially relevant when the inducing agents are amphipathic compounds whose head and tail regions are significantly different in size. Exogenous amphipathic molecules with a polar head group cross-sectional area that is much larger than that of the acyl chains produce a tendency towards echinocytosis (or stomatocytosis) if they preferentially intercalate into the outer (or inner) leaflet. Conversely, exogenous amphipathic molecules with a polar head group cross-sectional area that is much smaller than that of the acyl chains produce a tendency towards stomatocytosis (or echinocytosis) if they preferentially intercalate into the outer (or inner) leaflet.

The results of experimental [11, 28, 53, 109, 117, 180, 205, 231] and theoretical [21, 28, 83, 84, 102, 142, 187, 206, 231, 237] studies of fluid lipid vesicles strongly suggest
that the bending elasticity of the plasma membrane alone is insufficient to reproduce all shape classes of the SDE transformations. A fluid lipid vesicle is simply a closed bilayer composed of lipid molecules in the fluid phase. It is similar in behaviour to the plasma membrane and, thus, is a good model of the plasma membrane. Narrow-necked geometries are common in observed and predicted vesicle shapes; however, normal RBCs have never been found to adopt such shapes. This difference in behaviour between vesicle shapes and RBC shapes has been attributed to the (in-plane elasticities of the) membrane skeleton [96, 227, 234]: the membrane skeleton prevents the formation of narrow-necked shapes because the highly sheared neck region of a narrow-necked shape would incur a large cost in membrane skeletal shear energy.

1.4 Thesis objective

It is the main goal of this Thesis to demonstrate theoretically that both the bending elasticity of the plasma membrane and the in-plane elasticities of the membrane skeleton must be taken into account in order that the full sequence of SDE transformations should occur in response to induced changes in \( \overline{m}_o \). It will be shown that such a phenomenological model does successfully predict shapes and shape transformations closely resembling those of the SDE transformations (see Ref. 125 for a recent brief report of this work).

1.5 Thesis overview

We conclude this introduction with an overview of the remainder of this Thesis. It has been stated previously that a stable RBC shape is assumed to be a shape that minimises the overall mechanical energy of the plasma membrane and membrane skeleton. Therefore, one must first formulate an overall free energy functional \( F \) for the RBC membrane and then solve for the theoretical shape \( S \) that minimises \( F \) under appropriate conditions. Chapter 2 describes the formulation of \( F \) based on existing theoretical and experimental knowledge of the RBC and its membrane. In practice, \( F \) can only be minimised numerically. Chapter 3 describes the numerical technique used to minimise \( F \) and hence find \( S \). Chapter 4 describes the results of the numerical minimisation of \( F \) and gives important predictions regarding the observed RBC shapes and shape transformations. Finally, Chapter 5 gives
further comments on a few matters related to or arising from this work, and a summary of the important features and predictions of our model.
Chapter 2

Continuum Elastic Model of the RBC

This chapter describes the formulation of the overall free energy functional of the RBC membrane, $F[S, S_0]$, where the two surfaces $S$ and $S_0$ represent the theoretical RBC shape and the unstressed (or resting) shape of the membrane skeleton, respectively. $F[S, S_0]$ is composed of $F_{PM}[S]$ and $F_{MS}[S, S_0]$. The former is the free energy functional of the plasma membrane, whereas the latter is the free energy functional of the membrane skeleton. Sections 2.1 and 2.2 are devoted to discussions of the functional forms of $F_{PM}[S]$ and $F_{MS}[S, S_0]$, respectively. Section 2.3 presents some simple calculations to explain why the area $A$ and volume $V$ of a flaccid RBC are effectively constrained and, also, gives a brief discussion of the osmotic swelling of a RBC from the flaccid stage to the turgid stage. Section 2.4 gives a brief comparison of different models of the RBC membrane that include both bending and in-plane elasticities. Section 2.5 summarises the information presented in Sections 2.1 and 2.2 that is needed later. The important control parameters to emerge from the theoretical analyses in Sections 2.1 and 2.2 are $m_0$ and $V_{\text{rest}}$, respectively. $m_0$ is the mechanical parameter assumed to be driven by the inducing agents; $V_{\text{rest}}$ is a parameter describing the unknown shape $S_0$. In Chapter 3, we will show how to minimise $F[S, S_0]$ numerically for given values of $m_0$ and $V_{\text{rest}}$. The minimum-energy shapes shown in Chapter 4 constitute our predictions for stable RBC shapes.
CHAPTER 2. CONTINUUM ELASTIC MODEL OF THE RBC

2.1 Free energy of the plasma membrane

In our model, the overall elastic free energy functional for deformations of the plasma membrane, $F_{PM}$, has five components:


where the surface $S$ describes the geometry of the RBC membrane. The first two components will be discussed in Section 2.1.1 and the remaining three components in Section 2.1.3.

2.1.1 Constraint-compliance energy

$F_V$ arises from the osmotic balance between the interior and the exterior of the RBC, i.e., between the cytoplasm and the extracellular space. $F_A$ originates from the resistance of the plasma membrane to area dilation. The functional forms of $F_V$ and $F_A$ are

$$F_V[S] \approx \frac{K_V (V[S] - V_0)^2}{2V_0},$$

$$F_A[S] \approx \frac{K_A (A[S] - A_0)^2}{2A_0},$$

which are valid for small deviations of $V$ from $V_0$ and $A$ from $A_0$, respectively. The actual volume $V$ and area $A$ of a RBC with specified shape $S$ are given by the integrals

$$V[S] = \frac{1}{3} \int_S \mathbf{r} \cdot \mathbf{n}(\mathbf{r}) \, dA,$$

$$A[S] = \int_S dA,$$

where $\mathbf{r}$ is a point on $S$ and $\mathbf{n}$ is the outward unit normal to $S$ at the point $\mathbf{r}$. The constants $V_0$ and $A_0$ in Equations (2.2) and (2.3) are the preferred volume and area of $S$, respectively. $K_V$ is an "osmotic modulus" set by the osmolarity of the suspending medium [see Equation (2.39) in Section 2.3]. $K_A$ is the stretching (or area compressibility) modulus of the plasma membrane.

In practice, there are uncertainties in $V$ and $A$ arising from the finite thickness of the RBC membrane; however, these uncertainties are negligible because the thickness of the RBC membrane is much smaller than the RBC diameter.
The calculations in Section 2.3 show that the characteristic energies required to change $V$ and $A$ by factors of order unity are much larger than the other component energies of the overall free energy $F$. Thus, in practice, we can disregard the physical origin of $F_V$ and $F_A$ and treat them simply as fictitious energies for enforcing volume and area constraints:

\[ V[S] = V_0, \quad A[S] = A_0. \]  

(2.6) 

(2.7)

In this context, we regard the constants $K_V$ of $F_V$ and $K_A$ of $F_A$ as adjustable computational parameters. For our convenience later, we define the fictitious constraint-compliance energy,

\[ F_{\text{constraint}}[S] \equiv F_V[S] + F_A[S]. \]  

(2.8)

2.1.2 Constraint-compliance energy parameter values

Physically, $V_0$ is the volume at which the respective concentrations of all osmotically active molecules inside and outside the RBC equalise. We are interested in shape transformations where the osmolarity is always physiological; therefore, we set $V_0 = V_{\text{RBC}}$ in Equation (2.6). The area of a normal, discocytic RBC under physiological conditions is constrained by the hydrophobic effect to be $A = A_0 = A_{\text{RBC}}$. The transformation of the normal discocyte to another shape usually occurs with negligible change in $A_0$ from $A_{\text{RBC}}$; therefore, we set $A_0 = A_{\text{RBC}}$ in Equation (2.7). We choose the parameter values

\[ V_{\text{RBC}} = 100 \mu m^3, \quad A_{\text{RBC}} = 140 \mu m^2, \quad \frac{K_V}{\kappa_b} = 5 \times 10^4 \mu m^{-3}, \quad \frac{K_A}{\kappa_b} = 5 \times 10^4 \mu m^{-2}, \]

where the constant $\kappa_b$ is defined in Equation (2.9) of Section 2.1.3. The chosen values of $K_V$ and $K_A$ allow us to set values of $V$ and $A$ to within 0.02% error in our numerical minimisation.

2 Such fictitious energies for enforcing the volume and area constraints have been used by Wintz et al. [231] to predict starfish-shaped vesicles and Discher et al. [49] to simulate micropipette aspiration of a RBC.

3 In working with the ratios $K_V/\kappa_b$ and $K_A/\kappa_b$, we have chosen to let $\kappa_b$ set the energy scale of $F$. This overall scale disappears from the energy-minimisation problem; however, it does play a role in thermal fluctuations.
2.1.3 Bending energy

The functional forms of $F_{sc}$, $F_{ad}$ and $F_{g}$ are

$$F_{sc}[S] \approx \frac{K_b}{2} \int_S [C_1(r) + C_2(r) - C_0]^2 \, dA = \frac{K_b}{2} \int_S [2H(r) - C_0]^2 \, dA, \quad (2.9)$$

$$F_{ad}[S] \approx \frac{\kappa \pi (\Delta A[S] - \Delta A_0)^2}{2D^2A[S]}, \quad (2.10)$$

$$F_{g}[S] \approx \kappa_g \int_S C_1(r)C_2(r) \, dA, \quad (2.11)$$

where $H$ in Equation (2.9) is defined by

$$H(r) \equiv \frac{1}{2} [C_1(r) + C_2(r)]. \quad (2.12)$$

$F_{sc}$, Equation (2.9), is characterised by the two material parameters $\kappa_b$ and $C_0$. The elastic coefficient $\kappa_b$ is called the local bending modulus (or rigidity) of the plasma membrane. The constant $C_0$ is called the spontaneous curvature of the plasma membrane and is a measure of the difference in average molecular shape between molecules in the outer leaflet and molecules in the inner leaflet. $C_1$ and $C_2$ in Equation (2.9) are the two principal curvatures at the point $r$ on $S$. $H$ in Equations (2.9) and (2.12) is the mean curvature of $S$ at the point $r$.

$F_{ad}$, Equation (2.10), is characterised by the three material parameters $\kappa$, $D$ and $\Delta A_0$. The elastic coefficient $\kappa$ is called the area difference or non-local bending modulus (or rigidity) of the plasma membrane. The constant $D$ is the separation between the respective neutral surfaces of the two membrane leaflets. The constant $\Delta A_0$ is the preferred area difference between the neutral surfaces of the two leaflets. The functional $\Delta A$ in Equation (2.10) is the actual, geometrical area difference between the neutral surfaces of the two leaflets, induced when a flat bilayer is forced to bend in order to close. From geometry [23, pp. 224–225],

$$\Delta A[S] \approx D \int_S [C_1(r) + C_2(r)] \, dA = 2D \int_S H(r) \, dA. \quad (2.13)$$

$F_{g}$, Equation (2.11), is characterised by the material parameter $\kappa_g$, referred to as the Gaussian curvature bending modulus (or rigidity) of the plasma membrane.

The bending energy terms $F_{sc}$ and $F_{g}$ were introduced by Helfrich [85]. The sum of these two terms is called the spontaneous curvature model. Subsequent generalisation has

---

$^4$The neutral surface of a leaflet is the surface about which the net bending moment caused by the stress profile across the leaflet thickness vanishes.
led to the additional term $F_{ad}$. The sum of these three terms is called the *area difference elasticity* (ADE) model. The reader may consult Ref. 235 for an excellent introduction to the ADE model and Ref. 186 for a comprehensive review of the ADE model.

$F_{sc}$ is the total cost in energy of forcing the local mean curvature $H$ of the plasma membrane to depart from its preferred value, $C_0/2$. $C_0$ is assumed to be homogeneous over $S$, i.e., it does not depend on the local shape of the plasma membrane. $C_0$ is expected to be non-zero whenever there is an asymmetry between the two membrane leaflets, such as a difference between the compositions, or chemical environments, of the leaflets.

$F_{ad}$ is the energy incurred for the shape-dependent $\Delta A$ to be different from its preferred value, $\Delta A_0$. In contrast to the dependence of $F_{sc}$ on the local shape of the plasma membrane, $F_{ad}$ is non-local: it depends on the overall shape of the plasma membrane. The validity of $F_{ad}$ is based on the assumption that the time scale of equilibration of density within each leaflet of the plasma membrane is much smaller than the time scale of shape transformations of the RBC.

$F_g$ is the cost in energy of changing the topology of the plasma membrane and can be omitted: It can be shown by using the Gauss-Bonnet theorem that Equation (2.11) only depends on the topology of $S$, with $F_g = 4\pi \kappa_g (1 - g_S)$, where $g_S$ is the topological genus of $S$. For $S$ with the topology of a sphere, $g_S = 0$. Since we only consider deformations in which the topology of $S$ remains a sphere, $F_g = 4\pi \kappa_g$ is effectively a shape-independent constant, so Equation (2.11) will be omitted henceforth.

The three parameters $D$ and $\Delta A_0$ of $F_{ad}$ [Equation (2.10)] and $C_0$ of $F_{sc}$ [Equation (2.9)] are not generally measurable or otherwise known. However, they need not be independently specified because they appear in the form of a single combined parameter in the energy-minimisation problem. This combined parameter emerges naturally from adding $F_{ad}$ and $F_{sc}$ to form the overall free energy of bending of the plasma membrane [102]:

$$F_b[S] = F_{ad}[S] + F_{sc}[S] = \kappa_b \left\{ G[S] + \frac{\alpha_b}{2} \left( m[S] - \bar{m}_0 \right)^2 + w_0 \right\}, \quad (2.14)$$
CHAPTER 2. CONTINUUM ELASTIC MODEL OF THE RBC

where

\[
G[S] = 2 \int_S H^2(r) \, dA, \quad (2.15)
\]

\[
\alpha_b = \frac{\kappa}{\kappa_b}, \quad (2.16)
\]

\[
m[S] = \frac{\Delta A[S]}{2DR_A} = \frac{1}{R_A} \int_S H(r) \, dA \quad \text{[by Equation (2.13)]}, \quad (2.17)
\]

\[
\bar{m}_0 = \frac{2\pi R_A}{D} \left( \frac{\Delta A_0}{A} + \frac{DC_0}{\pi \alpha_b} \right), \quad (2.18)
\]

\[
\omega_0 = \left( 1 - \frac{1}{\pi \alpha_b} \right) \frac{C_0^2 A}{2} - \frac{C_0 \Delta A_0}{D} \quad . \quad (2.19)
\]

In Equations (2.17) and (2.18), \( R_A \) is the equivalent radius associated with the area \( A \), defined by

\[
A \equiv 4\pi R_A^2 . \quad (2.20)
\]

In other words, \( R_A \) is the radius of a sphere with the same area as the RBC. Note that \( D \), \( \Delta A_0 \), and \( C_0 \) are now combined into the two dimensionless constants \( \bar{m}_0 \) and \( \omega_0 \), given by Equations (2.18) and (2.19), respectively. It is clear from Equation (2.14) that \( \bar{m}_0 \) has influence over \( S \) through the product \( \bar{m}_0 m[S] \) and, therefore, must be specified. \( \bar{m}_0 \) is referred to as the reduced effective relaxed area difference between the neutral surfaces of the leaflets of the plasma membrane. It is the control parameter assumed, in the bilayer-couple picture, to be driven by the inducing agents. Terms proportional to \( \omega_0 \) and \( \bar{m}_0^2 \) in Equation (2.14) are irrelevant to the energy-minimisation problem, since they do not affect \( S \). Thus, they can be safely omitted from further considerations. This gives the following simplified form, suitable for minimisation:

\[
\frac{F_b[S]}{\kappa_b} = G[S] + \frac{\alpha_b}{2} \left( m^2[S] - 2\bar{m}_0 m[S] \right), \quad (2.21)
\]

where \( G[S] \) and \( m[S] \) are calculated from the shape \( S \). The dimensionless parameter \( \alpha_b \) is assumed to be unaffected (or only weakly affected) by the inducing agents.

It is clear from Equation (2.18) that \( \bar{m}_0 \) encapsulates the net effect of the two mechanisms by which an inducing agent drives shape transformations. In the first mechanism, the agent induces a change in the area of one leaflet of the plasma membrane relative to the other, which, in turn, gives rise to a change in \( \Delta A_0 \). In the second mechanism, the agent
induces a preferential change in the average molecular shape of the molecules in one leaflet of the plasma membrane, which, in turn, gives rise to a change in $C_0$. If these two mechanisms result in a net increase in $\bar{m}_0$, the agent is echinocytogenic. Conversely, if these two mechanisms result in a net decrease in $\bar{m}_0$, the agent is stomatocytogenic. We will explore the variation of the minimum-energy $S$ with $\bar{m}_0$ in Chapter 4.

### 2.1.4 Bending-energy parameter values

We choose the value

$$\kappa = \frac{4.0}{\pi} \times 10^{-19} \text{ J},$$

(2.22)

based on estimates given by studies of tether formation from the RBC membrane [95].

The value of $\kappa_b$ has been estimated by various experimental techniques, including local pulling of the RBC membrane using the tip of an atomic force microscope [182], flicker spectroscopy [30, 94, 162, 204, 240–242], micropipette aspiration of flaccid RBCs [60], and tether formation from the RBC membrane [95, 228]. These estimates are listed in Table 2.1. Only the tether-formation experiments use the full ADE model in the analysis. Thus, on the basis of the four estimates of $\kappa_b$ given by the tether-formation experiments, we choose the value

$$\kappa_b = 2.0 \times 10^{-19} \text{ J}.$$  

(2.23)

Substituting Equations (2.22) and (2.23) in Equation (2.16) gives

$$\alpha_b = \frac{2}{\pi}.$$  

(2.24)

Except for the flicker spectroscopy estimates, the estimates of $\kappa_b$ shown in Table 2.1 are in good agreement. The differences may be due to the different theoretical assumptions in the analyses of these experiments. In addition, it has been proposed [204] that the smaller estimates given by flicker spectroscopy of RBC membrane fluctuations at short wavelengths [241, 242] may be related to active (ATP-driven) motion of the RBC membrane. It has been shown (see, for example, Ref. 215) that fluctuations of the RBC membrane do depend on the intracellular ATP concentration; however, no experiments have been done to check whether $\kappa_b$ determined from short-wavelength fluctuations varies with the intracellular ATP concentration.
CHAPTER 2. CONTINUUM ELASTIC MODEL OF THE RBC

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Year</th>
<th>$\kappa_b \times 10^{-19}$ J</th>
<th>Method</th>
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<td>30</td>
<td>1975</td>
<td>0.13 – 0.3</td>
<td>Flicker spectroscopic analysis of:</td>
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<td></td>
<td></td>
<td></td>
<td>(1) correlation functions for thickness fluctuations at two</td>
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<td>different points</td>
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<td>241</td>
<td>1987</td>
<td>0.34 ±0.08</td>
<td>(2) Fourier modes (with wavelengths 0.5–1.0 $\mu$m) of RBC</td>
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<td>membrane deformation amplitudes in the normal direction</td>
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<td>1990</td>
<td>$\leq$ 2–3</td>
<td>(3) long-time decay of the autocorrelation function of thickness</td>
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<td>fluctuations</td>
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<td>1992</td>
<td>0.23 ±0.05</td>
<td>(4) Fourier modes (with wavelengths 0.25–3 $\mu$m) of RBC</td>
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<td>membrane deformation amplitudes in the normal direction</td>
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<td>1.4/4.3</td>
<td>(5) the thickness fluctuation profile along a diameter</td>
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<td>204</td>
<td>1995</td>
<td>2 – 7</td>
<td>(6) the first three azimuthal Fourier modes of fluctuations of</td>
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<td>the rim (wavelengths comparable to cellular dimensions)</td>
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<td>2003</td>
<td>1.0 – 1.9</td>
<td>(7) the power spectra and autocorrelation functions of</td>
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<td>(1) pulling tethers from RBC membranes at constant force</td>
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<td></td>
<td></td>
<td>(2) relaxation of tethering force at constant tether length</td>
</tr>
<tr>
<td>95</td>
<td>1997</td>
<td>2.0 ±0.6</td>
<td>(3) relaxation of tethering force at constant tether length</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.0</td>
<td>(4) pulling tethers from RBC membranes at constant velocity</td>
</tr>
<tr>
<td>182</td>
<td>2001</td>
<td>2.07 ±0.32</td>
<td>Pulling RBC membranes using an atomic force microscope</td>
</tr>
</tbody>
</table>

Table 2.1: Estimates of the value of $\kappa_b$.

### 2.2 Free energy of the membrane skeleton

In the commonly used [63] continuum elastic model of the RBC membrane, a variant of which we employ, the separation between the plasma membrane and membrane skeleton is neglected. This is a reasonable approximation because this separation is much smaller than the RBC diameter. Thus, the instantaneous configuration of the membrane skeleton is regarded as a two-dimensional elastic sheet positioned at the geometrical surface $S$. This is equivalent to assuming that the resultant strain field of any in-plane deformation of the membrane skeleton is defined over points $\mathbf{r}$ of $S$. The said instantaneous configuration and $S$ have the same geometry; therefore, for simplicity, we will use $S$ to refer to both of them. In the context used here, $S$ refers to the instantaneous configuration of the membrane skele-
It is known experimentally that, on coupling to the plasma membrane, the membrane skeleton, hence $S$, is constrained by the plasma membrane to have overall area $A_{RBC}$ but remains locally compressible.

It is necessary to define another two-dimensional elastic sheet $S_0$ to serve as the reference configuration for the membrane skeletal strain free energy. The points $r_0$ on $S_0$ are in one-to-one correspondence with the points $r(r_0)$ on $S$. The shape and area of $S_0$ are generally unknown and can be different from those of $S$. The volume of $S_0$, calculated from the shape and area of $S_0$, can also be different from that of $S$. We emphasise that the shape and area of $S_0$ cannot be determined by removing the membrane skeleton from the RBC membrane [105, 115, 122, 123, 208, 222], since they are set by the collective physico-chemical properties of the cytoplasm, plasma membrane and membrane skeleton. For example, it is known that the intracellular haemoglobin promotes spectrin dimer-dimer association and spectrin tetramer stability [133]. Therefore, the removal of haemoglobin will affect the shape and area of $S_0$.

$S$ and $S_0$ are assumed to be isotropic and hyperelastic, with $S_0$ further assumed to be homogeneous and unstressed. A review of the theory of finite elasticity for an isotropic hyperelastic sheet is given in Appendix B. By an unstressed $S_0$, we mean there is no residual stress field present when $S$ is allowed to relax back to $S_0$. In general, $S$ may be prevented from relaxing back to $S_0$ by the area and volume constraints on $S$, and the bending elasticity of the plasma membrane.

The strain of the isotropic hyperelastic $S$ caused by an in-plane deformation is completely specified by the two fields of strain invariants $\alpha(r_0)$ and $\beta(r_0)$:

$$\alpha = \lambda_1 \lambda_2 - 1,$$

$$\beta = \frac{1}{2} \left[ \left( \frac{\lambda_1}{\lambda_2} - 1 \right) + \left( \frac{\lambda_2}{\lambda_1} - 1 \right) \right] = \frac{\lambda_1^2 + \lambda_2^2}{2\lambda_1 \lambda_2} - 1,$$

where the two fields $\lambda_1(r_0)$ and $\lambda_2(r_0)$ are eigenvalues called the principal extension ratios or principal stretches. $\lambda_1$ and $\lambda_2$ describe the fractional elongation or contraction (along the two local principal axes of strain) of the infinitesimal neighbourhood of $r_0$ on $S_0$ as it deforms into the corresponding neighbourhood of the transformed point $r$ on $S$. Geometrically, $\alpha$ is the fractional change in area of a material element of $S_0$, i.e., it is a measure of area strain, and $\beta$ is a symmetric combination of the two possible fractional changes in
aspect ratio of that material element, i.e., it is a measure of shear strain. Since \( \lambda_1, \lambda_2 > 0, \alpha > -1 \) and \( \beta > 0 \).

The free energy functional of the membrane skeleton is given by

\[
F_{MS}[S, S_0] \equiv \oint_{S_0} f_{MS}(\alpha(r_0), \beta(r_0)) \, dA_0 ,
\]  

(2.27)

where \( f_{MS} \) is the membrane skeletal strain energy density with independent variables \( \alpha \) and \( \beta \). Note that the surface integral in Equation (2.27) is over the unstressed shape \( S_0 \). The functional form of \( f_{MS} \) is not known \textit{a priori} and can only be approximated by its Taylor expansion in terms of \( \alpha \) and \( \beta \). The particular form of the expansion we use is \cite{125}

\[
f_{MS}(\alpha, \beta) = f_{\text{stretch}}(\alpha) + f_{\text{shear}}(\alpha, \beta) ,
\]

(2.28)

where

\[
f_{\text{stretch}}(\alpha) = \frac{K_\alpha}{2} \left( \alpha^2 + a_3 \alpha^3 + a_4 \alpha^4 + \cdots \right) ,
\]

(2.29)

\[
f_{\text{shear}}(\alpha, \beta) = \mu \left( \beta + b_1 \alpha \beta + b_2 \beta^2 + \cdots \right)
\]

(2.30)

are the stretching and shear free energy densities, respectively. The coefficients \( K_\alpha \) and \( \mu \) are called, respectively, the \textit{stretching modulus} and the \textit{shear modulus} of the membrane skeleton. The former is also known as the \textit{area compressibility (or expansivity) modulus} of the membrane skeleton. The coefficients \( a_3, a_4, b_1, b_2 \) are dimensionless higher-order non-linear elastic moduli. Substituting Equations (2.28) to (2.30) in Equation (2.27) gives

\[
F_{MS}[S, S_0] = F_{\text{stretch}}[S, S_0] + F_{\text{shear}}[S, S_0] ,
\]

(2.31)

where

\[
F_{\text{stretch}}[S, S_0] = \oint_{S_0} f_{\text{stretch}} \, dA_0 = \frac{K_\alpha}{2} \oint_{S_0} \left( \alpha^2 + a_3 \alpha^3 + a_4 \alpha^4 + \cdots \right) \, dA_0 ,
\]

(2.32)

\[
F_{\text{shear}}[S, S_0] = \oint_{S_0} f_{\text{shear}} \, dA_0 = \mu \oint_{S_0} \left( \beta + b_1 \alpha \beta + b_2 \beta^2 + \cdots \right) \, dA_0
\]

(2.33)

are the stretching and shear free energy functionals, respectively.

We make the simplifying assumption that \( S_0 \) and \( S \) have the same area \( A_{RBC} \), which leaves us with the problem of characterising the unknown shape of \( S_0 \). (There are interesting consequences when \( S_0 \) and \( S \) differ in area, which will be discussed in Section 2.2.4.) We
have devised a method to vary systematically the shape of $S_0$ by a change in its volume, $V_{\text{rest}}$, while keeping its area fixed at $A_{\text{RBC}}$, to obtain a one-parameter family of oblate ellipsoidal $S_0$, as detailed in Section 3.7. At one extreme, $S_0$ is discocytic with the same volume $V_{\text{rest}} = 100 \, \mu m^3 = V_{\text{RBC}}$ as $S$. At the other extreme, $S_0$ is spherical with volume $V_{\text{rest}} = \frac{4}{3} \pi R_A^3 = 155.8 \, \mu m^3$, where $4\pi R_A^2 = A_{\text{RBC}} = 140 \, \mu m^2$. These two limiting shapes and some intermediate ones are shown in Figures 3.15 to 3.23. We will explore the variation of the minimum-energy $S$ with $V_{\text{rest}}$ in Chapter 4.

2.2.1 Membrane skeletal free energy parameter values

The values of the parameters of Equations (2.29) and (2.30) used in this Thesis are given in Table 2.2. The value of $\mu$ given in Table 2.2 is based on that of Ref. 86; however, one should be sceptical of all extant estimates of $\mu$ (see discussions in Sections 2.2.2 and 2.2.5). There are no experimental estimates of $K_\alpha$ for intact RBCs. There are recent estimates of $K_\alpha$ for bare membrane skeletons by Lenormand et al. [122, 123] (see further discussions in Section 2.2.5); however, they are not a good approximation to $K_\alpha$ for intact RBCs, since a bare membrane skeleton experiences chemical and physical conditions different from a membrane skeleton in an intact RBC. The ratio

$$\frac{K_\alpha}{\mu} \approx 2$$

has been predicted by models in which the membrane skeleton is approximated as a triangular network of springs [23, Ch. 3], [80]. This ratio has also been observed in recent experiments by Lenormand et al. [122, 123]; however, we do not regard this result as

<table>
<thead>
<tr>
<th>Elastic moduli</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_\alpha$</td>
<td>5.0 $\mu J m^{-2}$</td>
</tr>
<tr>
<td>$a_3$</td>
<td>$-2$</td>
</tr>
<tr>
<td>$a_4$</td>
<td>8</td>
</tr>
<tr>
<td>$\mu$</td>
<td>2.5 $\mu J m^{-2}$</td>
</tr>
<tr>
<td>$b_1$</td>
<td>0.7</td>
</tr>
<tr>
<td>$b_2$</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Table 2.2: Values of the membrane skeletal elastic moduli used in this Thesis.
definitive because of some open questions about the analysis. (see further discussions in Section 2.2.5). With these theoretical and experimental results in mind, we shall assume here that \( K_\alpha = 2\mu \), as reflected in the value of \( K_\alpha \) in Table 2.2. The moduli \( a_3, a_4, b_1 \) and \( b_2 \) have not been determined. The values of these four moduli given in Table 2.2 are guesses chosen to harden the membrane skeletal elasticity at large strain. The way these four values are chosen is discussed in Section 2.2.3.

### 2.2.2 Comparison with common large-deformation form

It is common in the treatment of RBC membrane mechanics to use the simplified free energy density [63],

\[
 f_{\text{MS}}(\alpha, \beta) = \frac{K_\alpha}{2} \alpha^2 + \mu \beta .
\]  

This expression is already non-linear in that, for small deformations \( \lambda_i \simeq 1 + E_i \), where \( |E_i| \ll 1 \) and \( i \in \{1, 2\} \), it reduces to the linear elasticity relation:

\[
 f_{\text{MS}}(\alpha, \beta) \simeq f_{\text{MS}}^{\text{linear}}(E_1, E_2) = \frac{K_\alpha}{2} (E_1 + E_2)^2 + \frac{\mu}{2} (E_1 - E_2)^2 .
\]  

The more general non-linear form, Equation (2.28), of course, reduces to Equation (2.35) as well. Note that Equation (2.35) identifies \( K_\alpha \) and \( \mu \) as the linear elastic moduli. For moderate to large deformations (operationally defined as \( |\lambda_i - 1| > 0.1 \) in one or both local principal directions), such as those expected to be found in the spicules of an echinocyte III, the linear relation is well beyond its domain of validity. Equation (2.34) is a particular form of extension of the linear relation into the high deformation regime. This extension is simple but has no firm experimental basis: The problem of determining the range of \( \alpha \) and the range of \( \beta \) for which Equation (2.34) holds remains unsolved, largely because experimental techniques for applying small, linear elastic deformations to the membrane skeleton and for determining membrane skeletal strains \( \lambda_i \) (hence \( \alpha \) and \( \beta \)) were not available until recently. The additional terms in Equation (2.28) are simply a way of providing additional freedom in fitting the theoretical elasticity to actual material properties of membrane skeletal regions with significant deviation of \( \lambda_i \) from unity. Since Equation (2.28) is new, there has yet to be any experimental determination of the range of \( \alpha \) and the range of \( \beta \) for which it holds.

In earlier work on RBCs, Equation (2.34) has often been used with the additional, unrealistic assumption of local area incompressibility of the membrane skeleton, which is equiv-
alent to $K_\alpha \to \infty$, or to $\alpha = 0$. For example, this approach was used to extract the value of $\mu$ from experiments on micropipette aspiration of RBCs (see the review in Ref. 87), leading to typical estimates of $\mu$ in the range of 6–9 $\mu$Jm$^{-2}$. The attractive feature of assuming the membrane skeleton to be locally incompressible is that it allows $\mu$ to be determined without the need to measure the variation of $\lambda_i$ over the aspirated region of the RBC. Recent experiments by Discher et al. [51, 52, 119] have since conclusively disproved the assumption of local area incompressibility of the membrane skeleton. In addition, they found [51, 119] that $\lambda_1$ and $\lambda_2$ in the aspirated region of the RBC actually depart significantly from unity, from which we infer that the true value of the linear elastic modulus $\mu$ should be equal to

![Elastic Free Energy Density of the Membrane Skeleton](image)

Figure 2.1: Comparison between (a) Equation (2.28) with the elastic moduli listed in Table 2.2 and (b) Equation (2.34) with $\mu = 6 \mu$Jm$^{-2}$ and the constraint $\alpha = 0$. 
or, more likely, lower than that given by the micropipette aspiration technique, since the micropipette aspiration experiments are really probing the non-linear elasticity at high deformation, not the linear elasticity at small deformation. Another recent development is a series of experiments that estimated $\mu$ in the linear, or purportedly linear, elastic deformation regime, as summarised in Table 2.3 and discussed in Section 2.2.5. These recent experiments suggest a value of the linear shear modulus $\mu$ in the range $1-10 \, \mu\text{Jm}^{-2}$, not inconsistent with the earlier estimates of $6-9 \, \mu\text{Jm}^{-2}$ from the high-deformation, micropipette aspiration experiments. In our calculations, we have assumed a value of $\mu = 2.5 \, \mu\text{Jm}^{-2}$ from the low end of this range, as shown in Table 2.2. This choice agrees with the recent measurements of Hénon et al. [86], which are specifically designed to probe the weak-deformation, linear elastic regime (but see further discussion in Section 2.2.5) and reflect our belief that there is significant hardening of the elastic moduli at high deformation. Finally, as discussed in Section 5.4, significantly higher values (in the range $6-9 \, \mu\text{Jm}^{-2}$) lead to problems in the shape-transformation sequence.

In order to fit both the low- and the high-deformation regimes, we propose in Equation (2.28) a form which incorporates the small-deformation limit by putting the linear elastic moduli, $\mu = 2.5 \, \mu\text{Jm}^{-2}$ and $K_\alpha = 5.0 \, \mu\text{Jm}^{-2}$, into the non-linear expression, Equation (2.34), but then “hardening” the elastic response at high deformation by adding two higher-order non-linear terms in order to reflect the larger effective moduli at high deformation. Figure 2.1 shows a graphical comparison between the curved surface described by Equation (2.28) and the straight line described by Equation (2.34) with the constraint $\alpha = 0$. The line is initially higher in energy density than the curved surface; however, the situation is eventually reversed as $\beta$ increases further.

### 2.2.3 Comparison with new large-deformation form

Discher et al. [52, 146] proposed another approximation to the true $f_{\text{MS}}$ at large deformation that takes into account the local area compressibility of the membrane skeleton:

$$f_{\text{MS}} = \frac{K_N}{2} \left[ (\lambda_1 \lambda_2)^2 + \frac{2}{n(\lambda_1 \lambda_2)^n} \right] + \frac{\mu}{2} \left( \lambda_1^2 + \lambda_2^2 \right)$$

or, equivalently,

$$f_{\text{MS}} = \frac{K_N}{2} \left[ (\alpha + 1)^2 + \frac{2}{n(\alpha + 1)^n} - 3 \right] + \mu N \beta (\alpha + 1), \quad (2.36)$$
where $K_N$ and $\mu_N$ are, respectively, the effective stretching and shear moduli at large deformation. Their operational definition of a large deformation is $|\lambda_{1,2} - 1| \gtrsim 0.5$, i.e., a uniaxial stretching or compression in a principal direction by at least 50%, given in a related work of Discher [119]. Note that $K_N$ and $\mu_N$ do not reduce to the linear elastic moduli in the limit $\lambda_i \to 1$. Discher et al. performed fluorescence imaging of the membrane skeletal deformation of the RBC projection aspirated into a micropipette. They determined experimentally that $K_N \approx 2 \mu_N$ and $1 \leq n < 2$; however, they did not determine the actual values of $K_N$ and $\mu_N$. They argue that

$$\mu \sim \frac{\mu_N K_N}{\mu_N + K_N},$$

where $\mu$ is the aforementioned older value of the shear modulus determined from micropipette aspiration of the RBC.

Figure 2.2 shows a graphical comparison between (a) Equation (2.28) with the parameter values shown in Table 2.2; (b) Equation (2.36) with $K_N / 2 = \mu_N = 9 \mu J m^{-2}$ (corresponding to $\mu \sim 6 \mu J m^{-2}$) and $n = 1$; and (c) Equation (2.34) with the same linear elastic moduli, $K_\alpha$ and $\mu$, as Equation (2.28). The use of linear elastic moduli in Equation (2.34) means that Equation (2.34) strictly describes the small deformation regime. Equation (2.36), on the other hand, strictly describes the large deformation regime, the limits of which have not been determined. As mentioned previously, the form of Equation (2.28) is meant to bridge the low- and high-deformation limits. In the absence of experimental measurements, we have chosen the values of the additional coefficients $a_3, a_4, b_1$ and $b_2$ so that the stretching component of Equation (2.28) coincides with that of Equation (2.36) at $\alpha = 1$ and the shear component of Equation (2.28) coincides with that of Equation (2.36) for $\lambda_1 \lesssim 0.3$ or $\lambda_2 \lesssim 0.3$. Equation (2.28) reduces to Equation (2.34) in the limit $\alpha \to 0$ and $\beta \to 0$ ($\lambda_1 \to 1$ and $\lambda_2 \to 1$).

### 2.2.4 Consequences of $S_0$ and $S$ differing in area

Mukhopadhyay et al. [150] have shown that a difference in area between $S_0$ and $S$ amounts to a renormalisation of $K_\alpha$ and $\mu$ in the case where the true $f_{MS}$ is approximated by Equation (2.34). They considered the deformation of $S_0$ to $S$ as a two step process, as shown in Figure 2.3. In the first step, a square area element $dA_0$ of $S_0$ with edge length $\frac{1}{b}$ is uniformly dilated to form a square area element $dA'_0$ of $S'_0$ with unit edge length, where the
CHAPTER 2. CONTINUUM ELASTIC MODEL OF THE RBC

Membrane Skeletal Stretch Energy Density

Membrane Skeletal Shear Energy Density

Figure 2.2: (Left) Comparison of the membrane skeletal stretch energy densities of (a) Equation (2.28), (b) Equation (2.36) and (c) Equation (2.34). (Right) Comparison of the membrane skeletal shear energy densities of (a) Equation (2.28), (b) Equation (2.36) and (c) Equation (2.34). The parameter values of Equation (2.28) are those in Table 2.2. The parameter values of Equation (2.36) are \( K_N/2 = \mu_N = 9 \ \mu\text{Jm}^{-2} \) and \( n = 1 \). The parameter values of Equation (2.34) are \( K_\alpha/2 = \mu = 2.5 \ \mu\text{Jm}^{-2} \).

intermediate surface \( S'_0 \) has the same area as \( S \). In the second step, \( dA'_0 \) is stretched to form a rectangular area element \( dA \) on \( S \) with edge lengths \( \lambda_1 \) and \( \lambda_2 \). Therefore, the local stretching and shear energies are, respectively,

\[
\Delta F_{\text{stretch}} = \frac{K_\alpha}{2} \alpha^2 dA_0 = \frac{K_\alpha}{2} \left( \frac{dA}{dA_0} \right)^2 dA_0 ,
\]

\[
\Delta F_{\text{shear}} = \mu \beta dA_0 = \frac{\mu}{2} \left[ \left( \frac{\lambda_1}{\lambda_2} - 1 \right) + \left( \frac{\lambda_2}{\lambda_1} - 1 \right) \right] dA_0 ,
\]
which can be rewritten as

\[ \Delta F_{\text{stretch}} = \frac{K_{\alpha}'}{2} \left( \frac{dA}{dA_0'} \right)^2 dA_0', \]

\[ \Delta F_{\text{shear}} = \frac{\mu'}{2} \left[ \left( \frac{\lambda_1}{\lambda_2} - 1 \right) + \left( \frac{\lambda_2}{\lambda_1} - 1 \right) \right] dA_0', \]

where

\[ K_{\alpha}' = b^2 K_{\alpha}, \]
\[ \mu' = \frac{\mu}{b^2}. \]

In other words, a difference in area between \( S_0 \) and \( S \) is equivalent to keeping the area of both the same, but renormalising the elastic moduli \( K_{\alpha} \) and \( \mu \) to \( K_{\alpha}' \) and \( \mu' \), respectively. The situation is not so straightforward if \( f_{\text{MS}} \) is approximated by Equation (2.28) instead of Equation (2.34).

![Figure 2.3: A square area element \( dA_0 \) with edge length \( \frac{1}{b} \) is uniformly dilated to form a square area element \( dA_0' \) with unit edge length. The dilated square area element \( dA_0' \) is then stretched to form a rectangular area element \( dA \) with edge lengths \( \lambda_1 \) and \( \lambda_2 \).](image)

### 2.2.5 Recent estimates of stretching and shear moduli

Table 2.3 shows a summary of recent estimates of \( K_{\alpha} \) and \( \mu \), obtained using techniques other than micropipette aspiration. The estimates of Hénon et al. [86], Guck et al. [76], and Lee and Discher [118] are based on intact RBCs, whereas the others are not.

We first discuss estimates not based on intact RBCs. The two experiments by Lenormand et al. [122, 123] are based on bare membrane skeletons; therefore, they may not be...
### Table 2.3: Recent estimates of $K_\alpha$ and $\mu$ using techniques other than micropipette aspiration.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Year</th>
<th>$K_\alpha$ ($\mu$Jm$^{-2}$)</th>
<th>$\mu$ ($\mu$Jm$^{-2}$)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hénon et al. [86]</td>
<td>1999</td>
<td>2.5 ± 0.4</td>
<td></td>
<td>Pulling two beads attached to a discocyteic or nearly spherical swollen RBC in diametrically opposite directions using optical tweezers.</td>
</tr>
<tr>
<td>Sleep et al. [193]</td>
<td>1999</td>
<td>200</td>
<td></td>
<td>Pulling two beads attached to a saponin-lysed spherical ghost in diametrically opposite directions using optical tweezers.</td>
</tr>
<tr>
<td>Guck et al. [76]</td>
<td>2001</td>
<td>13 ± 5</td>
<td></td>
<td>Applying optical stress fields using an optical stretcher, to stretch osmotically swollen spherical RBCs.</td>
</tr>
<tr>
<td>Lee &amp; Discher [118]</td>
<td>2001</td>
<td>1–10</td>
<td></td>
<td>Tracking thermal fluctuations of fluorescent beads 40 nm in diameter, attached to actin directly within ghosts or indirectly via glycophorin C outside RBCs.</td>
</tr>
<tr>
<td>Lenormand et al. [122]</td>
<td>2001</td>
<td>4.8 ± 2.7</td>
<td>2.4 ± 0.7</td>
<td>Pulling three beads attached to the periphery of a bare membrane skeleton in different directions using optical tweezers, at an osmolality of 25 mOsm/kg.</td>
</tr>
<tr>
<td>Lenormand et al. [123]</td>
<td>2003</td>
<td>9.7 ± 3.4</td>
<td>5.7 ± 2.3</td>
<td>Same as previous, but at an osmolality of 150 mOsm/kg.</td>
</tr>
</tbody>
</table>

very relevant for the case of intact RBCs. In the analysis of Lenormand et al., they assumed a homogeneous linear elastic stress field over the triangular region formed by the three beads attached to the periphery of a membrane skeleton. This assumption is questionable near the bead attachment points. The value of Sleep et al. [193] also may not be very relevant for the case of intact RBCs, since it is based on ghosts permeabilised by saponin. The analysis of Sleep et al. is based on the work of Parker and Winlove [160], who used Equation (2.34) with the unrealistic assumption of local area incompressibility of the membrane skeleton. The value of $\mu$ found by Sleep et al. is surprisingly large (it is even larger than those given by micropipette aspiration). We can only speculate as to the reason for this discrepancy. It may in part be due to the assumption of local area incompressibility, which is not realistic and certainly in contradiction of the direct measurements of Lenormand et
al. [122, 123]. Furthermore, the regions near the bead attachments points are highly stressed and likely to be in the large-deformation regime where the in-plane elastic moduli harden significantly.

We turn now to discuss estimates based on intact RBCs. Lee and Discher [118] found a broad, but still reasonable, range for $\mu$ through an analysis of the root-mean-squared in-plane displacements of beads attached to the membrane skeleton. They assumed that the bead displacements arise entirely from thermal fluctuations. It is not known if ATP-dependent fluctuations [215] are important for their analysis. Guck et al. [76] has found a good technique to realise small deformations of RBCs without applying point forces to the RBC membrane, as occurs in experiments based on optical tweezers, which is difficult to treat theoretically. They constructed a so-called optical stretcher. Unlike optical tweezers, the optical stretcher applies an optical stress field to the RBC membrane. This stress field is caused by the momentum transfer from the two opposed, unfocussed laser beams used to trap a RBC. Guck et al. used the optical stretcher to stretch osmotically swollen, spherical RBCs into ellipsoids. Their analysis of the cell deformation gives a value for $\mu$ (purportedly in the linear elastic regime) that is somewhat higher than the range found in micropipette aspiration, which operates in the non-linear elastic regime. This higher value may occur because of problems with their analysis: In treating the RBC membrane as a thin shell, they neglected the bending energy of the plasma membrane and did not separate the area compressibility of the plasma membrane from that of the membrane skeleton (these two area compressibilities operate at very different energy scales). In addition, they may have failed to take into account the important effects of $F_v$, the energy required to change the RBC volume from that set by the osmolarity of the suspending medium (see Section 2.3). A spherical, turgid RBC has an elevated internal pressure that puts the RBC membrane under isotropic tension. In deforming the RBC shape from a sphere to an ellipsoid, the RBC volume will decrease. When the volume of a turgid RBC is forced to decrease, there is a corresponding rise in the concentration of osmotically active molecules trapped inside the RBC. This increases the osmotic pressure difference across the RBC membrane according to Equation (2.38) and, hence, the isotropic tension the RBC membrane is subjected to. This neglect of the osmotic energy may artificially inflate the true value of $\mu$ to the higher value found by Guck et al. Finally, Hénon et al. [86] investigated stretching of discocytic-RBCs and nearly spherical RBCs using optical tweezers. Their value for $\mu$, although consistent
with our expectation, is also based on some questionable analysis. Specifically, they neglected the bending energy of the plasma membrane, assumed local area incompressibility of the membrane skeleton, approximated the RBC membrane as two parallel independent discs with no stress at the edge in the case of a discocytic RBC, and did not quantify the effect of point forces on the membrane skeletal deformation near the bead attachment points. All of these issues could affect the quoted results significantly.

In summary, we are somewhat sceptical of all new estimates of \( \mu \). We have chosen to use the value of Hénon et al. simply because it is the only one out of the three estimates based on intact RBCs that is lower than the value given by the micropipette aspiration technique.

### 2.3 Effective area and volume constraints

The van’t Hoff equation states that the osmotic pressure difference between the inside and outside of the RBC, \( \Delta P \), is given by [186]

\[
\Delta P = RT \left( \frac{n}{V} - c \right),
\]

where \( R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1} \) is the universal gas constant, \( T \) is the absolute temperature, \( n \) is the total number (measured in osmoles) of osmotically active molecules trapped within the RBC, and \( c \) is the total concentration (measured in osmoles per unit volume) of all osmotically active molecules in the suspending medium. Integration of Equation (2.38) over the volume from \( V_0 \) to \( V \) gives

\[
F_V = RT \left[ n \ln \left( \frac{V}{V_0} \right) - c(V - V_0) \right],
\]

which becomes Equation (2.2) in the limit of small deviations of \( V \) from \( V_0 \). The constants \( K_V \) and \( V_0 \) are set by the osmolarity of the suspending medium to be

\[
K_V = cRT,
V_0 = \frac{n}{c}.
\]

At \( c = 290 \text{ mosmol/ L} \) (physiological osmolarity), \( T = 300 \text{ K} \), \( V_0 = V_{RBC} = 100 \mu \text{m}^3 \), \( \kappa_b = 2.0 \times 10^{-19} \text{ J} \), \( K_A = 0.5 \text{ J m}^{-2} \) [110], \( A_0 = A_{RBC} = 140 \mu \text{m}^2 \) and \( \mu = K_\alpha/2 = 2.5 \times \ldots \)
which indicate that the energy scale $K_V V_0$ for a change in volume and the energy scale $K_A A_0$ for a change in area are much larger than the energy scale $\kappa_b$ for bending the plasma membrane, the energy scale $K_\alpha A_0$ for local area compression of the membrane skeleton, and the energy scale $\mu A_0$ for shearing the membrane skeleton. Consequently, bending of the plasma membrane and in-plane deformation of the membrane skeleton occur effectively at a fixed area and volume. This justifies our treatment of $K_V$ and $K_A$ as adjustable computational parameters for enforcing the volume and area constraints.

When $c$ is lowered from its physiological value (equivalent to raising $V_0$), there is an influx of water into the RBC. This raises the volume $V$ of a freely suspended RBC at fixed $A = A_{\text{RBC}}$ to $V \approx V_0$, which keeps $\Delta P \approx 0$. When the RBC is osmotically swollen to a sphere, however, an increase in $V$ to $V > 4/3 \pi R_A^3$ for $A_{\text{RBC}} = 4\pi R_A^2$ requires a corresponding increase in $A$ from $A_{\text{RBC}}$, which is opposed by the plasma membrane's resistance to area dilation. This gives rise to an elevated internal pressure ($\Delta P > 0$) and an elevated isotropic tension of the RBC membrane. Because $K_V$ and $K_A$ are so large, the constraint energies $F_V$ and $F_A$ overwhelm the membrane energies $F_b$ and $F_{\text{MS}}$ as soon as $V$ and $A$ exceed $V_0$ and $A_{\text{RBC}}$ by a small amount. Thus, $F_b$ and $F_{\text{MS}}$ may be neglected when a freely suspended RBC is osmotically swollen to a sphere. Shear deformation of a spherically swollen RBC can only be accomplished by forcing $V$ to decrease, $A$ to increase, or both, which implies either an increase in $F_V$, an increase in $F_A$, or both. A failure to include the large effects of $F_V$ and $F_A$ for a turgid RBC may lead the unwary investigator to conclude erroneously that $F_b$ and $F_{\text{MS}}$ are larger than their true values. Thus, great care must be taken when investigating aspects of $F_b$ and $F_{\text{MS}}$ through shear deformation of a spherically swollen RBC.

2.4 Comparison of models of the RBC membrane

Table 2.4 lists the references for models of the RBC membrane that include the effects of both bending and in-plane elasticities. Boal et al. constructed a hybrid model composed
of a continuum fluid membrane and a triangular network of (entropically) elastic springs. The former represents the plasma membrane, whereas the latter is a coarse-grained representation of the membrane skeleton. The fluid membrane and the spring network are attached at the junctions of the spring network. The fluid membrane is discretised more finely compared to the spring network. This so-called dual network model explicitly allows for relative motion between the plasma membrane and membrane skeleton. The other models do not make explicit such an allowance. Leibler and Maggs and Discher et al. constructed coarse-grained models that approximate the membrane skeleton as a triangular network of elastic springs, whereas the others constructed continuum elastic models. With the exception of the model of Stokke et al., all the continuum elastic models approximated the membrane skeleton as an isotropic elastic thin shell. Stokke et al. approximated the membrane skeleton as an ionic gel, instead.

We first compare the bending elasticity of these models. In modelling the RBC membrane, one must be aware that the bending elasticity of the RBC membrane comes mainly

<table>
<thead>
<tr>
<th>Year</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1977</td>
<td>Zarda et al. [63, 239]</td>
</tr>
<tr>
<td>1980</td>
<td>Pai and Weymann [159]</td>
</tr>
<tr>
<td>1980</td>
<td>Evans [59, 63]</td>
</tr>
<tr>
<td>1986</td>
<td>Stokke et al. [202, 203]</td>
</tr>
<tr>
<td>1986</td>
<td>McMillan et al. [140]</td>
</tr>
<tr>
<td>1990</td>
<td>Leibler and Maggs [120]</td>
</tr>
<tr>
<td>1992</td>
<td>Boal et al. [24]</td>
</tr>
<tr>
<td>1992</td>
<td>Peterson et al. [161, 162]</td>
</tr>
<tr>
<td>1996</td>
<td>Waugh [227]</td>
</tr>
<tr>
<td>1997</td>
<td>Iglič et al. [96–98]</td>
</tr>
<tr>
<td>1998</td>
<td>Discher et al. [49]</td>
</tr>
<tr>
<td>2002</td>
<td>Mukhopadhyay et al. [150]</td>
</tr>
<tr>
<td>2002</td>
<td>Lim et al. [125] (also this Thesis)</td>
</tr>
<tr>
<td>2003</td>
<td>Kuzman et al. [114]</td>
</tr>
</tbody>
</table>

Table 2.4: Models of the RBC membrane that include bending and in-plane elasticities.
CHAPTER 2. CONTINUUM ELASTIC MODEL OF THE RBC

from the plasma membrane. Early models, particularly those of Zarda et al., Pai and Weymann, and McMillan et al., did not give a satisfactory treatment of the physical origin of the bending elasticity. This is mainly because it was not realised at the time that the contribution of the membrane skeleton to the bending elasticity of the RBC membrane is negligible. These authors assumed that the entire RBC membrane is an isotropic elastic shell with a non-zero thickness (hence finite bending elasticity) and that the unstressed shape of the shell is also the shape that minimises the bending energy. It follows from the theory of thin plates that the bending energy in these models has the form

$$W_b = \frac{k_b}{2} \int_{S_0} \left[ (C_1 - \tilde{C}_1)^2 + 2\nu (C_1 - \tilde{C}_1)(C_2 - \tilde{C}_2) + (C_2 - \tilde{C}_2)^2 \right] dA_0,$$

where \(\nu\) is a material parameter and \(\tilde{C}_1(r_0)\) and \(\tilde{C}_2(r_0)\) are the principal curvatures at a point \(r_0\) on the unstressed shape \(S_0\) of the shell [cf. the constant \(C_0\) of \(F_{sc}\) in Equation (2.9)]. Note that \(W_b\) is an integration over the unstressed shape \(S_0\), not the actual shape \(S\) [cf. the functional form of \(F_{sc}\) in Equation (2.9)]. Strictly speaking, the functional form of \(W_b\) (without considering actual values of its parameters) describes the negligible bending energy of the membrane skeleton, not the plasma membrane. The plasma membrane is a fluid; therefore, it does not have an unstressed shape with well-defined \(\tilde{C}_1\) and \(\tilde{C}_2\). Evans proposed a more general form of \(W_b\) with \(\nu = 1\) in an attempt to include the area-difference bending energy of the plasma membrane, but still retained the inappropriate assumption that there is a reference shape for the bending elasticity with differing \(\tilde{C}_1\) and \(\tilde{C}_2\) that vary from point to point. Stokke et al. used a more primitive form of the bending energy (from which the functional forms given in Section 2.1 may be derived) that explicitly considers the contribution from each leaflet of the plasma membrane. Peterson et al. used two limiting forms of \(F_b\) [Equation (2.14)]: (a) \(\alpha_b = 0\), so that \(F_b = F_{sc}\), where \(F_{sc}\) is given by Equation (2.9); and (b) \(\alpha_b \to \infty\), so that \(F_b = \kappa_b G\) with the constraint \(m[S] = \bar{m}_0\), where \(G, m\) and \(\bar{m}_0\) are given by Equations (2.15), (2.17), and (2.18), respectively. Leibler and Maggs, Boal et al. (for the fluid membrane), Iglič [96], and Discher et al. also used the limiting form \(F_b = F_{sc}\). Boal et al. and Discher et al. further assume \(C_0 = 0\), so that \(F_b = F_{sc} = \kappa_b G\). It has been pointed out [75] that the methods used by Leibler and Maggs, Boal et al. and Discher et al. to calculate \(F_b\) for their triangular network models are incorrect. The discretised forms of \(F_b\) used by these authors are based on the condition that the triangular elements are equilateral and equal in area [75]. However, their models are unable to fulfil this condition. Finally,
Waugh, Iglič et al. [97, 98], Mukhopadhyay et al., Lim et al., and Kuzman et al. used the general form of $F_b$, Equation (2.14).

Next, we compare the in-plane elasticities of the continuum elastic models that approximate the membrane skeleton as an isotropic elastic thin shell. Zarda et al. approximated the true membrane skeletal free energy density $f_{MS}$ by

$$f_{MS} = \frac{\mu'}{2} \left( \beta + \beta^2 - \alpha - \frac{\alpha^2}{2} \right) + \frac{K_{\alpha}'}{2} \left( \alpha + \frac{\alpha^2}{2} \right)^2,$$

where $\mu'$ and $K_{\alpha}'$ are related to $\mu$ and $K_{\alpha}$. McMillan et al. also used this form, but further assumed local area incompressibility of the membrane skeleton ($\alpha = 0$). Pai and Weymann, Evans, Waugh, Iglič et al., and Mukhopadhyay et al. approximated the true $f_{MS}$ by Equation (2.34) and, with the exception of Mukhopadhyay et al., assumed local area incompressibility. Lim et al. approximated the true $f_{MS}$ by Equation (2.28). Peterson et al. and Kuzman et al. considered the linear elastic regime, with Peterson et al. further assuming local area incompressibility.

Note that there are additional theoretical studies dealing with only the in-plane elasticities of the membrane skeleton. Thus, the membrane skeleton is approximated as a network of elastic springs by Hansen et al. [80–82], as a C*-gel by Everaers et al. [64], and as a network of polymer chains by Boal [22] and Boey et al. [25]. Unlike the aforementioned studies, Kozlov and Markin [113] gave a purely analytic treatment of the electrical and mechanical properties of the membrane skeleton.

### 2.5 Overall free energy of the RBC membrane

The overall free energy of the RBC membrane, $F$, is given by

$$\frac{F[S, S_0]}{\kappa_b} = \frac{1}{\kappa_b} \left( F_{PM}[S] + F_{MS}[S, S_0] \right)$$

$$= \frac{1}{\kappa_b} \left( F_{\text{constraint}}[S] + F_b[S] + F_{\text{stretch}}[S, S_0] + F_{\text{shear}}[S, S_0] \right)$$

$$= \frac{1}{\kappa_b} \left( F_{\Psi}[S] + F_A[S] + F_b[S] + F_{\text{stretch}}[S, S_0] + F_{\text{shear}}[S, S_0] \right) \cdot$$
Substituting in the functional forms of $F_A$, $F_V$, $F_b$, $F_{\text{stretch}}$, and $F_{\text{shear}}$ from Sections 2.1 and 2.2 gives

$$
\frac{F[S, S_0]}{\kappa_b} = \frac{K_V (V[S] - V_{RBC})^2}{2 \kappa_b V_{RBC}} + \frac{K_A (A[S] - A_{RBC})^2}{2 \kappa_b A_{RBC}} + 2 \oint_S H^2(\mathbf{r}) \, dA \\
+ \frac{2 \pi \alpha_b}{A_{RBC}} \left( \oint_S H(\mathbf{r}) \, dA \right)^2 - 2 \bar{m}_0 \alpha_b \sqrt{\frac{\pi}{A_{RBC}}} \left( \oint_S H(\mathbf{r}) \, dA \right) \\
+ \frac{K_\alpha}{2 \kappa_b} \oint_{S_0} \left( \alpha^2 + a_3 \alpha^3 + a_4 \alpha^4 \right) \, dA_0 \\
+ \frac{\mu}{\kappa_b} \oint_{S_0} \left( \beta + b_1 \alpha \beta + b_2 \beta^2 \right) \, dA_0 ,
$$

(2.39)

where all the parameters are shown in Table 2.5. Details concerning the parametrisation of $S_0$ using $V_{\text{rest}}$ are given in Section 3.7. For convenience, we restate Equations (2.4), (2.5),

<table>
<thead>
<tr>
<th>Energy Components</th>
<th>Energy Parameters</th>
<th>Parameter Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_{\text{constraint}}/\kappa_b$</td>
<td>$K_V/\kappa_b$, $V_{RBC}$, $K_A/\kappa_b$, $A_{RBC}$</td>
<td>$5 \times 10^4 , \mu m^{-3}$, $100 , \mu m^3$, $5 \times 10^4 , \mu m^{-2}$, $140 , \mu m^2$</td>
</tr>
<tr>
<td>$F_b/\kappa_b$</td>
<td>$\alpha_b$, $\bar{m}_0$</td>
<td>$2/\pi$, variable</td>
</tr>
<tr>
<td>$F_{MS}/\kappa_b$</td>
<td>$K_\alpha/\kappa_b$, $a_3$, $a_4$, $\mu/\kappa_b$, $b_1$, $b_2$, $V_{\text{rest}}$</td>
<td>$25.0 , \mu m^{-2}$, $-2$, $8$, $12.5 , \mu m^{-2}$, $0.7$, $0.75$, variable</td>
</tr>
</tbody>
</table>

Table 2.5: Values of the energy parameters used in this Thesis.
CHAPTER 2. CONTINUUM ELASTIC MODEL OF THE RBC

(2.12), (2.25), and (2.26):

\[ V[S] = \frac{1}{3} \oint_{S} \mathbf{r} \cdot \mathbf{n}(\mathbf{r}) \, dA , \]
\[ A[S] = \oint_{S} dA , \]
\[ H(\mathbf{r}) = \frac{1}{2} \left[ C_{1}(\mathbf{r}) + C_{2}(\mathbf{r}) \right] , \]
\[ \alpha = \lambda_{1} \lambda_{2} - 1 , \]
\[ \beta = \frac{\lambda_{1}^{2} + \lambda_{2}^{2}}{2\lambda_{1} \lambda_{2}} - 1 . \]
Chapter 3

Numerical Minimisation of $F[S, S_0]$

This chapter describes the steps required to implement the numerical minimisation of $F[S, S_0]$, Equation (2.39), on a computer. One first decides on appropriate ways to discretize $S$ and $S_0$, and the components, $F_{\text{constraint}}[S] = F_V[S] + F_A[S]$, $F_b[S]$, $F_{\text{MS}}[S, S_0] = F_{\text{stretch}}[S, S_0] + F_{\text{shear}}[S, S_0]$ of $F$. It is clear from the functional dependence of the energies on $S$ and $S_0$ that the method used to approximate the energies will depend on the method used to approximate $S$ and $S_0$. We choose to use piecewise planar surfaces with triangular area elements (plaquettes), $\tilde{S}$ and $\tilde{S}_0$, to approximate $S$ and $S_0$, respectively.\(^1\) (We will use the tilda in this chapter to denote discretised objects and quantities.) Both $\tilde{S}$ and $\tilde{S}_0$ originate from an initial sphere $\tilde{S}_{\text{sphere}}$, the construction of which will be discussed in Section 3.1. In order to formulate the discretised bending energy, $\tilde{F}_b$, we will need to develop a plaquette representation of the mean curvature $H$. This is done in Section 3.2. $F_{\text{stretch}}$ and $F_{\text{shear}}$ are approximated by $\tilde{F}_{\text{stretch}}$ and $\tilde{F}_{\text{shear}}$, respectively. The calculation from $\tilde{S}_0$ and $\tilde{S}$ of $\tilde{F}_{\text{stretch}}$ and $\tilde{F}_{\text{shear}}$, and their sum $\tilde{F}_{\text{MS}}$ will be discussed in Section 3.3. $F_V$ and $F_A$ are approximated by $\tilde{F}_V$ and $\tilde{F}_A$, respectively. The calculation of $\tilde{F}_V$ and $\tilde{F}_A$, and their sum $\tilde{F}_{\text{constraint}}$ from the area $\tilde{A}$ and volume $\tilde{V}$ of $\tilde{S}$ will be discussed in Section 3.4. Once discrete representations of the surfaces and energies are available, the next step is to select an energy minimisation method. We choose a Monte Carlo method, namely, the standard Metropolis algorithm, as outlined in Section 3.5. Our method for analyzing the output of this algorithm is detailed in Section 3.6. We use this algorithm to obtain a family of $\tilde{S}_0$ parametrised by $V_{\text{rest}}$, as

\(^1\)Another method, which has only been applied to fluid vesicles [21], would be to parametrise the surface and then vary the shape parameters until the energy of the system is minimised.
discussed in Section 3.7. These \( \tilde{S}_0 \), in turn, are used as the unstressed membrane skeletal configurations in the determination of the shapes \( \tilde{S} \) that minimise \( \tilde{F} = \tilde{F}_b + \tilde{F}_{MS} + \tilde{F}_{\text{constraint}} \). The results are given in Chapter 4.

### 3.1 Construction of initial sphere \( \tilde{S}_{\text{sphere}} \)

\( \tilde{S}_{\text{sphere}} \) is built up from an icosahedron by utilising a standard method of constructing a sphere in computer graphics. The icosahedron is based on three identical, intersecting Golden Rectangles. The ratio of the width to the height of each Golden Rectangle is the Golden Ratio \( \frac{1}{2}(\sqrt{5} + 1) \). The width and height of each rectangle are then set by giving the length of the diagonal from the origin to the corner of each rectangle. This diagonal length is \( R_A \), the radius of a sphere of area \( A_{RBC} = 4\pi R_A^2 \).

Once the initial geometry is established, each triangle of the icosahedron is then divided into four smaller ones with the midpoints of the edges of the initial triangle taken to be the

![Figure 3.1: The construction of an icosahedron with 12 vertices and 20 triangular faces is based on three intersecting Golden Rectangles.](image)
new vertices. These new vertices are translated radially onto the spherical surface and also combined with the initial vertices to form a new mesh. This procedure is repeated for the triangles of the new mesh until the desired level of mesh refinement is reached. Note that the 12 initial vertices retain their five-fold connectivity, whereas all the others have a six-fold connectivity; the former are referred to as defective vertices and the latter regular vertices. Consider an initial edge with vertex coordinates \((x_1, y_1, z_1)\) and \((x_2, y_2, z_2)\). After a mesh refinement, the new vertex on the spherical surface corresponding to this edge has coordinates

\[
\frac{R_A(x_1 + x_2, y_1 + y_2, z_1 + z_2)}{\sqrt{2(x_1x_2 + y_1y_2 + z_1z_2 + R_A^2)}}.
\]

Let the number of mesh refinements (or generations of triangle divisions) be \(N_{\text{div}}\). The number of triangles \(N_t\) and the corresponding number of vertices \(N_v\) are then given by\(^2\)

\[
\begin{align*}
N_t &= 20 \times 4^{N_{\text{div}}} , \\
N_v &= \frac{3}{6} (N_t - 20) + \frac{3}{5} \times 20 = \frac{N_t}{2} + 2 .
\end{align*}
\]

Tabulated values of \(N_t\) and \(N_v\) for the first five mesh refinements are shown in Table 3.1.

<table>
<thead>
<tr>
<th>(N_{\text{div}})</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>(N_t)</td>
<td>80</td>
<td>320</td>
<td>1280</td>
<td>5120</td>
<td>20480</td>
</tr>
<tr>
<td>(N_v)</td>
<td>42</td>
<td>162</td>
<td>642</td>
<td>2562</td>
<td>10242</td>
</tr>
</tbody>
</table>

Table 3.1: The number of triangles, \(N_t\), and the number of vertices, \(N_v\), for the first five mesh refinements.

Information pertaining to the mesh is stored in three files. The first contains the vertex coordinates calculated to a precision of 16 decimal places; the second, the vertex indices of each triangle, ordered anti-clockwise as seen from the outside; and, the third, the indices of the five or six nearest-neighbour vertices of each vertex, also ordered anti-clockwise as

\(^2\)A well known result of topology states that \(N_t - N_e + N_v = 2(1 - g)\) where \(N_e\) is the number of edges and \(g\) the topological genus of the surface. All the surfaces in this thesis are of genus \(g = 0\).
Figure 3.2: Left to right - the cluster about a vertex \( k \) for \( N_{\text{div}} \geq 1 \) consists of 20 triangles if \( k \) is a defective vertex, 23 triangles if \( k \) is a regular vertex next to a defective vertex, or 24 triangles otherwise. When \( k \) is displaced, the nearest triangles (coloured grey) affect \( F_{\text{MS}} \) and \( F_{\text{constraint}} \) (through \( \tilde{A} \) and \( \tilde{V} \)), while \( \tilde{F}_b \) is affected by all the triangles.

seen from the outside. The information within these files completely specifies the group of nearest and second-nearest triangles about each vertex (Figure 3.2), which we refer to as a cluster. The geometries of these triangles are required for calculating the change in energy caused by a movement of the vertex.

This mesh refinement method is applied again to \( \tilde{S}_0 \) and \( \tilde{S} \) later on, but without projecting the newly created vertices of \( \tilde{S}_0 \) and \( \tilde{S} \) onto \( S_0 \) and \( S \), respectively, since \( S_0 \) (during its construction) and \( S \) are not known a priori. In practice, the number of mesh refinements required for sufficient numerical accuracy (see Sections 3.2.1, 3.3.1 and 3.4.1) is three for \( \tilde{S} \)'s with smooth morphologies, such as axisymmetric and non-axisymmetric discocytes, the stomatocyte I and the knizocyte; and the number of mesh refinements required is four for \( \tilde{S} \)'s with sharper features, such as echinocytes I, II and III, and stomatocytes II and III.

### 3.2 Discretisation of \( F_b \)

The discretised surface, \( \tilde{S} \), is piecewise planar. Therefore, it intrinsically lacks a well-defined mean curvature. This is the most problematic part in formulating a sensible \( F_b \).

The earliest, and best known, model was proposed by Kantor and Nelson [108] in their
Figure 3.3: $\Gamma_k$ (dark grey area) in relation to the cluster about a vertex shown in Figure 3.2. Its perimeter is formed by connecting the centroids of the nearest neighbour triangles and the midpoints of the edges of these triangles. One of its nearest neighbours, $\Gamma_{k'}$, is also shown.

pioneering study of the statistical mechanics of tethered networks. This model has since been shown to be flawed [75] but remains widely used. We do not use the model of Kantor and Nelson. Instead, our method for discretising $F_b$ is based on that of Ref. 107, which correctly represents $F_b$ [75].

Equation (2.39) contains two surface integrals over $S$ that must be approximated:

$$M = \int_S H \, dA,$$

$$N = \int_S H^2 \, dA,$$

where $M$ and $N$ are known as the total mean curvature and the total mean curvature squared, respectively.

We define a surface $\Gamma_k$ about each vertex $k$, as shown in Figure 3.3. There are $N_v$ $\Gamma_k$'s, each with an area $A_k$ [defined later in Equation (3.6)], and their union $\bigcup_{k=1}^{N_v} \Gamma_k$ covers $\tilde{S}$. We associate each vertex $k$ with the contribution to $M$ (or $N$) of $S_k$, the element of $S$ above $\Gamma_k$. Thus, we write

$$M = \sum_{k=1}^{N_v} \int_{S_k} H \, dA = \sum_{k=1}^{N_v} M_k, \quad (3.1)$$

$$N = \sum_{k=1}^{N_v} \int_{S_k} H^2 \, dA = \sum_{k=1}^{N_v} N_k, \quad (3.2)$$
where
\[
M_k[S_k] = \int_{S_k} H \, dA,
\]
\[
N_k[S_k] = \int_{S_k} H^2 \, dA,
\]
are the local integrated mean curvature and the local integrated mean curvature squared, respectively. We define a local average of the mean curvature (average of \(H\) over \(S_k\)) by
\[
\overline{H}_k \equiv \frac{1}{A_k} \int_{S_k} H \, dA = \frac{M_k}{A_k},
\]
where \(A_k\) is the area of \(S_k\). As long as the variation of \(H\) over \(S_k\) is small, \(M_k, \overline{H}_k\) and \(N_k\) may be approximated by [107, 111]
\[
M_k \approx \int_{\Gamma_k} H \, dA , \tag{3.3}
\]
\[
\overline{H}_k \approx \frac{M_k}{A_k}, \tag{3.4}
\]
\[
N_k \approx \overline{H}_k^2 \int_{\Gamma_k} dA = \frac{(\overline{H}_k A_k)^2}{A_k} = \frac{M_k^2}{A_k}. \tag{3.5}
\]

The area \(A_k\) of \(\Gamma_k\) is simply
\[
A_k = \left\{ \begin{array}{ll}
\frac{1}{3} \sum_{j=1}^{5} A_{k,j} & \text{for defective vertices} \\
\frac{1}{3} \sum_{j=1}^{6} A_{k,j} & \text{for regular vertices}
\end{array} \right., \tag{3.6}
\]
where \(A_{k,j}\) is the area of the nearest neighbour triangle with unit normal \(\mathbf{n}_{k,j}\), as illustrated in Figures 3.2 and 3.4. It now remains to determine \(M_k\). It turns out that a good approximation to \(M_k\) can be obtained through a subtle limiting process that assumes the curvature of \(S\) becomes localised in the edges of \(\widetilde{S}\). This approximation becomes exact in the limit of infinitely fine triangulation. One imagines two triangles with a common edge of length \(l_{k,j}\) being separated and attached tangentially to a cylinder with radius \(\varepsilon_{k,j}\) and length \(l_{k,j}\), as shown in Figure 3.4, so that an angle of \(\theta_{k,j}\) is subtended at the axis of the cylinder. The joint surface consisting of the two triangles and the curved section (light grey area) of the
Figure 3.4: The arrangement of the basic geometrical entities that allows an integrated mean curvature to be defined at the common edge of two neighbouring triangles.

cylinder corresponding to $\theta_{k,j}$ has an integrated mean curvature

$$M_{k,j} = \frac{1}{2} \left( \frac{1}{\varepsilon_{k,j}} + 0 \right) \times \frac{l_{k,j} \varepsilon_{k,j} \theta_{k,j}}{\text{area of curved section}} = \frac{1}{2} l_{k,j} \theta_{k,j} ;$$

which arises entirely from the curved section of the cylinder. Equation (3.7) is independent of $\varepsilon_{k,j}$; therefore, it continues to hold in the limit $\varepsilon_{k,j} \to 0$, where the triangles reattach at their common edge. By summing $M_{k,j}$ of every edge connected to a vertex and substituting Equation (3.7) in the sum,

$$M_k = \begin{cases} 
\frac{1}{2} \sum_{j=1}^{5} M_{k,j} = \frac{1}{2} \sum_{j=1}^{5} l_{k,j} \theta_{k,j} & \text{for defective vertices} \\
\frac{1}{2} \sum_{j=1}^{6} M_{k,j} = \frac{1}{2} \sum_{j=1}^{6} l_{k,j} \theta_{k,j} & \text{for regular vertices}
\end{cases} ,$$

Here, treating the curvature as being concentrated at the edges is of purely mathematical relevance. Such a treatment, however, can also be of physical significance. An amusing example can be found in the theory of crumpled sheets, where the bending energy of a crumpled elastic sheet is indeed concentrated at the creases [74].
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$  

where the factor of $\frac{1}{2}$ in the first equality accounts for the fact that an edge is shared by two vertices. The angle $\theta_{k,j}$ is also the angle between the two unit normals $\mathbf{n}_{k,j-1}$ and $\mathbf{n}_{k,j}$ of each pair of adjoining triangles. It can thus be calculated from the relation $\cos \theta_{k,j} = \mathbf{n}_{k,j-1} \cdot \mathbf{n}_{k,j}$.

Using Equations (3.1), (3.2), and (3.5), we approximate $F_b$ by

$$\tilde{F}_b = \frac{2}{\kappa_b} \sum_{k=1}^{N_v} M_k^2 + \frac{2\pi \alpha_b}{A_{RBC}} \left( \sum_{k=1}^{N_v} M_k \right)^2 - 2\bar{m}_0 \alpha_b \sqrt{\frac{\pi}{A_{RBC}}} \left( \sum_{k=1}^{N_v} M_k \right),$$  \hspace{1cm} (3.9)

where $M_k$ and $A_k$ are given by Equations (3.8) and (3.6), respectively. Strictly speaking, $A_{RBC}$ of Equation (3.9) should be replaced by $\tilde{A}$ of Equation (3.14). Given that $\tilde{A}$ is intended to be kept close to $A_{RBC}$, however, the minute difference between the two will not have any significant effect on Equation (3.9).

3.2.1 Test 1

We illustrate the preceding remarks by showing a direct test of the discretised bending energy, $\tilde{F}_b$, for a sphere. This simple geometry allows us to calculate the exact value of $F_b$ from Equation (2.14) and to compare the exact value with the approximate value given by Equation (3.9). This test serves two purposes: (i) to check that the routine in our computer programme for calculating the initial bending energy is correct; and (ii) to check the quality of the convergence $\tilde{F}_b \rightarrow F_b$ with increasing number of mesh refinements, measured by $N_{\text{div}}$ or $N_t$. We have examined refinements in the range $1 \leq N_{\text{div}} \leq 5$ in three cases, corresponding to different values of $\bar{m}_0$ and $\alpha_b$:

1. $\bar{m}_0 = 0, \alpha_b = 0$: $F_b = 8\pi \kappa_b$;
2. $\bar{m}_0 = 0, \alpha_b = 1$: $F_b = 8\pi (1 + \pi) \kappa_b$;
3. $\bar{m}_0 = 1, \alpha_b = 1$: $F_b = 8\pi \left( \frac{1}{2} + \pi \right) \kappa_b$.

Table 3.2 shows the approximate values, $\tilde{F}_b$, as a function of $N_t$, and the exact values, $F_b$. Figure 3.5 shows semilog plots of the absolute percentage difference,

$$\sigma_b \equiv \frac{|\tilde{F}_b - F_b|}{F_b} \times 100,$$
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against $N_t$ for the three cases. Note that $\sigma_b < 1\%$ for $N_t \geq 320$. The sphere is a particularly smooth shape; $\widetilde{F}_b$ of shapes that are less smooth may converge to $F_b$ more slowly. Thus, we conclude that we will require $N_t \geq 1280$ to achieve a convergence error of 1% or less for all shapes.

<table>
<thead>
<tr>
<th>$N_{div}$</th>
<th>$N_t$</th>
<th>Case 1 ($\times 10\kappa_b$)</th>
<th>Case 2 ($\times 10\kappa_b$)</th>
<th>Case 3 ($\times 10\kappa_b$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\widetilde{F}_b$</td>
<td>1</td>
<td>80</td>
<td>2.585040</td>
<td>10.10188</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>320</td>
<td>2.532096</td>
<td>10.32859</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1280</td>
<td>2.518041</td>
<td>10.38864</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5120</td>
<td>2.514470</td>
<td>10.40386</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>20480</td>
<td>2.513573</td>
<td>10.40768</td>
</tr>
<tr>
<td>$F_b$</td>
<td>–</td>
<td>–</td>
<td>2.513274</td>
<td>10.40896</td>
</tr>
</tbody>
</table>

Table 3.2: A comparison between the theoretical values of $F_b$ and the numerical values of $\widetilde{F}_b$ for a spherical surface for three different sets of parameters.

3.3 Discretisation of $F_{MS}$

Consider the $N_t$ triangles $T_i^0$ of $\tilde{S}_0 = \bigcup_{i=1}^{N_t} T_i^0$ and the $N_t$ triangles $T_i$ of $\tilde{S} = \bigcup_{i=1}^{N_t} T_i$. For each local deformation $T_i^0 \rightarrow T_i$, as depicted in Figure 3.6, the energy stored in $T_i$ is

$$\Delta_i\tilde{F}_{\text{MS}}[T_i, T_i^0] = \Delta_i\tilde{F}_{\text{stretch}}[T_i, T_i^0] + \Delta_i\tilde{F}_{\text{shear}}[T_i, T_i^0],$$

where

$$\Delta_i\tilde{F}_{\text{stretch}} = \frac{K\alpha}{2} \int_{T_i^0} \left( \alpha^2 + a_3 \alpha^3 + a_4 \alpha^4 \right) dA_0,$$  \hspace{1cm} (3.10)

$$\Delta_i\tilde{F}_{\text{shear}} = \mu \int_{T_i^0} \left( \beta + b_1 \alpha \beta + b_2 \beta^2 \right) dA_0,$$  \hspace{1cm} (3.11)

and $\alpha[T_i, T_i^0]$ and $\beta[T_i, T_i^0]$ are calculated using both $T_i$ and $T_i^0$. We take the strains to be constant over each triangle, i.e., $\alpha[T_i, T_i^0] \simeq \alpha_i$ and $\beta[T_i, T_i^0] \simeq \beta_i$. Thus, Equations (3.10)
Figure 3.5: Semilog plots of $\sigma_b$ against $N_t$ for the three test cases whose results are given in Table 3.2. The data points are joined by straight line segments to aid inspection.
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Figure 3.6: The deformation of the $i$th triangle from its original, unstressed geometry $T^0_i$ on $\tilde{S}_0$ to its final geometry $T_i$ on $\tilde{S}$, with corresponding changes to its edge vectors, $\mathbf{l}^0_{i,1} \rightarrow \mathbf{l}_{i,1}$ and $\mathbf{l}^0_{i,2} \rightarrow \mathbf{l}_{i,2}$. The normal vector $\mathbf{N}_i$ is the cross product of $\mathbf{l}_{i,1}$ with $\mathbf{l}_{i,2}$.

and (3.11) become

\[
\Delta_i \bar{F}_{\text{stretch}} \simeq \frac{K\alpha}{2} \left( \alpha_i^2 + a_3 \alpha_i^3 + a_4 \alpha_i^4 \right) \Delta_i A_0 ,
\]

\[
\Delta_i \bar{F}_{\text{shear}} \simeq \mu \left( \beta_i + b_1 \alpha_i \beta_i + b_2 \beta_i^2 \right) \Delta_i A_0 ,
\]

where $\Delta_i A_0$ is the area of $T^0_i$. Thus, $F_{\text{MS}}$ is approximated by

\[
\bar{F}_{\text{MS}} = \bar{F}_{\text{stretch}} + \bar{F}_{\text{shear}} ,
\]

where

\[
\bar{F}_{\text{stretch}} = \sum_{i=1}^{N_t} \Delta_i \bar{F}_{\text{stretch}} ,
\]

\[
\bar{F}_{\text{shear}} = \sum_{i=1}^{N_t} \Delta_i \bar{F}_{\text{shear}} .
\]

$\alpha_i$ and $\beta_i$ are given by

\[
\alpha_i = \lambda_x \lambda_y - 1 ,
\]

\[
\beta_i = \frac{1}{2} \left[ \frac{\lambda_x^2}{\lambda_x \lambda_y} + \frac{\lambda_x \lambda_y}{\lambda_x^2} \left( 1 + \tan^2 \phi \right) - 2 \right] ,
\]
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

Figure 3.7: An initial triangle is imagined to be contained within a larger rectangle. Deformation of the initial triangle into the final triangle is then accomplished by stretching (or compressing) the rectangle along the lines $0\overline{1}$ and $\overline{32}$ by factors of $\lambda_x$ and $\lambda_y$, respectively, followed by shearing through an angle $\varphi$.

where the dependence of $\lambda_x$, $\lambda_y$, and $\varphi$ on $i$ has been suppressed, and $\varphi$ is as defined in Figure 3.7. The four quantities $\lambda_x\lambda_y$, $\lambda_x^2$, $\tan^2\varphi$, and $\Delta_2A_0$ are calculated from the initial edge vectors, $I_1^0$ and $I_2^0$, and final edge vectors, $I_1$ and $I_2$ (the subscript $i$ of all the edge vectors has been omitted for brevity) according to:

$$\lambda_x\lambda_y = \frac{|I_1 \times I_2|}{|I_1^0 \times I_2^0|}, \quad \lambda_x^2 = \frac{|I_1|^2}{|I_1^0|^2}, \quad \tan^2\varphi = \frac{P}{Q}, \quad \Delta_2A_0 = \frac{1}{2}|I_1^0 \times I_2^0|,$$

where

$$P = \left(\frac{I_1^0 \cdot I_2^0}{|I_1^0|^2} - \frac{I_1 \cdot I_2}{|I_1|^2}\right)^2 |I_1|^2, \quad Q = |I_2| - \left(\frac{I_1 \cdot I_2}{|I_1|^2}\right)|I_1|^2.$$

3.3.1 Test 2

As a partial test of the routine in our computer programme for calculating the initial value of $\widetilde{F}_{MS}$, and also of the convergence $\widetilde{F}_{MS} \rightarrow F_{MS}$, values of $\widetilde{F}_{MS}$ from Equation (3.12) were computed for $1 \leq N_{div} \leq 5$ for the uniform compression of an initial sphere. For uniform compression, $\widetilde{F}_{MS} = \widetilde{F}_{\text{stretch}}$. The relevant parameters were set to $\mu = 0$, $K_\alpha = 1$, $a_3 = -2$, $a_4 = 3$, $A_0 = 1.5$, and $A = 1$ (all in arbitrary units). They give $\alpha = \frac{A}{A_0} - 1 = -\frac{1}{3}$ and

$$F_{MS} = \frac{K_\alpha}{2} \left(\alpha^2 + a_3\alpha^3 + a_4\alpha^4\right)A_0 = \frac{1}{6} = 0.16.$$
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Values of $\tilde{F}_{MS}$ as a function of $N_t$ and the corresponding absolute percentage difference,

$$\sigma_{MS} = \left| \frac{\tilde{F}_{MS} - F_{MS}}{F_{MS}} \right| \times 100,$$

are shown in Table 3.3. The data in Table 3.3 show that obtaining estimates of $F_{MS}$ (at least, in the case of vanishing shear deformation) with 99% accuracy or better requires $N_t \geq 1280$.

<table>
<thead>
<tr>
<th>$N_t$</th>
<th>$\tilde{F}_{MS}$</th>
<th>$\sigma_{MS}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>0.154724</td>
<td>7.165468</td>
</tr>
<tr>
<td>320</td>
<td>0.163530</td>
<td>1.882182</td>
</tr>
<tr>
<td>1280</td>
<td>0.165873</td>
<td>0.476493</td>
</tr>
<tr>
<td>5120</td>
<td>0.166468</td>
<td>0.119499</td>
</tr>
<tr>
<td>20480</td>
<td>0.166617</td>
<td>0.029898</td>
</tr>
<tr>
<td>exact</td>
<td>0.166666</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.3: $\tilde{F}_{MS}$ ($\tilde{F}_{stretch}$) as a function of $N_t$ and the corresponding $\sigma_{MS}$ for uniform compression of a sphere.

3.4 Discretisation of $F_{constraint}$

The first step in approximating $F_{constraint}$ is to calculate the volume $\tilde{V}$ and area $\tilde{A}$ of $\tilde{S}$. From elementary vector algebra, $V$ [Equation (2.40)] and $A$ [Equation (2.41)] are approximated respectively by

$$\tilde{V} = \frac{1}{6} \sum_{i=1}^{N_t} \mathbf{R}_i \cdot \left( \mathbf{l}_{i,1} \times \mathbf{l}_{i,2} \right),$$

$$\tilde{A} = \frac{1}{2} \sum_{i=1}^{N_t} \left| \mathbf{l}_{i,1} \times \mathbf{l}_{i,2} \right|,$$

where $\mathbf{R}_i$, $\mathbf{l}_{i,1}$ and $\mathbf{l}_{i,2}$ are as shown in Figure 3.6. To calculate $\tilde{V}$ correctly from Equation (3.13) for all $\tilde{S}$ of topological genus 0, it is crucial to ensure that the cross product of $\mathbf{l}_{i,1}$ and $\mathbf{l}_{i,2}$ (shown as the normal $\mathbf{N}_i$ in Figure 3.6) points outward. The extra factor of $\frac{1}{2}$ in
Equations (3.13) and (3.14) compared to Equations (2.40) and (2.41) accounts for the fact that the parallelogram defined by \( l_{i,1} \) and \( l_{i,2} \) has twice the area of the triangle \( T_i \). Once \( \tilde{V} \) and \( \tilde{A} \) are found using Equations (3.13) and (3.14), they are substituted in

\[
\tilde{F}_V = \frac{K_V (\tilde{V} - V_{RBC})^2}{2V_{RBC}},
\]

(3.15)

\[
\tilde{F}_A = \frac{K_A (\tilde{A} - A_{RBC})^2}{2A_{RBC}},
\]

(3.16)

to give the approximate \( F_V \) and \( F_A \), respectively, and the approximate \( F_{\text{constraint}} \),

\[
\tilde{F}_{\text{constraint}} = \tilde{F}_V + \tilde{F}_A.
\]

(3.17)

### 3.4.1 Test 3

We end this Section by showing the results of testing the routines in our computer programme for calculating the initial values of \( \tilde{A}, \tilde{V}, \) and \( \tilde{F}_{\text{constraint}} \) for a sphere, again for

\[1 \leq N_{\text{div}} \leq 5\]

This is also a test of the convergence of \( \tilde{A}, \tilde{V}, \) and \( \tilde{F}_{\text{constraint}} \). By setting

\[K_A = A = 2, A_{RBC} = 1, K_V = 6\sqrt{2\pi} \text{ and } V_{RBC} = \frac{1}{3} \sqrt{\frac{2}{\pi}}\]

(all in arbitrary units), one gets

\[V = \frac{1}{3} \sqrt{\frac{2}{\pi}} = 0.265962 \text{ and } F_A = F_V = 1\].

Comparison of \( \tilde{A}, \tilde{V}, \tilde{F}_A, \) and \( \tilde{F}_V \) from Table 3.4 with their respective theoretical values again shows that \( N_1 \geq 1280 \) is required for numerical accuracy.

<table>
<thead>
<tr>
<th>(N_1)</th>
<th>(\tilde{A})</th>
<th>(\tilde{V})</th>
<th>(\tilde{F}_A)</th>
<th>(\tilde{F}_V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>1.856691</td>
<td>0.232305</td>
<td>0.733919</td>
<td>1.269108</td>
</tr>
<tr>
<td>320</td>
<td>1.962356</td>
<td>0.256962</td>
<td>0.926130</td>
<td>1.068824</td>
</tr>
<tr>
<td>1280</td>
<td>1.990470</td>
<td>0.263673</td>
<td>0.981031</td>
<td>1.017286</td>
</tr>
<tr>
<td>5120</td>
<td>1.997610</td>
<td>0.265387</td>
<td>0.995226</td>
<td>1.004326</td>
</tr>
<tr>
<td>20480</td>
<td>1.999402</td>
<td>0.265818</td>
<td>0.998804</td>
<td>1.001082</td>
</tr>
<tr>
<td>exact</td>
<td>2</td>
<td>0.265962</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3.4: \( \tilde{A}, \tilde{V}, \tilde{F}_A, \) and \( \tilde{F}_V \) as a function of \( N_1 \), calculated using Equations (3.13) to (3.16).
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

3.5 Minimisation by the Metropolis algorithm

At this point, we have all the necessary preparation for calculating the approximate total free energy,

$$\tilde{F} [\tilde{S}, \tilde{S}_0] = \tilde{F}_{\text{RBC}} [\tilde{S}, \tilde{S}_0] + \tilde{F}_{\text{constraint}} [\tilde{S}],$$

(3.18)

where

$$\tilde{F}_{\text{RBC}} [\tilde{S}, \tilde{S}_0] = \tilde{F}_b [\tilde{S}] + \tilde{F}_{\text{MS}} [\tilde{S}, \tilde{S}_0].$$

(3.19)

$\tilde{F}_{\text{constraint}}$ is kept small by means of large values of $K_A$ and $K_V$, so that $\tilde{F} \simeq \tilde{F}_{\text{RBC}}$. We proceed to the final step in minimising $\tilde{F}$, implementing the Metropolis algorithm (see, e.g., Ref. 1 or 155) to carry out the actual minimisation. For computational efficiency, the twelve defective vertices and the $N_v - 12$ regular vertices are separated at the beginning by numbering the former as the first twelve followed by the latter in the array of all vertex indices. Then, each vertex on $\tilde{S}$ is picked sequentially from the array and subjected to a trial move. In each trial move, the vertex is displaced randomly by a small amount through the use of a pseudo-random number generator (the simple multiplicative congruential generator, $\text{ran0}$, the algorithm of which is shown on p. 279 of Ref. 168) to produce a slightly perturbed surface, $\tilde{S}_{\text{new}}$, from $\tilde{S}$. This vertex displacement gives rise to a change in $\tilde{F}$,

$$\Delta \tilde{F} \equiv \tilde{F} [\tilde{S}_{\text{new}}, \tilde{S}_0] - \tilde{F} [\tilde{S}, \tilde{S}_0],$$

which is calculated from the new and old geometries of the triangles of the cluster about the displaced vertex (cf. Figure 3.2). Geometrical changes of the nearest neighbour triangles of the cluster (coloured grey) affect $\tilde{F}_{\text{MS}}$ and $\tilde{F}_{\text{constraint}}$ (through $\tilde{A}$ and $\tilde{V}$). $\tilde{F}_b$, on the other hand, is affected by all the triangles of the cluster because of its reliance on $A_k$ [Equation (3.6)] and $M_k$ [Equation (3.8)] of $\Gamma_k$ as well as on $A_{k'}$ and $M_{k'}$ of each of the neighbouring $\Gamma_{k'}$'s (shown in Figure 3.3). The last part of the trial move consists of a test to reject or accept the move:

1. If the Boltzmann factor, $\exp \left( -\Delta \tilde{F}/k_B T \right)$, is less than a randomly chosen number in the interval [0, 1], the move is rejected and the moved vertex reverts to its old coordinates.

2. Otherwise, the move is accepted and all related computational variables are updated to reflect the assignment $\tilde{S} = \tilde{S}_{\text{new}}$. 
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

In the above, $k_B$ is Boltzmann's constant and $T$ is a user-defined minimisation parameter, referred to loosely as the “temperature.” $T$ can certainly be made physical by equating it to the temperature of the RBC's environment, in which case $\tilde{F}$ includes a thermal correction to the $T = 0$ minimum mechanical energy $\tilde{F}_{\text{min}}$. For the most part, we are more interested in the shape $S_{\text{min}}$ corresponding to $\tilde{F}_{\text{min}}$ than $\tilde{F}_{\text{min}}$ itself. Neither $\tilde{F}_{\text{min}}$ or $S_{\text{min}}$ is directly attainable from the Metropolis algorithm, since the algorithm relies on the use of a non-zero $T$ and, hence, cannot enforce strict minimisation. However, a good approximation of both can be had by setting $T$ to a value much lower than is physiologically realistic. By using a very small $T$ in the Boltzmann factor, trial moves that raise $\tilde{F}$ (positive $\Delta\tilde{F}$) substantially are heavily penalised, while moves that lower $\tilde{F}$ (negative $\Delta\tilde{F}$) are always accepted. For a sufficiently large number of sweeps (a sweep being a pass over all $N_v$ vertices), $\tilde{F}_{RBC}$ will converge to, and then fluctuate about, the thermal ensemble average $\langle \tilde{F}_{RBC} \rangle_T$. In general, the initial $\tilde{F}_{RBC}$ may be higher or lower than $\langle \tilde{F}_{RBC} \rangle_T$, depending on the starting shape. Here, the usual situation is the former. In principle, one varies $T$ and $N_t$ to obtain $\langle \tilde{F}_{RBC} \rangle_T$ as a function of $T$ and $N_t$. This allows $\tilde{F}_{\text{min}}$ to be estimated from the extrapolation of $\langle \tilde{F}_{RBC} \rangle_T$ to $T = 0$ and $N_t^{-1} = 0$. In practise, the operation of varying $N_t$ is only feasible for smooth shapes, with the feasible values of $N_t$ being 1280 and 5120. $N_t = 5120$ is effectively the only choice for shapes with sharper features, since the case of $N_t \geq 20480$ is too time-consuming. The probability of acceptance of a trial move is kept at $(50 \pm 2)\%$ by adjusting the step size of the trial moves throughout a minimisation run. Another important computational parameter is $N_{\text{sweep}}$, the total number of sweeps for the initial convergence of $\tilde{F}_{RBC}$ to $\langle \tilde{F}_{RBC} \rangle_T$ and the subsequent gathering of sufficient statistics to estimate $\langle \tilde{F}_{RBC} \rangle_T$. The values of $N_{\text{sweep}}$ and $T$ used are dependent upon the size of the system (as measured by $N_t$, for example) and the type of operation under consideration. There are two types of operations: (i) the preparation of the set of reference surfaces $\tilde{S}_0$ (discussed in Section 3.7), and (ii) the production of the minimum energy $\tilde{S}$ for a given $\tilde{S}_0$ (discussed in Chapter 4); the exact values of $N_{\text{sweep}}$ and $T$ for each will be given as the need arises.
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$  

3.5.1 Test 4

In the last three tests (Sections 3.2.1, 3.3.1 and 3.4.1), we have confirmed that the routines in our computer programme for calculating the initial values of $\tilde{F}_b$, $\tilde{F}_{\text{stretch}}$, $\tilde{F}_{\text{constraint}}$, $\tilde{A}$ and $\tilde{V}$ are correctly implemented. However, we have not tested the routine for calculating the initial value of $\tilde{F}_{\text{shear}}$.

We now perform a test with two parts. Firstly, we exploit the routines for calculating the initial energies to ensure that our implementation of the Metropolis algorithm is correctly calculating and updating all the energies except $\tilde{F}_{\text{shear}}$. Secondly, we use hand calculations to ensure that the routine for calculating the initial $\tilde{F}_{\text{shear}}$ and the routine for calculating and updating $\tilde{F}_{\text{shear}}$ in the Metropolis algorithm are correctly programmed.

In this test, an initial icosahedron ($N_t = 20$) is deformed through Monte Carlo moves of its 12 vertices and all the energy parameters are set to non-zero values. The simplicity of the icosahedron allows us to check all the energies using hand calculations, if so desired, within a reasonable time. Setting all the parameters to non-zero values exposes as much as possible the energy calculation routines to scrutiny. The parameter values used are shown in Table 3.5.

In the first part of the test, two consecutive runs with $N_{\text{sweep}} = 100$ each at $\kappa_b/k_BT = 1$ are carried out. We look for agreement between (i) the final energies of the first run, which are generated by the Metropolis algorithm, and (ii) the initial energies of the second run, which are produced by the routines for calculating the initial energies. Given that the routines for calculating the initial $\tilde{F}_b$, $\tilde{F}_{\text{MS}}$ (excluding the shear component), and $\tilde{F}_{\text{constraint}}$ have been shown previously to be implemented correctly, this agreement will give us confidence that the energies are being updated correctly in the minimisation step, barring subtle errors in the data output routine that give apparently correct results when compounded. The energies, area and volume after the first run are given in the middle column of Table 3.6. They agree with those calculated at the beginning of the second run, shown in the last column of Table 3.6, except for the discrepancy in the last two to four significant figures due to computer round-off error. Thus, the routines for minimising $\tilde{F}_b$, $\tilde{F}_{\text{stretch}}$ and $\tilde{F}_{\text{constraint}}$ appear to have been implemented correctly.

Table 3.6 also shows that $\tilde{F}_{\text{shear}}$ after the first run agrees with that at the beginning of the second, i.e., the routine for calculating the initial $\tilde{F}_{\text{shear}}$ and the routine for minimising...
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

<table>
<thead>
<tr>
<th>Energy Components</th>
<th>Energy Parameters</th>
<th>Test 4 Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_b / \kappa_b$</td>
<td>$\alpha_b, m_0$</td>
<td>1</td>
</tr>
<tr>
<td>$F_{MS} / \kappa_b$</td>
<td>$K_{\alpha}/\kappa_b, a_3, a_4, \mu/\kappa_b, b_1, b_2, S_0$</td>
<td>2</td>
</tr>
<tr>
<td>$F_{constraint} / \kappa_b$</td>
<td>$K_A/\kappa_b$, $A_{RBC}$, $K_V/\kappa_b$, $V_{RBC}$</td>
<td>$5 \times 10^4, 4\pi, 5 \times 10^4, 2\pi/3$</td>
</tr>
</tbody>
</table>

Table 3.5: Values of the energy parameters used in Tests 4, all in arbitrary units.

<table>
<thead>
<tr>
<th></th>
<th>At end of first run</th>
<th>At start of second run</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\tilde{F}_b / \kappa_b$</td>
<td>$1.1729282546873219 \times 10^2$</td>
<td>$1.1729282546873236 \times 10^2$</td>
</tr>
<tr>
<td>$\tilde{F}_{stretch} / \kappa_b$</td>
<td>$6.1895518024617813$</td>
<td>$6.1895518024617804$</td>
</tr>
<tr>
<td>$\tilde{F}_{shear} / \kappa_b$</td>
<td>$1.0085074230422778 \times 10^1$</td>
<td>$1.0085074230422775 \times 10^1$</td>
</tr>
<tr>
<td>$\tilde{A}$</td>
<td>$1.1546974470083848 \times 10^1$</td>
<td>$1.1546974470083859 \times 10^1$</td>
</tr>
<tr>
<td>$\tilde{V}$</td>
<td>$2.6365688089467745$</td>
<td>$2.6365688089467727$</td>
</tr>
<tr>
<td>$\tilde{F}_A / \kappa_b$</td>
<td>$2.0673600414426228 \times 10^3$</td>
<td>$2.0673600414425814 \times 10^3$</td>
</tr>
<tr>
<td>$\tilde{F}_V / \kappa_b$</td>
<td>$3.5087974535243766 \times 10^3$</td>
<td>$3.5087974535243516 \times 10^3$</td>
</tr>
</tbody>
</table>

Table 3.6: Comparison of results at the end of the first run and those at the beginning of the second run of Test 4.

$\tilde{F}_{shear}$ are producing consistent results. The second part of this test is to verify the accuracy of $\tilde{F}_{shear}$. This is done by comparing the final $\tilde{F}_{shear}$ of the second run with that calculated
### Table 3.7: Comparison of results at the end of the second run of Test 4 and those calculated by entering the final data of the second run into Matlab.

<table>
<thead>
<tr>
<th></th>
<th>At end of second run</th>
<th>Calculated using Matlab</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\bar{F}_{\text{stretch}}/\kappa_b)</td>
<td>(1.457767399094672 \times 10^1)</td>
<td>(1.457767399094673 \times 10^1)</td>
</tr>
<tr>
<td>(\bar{F}_{\text{shear}}/\kappa_b)</td>
<td>(4.5697778044045165 \times 10^1)</td>
<td>(4.569777804404459 \times 10^1)</td>
</tr>
</tbody>
</table>

Table 3.7: Comparison of results at the end of the second run of Test 4 and those calculated by entering the final data of the second run into Matlab.

from entering the data describing the deformed icosahedron into Matlab, i.e., a “hand” calculation. These data consist of the instantaneous vertex coordinates (Table E.1), the initial vertex coordinates (Table E.2), and the vertex indices of each triangle (Table E.3). The content of the Matlab script file “pxratios.m” used for calculating \(\bar{F}_{\text{MS}}\) is given in Appendix D. The values of \(\bar{F}_{\text{stretch}}\) and \(\bar{F}_{\text{shear}}\) after the second run are shown in the middle column of Table 3.7. They are in good agreement with the values given by Matlab, shown in the last column of Table 3.7. Thus, the routine for calculating the initial \(\bar{F}_{\text{shear}}\) and the routine for minimising \(\bar{F}_{\text{shear}}\) appear to have been programmed correctly.

#### 3.6 Analysis of raw data

In this section, we treat a simplified example to show how to analyse the raw data generated by the Metropolis algorithm. We carried out a complete minimisation using the set of parameters given in Table 3.5. These parameters have been chosen to simplify the form of \(F\). In particular, \(F_b\) has no contribution from the area-difference elasticity and, consequently, reduces to \(\kappa_b G\), where \(G\) is given by Equation (2.15). We found that \(\bar{S}\), the surface that minimises \(\bar{F}_{\text{RBC}} = \bar{F}_b + \bar{F}_{\text{MS}}\), is an axisymmetric oblate ellipsoid, as shown in Figure 3.8. This shape is similar to the discocytic shape of the normal RBC.

First, we discuss the method to ensure statistical independence in our analysis. Tables 3.9 to 3.12 show the statistics of the distributions of \(\bar{F}_b\), \(\bar{F}_{\text{stretch}}\), \(\bar{F}_{\text{shear}}\) and \(\bar{F}_{\text{RBC}}\) for given values of \(N_t\) and \(\kappa_b/k_B T\). The values of \(N_t\) used are 320, 1280 and 5120. The values of \(\kappa_b/k_B T\) used are between \(1 \times 10^3\) and \(1 \times 10^5\). The 48 averages are based on runs of \((4-19) \times 10^6\) sweeps each, with each run sampled at a separation of 5000 sweeps. To ensure adequate equilibration, a variable number of initial data points are eliminated from the data set used to determine each average, leaving \(N_{\text{eq}}\) data points for each energy for
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

<table>
<thead>
<tr>
<th>Energy Components</th>
<th>Energy Parameters</th>
<th>Parameter Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_b/\kappa_b$</td>
<td>$\alpha_b$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$\overline{m}_0$</td>
<td>0</td>
</tr>
<tr>
<td>$F_{MS}/\kappa_b$</td>
<td>$K_{\alpha}/\kappa_b$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$a_3$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$a_4$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$\mu/\kappa_b$</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>$b_1$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$b_2$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$S_0$</td>
<td>sphere with area $A_{RBC}$</td>
</tr>
<tr>
<td>$F_{\text{constraint}}/\kappa_b$</td>
<td>$K_A/\kappa_b$</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>$A_{RBC}$</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>$K_V/\kappa_b$</td>
<td>$5 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>$V_{RBC}$</td>
<td>95</td>
</tr>
</tbody>
</table>

Table 3.8: Values of the energy parameters (in arbitrary units) used in the minimisation runs of Section 3.6.

For a given $T$ and $N_t$. These $N_{eq}$ points are subsequently used to determine the unnormalised autocorrelation functions, $AC_b(m)$, $AC_{\text{stretch}}(m)$, $AC_{\text{shear}}(m)$ and $AC_{\text{RBC}}(m)$ for $\bar{F}_b$, $\bar{F}_\text{stretch}$, $\bar{F}_\text{shear}$ and $\bar{F}_{\text{RBC}}$, respectively, to check these points' statistical independence. The variable $m$ of the autocorrelation functions is the separation between two data points in multiples of 5000 sweeps, the meaning of which will be made precise below. For the set of data points $\{F_i; i = 1, \ldots, N_{eq}\}$ of $\bar{F}_{\text{RBC}}$, $AC_{\text{RBC}}$ is defined by

$$AC_{\text{RBC}}(m) \equiv \frac{1}{N_{eq} - m} \sum_{i=1}^{N_{eq}-m} (F_i - \overline{F}_i)(F_{i+m} - \overline{F}_{i+m})$$  \hspace{1cm} (3.20)

$$= \frac{1}{N_{eq} - m} \sum_{i=1}^{N_{eq}-m} F_i F_{i+m} - \overline{F}_i \overline{F}_{i+m}$$  \hspace{1cm} (3.21)
Figure 3.8: Minimum-energy shapes with $N_i = 1280$ (panels on the left) and $N_i = 5120$ (panels on the right) at $\kappa_b/k_B T = 10000$.

where

\begin{align}
\overline{F}_i(m) &= \frac{1}{N_{eq} - m} \sum_{i=1}^{N_{eq}-m} F_i, \\
\overline{F}_{i+m}(m) &= \frac{1}{N_{eq} - m} \sum_{i=1}^{N_{eq}-m} F_{i+m},
\end{align}

and, as a rule of thumb to ensure the accuracy of $AC_{RBC}$, $\overline{F}_i$ and $\overline{F}_{i+m}$ at large $m$, $m$ is restricted to $0 \leq m \leq N_{eq} - 100$. The other 3 autocorrelation functions are defined similarly. As an example, consider the case where $N_i = 1280$ and $\kappa_b/k_B T = 10000$. The four corresponding autocorrelation functions plotted in Figure 3.9 show very clearly that there is no correlation between data points with a point-to-point separation of 5000 sweeps ($m = m_{\text{indep}} = 1$) or more. Thus, it follows that all $N_{eq}$ points can be used to calculate the standard deviation of the corresponding energy and the standard error of the corresponding average energy. However, for the largest system size at very low $T$, the energies remain
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

Figure 3.9: Plots of $AC_b$, $AC_{\text{stretch}}$, $AC_{\text{shear}}$ and $AC_{\text{RBC}}$ as functions of $m$, for $N_t = 1280$ and $\kappa_b/k_B T = 10000$ [see Equation (3.21)].
correlated over a few multiples of 5000 sweeps, in which case those points used for calculating each average are selected from the pool of \( N_{eq} \) with a point-to-point separation of \( 5000 \times m_{\text{indep}} \) sweeps, where \( m_{\text{indep}} > 1 \) is sufficient for the correlation to vanish. Thus, in general, the number of points used for each average is \( N_{\text{eq}}' = N_{eq}/m_{\text{indep}}, m_{\text{indep}} \geq 1 \).

Next, we examine the shape of each sample distribution. Tables 3.9 to 3.12 include two additional statistics of each sample distribution: skewness and kurtosis, defined by (see, e.g., Section 14.1 of Ref. 168)

\[
\text{Skew} = \frac{M_3}{M_2^{3/2}} \quad \text{and} \quad \text{Kurt} = \frac{M_4 - 3M_2^2}{M_2^2},
\]

respectively, where \( M_i \) is the \( i \)th moment of the distribution. The significance of these statistics is that they provide some quantitative measure of how closely each sample distribution resembles a normal distribution. A normal distribution has Skew = Kurt = 0 and standard deviations of \( \sqrt{15}/N \) and \( \sqrt{96}/N \) for Skew and Kurt, respectively, where \( N \) is the number of sample points. By substituting in \( N = N_{\text{eq}}' \) from Tables 3.9 to 3.12, it can be seen that the Skew and Kurt of all the sample distributions are within two standard deviations of those for the corresponding normal distributions. Therefore, it is reasonable to regard all the samples as being normally distributed. (That the samples are normally distributed may be predicted physically from Gaussian fluctuations of the vertices at low \( T \), as discussed at the end of this Section.)

The analysis so far has focussed on the statistics of single minimisation runs at particular values of \( N_t \) and \( T \). We now describe how to extract from such data a reliable estimate of the minimum overall mechanical energy \( F_{\text{RBC}} \) and its components in the continuum limit. This requires two levels of extrapolation, \( T \to 0 \) and \( N_t \to \infty \). We first extrapolate the four groups of average energies given in Tables 3.9 to 3.12 to \( T = 0 \) for a given \( N_t \) and then extrapolate the zero-\( T \) energies to \( N_t^{-1} = 0 \). The best fit to the \( T \)-dependence of each of \( \langle F_b \rangle_T, \langle F_{\text{stretch}} \rangle_T, \langle F_{\text{shear}} \rangle_T \) and \( \langle F_{\text{RBC}} \rangle_T \) is chosen to be a straight line of the form \( b_0 (k_B T / \kappa_b) + c_0 \), where \( c_0 = \langle F_b \rangle_{T=0}, \langle F_{\text{stretch}} \rangle_{T=0}, \langle F_{\text{shear}} \rangle_{T=0} \) or \( \langle F_{\text{RBC}} \rangle_{T=0} \). (That the average energies are linearly dependent on \( T \) also arises physically from Gaussian fluctuations of the vertices at low \( T \), as discussed at the end of this Section.) The best fit to the \( N_t \)-dependence of each of \( \langle F_b \rangle_{T=0}, \langle F_{\text{stretch}} \rangle_{T=0}, \langle F_{\text{shear}} \rangle_{T=0} \) and \( \langle F_{\text{RBC}} \rangle_{T=0} \) is chosen to be a second order polynomial of the form \( a_\infty (N_t^{-1})^2 + b_\infty (N_t^{-1}) + c_\infty \), where \( c_\infty = \langle F_b \rangle_{T=0, N_t \to \infty}, \langle F_{\text{stretch}} \rangle_{T=0, N_t \to \infty}, \langle F_{\text{shear}} \rangle_{T=0, N_t \to \infty} \) or \( \langle F_{\text{RBC}} \rangle_{T=0, N_t \to \infty} \). (That the
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$  

<table>
<thead>
<tr>
<th>$N_t$</th>
<th>$\kappa_b/k_B T$</th>
<th>$N_{\text{sweep}}$</th>
<th>$m_{\text{indep}}$</th>
<th>$N_{eq}$</th>
<th>$\langle F_b \rangle_T$</th>
<th>std.</th>
<th>Skew</th>
<th>Kurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>1000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>46.76953 $\pm$ 0.00063</td>
<td>0.017</td>
<td>0.1197</td>
<td>-0.0123</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>$4 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>46.73275 $\pm$ 0.00039</td>
<td>0.010</td>
<td>0.1827</td>
<td>-0.0727</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>600</td>
<td>46.71017 $\pm$ 0.00027</td>
<td>0.0065</td>
<td>0.1362</td>
<td>-0.0902</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>500</td>
<td>46.70271 $\pm$ 0.00019</td>
<td>0.0042</td>
<td>0.1363</td>
<td>0.2432</td>
</tr>
<tr>
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<td>5000</td>
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<td>1800</td>
<td>48.15124 $\pm$ 0.00014</td>
<td>0.0060</td>
<td>0.0886</td>
<td>-0.1413</td>
</tr>
<tr>
<td></td>
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<td>1200</td>
<td>48.11956 $\pm$ 0.00011</td>
<td>0.0039</td>
<td>0.2042</td>
<td>-0.0582</td>
</tr>
<tr>
<td></td>
<td>20000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>1100</td>
<td>48.103839 $\pm$ 0.000076</td>
<td>0.0025</td>
<td>0.2637</td>
<td>0.3644</td>
</tr>
<tr>
<td></td>
<td>50000</td>
<td>$16 \times 10^6$</td>
<td>1</td>
<td>800</td>
<td>48.094352 $\pm$ 0.000056</td>
<td>0.0015</td>
<td>0.1258</td>
<td>-0.1814</td>
</tr>
<tr>
<td></td>
<td>100000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>1100</td>
<td>48.091231 $\pm$ 0.000033</td>
<td>0.0011</td>
<td>0.1439</td>
<td>-0.1749</td>
</tr>
<tr>
<td>5120</td>
<td>10000</td>
<td>$7 \times 10^6$</td>
<td>6</td>
<td>234</td>
<td>48.56561 $\pm$ 0.000032</td>
<td>0.0049</td>
<td>0.2259</td>
<td>0.0676</td>
</tr>
<tr>
<td></td>
<td>20000</td>
<td>$8 \times 10^6$</td>
<td>10</td>
<td>160</td>
<td>48.50136 $\pm$ 0.000023</td>
<td>0.0029</td>
<td>-0.0504</td>
<td>-0.3432</td>
</tr>
<tr>
<td></td>
<td>40000</td>
<td>$8 \times 10^6$</td>
<td>6</td>
<td>237</td>
<td>48.46977 $\pm$ 0.000012</td>
<td>0.0020</td>
<td>0.0331</td>
<td>-0.3433</td>
</tr>
</tbody>
</table>

Table 3.9: The mean ± standard error of the mean, standard deviation, skewness and kurtosis of each sample distribution of $F_b$ for a given $N_t$ and $T$. Skewness and kurtosis are defined in Equation (3.24).

<table>
<thead>
<tr>
<th>$N_t$</th>
<th>$\kappa_b/k_B T$</th>
<th>$N_{\text{sweep}}$</th>
<th>$m_{\text{indep}}$</th>
<th>$N_{eq}$</th>
<th>$\langle F_{\text{stretch}} \rangle_T$</th>
<th>std.</th>
<th>Skew</th>
<th>Kurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>1000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>0.5549 $\pm$ 0.0011</td>
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<td>-0.2329</td>
</tr>
<tr>
<td></td>
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<td>$4 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>0.52368 $\pm$ 0.00074</td>
<td>0.020</td>
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<td>-0.1184</td>
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<tr>
<td></td>
<td>5000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>600</td>
<td>0.50543 $\pm$ 0.00052</td>
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<td>-0.3546</td>
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<td>500</td>
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<td>0.0090</td>
<td>-0.0094</td>
<td>-0.1071</td>
</tr>
<tr>
<td>1280</td>
<td>5000</td>
<td>$19 \times 10^6$</td>
<td>1</td>
<td>1800</td>
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<td>0.0335</td>
<td>-0.0391</td>
</tr>
<tr>
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<td>-0.0811</td>
</tr>
<tr>
<td></td>
<td>50000</td>
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<td>1</td>
<td>800</td>
<td>0.53439 $\pm$ 0.00014</td>
<td>0.0039</td>
<td>0.0406</td>
<td>-0.1374</td>
</tr>
<tr>
<td></td>
<td>100000</td>
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<td>1</td>
<td>1100</td>
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<td>-0.1414</td>
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<td>5120</td>
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<td>6</td>
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<td>0.64019 $\pm$ 0.00062</td>
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<td>0.0814</td>
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<td>0.0304</td>
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Table 3.10: The mean ± standard error of the mean, standard deviation, skewness and kurtosis of each sample distribution of $F_{\text{stretch}}$ for a given $N_t$ and $T$. Skewness and kurtosis are defined in Equation (3.24).
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

Table 3.1: The mean ± standard error of the mean, standard deviation, skewness and kurtosis of each sample distribution of $\tilde{F}_{\text{shear}}$ for a given $N_t$ and $T$. Skewness and kurtosis are defined in Equation (3.24).

<table>
<thead>
<tr>
<th>$N_t$</th>
<th>$\kappa_b/k_B T$</th>
<th>$N_{\text{swep}}$</th>
<th>$m_{\text{indep}}$</th>
<th>$N'_{eq}$</th>
<th>$\langle \tilde{F}_{\text{shear}} \rangle_T$</th>
<th>std.</th>
<th>Skew</th>
<th>Kurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>1000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>1.4842 ± 0.0012</td>
<td>0.032</td>
<td>0.0475</td>
<td>0.1035</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>$4 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>1.43227 ± 0.00080</td>
<td>0.021</td>
<td>-0.0120</td>
<td>0.0007</td>
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<td>5000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>600</td>
<td>1.40065 ± 0.00056</td>
<td>0.014</td>
<td>0.0794</td>
<td>-0.2447</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>500</td>
<td>1.39097 ± 0.00042</td>
<td>0.0094</td>
<td>-0.0411</td>
<td>-0.0837</td>
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<tr>
<td>1280</td>
<td>5000</td>
<td>$19 \times 10^6$</td>
<td>1</td>
<td>1800</td>
<td>1.38894 ± 0.00030</td>
<td>0.013</td>
<td>0.0416</td>
<td>-0.1402</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>$16 \times 10^6$</td>
<td>1</td>
<td>1200</td>
<td>1.34957 ± 0.00026</td>
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<tr>
<td></td>
<td>20000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>1100</td>
<td>1.32979 ± 0.00019</td>
<td>0.0064</td>
<td>0.1507</td>
<td>-0.1715</td>
</tr>
<tr>
<td></td>
<td>50000</td>
<td>$16 \times 10^6$</td>
<td>1</td>
<td>800</td>
<td>1.31817 ± 0.00014</td>
<td>0.0041</td>
<td>0.0263</td>
<td>0.0412</td>
</tr>
<tr>
<td></td>
<td>100000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>1100</td>
<td>1.314181 ± 0.000083</td>
<td>0.0028</td>
<td>0.0006</td>
<td>-0.3034</td>
</tr>
<tr>
<td>5120</td>
<td>1000</td>
<td>$7 \times 10^6$</td>
<td>6</td>
<td>234</td>
<td>1.44855 ± 0.00059</td>
<td>0.0090</td>
<td>0.0842</td>
<td>-0.2986</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>$8 \times 10^6$</td>
<td>10</td>
<td>160</td>
<td>1.37077 ± 0.00058</td>
<td>0.0073</td>
<td>0.3498</td>
<td>-0.3393</td>
</tr>
<tr>
<td></td>
<td>40000</td>
<td>$8 \times 10^6$</td>
<td>6</td>
<td>267</td>
<td>1.33211 ± 0.00028</td>
<td>0.0046</td>
<td>-0.0229</td>
<td>-0.2462</td>
</tr>
</tbody>
</table>

Table 3.12: The mean ± standard error of the mean, standard deviation, skewness and kurtosis of each sample distribution of $\tilde{F}_{\text{RBC}}$ for a given $N_t$ and $T$. Skewness and kurtosis are defined in Equation (3.24).

<table>
<thead>
<tr>
<th>$N_t$</th>
<th>$\kappa_b/k_B T$</th>
<th>$N_{\text{swep}}$</th>
<th>$m_{\text{indep}}$</th>
<th>$N'_{eq}$</th>
<th>$\langle \tilde{F}_{\text{RBC}} \rangle_T$</th>
<th>std.</th>
<th>Skew</th>
<th>Kurt</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>1000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>48.80867 ± 0.00057</td>
<td>0.015</td>
<td>0.1581</td>
<td>0.3220</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>$4 \times 10^6$</td>
<td>1</td>
<td>700</td>
<td>48.68870 ± 0.00030</td>
<td>0.0079</td>
<td>0.1422</td>
<td>-0.1678</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>600</td>
<td>48.61625 ± 0.00012</td>
<td>0.0030</td>
<td>-0.0093</td>
<td>0.2037</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>500</td>
<td>48.592246 ± 0.000069</td>
<td>0.0015</td>
<td>0.0614</td>
<td>-0.2907</td>
</tr>
<tr>
<td>1280</td>
<td>5000</td>
<td>$19 \times 10^6$</td>
<td>1</td>
<td>1800</td>
<td>50.11972 ± 0.00015</td>
<td>0.0062</td>
<td>-0.0463</td>
<td>-0.1687</td>
</tr>
<tr>
<td></td>
<td>10000</td>
<td>$16 \times 10^6$</td>
<td>1</td>
<td>1200</td>
<td>50.023678 ± 0.000090</td>
<td>0.0031</td>
<td>0.1312</td>
<td>0.0714</td>
</tr>
<tr>
<td></td>
<td>20000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>1100</td>
<td>49.975725 ± 0.000048</td>
<td>0.0016</td>
<td>-0.0097</td>
<td>-0.2412</td>
</tr>
<tr>
<td></td>
<td>50000</td>
<td>$16 \times 10^6$</td>
<td>1</td>
<td>800</td>
<td>49.946909 ± 0.000023</td>
<td>0.00064</td>
<td>0.1147</td>
<td>-0.2861</td>
</tr>
<tr>
<td></td>
<td>100000</td>
<td>$6 \times 10^6$</td>
<td>1</td>
<td>1100</td>
<td>49.9373269 ± 0.000009</td>
<td>0.00032</td>
<td>0.0270</td>
<td>-0.2310</td>
</tr>
<tr>
<td>5120</td>
<td>10000</td>
<td>$7 \times 10^6$</td>
<td>6</td>
<td>234</td>
<td>50.65435 ± 0.00041</td>
<td>0.0062</td>
<td>-0.0207</td>
<td>-0.2611</td>
</tr>
<tr>
<td></td>
<td>20000</td>
<td>$8 \times 10^6$</td>
<td>10</td>
<td>160</td>
<td>50.46296 ± 0.00023</td>
<td>0.0029</td>
<td>-0.2638</td>
<td>-0.6541</td>
</tr>
<tr>
<td></td>
<td>40000</td>
<td>$8 \times 10^6$</td>
<td>6</td>
<td>267</td>
<td>50.367531 ± 0.000096</td>
<td>0.0016</td>
<td>-0.2285</td>
<td>-0.1041</td>
</tr>
</tbody>
</table>
zero-\(T\) energies are second order polynomials in \(N_t^{-1}\) is not something we know analytically; however, the graphs in Figure 3.13 seem to show that it is reasonable.) The fitting parameters \(b_0, c_0, a_\infty, b_\infty\) and \(c_\infty\) are found from the method of least squares, the details of which are given in Appendix C. This method assumes that each measurement of the dependent variable is drawn from a Gaussian distribution with a to-be-fitted mean and a standard deviation corresponding to that measurement’s uncertainty. For the \(T\)-fitting, the use of the method of least squares is justified since the measurements are averages of Gaussian random variables and, hence, themselves Gaussian random variables according to the central limit theorem. As for the \(N_t^{-1}\)-fitting, the use of the method of least squares is essentially a matter of convenience. Table 3.13 shows the parameters for the \(T\)-fittings of the average energies from Tables 3.9 to 3.12. Table 3.14 shows the parameters for the subsequent \(N_t^{-1}\)-fittings of the values of \(c_T\) from Table 3.13. Figures 3.10, 3.11 and 3.12 illustrate graphically the \(T\)-fittings for \(N_t = 320, 1280\) and 5120, respectively. Figure 3.13 illustrates graphically the respective \(N_t^{-1}\)-fittings for \(\langle \tilde{F}_b \rangle_{T=0}, \langle \tilde{F}_{\text{stretch}} \rangle_{T=0}, \langle \tilde{F}_{\text{shear}} \rangle_{T=0}\) and \(\langle \tilde{F}_{\text{RBC}} \rangle_{T=0}\) from Table 3.14. Table 3.13 also shows the minimum \(\chi^2, \chi^2_{\text{min}},\) for each \(T\)-fitting and the goodness-of-fit probability, \(Q,\) which measures the likelihood of \(\chi^2\) of the current data set being equal to or exceeded by the \(\chi^2_{\text{min}}\) value of another data set with

<table>
<thead>
<tr>
<th>(\langle \tilde{F}_b \rangle_T)</th>
<th>(N_t)</th>
<th>(\frac{3}{2}N_t)</th>
<th>(b_0)</th>
<th>(c_0 = \langle \tilde{F} \rangle_{T=0})</th>
<th>(\chi^2_{\text{min}})</th>
<th>(Q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(320)</td>
<td>(-)</td>
<td>(74.50 \pm 0.64)</td>
<td>(46.69528 \pm 0.00020)</td>
<td>(0.4885)</td>
<td>(0.7833)</td>
<td></td>
</tr>
<tr>
<td>(1280)</td>
<td>(-)</td>
<td>(315.59 \pm 0.66)</td>
<td>(48.088064 \pm 0.000031)</td>
<td>(0.8487)</td>
<td>(0.8378)</td>
<td></td>
</tr>
<tr>
<td>(5120)</td>
<td>(-)</td>
<td>(1276.3 \pm 4.4)</td>
<td>(48.43781 \pm 0.00019)</td>
<td>(1.7664)</td>
<td>(0.1838)</td>
<td></td>
</tr>
<tr>
<td>(\langle \tilde{F}_{\text{stretch}} \rangle_T)</td>
<td>(320)</td>
<td>(-)</td>
<td>(62.5 \pm 1.2)</td>
<td>(0.49251 \pm 0.00040)</td>
<td>(0.9321)</td>
<td>(0.6275)</td>
</tr>
<tr>
<td>(1280)</td>
<td>(-)</td>
<td>(251.0 \pm 1.4)</td>
<td>(0.529413 \pm 0.000075)</td>
<td>(0.7169)</td>
<td>(0.8692)</td>
<td></td>
</tr>
<tr>
<td>(5120)</td>
<td>(-)</td>
<td>(994.7 \pm 8.9)</td>
<td>(0.54083 \pm 0.00043)</td>
<td>(0.2869)</td>
<td>(0.5922)</td>
<td></td>
</tr>
<tr>
<td>(\langle \tilde{F}_{\text{shear}} \rangle_T)</td>
<td>(320)</td>
<td>(-)</td>
<td>(103.7 \pm 1.3)</td>
<td>(1.38038 \pm 0.00043)</td>
<td>(0.9962)</td>
<td>(0.6077)</td>
</tr>
<tr>
<td>(1280)</td>
<td>(-)</td>
<td>(393.2 \pm 1.5)</td>
<td>(1.310248 \pm 0.000078)</td>
<td>(0.5385)</td>
<td>(0.9104)</td>
<td></td>
</tr>
<tr>
<td>(5120)</td>
<td>(-)</td>
<td>(1552.2 \pm 8.6)</td>
<td>(1.29329 \pm 0.00042)</td>
<td>(0.0592)</td>
<td>(0.8077)</td>
<td></td>
</tr>
<tr>
<td>(\langle \tilde{F}_{\text{RBC}} \rangle_T)</td>
<td>(320)</td>
<td>(243)</td>
<td>(240.68 \pm 0.48)</td>
<td>(48.568167 \pm 0.000093)</td>
<td>(0.7581)</td>
<td>(0.6845)</td>
</tr>
<tr>
<td>(1280)</td>
<td>(963)</td>
<td>(959.77 \pm 0.55)</td>
<td>(49.927727 \pm 0.000012)</td>
<td>(0.6261)</td>
<td>(0.8904)</td>
<td></td>
</tr>
<tr>
<td>(5120)</td>
<td>(3843)</td>
<td>(3822.7 \pm 5.1)</td>
<td>(50.27195 \pm 0.00018)</td>
<td>(0.4333)</td>
<td>(0.5104)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.13: The parameters from fitting \(\langle \tilde{F}_b \rangle_T, \langle \tilde{F}_{\text{stretch}} \rangle_T, \langle \tilde{F}_{\text{shear}} \rangle_T\) and \(\langle \tilde{F}_{\text{RBC}} \rangle_T\) to straight lines in \(k_B T / \kappa_b\) for a given \(N_t\).
**CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$**

<table>
<thead>
<tr>
<th>$\langle \widetilde{F}<em>b \rangle</em>{T=0}$</th>
<th>$a_{\infty}$</th>
<th>$b_{\infty}$</th>
<th>$c_{\infty} = \langle \tilde{F} \rangle_{T=0, N_t \to \infty}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$900 \pm 120$</td>
<td>$-597.78 \pm 0.45$</td>
<td>$48.55453 \pm 0.00028$</td>
<td></td>
</tr>
<tr>
<td>$1280 \pm 260$</td>
<td>$-20.7 \pm 1.0$</td>
<td>$0.54483 \pm 0.00061$</td>
<td></td>
</tr>
<tr>
<td>$330 \pm 260$</td>
<td>$28.62 \pm 0.98$</td>
<td>$1.28769 \pm 0.00060$</td>
<td></td>
</tr>
<tr>
<td>$2520 \pm 110$</td>
<td>$-589.94 \pm 0.42$</td>
<td>$50.38708 \pm 0.00026$</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.14: The parameters from fitting $\langle \widetilde{F}_b \rangle_{T=0}$, $\langle \widetilde{F}_{\text{stretch}} \rangle_{T=0}$, $\langle \widetilde{F}_{\text{shear}} \rangle_{T=0}$ and $\langle \widetilde{F}_{\text{RBC}} \rangle_{T=0}$ from Table 3.13 to second order polynomials in $N_t^{-1}$.

the same number of points and drawn from the same probability distribution. $\chi^2_{\text{min}}$ is expected to be 2, 3 and 1 with standard deviations 2, $\sqrt{6}$ and $\sqrt{2}$ for $N_t = 320$, 1280 and 5120, respectively. Now, since the $\chi^2_{\text{min}}$ values from Table 3.13 are all within one standard deviation of the expected values, we conclude that the chosen form of the lines of best fit are reasonable.

We will now comment on the physical reasons behind the sample energies in Tables 3.9 to 3.12 being normally distributed and the averages of these sample energies being linearly dependent on $T$. Note that the respective lines of best fit of $\langle \widetilde{F}_b \rangle_T$, $\langle \widetilde{F}_{\text{MS}} \rangle_T \equiv \langle \widetilde{F}_{\text{stretch}} \rangle_T + \langle \widetilde{F}_{\text{shear}} \rangle_T$ and $\langle \widetilde{F}_{\text{RBC}} \rangle_T$ according to the parameter values given in Table 3.13 correspond to the following approximate forms:

\[
\langle \widetilde{F}_b \rangle_T \simeq \langle \widetilde{F}_b \rangle_{T=0} + \frac{1}{2} N_v \left( \frac{k_B T}{\kappa_b} \right),
\]

\[
\langle \widetilde{F}_{\text{MS}} \rangle_T \simeq \langle \widetilde{F}_{\text{MS}} \rangle_{T=0} + N_v \left( \frac{k_B T}{\kappa_b} \right),
\]

\[
\langle \widetilde{F}_{\text{RBC}} \rangle_T \simeq \langle \widetilde{F}_{\text{RBC}} \rangle_{T=0} + \frac{3}{2} N_v \left( \frac{k_B T}{\kappa_b} \right),
\]

where $\langle \widetilde{F}_{\text{MS}} \rangle_{T=0} \equiv \langle \widetilde{F}_{\text{stretch}} \rangle_{T=0} + \langle \widetilde{F}_{\text{shear}} \rangle_{T=0}$ and $N_v$, the number of vertices, takes on the values 162, 642 and 2562 (Table 3.1) for $N_t = 320$, 1280 and 5120, respectively. We may understand this behaviour as follows. Each vertex carries three degrees of freedom, one for each of its three directions of motion. Thus, the overall number of degrees of freedom is $3N_v$. This is, then, the dimensionality of the space of small deviations about the discretized $T = 0$ minimum-energy solution. For low temperatures and in the absence of soft modes, these degrees of freedom may be regarded as harmonic. Each harmonic degree
Figure 3.10: Plots of $\langle \tilde{F}_b \rangle_T$, $\langle \tilde{F}_{\text{stretch}} \rangle_T$, $\langle \tilde{F}_{\text{shear}} \rangle_T$ and $\langle \tilde{F}_{RBC} \rangle_T$ from Tables 3.9 to 3.12 against $k_B T$ and their respective lines of best fit, at $N_t = 320$. 
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

Figure 3.11: Plots of $\langle \tilde{F}_b \rangle_T$, $\langle \tilde{F}_{\text{stretch}} \rangle_T$, $\langle \tilde{F}_{\text{shear}} \rangle_T$ and $\langle \tilde{F}_{\text{RBC}} \rangle_T$ from Tables 3.9 to 3.12 against $k_B T$ and their respective lines of best fit, at $N_t = 1280$. 
Figure 3.12: Plots of $\langle \vec{F}_b \rangle_T$, $\langle \vec{F}_{\text{stretch}} \rangle_T$, $\langle \vec{F}_{\text{shear}} \rangle_T$ and $\langle \vec{F}_{\text{RBC}} \rangle_T$ from Tables 3.9 to 3.12 against $k_B T$ and their respective lines of best fit, at $N_t = 5120$. 
Figure 3.13: Plots of $\langle \bar{F}_b \rangle_{T=0}$, $\langle \bar{F}_{\text{stretch}} \rangle_{T=0}$, $\langle \bar{F}_{\text{shear}} \rangle_{T=0}$ and $\langle \bar{F}_{\text{RBC}} \rangle_{T=0}$ against $N_t^{-1}$ and their respective lines of best fit.
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

of freedom contributes $\frac{1}{2}k_BT$ to the average energy. Thus, the overall thermal energy is $\frac{3}{2}N_vk_BT$, as shown in the last three rows of Table 3.13 and in Equation (3.27). The division of this total thermal-fluctuation energy into a part associated with the bending motion and a part associated with the in-plane, membrane skeletal motion is approximate for finite mesh size. However, the bending energy suppresses local height fluctuations, so we expect that the in-plane degrees of freedom, which contribute to the membrane skeletal fluctuations, separate increasingly well from the out-of-plane degrees of freedom as the mesh becomes finer. There are two in-plane degrees of freedom per vertex, thus leading to Equation (3.26). The remaining single degree of freedom per vertex must appear in the bending energy, as illustrated by Equation (3.25). Knowing the analytic behaviour of the vertices at low $T$ allows us to perform statistical mechanical calculations of the probability distributions of the free energies. Because of the harmonicity of the degrees of freedom, these probability distributions will be Gaussian in form, as exhibited by the sample energies of Tables 3.9 to 3.12.

Equations (3.25) to (3.27) also tell us that all the energies become infinite as the degrees of freedom, $N_v$, become infinite in the continuum limit, i.e., the mesh becomes infinitely fine. This increase to infinity can be seen if we compare the energies for different $N_v$ and a fixed $T$ in each of Tables 3.9 to 3.12. In reality, the physical system we are dealing with is discrete. Thus, the approach to infinity for $F_b$ and $F_{MS}$ (hence $F_{RBC}$) are prevented by the cut-off at small length scales imposed by the size of the lipid molecules in the plasma membrane and the size of the spectrin tetramers of the membrane skeleton, respectively.

3.6.1 Test 5

As a final test, we compare our results in Table 3.14 with the solutions obtained independently by Mukhopadhyay [149] using an entirely different, quasi-analytic method [150]. This test is made possible by the natural axisymmetry of the minimum-energy shapes that we found (see Figure 3.8), as the method of Mukhopadhyay is restricted to axisymmetric shapes. For axisymmetric shapes, the differential equations governing the mechanics of the RBC membrane are simple enough to write down explicitly [150]. The shape problem thus reduces to a set of coupled ordinary differential equations that can be solved numerically to find the minimum free energy and the corresponding axisymmetric shape. This compar-
ison is, in principle, a strong check on the correctness of our computer programme, since Mukhopadhyay’s minimisation method is entirely different from ours.

The values of $\langle \tilde{F}_b \rangle_{T=0,N_t \to \infty}$, $\langle \tilde{F}_{\text{stretch}} \rangle_{T=0,N_t \to \infty}$, $\langle \tilde{F}_{\text{shear}} \rangle_{T=0,N_t \to \infty}$ and $\langle \tilde{F}_{\text{RBC}} \rangle_{T=0,N_t \to \infty}$ from Table (3.14) are our estimates of the minimum $F_b$, $F_{\text{stretch}}$, $F_{\text{shear}}$ and $F_{\text{RBC}}$, respectively. Table 3.15 shows a comparison of these estimates with corresponding results obtained by Mukhopadhyay [149] from solving the differential equations governing axisymmetric shapes. The two sets of results show agreement at the $10^{-3}$ level for the total energy $F_{\text{RBC}}$ as well as the bending component $F_b$ (which constitutes the largest contribution to the total energy). This agreement gives us confidence that our programme does not contain major errors. On the other hand, closer examination does reveal two troubling features. First, the minimum $F_{\text{RBC}}$ found by Mukhopadhyay is lower than what we find by an amount that is appreciably larger than our estimated uncertainty. Second, when we look not at $F_{\text{RBC}}$ but at the components, we see appreciable discrepancies between our values and those of Mukhopadhyay, up to 7% for the case of the stretching energy.

We have discussed these discrepancies extensively with Mukhopadhyay but still do not have a fully satisfactory explanation of them. It is important to note in this context that Mukhopadhyay’s solutions are given by a shooting method and an iterative convergence routine. The whole process is exceptionally delicate and unstable. Slightly different starting conditions can lead to convergence to quite different apparent solutions of the mechanical shape equations. Mukhopadhyay has tested these solutions and they do appear to satisfy the differential equations to high accuracy. The values quoted in Table 3.15 correspond to the lowest-energy solution obtained. Some other solutions have distinctly higher ener-

<table>
<thead>
<tr>
<th></th>
<th>extrapolated values from Table 3.14</th>
<th>components of lowest extremum from Ref. 149</th>
</tr>
</thead>
<tbody>
<tr>
<td>minimum $F_b$</td>
<td>$48.55453 \pm 0.00028$</td>
<td>$48.60$</td>
</tr>
<tr>
<td>minimum $F_{\text{stretch}}$</td>
<td>$0.54483 \pm 0.00061$</td>
<td>$0.51$</td>
</tr>
<tr>
<td>minimum $F_{\text{shear}}$</td>
<td>$1.28769 \pm 0.00060$</td>
<td>$1.24$</td>
</tr>
<tr>
<td>minimum $F_{\text{RBC}}$</td>
<td>$50.38708 \pm 0.00026$</td>
<td>$50.35$</td>
</tr>
</tbody>
</table>

Table 3.15: Comparison of values of the minimum $F_b$, $F_{\text{stretch}}$, $F_{\text{shear}}$ and $F_{\text{RBC}}$ estimated in Test 5 with the those estimated in Ref. 149.
gies and may correspond to stationary- but not minimum-energy solutions; others, however, have energies quite close to the lowest-energy solution but with appreciable variations in the stretch and shear components, comparable to the discrepancies between our solution and Mukhopadhyay's (the overall shapes of these solutions are all very similar; they differ mainly in the distribution of in-plane elastic energies). It is possible that these multiple, nearby low-energy solutions are real; however, it is also possible that they are computational artifacts. If they are real, then the computational energy landscape near the absolute minimum is rather complex, and it is surprising that we do not find some comparable thermal wandering of the Monte Carlo results over a wider range. It remains possible, of course, that further exploration would reveal a solution of even lower energy with stretch and shear energies closer to what we find. Of course, this would make the \(10^{-3}\) disagreement of the overall energy worse. On the other hand, if the multiple solutions are in some way a computational artifact of the instabilities in the shooting method itself, then the range of the low-energy solutions found may be a better measure of the uncertainties of Mukhopadhyay's solution than the apparent convergence of the single run illustrated here. In this case, the level of agreement of our results with his is rough but acceptable. In summary, then, the agreement illustrated in Table 3.15, while imperfect, does certainly appear to rule out gross programming errors. To the extent that the discrepancies between the two methods are outside of the estimated uncertainties, it remains unclear which set of values should be regarded as the more reliable. Thus, in our opinion, the two sets of values are not so different as to cause major concerns.

3.7 Construction of reference shapes \(\tilde{S}_0\)

Evaluation of the membrane skeletal free energy \(F_{MS}[S, S_0]\) requires as input the unknown shape of the putative relaxed or unstressed membrane skeleton, as discussed in Section 2.2. It is not feasible to test the behaviour of the RBC shape evolution for all possible reference shapes \(S_0\); on the other hand, we do wish to explore some effects of changing \(S_0\), since there is no clear choice of \(S_0\). As a compromise, we have chosen to study a one-parameter family of plausible reference shapes \(S_0\) that interpolates, in a somewhat arbitrary manner, between two simple limits: the symmetrical hypothesis that \(S_0\) is a sphere and the simple assumption that \(S_0\) is, effectively, just the discocytic shape of the normal, resting RBC.
A handy observation was made in this connection in the course of performing Test 5 in Section 3.6.1. It turns out that just such a sequence is generated by the minimisation of $F_{\text{RBC}}$ in the simple case where $F_0 = \kappa_b G$ (the area difference elasticity is neglected), the elastic moduli $K_\alpha$ and $\mu$ are much weaker than those of the RBC, $a_3 = a_4 = b_1 = b_2 = 0$, and $S_0$ is a sphere. This minimisation results in axisymmetric oblates with reduced volumes in the range $0.61 \leq v \leq 1$, such as those shown in Figures 3.15 to 3.23. At the lowest reduced volume, $v = 0.61$, these shapes are discocytic and quite similar to the normal RBC [Figure 4.81(d)]; as $v$ approaches 1, these shapes approach a sphere. Thus, we have a simple way of constructing a one-parameter shape class with the desired limits. It is known from earlier work [187] that such a sequence arises from minimisation of the bending energy, $\kappa_b G$, alone. The reason for adding a weak membrane skeletal elasticity is numerical: Without the in-plane elastic energies, the triangulated network represents only the shape of a closed fluid membrane, so there is no driving force to keep the triangular elements fairly regular during the Monte Carlo minimisation. As a result, the triangular elements have a tendency to become thin and needle-like, so that they do not accurately represent a smooth surface. Introducing a weak $F_{\text{MS}}$ is an effective way of keeping the shapes of the triangular elements regular, as Figures 3.15 to 3.23 clearly show.

Substituting $\tilde{S}_0$ for $\tilde{S}$ and $\tilde{S}_{\text{sphere}}$ for $\tilde{S}_0$ in Equations (3.18) and (3.19), the reference shapes $\tilde{S}_0$ are obtained from minimising

$$
\bar{F} \left[ \tilde{S}_0, \tilde{S}_{\text{sphere}} \right] = \bar{F}_b \left[ \tilde{S}_0 \right] + \bar{F}_{\text{MS}} \left[ \tilde{S}_0, \tilde{S}_{\text{sphere}} \right] + \bar{F}_{\text{constraint}} \left[ \tilde{S}_0 \right]
$$

at different volumes $V_{\text{rest}}$ for a fixed $\tilde{S}_{\text{sphere}}$, defined in Section 3.1 as the piecewise-planar approximation to a sphere with area $A_{\text{RBC}}$. The energy parameters used are given in Table 3.16. The triangulation of $\tilde{S}_0$ is based on $N_l = 1280$ or 5120, or both. The temperature is set to $\kappa_b/k_B T = 10^5$ for $N_l = 1280$ or $\kappa_b/k_B T = 10^6$ for $N_l = 5120$. Figures 3.15 to 3.23 show the top and side views of all the reference shapes prepared in this manner.

We comment that an arbitrary $S_0$ actually belongs to a class of equivalent shapes. This is also the case for each $S_0$ of the one-parameter family we used. For a given $S$, a replacement of $S_0$ by any other shape of its equivalence class causes no change in $F_{\text{MS}}$. An equivalence class may include both axisymmetric and non-axisymmetric shapes. Figure 3.14 shows a

---

4This is related to the symmetry of reparametrisation invariance of $S$, according to which a reparametrisation of $S$ does not affect $G[S]$. 
CHAPTER 3. NUMERICAL MINIMISATION OF $F[S, S_0]$

few axisymmetric shapes of the equivalence classes for a sphere and a discocyte. Strictly speaking, therefore, $V_{\text{rest}}$ uniquely characterises the equivalence class for $S_0$, not $S_0$ itself.

<table>
<thead>
<tr>
<th>Energy Components</th>
<th>Energy Parameters</th>
<th>Parameter Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_b / \kappa_b$</td>
<td>$\alpha_b$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$m_0$</td>
<td>0</td>
</tr>
<tr>
<td>$F_{MS} / \kappa_b$</td>
<td>$K_\alpha / \kappa_b \ (\mu m^{-2})$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$a_3$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$a_4$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$\mu / \kappa_b \ (\mu m^{-2})$</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>$b_1$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$b_2$</td>
<td>0</td>
</tr>
<tr>
<td>reference shape</td>
<td></td>
<td>sphere with area $A_{\text{RBC}}$</td>
</tr>
</tbody>
</table>

| $F_{\text{constraint}} / \kappa_b$ | $K_A / \kappa_b \ (\mu m^{-2})$ | $10^4$         |
|                                     | $A = A_{\text{RBC}} \ (\mu m^2)$ | 140            |
|                                     | $K_V / \kappa_b \ (\mu m^{-3})$  | $10^4$         |
|                                     | $V_{\text{rest}} = V_{\text{RBC}} \ (\mu m^3)$ | 100, 110, 114, 116, 118, 130 |
|                                     |                                 | 148, 152, 154, 155.8 (sphere) |

Table 3.16: Values of the energy parameters used in constructing the reference shapes $\tilde{S}_0$.

Figure 3.14: Some axisymmetric shapes in the equivalence classes for a sphere and a discocyte.
Figure 3.15: \( \tilde{S}_0 \) at \( V_{\text{rest}} = 100 \, \mu m^3 \) for \( N_t = 1280 \) (left) and 5120 (right).

Figure 3.16: \( \tilde{S}_0 \) at \( V_{\text{rest}} = 110 \, \mu m^3 \) for \( N_t = 1280 \) (left) and 5120 (right).
Figure 3.17: \( \tilde{S}_0 \) at \( V_{\text{rest}} = 114 \, \mu m^3 \) (left) and \( V_{\text{rest}} = 116 \, \mu m^3 \) (right) for \( N_t = 1280 \).

Figure 3.18: \( \tilde{S}_0 \) at \( V_{\text{rest}} = 118 \, \mu m^3 \) for \( N_t = 1280 \).
Figure 3.19: \( \tilde{S}_0 \) at \( V_{\text{rest}} = 130 \mu \text{m}^3 \) for \( N_t = 1280 \) (left) and 5120 (right).

Figure 3.20: \( \tilde{S}_0 \) at \( V_{\text{rest}} = 148 \mu \text{m}^3 \) for \( N_t = 1280 \) (left) and 5120 (right).
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Figure 3.21: $\tilde{S}_0$ at $V_{\text{rest}} = 152 \, \mu m^3$ for $N_t = 1280$ (left) and 5120 (right).

Figure 3.22: $\tilde{S}_0$ at $V_{\text{rest}} = 154 \, \mu m^3$ for $N_t = 1280$ (left) and 5120 (right).
Figure 3.23: $\tilde{S}_0$ at $V_{\text{rest}} = 155.8 \, \mu\text{m}^3$ for $N_t = 1280$ (left) and 5120 (right).
Chapter 4

Results of Numerical Minimisation

This chapter describes the results of numerical minimisation of the overall free energy functional of the RBC membrane, $F[S, S_0]$, for given values of the parameters $V_{\text{rest}}$ and $\bar{m}_0$. $V_{\text{rest}}$ describes the unstressed shape of the membrane skeleton. $\bar{m}_0$ describes the bending tendency of the plasma membrane and is assumed to be the parameter driven by the inducing agents. Section 4.1 is a general discussion of all the different shapes and shape transformations given by our numerical minimisation technique, and the classification of these shapes. Based on the results given in Section 4.1, we make specific predictions in Section 4.2 that are of direct relevance to the observed RBC shapes and shape transformations.

4.1 Predicted shapes and shape transformations

Figures 4.1, 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7 summarise graphically the raw results of energy minimisation at $V_{\text{rest}} = 100, 110-118, 130, 148, 152, 154$ and $155.8 \mu m^3$, respectively. Each figure indicates the values of $\bar{m}_0$ probed and the corresponding stable or metastable shape(s). The shapes we find as minimisers of $F$ are grouped into 16 different classes. Table 4.1 lists the names of these classes, their corresponding abbreviations, and figure references for their stability diagrams (to be defined shortly) and representative shapes. These classes are distinguished principally by symmetry. The exceptions are NAS, NAS-3 and SS, which are grouped according to their respective surface features. NAS actually includes symmetric shapes; however, it is hard to determine the values of $\bar{m}_0$ that separate
CHAPTER 4. RESULTS OF NUMERICAL MINIMISATION

<table>
<thead>
<tr>
<th>Shape Class</th>
<th>Abbreviation</th>
<th>Figure No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Axisymmetric Stomatocyte with</td>
<td>NAS-3</td>
<td>4.8</td>
</tr>
<tr>
<td>triangular invagination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Axisymmetric Stomatocyte with</td>
<td>NAS'</td>
<td>4.9</td>
</tr>
<tr>
<td>shallow invagination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Axisymmetric Stomatocyte</td>
<td>NAS</td>
<td>4.10 to 4.27</td>
</tr>
<tr>
<td>Axisymmetric Stomatocyte</td>
<td>AS</td>
<td>4.28 to 4.36</td>
</tr>
<tr>
<td>Axisymmetric Discocyte</td>
<td>AD</td>
<td>4.37 to 4.40</td>
</tr>
<tr>
<td>Non-Axisymmetric Discocyte</td>
<td>NAD</td>
<td>4.37 and 4.44</td>
</tr>
<tr>
<td>Echinocyte I with 9 bulges</td>
<td>E1-9</td>
<td>4.37, 4.41 to 4.43</td>
</tr>
<tr>
<td>Echinocyte I with 10 bulges</td>
<td>E1-10</td>
<td>4.53</td>
</tr>
<tr>
<td>Echinocyte I with 11 bulges</td>
<td>E1-11</td>
<td>4.54</td>
</tr>
<tr>
<td>Echinocyte I with 12 bulges</td>
<td>E1-12</td>
<td>4.55</td>
</tr>
<tr>
<td>Spiculated Shape</td>
<td>SS</td>
<td>4.56 to 4.79</td>
</tr>
<tr>
<td>Spiculated Shape II</td>
<td>SS2</td>
<td>4.52</td>
</tr>
<tr>
<td>Knizocyte</td>
<td>K</td>
<td>4.37, 4.45 and 4.46</td>
</tr>
<tr>
<td>Knizo-Echinocyte I, sub class A</td>
<td>KE1 A</td>
<td>4.37, 4.47</td>
</tr>
<tr>
<td>Knizo-Echinocyte I, sub-class B</td>
<td>KE1-B</td>
<td>4.48, 4.49 and 4.50</td>
</tr>
<tr>
<td>Knizo-Echinocyte I, sub-class C</td>
<td>KE1-C</td>
<td>4.51</td>
</tr>
</tbody>
</table>

Table 4.1: Abbreviations of names of shape classes and the figure number(s) corresponding to each class.

these symmetric shapes from the asymmetric ones. Therefore, we have chosen to group them together. NAS-3 and SS do not have obvious symmetries. In principle, shapes in the SS class could be divided further into sub-classes according to the number and arrangement of spicules; however, the number of such sub-classes is very large and the transitions between them are delicate and hysteretic, so we have chosen to group them together. Shape classes are represented by vertical lines in Figures 4.1 to 4.7, with solid and dashed vertical lines denoting symmetric and asymmetric classes, respectively. Representative shapes of a class are numbered in Figures 4.1 to 4.7 and shown separately. For example, point 1 of the class NAS in Figure 4.1 indicates the representative shape NAS(1) shown in Figure 4.11.
Figure 4.1: Predicted shape transformations as a function of $m_0$ at $V_{rest} = 100 \, \mu m^3$. 
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\[ \bar{m}_0 \]

150
100
50

\[ \kappa_b/k_B: 500 \rightarrow 1000 \]

\[ \text{stability boundary undetermined} \]

\[ V_{\text{rest}} = 110 \, \mu m^3 \]

\[ V_{\text{rest}} = 114 \, \mu m^3 \]

\[ V_{\text{rest}} = 116 \, \mu m^3 \]

\[ V_{\text{rest}} = 118 \, \mu m^3 \]

\textit{dct} discontinuous transition

○ stability boundary

• \( \kappa_b/k_B: 500 \rightarrow 1000 \)

? stability boundary undetermined

Figure 4.2: Predicted shape transformations as a function of \( \bar{m}_0 \) at \( V_{\text{rest}} = 110, 114, 116 \) and 118 \( \mu m^3 \).
Figure 4.3: Predicted shape transformations as a function of $\bar{m}_0$ at $V_{\text{rest}} = 130 \, \mu m^3$. 
CHAPTER 4. RESULTS OF NUMERICAL MINIMISATION

Figure 4.4: Predicted shape transformations as a function of $\bar{m}_0$ at $V_{\text{rest}} = 148 \, \mu m^3$. 

- **ct**: continuous transition
- **dct**: discontinuous transition

- Stability boundary
- $\kappa_b/k_B T$: 500 → 1000
- $\kappa_b/k_B T$: 500 → 1000 → 5000
- $\kappa_b/k_B T$: 48.3 → 500 → 1000
- Stability boundary undetermined
Figure 4.5: Predicted shape transformations as a function of $\bar{m}_0$ at $V_{rest} = 152 \mu m^3$. A shape in the AD class near the upper stability boundary has very slight undulations on the rim. We, however, have not grouped it under E1-9 because we have not been able to exclude thermal fluctuations as the cause of these undulations.
CHAPTER 4. RESULTS OF NUMERICAL MINIMISATION

possibly EI-9

ct continuous transition
dct discontinuous transition

○ stability boundary
• $\kappa_b/k_B T$: 500 $\rightarrow$ 1000
• $\kappa_b/k_B T$: 500 $\rightarrow$ 1000 $\rightarrow$ 5000
• $\kappa_b/k_B T$: 48.3 $\rightarrow$ 500 $\rightarrow$ 1000
? stability boundary undetermined

Figure 4.6: Predicted shape transformations as a function of $\overline{m_0}$ at $V_{\text{rest}} = 154 \mu m^3$. A shape in the AD class near the upper stability boundary has very slight undulations on the rim. We, however, have not grouped it under EI-9 because we have not been able to exclude thermal fluctuations as the cause of these undulations.
CHAPTER 4. RESULTS OF NUMERICAL MINIMISATION

$N_t = 5120$

\[ N_t = 1280 \]

\[ N_t = 5120 \]

$\bar{m}_0$

$150$

$100$

$50$

$0$

$-50$

$5$

$ct$ continuous transition

$dct$ discontinuous transition

○ stability boundary

● $\kappa_b/k_B T$: 500 $\rightarrow$ 1000

● $\kappa_b/k_B T$: 500 $\rightarrow$ 1000 $\rightarrow$ 5000

○ $\kappa_b/k_B T$: 48.3 $\rightarrow$ 500 $\rightarrow$ 1000

? stability boundary undetermined

Figure 4.7: Predicted shape transformations as a function of $\bar{m}_0$ at $V_{rest} = 155.8 \ \mu m^3$. Three shapes in the AS class near the lower stability boundary may be slightly non-axisymmetric. We, however, have not grouped them under NAS because we have not been able to exclude surface triangulation roughness as the cause of this asymmetry.
Monte Carlo minimisation of $F$ for a particular $V_{\text{rest}}$ and $m_0$ gives one or several locally stable $S$'s (for example, all three of NAS, AS and AD in Figure 4.1 may be locally stable at a given $m_0$). One of these is the stable $S$ corresponding to the absolute minimum of $F$; the others are metastable $S$'s corresponding to local minima of $F$. Let the energy barrier between any two minima be $\Delta F_{\text{min}}$. In order to have local stability, the $\Delta F_{\text{min}}$'s between all the local minima must, of course, be larger than $k_B T$ in the Boltzmann factor $\exp\left(-\Delta F_{\text{min}}/k_B T\right)$ corresponding to the computational temperature $T$; however, these $\Delta F_{\text{min}}$'s may or may not be larger than $k_B T_{\text{room}}$, where $T_{\text{room}}$ is the physical, room temperature. When the barriers are smaller than $k_B T_{\text{room}}$, then we expect to see all the shapes as part of a single thermal ensemble. On the other hand, when the barriers are larger than $k_B T_{\text{room}}$, then we expect hysteresis to occur in RBC shape transformations, i.e., shape sequences that depend on the transformation history. In this connection, it is useful to note that the energy scale in $F$ is set by the bending modulus $K_0$, which is about $50k_B T_{\text{room}}$; thus, significant hysteresis would be expected.

As the parameters $V_{\text{rest}}$ and $m_0$ change, so also do the locally stable $S$'s. Thus, each shape class is associated with a collection of values of the pair $V_{\text{rest}}$ and $m_0$. We refer to this collection as the \textit{region of stability} (in the parameter space defined by $V_{\text{rest}}$ and $m_0$). We will subsequently explore the region of stability of each class. We refer to a plot of a region of stability as a \textit{stability diagram}. Regions of stability of different classes generally overlap. This overlap gives rise to hysteresis of shape transformations as $V_{\text{rest}}$ and $m_0$ are varied. The boundaries of each region of stability are the locus of points (analogous to spinodal points in the theory of phase transitions) at which solutions belonging to that particular class disappear. Typically, but not always, these boundaries mark the onset of mechanical instabilities. In crossing a boundary by slowly varying $V_{\text{rest}}$ and $m_0$ in the Monte Carlo minimisation, we may encounter a situation in which the shape class we are following abruptly vanishes, the value of the minimum of $F$ decreases discontinuously, and a shape with different symmetry appears. We refer to such boundaries as \textit{limits of metastability} or \textit{metastability boundaries}. These abrupt shape transformations, which we label as \textit{discontinuous (dct)} in Figures 4.1 to 4.7, are analogous to first-order phase transitions. Discontinuous shape transformations from one class to the other are possible only if the regions of stability of the two classes overlap. Thus, the energy solutions for both classes will cross. An intersection point of this crossing is analogous to a phase boundary point of first-order phase transitions. Unlike a
CHAPTER 4. RESULTS OF NUMERICAL MINIMISATION

first-order phase boundary point, however, this intersection point does not have any special significance in the laboratory because the energy barrier between the two energy solutions is generally high. A minority of the boundaries of stability regions are characterised by a different behaviour. At these boundaries, there is no discontinuity in the value of the minimum of $F$. Instead, there is a continuous symmetry breaking as when undulations appear at the periphery of the discocytic shape in passing from the AD class to the E1-9 class. Such transformations are analogous to second-order phase transitions and we refer to them as continuous (ct) in Figures 4.1 to 4.7. The boundaries at which one class transforms continuously into another class is analogous to the boundaries of second-order phase transitions and the two classes involved do not have overlapping regions of stability.

We have studied in detail the cases of $V_{\text{rest}} = 100, 130, 148, 154$ and $155.8 \, \mu m^3$, the results of which are reported in Figures 4.1, 4.3, 4.4, 4.6 and 4.7, respectively. These figures have additional information showing how the main shape classes transform. The cases of $V_{\text{rest}} = 110, 114, 116$ and $118 \, \mu m^3$, shown in Figure 4.2, have only been explored to the extent that it allows us to determine the lower stability boundary points of the AD and AS classes. The case of $V_{\text{rest}} = 152 \, \mu m^3$, shown in Figure 4.5, have only been explored to the extent that it allows us to determine the boundary points of shape classes that are not spiculated. Note that the sequence of shape classes encountered on increasing $E_0$ can be rather different from that encountered on decreasing $\bar{m}_0$ because of metastability effects.

Each point $(V_{\text{rest}}, \bar{m}_0)$ in Figures 4.1 to 4.7 is associated with the three computational parameters $N_t$, $\kappa_b/k_B T$ and $N_{\text{sweep}}$ (not shown in Figures 4.1 to 4.7). They are, respectively, the number of triangles used to approximate $S$, the normalised inverse computational temperature at which $S$ is equilibrated and the number of Monte Carlo sweeps used. We have used either 1280 or 5120 triangles to represent $S$, except for one point in Figure 4.6, where we have used both. Generally, we have chosen to use 1280 triangles to represent smooth $S$'s and to use 5120 triangles to represent $S$'s with sharper surface features. The highest equilibration temperature for each point is either $\kappa_b/k_B T = 500$ or $\kappa_b/k_B T = 48.3$ (room temperature). The former is used mainly in the determination of the regions of stability for the major non-spiculated shape classes. In the case of the SS and SS2 classes, it is used only in the determination of the lower stability boundaries. Exploration of the region where $\bar{m}_0 \geq 100$ is complicated by the occurrence of numerous locally stable spiculated shapes for the same $V_{\text{rest}}$ and $\bar{m}_0$. In order to make our computation time feasible, we did not system-
attractively explore this region. Instead, we limited our search to shapes at or near the absolute minima by equilibrating at $\kappa_b/k_B T = 48.3$ (room temperature) before lowering $\kappa_b/k_B T$ to 500, then 1000. The shapes obtained in this manner belong to the spiculated shape classes (SS and SS2). In general, $N_{\text{sweep}} \geq 2 \times 10^6$ for the equilibration at each temperature.

Some data points in the SS class are associated with double-headed arrows. The tails of these arrows indicate the starting shapes. Those data points in the SS class not associated with double-headed arrows are the initial data that made us realise the complication arising from the multiplicity of locally stable spiculated shapes; we did not record the starting shapes of these initial data.

Some parts of the NAS-3, NAS', E1-10, E1-11, E-12 and KE1-C shape classes are not important and, thus, are not thoroughly explored.

We comment further on each shape class in the following sections. Sections 4.1.1 to 4.1.13 give the stability diagrams of individual shape classes and the representative shape(s) of each class. The chosen shapes have the best, i.e., largest, values of $N_t$ and $\kappa_b/k_B T$ for a given $V_{\text{rest}}$ and $m_0$. Actual data points are shown as circles in the stability diagrams. The stability boundary or boundaries of each class have an uncertainty in $m_0$ of $\pm 5$, which is the step size that we used. Crossing a stability boundary constitutes a transformation from one class to another, with the new class varying with $V_{\text{rest}}$. This variation of the new class is indicated by the labels of the actual data points just outside a stability boundary.

4.1.1 NAS-3

A locally stable non-axisymmetric stomatocyte with a triangular invagination is found at $V_{\text{rest}} = 148 \mu m^3$ and $m_0 = -60$, as shown in Figure 4.8. It has no obvious symmetry. The NAS-3 shape transforms discontinuously into the NAS class on crossing the upper stability boundary. The NAS-3 shape is similar in appearance to the experimentally observed triangular stomatocyte shown in Figure 1.3 (b).

4.1.2 NAS'

A locally stable shallow, non-axisymmetric stomatocyte with an ellipsoidal invagination is found at $V_{\text{rest}} = 100 \mu m^3$ and $m_0 = -60$, as shown in Figure 4.9. It is mirror symmetric with two mirror planes. This shape transforms continuously into the axisymmetric AS class on
crossing the upper stability boundary and discontinuously into the NAS class on crossing the lower stability boundary.

### 4.1.3 NAS

The stability diagram of the NAS shape class is shown in Figure 4.10. The representative shapes NAS(1), NAS(2), ..., NAS(17) are shown in Figures 4.11 to 4.27. The NAS class is a large collection of locally stable non-axisymmetric stomatocytes with similar changes in surface features as \( \overline{m}_0 \) is varied, notably the narrowing and elongation of the initially oval invagination as \( \overline{m}_0 \) decreases. The NAS class includes some mirror-symmetric shapes with one or two mirror planes. We have not attempted to isolate these symmetric shapes from the non-symmetric ones because the continuous transformations between symmetric and non-symmetric shapes make it difficult to determine their stability boundaries. Shapes in the upper portion of the stability diagram, such as NAS(1), NAS(2), NAS(4), NAS(7), NAS(8), NAS(11), NAS(12) and NAS(15), shown in Figures 4.11, 4.12, 4.14, 4.17, 4.18, 4.21, 4.22 and 4.25, respectively, resemble the stomatocyte I (\( \text{Figure 1.2b} \)). Consider the shapes at \( \overline{m}_0 = -60 \), namely, NAS(3), NAS(6), NAS(10), NAS(14) and NAS(17), shown in Figures 4.13, 4.16, 4.20, 4.24 and 4.27, respectively. A comparison of these shapes reveals that only those with volumes in the range \( 130 \mu m^3 < V_{\text{rest}} < 155.8 \mu m^3 \) will exhibit the characteristic curved invagination of the stomatocyte III (\( \text{cf. Figure 1.2a} \)). In other words, the reference shape \( S_0 \) must be highly inflated, but not spherical, for shapes resembling the stomatocyte III to occur. Upon crossing the upper stability boundary, the NAS shape class transforms discontinuously into the axi- and up-down symmetric AD class, if \( 100 \mu m^3 \leq V_{\text{rest}} < V_{\text{AD/AS}} \), or the axisymmetric AS class, if \( V_{\text{AD/AS}} < V_{\text{rest}} < 155.8 \mu m^3 \). The transition point \( V_{\text{AD/AS}} \) is somewhere in the interval \( 130 \mu m^3 < V_{\text{rest}} < 148 \mu m^3 \). The transformation into the AS class is continuous at \( V_{\text{rest}} = 155.8 \mu m^3 \), which corresponds to a spherical \( S_0 \).

### 4.1.4 AS

The stability diagram of the AS shape class is shown in Figure 4.28. The representative shapes AS(1), AS(2), ..., AS(8) are shown in Figures 4.29 to 4.36. The AS class, which strongly resembles the stomatocyte I (\( \text{cf. Figure 1.2c} \)), is characterised by its axisymmetry and lack of up-down symmetry. On crossing the upper stability boundary, the AS class
CHAPTER 4. RESULTS OF NUMERICAL MINIMISATION

transforms discontinuously into the axi- and up-down symmetric AD class, except when $S_0$ is spherical, in which case the AS class transforms continuously into the K class. The notable feature in the transformation from AS to K is that the convex side of AS turns into the pinch of K, which corresponds to a change in shape symmetry from the axisymmetry of AS to the mirror symmetry of K. On crossing the lower stability boundary, the AS class transforms discontinuously into the NAS class if $100 \mu m^3 < V_{\text{rest}} < 155.8 \mu m^3$, continuously into the mirror-symmetric NAS' class in the vicinity of $V_{\text{rest}} = 100 \mu m^3$, and continuously into the NAS class if $S_0$ is spherical.

4.1.5 AD

The stability diagram of the AD shape class is shown in Figure 4.37. The representative shapes AD(1), AD(2), ..., AD(8) are shown in Figures 4.38 to 4.40. The AD class is characterised by its axi- and up-down symmetries. It includes a subset of self-intersecting shapes located in the lower left corner of the stability diagram, where $V_{\text{rest}} \lesssim 114 \mu m^3$. These self-intersecting shapes are not physical; they come about because we did not implement global self-avoidance of $S$. Upon crossing the lower stability boundary, AD transforms discontinuously into NAS if $114 \mu m^3 < V_{\text{rest}} < V_{\text{NAS/AS}}$, or the axisymmetric AS if $V_{\text{NAS/AS}} < V_{\text{rest}} < 154 \mu m^3$. The transition point $V_{\text{NAS/AS}}$ between these two regions is somewhere in the interval $130 \mu m^3 < V_{\text{rest}} < 148 \mu m^3$. AD transforms continuously into the mirror- and up-down symmetric NAD with decreasing $\overline{m}_0$ in a narrow region about $V_{\text{rest}} = 154 \mu m^3$. AD transforms continuously into the 9-fold and up-down symmetric E1-9 upon crossing the upper stability boundary. Since we did not investigate shape transformations in the narrow band defined by $154 \mu m^3 < V_{\text{rest}} < 155.8 \mu m^3$, we have no information on the shapes and the type of shape transformations that occur in that band. Therefore, the continuous transformations depicted in Figure 4.37 between AD and K, and between AD and KE1-A, should be regarded as guesses.

Figures 4.38 to 4.40 show very clearly the progressive disappearance of the two dimples and the general flattening of the AD shape with increasing $\overline{m}_0$. Flattening of a discocytic RBC has also been observed experimentally in the shape transformation from a discocyte to an echinocyte I induced by a change in conformation of the transmembrane band 3 proteins [19, 90].
4.1.6 NAD

The stability diagram of the NAD shape class is shown in Figure 4.37. The representative shape NAD(1) is shown in Figure 4.44. The NAD class is characterised by mirror and up-down symmetries. The NAD stability diagram consists of a small region about $V_{\text{rest}} = 154 \mu m^3$ that joins continuously onto the lower right corner of the much larger AD stability diagram. NAD transforms continuously into the axi- and up-down symmetric AD with increasing $\bar{m}_0$, and discontinuously into the axisymmetric AS upon crossing the lower stability boundary.

NAD(1) is similar in appearance to the experimentally observed asymmetric discocyte shown in Figure 1.3 (a). Note, however, that the experimental shape does not occur naturally. The experimental shape is obtained by treating an osmotically swollen RBC with diamide and then osmotically shrinking it back to the normal volume [67]. The diamide treatment cross-links the spectrin tetramers of the membrane skeleton and has two effects [67]: fixing $S_0$ close to a sphere and increasing $K_\alpha$ and $\mu$. The fact that we found a NAD shape at lower, physiological values of $K_\alpha$ and $\mu$ only when $S_0$ is nearly spherical suggests that a requirement for the production of a locally stable NAD is a nearly spherical $S_0$. Separately, we obtained another locally stable NAD shape with the same physical and computational parameters as NAD(1), except that the values of $K_\alpha$ and $\mu$ are doubled. This shape, shown together with NAD(1) in Figure 4.44, has a more pronounced asymmetry in its rim thickness than NAD(1), which indicates that an increase in $K_\alpha$ and $\mu$ enhances the NAD shape asymmetry.

4.1.7 E1-9

The stability diagram of the E1-9 shape class is shown in Figure 4.37. The representative shapes E1-9(1), E1-9(2), ..., E1-9(7) are shown in Figures 4.41 to 4.43. The E1-9 class is characterised by its 9-fold rotational and up-down symmetries. The 9-fold symmetry is due to the nine bulges that develop on the rim. The E1-9 shapes are effectively discocytes on which a 9-fold undulation of the rim has broken the axisymmetry. The E1-9 stability diagram abuts the top of the AD stability diagram. E1-9 transforms continuously into the axi- and up-down symmetric AD with decreasing $\bar{m}_0$ and discontinuously into the SS class upon crossing the upper stability boundary. The range of $\bar{m}_0$ values over which E1-9 is
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locally stable decreases with increasing $V_{\text{rest}}$ and eventually vanishes when $S_0$ becomes spherical.

4.1.8 E1-10, E1-11 and E1-12

The incomplete stability diagrams of the E1-10, E1-11 and E1-12 classes, each accompanied by a representative shape, are shown in Figures 4.53, 4.54 and 4.55. E1-10, E1-11 and E1-12 are characterised by 10-, 11-, and 12-fold rotational symmetries, respectively, in addition to up-down symmetry. These three classes appear in shape transformations with decreasing $\bar{m}_0$, starting from the SS class. The E1-10/11/12 shapes are effectively discocytes on which a 10/11/12-fold undulation of the rim has broken the axisymmetry. The sketchy data suggest that the trend in the series of discontinuous transformations from E1-12 to E1-9 is a decrease in the number of bulges on the rim with decreasing $\bar{m}_0$.

4.1.9 K and KE1-A

The stability diagrams of the K and KE1-A shape classes are shown in Figure 4.37. The representative shapes K(1) and K(2) of the K class are shown in Figures 4.45 and 4.46. Both the K and KE1-A classes are characterised by mirror symmetry. At high $\bar{m}_0$, the K class may also have three-fold symmetry. The K class changes from monoconcave at low $\bar{m}_0$, such as K(2), to triconcave at high $\bar{m}_0$, such as K(1). Note the resemblance of these shapes to the experimentally observed knizocyte shown in Figure 1.3 (c). The representative shape KE1-A(1) of KE1-A is shown in Figure 4.47. These two classes are rather special in that their stability requires a spherical or nearly spherical $S_0$. The K class transforms continuously into the KE1-A class with increasing $\bar{m}_0$. This transformation is accompanied by the development of undulations on the three edges of $S$. The K class transforms continuously into the axisymmetric AS class with decreasing $\bar{m}_0$. The KE1-A class transforms discontinuously into the KE1-B class upon crossing the upper KE1-A stability boundary.

4.1.10 KE1-B

The stability diagram of the KE1-B shape class is shown in Figure 4.48. The representative shapes KE1-B(1) and KE1-B(2) are shown in Figures 4.49 and 4.50, respectively. KE1-B
is initially mirror-symmetric with a flattened, ellipsoidal base. This mirror symmetry is lost as nine bulges develop on the rim of the base with increasing $\bar{m}_0$. KE1-B is also a special class, like K and KE1-A, that requires a spherical or nearly spherical $S_0$ in order to be locally stable. The constant surface feature of the KE1-B class is its pinch with three bulges. KE1-B transforms discontinuously into SS2 and the mirror-symmetric KE1-A upon crossing the upper and lower stability boundaries, respectively.

4.1.11 KE1-C

The incomplete stability diagram of KE1-C, together with a representative shape, are shown in Figures 4.51. The KE1-C shape has mirror symmetry. It may also possess three-fold symmetry. The KE1-C class is another special class that requires a spherical or nearly spherical $S_0$ in order to be locally stable. It appears in shape transformations with decreasing $\bar{m}_0$, starting from the SS class. The KE1-C shape is characterised by the four bulges on each of its three edges and its mirror symmetry. KE1-C transforms discontinuously into KE1-B upon crossing the lower stability boundary.

4.1.12 SS2

The incomplete stability diagram of the SS2 class, together with a representative shape, are shown in Figures 4.52. The SS2 class may possess mirror symmetry. It is yet another special class that requires a spherical or nearly spherical $S_0$ in order to be locally stable. The SS2 shape is characterised by the emergence of a cluster of six spicules and a flattened base with nine bulges. Five of the six spicules are arranged in a pentagonal fashion, with the remaining one located at the centre of the pentagon. SS2 transforms discontinuously into KE1-B upon crossing the lower stability boundary.

4.1.13 SS

The stability diagram of the SS shape class is shown in Figure 4.56. The representative shapes SS(1), SS(2), ..., SS(23) are shown in Figures 4.57 to 4.79. The SS class is a large collection of locally stable shapes with spicules and no obvious shape symmetry. As $\bar{m}_0$ increases, the spicules become shorter, sharper and more numerous, while the main body
changes from a disc to an oval. This behaviour mirrors that observed experimentally during echinocytosis; however, comparison between the shape sequences at different $V_{\text{rest}}$ shows that $V_{\text{rest}}$, i.e., the shape of $S_0$, drastically affects the distribution of spicules on the main body. Three effects of $V_{\text{rest}}$ are particularly noteworthy:

1. Spicules tend to appear at locations on $S$ where the corresponding locations on $S_0$ have a large positive mean curvature. Thus, as $S_0$ becomes more deflated with decreasing $V_{\text{rest}}$, spicules increasingly tend to congregate at the rim of $S$, which corresponds to the part of $S_0$ where the mean curvature is most positive. Comparison of the shapes at $\bar{v}_0 = 140$, namely, SS(1), SS(5), SS(10), SS(13) and SS(17), shown in Figures 4.57, 4.61, 4.66, 4.69 and 4.73, respectively, shows that $S_0$ must be highly inflated for the spicules on $S$ to be regularly spaced like those on an echinocyte III.

2. The stage where the main body of a spiculated $S$ is discoidal vanishes when $S_0$ is spherical. In other words, there are no stable shapes resembling echinocyte II, which has a discoidal main body, when $S_0$ is spherical.

3. If $V_{\text{rest}} < 154 \mu m^3$, the SS class transforms discontinuously into an echinocyte I-like class (EI-10/11/12) on crossing the lower stability boundary, as indicated in Figure 4.56. Otherwise, the SS class transforms discontinuously into the AD or the KE1-C class. Therefore, $V_{\text{rest}} < 154 \mu m^3$ is required for our predicted shape transformations to agree with the experimental observation that an echinocyte II transforms into an echinocyte I.

### 4.2 Significant predictions

#### 4.2.1 Most likely unstressed shape of the membrane skeleton

Figure 4.80 shows the combined stability diagram of the NAS, AS, AD, NAD, EI-9, K, KE1-A, KE1-B and SS classes. We deduce from Figure 4.80 and our results in the previous sections that the most likely $V_{\text{rest}}$ of the RBC is in a narrow range approximately centred about $V_{\text{rest}} = 148 \mu m^3 (\nu = 0.9502)$. The lower limit, at approximately $144 \mu m^3 (\nu = 0.9247)$, is the intersection point of the upper stability boundaries of NAS and AS. The upper limit, at approximately $152 \mu m^3 (\nu = 0.9758)$, is the intersection point of the
lower stability boundaries of E1-9 and SS. Within this range, the predicted shapes and shape transformations are consistent with those of the experimental SDE transformations. This is illustrated clearly in Figure 4.81, where laboratory images of shapes from the SDE transformations are compared with the seven representative predicted shapes NAS(10), NAS(7), AS(3), AD(5), E1-9(3), SS(11) and SS(10) from the sequence of shape transformations at $V_{\text{rest}} = 148 \ \mu m^3$, obtained by increasing $\bar{m}_0$ from -60 to $\bar{m}_0 = 140$. The unstressed shape $S_0$ with $V_{\text{rest}} = 148 \ \mu m^3$ is shown in Figure 3.20.

The lower limit does not depend on the higher-order non-linear terms with unknown coefficients $a_3$, $a_4$, $b_1$ and $b_2$ in Equations (2.29) and (2.30). This is because the strains experienced by the two locally stable shapes at this limit remain in the linear elastic regime. On the other hand, the upper limit is dependent on these higher-order non-linear terms, since shapes in the SS class experience large, non-linear strains. This dependence, however, is not expected to be strong: the upper limit is set by the lower stability boundary of SS, where the corresponding shapes are the least strained of the class. Any change in the other parameters of $F$ will, of course, shift the positions of both limits.

Various authors [63, 67, 140, 239] have speculated that the unstressed shape of the membrane skeleton is either a sphere ($v = 1$) or a replica of the discocytic shape of the RBC ($v \approx 0.6$); however, these speculations do not seem to be based on strong evidence. Our results suggest that both scenarios are unlikely. If the unstressed shape were discocytic, we predict from our results at $V_{\text{rest}} = 100 \ \mu m^3$ (Figure 4.1) that the stomatocyte I stage would not be part of the main sequence of shape transformations and that there would not be spiculated RBC shapes resembling echinocytes II and III. While these predictions depend on the shape of $S_0$, they do not depend on $a_3$, $a_4$, $b_1$ and $b_2$. If the unstressed shape were a perfect sphere, we predict from our results at $V_{\text{rest}} = 155.8 \ \mu m^3$ (Figure 4.7) that the discocyte would be unstable and not occur in the main sequence of shape transformations, and that there would not be spiculated RBC shapes resembling echinocytes I and II. These predicted shape disappearances are the direct result of $S_0$ becoming a sphere and do not depend on $a_3$, $a_4$, $b_1$ and $b_2$. Our results suggest that the most likely unstressed shape of the membrane skeleton is an oblate spheroid (a shape that results from flattening the two poles of a sphere) with a reduced volume in the range $0.9247 \leq v \leq 0.9758$. Such a shape is not unreasonable. It is known that an immature RBC (reticulocyte) has a highly irregular, folded shape [14]. Therefore, we expect the unstressed shape of its membrane skeleton
to be highly irregular as well. As the RBC matures and enters the circulatory system, the cumulative effect of the incessant deformation it experiences would have molded the unstressed shape of its membrane skeleton into a regular, roughly spherical shape.

4.2.2 Hysteresis of RBC shape transformations

It is clear from inspection of Figures 4.1 to 4.7 and 4.80 that there is significant overlap between the predicted stability diagrams of the various shape classes. In other words, there is more than one local minimum of the free energy $F$ for $V_{\text{rest}}$ and $\bar{m}_0$ in any region of overlap, with each local minimum giving rise to a locally stable shape. A pertinent experimental example that has received scant attention is the occasional transformation of a stomatocyte II to a triangular stomatocyte instead of a stomatocyte III [13], which indicates that both the triangular stomatocyte and the stomatocyte III have overlapping regions of stability. Indeed, the theoretical counterpart to the triangular stomatocyte, NAS-3 of Figure 4.8, is found at the same $V_{\text{rest}}$ and $\bar{m}_0$ as the theoretical counterpart to the stomatocyte III, NAS(10) of Figure 4.20.

The possibility of multiple locally stable shapes implies that a full cycle of shape transformations involving an increase in $m_0$ followed by a decrease in $\bar{m}_0$ to the starting value is not always reversible, since some shapes in the transformations with increasing $\bar{m}_0$ are not accessible in the transformations with decreasing $\bar{m}_0$, and vice versa. There are currently no experimental studies of hysteresis of RBC shape transformations. Thus, it is not known if hysteresis exists in the experimental SDE transformations. What we do know, as mentioned previously in Chapter 1, is that qualitative observations of RBC shape transformations suggest that every normal RBC can be made to cycle through each of the non-terminal stages of shape transformations in a reversible fashion. These observations are still consistent with our predictions, in that every major class is apparently reversible when $V_{\text{rest}}$ is in the predicted range for the RBC, but not every shape. We hope that our work will stimulate a systematic experimental study of hysteresis of RBC shape transformations.

4.2.3 Strain distribution over the membrane skeleton

Figures 4.82 to 4.88 show the predicted strain fields $\alpha$ and $\beta$ for the seven shapes of Figure 4.81 at $V_{\text{rest}} = 148 \, \mu m^3$. Stomatocytic shapes are typically highly dilated at the centre of
the invagination and highly sheared and compressed near the rim of the invagination. The rest of the surface is more or less unstrained. Spiculated shapes, on the other hand, typically have large dilation at the top of each spicule, large shear strain in the spicule neck region, and significant compression in the main body.

The qualitative behaviour of the membrane skeletal strain distribution of an echinocyte III can, in fact, be predicted without resorting to theory. For well-defined spicules of an echinocyte III, the uniformity of their size, shape, and distribution over the RBC membrane makes consideration of the strain behaviour of the underlying membrane skeleton easier, since one needs to examine only the local neighbourhood of a spicule instead of the entire RBC membrane. Figure 4.89 shows the expected qualitative strain distribution over the membrane skeleton when it is deformed from its locally flat and circular unstressed shape into a spicule. The unstressed shape is marked by concentric circles spaced equally apart in the radial direction to allow visualisation of the strain that develops in the membrane skeleton as the RBC membrane is forced outward by an inducing agent in the direction perpendicular to the plane of the membrane skeleton. It is easy to see that, since the pole (central region of 7) and the base (outer area of region 1) of the spicule retain their respective basic shapes, the deformation there is purely a change in area. The base suffers an area compression since the outer region of the unstressed shape must be radially contracted for the out-of-plane deformation of the RBC membrane to occur. Conservation of area of the RBC membrane dictates that the loss of area from the outer region of the membrane skeleton be compensated by an equal gain in area in the inner region of the membrane skeleton, so that there is a change from area compression at the base to an area expansion at the pole. For the intermediate region between the pole and the base, the change in area is also accompanied by shear deformation, since its basic shape is modified along with its area. Note that the expected membrane skeletal strain in the vicinity of the spicule pole bears striking qualitative similarity to the observed membrane skeletal strain in the vicinity of the tip of a RBC tongue aspirated into a micropipette (see, for example, Ref. 119).
Figure 4.8: Incomplete stability diagram of NAS-3 and the locally stable shape found at $V_{\text{rest}} = 148 \, \mu m^3$ and $m_0 = -60$. 

Non-Axisymmetric Stomatocyte with triangular invagination (NAS-3)
Figure 4.9: Incomplete stability diagram of NAS' and the locally stable shape found at $V_{\text{rest}} = 100 \mu m^3$ and $\bar{m}_0 = -60$. 

Non-Axisymmetric Stomatocyte with shallow invagination (NAS')
Figure 4.10: Stability diagram of the shape class NAS. The representative shapes NAS(1) to NAS(17) are shown in Figures 4.11 to 4.27.
Figure 4.11: Shape NAS(1) at $V_{\text{ref}} = 100 \, \mu m^3$ and $m_0 = 10$. 
Figure 4.12: Shape NAS(2) at $V_{\text{rest}} = 100 \, \mu m^3$ and $\bar{m}_0 = -5$. 
Figure 4.13: Shape NAS(3) at $V_{\text{rest}} = 100 \, \mu m^3$ and $m = -60$. 
Figure 4.14: Shape NAS(4) at $V_{\text{rest}} = 130 \, \mu m^3$ and $\bar{m}_0 = 0$. 
Figure 4.15: Shape NAS(5) at $V_{\text{test}} = 130 \, \mu\text{m}^3$ and $\bar{m}_0 = -45$. 
Figure 4.16: Shape NAS(6) at $V_{\text{rest}} = 130 \, \mu m^3$ and $\bar{m}_0 = -60$. 
Figure 4.17: Shape NAS(7) at $V_{\text{rest}} = 148 \, \mu m^3$ and $m_0 = -5$. 
Figure 4.18: Shape NAS(8) at $V_{\text{rest}} = 148 \, \mu m^3$ and $\bar{m}_0 = -25$. 
Figure 4.19: Shape NAS(9) at $V_{\text{rest}} = 148 \mu m^3$ and $m_0 = -40$. 
Figure 4.20: Shape NAS(10) at $V_{\text{rest}} = 148 \, \mu m^3$ and $m_e = -60$. 
Figure 4.21: Shape NAS(11) at $V_{\text{rest}} = 154 \, \mu\text{m}^3$ and $\bar{m}_0 = -5$. 
Figure 4.22: Shape NAS(12) at $V_{\text{rest}} = 154 \, \mu m^3$ and $\bar{m}_0 = -20$. 
Figure 4.23: Shape NAS(13) at $V_{\text{rest}} = 154 \ \mu m^3$ and $\bar{m}_0 = -40$. 
Figure 4.24: Shape NAS(14) at $V_{\text{rest}} = 154 \, \mu m^3$ and $\bar{m}_0 = -60$. 
Figure 4.25: Shape NAS(15) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = -20$. 
Figure 4.26: Shape NAS(16) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = -40$. 
Figure 4.27: Shape NAS(17) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = -60$. 
Figure 4.28: Stability diagram of the shape class AS. The representative shapes AS(1) to AS(8) are shown in Figures 4.29 to 4.36.
Figure 4.29: Shape AS(1) at $V_{\text{rest}} = 100 \, \mu m^3$ and $\overline{m}_0 = -20$. 
Figure 4.30: Shape AS(2) at $V_{\text{rest}} = 100 \, \mu m^3$ and $\tilde{m}_0 = -55$. 
Figure 4.31: Shape AS(3) at $V_{\text{rest}} = 148 \mu m^3$ and $\overline{m}_0 = 5$. 
Figure 4.32: Shape AS(4) at $V_{\text{rest}} = 148 \, \mu m^3$ and $\bar{m}_0 = -20$. 
Figure 4.33: Shape AS(5) at $V_{\text{rest}} = 154 \mu m^3$ and $m_0 = 45$. 
Figure 4.34: Shape AS(6) at $V_{\text{rest}} = 154 \, \mu m^3$ and $\bar{m}_0 = -15$. 
Figure 4.35: Shape AS(7) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = 50$. 
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Figure 4.36: Shape AS(8) at \( V_{\text{rest}} = 155.8 \mu m^3 \) and \( \bar{m}_0 = 0 \).
Self-intersecting Axisymmetric Discocyte

Figure 4.37: Combined stability diagram of the shape classes AD, NAD, E1-9, K and KE1-A. The representative shapes AD(1) to AD(8), NAD(1), E1-9(1) to E1-9(7), K(1) and K(2), and KE1-A(1) are shown in Figures 4.38 to 4.40, 4.44, 4.41 to 4.43, 4.45 and 4.46, and 4.47, respectively. Note that the transformation between AD and E1-9, and between AD and NAD, is continuous.
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Figure 4.38: Shapes AD(1) (left), AD(2) (middle) and AD(3) (right) at $m_0 = 80$, 15 and -55, respectively, and $V_{rest} = 100 \mu m^3$.

Figure 4.39: Shapes AD(4) (left), AD(5) (middle) and AD(6) (right) at $m_0 = 95$, 10 and -5, respectively, and $V_{rest} = 148 \mu m^3$. 
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Figure 4.40: Shapes AD(7) (left) and AD(8) (right) at $\bar{m}_0 = 110$ and 50, respectively, and $V_{\text{rest}} = 154 \, \mu m^3$.

Figure 4.41: Shapes E1-9(1) (left) and E1-9(2) (right) at $\bar{m}_0 = 125$ and 85, respectively, and $V_{\text{rest}} = 100 \, \mu m^3$. 
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Figure 4.42: Shapes E1-9(3) (left), E1-9(4) (middle) and E1-9(5) (right) at $\bar{m}_0 = 120, 110$ and 100, respectively, and $V_{\text{rest}} = 148 \, \mu m^3$.

Figure 4.43: Shapes E1-9(6) (left) and E1-9(7) (right) at $\bar{m}_0 = 120$ and 115, respectively, and $V_{\text{rest}} = 154 \, \mu m^3$. 
Figure 4.44: Shape NAD(1) (left) and a NAD shape with twice the values of $K_\alpha$ and $\mu$ (right). Both shapes are at $V_{\text{rest}} = 154 \mu m^3$ and $\bar{m}_0 = 15$. 
Figure 4.45: Shape K(1) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $m_0 = 80$. 
Figure 4.46: Shape K(2) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = 60$. 
Figure 4.47: Shape KE1-A(1) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = 95$. 
Figure 4.48: Stability diagram of the shape class KE1-B. The representative shapes KE1-B(1) and KE1-B(2) are shown in Figures 4.49 and 4.50, respectively.
Figure 4.49: Shape KE1-B(1) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = 105$. 
Figure 4.50: Shape KE1-B(2) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar{m}_0 = 95$. 
Figure 4.51: Incomplete stability diagram of the shape class KE1-C and a representative shape at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\varpi_0 = 100$. 
Figure 4.52: Incomplete stability diagram of the shape class SS2 and a representative shape at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $m_0 = 110$. 
Figure 4.53: Incomplete stability diagram of E1-10 and a representative shape at $V_{\text{rest}} = 100 \, \mu m^3$ and $\bar{m}_0 = 95$. 

Echinocyte I with 10 bulges (E1-10)
Figure 4.54: Incomplete stability diagram of E1-11 and a representative shape at $V_{\text{rest}} = 130 \, \mu m^3$ and $m_0 = 110$. 
Figure 4.55: Incomplete stability diagram of E1-12 and a representative shape at $V_{\text{rest}} = 100\ \mu m^3$ and $\bar{m}_0 = 110$. 

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Figure 4.56: Stability diagram of the shape class SS. The representative shapes SS(1) to SS(23) are shown in Figures 4.57 to 4.79.
Figure 4.57: Shape SS(1) at $V_{\text{rest}} = 100 \mu m^3$ and $\bar{m}_0 = 140$. 
Figure 4.58: Shape SS(2) at $V_{\text{rest}} = 100 \, \mu m^3$ and $\bar{m}_0 = 130$. 
Figure 4.59: Shape SS(3) at $V_{\text{test}} = 100 \, \mu m^3$ and $\bar{m}_0 = 120$. 
Figure 4.60: Shape SS(4) at $V_{\text{rest}} = 100 \, \mu m^3$ and $\bar{m}_0 = 115$. 
Figure 4.61: Shape SS(5) at $V_{\text{rest}} = 130 \, \mu m^3$ and $\bar{m}_0 = 140$. 
Figure 4.62: Shape SS(6) at $V_{\text{rest}} = 130 \, \mu m^3$ and $\overline{m}_0 = 130$. 
Figure 4.63: Shape SS(7) at $V_{\text{rest}} = 130 \, \mu m^3$ and $m_0 = 125$. 
Figure 4.64: Shape SS(8) at $V_{\text{rest}} = 130 \, \mu\text{m}^3$ and $\overline{m}_0 = 115$. 
Figure 4.65: Shape SS(9) at $V_{\text{rest}} = 148 \, \mu m^3$ and $\bar{m}_0 = 145$. 
Figure 4.66: Shape SS(10) at $V_{\text{rest}} = 148 \, \mu m^3$ and $\bar{m}_0 = 140$. 
Figure 4.67: Shape SS(11) at $V_{\text{rest}} = 148 \, \mu\text{m}^3$ and $\bar{w}_0 = 125$. 
Figure 4.68: Shape SS(12) at $V_{\text{rest}} = 148 \, \mu m^3$ and $m_0 = 115$. 
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Figure 4.69: Shape SS(13) at $V_{\text{rest}} = 154 \, \mu m^3$ and $\overline{m}_0 = 140$. 
Figure 4.70: Shape SS(14) at $V_{\text{rest}} = 154 \mu m^3$ and $\bar{m}_0 = 125$. 
Figure 4.71: Shape SS(15) at $V_{\text{rest}} = 154 \, \mu m^3$ and $\bar{m}_0 = 110$. 
Figure 4.72: Shape SS(16) at $V_{\text{rest}} = 154 \, \mu m^3$ and $m_0 = 105$. 
Figure 4.73: Shape SS(17) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\bar m_0 = 140$. 
Figure 4.74: Shape SS(18) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $m_0 = 120$. 
Figure 4.75: Shape SS(19) at $V_{\text{rest}} = 155.8 \, \mu\text{m}^3$ and $m_0 = 115$. 
Figure 4.76: Shape SS(20) at $V_{\text{rest}} = 155.8 \ \mu m^3$ and $\bar{m}_0 = 120$. This shape is similar to SS(18) of Figure 4.74, but has a slightly lower value of $F$. 
Figure 4.77: Shape SS(21) at $V_{\text{rest}} = 155.8 \, \mu\text{m}^3$ and $\bar{m}_b = 115$. 
Figure 4.78: Shape SS(22) at $V_{\text{rest}} = 155.8 \, \mu m^3$ and $\overline{m}_0 = 110$. 
Figure 4.79: Shape SS(23) at $V_{\text{fin}} = 155.8 \, \mu \text{m}^3$ and $m_0 = 105$. 
Figure 4.80: Combined stability diagram of the NAS, AS, AD, NAD, E1-9, K, KE1-A, KE1-B and SS classes. The shapes NAS(10), NAS(7), AS(3), AD(5), E1-9(3), SS(11) and SS(10) from the sequence of shape transformations with increasing $\bar{m}_0$ at $V_{\text{rest}} = 148 \, \mu m^3$ are compared with laboratory images of the SDE transformations in Figure 4.81.
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Figure 4.88: The fields of $\alpha$ and $\beta$ over the shape SS(10) of Figure 4.66.
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Figure 4.89: The expected membrane skeletal strain in the neighbourhood of a spicule of an echinocyte III.
Chapter 5

Discussion, Conclusions and Outlook

This Chapter contains an assortment of remarks on the significance of the results presented in Chapter 4, their extensions and limitations, and a general summary.

5.1 Surfaces of minimum free energy, trajectories in phase space and the SDE transformations

Our computer programme allows us to calculate numerically the functional dependence of $F_{\text{min}}$, the local minimum of $F$, on all the RBC mechanical parameters. A plot of this functional dependence in the multi-dimensional space defined by $F_{\text{min}}$ and all the parameters will generally consist of multiple generalised surfaces. In this Thesis, we have fixed most parameters at reasonable physical values and have explored the phase plane defined by the two parameters $V_{\text{rest}}$ and $\bar{m}_0$, which we regard as the most important unknown or unfixed parameters. For each point $(V_{\text{rest}}, \bar{m}_0)$ in the phase plane, there may be more than one $F_{\text{min}}$ and, hence, more than one minimum-energy shape. This gives rise to multiple surfaces in the space defined by $F_{\text{min}}$, $V_{\text{rest}}$ and $\bar{m}_0$. Each surface corresponds to a distinct shape class, often but not always characterised by a particular shape symmetry. These surfaces can intersect one another, as they do for discontinuous shape transformations, or they can join at some singular boundary, as they do for continuous shape transformations. Projection of each surface down onto the phase plane defines the region of stability of the corresponding shape class, a plot of which constitutes the corresponding stability diagram.
When the RBC is exposed to non-physiological physical, chemical or biochemical conditions, it is reasonable to suppose that the parameters that enter its shape mechanics change in a smooth way. We have focussed particularly on the parameter $\overline{m}_0$ that controls the tendency of the RBC membrane to bulge outward (as $\overline{m}_0$ increases) or bulge inward (as $\overline{m}_0$ decreases). It is our central hypothesis that, to a good approximation, change in this single parameter is the principal driving force for RBC shape transformations. As $\overline{m}_0$ changes smoothly, the trajectory $(V_{\text{rest}}, \overline{m}_0, F_{\text{min}})$ generally follows a single surface of minimum $F$, since the different energy minima at each point of the phase plane are typically separated by barriers that are significantly larger than $k_BT_{\text{room}}$, the energy scale of thermal fluctuations; however, this condition breaks down near metastability boundaries (the loci of mechanical instability at which discontinuous shape transformations occur). When the trajectory reaches the edge of a surface (or close to the edge, in the case of discontinuous transformations at $T > 0$), the RBC must adopt a new minimum-energy shape. In the case of discontinuous shape transformations, the trajectory will reach close to the edge of the surface and will then drop down from one minimum into another, lower minimum nearby, as soon as the energy barrier between the two becomes comparable to $k_BT_{\text{room}}$. Often there is only one energy minimum nearby; when there is more than one, then it is the geometry of the full energy landscape of $F$ (not just the surfaces of minimum $F$) that determines which minimum to drop into. This is generally trajectory dependent. In the case of continuous shape transformations, the trajectory passes smoothly from one surface to the next with no discontinuity in $F_{\text{min}}$.

It follows from the picture above that, as the experimenter changes in a continuous way the conditions to which the RBC is subject (thereby moving smoothly across the phase space of driving parameters), the RBC responds by smooth changes of shape, within a given shape class, punctuated by abrupt changes of shape class, when its trajectory in phase space approaches the boundaries of each region of stability.

Figure 5.1 shows schematically the sequence of surfaces of $F_{\text{min}}$ as a function of $\overline{m}_0$ that we found in the vicinity of $V_{\text{rest}} = 148 \, \mu m^3$. There are five dominant surfaces corresponding to the five major shape classes. All transformations are discontinuous with the exception of the transformation between AD and E1-9 and the transformation (not shown) on the NAS surface between shapes with two mirror planes and those with only one. These exceptional transformations are predicted to be continuous.
Figure 5.1: A schematic illustration of the way the main surfaces of minimum $F$ intersect in the vicinity of $V_{\text{rest}} = 148 \mu\text{m}^3$. This arrangement of surface intersections is the only one that will allow every major shape class to appear and be reversible. For clarity, we have omitted the intermediate shape class(es) in the transformation from SS to E1-9. Dashed lines denote discontinuous changes in the value of $F_{\text{min}}$ during shape transformations.
The sequence of shape transformations for $V_{\text{rest}} = 148 \mu m^3$ corresponds precisely to the experimentally observed SDE transformations induced by echinocytogenic or stomatocytogenic agents. The fitting necessary to achieve this agreement is primarily in the choice of $V_{\text{rest}}$; all other parameters, with the exception of the four unknown higher-order non-linear elastic moduli $a_3$, $a_4$, $b_1$, and $b_2$, are set to reasonable values based on experiments on intact RBCs, as explained in Chapter 2. The ability of our model to achieve this level of agreement is the central and major contribution of this Thesis. It goes well beyond previous work in the field.

It would be interesting to know how robust this agreement is with respect to the other parameters (listed in Table 2.5) of our model. Unfortunately, this extended parameter space is very large; therefore, we have not been able to explore it in detail. It must suffice for present purposes to frame the discussion: On the one hand, small changes in these parameters will leave the general sequence of shape transformations intact, although numerical values of stability boundaries will certainly shift. Some features are more sensitive than others. We have already seen, for example, the relatively narrow range of $V_{\text{rest}}$ that permits the full SDE transformations to appear. On the other hand, some limited spot checks suggest that large changes in the parameters can easily eliminate parts of the SDE transformations or introduce other shape classes not generally seen in experiment or both. We have tested the effects of varying the shear modulus, $\mu$, the discussion of which is given in Section 5.4. We have also tested the effects of setting the coefficients $a_3$, $a_4$, $b_1$, and $b_2$ to zero, the discussion of which is given in Section 5.5. It is likely that changes in one parameter can to some extent be compensated for by correlated changes in other parameters; however, we have not undertaken a systematic study of such effects.

Some level of robustness is desirable, since our simple picture that all agents act strictly through their effect on $m_0$ is certainly an oversimplification. Presumably, agents (such as a change in pH) do cause changes in other mechanical parameters through, e.g., their effect on the charge states of membrane skeletal proteins, which, in turn, would affect membrane skeletal elastic constants. Changing the concentration of a single biochemical agent would, thus, drive $F_{\text{min}}$ along a trajectory in a multi-dimensional phase space of mechanical parameters. The calculations of our model simplify this picture by projecting all these changes to a single dimension, namely, $m_0$.

Finally, as illustrated in Figure 5.1, the metastability of the discontinuous shape trans-
formations means that significant hysteresis is expected in the details of a sequence of shape transformations, depending on whether \( \bar{m}_0 \) is increasing or decreasing (see also Figures 4.1 to 4.7 in Chapter 4). This is a prediction of our model that, in principle, can be tested.

### 5.2 Relation to the bilayer-couple hypothesis

In 1974, Sheetz and Singer [189] proposed the bilayer-couple hypothesis as an explanation for the SDE transformations. The original proposal was formulated in connection with a pure bending model of the plasma membrane, with no role for the membrane skeletal mechanics in the shape transformations. We now know that, without the membrane skeleton, the region of high \( \bar{m}_0 \) in phase space would be dominated by budding and vesiculated shapes [96, 227, 234] rather than echinocytes, so that, in its original form, the bilayer-couple mechanism was seriously incomplete. On the other hand, the notion that the bending tendency, expressed quantitatively as the parameter \( \bar{m}_0 \) in our model, is the basic driving force behind the SDE transformations is certainly correct, in our view. Thus, in the broader sense, this Thesis should be regarded as a validation of the original bilayer-couple mechanism. Although this mechanism was proposed nearly 30 years ago, there has not been until now a quantitative demonstration that the observed shapes and shape transformations arise from the bilayer-couple mechanism. The main reason for this 30-year hiatus has been the lack on the one hand of a specific mechanical model for the shape mechanics and on the other of sufficient computational power for testing different models. Realistic model testing, as we have done in this Thesis, involves intensive calculations of shapes that need not be axisymmetric, such as echinocytes, based on the mechanics built into each model. Common computers available before the late 1990's, however, simply were not powerful enough to perform such a task in a reasonable time.

Our ability to explore quantitatively the implications of a specific mechanical model has made possible a remarkable level of validation of the bilayer-couple mechanism. The action of specific chemical agents on the mechanical moduli of the RBC membrane remains to be elucidated. As we have stressed in the previous Section, the actual trajectory through the mechanical phase space may well be more complex than the simple, one-dimensional scans we have explored in this Thesis. Nevertheless, the fact that the shapes and shape transformations seen in experiments do appear in the membrane mechanics in a detailed
and highly non-trivial way would seem to provide very strong evidence for the validity of the bilayer-couple mechanism.

Further testing of the bilayer-couple mechanism and the mechanical model will need to focus on quantifying the link between the concentration of an echinocytogenic (or stomatocytogenic) agent and specific changes in the mechanical moduli. We hope and believe that detailed tests of this type will be forthcoming and that it will pin down further the values of the parameters that govern the shape mechanics. We believe that the interplay between theory and experiment will play a central role in this effort. In particular, it will be important to have a computer programme that is able to predict shapes based on given mechanical-parameter inputs. We hope that we have supplied a model for such a programme.

5.3 Effects of thermal fluctuations on shape transformations between discocyte and echinocyte

Anecdotal experimental observations [62] of the shape transformation at room temperature from a discocyte to an echinocyte I have suggested that the RBC membrane may experience significant thermal fluctuations in this transformation. We suspect that significant thermal fluctuations of the RBC membrane do occur for this transformation and that these fluctuations are the result of a low-lying shape eigenmode becoming soft in the vicinity of a mechanical instability of the RBC membrane.

We have seen in our results (not shown) that an E1-9 shape appears to be very susceptible to thermal fluctuations when it is near the continuous stability boundary between the AD class (theoretical equivalent of a discocyte) and the E1-9 class (theoretical equivalent of an echinocyte I). The 9 bulges on the rim of the E1-9 shape are clearly visible and regular in size at very low $T$. When $T$ is increased to room temperature, however, the overall shape becomes an irregular discocyte and the bulges are no longer distinguishable. In order to see clear bulges on the rim at room temperature, one needs to increase $\bar{m}_0$ to move away from the continuous boundary. In the near future, we will perform a more systematic study of the effects of thermal fluctuations on the transformation between the AD and E1-9 classes.
5.4 Effects of varying $\mu$

We have tested the effects of varying the shear modulus. We found that an increase in the value of the linear-elastic shear modulus $\mu$ from 2.5 $\mu$Jm$^{-2}$ to 6–9 $\mu$Jm$^{-2}$ (estimates from micropipette aspiration of RBCs) will cause the region of stability of the NAD class to spread to lower values of $V_{\text{rest}}$, in addition to shifting the stability boundaries of every shape class. Thus, the AS $\rightarrow$ AD shape class transformation on increasing $\mu$ for $144 \mu m^2 \lesssim V_{\text{rest}} \lesssim 152 \mu m^2$ (see Figure 4.81) is replaced by the AS $\rightarrow$ NAD $\rightarrow$ AD shape class transformations. This sequence of shape class transformations for the aforementioned higher values of $\mu$ is inconsistent with observed shape class transformations. On the other hand, lowering the value of $\mu$ by 50% to 1.25 $\mu$Jm$^{-2}$ will cause the disappearance of shapes resembling echinocytes I, II and III, in addition to shifting the stability boundaries of every shape class. Instead of echinocytic shapes, shapes with several long arms appear on increasing $\mu$. In particular, the AD $\rightarrow$ E1-9 shape class transformation for $\mu = 2.5 \mu$Jm$^{-2}$ is replaced by a transformation from the AD class to a class resembling a starfish. Thus, the shape class transformations for the aforementioned lower value of $\mu$ are also inconsistent with observed shape class transformations.

5.5 Higher-order non-linear elastic terms

A crucial part of our continuum elastic model is the inclusion of higher-order non-linear elastic terms in the Taylor expansion of the membrane skeletal strain energy density, $f_{\text{MS}}$, to harden further the membrane skeletal elasticities at moderately large values of $\alpha$ and $\beta$. As mentioned previously in Chapter 2, the coefficients $a_3$, $a_4$, $b_1$ and $b_2$ of these additional terms are unknown and reasonable guesses of their values have been used here. These additional terms affect only the finer details of highly strained shapes. Specifically, they affect the size and shape of spicules of shapes in the SS class, and the size, shape and orientation of the invaginations of shapes corresponding to the lower half of the NAS stability diagram. It may be possible to extract the values of $a_3$, $a_4$, $b_1$ and $b_2$ using techniques based on the optical tweezers [86, 193] or the optical stretcher [76].

We have tested the effects of eliminating these higher-order terms. This is accomplished by setting $a_3$, $a_4$, $b_1$ and $b_2$ to zero. We found that the elimination of these terms has no
drastic effect on shapes with surface features that are rather smooth: shapes resembling the stomatocytes III, II and I, discocyte and echinocyte I of the experimental SDE transformations continue to remain locally stable. This insensitivity to the higher-order terms (or, more precisely, the variation of the in-plane elastic free energy density, \( f_{MS} \), in the large deformation regime) is not unexpected, since most of the membrane skeletons of these shapes still do not experience large and rapidly-varying local area and shear deformations when the higher-order terms are present (cf. Figures 4.82 to 4.86). The effect of eliminating the higher-order terms on shapes in the spiculated-shape classes is significant. In the absence of non-linear hardening of the membrane skeletal elasticity, the bending elasticity of the plasma membrane begins to dominate for spiculated RBCs and, consequently, forces a large part of the membrane skeleton to undergo large and rapidly-varying local area and shear deformations. (This can be seen in Figures 4.87 and 4.88, which are examples of the general case in which the higher-order terms are present.) The RBC shape is very sensitive to the variation of \( f_{MS} \) in the large deformation regime. If the higher-order terms are eliminated, thereby weakening the in-plane elasticities, we would expect the shape to have a bending-dominated behaviour, typified by the formation of small spherical buds that are joined via narrow necks to a larger body. Unfortunately, we were not able to study locally stable shapes that result from elimination of the higher-order terms. This is because the shapes we obtained were non-physical, numerical artifacts whose triangular surface elements failed the checks for shape regularity in our programme. This comes about because we are effectively constrained to use a maximum of 5120 triangles to represent \( S \), which would be insufficient if the buds (in the absence of the higher-order terms) were smaller in size than a spicule (in the presence of the higher-order terms).

5.6 Experimental quantitation of \( \bar{m}_0 \)

Our model predicts the quantitative correspondence between a shape and a value of \( \bar{m}_0 \). In order to test our model in a shape transformation experiment, it is necessary to have a technique for determining the value of \( \bar{m}_0 \) for an actual shape. Very recently, Kuzman et al. [114] estimated the value of \( \Delta a_0 \equiv \bar{m}_0/4\pi \) for echinocyte III shapes using the micropipette aspiration technique and an approximate, single-spicule continuum mechanical model. They found that \( 12 \leq \Delta a_0 \leq 18 \) or, equivalently, \( 150.8 \leq \bar{m}_0 \leq 226.2 \). The lower
limit of this range is in rough agreement with the value of $\bar{m}_0 = 140$ for the echinocyte III-like SS(10) shape of Figure 4.66. This agreement is somewhat fortuitous, considering that their model assumes linear in-plane elasticities and has slightly different parameter values. The parameters that differ in values from ours are the volume $V_{\text{RBC}}$, the shear modulus $K_\alpha = 2\mu$ and the non-local bending modulus $\bar{K}$. They used $V_{\text{RBC}} = 109 \text{ m}^3$, $\mu = 6 \text{ m}^{-2}$ and $\bar{K} = 8/\pi \times 10^{-19} \text{ J}$, whereas we used $V_{\text{RBC}} = 100 \text{ m}^3$, $\mu = 2.5 \text{ m}^{-2}$ and $\bar{K} = 4/\pi \times 10^{-19} \text{ J}$. Their use of linear in-plane elasticities with larger values of $\mu$ and $K_\alpha$ should be regarded as an effective form of the in-plane elasticities at large deformation; however, this effective form may not be adequate for capturing the variation of $f_{\text{MS}}$ over the membrane skeleton of an echinocyte III. There are also several older studies [37, 66, 116] that estimated the fractional area difference between leaflets of the plasma membrane. These estimates are, at best, semi-quantitative. Furthermore, the quantity that has been estimated in these studies is the actual (fractional) area difference, not the preferred (fractional) area difference. Specifically, Lange and Slayton [116] estimated that the actual area difference required for echinocytosis is about 1%, Ferrell et al. [66] estimated that the actual area difference required to induce stage III echinocytes is $1.7 \pm 0.6\%$, and Chi and Wu [37] estimated that the actual area difference required to induce stage III echinocytes is $3.2 \pm 0.2\%$. All these estimates are of the order of a few percent. Our theory predicts a preferred area difference for stage III echinocytes that is also of the order of a few percent: The fractional preferred area difference between the neutral surfaces of the leaflets of the plasma membrane, $\bar{\Delta}a_0$, is defined to be

$$\bar{\Delta}a_0 = \frac{\Delta A_0}{A} + \frac{DC_0}{\pi \alpha_b} = \frac{D}{2\pi R_A} \bar{m}_0,$$

based on Equation (2.18). The SS(10) shape in Figure 4.66 with $\bar{m}_0 = 140$ is our theoretical equivalent of the experimental echinocyte III shape. Assuming $D = 3$ nm and $4\pi R_A^2 = 140 \text{ m}^2$, $\bar{\Delta}a_0 = 2.003\%$ for the SS(10) shape. One cannot meaningfully compare the predicted preferred area difference with the experimental estimates of the actual area difference. Nevertheless, the magnitudes of the four values above do suggest that a small area difference between the two leaflets is sufficient to cause large qualitative changes in shape.

Ideally, one would like to be able to control systematically $\bar{m}_0$ of the RBC. This has not been achieved. In order to do so, one must have an intimate understanding of the
physico-chemical interactions between an inducing agent and the plasma membrane. Comprehensive reviews of the biochemical effects of various chemical agents on the plasma membrane may be found in Refs. 27, 47 and 184. An obvious concern is the ATP-dependent enzymes in the plasma membrane that regulate the transmembrane lipid distribution asymmetry (discussed in more detail in Section A.2). An agent may temporarily disrupt the normal operations of these enzymes and, thus, induce a transient transmembrane lipid redistribution. It has been shown, for example, that the stomatocytogenic agent CPZ induces such an effect [36, 178, 185]. Thus, one needs to take into account the time required for the transmembrane lipid distribution to stabilise. The final shape will be either more or less extreme than the initial shape, depending on how the transmembrane lipid composition is ultimately re-distributed. Another consideration is that transient, mechanically-driven phospholipid translocation between leaflets of a fluid bilayer may occur. This phenomenon has been observed in the membranes of phospholipid vesicles [169, 170, 207] and in the RBC plasma membranes [4, 114]. In the latter case, it results in the discocyte-echinocyte transformation. Mechanically induced echinocytes are driven back to discocytes by the ATP-dependent enzymes in the plasma membrane that maintain the transmembrane lipid distribution asymmetry. The characteristic time for this shape reversal is found to be about 15 min. Thus, whenever mechanically-driven interleaflet transport occurs, the time scale of any measurement related to the shape must be much shorter or much longer than this characteristic time to ensure stability of the experimental conditions. All these effects will complicate quantitative experimental tests of the relation between $\bar{m}_0$ and the observed shape.

5.7 Lateral inhomogeneity of the plasma membrane

We have used the ADE model to describe the bending energy of the plasma membrane. This is tantamount to assuming that the components of the plasma membrane are uniformly distributed in the plane of the plasma membrane at length scales larger than about 100 nm [150]. There are some indications that this assumption may not hold. Rodgers and Glaser [175–177, 230] have observed regions of inhomogeneity larger in size than 100 nm in the membranes of rabbit RBCs, as shown in Figure A.2 of Appendix A.2. They found that domains enriched in either phosphatidylcholine or phosphatidylserine form in intact
membranes not subjected to an inducing agent, which suggests that these lipid domains occur naturally. In addition, when they exposed intact membranes to chlorpromazine (CPZ), a fluorescent and stomatocytogenic compound, they observed the formation of CPZ-enriched domains. Rodgers and Glaser osmotically swelled the CPZ-laced RBCs to spheres because they needed simple cell geometries for fluorescence imaging. Thus, it is not known if the CPZ-enriched domains preferentially form in the invaginations of stomatocytes.

The results of Rodgers and Glaser suggest that the model of the plasma membrane should take into account the effects of coupling between curvature and membrane composition. This improvement is not expected to be difficult to accomplish theoretically and will result in more parameters than the ADE model. Such a model, however, will not have much practical value until its parameters can be estimated through systematic and quantitative studies of the coupling between curvature and membrane composition for RBCs in all stages of the SDE transformations. No such studies have been performed to date.

### 5.8 Pros and cons of Monte Carlo minimisation

The trapping of a physical system in a metastable configuration at low temperatures is commonly considered to be an undesirable problem of conventional Monte Carlo methods [155]. We, however, regard it as an advantage, since it enables us to obtain the stability diagrams of various shape classes. The second advantage of the Monte Carlo method is that it is analytically simpler, since we do not need to carry out separate calculations for the stationarity and stability of a shape. The third advantage is that we can use it to examine the effects of thermal fluctuations (though not ATP-driven fluctuations [215]).

The use of a Monte Carlo method to minimise $F$ has two disadvantages. Firstly, the boundaries of the stability diagrams become temperature dependent and, consequently, not precisely defined. Secondly, the computation time required is longer compared to that of a conventional minimisation technique because of three factors: not all trial moves are successful and the successful ones do not always lower $F$; the step size in a trial move is small at low temperatures as a result of our keeping the probability of acceptance of a trial move at about 50%; and a large number of trial moves are required to ensure shape relaxation and statistical accuracy of the average energies.
5.9 Membrane mechanics of RBCs of other mammals

The structures of normal human RBCs and those of other mammals are similar. Their similarities include the quasi-two-dimensional structural organisation of their membranes, their discocyte shape under physiological conditions\(^1\), and their gross shapes\(^2\) and shape transformations (spiculation or cupping) induced by an echinocytogenic or stomatocytogenic agent [99-101, 194, 195]. These similarities should allow the model proposed in this Thesis to be used in analysing the biomechanics of other mammalian RBCs, provided that the mechanical parameters specific to each species are known. The shear moduli for a few mammals other than human have been estimated. They include the rabbit, the rat, the oppossum and the llama (whose RBCs are not discocyte) [225, 226]. These moduli were obtained using the micropipette aspiration technique and under the assumption of local area incompressibility of the membrane skeleton. As mentioned previously in Section 2.2.2, the micropipette aspiration technique probes the non-linear in-plane elasticities at high deformation. Therefore, the measured shear moduli for the aforementioned mammals should not be equated to the \(\mu\) of Equation (2.30), since they are not the linear-elastic shear moduli. Note also that the assumption of local area incompressibility of the membrane skeleton is now known to be unrealistic, as mentioned previously in Section 2.2.2. The RBC area and volume for rabbit, mouse, rat and hamster have also been measured [226]. There are currently no measurements of the other mechanical parameters (see Table 2.5) for mammals other than human. It is hoped that our work will stimulate experiments to probe in detail the membrane mechanics of other mammalian RBCs. However, at present the ingredients for testing our approach on other mammalian species do not appear to be available.

5.10 Summary

To summarise, we have proposed a continuum elastic model of the RBC that is the most general and realistic to date. The essential features of this model are:

1. The implementation of the computational surface in three dimensions without any prior assumption of shape symmetry.

\(^1\)Except for a few mammalian species.
\(^2\)Differences do exist in the finer surface features.
2. The inclusion of the plasma membrane bending elasticity and the membrane skeletal stretching and shear elasticities.

3. The allowance for local area compressibility of the membrane skeleton.

4. The inclusion of higher-order non-linear elastic terms in the Taylor expansion of the membrane skeletal strain energy density \( f_{\text{MS}} \).

The total free energy \( F \) of the model contains two unknown parameters, \( V_{\text{rest}} \) and \( \bar{m}_0 \). The former governs the unstressed shape of the membrane skeleton, whereas the latter governs the bending tendency of the plasma membrane. We assume that an observed RBC shape is a locally stable shape that minimises \( F \) for some given values of \( V_{\text{rest}} \) and \( \bar{m}_0 \). In practice, the minimisation of \( F \) can only be performed numerically. We have chosen to minimise \( F \) numerically using a Monte Carlo technique. By minimising \( F \) at increasing or decreasing values of \( \bar{m}_0 \) for a given \( V_{\text{rest}} \), we have obtained results in the form of the shape transformations as a function of \( \bar{m}_0 \) at fixed values of \( V_{\text{rest}} \), the stability diagrams of the various shape classes found, and the locally stable shape(s) contained within each class. The main objective of the exploration of values of \( V_{\text{rest}} \) and \( \bar{m}_0 \) is to search for an unstressed shape of the membrane skeleton that gives the right RBC shapes and shape transformations as a function of \( \bar{m}_0 \). We have estimated that the unstressed shapes with volumes in the range \( 144 \, \mu\text{m}^3 \lesssim V_{\text{rest}} \lesssim 152 \, \mu\text{m}^3 \), or reduced volumes in the range \( 0.9245 \lesssim v \lesssim 0.9758 \) (assuming \( A_{\text{RBC}} = 140 \, \mu\text{m}^2 \)), will give the desired results. In addition, our results suggest that there will generally be hysteresis in a sequence of shape transformations that involves more than one shape class. Finally, we have made the first comprehensive predictions of the area and shear strain fields over every RBC shape of the SDE transformations.
Appendix A

Review of RBC Membrane Structural Organisation

The RBC membrane is a multi-layered composite material. Its structural elements, their organisation into cellular structures, and the relation of the cellular structures to the membrane’s overall physico-chemical properties have been and continue to be the subject of a multitude of studies. For a taste of the breadth of the field, one may consult recent monographs, such as Refs. 6, 91, 130 and 147, and reviews such as Refs. 10, 16, 45, 48, 50, 55, 70, 135, 139, 144, 146, 157 and 197. The composite consists of a plasma membrane with an undercoat of membrane skeleton. The former functions as an osmotic barrier that inhibits the passage of large or charged solute molecules; the latter’s elastic support prevents the RBC membrane from disintegrating in circulatory shear flow. In the following, we review the structural organisation of the membrane skeleton in Section A.1 and the structural organisation of the plasma membrane in Section A.2.

A.1 The membrane skeleton

The membrane skeleton, which appears as a dense, convoluted meshwork in electron micrographs (Figure A.1), is a quasi-two-dimensional multi-protein network with F-actin, band 4.1 (or, more precisely, band 4.1R, where R denotes the RBC), ankyrin (band 2.1), and
negatively charged spectrin as its major structural components [10, 135, 146].

Spectrins are composed of α- and β-subunits. An α-subunit associates laterally with a β-subunit in an anti-parallel fashion to form a coiled heterodimer. There are approximately $2 \times 10^5$ spectrin heterodimers per RBC [8]. Two heterodimers may associate head to head to form a 200 nm long heterotetramer, with the C-terminal domain of the β-subunit (and N-terminal domain of the α-subunit) of one heterodimer linked to the N-terminal domain of the α-subunit (and C-terminal domain of the β-subunit) of the other heterodimer. While the tetrameric form of spectrin is the prevalent form in the RBC, higher oligomeric forms such as hexamers and octamers also exist [134]. The relative amounts of dimers, tetramers and other oligomers in the RBC have been estimated to be 5–10%, 45–55% and 25–35%, respectively [134]. Each β-subunit of spectrin has sites for binding to actin and

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So named because it was the first protein to be purified from red cell “ghosts” [147, p. 157].
ankyrin, located at its N- and C-terminal regions, respectively. Binding of filamentous spectrin tetramers (and the higher oligomers) to short, stiff, 40 nm long actin segments and band 4.1 molecules at junctional complexes gives rise to the protein network. The dense packing and convoluted nature of the spectrin filaments in situ makes it difficult to identify the structural arrangement at the junctional complexes. Consequently, there has yet to be a consensus on the number of spectrin filaments joined to each actin segment. On the one hand, it is possible for an actin segment to accommodate a maximum of approximately 6 spectrins [10], and early experiments based on spread isolated skeletons found 5 to 7 spectrins to each actin segment [31, 131, 190]. On the other hand, later experiments based on unspread, RBC membrane-associated skeletons by Ursitti et al. [20, 217, 218] found a lower estimate of 3 to 4 spectrins per actin segment, although this reduction may be due to damage during preparation of the membrane [9].

The protein network is tethered to the cytoplasmic face of the plasma membrane primarily by protein linkages between the network and the transmembrane proteins band 3 (otherwise known as Anion Exchanger 1 or AE1) and glycophorin C (GPC) of the plasma membrane. Binding of ankyrin to β-spectrin and band 3 constitutes the main linkage. There are approximately $10^5$ ankyrin monomers and $10^6$ band 3 monomers per RBC [8, 39], with one ankyrin predominantly associating with two band 3 dimers to form an ankyrin-mediated band 3 pseudo-tetramer [10, 221]. Band 3 has two sites for association with ankyrin and ankyrin also has two sites for association with band 3 [221]. It is not clear at present how the sites on band 3 and ankyrin interact; but, these interactions are suspected to be related to the observed populations of high- and low-affinity binding between band 3 and ankyrin [221]. While the nature of the band 3-ankyrin interaction remains to be clarified, it is expected that about 40% of the band 3 monomers will be bound to the membrane skeleton via ankyrin and will not diffuse laterally. This expected percentage is roughly consistent with the experimental estimate of laterally immobile band 3 monomers$^2$, based on fluorescence recovery after photobleaching (FRAP) and single particle tracking (SPT) techniques (see Ref. 212 and references therein). In SPT of band 3 in ghosts in a hypotonic medium, diffusional immobile band 3 molecules showed only oscillatory motion [211, 212], which reflects the

$^2$The immobile band 3 molecules include those not bound to ankyrin but still immobilised by non-specific interactions with the membrane skeleton. Raising the temperature or lowering the ionic strength will free these molecules and decrease the immobile population [73, 214].
ATP- and thermally driven fluctuations of the membrane skeleton [215]. In addition to the main β-spectrin-ankyrin-band 3 links, secondary attachments of the membrane skeleton to the plasma membrane occur at the junctional complexes, with band 4.1 monomers binding to actin segments and GPC monomers [233]. There are approximately $2 \times 10^5$ band 4.1 monomers [8], $3 \times 10^4$ actin segments [8] and $1.5 \times 10^5$ GPC monomers [196] per RBC. These numbers, together with the finding that all or nearly all ($\geq 90\%$) of GPC monomers are laterally immobile as determined by SPT [72], give rise to the current thinking that GPC monomers bind to band 4.1 monomers in a 1:1 ratio and 3 band 4.1 monomers bind to each of the two sides of an actin segment [10, 165], thus keeping the actin segments approximately tangential to the plasma membrane [165] and, under deformation, weakly aligned with extension of the protein network [164]. A recent study found that the dissociation of GPC from the membrane skeleton has little or no effect on membrane stability under ektacytometry [34], which suggests that the spectrin-ankyrin-band 3 links bear most, if not all, of the stress required to prevent membrane skeletal fluctuations from separating the membrane skeleton from the plasma membrane (this can be tested, perhaps, by measuring the energy or force required to pull a tether from an RBC with severed band 4.1-GPC links, and then making a comparison with the known force or energy required to pull a tether from an RBC with intact band 4.1-GPC links [88, 95, 228]).

Apart from the two protein linkages above, an additional minor coupling between the membrane skeleton and the plasma membrane is provided by the association of spectrin with phospholipids of the plasma membrane. Some studies have found a special affinity of spectrin to negatively-charged phosphatidyserine (PS) compared to neutral phospholipids, whereas other studies did not find that to be the case (see, e.g., Ref. 158). Maksymiw et al. [136], who observed a preferential binding between spectrin and PS, suggest that the coupling arises from electrostatic attraction between positively charged binding sites on spectrin and PS in the inner leaflet of the plasma membrane. O’Toole et al. [158] found that spectrin did present positive charges to the lipids but did not detect a preferential binding between spectrin and PS.

Spectrin dimers and tetramers exist in a state of dynamic equilibrium in situ [132] (and also in solution [216]). The tetrameric form is highly favoured under physiological conditions [132, 133], due largely to the promotion of dimer-dimer association and the stabilisation of the tetramers by intracellular hæmoglobin [133], with a molar ratio of dimers to
tetramers of 0.1–0.2 [134]. Membrane skeletal mechanical stability is therefore enhanced by the presence of haemoglobin [133], presumably as a consequence of a corresponding increase in the connectivity of the protein network. The spectrin dimer-tetramer equilibrium is also affected by the ionic strength of the suspending medium, with low ionic strength (hypotonic solution) promoting the reversible dissociation of tetramers into dimers and reducing the structural stability of the membrane skeleton [132], presumably caused by a corresponding reduction in the connectivity of the protein network. However, the dissociation of tetramers into dimers due to low ionic strength is inhibited by the presence of haemoglobin [133]. When haemoglobin is removed from RBCs, the molar ratio of dimers to tetramers increases to 1 for the resultant ghosts in an isotonic solution [133]. Studies of spectrin in solution [216] have shown that temperature < 40 °C does not affect the preferred polymeric state of the spectrin dimer-tetramer equilibrium, but it does affect the rate at which the equilibrium is attained. As the temperature is lowered, a longer time is required for the inter-conversions between the two species to come into equilibrium. At and below ~ 4 °C, the rate of conversion from one species to the other is so small that each species is kinetically trapped. There does not appear to be a similar systematic study of the effect of temperature on the spectrin dimer-tetramer equilibrium in situ; nevertheless, it appears to be well accepted that the situation in situ mirrors that in solution. In fact, it is standard practice to exploit the kinetic trapping of spectrin at low temperature to prevent tetramers from dissociating into dimers during the extraction of spectrin from the RBC membrane, e.g., Refs. 133 and 2 from 1984 and 2002, respectively. A recent study [2] has shown that spectrin dimers can also form by the rupturing of tetramers in situ by shear forces well below that experienced by the RBC in the circulation, as applied by an ektacytometer. Another contributor to the mechanical stability of the RBC membrane is the level of phosphorylation (covalent attachment of phosphate groups) of P-spectrin in situ. Manno et al. found that an increase in β-spectrin phosphorylation decreases membrane mechanical stability, while a decrease in phosphorylation has the opposite effect [138]. The molecular mechanism governing this behaviour is not clear. A possibility that they suggested is phosphorylation-induced changes in spectrin oligomerisation in situ.

Band 4.1 has the capacity to compete with ankyrin to bind with band 3. Band 4.1-band 3 binding is accompanied by a reduction in ankyrin-band 3 binding affinity and, consequently, a reduction in membrane mechanical stability and an increase in membrane deformability
under ektacytometry [3]. However, the interaction between band 4.1 and band 3 does not appear to involve the displacement of GPC by band 3 at band 4.1-linked junctional complexes [233] of the membrane skeleton.

A.2 The plasma membrane

The plasma membrane is organised into a fluid bilayer structure with a highly heterogeneous distribution of phospholipids, cholesterol, and membrane-bound proteins in the lateral and transverse directions.

There are a total of \((4.00 \pm 0.17) \times 10^{-16}\) moles of phospholipid molecules in the plasma membrane [54], with phosphatidylcholine (PC or lecithin), sphingomyelin (SM), phosphatidylethanolamine (PE) and phosphatidylserine (PS) constituting the four major classes at mole % of \((29.2 \pm 1.5)\), \((25.4 \pm 1.4)\), \((27.5 \pm 1.5)\), and \((14.8 \pm 1.7)\), respectively (see Refs. 42 and 219 for reviews of studies of RBC lipid composition). In the transverse direction, there exists a well known asymmetric transmembrane lipid distribution: out of a total of \(61 \times 10^6\) and \(70 \times 10^6\) molecules of SM and PC, respectively, 82% of the former and 76% of the latter accumulate in the outer leaflet, whereas 80% of the \(66 \times 10^6\) molecules of PE and all \(36 \times 10^6\) molecules of PS are located in the inner leaflet [91, Ch. 1]. In comparison to the other three classes of lipids, the acyl chain composition of SM shows a very unusual asymmetry: about 73% of SM molecules in the inner leaflet have acyl chains of less than 20 carbon atoms, whereas about 69% of those in the outer leaflet have acyl chains of 20 carbon atoms or more. There is also a slight asymmetry in the transmembrane distribution of the molecular species of PE. For PC, on the other hand, there is essentially no compositional difference between its inner and outer leaflet populations. At physiological pH, SM, PC and PE are zwitterionic, while PS is negatively charged. This charge distribution among the lipids combines with the transmembrane lipid asymmetry to give a neutral outer leaflet and a negatively charged inner leaflet. The transmembrane lipid asymmetry is maintained by enzymes acting as phospholipid pumps and, perhaps, the interaction between PS in the inner leaflet and spectrin of the membrane skeleton [91, Chs. 1 and 2], [5, 16, 44, 45, 243].

The phospholipid pumps are categorised according to their functions as flippases, floppases and scramblases, with the first two being ATP-dependent. While their effects are clear, the identities of these enzymes remain elusive. Under physiological conditions, with
APPENDIX A. REVIEW OF RBC MEMBRANE STRUCTURAL ORGANISATION

Free cytoplasmic Ca\(^{2+}\) concentration measured to be 10–30 nM [26, 124, 174], the unidirectional flippases rapidly and selectively pump PE and PS from the outer to the inner leaflet (rate for PS > rate for PE in general), the unidirectional floppases slowly and non-selectively pump all phospholipids (perhaps with the exception of SM) from the inner to the outer leaflet, and the bi-directional scramblases are inactive [91, Chs. 1 and 2], [5, 16, 44, 45, 243]. Estimation of the half-times of the flippase-primed inward translocation of PS and PE, and the half-times of the floppase-primed outward translocation of PS, PE and PC, is complicated by the unavoidable uncertainty with the ability of the particular phospholipid analogue used to mimic the biochemical characteristics of its endogenous counterpart. With this caveat in mind, the half-times at 37 °C of the active inward translocation of PS analogues have been estimated to be ~5 min [18, 40, 41, 148] and those of PE analogues from ~5 to ~50 min [18, 40, 41, 65, 148], whereas the half-times at 37 °C of the active outward translocation of PS, PE and PC have been estimated to be 57.7 min, 77.0 min, and 230 min, respectively [18], or all virtually identical at ~1.5 hr [41]. In a contrary view, endogenous PS is thought to be unaffected by the floppases, since it resides exclusively in the inner leaflet [91, Ch. 1]. For PC, the half-times of its inward translocation by transmembrane diffusion at 37 °C are 3–26 hr, depending on the composition of its acyl chains [143]. SM, in accordance with the highly asymmetric transmembrane distribution of its molecular species, appears to have very little exchange between its inner and outer leaflet populations [91, Ch. 1]. Scramblases are activated, and flippases deactivated, by a high concentration of Ca\(^{2+}\), with the subsequent bi-directional flow of lipids distorting the transmembrane lipid asymmetry [91, Chs. 1 and 2], [5, 16, 44, 45, 243]. Reinhart and Chien [173] found that a 2-min exposure to a high concentration (2.2 mM) of extracellular Ca\(^{2+}\) caused blister-like lesions, with a diameter of about 100 nm, dispersed over the plasma membrane. It will be interesting to see whether the proteins that constitute the scramblases are contained within these lesions. The lipid asymmetry is important for the maintenance of the RBC’s normal discoid shape and mechanical stability: a recent study by Manno et al. [137] reported that the alteration of the lipid asymmetry of resealed ghosts, by deactivating the flippases by ATP deprivation during the preparation of the ghosts, led to echinocytosis (cf. Table 1.2) and a greater susceptibility to fragmentation under shear deformation in an ektacytometer. Manno et al. suggested that the translocation of PS to the outer leaflet when the flippases were deactivated, reduced the binding between PS in the
inner leaflet and spectrin, hence reducing the mechanical stability of the RBC membrane.

There are $\sim 190 \times 10^6$ cholesterol molecules in the plasma membrane [54, 116], each with a cross-sectional area of $\sim 0.39$ nm$^2$ [43, 116]. Cholesterol is believed to be also distributed asymmetrically among the two leaflets, although experiments so far have given conflicting results as to which leaflet it is concentrated in. These results have suggested that cholesterol may be mostly in the outer leaflet, mostly in the inner leaflet, or nearly equally distributed across the plasma membrane with a slight preference for the outer leaflet [201].

Given its affinity for SM [157, 223], however, cholesterol seems more likely to prefer the outer leaflet. Similarly, it remains uncertain whether cholesterol diffuses across the plasma membrane in a matter of seconds or less, minutes, or hours [201]. The smallest and most recent estimate of the inward or outward transmembrane diffusion half-time is $< 1$ s at 37°C, as inferred from a study of cholesterol extraction from the plasma membrane using cyclodextrin [201]. This estimate is believed to be the best yet [200], and suggests a fast transmembrane equilibration of cholesterol, which in turn suggests that cholesterol is more likely to be distributed almost symmetrically, with a slight preference for the outer leaflet, as found in an earlier study [116].

The most abundant and best-studied transmembrane proteins of the plasma membrane are band 3 and glycophorins A, B, C and D (or GPA, GPB, GPC and GPD, respectively) [35, 78, 146, 209]. GPA, GPB, GPC and GPD are known formerly as the sialoglycoproteins $\alpha$, $\delta$, $\beta$ and $\gamma$, respectively. Band 3 and the glycophorins constitute approximately 25% and 2%, respectively, of total RBC membrane proteins. Band 3 and GPC, as mentioned in the previous section, are the sites to which the membrane skeleton is attached. GPB is present in the RBC at approximately $3 \times 10^5$ copies and appears to have no structural function. GPD is present in the RBC at approximately $8 \times 10^4$ copies [196] and its structural role is not clear. GPA, present at approximately $10^6$ copies per RBC, can bind to band 3. GPA has very little or no interaction with the membrane skeleton in its native state, but can be induced to associate with the membrane skeleton by the binding of a ligand to its exoplasmic domain, which decreases the RBC deformability under ektacytometry. The nature of this ligand-induced association is not clear. The glycophorins, being small in mass fraction relative to total RBC proteins and with each having only one, $\alpha$-helical transmembrane domain, are expected to have hydrophobic domains that are small in cross-sectional area. Consequently, local geometrical distortions of the plasma membrane bilayer by these proteins [238, Ch. 7]
are expected to have negligible geometrical effect at larger length scales at which a curvature can be meaningfully ascribed to the plasma membrane. Out of the four glycophorins, it appears that only for GPA has the cross-sectional area been estimated [163], with a value of approximately $A_{\text{GPA}} = 1.77 \, \text{nm}^2$. Summing over the approximately $10^6$ copies of GPA per RBC, this translates into about 1.3% of $A_{\text{RBC}}$, assuming $A_{\text{RBC}} = 140 \, \mu\text{m}^2$ (Table 1.1). Indeed, it is found that RBCs completely lacking in GPA have normal discocytic morphology, no clinically significant anaemia and, under ektacytometry, normal deformability and mechanical stability [35, 210].

On the other hand, band 3, being large in mass fraction relative to total RBC proteins and possessing multiple transmembrane domains [78, 209], has a large cross-sectional area of 27.5 nm$^2$ for the membrane domain of a band 3 dimer [224], which constitutes about 10% of $A_{\text{RBC}} = 140 \, \mu\text{m}^2$ when summed over the approximately $10^6$ copies of band 3 monomers per RBC. Thus, geometrical effects due to the interaction between band 3 and lipids of the plasma membrane are expected to affect the curvature of the plasma membrane appreciably. In those cases of hereditary spherocytosis (HS) that involve a genetic disorder that leads to a gross reduction in the population of band 3 not attached to the membrane skeleton, it is strongly suspected that the affected RBCs spontaneously vesiculate externally until they assume a spherical shape [103]. This is reminiscent of spheroechinoctysosis and suggests that (i) the plasma membrane has a large tendency to curve outward in the absence of band 3 and (ii) band 3 stabilises the plasma membrane by imposing a counter-balancing negative curvature [220] (perhaps involving moderation of the shape of lipids in the neighbourhood of band 3 [70]). For RBCs with the normal complement of freely diffusing and skeletally attached populations of band 3, experiments have suggested that a change in the conformation [19, 71, 90], or polymeric state of association [220], of band 3 will alter the bending tendency of the plasma membrane, leading to shape transformations. In sum, band 3 does not merely facilitate the transfer of in-plane stresses to the membrane skeleton, but is very likely also involved in regulating the bending elasticity of the plasma membrane.

It is instructive to estimate the ratio $E_{\text{GPC}}/E_{\text{B3}}$, where $E_{\text{GPC}}$ and $E_{\text{B3}}$ are the energy costs of fully exposing the hydrophobic domains of all the skeletally attached GPC monomers and band 3 dimers, respectively, to water. For a GPC monomer, it is reasonable to assume that its hydrophobic domain is cylindrically shaped, with a cross-sectional area comparable
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Therefore,\\

\[ E_{\text{GPC}} \sim N_{\text{GPC}} \times 2\pi R_{\text{GPC}} h \times \sigma \]

\[ = 1.5 \times 10^5 \times 2 \sqrt{\pi (1.77 \text{ nm}^2)} \, h \sigma ,\]

(A.1)

where \( N_{\text{GPC}} \) is the number of GPC molecules attached to the membrane skeleton, \( R_{\text{GPC}} \) is the radius of the cylinder approximating the hydrophobic domain of GPC, \( h \) is the thickness of the hydrophobic region of the plasma membrane and \( \sigma \) is the effective free energy cost of forcing bulk water to sit against a hydrophobic surface [235]. At a resolution of 2 nm, a band 3 dimer in projection consists of a \( 4 \times 5 \text{ nm} \) central domain flanked by two \( 1.5 \times 2.5 \text{ nm} \) peripheral domains [224]. Approximating each of these three domains as a cylinder,

\[ E_{\text{B3}} \sim N_{\text{B3}} \times 2\pi (R_c + 2R_p) h \times \sigma \]

\[ = 2 \times 10^5 \times 2 \sqrt{\pi \left( \sqrt{20 \text{ nm}^2} + 2\sqrt{3.75 \text{ nm}^2} \right)} h \sigma ,\]

(A.2)

where \( N_{\text{B3}} \) is the number of band 3 dimers attached to the membrane skeleton, \( R_c \) is the radius of the cylinder approximating the central domain and \( R_p \) is the radius of the cylinder approximating each peripheral domain. Dividing Equation (A.1) by Equation (A.2) then yields the estimate

\[ \frac{E_{\text{GPC}}}{E_{\text{B3}}} \sim 0.12.\]

(A.3)

Given this ratio, it would seem plausible that the spectrin-ankyrin-band 3 links bear most of the stress required to keep the membrane skeleton-plasma membrane coupling intact, as suggested previously.

In the lateral direction of the plasma membrane, it was initially thought that all the membrane components freely diffuse to give a completely random and homogeneous mixture of lipids, cholesterol and proteins. This ideal mixing was the central tenet of the first model of plasma membranes, the fluid mosaic model [192]. It has since been realised that there is more to this simplistic picture wherein the mobility of the membrane components is limited by diffusion only. The latest studies indicate that there is substantial lateral patchiness or ordering of the plasma membrane (see, e.g., Refs. 55, 139, 91 (Chs. 5 and 6)), the properties of which are difficult to characterise. These patches or domains may be transient or stable, and their linear dimensions vary from tens of nm to hundreds of nm.
At length scales of 50–70 nm, there may be formation of *rafts* [77, 157, 167, 181, 191]. These are micro-domains in the outer leaflet which are enriched in cholesterol, SM and certain proteins, including those anchored to glycosyl-phosphatidyl-inositol (GPI).

In SPT of band 3 molecules in ghosts in a hypotonic medium [211, 212], mobile band 3 molecules have been shown to be temporarily trapped within corrals 50–200 nm in diameter\(^3\), with an average hopping time between two adjacent corrals of 350 ms. These corrals have been shown to arise from steric hindrance of the cytoplasmic domain of band 3 by the underlying spectrin filaments of the membrane skeleton. Within a corral, a band 3 molecule diffuses almost freely, slowed only by the presence of other membrane proteins.

In an experiment by Rodgers and Glaser that probed the plasma membranes of intact rabbit erythrocytes [175], lipid domains several hundred nm in size have been directly visualised using FDIM (fluorescence digital imaging microscopy). Two varieties of domains have been detected with the aid of fluorescent PC and PS derivatives, with one enriched in PC and the other enriched in PS (presumably, the latter was confined exclusively to the inner leaflet). Figure A.2 (A *Left*) shows an image of PC-enriched domains on intact cells obtained by Rodgers and Glaser. Interestingly, when ghosts were used instead of intact cells [175, 176], domains as large as several \(\mu\)m enriched in PC, PS and PE, respectively, were observed using fluorescent PC, PS and PE derivatives. Moreover, in comparison to the PC- and PS-enriched domains on intact cells [175], those on the ghosts [175, 176] appear to be more enriched in their respective lipid headgroup species.\(^4\) Figure A.2 (C) shows an image of domains on a ghost that are larger and more enriched in PC compared to those on an intact cell. The sizes and enrichment of the PC-, PS- and PE-enriched domains on ghosts were, qualitatively, insensitive to temperature variations (based on observations at 4 and 37 °C), but strongly dependent on the ionic strength of the buffer, with low ionic strength promoting smaller and less enriched domains. They argued that these domains were unlikely the manifestation of co-existence of gel and liquid phases of the lipids, since

---

\(^3\)These measurements were taken at low ionic strength conditions, under which spectrin tetramers tend to dissociate into dimers that would impose less steric restriction on band 3 molecules than the tetramers. For the RBC in physiological conditions, which favours the tetrameric form of spectrin over the dimeric form, the corral diameter is thus expected to be smaller.

\(^4\)Actually, Ref. 175 only shows a representative image for PC-enriched domains on intact cells. While it does not show any image of PS-enriched domains on intact cells, its text does mention that the distributions of their size and enrichment are similar to those of the PC-enriched domains.
Figure A.2: (A Left) Fluorescence image of PC-enriched domains on an intact discocytic rabbit RBC. (A Right) Image of the same RBC under bright field illumination at the same plane of focus with the same objective. (B) Image of domains enriched in CPZ (a fluorescent and stomatocytogenic compound) on an intact spherical rabbit RBC. (C) Image of PC-enriched domains on a discocytic ghost. (D) Image of PC-enriched domains on a discocytic ghost after treatment to induce aggregation of intramembrane particles. (E) Colour scheme representing the lipid enrichment level, with red being the highest. The horizontal white bar underneath (C) equals 2.5 \( \mu \text{m} \). Reprinted from Fig. 1 of Ref. 175 with permission of the authors.

The gel-liquid phase transition would be expected to be strongly temperature dependent. For vesicles made of lipids extracted from rabbit RBCs, lipid phase transition was not evident and there was no visible domain formation with a change in ionic strength or temperature. Thus, they hypothesized that the membrane skeletons underneath the plasma membranes of the intact cells and ghosts prevented the free lateral diffusion of certain membrane proteins with an affinity for a particular lipid headgroup. The aggregation of these proteins would, in
turn, attract their preferred lipids to their neighbourhoods and give rise to domains enriched in these proteins and lipids.\(^5\) Such a mechanism of lipid domain formation was suggested to be at work in the case of the ghosts, where domains enriched in band 3 proteins were found to be correlated with domains enriched in PC and also with artificially induced areas of high spectrin concentration [176]. Rodgers and Glaser suggested that the domains corresponded to the immobile fraction of band 3 found by Golan and Veatch [73], based on the similarity in behaviour between the decrease in the size and enrichment of the domains with decreasing ionic strength on the one hand, and the decrease in the immobile fraction of band 3 with decreasing ionic strength on the other. However, contradiction arises on examining the temperature behaviour of the domains and the immobile fraction of band 3. Specifically, Golan and Veatch found that the immobile fraction of band 3 increased with decreasing temperature, which would increase the domain size and enrichment according to the argument of Rodgers and Glaser above, whereas Rodgers and Glaser themselves found that the domain size and enrichment were insensitive to temperature. An alternative explanation is that the dependence of the domain size and enrichment on ionic strength is an effect of ionic strength on the membrane skeleton’s spectrin dimer-tetramer equilibrium: the dissociation of spectrin tetramers to dimers at low ionic strength is expected to reduce the number of corrals encountered by the freely diffusing population of band 3, giving them more time and roaming space (an increase in entropy in other words) to escape before their preferred lipids (PC) have sufficient time to cluster around them. As a result, there is less aggregation of band 3, and PC about band 3, hence smaller and less enriched lipid domains. The involvement of spectrin could also partially account for the difference in domain size and enrichment between intact cells and ghosts, in addition to other factors such as fluorescence quenching of the fluorescent lipid derivatives by hemoglobin, and altered transmembrane lipid asymmetry and diffusion. In intact cells, the spectrin tetramers prevent the diffusion of free band 3 molecules over large distances. Therefore, they and their ankyrin-bound counterpart become localised and more or less evenly distributed over the plasma membrane, except for those occasions when small in-plane deformations of the membrane skeleton caused by thermal and ATP-driven fluctuations bring about a small difference in the concentration of band 3, and in the concentration of PC, between the com-

\(^5\)It remains to be seen whether other effects, such as the elimination of the transmembrane lipid asymmetry, also contribute to the absence of domains on vesicles made of rabbit RBC lipid extracts.
pressed and expanded regions of the membrane skeleton (cf. Figure A.2 (A Left)). Then, with the removal of intracellular haemoglobin and its protective effect on spectrin tetramers in the preparation of ghosts, the spectrin dimer-tetramer equilibrium is shifted towards the dimers. This amounts to the creation of regions of high and low tetramer concentrations with a more pronounced difference in concentration compared to that between regions of high and low tetramer concentrations in intact cells; they also correspond, respectively, to regions of relatively more pronounced high and low band 3 concentrations. The regions of high band 3 concentration on ghosts at or near isotonic ionic strength are also likely to be larger in size in comparison to those on intact cells, thus leading to larger and more enriched PC-enriched domains on ghosts (cf. Figure A.2 (C)). However, as mentioned earlier, when more and more spectrin tetramers dissociate into dimers when the ionic strength is lowered, shrinking the regions of high band 3 concentration and reducing the number of band 3 molecules trapped in these regions in the process, the PC-enriched domains on ghosts will also shrink and become less enriched. Furthermore, the temperature insensitivity of the domain size and enrichment may be due partly to the temperature insensitivity of the preferred polymeric state of association of spectrin (in addition to the absence of a gel-liquid phase transition). Rodgers and Glaser did not address the question of whether the domains on intact cells would show similar dependence on ionic strength and temperature, though it is reasonable to assume that they would, but to a lesser degree because of the protective effect on spectrin of haemoglobin.

Virtanen et al. [197, 223] recently proposed a superlattice model of plasma membrane organisation, in which it is assumed that there is only short range order among the lipids. In rationalising the incompatibility between this assumption and the large domains on ghosts observed by Rodgers and Glaser, they argued for two alternative causes of lipid domains on ghosts: (i) Ghosts easily lose their transmembrane lipid asymmetry, so the domains might form due to compositional changes of the outer and inner leaflets. (ii) The fluorescent lipid derivatives used are different in structure and conformation from natural lipids because of the extra polar molecule incorporated into one acyl chain of each lipid derivative, so it is possible that the derivatives also do not resemble their natural counterparts in their interactions. Domains could then be induced artefactually by the altered interactions of the lipid derivatives. Checks by Rodgers and Glaser, while not completely ruling out (ii), found the domains on ghosts to be independent of labelling within experimental conditions.
Appendix B

Isotropic Hyperelastic Surfaces

This appendix gives a brief review of the continuum elastic theory of isotropic hyperelastic surfaces. The theory is a straightforward adaptation of the continuum elastic theory of isotropic hyperelastic solids, the in-depth discussions of which may be found, for example, in Refs 7, 17, 93, 121, 156 and 213.

B.1 Deformation gradient tensor

Suppose a smooth elastic surface $S_A$ is deformed into another surface $S_X$. Under this deformation, the neighbourhood of a point $A_0$ on $S_A$ is mapped into its corresponding image on $S_X$. We consider only the class of deformations under which the mapping is one-to-one, since problems of this class are physically most relevant. Therefore, every point in the neighbourhood of $A_0$ is mapped into a different point in the image neighbourhood; in particular, $A_0$ is mapped into the point $X_0$ on $S_X$. The points on $S_A$ and any coordinate system used to represent them are described as Lagrangian or material, while the points on $S_X$ and any coordinate system used to represent them are described as Eulerian or spatial. In anticipation of the deformation being generally non-uniform, we concentrate our treatment on neighbourhoods which are small enough that neither the original neighbourhood on $S_A$ nor its image on $S_X$ are significantly curved, i.e., they should be well approximated by the tangent planes at $A_0$ and $X_0$, respectively. With this understanding, we can now introduce local orthogonal coordinate systems $(^1a, ^2a)$ on $S_A$ and $(^1x, ^2x)$ on $S_X$, with origins at $A_0$.
APPENDIX B. ISOTROPIC HYPERELASTIC SURFACES

and \( X_0 \), respectively. Note that:

1. \( X_0 \) is generally translated with respect to \( A_0 \).

2. The orientation of the neighbourhood of \( X_0 \) with respect to the corresponding neighbourhood of \( A_0 \) will generally be rotated.

3. The coordinates \( \alpha a \) and \( x \), where \( \alpha, i \in \{1, 2\} \), are arbitrary in the sense that there is no assumption, for example, that the \( 1a \) coordinate maps into the \( 1x \) coordinate under the deformation. Note the use of Greek and Roman indices to denote material and spatial quantities, respectively.

The transformation

\[
i_x = \mathbf{x}(\alpha a)
\]  

(B.1)

represents the local deformation which maps the material point \( A \) with coordinates \( \alpha a \) into the spatial point \( X \) with coordinates \( x \). Equation (B.1) has the same form as the coordinate transformation of a single point, but governs a completely different physical situation. By Equation (B.1), a material point \( A' \) at \( \alpha a + da^\alpha \) which is differentiably close to \( A \) is mapped into a spatial point \( X' \) at

\[
i_x + dx^i = \mathbf{x}(\alpha a + da^\alpha)
\]  

(B.2)

which is differentiably close to \( X \). Each function \( \mathbf{x} \) is required to be single-valued and at least once continuously differentiable with respect to \( \alpha a \) to allow a Taylor expansion of Equation (B.2) to first order about \( A \). The expansion leads to the local linear mapping of \( da^\alpha \) to

\[
dx^i = \frac{\partial (x)}{\partial (\alpha a)} dx^\alpha + \cdots
\]  

(B.3)

on taking the difference between Equations (B.2) and (B.1).

By using the contemporary absolute tensor notation, Equation (B.3) may be written in a more general form that does not refer to a particular coordinate system:

\[
dx = \mathbf{F}|_A da + \cdots,
\]  

(B.4)

1For notational clarity, we depart from convention to write the indices before the components of tensors of any order that are intrinsically observer-dependent, i.e., tensors that are not objective in continuum mechanics, as defined in Section B.2.
where bold lowercase letters denote tensors of order one (commonly called vectors) and bold uppercase letters denote tensors of order two (commonly called tensors). Equation (B.4) describes the deformation of the material line element $da$ between two material points to the spatial line element $dx$ between two spatial points without reference to any coordinate system. $F = \nabla \otimes \mathbf{x}$ is called the deformation gradient tensor.

Now, each material point $A$ (respectively spatial point $X$) has associated with it a basis of two covariant vectors, both tangential to $S_A$ at $A$ (respectively $S_X$ at $X$). Our choice of material and spatial bases are the sets of orthonormal vector fields $\{\mathbf{a}_\alpha(A)\}$ and $\{\mathbf{x}_i(X)\}$, respectively. For simplicity, each $\mathbf{a}_\alpha$ is required to be tangential to the $a^\alpha$ coordinate line passing through $A$, and each $\mathbf{x}_i$ is required to be tangential to the $i^\mathbf{x}$ coordinate line passing through $X$. Then, when expressed in terms of these bases, $da$ and $dx$ are the contravariant vector components of $da = da^\alpha a_\alpha$ and $dx = dx^i x_i$, respectively, and the decomposition of $F$ into its base tensors $x_i \otimes a^\alpha$ is

$$F = F_{i.\alpha} x_i \otimes a^\alpha, \quad \text{where} \quad F_{i.\alpha} = \frac{\partial(i^x)}{\partial(a^\alpha)}. \quad \text{(B.5)}$$

The contravariant base vectors $a^\alpha$ are related to $a_\alpha$ by $a^\alpha = A^{\alpha\beta} a_\beta$, where $A^{\alpha\beta}$ are the components of the local metric tensor on $S_A$ (in this case, it is simply the identity tensor). With $F$ being the link between points in two configurations, as shown by the indices $i$ and $\alpha$ of its components $F_{i.\alpha}$, it is thus variously described as a two-point, mixed Eulerian-Lagrangian, bi-, or mixed-base, tensor. Now, to guarantee the existence of the corresponding inverse mapping

$$a^\alpha = a^\alpha g(i^x)$$

with deformation gradient tensor $G = F^{-1}$, we require the function $a^\alpha g$ to be at least once continuously differentiable with respect to $i^x$ and $\det F \equiv |F_{i.\alpha}| \neq 0$. Decomposition of $G$ then gives

$$G = G^{\alpha}_{i.} a_\alpha \otimes x^i, \quad \text{where} \quad G^{\alpha}_{i.} = \frac{\partial(a^\alpha)}{\partial(i^x)}$$

and $x^i = X^{ij} x_j$, with $X^{ij}$ being the components of the local metric tensor on $S_X$ (here, it is the identity tensor). Note the following properties of $F$ ($G$, of course, has analogous

---

As alluded to earlier in footnote 1, tensors in continuum mechanics can be intrinsically observer-dependent, in which case they are called not objective, or not, in which case they are called objective. This distinction will be made precise in Section B.2.
properties):

1. The components of $F$ are real since the deformation is physical.

2. $F$ depends on $\mathcal{A}$ and will generally differ from point to point over $S_A$.

3. $F$ is non-singular ($\det F \neq 0$) because the transformation of $S_A$ into $S_X$ is one-to-one, and there is no loss of generality in assuming $\det F > 0$, the justification being that $\det F$ is physically the ratio of the area of a material surface element to the area of the corresponding spatial surface element. Transformations with $\det F > 0$ are known as proper transformations, which have the property of mapping a right-handed set of coordinates to another right-handed set, hence preserving the orientation of any closed curve on $S_A$.

4. The components of $F$ are not assumed to be small or to differ by a small amount from unity, i.e., the material may be strongly strained.

5. $F$ is generally not symmetric, so that, although it has eigenvectors, those eigenvectors are generally not orthogonal.

6. The deformation gradients of any sequence of deformations compose multiplicatively, so that a deformation with deformation gradient $F_1$ followed by another with deformation gradient $F_2$ results in an overall local linear deformation

$$dx = F_2 \big|_{\mathcal{A}'} F_1 \big|_{\mathcal{A}} \, d\mathcal{A},$$

where the point $\mathcal{A}'$ is the image of $\mathcal{A}$ under $F_1$.

By the continuity of $F^i_\alpha$ in Equation (B.5) and by choosing a sufficiently small neighbourhood, it is a good approximation to assume that $F^i_\alpha$ is locally constant. The deformation of the material is then said to be locally uniform (or locally homogeneous).

### B.2 Rotations and material objectivity

It will be important in subsequent discussions to be familiar with operations involving rigid rotations of the neighbourhood of a point by a rotation tensor $R$. $R$ is defined to be an
orthogonal tensor that is additionally proper, \( i.e., \det R = +1 \). By the orthogonality of \( R \), the mapping of two arbitrary vectors \( u \) and \( v \) in an infinitesimal neighbourhood to \( Ru \) and \( Rv \), respectively, of the corresponding rotated neighbourhood preserves the dot product \( u \cdot v \), \( i.e., Ru \cdot Rv = u \cdot v \). Therefore, the angle between two vectors and the length of a vector are invariant under \( R \). In addition, \( R^{-1} = R^T \), where the superscript \( T \) denotes the transpose. Physically, \( R \) rotates a vector anti-clockwise by an angle \( \theta \) about an axis. \( R \) is a two-point tensor, which can be verified easily in the simplest case where the material surface \( S_A \) is subjected to a rigid body rotation \( F = R = \overline{R} \), a constant tensor that does not vary from point to point.

A general rigid body motion of a neighbourhood involves a rotation \( R \) and a translation \( c \). Under a rigid body motion, a point in the neighbourhood with position vector \( r \) is mapped to a different point with position vector

\[
 r' = Rr + c. \tag{B.6}
\]

Tensor fields over the neighbourhood that are independent of \( c \) of Equation (B.6) are said to be \textit{objective} or \textit{indifferent} (to different regions of space).

Next, we examine how objective tensor fields over a neighbourhood transform under \( R \) to preserve distances and orientations. Ogden [156] distinguishes between \textit{Eulerian}, \textit{Lagrangian}, \textit{and two-point} objectivity, corresponding to transformations of spatial tensor fields under a spatial rigid rotation \( R_S \), transformations of material tensor fields under a material rigid rotation \( R_M \), and transformations of two-point tensor fields under a spatial rigid rotation \( R_S \), respectively.

Under a rotation \( R_S \) of the spatial neighbourhood formed under \( F \), the spatial tensor fields that satisfy Eulerian objectivity and the two-point tensor \( F \) that satisfies two-point objectivity must transform according to the transformation rules of Table B.1. Note that the spatial scalar field \( f_S \), material tensor fields of all orders, and the material part of \( F \) are invariant under \( R_S \). For spatial vector fields \( u_S \) and \( v_S \), the transformation rule for spatial vectors ensures \( u_S \cdot v_S = u'_S \cdot v'_S \), \( i.e., \) the invariance of the vector dot product. For a spatial second-order tensor field \( T_S \), if \( T_S \) maps \( u_S \) to \( v_S \) by \( T_S u_S = v_S \), the transformation rule for \( T_S \) ensures \( T'_S u'_S = v'_S \).

Under a rotation \( R_M \) of a material neighbourhood before \( F \) is applied, the material tensor fields that satisfy Lagrangian objectivity must transform according to the transformation
APPENDIX B. ISOTROPIC HYPERELASTIC SURFACES

<table>
<thead>
<tr>
<th>spatial scalar field</th>
<th>$f_S \rightarrow f_S' = f_S$</th>
</tr>
</thead>
<tbody>
<tr>
<td>spatial vector field</td>
<td>$v_S \rightarrow v_S' = R_S v_S$</td>
</tr>
<tr>
<td>spatial tensor field</td>
<td>$T_S \rightarrow T_S' = R_S T_S R_S^T$</td>
</tr>
<tr>
<td>two-point tensor field</td>
<td>$F \rightarrow F' = R_S F$</td>
</tr>
</tbody>
</table>

Table B.1: Transformation rules under a spatial rotation $R_S$ for arbitrary spatial tensor fields that satisfy Eulerian objectivity and the two-point tensor field $F$ that satisfies two-point objectivity.

<table>
<thead>
<tr>
<th>material scalar field</th>
<th>$f_M \rightarrow f_M' = f_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>material vector field</td>
<td>$v_M \rightarrow v_M' = R_M v_M$</td>
</tr>
<tr>
<td>material tensor field</td>
<td>$T_M \rightarrow T_M' = R_M T_M R_M^T$</td>
</tr>
</tbody>
</table>

Table B.2: Transformation rules under a material rotation $R_M$ for arbitrary material tensor fields that satisfy Lagrangian objectivity.

The rules of Table B.2. Note that the spatial tensor fields formed under $F$ and the spatial tensor fields formed under $F' = FR_M$ are in general different. The transformation $F \rightarrow F' = FR_M$ is related to the definition of an isotropic material, the discussion of which will be given in Section B.6.

Historically, most authors (see, for example, Refs. 7, 121 and 213) have adopted the definition of objectivity in the influential work of Truesdell [213], who implicitly assumed Eulerian objectivity and, thus, did not make a distinction between the three types of objectivity as Ogden. By assuming only Eulerian objectivity, it is argued that the two-point tensor field $F$ is not objective since it transforms like a spatial vector field instead of a spatial second-order tensor field.

B.3 Strain measures

A crucial part of the analysis of finite deformation is the determination of a good measure of strain, the relative change in length between a spatial line element $dx$ and its corresponding material line element $da$. A strain measure must meet two requirements: it should vanish when there is no deformation and be objective. The latter is imposed by the principle of...
material objectivity discussed in Sections B.2 and B.5.

$F$ does not satisfy the first requirement since $F = I$ when there is no deformation, where $I$ is the identity tensor. As for the second, it has been mentioned in Section B.2 that the transformation $F \rightarrow F' = R_s F$ has historically not been thought of as objective. Therefore, $F$ is commonly deemed unsuitable as a strain measure. It is possible, however, to construct various reasonable strain measures using tensors derived from the polar decomposition of $F$.

The polar decomposition theorem asserts that $F$, being non-singular, can be uniquely represented as a multiplicative composition of a local rotation and a local stretch in either of two alternative forms:

$$ F = RU = VR, \quad (B.7) $$

where $R$ is a rotation tensor (it is, therefore, proper and orthogonal) and $U$ and $V$ are symmetric and positive definite tensors. In general, $R$, $U$ and $V$ can vary from point to point. The tensor $U$ acts on the neighbourhood of a material point and is called the right (or material) stretch tensor. The tensor $V$ acts on the neighbourhood of a material point that has been rotated by $R$ and is called the left (or spatial) stretch tensor. In the special case of $F = R$, corresponding to a rigid body rotation of the entire material surface $S_d$, the polar decomposition of $F$ gives $U = V = I$.

Note that $U$, being symmetric, has positive eigenvalues $\lambda_\alpha$, eigenvectors $\hat{a}_\alpha$ that form a material orthonormal basis, and a special representation in terms of $\lambda_\alpha$ and $\hat{a}_\alpha \otimes \hat{a}_\alpha$ of the form

$$ U = \sum_{\alpha=1}^{2} \lambda_\alpha \left( \hat{a}_\alpha \otimes \hat{a}_\alpha \right). \quad (B.8) $$

$V = RUR^T$ has the same eigenvalues as $U$, eigenvectors $\hat{x}_i = \alpha = R\hat{a}_\alpha$ that form a spatial orthonormal basis, and a special representation in terms of $\lambda_\alpha$ and $\hat{x}_\alpha \otimes \hat{x}_\alpha$ that is similar to Equation (B.8). The eigenvalues $\lambda_\alpha$ are called principal extension ratios or principal stretches. The orthonormal basis $\{\hat{a}_\alpha(\mathcal{A})\}$ defines the principal material directions (or axes) of $U$, whereas the orthonormal basis $\{\hat{x}_\alpha(\mathcal{X})\}$ defines the principal spatial directions (or axes) of $V$. The special representation of $U$ in Equation (B.8) is known as the spectral representation (or decomposition) of $U$ and its components $\hat{U}_{\alpha\beta}^\alpha$ form a diagonal matrix

$$ \left[ \hat{U}_{\alpha\beta}^\alpha \right] = \left[ \begin{array}{cc} \lambda_1 & 0 \\ 0 & \lambda_2 \end{array} \right] = \left[ \lambda_\alpha \delta_{\alpha\beta}^\alpha \right]. $$
In general, in an arbitrary orthornormal basis \( \{a_\alpha(\mathcal{A})\} \) related to \( \{\widehat{a}_\alpha(\mathcal{A})\} \) by a rotation \( \mathbf{M} \) about \( \mathcal{A} \), i.e.,
\[
a_\alpha = \mathbf{M} \widehat{a}_\alpha,
\]
U has representation
\[
U = U^\gamma_\mu a_\gamma \otimes a^\mu,
\]
with components \( U^\gamma_\mu \) that can be shown to be related to \( \widehat{U}_\gamma^\alpha_\beta \) by the matrix equation
\[
\begin{bmatrix}
U^\gamma_\mu
\end{bmatrix}
= \begin{bmatrix}
(M^T)^\gamma_\alpha_\mu
\end{bmatrix}
\begin{bmatrix}
\widehat{U}_\gamma^\alpha_\beta
\end{bmatrix}
\begin{bmatrix}
M^\beta_\mu
\end{bmatrix}
= \begin{bmatrix}
(M^T)^\gamma_\alpha_\mu
\end{bmatrix}
\begin{bmatrix}
\lambda_\alpha \delta_\gamma^\alpha_\beta
\end{bmatrix}
\begin{bmatrix}
M^\beta_\mu
\end{bmatrix}.
\]
Note that the rotation of \( \widehat{a}_\alpha \) into \( a_\alpha \) by \( \mathbf{M} \) is a coordinate transformation, not a rigid rotation of some region of the surface. Therefore, \( \mathbf{M} \) is not a two-point tensor.

The two-point tensor \( \mathbf{R} \) has representation
\[
\mathbf{R} = R^i_\gamma x_i \otimes a^\gamma.
\]
Therefore, it follows that
\[
\begin{bmatrix}
F^i_\mu
\end{bmatrix}
= \begin{bmatrix}
R^i_\gamma
\end{bmatrix}
\begin{bmatrix}
(M^T)^\gamma_\alpha_\mu
\end{bmatrix}
\begin{bmatrix}
\lambda_\alpha \delta_\gamma^\alpha_\beta
\end{bmatrix}
\begin{bmatrix}
M^\beta_\mu
\end{bmatrix}.
\tag{B.9}
\]
Note that:

1. In practice, one must choose a set of material and spatial bases to describe \( \mathbf{F} \). Equation (B.9) tells us that for arbitrary material and spatial bases \( \{a_\alpha(\mathcal{A})\} \) and \( \{x_i(X)\} \), the components of \( \mathbf{F} \) result from four steps: an initial rotation of the local axes of a material neighbourhood until \( a_\alpha \) coincide with the local principal axes, a pure stretch \( \lambda_\alpha \) of the rotated neighbourhood in the direction of each of the principal axes, a rotation of the local axes counter to the initial one, and a rigid rotation of the stretched neighbourhood. This situation is depicted in Figure B.1.

2. The eigenvalues \( F_1 \) and \( F_2 \) of \( \mathbf{F} = \mathbf{R} \mathbf{U} \) are generally different from the eigenvalues \( \lambda_1 \) and \( \lambda_2 \) of \( \mathbf{U} \), since \( \mathbf{R} \neq \mathbf{I} \) in general.

3. To find the representations for \( \mathbf{R}, \mathbf{M}, \mathbf{U}, \) and \( \mathbf{V} \), \( \lambda_\alpha \) and \( \widehat{a}_\alpha \) of \( \mathbf{U} \) must first be determined. By the reason described previously, \( \lambda_\alpha \) and \( \widehat{a}_\alpha \) cannot be determined directly from \( \mathbf{F} \). However, one can proceed as follows:
Figure B.1: A comparison of transformations of two surface elements, $dS_A^{(1)} \rightarrow dS_X^{(1)}$ and $dS_A^{(2)} \rightarrow dS_X^{(2)}$, under a deformation $F = RU$. The material elements $dS_A^{(1)}$ and $dS_A^{(2)}$ are rectangular and oriented at right angles to the local material axes and local principal material axes, respectively. Unlike the transformation $dS_A^{(1)} \rightarrow dS_X^{(1)}$, no shearing is involved in the transformation $dS_A^{(2)} \rightarrow dS_X^{(2)}$, so $dS_X^{(2)}$ is rectangular.
(a) Use the base tensors $a_\alpha \otimes a_\beta$ to construct the representation of the positive definite real symmetric tensor
\[ C \equiv F^T F = U^T U = U^2, \] (B.10)
where we have made use of the orthogonality of $R$ and the symmetry of $U$. The tensor $C$ is known as the right Cauchy-Green tensor (or Green deformation tensor).

(b) The eigenvalues of $C$ are $\lambda_1^2$ and $\lambda_2^2$; thus, the positive square roots of these eigenvalues identify $\lambda_1$ and $\lambda_2$.

(c) $C$ and $U$ have common normalised eigenvectors $\hat{a}_1$ and $\hat{a}_2$.

(d) With knowledge of $a_\alpha$ and $\hat{a}_\alpha$, the representation of $M$ using the base tensors $a_\alpha \otimes a_\beta$ can be constructed.

(e) With knowledge of the spectral representation of $U$ in terms of $\lambda_\alpha$ and $\hat{a}_\alpha \otimes \hat{a}_\alpha$, the representation of $U$ using the base tensors $a_\alpha \otimes a_\beta$ can be constructed.

(f) Finally, the representation of $R$ using $x_i \otimes a^\alpha$ can be constructed from the relation $R = F U^{-1}$, where $U^{-1}$ has the same eigenvectors as $U$ and eigenvalues $\lambda_1^{-1}$ and $\lambda_2^{-1}$, and the representation of $V$ using $x_i \otimes x^j$ can be constructed from the relation $V = R U R^T$.

4. The principal invariants of $C$ are defined by $I_C \equiv \text{Tr } C$ and $II_C \equiv \text{det } C$. By the symmetry of $C$,
\[ I_C = \lambda_1^2 + \lambda_2^2, \]
\[ II_C = \lambda_1^2 \lambda_2^2, \]
which are symmetric in the indices 1 and 2.

5. We can repeat the argument above for $V$. Doing so will bring into consideration the counterpart to $C$, the left Cauchy-Green tensor (or Finger deformation tensor)
\[ B \equiv F F^T = V V^T = V^2, \] (B.11)
which is also positive definite, real, and symmetric. The principal invariants of $B$, $I_B \equiv \text{Tr } B$ and $II_B \equiv \text{det } B$, are the same as those of $C$:
\[ I_B = \lambda_1^2 + \lambda_2^2 = I_C, \]
\[ II_B = \lambda_1^2 \lambda_2^2 = II_C. \]
The tensors \( U \) and \( V \) are important as the basic quantities in various definitions of strain tensors, which can be divided into two groups. A general material strain tensor of order \( \alpha \) is defined by

\[
E_M^{(\alpha)}(U) \equiv \begin{cases} 
\frac{1}{\alpha} (U^\alpha - I) & \text{for } \alpha > 0 \\
\ln U & \text{for } \alpha = 0
\end{cases},
\]

where \( \ln U \) is defined by a power series expansion

\[
\ln U = \ln(I + (U - I)) = (U - I) - \frac{1}{2} (U - I)^2 + \frac{1}{3} (U - I)^3 + \cdots.
\]

Similarly, a general spatial strain tensor of order \( m \) is defined by

\[
E_S^{(m)}(V) \equiv \begin{cases} 
\frac{1}{m} (V^m - I) & \text{for } m < 0 \\
\ln V & \text{for } m = 0
\end{cases}.
\]

The material strain tensors of order 0, 1, and 2 are known as the Hencky strain tensor, Biot strain tensor, and Green-Lagrange strain tensor, respectively, with the last being the most commonly used material strain tensor. The spatial strain tensors of order 0, \(-1\), and \(-2\) are known as the spatial Hencky strain tensor, spatial Biot strain tensor, and Euler-Almansi strain tensor, respectively, with the last being the most commonly used spatial strain tensor.

The Green-Lagrange strain tensor

\[
E = E_M^{(2)} = \frac{1}{2} (U^2 - I) = \frac{1}{2} (C - I) = \frac{1}{2} (F^TF - I)
\]

is symmetric. It has the same orthonormal eigenvectors \( \hat{a}_\alpha \) as \( U \) and eigenvalues

\[
E_\alpha = \frac{1}{2} (\lambda_\alpha^2 - 1),
\]

which are referred to as the principal strains. The principal invariants of \( E \) are

\[
I_E \equiv \text{Tr } E = E_1 + E_2 = \frac{1}{2} (\lambda_1^2 + \lambda_2^2) - 1,
\]

\[
II_E \equiv \det E = E_1 E_2 = \frac{1}{4} (\lambda_1^2 - 1)(\lambda_2^2 - 1),
\]
both of which vanish when $E = 0$ and are symmetric in the indices 1 and 2. In the decomposition of $E$ into its base tensors $a_\alpha \otimes a^\beta$, the components of $E$ are

$$E_{\cdot \beta}^{\alpha} = \frac{1}{2} \left( F_i^{\alpha \beta} F_i^{\cdot \beta} - \delta^\alpha_{\cdot \beta} \right) = \frac{1}{2} \left( F_i^{\cdot \beta} a_i^{\cdot \alpha} - \delta^\alpha_{\cdot \beta} \right) = \frac{1}{2} \left( \frac{\partial (t^\alpha)}{\partial (\alpha a)} \frac{\partial (t^\beta)}{\partial (\beta a)} - \delta^\alpha_{\beta} \right).$$

Although $E^{\alpha \beta}$ is expressed in terms of the mixed-base $F_i^{\cdot \alpha}$, the sum over the index $i$ eliminates the dependence on the spatial base vectors $x_i$. In introductory texts on continuum elasticity, it is common to motivate the definition of $E$ by an alternative way that is physically more insightful, as shown below. Consider the square of the length of the material line element,

$$da^2 \equiv da \cdot da = da \cdot (Ida),$$

and the square of the length of the spatial line element,

$$dx^2 \equiv dx \cdot dx = (Fda) \cdot (Fda) = da \cdot (F^T F da).$$

Their difference gives the definition of $E$:

$$dx^2 - da^2 = da \cdot (F^T F da) - da \cdot (Ida) = da \cdot [(F^T F - I) da] \equiv da \cdot (2Eda).$$

### B.4 Hyperelastic materials

There are two classes of elastic materials in continuum mechanics: *Cauchy elastic materials* and *Green elastic materials* (or *hyperelastic materials*). Cauchy elasticity, unlike Green elasticity, is non-conservative, in that the stress is not assumed to be derivable from a scalar potential. Here, we are interested in Green elasticity and must, therefore, specialise by assuming that the surface under consideration is hyperelastic.

Green elasticity postulates the existence of a free energy area density $f$ of the hyperelastic surface, so that the work done on the surface to deform it from $S_A$ to $S_X$ is given by the difference in the free energy $F$ of the surface,

$$F[S_X, S_A] - F[S_A, S_A] = \oint_{S_A} f(A) dS_A,$$
where \( A \) is a point on the material surface \( S_A \), which we shall assume to be unstressed. 

\( f(A) \), commonly called the *strain-energy function* or *stored-energy function*, is the local free energy density associated with the deformation of the neighbourhood \( dS_A \) of \( A \) on \( S_A \) into the corresponding neighbourhood on \( S_X \). The local strain of the surface is fully characterised by \( F \); hence, we write

\[
f(A) = f[F(A)],
\]

*i.e.*, the local free energy density is a functional of the local deformation gradient tensor. At this point, the dependence \( f[F] \) is quite general; no assumptions about the material properties of the surface have yet been made beyond the obvious statement \( f[I] = 0 \), *i.e.*, that the unstressed surface corresponds to its reference free energy. We now explore two physical hypotheses and the constraints that they imply for the functional dependence \( f[F] \).

### B.5 Objectivity of hyperelastic materials

Firstly, the material properties of *all* materials must obey Eulerian objectivity according to the fundamental axiom of mechanics known as the *principle of material frame indifference* (or *principle of material objectivity*). This implies that the particular elastic response \( f[F] \) of the surface must be a spatial scalar field that remains invariant under *all* spatial rotations. One may also argue that spatial rotations preserve distance relations between nearby points and cannot, therefore, cost elastic energy. This hypothesis means that \( f[F] \) must satisfy the condition

\[
f[R_S F] = f[F] \quad (B.14)
\]

for any deformation \( F \) and any rotation \( R_S \), *i.e.*, whatever elastic energy density the deformation \( F \) entails, it is unchanged by a subsequent local rotation. Two comments:

1. The special case of Equation (B.14), where \( f[R_S] = f[I] = 0 \) for \( F = I \), is the requirement that pure rotations do not change the (vanishing) energy density.

2. The superficially similar statement \( f[F R_M] = f[F] \), where \( R_M \) is a material rotation, is generally *not* true unless the material is assumed to be isotropic (to be discussed in Section B.6). The reason is simply that, for an anisotropic material, local rotation prior to a local stretch will result in an inequivalent deformation.
For $f$ to remain invariant under the transformation $F \to F' = R_S F$, $f$ must depend on the bilinear combination $F^T F$, i.e., the unique solution of Equation (B.14) is

$$f[F] = f_1[F^T F] = f_1[U^T U] = f_1[U^2] = f_1[C] = f_2[E].$$

It is clear, then, that $f_1[F^T F'] = f_1[F^T F]$ by the orthogonality of $R_S$. Note that any functional $f_1$ of the Cauchy-Green tensor $C$ or, equivalently, any functional $f_2$ of the Green-Lagrange strain tensor $E$ will satisfy Equation (B.14). Since $f_1$ and $f_2$ are required to remain invariant under $R_S$, they are the spatial scalar fields of Table B.1. Now, the case of no deformation is equivalent to $F = C = I$ or $E = 0$. Therefore, $f[F = I] = f_1[C = I] = f_2[E = 0] = 0$. If, in addition, the unstressed surface is to correspond to an energy minimum, then linear terms in the Taylor expansion of $f_1$ about $C = I$ or $f_2$ about $E = 0$ must be excluded.

### B.6 Isotropy of hyperelastic materials

The second hypothesis is the assumption that the surface under consideration is isotropic. In general, the assumption of isotropy holds only for some materials. The free energy density of a hyperelastic material that is also isotropic must be invariant under a local rotation before any deformation is applied. In other words, $f$ must satisfy the additional condition

$$f[FR_M] = f[F]$$

for any rotation $R_M$ and any deformation $F$. For $F \to F' = FR_M$, the corresponding transformations of $C$ and $E$ are, respectively,

$$C \to C' = R_M^T CR_M,$$
$$E \to E' = R_M^T ER_M.$$

It follows that $f_1$ and $f_2$ must satisfy

$$f_1[R_M^T CR_M] = f_1[C], \quad (B.16)$$
$$f_2[R_M^T ER_M] = f_2[E]. \quad (B.17)$$

Note that Equation (B.16) contains the statement

$$f_1[B] = f_1[C], \quad (B.18)$$
which may be verified by replacing $\mathbf{R}_M^T$ in Equation (B.16) with $\mathbf{R}$ from the polar decomposition of $\mathbf{F}$. Equation (B.18) tells us that $f_1$ can only depend on the eigenvalues $\lambda_1^2$ and $\lambda_2^2$ common to $\mathbf{B}$ and $\mathbf{C}$. Furthermore, according to Equation (B.16), $f_1$ can only depend on symmetric combinations of $\lambda_1^2$ and $\lambda_2^2$, since such combinations are rotationally invariant. Two natural and independent symmetric combinations of $\lambda_1^2$ and $\lambda_2^2$ are the principal invariants $I_B = \lambda_1^2 + \lambda_2^2 = I_C$ and $II_B = \lambda_1^2\lambda_2^2 = II_C$. Therefore, we conclude that a general form of $f_1$ for an isotropic hyperelastic material is $f_1(I_C, II_C)$. It follows from the equality $f_1[C] = f_2[E]$ that a general form of $f_2$ is $f_2(I_E, II_E)$. Note that the variables $I_C$ and $II_C$ have no special significance, so any symmetric function $f_1'$ of $\lambda_1^2$ and $\lambda_2^2$, 

$$f_1' (\lambda_1^2, \lambda_2^2) = f_1' (\lambda_2^2, \lambda_1^2) = f_1(I_C, II_C),$$

is an acceptable energy density. Similarly, any symmetric function $f_2'$ of the principal strains $E_1$ and $E_2$, 

$$f_2' (E_1, E_2) = f_2' (E_2, E_1) = f_2(I_E, II_E),$$

is also an acceptable energy density.

In the linear elastic regime where the principal strains of $\mathbf{E}$ are weak, i.e., $|E_\alpha| \ll 1$, and if $S_A$ is the unstressed configuration, it can easily be shown that the Taylor expansion of $f_2'$ up to quadratic order in $E_\alpha$ (the harmonic approximation of $f_2'$) can be rewritten in the form

$$f_2' (E_1, E_2) = \frac{K_\alpha}{2} (E_1 + E_2)^2 + \frac{\mu}{2} (E_1 - E_2)^2 + \cdots \quad \text{(B.19)}$$

or, equivalently,

$$f_2 (I_E, II_E) = \frac{K_\alpha I_E^2}{2} + \frac{\mu}{2} (I_E^2 - 4II_E) + \cdots.$$
combinations
\[
\alpha = \lambda_1 \lambda_2 - 1 = \sqrt{I_C} - 1,
\]
\[
\beta = \frac{1}{2} \left[ \left( \frac{\lambda_1}{\lambda_2} - 1 \right) + \left( \frac{\lambda_2}{\lambda_1} - 1 \right) \right] = \frac{\lambda_1^2 + \lambda_2^2}{2\lambda_1 \lambda_2} - 1 = \frac{H_C}{2\sqrt{I_C}} - 1,
\]

where \(\alpha\) is the fractional change in area of a surface element under deformation and \(\beta\) is a symmetric combination of the two possible fractional changes in aspect ratio of that surface element. Taylor expansion of \(f\) in terms of the strain invariants \(\alpha\) and \(\beta\) then gives
\[
f(\alpha, \beta) = \frac{K_\alpha}{2} \left( \alpha^2 + a_3 \alpha^3 + a_4 \alpha^4 + \cdots \right) + \mu \left( \beta + b_1 \alpha \beta + b_2 \beta^2 + \cdots \right).
\]

Note that every term in \(f\) in Equation (B.20) is inherently non-linear elastic. At weak non-linear deformation, the dominant non-linear elastic terms are those involving \(\alpha^2\) and \(\beta\). As the deformation becomes stronger, terms of higher order than \(\alpha^2\) and \(\beta\) become non-negligible and, thus, must be taken into account as higher-order corrections to the weak non-linear deformation limit. These higher-order terms are important for expressing the kind of “hardening” of the elasticity typical of polymeric materials as the polymer chains approach full extension. The dimensionless coefficients \(a_3\), \(a_4\), \(b_1\), \(b_2\), and so forth, are dimensionless higher-order non-linear elastic moduli. It is legitimate to use identical \(K_\alpha\) and \(\mu\) in Equations (B.19) and (B.20) since
\[
\alpha = (E_1 + E_2) + O(E_\alpha^2),
\]
\[
\beta = \frac{1}{2} (E_1 - E_2)^2 + O(E_\alpha^3)
\]
when the deformation is very weak and effectively linear, so that
\[
f(\alpha, \beta) = \frac{K_\alpha}{2} \alpha^2 + \mu \beta + \cdots
\]
\[
= \frac{K_\mu}{2} (E_1 + E_2)^2 + \frac{\mu}{2} (E_1 - E_2)^2 + \cdots = f_2'(E_1, E_2).
\]
Appendix C

Polynomial Fit by the Method of Least Squares

C.1 Determination of fitting parameters

Suppose an independent variable, \( x \), is varied to give a set of measurements, \( y_i, i = 1, \ldots, N \), each with an uncertainty \( \sigma_i \). We wish to fit to the \( N \) data points \((x_i, y_i)\) a polynomial of degree \( n \),

\[
y(x) = a_1 + a_2x + \cdots + a_{n+1}x^n = \sum_{\mu=1}^{n+1} a_\mu x^{\mu-1},
\]

where \( n + 1 < N \). Assuming each \( y_i \) is drawn from a Gaussian distribution with mean \( y(x_i) \) and standard deviation \( \sigma_i \), the probability distribution for observing the set of \( y_i \)'s is thus the product of the individual Gaussian distributions:

\[
P(a_0, \ldots, a_n) \propto \prod_{i=1}^{N} \exp \left\{ -\frac{1}{2} \left[ \frac{y_i - y(x_i)}{\sigma_i} \right]^2 \right\} = \exp \left\{ -\frac{1}{2} \sum_{i=1}^{N} \left[ \frac{y_i - y(x_i)}{\sigma_i} \right]^2 \right\}. \tag{C.2}
\]

Equation (C.1) is a more likely fit of the data points the higher \( P \) is; therefore, \( P \) is to be maximised. The maximisation is equivalent to minimising the sum in the exponential of Equation (C.2),

\[
\chi^2(a_0, \ldots, a_n) \equiv \sum_{i=1}^{N} \left[ \frac{y_i - y(x_i)}{\sigma_i} \right]^2. \tag{C.3}
\]
From partial differentiation of $\chi^2$ with respect to each fitting parameter $a_v$ and setting the resultant derivative to zero, we obtain a set of $n + 1$ equations:

$$\sum_{i=1}^{N} \frac{y_i}{\sigma_i^2} x_i^{v-1} = \sum_{i=1}^{N} \left( \frac{x_i^{v-1}}{\sigma_i^2} \sum_{\mu=1}^{n} a_\mu x_i^{\mu-1} \right), \quad v = 1, 2, \ldots, n + 1,$$

or, in matrix notation,

$$\begin{bmatrix} \sum_{i=1}^{N} \frac{y_i}{\sigma_i^2} x_i^{n} \\ \sum_{i=1}^{N} \frac{y_i}{\sigma_i^2} x_i^{n-1} \\ \vdots \\ \sum_{i=1}^{N} \frac{y_i}{\sigma_i^2} x_i^{2} \end{bmatrix} = \begin{bmatrix} \sum_{i=1}^{N} \frac{1}{\sigma_i^2} x_i^{n} \\ \sum_{i=1}^{N} \frac{1}{\sigma_i^2} x_i^{n-1} \\ \vdots \\ \sum_{i=1}^{N} \frac{1}{\sigma_i^2} x_i^{2} \end{bmatrix} \begin{bmatrix} a_1 \\ a_2 \\ \vdots \\ a_{n+1} \end{bmatrix}. \quad (C.4)$$

For brevity, we write Equation (C.4) as

$$[\beta_v] = [\alpha_{\nu \mu}] [a_\mu],$$

where

$$\beta_v \equiv \sum_{i=1}^{N} \frac{y_i}{\sigma_i^2} x_i^{v-1} \quad \text{and} \quad \alpha_{\nu \mu} \equiv \sum_{i=1}^{N} \frac{x_i^{\nu-1}}{\sigma_i^2} x_i^{\mu-1}. \quad (C.5)$$

The unknown parameters $a_\mu$ are thus given by

$$[a_\mu] = [\alpha_{\nu \mu}]^{-1} [\beta_v] = [\epsilon_{\nu \mu}] [\beta_v], \quad \text{where} \quad [\epsilon_{\nu \mu}] = [\alpha_{\nu \mu}]^{-1}.$$

More explicitly,

$$a_\mu = \sum_{\nu=1}^{n} \left( \epsilon_{\nu \mu} \sum_{i=1}^{N} \frac{y_i x_i^{\nu-1}}{\sigma_i^2} \right)$$

by substituting in the expression for $\beta_v$ from Equation (C.5). Substituting these values of $a_\mu$ in Equation (C.3) will give the minimum $\chi^2, \chi^2_{\text{min}}$. For later use, we calculate the partial derivative of $a_\mu$ with respect to $y_j$:

$$\frac{\partial a_\mu}{\partial y_j} = \sum_{\nu=1}^{n} \epsilon_{\nu \mu} \frac{x_j^{\nu-1}}{\sigma_j^2}.$$

(C.6)
C.2 Uncertainties in fitting parameters

The \( n \) parameters \( a_\mu \) calculated in the previous Section have individual uncertainties \( \sigma_\mu \) due to the individual uncertainties \( \sigma_i \) of \( y_i \). These \( n \) uncertainties may be determined from the covariance matrix of \( a_\mu \), \([\xi_{\mu \nu}]\), the elements of which are defined by

\[
\xi_{\mu \nu} = \sum_{i=1}^{N} \frac{\partial a_\mu}{\partial y_i} \frac{\partial a_\nu}{\partial y_i} \sigma_i^2
\]

\[
= \sum_{i=1}^{N} \left( \sum_{\delta=1}^{n} \xi_{\mu \delta} \frac{x_{i \delta}^2}{\sigma_i^2} \right) \left( \sum_{\gamma=1}^{n} \xi_{\nu \gamma} \frac{x_{i \gamma}^2}{\sigma_i^2} \right) \sigma_i^2
\]

by Equation (C.6)

\[
= \sum_{\delta=1}^{n} \xi_{\mu \delta} \sum_{\gamma=1}^{n} \xi_{\nu \gamma} \left( \sum_{i=1}^{N} \frac{x_{i \delta}^2 x_{i \gamma}^2}{\sigma_i^2} \right)
\]

by Equation (C.5)

\[
= \sum_{\delta=1}^{n} \xi_{\mu \delta} \sum_{\gamma=1}^{n} \xi_{\nu \gamma} \alpha_{\delta \gamma}
\]

Thus, the uncertainties \( \sigma_\mu \) are given by the diagonal elements of \([\xi_{\mu \nu}]\):

\[
\sigma_\mu^2 = \xi_{\mu \mu}.
\]

C.3 Goodness of fit

Lastly, the goodness of the fitting function \( y(x_i) \) in modelling the data is to be tested. Knowing that \( \chi^2 \) is a chi-square random variable, its mean and standard deviation are expected [168, p. 661] to be \( d \) and \( \sqrt{2d} \), respectively, where \( d = N - n - 1 \) is the degree of freedom. The fit is then deemed a good one if \( \chi_{\text{min}}^2 \) is within one standard deviation of the expected mean. In addition, one can also calculate the probability \( Q \) that the \( \chi^2 \) value from another, random set of \( N \) data points will be greater than or equal to \( \chi_{\text{min}}^2 \) of the current set:

\[
Q(\chi^2 \geq \chi_{\text{min}}^2; d) = G(\chi_{\text{min}}^2; d, \frac{d}{2})
\]
APPENDIX C. POLYNOMIAL FIT BY THE METHOD OF LEAST SQUARES

where $G$ is the complementary incomplete gamma function defined by [168, p. 216]

$$G(x; a) = \frac{1}{\Gamma(a)} \int_{x}^{\infty} t^{a-1} e^{-t} \, dt, \quad a > 0,$$

with $\Gamma(a)$ being the gamma function.
Appendix D

Matlab File

The m-file `pxratios.m`:

```matlab
% Read the file which stores the vertex indices of each triangle
fid = fopen('rbc.indsl', 'r');
nt = fscanf(fid, '%d', 1);
ncol = fscanf(fid, '%d\n', 1);
t = zeros(nt, ncol);
for i = 1:nt,
    t(i,:) = fscanf(fid, '%d %d %d\n', [1,ncol]);
end
t = t + 1;
fclose(fid);

% Read the file which stores the coordinates of the vertices of the
% initial surface
fid = fopen('rbc.restl', 'r');
nv = fscanf(fid, '%d', 1);
dim = fscanf(fid, '%d\n', 1);
r0 = zeros(nv, dim);
for i = 1:nv,
    r0(i,:) = fscanf(fid, '%e %e %e\n', [1, dim]);
end
```
APPENDIX D. MATLAB FILE

fclose(fid);

% Read the file which stores the coordinates of the vertices of the
% instantaneous surface
fid = fopen('rbc.coords', 'r');
nv = fscanf(fid, '%d', 1);
dim = fscanf(fid, '%d
f', 1);
r = zeros(nv, dim);
for i = 1:nv,
    r(i,:) = fscanf(fid, '%e %e %e
l', [1, dim]);
end
fclose(fid);

% Initialize variables for calculating the membrane skeleton elastic
% energies
area_energy = zeros(nt,1); area_energy_density = zeros(nt,1);
shear_energy = zeros(nt,1); shear_energy_density = zeros(nt,1);
lxly = zeros(nt,1);
lx_sq = zeros(nt,1);
tan_sq_phi = zeros(nt,1);
da0 = zeros(nt,1);
alpha = zeros(nt,1);
beta = zeros(nt,1);
lambda1 = zeros(nt,1);
lambda2 = zeros(nt,1);

% Prompt user for values of elastic moduli
K_alpha = input('Stretch modulus: ');
a3 = input(' a3: ');
a4 = input(' a4: ');
mu = input(' Shear modulus: ');
b1 = input(' b1: ');
b2 = input(' b2: ');

% Calculate the membrane skeleton elastic energies
for i = 1:nt,

% The 2 edge vectors of each initial triangle and their cross product
x0_1 = r0(t(i,2,:)) - r0(t(i,1,:)); % first edge vector
x0_2 = r0(t(i,3,:)) - r0(t(i,1,:)); % second edge vector
n0 = cross(x0_1, x0_2); % edge vector cross product

norm_n0 = norm(n0); % magnitude of edge vector cross product
norm_x0_1_sq = norm(x0_1)^2; % magnitude of first edge vector squared
dotprod_x0 = sum(x0_1.*x0_2); % magnitude of edge vector dot product

% The 2 edge vectors of each instantaneous triangle and their cross product
x1 = r(t(i,2,:)) - r(t(i,1,:)); % first edge vector
x2 = r(t(i,3,:)) - r(t(i,1,:)); % second edge vector
n = cross(x1, x2); % edge vector cross product

norm_n = norm(n); % magnitude of edge vector cross product
norm_x1_sq = norm(x1)^2; % magnitude of first edge vector squared
dotprod_x = sum(x1.*x2); % magnitude of edge vector dot product

% Assign values to frequently-used variables in the calculation of
% alpha and beta
lxly(i) = norm_n/norm_n0;
lx_sq(i) = norm_x1_sq/norm_x0_1_sq;
P = (dotprod_x0/norm_x0_1_sq - dotprod_x/norm_x1_sq)^2*norm_x1_sq;
Q = norm(x2 - (dotprod_x/norm_x1_sq)*x1)^2;
tan_sq_phi(i) = P/Q;
dA0(i) = 0.5*norm_n0;

%Calculate the two fields of local strain invariants, alpha and beta
alpha(i) = lxly(i) - 1;
beta(i) = 0.5*(lx_sq(i)/lxly(i) + ...
lxly(i)/lx_sq(i)*(1 + tan_sq_phi(i))) - 1;

%Calculate the stretch and shear energies of each triangle
area_energy_density(i) = 0.5*K_alpha*(alpha(i)^2 + a3*alpha(i)^3 + ...
a4*alpha(i)^4);
area_energy(i) = area_energy_density(i)*dA0(i);
shear_energy_density(i) = mu*(beta(i) + b1*alpha(i)*beta(i) + ...
b2*beta(i)^2);
shear_energy(i) = shear_energy_density(i)*dA0(i);

%Calculate the two fields of local principal stretches, lambda1 and lambda2
lambda1(i) = sqrt((beta(i) + 1 - sqrt((beta(i) + 1)^2 - 1))*...
(alpha(i) + 1));
lambda2(i) = sqrt((beta(i) + 1 + sqrt((beta(i) + 1)^2 - 1))*...
(alpha(i) + 1));
end

%Calculate and display the total stretch, shear and elastic energies
tot_area_energy = sum(area_energy);
tot_shear_energy = sum(shear_energy);
tot_energy = tot_area_energy + tot_shear_energy;
disp(['Total stretch energy = ', num2str(tot_area_energy)]);
disp(['Total shear energy = ', num2str(tot_shear_energy)]);
disp(['Total elastic energy = ', num2str(tot_energy)]);
APPENDIX D. MATLAB FILE

% Display the raw instantaneous surface

x = r(:,1);
y = r(:,2);
z = r(:,3);

figure(1), clf
trimesh(t,x,y,z,'EdgeColor','red');
axis image, rotate3d on
xlabel('x (\mu m)'), ylabel('y (\mu m)'), zlabel('z (\mu m)')
title('RBC shape')

% Centre the instantaneous surface at the origin through the diagonalization of the moment of inertia tensor

xcm = sum(x)/nv; xnew = x - xcm;
ycm = sum(y)/nv; ynew = y - ycm;
zcm = sum(z)/nv; znew = z - zcm;

Ixx = sum(ynew.^2 + znew.^2);
Iyy = sum(znew.^2 + xnew.^2);
Izz = sum(xnew.^2 + ynew.^2);
Ixy = -sum(xnew.*ynew);
Iyz = -sum(ynew.*znew);
Izx = -sum(znew.*xnew);

I = [Ixx Ixy Izx;
     Ixy Iyy Iyz;
     Izx Iyz Izz];

[V,Ip] = eig(I);
v1 = V(:,1)./norm(V(:,1));
v2 = V(:,2)./norm(V(:,2));
v3 = V(:,3)./norm(V(:,3));
Ip = Ip./max(max(Ip))
rnew = [xnew ynew znew]*[v1 v2 v3];
xnew = rnew(:,1);
ynew = rnew(:,2);
znew = rnew(:,3);

% ............................
% Colour-coded display of alpha and beta
% ............................
figure(3), clf, colormap('default')
subplot(121)
trisurf(t,xnew,ynew,znew,alpha);
colorbar('horiz');
axis image, rotate3d on, hidden off
xlabel('x (\mum)'), ylabel('y (\mum)'), zlabel('z (\mum)')
title('RBC shape: field of \alpha')
rotate3d on

subplot(122)
trisurf(t,xnew,ynew,znew,beta);
colorbar('horiz');
axis image, rotate3d on, hidden off
xlabel('x (\mum)'), ylabel('y (\mum)'), zlabel('z (\mum)')
title('RBC shape: field of \beta')
rotate3d on

% ............................
% Colour-coded display of the energy densities
% ............................
figure(4), clf
subplot(121)
trisurf(t,xnew,ynew,znew,...
  shear_energy_density./{(area_energy_density+shear_energy_density)});
colorbar('horiz')
axis image, rotate3d on, hidden off
xlabel('x (\mum)'), ylabel('y (\mum)'), zlabel('z (\mum)')
title('RBC shape: fractional shear energy density')
rotate3d on
APPENDIX D. MATLAB FILE

```matlab
subplot(1,2,1)
trisurf(t,xnew,ynew,znew,area_energy_density + shear_energy_density);
colorbar('horizontal')
axis image, rotate3d on, hidden off
xlabel('x (\mu m)'), ylabel('y (\mu m)'), zlabel('z (\mu m)')
title('RBC shape: total elastic energy density')
rotate3d on
```
Appendix E

Test 4 Supplementary Data

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<tr>
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<th>y</th>
<th>z</th>
</tr>
</thead>
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<td>3.2392113818130391e-01</td>
</tr>
</tbody>
</table>

Table E.1: The instantaneous vertex coordinates (x, y and z from left to right) at the end of the second run of Test 4, with the vertices implicitly numbered (starting at 0) from top to bottom.
Table E.2: The initial vertex coordinates \((x, y, z)\) from left to right of Test 4, with the vertices implicitly numbered (starting at 0) from top to bottom.

<table>
<thead>
<tr>
<th>x</th>
<th>y</th>
<th>z</th>
<th>x</th>
<th>y</th>
<th>z</th>
</tr>
</thead>
<tbody>
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<td>5.257311211913359e-01</td>
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<td>5.257311211913359e-01</td>
<td>0.0000000000000000e+00</td>
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</tr>
<tr>
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</tr>
</tbody>
</table>
Table E.3: The indices of the vertices of the triangles in Test 4 (left to right corresponds to anti-clockwise order), with the triangles implicitly numbered (starting at 0) from top to bottom.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<tr>
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<tr>
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Bibliography


BIBLIOGRAPHY


