Impact of cellular and medium properties on depletion-mediated red cell aggregation

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NANYANG
TECHNOLOGICAL
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**IMPACT OF CELLULAR AND MEDIUM
PROPERTIES ON DEPLETION-MEDIATED RED
CELL AGGREGATION**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2011

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CELL AGGREGATION**

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School of Chemical and Biomedical Engineering

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Table of Contents

ACKNOWLEDGEMENT.....	I
LIST OF PUBLISHED WORKS	II
TABLE OF CONTENTS	III
LIST OF TABLES	VI
LIST OF FIGURES.....	VII
LIST OF SYMBOLS	X
ABSTRACT	1
CHAPTER 1 INTRODUCTION AND BACKGROUND	2
1.1 RED CELL AGGREGATION; AN OVERVIEW	2
1.2 EFFECTS OF AGGREGATION ON BLOOD FLOW	5
1.3 MECHANISMS OF AGGREGATION.....	8
1.4 METHODS OF MEASURING AGGREGATION	9
1.4.1 <i>Light transmission/reflection</i>	9
1.4.2 <i>Ultrasound backscattering</i>	9
1.4.3 <i>Microscopic observation</i>	9
1.4.4 <i>Measurement of erythrocyte sedimentation rate</i>	10
1.4.5 <i>Measurement of low shear viscosity</i>	10
1.5 AGGREGABILITY	11
1.6 EFFECT OF PATHOLOGICAL CONDITIONS IN RBC AGGREGATION	13
1.7 HYPOTHESES AND SPECIFIC AIMS	14
1.8 STRUCTURE OF THE THESIS	15
CHAPTER 2 THEORETICAL CONSIDERATIONS.....	16
2.1 INTRODUCTION.....	16
2.2 DEPLETION INTERACTIONS	17
2.3 CALCULATIONS OF THE PENETRATION DEPTH	21
2.4 STERIC INTERACTIONS	22
2.5 ELECTROSTATIC INTERACTIONS	24
2.5.1 <i>Double layer and mobile charge distribution</i>	24
2.5.2 <i>Electrostatic potential</i>	26
2.5.3 <i>Electrostatic interaction energy</i>	29
2.6 ELECTROPHORETIC MOBILITY	31
2.6.1 <i>Electric potential</i>	34
2.6.2 <i>Velocity profile</i>	35
2.6.3 <i>Electrophoretic mobility ratio</i>	38
2.7 CONCLUDING REMARKS	39
CHAPTER 3 CAN DEPLETION PLAY A ROLE? EXPERIMENTAL APPROACH.	40

3.1	INTRODUCTION.....	40
3.2	MATERIAL AND METHOD.....	42
3.2.1	<i>Sample preparation</i>	42
3.2.2	<i>Miscellaneous techniques</i>	42
3.3	THEORETICAL CONSIDERATIONS	44
3.3.1	<i>Viscosity-corrected mobility</i>	44
3.4	RESULTS.....	46
3.4.1	<i>Depletion layer thickness versus Debye-Hückel length</i>	46
3.4.2	<i>Experimental and theoretical mobility ratios</i>	49
3.5	DISCUSSION.....	54
CHAPTER 4 IMPACT OF CELLULAR PROPERTIES ON RED CELL-CELL AFFINITY IN PLASMA-LIKE MEDIUM- THEORETICAL APPROACH CONSIDERING ELECTROSTATIC AND DEPLETION INTERACTIONS WITH UNIFORM GLYCOCALYX STRUCTURE.....		56
4.1	INTRODUCTION.....	56
4.2	MODEL DESCRIPTION.....	58
4.2.1	<i>Assumptions</i>	58
4.2.2	<i>Polymer penetration into the glycocalyx</i>	59
4.2.3	<i>Physiochemical properties of cells and suspensions</i>	60
4.2.4	<i>Total interaction energies</i>	61
4.2.4.1	<i>Electrostatic energy</i>	61
4.2.4.2	<i>Depletion energy</i>	63
4.2.4.3	<i>Total interaction energy</i>	63
4.3	RESULTS.....	64
4.3.1	<i>Initial considerations</i>	64
4.3.2	<i>Impact of glycocalyx volume fraction on cell-cell affinity</i>	65
4.3.3	<i>Impact of glycocalyx thickness on cell-cell affinity</i>	67
4.3.4	<i>Red blood cell affinity</i>	67
4.4	DISCUSSION.....	70
CHAPTER 5 IMPACT OF CELLULAR PROPERTIES ON RED CELL-CELL AFFINITY IN PLASMA-LIKE MEDIUM- THEORETICAL APPROACH CONSIDERING ELECTROSTATIC, DEPLETION AND STERIC INTERACTIONS WITH VARIOUS GLYCOCALYX STRUCTURES		71
5.1	INTRODUCTION.....	71
5.2	MODEL DESCRIPTION.....	72
5.2.1	<i>Glycocalyx Structure</i>	72
5.2.2	<i>Glycocalyx volume fraction and steric interactions</i>	75
5.2.2.1	<i>Uniform segment distribution</i>	76
5.2.2.2	<i>Linear segment distribution</i>	77
5.2.2.3	<i>Pseudo-tail segment distribution</i>	77
5.2.3	<i>Penetration depth calculation</i>	78
5.2.4	<i>Electrostatic energy</i>	80
5.2.4.1	<i>Linear charge distribution profile:</i>	81
5.2.4.2	<i>Pseudo-tail charge distribution profile:</i>	82

5.3	RESULTS.....	84
5.3.1	<i>Electrostatic interactions</i>	84
5.3.1.1	Impact of charge distribution on the electrostatic potential	84
5.3.1.2	Impact of ionic strength on the electrostatic potential	85
5.3.1.3	Impact of ionic strength and charge distribution on the electrostatic energy...	86
5.3.2	<i>Steric interactions</i>	87
5.3.2.1	Impact of glycocalyx segment distribution on the steric interactions.....	87
5.3.2.2	Impact of glycocalyx volume fraction on the steric interactions	88
5.3.3	<i>Penetration depth</i>	90
5.3.4	<i>Depletion interactions</i>	94
5.3.5	<i>Total interaction energy</i>	96
5.3.6	<i>Validation of the results with experimental data</i>	102
5.3.6.1	Buxbaum's data	102
5.3.6.2	Chien's data	111
5.3.6.3	Neu's data	117
5.4	DISCUSSION.....	120
CHAPTER 6 CONCLUSION AND FUTURE DIRECTIONS		122
REFERENCES		124

List of Tables

TABLE 3-1 INTRINSIC VISCOSITIES FOR VARIOUS FRACTIONS OF DEXTRAN	45
TABLE 3-2 PHYSIOCHEMICAL PROPERTIES OF DEXTRAN	47
TABLE 4-1 ESTIMATED VALUES FOR RADIUS OF GYRATION AND SECOND VIRIAL COEFFICIENT FOR DIFFERENT MOLECULAR WEIGHT DEXTRANS	61
TABLE 5-1 NAME AND NUMBER OF GLYCOPROTEINS PER CELL [147]	73
TABLE 5-2 COMPARISON WITH EXPERIMENTAL DATA, DEXTRAN 70kDa, UNIFORM	107
TABLE 5-3 COMPARISON WITH EXPERIMENTAL DATA, DEXTRAN 70kDa, LINEAR	107
TABLE 5-4 COMPARISON WITH EXPERIMENTAL DATA, DEXTRAN 70kDa, PSEUDO-TAIL	108
TABLE 5-5 COMPARISON WITH EXPERIMENTAL DATA, DEXTRAN 150kDa, UNIFORM	108
TABLE 5-6 COMPARISON WITH EXPERIMENTAL DATA, DEXTRAN 150kDa, LINEAR	109
TABLE 5-7 COMPARISON WITH EXPERIMENTAL DATA, DEXTRAN 150kDa, PSEUDO-TAIL	109
TABLE 5-8 SUMMARY OF COMPARISON	110
TABLE 5-9 COMPARISON PARAMETERS FOR DEXTRAN 80kDa	115
TABLE 5-10 COMPARISON PARAMETERS FOR DEXTRAN 150kDa	116
TABLE 5-11 COMPARISON PARAMETERS FOR DEXTRAN 500kDa	116

List of Figures

FIGURE 2-1 SCHEMATIC REPRESENTATION OF THE DEPLETION PHENOMENA; COLLOIDAL PARTICLES ARE PRESENTED IN BLUE AND NON-ADSORBING POLYMERS IN GREEN. TOP FRAME: COLLOIDAL PARTICLES WITH THEIR POLYMER-DEPLETED REGIONS (DASHED LINES). MIDDLE FRAME: APPROACHING PARTICLES. BOTTOM FRAME: POLYMER-DEPLETED ZONES OF THE PARTICLES ARE OVERLAPPED, RESULTING IN DEPLETION ATTRACTIVE FORCE BETWEEN PARTICLES	17
FIGURE 2-2 ILLUSTRATION OF THE DEPLETED LAYER NEAR THE CELL SURFACE; δ : GLYCOCALYX THICKNESS, p : PENETRATION DEPTH OF THE FREE POLYMER INSIDE THE GLYCOCALYX STRUCTURE, Δ : DEPLETION LAYER THICKNESS. FREE POLYMER CONCENTRATION CHANGES FROM ZERO AT $\delta - p$ TO BULK POLYMER CONCENTRATION AT $\delta - p + \Delta$	18
FIGURE 2-3 (A) SCHEMATIC ILLUSTRATION OF ION DISTRIBUTION AROUND NEGATIVE CELL SURFACE, (B) POTENTIAL VARIATION AS A FUNCTION OF DISTANCE FROM CELL SURFACE	25
FIGURE 2-4 MODEL OF TWO CELLS; CELLS ARE LOCATED AT DISTANCE $2d$ FROM EACH OTHER. GLYCOCALYX LAYER OF THE CELLS HAVE THE FIXED CHARGE DENSITY OF ρ_{fix1} AND ρ_{fix2} WHICH IS ZERO OUTSIDE OF THE GLYCOCALYX LAYER. ρ_{mx} IS THE CHARGE DENSITY DUE TO THE FREE IONS IN THE ELECTROLYTE CALCULATED BY EQUATION (2.5-2)	27
FIGURE 2-5 SCHEMATIC STRUCTURE OF THE FLOW CHANNEL WITH THICKNESS $2d$; ATTACHED TO THE WALL IS A GLYCOCALYX-LIKE STRUCTURE WITH THICKNESS δ WHERE $\delta \ll 2d$. FLUID FLOWS IN THE Y- DIRECTION DUE TO PRESSURE OR POTENTIAL GRADIENT ACROSS THE LENGTH OF THE CHANNEL. VELOCITY PROFILE IN THE Y-DIRECTION IS A FUNCTION OF DISTANCE FROM CHANNEL WALLS	32
FIGURE 3-1 DEPLETION LAYER THICKNESS AS A FUNCTION OF POLYMER MASS AND CONCENTRATION (FROM [135] WITH PERMISSION).....	46
FIGURE 3-2 FREE POLYMER CONCENTRATION PROFILE FOR DIFFERENT FRACTIONS OF DEXTRAN AT BULK CONCENTRATION OF 0.9G/DL (FROM [135] WITH PERMISSION).....	48
FIGURE 3-3 CALCULATED POLYMER CONCENTRATION AT THE EDGE OF GLYCOCALYX AS A FUNCTION OF BULK POLYMER CONCENTRATION FOR DIFFERENT DEXTRAN FRACTIONS (FROM [135] WITH PERMISSION)	49
FIGURE 3-4 MEASURED ELECTROPHORETIC MOBILITY VALUES (LEFT AXIS) AND SAMPLE VISCOSITY (RIGHT AXIS) AS A FUNCTION OF DEXTRAN MOLECULAR MASS (FROM [135] WITH PERMISSION) ...	50
FIGURE 3-5 EXPERIMENTAL (SYMBOLS) AND THEORETICAL (SOLID LINES) OF MOBILITY RATIO AS A FUNCTION OF POLYMER SIZE AND CONCENTRATION FOR (A) $I = 15 \text{ mM}$ AND (B) $I = 5 \text{ mM}$ (FROM [135] WITH PERMISSION).....	52
FIGURE 4-1 SCHEMATIC ILLUSTRATION OF THE CONCENTRATION PROFILES FOR A SYSTEM OF TWO IDENTICAL CELLS; UNIFORM CONCENTRATION PROFILE ϕ_a HAS BEEN CHOSEN FOR THE GLYCOCALYX WHILE LINEAR PROFILE $\phi_{fx} = \phi_{bx} + p - \delta\Delta$ HAS BEEN SELECTED FOR THE FREE POLYMER- CELLS ARE LOCATED AT A DISTANCE $2d$. SUBSCRIPTS A, B AND F RELATE TO THE ATTACHED, BULK AND FREE POLYMER RESPECTIVELY.	59
FIGURE 4-2 CHARGE DISTRIBUTION FOR DIFFERENT CELL-CELL DISTANCES: (A) $2d > 2\delta$, (B) $2d < 2\delta$. IN THE $[2d - \Delta, \Delta]$ REGION, THE CHARGE DENSITY IS TWICE OF THE CELL'S CHARGE DENSITY DUE TO THE OVERLAP OF CHARGED LAYERS.....	62
FIGURE 4-3 (A) CALCULATED DEPENDENCE OF INTERACTION ENERGY ON MOLECULAR MASS AND CONCENTRATION OF DEXTRAN, (B) CELL-CELL DISTANCE AS A FUNCTION OF POLYMER WEIGHT AND CONCENTRATION (FROM [143] WITH PERMISSION).....	64
FIGURE 4-4 (A) INTERACTION ENERGY AS A FUNCTION OF MOLECULAR MASS AND GLYCOCALYX VOLUME FRACTION, (B) CALCULATED PENETRATION DEPTH AS A FUNCTION OF POLYMER MOLECULAR MASS AND GLYCOCALYX VOLUME FRACTION. BULK POLYMER CONCENTRATION IS CONSIDERED	

CONSTANT AND EQUAL TO 1gr/dl AND GLYCOCALYX THICKNESS IS 5nm (FROM [143] WITH PERMISSION)	66
FIGURE 4-5 INTERACTION ENERGY AS A FUNCTION OF MOLECULAR MASS AND GLYCOCALYX THICKNESS (FROM [143] WITH PERMISSION)	67
FIGURE 4-6 LINES OF CONSTANT TOTAL INTERACTION ENERGY ($\mu\text{J/m}^2$) FOR GLYCOCALYX THICKNESS OF 6nm $\phi a = 0$, $\sigma = 0.036\text{ C/m}^2$; MINIMUM ENERGY OCCURS AT MOLECULAR MASS OF 312 KDa AND CONCENTRATION OF 3.16 G/DL.....	68
FIGURE 4-7 LINES OF CONSTANT TOTAL INTERACTION ENERGY ($\mu\text{J/m}^2$) FOR GLYCOCALYX THICKNESS OF 5nm $\phi a = 0$, $\sigma = 0.036\text{ C/m}^2$; MINIMUM ENERGY OCCURS AT MOLECULAR MASS OF 216 KDa AND CONCENTRATION OF 3.6 G/DL.....	68
FIGURE 4-8 LINES OF CONSTANT TOTAL INTERACTION ENERGY (mJ/m^2) FOR GLYCOCALYX THICKNESS OF 4nm $\phi a = 0$, $\sigma = 0.036\text{ C/m}^2$; MINIMUM ENERGY OCCURS AT MOLECULAR MASS OF 149 KDa AND CONCENTRATION OF 4.11 G/DL	69
FIGURE 5-1- SCHEMATIC REPRESENTATION OF RED CELL MEMBRANE [146].....	73
FIGURE 5-2 SCHEMATIC REPRESENTATION OF GLYCOCALYX STRUCTURE COMPOSING OF SMALL GLUCOSE SEGMENTS; X-AXIS IS NORMAL TO THE CELL SURFACE WITH ITS ORIGIN LOCATED AT OUTER LAYER OF THE LIPID BILAYER	74
FIGURE 5-3 SEGMENT DISTRIBUTION AS A FUNCTION OF DISTANCE FROM THE CELL SURFACE FOR UNIFORM, LINEAR AND PSEUDO-TAIL PROFILES	75
FIGURE 5-4 SCHEMATIC REPRESENTATION OF UNIFORM SEGMENT DISTRIBUTION- IN THE PENETRATION ZONE ($2d - \delta$ TO δ) THE SEGMENT DENSITY OF TWO CELLS, ARE ADDED TOGETHER.....	76
FIGURE 5-5 FREE AND ATTACHED POLYMER PROFILES, SUBSCRIPTS A, B AND F ARE RELATED TO THE ATTACHED, BULK AND FREE POLYMERS, RESPECTIVELY.....	79
FIGURE 5-6 TRAPEZOIDAL INTEGRATION; THE INTEGRAL IS ESTIMATED BY THE SUM OF THE AREA OF THE THIN TRAPEZOIDS COVERING THE SURFACE AREA UNDER THE CURVE $F(X)$	82
FIGURE 5-7 ELECTROSTATIC POTENTIAL AS A FUNCTION OF CELL-CELL DISTANCE FOR VARIOUS CHARGE DISTRIBUTIONS; THREE CELL-CELL DISTANCE HAVE BEEN CONSIDERED HEREIN: $2d < 2\delta$ (7NM), $2d = 2\delta$ (10NM) AND $2d > 2\delta$ (15NM)	84
FIGURE 5-8 IMPACT OF VARIOUS IONIC STRENGTHS ON THE ELECTROSTATIC POTENTIAL DISTRIBUTION FOR CELL-CELL DISTANCE OF 15 NM	85
FIGURE 5-9 ELECTROSTATIC ENERGY AS A FUNCTION OF CELL-CELL DISTANCE FOR VARIOUS IONIC STRENGTHS AND CHARGE DISTRIBUTIONS FOR CELL-CELL DISTANCES LESS THAN 15NM.....	86
FIGURE 5-10 STERIC ENERGY AS A FUNCTION OF CELL-CELL DISTANCE FOR VARIOUS DISTRIBUTIONS...	88
FIGURE 5-11 STERIC ENERGY AS A FUNCTION OF GLYCOCALYX VOLUME FRACTION; IMPACT OF VARIOUS SEGMENT DISTRIBUTIONS AND FLORY INTERACTION PARAMETERS ON THE STERIC INTERACTION IS PRESENTED FOR CELL-CELL DISTANCE OF 9.5 NM	89
FIGURE 5-12 PENETRATION DEPTH VS. POLYMER CONCENTRATION; IMPACT OF SEGMENT DISTRIBUTION FOR 500kDa DEXTRAN, (A) $\phi a = 0.077$, (B) $\phi a = 0.004$	91
FIGURE 5-13 PENETRATION DEPTH VS. POLYMER MASS FOR $\phi a = 0.004$ AND $c = 1\text{g/dl}$	92
FIGURE 5-14 PENETRATION DEPTH VS. VOLUME FRACTION; IMPACT OF SEGMENT DISTRIBUTION FOR $M = 500\text{kDa}$ AND $c = 1\text{g/dl}$	93
FIGURE 5-15 DEPLETION ENERGY AS A FUNCTION OF CELL-CELL DISTANCE FOR 1G/DL OF 500KDa DEXTRAN (A) $\phi a = 0.004$ AND (B) $\phi a = 0.077$	95
FIGURE 5-16 INTERACTION ENERGY VS. CELL-CELL DISTANCE FOR 1G/DL OF DEXTRAN 500KDa; THE IMPACT OF FLORY INTERACTION PARAMETER (0.4 AND 0.48) AND GLYCOCALYX STRUCTURE ON THE INTERACTION ENERGY IS PRESENTED.	96
FIGURE 5-17 INTERACTION ENERGY AS A FUNCTION OF POLYMER CONCENTRATION FOR UNIFORM DISTRIBUTION FOR DEXTRAN 40, 70, 250 AND 500KDa (A) $\chi = 0.4$, $\phi a = 0.004$, (B) $\chi = 0.48$, $\phi a = 0.004$, (C) $\chi = 0.4$, $\phi a = 0.077$ (D) CELL-CELL DISTANCE AT WHICH MINIMUM INTERACTION ENERGY IS FOUND FOR $\chi = 0.4$, $\phi a = 0.077$	98

FIGURE 5-18 INTERACTION ENERGY AS A FUNCTION OF POLYMER CONCENTRATION FOR LINEAR DISTRIBUTION FOR DEXTRAN 40, 70, 250 AND 500kDa (A) $\chi = 0.4, \phi a = 0.004$, (B) $\chi = 0.48, \phi a = 0.004$, (C) $\chi = 0.4, \phi a = 0.077$ (D) CELL-CELL DISTANCE AT WHICH MINIMUM INTERACTION ENERGY IS FOUND FOR $\chi = 0.4, \phi a = 0.077$	99
FIGURE 5-19 (A) INTERACTION ENERGY AS A FUNCTION OF POLYMER CONCENTRATION FOR PSEUDO-TAIL DISTRIBUTION FOR $\chi = 0.48, \phi a = 0.077$ AND DEXTRAN 40, 70, 250 AND 500 kDa, (B) CELL-CELL DISTANCE AT WHICH MINIMUM INTERACTION ENERGY IS FOUND.....	101
FIGURE 5-20 CALCULATED RESULTS (SOLID LINES), EXPERIMENTAL DATA BY BUXBAUM <i>ET AL.</i> (POINTS) AND QUADRATIC FITTING OF EXPERIMENTAL RESULT (DASHED LINE) FOR (A) DEXTRAN 70kDa AND (B) DEXTRAN 150kDa	103
FIGURE 5-21 FITTED DATA FOR DEXTRAN 70kDa; EXPERIMENTAL DATA (DASHED LINE), $\sigma = 0.02C/m^2$ (DOTTED LINE), $\sigma = 0.036C/m^2$ (SOLID LINE), $\delta = 4\text{NM}$ (BLUE LINE), $\delta = 5\text{NM}$ (GREEN LINE), $\delta = 6\text{NM}$ (RED LINE), (A) UNIFORM, (B) LINEAR, (C) PSEUDO-TAIL	105
FIGURE 5-22 FITTED DATA FOR DEXTRAN 150kDa; EXPERIMENTAL DATA (DASHED LINE), $\sigma = 0.02C/m^2$ (DOTTED LINE), $\sigma = 0.036C/m^2$ (SOLID LINE), $\delta = 4\text{NM}$ (BLUE LINE), $\delta = 5\text{NM}$ (GREEN LINE), $\delta = 6\text{NM}$ (RED LINE), (A) UNIFORM, (B) LINEAR, (C) PSEUDO-TAIL	106
FIGURE 5-23 CALCULATED AND EXPERIMENTAL RESULTS FOR AGGREGATION ENERGY FOR SAMPLES CONTAINING DEXTRAN 80kDa. (A) UNIFORM, (B) LINEAR, (C) PSEUDO-TAIL AND (D) EXPERIMENTAL RESULTS BY CHIEN <i>ET AL</i> [152] WITH PERMISSION	112
FIGURE 5-24 CALCULATED AND EXPERIMENTAL RESULTS FOR AGGREGATION ENERGY FOR SAMPLES CONTAINING DEXTRAN 150kDa. (A) UNIFORM, (B) LINEAR, (C) PSEUDO-TAIL AND (D) EXPERIMENTAL RESULTS BY CHIEN <i>ET AL</i> [152] WITH PERMISSION	113
FIGURE 5-25 CALCULATED AND EXPERIMENTAL RESULTS FOR AGGREGATION ENERGY FOR SAMPLES CONTAINING DEXTRAN 500kDa. (A) UNIFORM, (B) LINEAR, (C) PSEUDO-TAIL AND (D) EXPERIMENTAL RESULTS BY CHIEN <i>ET AL</i> [152] WITH PERMISSION	114
FIGURE 5-26 (A) EXPERIMENTAL VALUES OF AGGREGATION INDEX FOR HIGH MOLECULAR MASS DEXTRAN 500kDa, 2MDa AND 28 MDa BY NEU <i>ET AL</i> [126], (B) CALCULATED INTERACTION ENERGY FOR THE SAME MOLECULAR MASS DEXTRANS	118

List of Symbols

δ	Glycocalyx thickness
ε	Relative permittivity of the medium
ε_0	Permittivity of the vacuum
ζ	Zeta potential
$[\eta]$	Intrinsic viscosity
κ	Debye-Hückel parameter
κ^{-1}	Debye-Hückel length
μ	Fluid viscosity
μ_0	Viscosity of polymer-free solution
μ_b	Bulk viscosity
μ_δ	Viscosity of fluid in the glycocalyx layer
μ_{sp}	Specific viscosity
ρ_a	Attached layer segment density
$\hat{\rho}_d$	Normalized segment density
$\hat{\rho}'_d$	Mirror image of $\hat{\rho}_d$
ρ_{fix}	Fixed charge density
ρ_m	Mobile charge density
ρ_T	Total charge density
σ	Surface charge density
$\bar{\varphi}_a$	Average attached polymer (glycocalyx) volume fraction
φ_a	Attached polymer (glycocalyx) volume fraction
φ_b	Bulk polymer volume fraction
φ_f	Free polymer volume fraction
χ	Flory interaction parameter
ψ	Electric potential

Δ_0	Depletion layer thickness for very dilute polymer solution
Δ	Depletion layer thickness
Π	Osmotic pressure
ψ	Electric potential
a	Sphere radius
a	Friction factor
a_p	Friction factor in the presence of polymer
b	Electrophoretic mobility
b_0	Electrophoretic mobility in polymer-free solution
b_p	Electrophoretic mobility in polymer solution
$\tilde{b}_p$	Viscosity-corrected mobility in polymer solution
$c(x)$	Polymer concentration
c_b	Bulk polymer concentration
d	Half of cell-cell distance
e	Electron charge
f	Effective Stokes friction coefficient
i	Number of polymer segments per chain
k_B	Boltzmann constant
k'	Huggins constant
l	Length of flow chamber
n_i	Concentration of ion i
p	Penetration depth
r_{seg}	Radius of dextran segment
u_e	Electro-osmotic velocity
$u(x)$	Fluid velocity in the y direction as a function of x
v	Number of polymer chains per unit area of cell surface
v_1	Molar volume of solvent

v_s	Volume of polymer segment
w	Width of the flow chamber
z_i	Valence of ion i
B	Second virial coefficient
E	Electrostatic free energy
E	Electric field
G_{mix}	Mixing free energy
I	Ionic strength
I	Electric current
J	Volume flow
L_{11}	Electrical conductivity
L_{12}	Streaming current
L_{21}	Electro-osmotic flow
L_{22}	Hydraulic conductivity
M	Polymer molecular mass
N_A	Avogadro's Number
N_v	Average polymer segment density
R	Gas constant
R_g	Radius of gyration of the polymer
T	Absolute temperature
W_D	Depletion interaction energy
W_E	Electrostatic interaction energy
W_S	Steric interaction energy
W_T	Total interaction energy

Abstract

The reversible red blood cell aggregation induced by polymers or proteins continues to be of biological and biophysical interest and the details regarding this process are still being explored.

A depletion mechanism has been proposed for dextran-mediated RBC aggregation and its applicability to high molecular weights has been recently documented. In part of this study, the depletion layers around the cell surface for various polymer sizes (40kDa-28MDa), ionic strengths (5 and 15mM) and polymer concentrations ($\leq 0.9g/dl$) are measured by means of electrophoretic mobility. The results indicate that the impact of bulk viscosity on the red cell mobility is reduced by increasing the polymer size, and mobility values are higher than that predicted by Helmholtz-Smoluchowski equation. These results agree with the concept of polymer depletion near the cell surface and support a depletion mechanism for dextran-mediated red cell aggregation.

Furthermore, the interaction energies between cells have been calculated by considering cellular and medium properties. The impact of glycocalyx thickness, volume fraction, structure and charge density as well as bulk polymer size and concentration on the cell-cell affinity are calculated. The theoretical model predicts a non-monotonic dependence of cell-cell affinity to polymer size and concentration which suggests an optimum dextran size and concentration for inducing adhesion. These results agree quantitatively with recent experimental observations.

Our findings provide important new insights into polymer-RBC interactions and confirm the concept of depletion mechanism for RBC aggregation which may help in understanding how cellular properties control *in-vivo* RBC interactions.

Chapter 1 Introduction and Background

1.1 Red cell aggregation; an overview

Erythrocytes or red blood cells (RBCs) are biconcave hemoglobin containers with the diameter of $6 - 8 \mu m$ and thickness of about $2 \mu m$ [1]. They flow with the blood in the body and act as small exchange units for transferring oxygen and carbon dioxide in the tissues and organs. Red cells grow in bone marrow where they have nuclei and their shape is irregular. Then as they grow mature, they expel their nuclei, enter the blood and start circulation for 120 days before swelling into spherical shape and becoming hemolyzed. In their 120-day life-span, they go through microcirculation more than 1.2×10^5 times [2]. Red blood cells occupy about 40 – 50% and 36 – 46% of the blood volume in men and women, respectively [3] ranging from 4.5 to 6 million per cubic millimeter of blood. Being the main cellular constituent, RBC shape and behavior affects the flow properties of blood greatly.

Red cell aggregation is one of these behaviors. In stasis or during the low shear rate flow and in the presence of macromolecules like fibrinogen, erythrocytes attach loosely together in a linear characteristic face-to-face morphology often referred to as rouleaux [4, 5]. Subsequently, these linear aggregates attach to each other to form denser, nonlinear three dimensional structures, if they have sufficient time.

Aggregation occurs only in the presence of an aggregant in the form of a polymer or protein. In fact, if the cells are washed and resuspended in a polymer free solution, no aggregation can be observed [5, 6]. Although in plasma suspensions, fibrinogen and macroglobulins act as the aggregating agent [7-9], in phosphate buffered saline, other

macromolecules such as dextran (70kDa), polyvinylpyrrolidone (360kDa), sodium heparin (17kDa) and poly-l-glutamic acid (61kDa) can cause aggregation.

In normal conditions, the attractive forces in such aggregates are weak and with slight increase in shear rate to $20 - 40 \text{ s}^{-1}$, they break down into individual cells [6]. On the other hand abnormal increase in RBC aggregation has been observed in several diseases associated with vascular disorders [10-14]. Diabetes, sepsis, hypertension and sickle cell anemia are some of these disorders.

Factors affecting aggregation can be classified in two main groups, intrinsic and extrinsic. While the former includes all the cellular properties of erythrocytes such as surface charge density, stiffness, shape and deformability, the latter takes account of the suspending phase properties such as size and concentration of polymers as well as ionic strength, hematocrit and shear rate.

The first observation of aggregation goes back to the Dutch scientist, Anthoni van Leeuwenhoek, in 1674 [15, 16]. He noticed the increased extent of aggregation in pregnancy and in some infections. In the mid 1700's, John Hunter reported the aggregation of red cells, and stated that in inflammatory conditions the attraction between cells increases [17]. It was already known to ancient Greeks that the rate of separation and settling of blood elements was associated with diseased states. Once aggregation was observed, an interest in interconnecting the aggregation phenomena with the settling rate of blood raised. Soon it was realized that there is a relation between the increased aggregation and settling rate of blood. Robin Fåhræus worked comprehensively on settling of blood in 1910's which led to the development of Erythrocyte Sedimentation Rate test (ESR) which is used today as a diagnostic tool and as a measure of aggregation extent [18]. In 1921 Swift tried to make connection

between diseased states like Hodgkin's disease, leukemia, cancer, sarcoma and the extent of rouleaux formation [19]. Interestingly, what he observed was the reduced extents of rouleaux formation relative to normal healthy conditions. In 1937, Kegerreis studied the hydrodynamics of rouleaux formation in-vitro between a cover glass and a glass slide and tried to artificially produce aggregates with discocyte particles [20]. In 1950, Hardwicke published an article in Nature about the effects of various dextran molecular sizes on the sedimentation rates of RBCs. He found out that adding dextran to the blood increases sedimentation rate of erythrocytes and this rate is higher for larger dextrans (100-600kDa). He also mentioned that for dextrans with molecular mass less than 60kDa there is no increase in the sedimentation rate [21].

Studies on red cell aggregation carried on enormously in the twentieth century with hundreds of publications dealing with the effect of aggregation on blood flow and blood viscosity [22-29], effects of pathological conditions on the extent of erythrocyte aggregation [30-33], effects of cellular factors on aggregation [34-38], mechanisms of aggregation [39-43] and modeling and simulating the aggregation process [44-49].

1.2 Effects of aggregation on blood flow

Blood is a shear-thinning non-Newtonian fluid, i.e. its viscosity decreases with increasing the shear rate. This property of blood is a direct consequence of RBC behavior in different shear rates. In low shear rates or during stasis, erythrocytes stick together to form aggregates. This leads to higher viscosity of blood. By increasing the rate of flow, aggregates start to break down, resulting in decreasing the blood viscosity. By further increase of shear rate, red blood cells deform and align with the flow, making the blood viscosity even lower [1]. Thus RBC aggregation is the main determinant of low-shear blood viscosity. Effects of RBC aggregation on blood flow has been investigated both *in-vitro* and *in-vivo*.

The effect of aggregation on blood flow *in-vitro* was first published by Robin Fåhræus [25]. He studied the flow of RBC suspensions in glass capillaries when he observed that by increasing the RBC aggregation, more red cells migrate to the center of the tube and a cell-free layer is created around the capillary wall. This migration of cells toward the center results in the minimal energy dissipation.

Now it is known that the effect of aggregation on blood flow depends strongly on the orientation of the capillary. For vertical capillaries, axial migration of red cells results in lower viscosity and flow resistance [3, 50], whereas in horizontal capillaries, sedimentation of aggregates results in higher apparent viscosity [51, 52]. Although *in-vitro* studies have been performed in cylindrical glass tubes, it is difficult to correlate the results to *in-vivo* conditions due to the complexion of the microcirculation geometry and different orientation of blood vessels.

Due to lower shear rates, the aggregation *in-vivo* is most likely to occur in the venous side of circulatory system; the blunted velocity profile has been observed in venules

with enhanced aggregation [53]. In the arterial side of circulation high shear rates prevent aggregate formation. However, aggregation might happen in the middle section of the artery's diameter due to relatively lower shear forces and higher density of red blood cells, which is a consequence of cell migration [54].

In in-vivo investigations of aggregation effect on the blood flow, usually a high molecular weight polymer or protein is infused to the circulation to induce the aggregation. Investigations of in-vivo aggregation are performed by using intravital microscopy technique or studying the blood flow in an isolated organ or vessel. Studies using intravital microscopy of rat [55] and rabbit [56] mesentery after infusing high molecular weight dextran (500kDa and 70kDa respectively) have shown increased flow resistance due to RBC aggregation, independent of increased plasma viscosity, both of which are the result of adding dextran [55]. However, it has also been observed that the thickness of cell-free layer increases with enhanced aggregation in micro-vessels [56]. Hence, a decreased hydrodynamic resistance would be expected. The latter two observations are counteracting: increasing flow resistance due to blunted velocity profile, and decreasing flow resistance due to axial migration of red cells. Therefore, the net effect of RBC aggregation on blood flow is determined by summing up these two opposite effects [57, 58] and various extents of aggregation may affect the flow in different ways [59].

The impact of aggregation on the functional capillary density, i.e. capillaries with RBC flow, has been studied by Kim *et al* [57]. He mentioned that in arteries with reduced pressure, red cell aggregation results in increasing number of erythrocyte-free capillaries. The impact of RBC aggregation of the reduced functional capillary density has also been reported in another way by McHedlisvili *et al* [60] in that capillaries become filled with non-flowing RBC.

Red cell aggregation is considered as one of the determinants of the tissue hematocrit. The microcirculatory hematocrit is characterized by plasma skimming and Fahraeus effect [61], both of which depend on the axial migration of red cells, and as described before, axial migration is promoted by red cell aggregation.

Axial cell migration can also affect the shear stress on the vessel walls by producing a cell-free region with low viscosity, near the endothelium. Since the vascular functions are strongly dependent on the shear stress on the endothelial cells [62], RBC aggregation can alter the mechanisms controlling vessel dimensions [54].

1.3 Mechanisms of aggregation

Currently there are two coexisting models dealing with RBC aggregation mechanisms: bridging model and depletion model.

In bridging model, macromolecules adsorb onto the surface of two adjacent RBCs and form so-called bridges between them. Aggregation occurs when the attractive forces due to adsorption exceed disaggregation forces due to electrostatic repulsion, membrane strain and blood shear [40, 42, 63]. This model seems similar to other cell-cell interactions like agglutination, but the difference is that the attractive energy is much lower in the case of aggregation which makes it reversible. The formulation of this mechanism has been done by Chien by using a force-balance equation [64]. However, Brooks *et al* have suggested that this model may not be appropriate for aggregations induced by proteins [65].

Depletion model, on the other hand, proposes almost opposite mechanism. In the depletion mechanism, lower polymer concentration near the cell surface relative to the bulk phase results in an osmotic gradient. This gradient acts as a driving force to expel the fluid away from intercellular gap and bringing cells close together. According to this theory, aggregation happens when depletion attractive force is greater than abovementioned disaggregation forces. Depletion interactions are well-known in colloidal chemistry and several reports about “depletion flocculation” can be found in the literature [66-69]. The applicability of such interactions in the field of red cell aggregation was introduced by van Oss *et al* [43] and by Evans and coworkers [70]. So far less attention has directed into this field and there are only a few reports concerning depletion interaction in red cell aggregation [39, 71-73].

1.4 Methods of measuring aggregation

Different methods have been utilized extensively to measure the extent of aggregation.

1.4.1 Light transmission/reflection

In this method the transmitted or reflected light through or from an erythrocyte suspension is measured [74-78]. By increasing the rouleaux formation, light transmission through sample increases and in contrast light reflection decreases. The aggregation can be quantified either on stasis or under low shear rates by measuring the changes in transmitted or reflected light. In both stasis and low shear rate measurements, a high shear rate is applied to the system to disperse all the pre-existing aggregates following with an abrupt decrease to zero or desired low shear rate. In addition to what mentioned above, with this method it is possible to find kinetic parameters of aggregation as well as disaggregation shear rate.

1.4.2 Ultrasound backscattering

In the ultrasound backscattering technique, the backscattering coefficient of the RBC suspension is measured. Since the backscattering magnitude is proportional to the volume of cells or aggregates, with increasing the extent of aggregation, higher backscattering coefficient is obtained that can be used as a measure of aggregation. Conversely, during disaggregation with increasing shear rate, backscattering coefficient decreases [79-83].

1.4.3 Microscopic observation

Low hematocrit sample (about 1%) with a known cell-count is placed in a hemocytometer chamber. The sample is then left undisturbed for few minutes. Microscopic aggregation index is found by counting the number of cell units (both

mono-dispersed and aggregated) and dividing it to the mono-dispersed cell count [27, 84, 85]. Microphotographs of blood in parallel plate chamber has also been used to measure the size and “surface to volume ratio” of aggregates [11].

1.4.4 Measurement of erythrocyte sedimentation rate

Measurement of sedimentation rate of red blood cells has long been employed as a measure of presence of plasma proteins. According to Stokes’ law, the rate of settling of a particle in a viscous fluid due to gravity is proportional to the square radius of the particle [86]. Higher degrees of aggregation increase the particle size and the settling rate, hence sedimentation rate is considered as a measure of aggregation [87-91].

1.4.5 Measurement of low shear viscosity

At low shear rates below $5s^{-1}$, RBC aggregation is the main determinant of blood viscosity. When the aggregation proceeds, the low shear rate viscosity increases more than the viscosity of high shear rates [41]. So measurement of low shear blood viscosity for different RBC suspensions can be a measure of the aggregation extents. However, caution should be made with this technique in that slight decrease in cell deformability can also cause higher values of low-shear viscosity [85, 92].

1.5 Aggregability

Aggregation occurs in the presence of a polymer or protein and any change in the type and concentration of the polymer affects the extent of aggregation. However, alterations of the cellular properties can also influence the RBC aggregation regardless of the surrounding medium. Term “aggregability” is used in the literature to distinguish between cellular intrinsic characteristics and surrounding phase properties [6, 7, 93, 94]. “RBC aggregability” is different from the term “RBC aggregation” in that “RBC aggregation” is the observed/measured aggregation in any medium, while “RBC aggregability” is the observed/measured aggregation of a group of washed cells in a specified medium *compared* to the aggregation of other washed cell group in the same medium.

Studying the effects of cellular factors on the aggregation was prompted by Nordt [35]. He studied the aggregation behavior of density separated cells and observed that aged, denser cells show more degree of aggregation related to younger cells in autologous plasma. These observations have also been confirmed by others [34, 36] and it is shown that there is a correlation between extent of aggregation in autologous plasma and polymer suspensions which indicates an intrinsic aggregation potential of the cells.

Impact of the cell age on the aggregation tendency has been studied by Nordt [35], Nash et al [36] and Whittingstall et al [95]. In their studies after centrifugation, top 10% and bottom 10% were collected representing younger and older cells, respectively [96]. Aggregation was measured in autologous plasma and polymer solutions of 3% dextran 70kDa, 0.5% PVP 360kDa, 0.6% poly-L-glutamic acid 61.2kDa and 6% sodium heparin. In all of the samples, older cells showed

significantly higher aggregation with BOT/TOP ratio of 2.6, 2.7, 3.1, 1.7 and 2.1 respectively.

Sowemimo et al [34], Whittingstall et al [95], Nash et al [36] and Rampling and co-workers [97] studied donor-specific effects on the aggregation. After washing, the erythrocytes of different healthy adults were used in autologous plasma and PBS solution containing various polymers. 3% dextran 70kDa, 0.5% PVP 360kDa, 0.6% poly-L-glutamic acid 61.2kDa and 6% sodium heparin 17kDa were used as the aggregant. Aggregation was measured using Myrenne aggregometer, sedimentation rate, and low shear viscosity. The results indicated great difference in the extent of aggregation for different cells suspended in the same medium. For instance, the difference of aggregation for cells of different donors suspended in the plasma and dextran solution was approximately five and two folds, respectively [34]. Furthermore, it was realized that cells which exhibit more aggregation in the autologous plasma, show greater aggregation in any other polymer solution too.

The impact of neuraminidase treatment on the aggregability of red cells has been studied by Nash et al [36] who observed that neuraminidase treated cells in 3% dextran 70kDa exhibit markedly elevated levels of aggregation while treated cells in autologous plasma show slightly reduced aggregation. Rampling and Pearson [98] also observed that neuraminidase treated cells exhibit increased aggregation in dextran 40 and 70kDa, while this treatment has no effect on the fibrinogen-induced aggregation. The later observation indicates that the impact of cellular factors on the red cell aggregation might be related to the aggregant type.

1.6 Effect of pathological conditions in RBC aggregation

The impact of pathological conditions on the red cell aggregation has been studied enormously. Various clinical states have been studied using whole blood or erythrocytes in plasma and almost all of them indicate greater aggregation of RBCs in autologous plasma [10, 99]. These variations in extent of aggregation can be caused by changes in plasma proteins or cellular properties.

Fibrinogen is the major macromolecule in plasma which contributes in the aggregation and strong correlation between the extent of aggregation and fibrinogen concentration is observed [100]. In pathological conditions like inflammation, concentration of fibrinogen as well as its effectiveness in making aggregates can be altered [101, 102].

Alterations in red cell aggregability has been observed in different pathological conditions like diabetes [30], thalassemia major [31], obesity [32], schizophrenia [33] and sepsis [12]. The reasons behind these alterations are not clearly known yet. However it has been suggested that the variations in aggregability is due to changes in the membrane compositions as well as the sialic acid content of the cell [33].

In physiological aggregation, cells stick to each other in a characteristic face-to-face morphology followed by branching and creating three dimensional structures. However, in pathological conditions more irregular shaped are observed. The cells in pathologic aggregates exhibit increased adhesion strength, while the size of the aggregate might remain intact or even decreased [103, 104].

1.7 Hypotheses and specific aims

Polymer depletion is a likely mechanism of red blood cell aggregation. However, its relevance to red cell-red cell adhesion requires more investigations. Furthermore, the role of depletion interactions in increased RBC adhesion in pathological states needs to be clearly identified.

The main hypotheses of this project are:

- 1- Depletion interaction is a determinant of the RBC adhesiveness
- 2- Depletion interaction is a determinant of the altered RBC adhesiveness and aggregability in pathological conditions

In order to test the importance of depletion interactions in RBC-RBC interactions two specific aims have been determined:

- 1- Identification of polymer depletion layer around the cell surface
- 2- Presenting theoretical frameworks to study the impact of various cellular and medium parameters on the red cell-red cell adhesion, induced by depletion interactions

1.8 Structure of the thesis

This thesis consists of six chapters. After introduction and literature review in Chapter 1, the required theoretical knowledge for this project is presented in chapter 2.

In chapter 3, the polymer-depleted layer around the cell surface and the impact of high molecular weight dextran on the formation of the depletion layer is investigated by means of electrophoretic mobility measurements.

In chapter 4, a simplified model of red cell interactions is made by considering electrostatic and depletion interactions for a uniform distribution of glycocalyx segments and surface charge density.

Chapter 5 enhances the previous chapter by presenting a more comprehensive model on red cell interactions considering steric interactions as well as different distributions for the glycocalyx structure.

Finally, chapter 6 is the concluding chapter and presents possible future directions of this project.

At the end, the findings of this project will benefit us in having a better insight into the depletion-mediated RBC adhesion and aggregation.

Chapter 2 Theoretical Considerations

2.1 Introduction

In order to investigate the energetic of the cell-cell adhesion and the impact of various parameters on the extent of it, it is essential to consider all major forces involving during the cell-cell interaction. Throughout this thesis, the following three major forces will be considered:

- 1- Electrostatic interactions arising from negative surface charge density of cells which are mainly due to COO⁻ groups of neuraminic acids
- 2- Depletion interactions which occur as a result of lower polymer concentration around the cell surface relative to the bulk phase.
- 3- Steric interaction that arise from spatial hindrance of glycocalices when the distance between cells become less than twice of the glycocalyx thickness.

In order to understand the energetic of the cell-cell adhesion, it is important to have a comprehensive insight into all of these forces. Furthermore it is critical to know how different cell parameters and physiochemical properties of the cell and surrounding medium alter these forces and total interaction energy between cells. In this chapter, first the theoretical concepts behind electrostatic, depletion and steric interactions will be elucidated. Next, the theory and application of Electrophoretic Mobility Measurement (EPM) as a tool to study the polymer-cell surface interface will be discussed. The content of this chapter will be the theoretical foundation and used later in other chapters.

2.2 Depletion Interactions

The idea of introducing depletion interactions in red cell aggregation has come from colloidal chemistry. When a polymer chain approaches a surface it experiences a loss in conformational entropy. Now there will be two options for the polymer; one option is adsorption to the surface and the other is the displacement of the center of mass of the polymer from the interface, thereby creating a polymer-depleted region around the surface of the particle. If in the latter case two particles approach each other so that their depletion layers overlap, solvent will tend to diffuse out of the depletion zone due to the difference in polymer concentration between the depletion region and the bulk phase. This results in an attractive depletion force between the two surfaces, pulling particles together [39]. This phenomenon is depicted schematically in Figure 2-1.

Figure 2-1 Schematic representation of the depletion phenomena; colloidal particles are presented in blue and non-adsorbing polymers in green. Top frame: colloidal particles with their polymer-depleted regions (dashed lines). Middle frame: approaching particles. Bottom frame: polymer-depleted zones of the particles are overlapped, resulting in depletion attractive force between particles

Depletion interaction is a well-known phenomenon in colloidal chemistry [66-69]. It was first introduced by Asakura and Oosawa in 1950s [105] who suggested that the depletion forces can lead to reversible flocculation and phase separation in colloid-polymer mixtures.

In order to study the depletion interactions, it is necessary to distinguish between *hard* and *soft* surfaces. Hard surfaces are smooth and the free polymer cannot penetrate into it. On the other hand soft surfaces have attached polymeric layer on the surface in which free polymer can penetrate fully or in part [69, 106]. Accordingly, the red cell surface with its attached glycocalyx layer is considered as a soft surface. Figure 2-2 schematically shows the polymer profile in the depleted layer of a soft surface. In this figure Δ , p and δ are the depletion layer thickness, penetration depth and glycocalyx thickness, respectively.

Figure 2-2 Illustration of the depleted layer near the cell surface; δ : glycocalyx thickness, p : penetration depth of the free polymer inside the glycocalyx structure, Δ : depletion layer thickness. Free polymer concentration changes from zero at $\delta - p$ to bulk polymer concentration at $\delta - p + \Delta$

To find the thickness of depletion layer, an approach introduced by Vincent [68] is used in which depletion layer thickness is a function of bulk osmotic pressure, molecular weight, concentration and physiochemical properties of the polymer:

$$\Delta = -0.5 \left(\frac{\Pi}{D} - \sqrt{\left(\frac{\Pi}{D} \right)^2 + 4\Delta_0^2} \right) \quad (2.2-1)$$

The parameter D is a function of bulk polymer concentration c_b .

$$D = \frac{2k_B T}{\Delta_0^2} \left(\frac{c_b N_a}{M} \right)^{\frac{2}{3}} \quad (2.2-2)$$

In this equation k_B , N_a , T and M are Boltzmann constant, Avogadro's number, absolute temperature and polymer molecular mass, respectively. Δ_0 is the depletion layer thickness for a very dilute polymer solution and is equal to $1.4 R_g$ where R_g is the radius of gyration of the polymer [68].

The parameter Π in (2.2-1) is the osmotic pressure of the bulk phase and is calculated using the virial equation ignoring coefficients higher than 2, [4, 107] i.e.:

$$\Pi = \frac{c_b}{M} RT + B c_b^2 \quad (2.2-3)$$

where R is the gas constant and equal to $8.314472 \frac{J}{mol K}$ and B is second virial coefficient.

The depletion interaction energy for the case of hard particles in a distance of less than 2Δ where there is no free polymer between them is [69]:

$$W_{D_s} = 2\pi a \Pi (\Delta - d) \quad (2.2-4)$$

This equation had been found by assuming a linear profile for free polymer in the depleted region and is correct only if the particle radius a is much larger than distance between cells ($2d$) as well as depletion layer thickness Δ . In the case of red blood cells where the size of cells is much larger than the glycocalyx and depletion layer thickness, one can ignore the curvature of cell and assume it being a flat surface. In

that case it is possible to use the reverse of Derjaguin approximation to find the depletion energy of flat surfaces.

The Derjaguin approximation [108] relates the potential energy of a sphere to that of a flat surface by:

$$W_s(h) = \pi a \int_h^\infty W_p(\xi) d\xi \quad (2.2-5)$$

In this equation, W is any kind of potential energy, h is the distance between two particles and subscripts s and p refer to sphere and flat plate respectively. The potential of flat plate then will be:

$$W_p(h) = -\frac{dW_s(h)}{dh} \quad (2.2-6)$$

Referring back to equation (2.2-4) and using the reverse Derjaguin approximation of equation (2.2-6) one can find the depletion interaction energy for a flat hard surface as:

$$W_D = -2\Pi(\Delta - d) \quad (2.2-7)$$

where d is half of the distance between surfaces. Eventually when there is a soft surface with the penetration depth of p into the glycocalyx layer, equation (2.2-7) becomes [4]:

$$W_D(d) = -2\Pi(\Delta + \delta - p - d) \quad (2.2-8)$$

2.3 Calculations of the penetration depth

An important parameter to be considered in calculating the depletion interaction energies is the magnitude of the penetration depth. The penetration depth can be evaluated by assuming that penetration continues until a point where the osmotic pressure in the attached layer is equal with the bulk osmotic pressure.

The Flory-Krigbaum theory which deals with non-ideal polymer coils approaching each other, proposes the following equation for mixing free energy of the system per unit of surface area [69]:

$$\Delta G_{mix} = \frac{2kT}{v_1} \left(\frac{1}{2} - \chi \right) \int_{\delta-p}^{\delta} \varphi_a(x) \varphi_f(x) dx \quad (2.3-1)$$

where χ , v_1 , k , T , φ_a and φ_f are Flory interaction parameter, molar volume of solvent, Boltzmann constant, absolute temperature, attached and free polymer volume fraction, respectively. This equation can be calculated only when the volume fraction profiles are known.

The penetration is then derived by differentiating ΔG_{mix} with respect to p and setting the result equal to the bulk osmotic pressure Π , i.e. :

$$\frac{d(\Delta G_{mix})}{dp} = \Pi \quad (2.3-2)$$

As before the osmotic pressure is found by equation (2.2-3).

2.4 Steric Interactions

“Steric stabilization” is a term which refers to stabilization of colloidal particles by means of nonionic macromolecules. When two cells with anchored macromolecules of thickness δ approach each other and the distance between them ($2d$) becomes less than twice of glycocalyx thickness, attached macromolecules start to interpenetrate. Polymer segments of each surface interact with the segments of the other surface and it results in increasing the mixing energy of the system.

For the cell-cell distance $d > \delta$, no interactions take place between attached layers. But when the distance is decreased to $\frac{\delta}{2} < d < \delta$, if there is sufficient time for relaxation of attached polymers, segment interpenetration takes place. If the distance is further decreased to $d < \frac{\delta}{2}$, the polymer segments should stay in shorter distance, so in addition to the segment interpenetration, compression of attached molecules also occurs.

Segment interpenetration gives rise to the mixing free energy while compression increases the elastic free energy of the system. Hence, two main domains can be identified:

1- Interpenetration domain $\frac{\delta}{2} < d < \delta$

2- Interpenetration + compression domain $d < \frac{\delta}{2}$

According to Napper [109] it is unlikely for the elastic term to have any effect in interpenetration domain. The reason is that the peripheral regions of chains which take part in interpenetration have small volume fraction.

Using Flory-Krigbaum theory [110], the repulsive potential energy per unit area of the cell surface is described by:

$$W_s = 2kT(0.5 - \chi) \left(\frac{v_s^2}{v_1} \right) v^2 i^2 R \quad (2.4-1)$$

where χ is the Flory interaction parameter, v_1 is the volume of solvent molecule, v_s is the volume of one polymer segment, v is the number of chains per unit area and i is the number of segments per chain. Parameter R is defined as

$$R = \int_0^{2d} \hat{\rho}_d^2 dx - \int_0^\infty \hat{\rho}_\infty^2 dx + \int_0^{2d} \hat{\rho}_d \hat{\rho}'_d dx \quad (2.4-2)$$

where $\hat{\rho}_d$ is normalized segment density profile so that summation of segments in the glycocalyx thickness is equal to one, i.e. $\int_0^\delta \hat{\rho}_d dx = 1$ and $\hat{\rho}'_d$ is the mirror image of $\hat{\rho}_d$. To derive the normalized segment density to be used in equation (2.4-2), one needs to find the value of A so that $\int_0^\delta A \rho_a dx = 1$, where ρ_a is the attached layer segment density.

R is a parameter which takes polymer segment distribution into account in the direction normal to the cell surface. The first two terms of equation (2.4-2), represent the compression of polymers while the last term describes the interpenetration zone. Hence, if only the interpenetration domain $\frac{\delta}{2} < d < \delta$ is on focus, the first two terms will be zero and only the last term needs to be considered.

2.5 Electrostatic Interactions

2.5.1 Double layer and mobile charge distribution

In colloidal chemistry, electrostatic repulsion is of great importance in stabilizing the colloidal solution. Electrostatic interactions between erythrocytes arise from their negative charge density which is mainly caused by dissociated COO⁻ groups of neuraminic acids in glycoproteins of the cell membrane. This is a relatively strong repulsive force and prevents the cells from attaching into each other. The magnitude of surface charge, e.g. dissociated COO⁻ groups of neuraminic acids per cell, can be calculated by neuraminidase treatment of cells [111].

When cells with their negative potential are suspended in an electrolyte, they affect the ion distribution in the solution to form an electrical double layer. The counter ions in the electrolyte approach the cell surface to neutralize the surface charge and as a consequence of the thermal motion, they distribute in a finite space around the cell. Due to diffusive manners that counter ions distribute, this double layer is also called diffuse double layer [112]. The absolute electric potential declines from ψ_0 on the surface to zero in the bulk. Both the ion distribution and potential decay is shown schematically in Figure 2-3.

The distribution of the free ions around the cell surface obeys the Boltzmann's law:

$$n_i(x) = n_i^\infty e^{\left(-\frac{z_i e \psi(x)}{kT}\right)} \quad (2.5-1)$$

where $n_i(x)$ is the concentration of i^{th} ion at position x , while n_i^∞ represents the same parameter at bulk phase, e is the electron charge, z_i is the valence of i^{th} ion, $\psi(x)$ is the electric potential at position x and finally k is the Boltzmann's constant

which in multiplication with absolute temperature (T) represents the thermal energy of ions.

Figure 2-3 (a) Schematic illustration of ion distribution around negative cell surface, (b) potential variation as a function of distance from cell surface

Charge density at each position is related to ion concentration by

$$\rho_m(x) = \sum_{i=1}^N z_i e n_i(x) = \sum_{i=1}^N z_i e n_i^{\infty} e^{\left(-\frac{z_i e \psi(x)}{kT}\right)}. \quad (2.5-2)$$

The exponential function e^x is expanded by the Taylor series as:

$$e^x = \sum_{n=0}^{\infty} \frac{x^n}{n!}. \quad (2.5-3)$$

For small values of x one can neglect the terms for $n \geq 2$. Back to equation (2.5-2), if

the potential is small (so that $\left|\frac{z_i e \psi(x)}{kT}\right| \ll 1$), charge density of free ions will be:

$$\rho_m(x) = -\varepsilon \varepsilon_0 \kappa^2 \psi(x) \quad (2.5-4)$$

where ε and ε_0 are the relative permittivity of the medium and vacuum permittivity. κ is the Debye-Hückel parameter and is equal to:

$$\kappa = \sqrt{\frac{1}{\varepsilon\varepsilon_0 kT} \sum_{i=1}^N z_i^2 e^2 n_i^\infty}. \quad (2.5-5)$$

The value of $1/\kappa$ with the dimensions of length is measure of the double layer thickness and is called Debye-Hückel length.

For an electrolyte with ionic strength of I , an alternative equation (2.5-6) can be used to find the Debye parameter [113]. In this equation I is in units of mol/m^3 .

$$\kappa = \sqrt{\frac{2N_A e^2}{\varepsilon\varepsilon_0 kT} I} \quad (\text{m}^{-1}) \quad (2.5-6)$$

2.5.2 *Electrostatic potential*

In order to calculate the electrostatic energy between cells, one needs to find the potential distribution $\psi(x)$ first. The Poisson equation relates the electric potential of each point to the charge density of the same point, i.e.:

$$\nabla^2 \psi(r) = -\rho_T(r)/\varepsilon\varepsilon_0 \quad (2.5-7)$$

where $\rho_T(r)$ is the summation of both mobile and fixed charges. Our model of two cells with the fixed charge density of $\rho_{fix1}(x)$ and $\rho_{fix2}(x)$ is shown in the Figure 2-4. The origin of coordinate system is set on the surface of one cell and the other cell is on the distance of $2d$ from it. Generally different fixed charge distributions can be chosen for the cells. The mobile charge distribution $\rho_m(x)$ can be found using equation (2.5-4).

Figure 2-4 Model of two cells; Cells are located at distance $2d$ from each other. Glycocalyx layer of the cells have the fixed charge density of ρ_{fix1} and ρ_{fix2} which is zero outside of the glycocalyx layer. $\rho_m(x)$ is the charge density due to the free ions in the electrolyte calculated by equation (2.5-2)

Equation (2.5-7) should be solved with appropriate boundary conditions to find the potential distribution as a result of fixed and mobile charge distributions. In our one-dimensional case in which the charge density only varies in the direction x normal to the cell surface, Poisson equation (2.5-7) becomes:

$$\psi''(x) = -\rho_m(x)/\epsilon\epsilon_0 \quad (2.5-8)$$

Combining Poisson and Boltzmann equations and making it linear for small values of ψ yields:

$$\psi''(x) = \kappa^2\psi(x) - \frac{\rho_{fix}(x)}{\epsilon\epsilon_0}. \quad (2.5-9)$$

For the sake of simplicity, it is assumed that fixed charge densities of two cells are the same, or $\rho_{fix1}(x) = \rho_{fix2}(x) = \rho_{fix}(x)$. These assumptions provide symmetry in the system and allow calculating the potential of one half of the system only. In this case, the fixed charge density of half of the system would be:

$$\rho_{fix}(x) = \begin{cases} \rho_{fix}(x) & 0 < x \leq \delta \\ 0 & \delta < x < d \end{cases} \quad (2.5-10)$$

Equation (2.5-9) needs to be solved with appropriate boundary condition. The boundary conditions to solve equation (2.5-9) are:

$$\psi(x)|_{x=\delta^+} = \psi(x)|_{x=\delta^-} \quad (1)$$

$$\left. \frac{d\psi(x)}{dx} \right|_{x=\delta^+} = \left. \frac{d\psi(x)}{dx} \right|_{x=\delta^-} \quad (2)$$

$$\left. \frac{d\psi(x)}{dx} \right|_{x=d} = 0 \quad (3) \quad (2.5-11)$$

$$\left. \frac{d\psi(x)}{dx} \right|_{x=0} = 0 \quad (4)$$

The first and second boundary conditions come from the continuity of the potential on the edge of glycocalyx. The third boundary condition is due to the symmetry of the system, if the fixed charged density of two cells is considered to be the same. The last boundary condition comes from Poisson equation on the cell surface, by assuming that the surface charge density at $x = 0$ is zero and all the charges are distributed throughout the glycocalyx thickness.

Solving the second order differential equation (2.5-9) with boundary conditions (2.5-11) results to the flowing equation for potential distribution in the left half of the system [114, 115]:

$$\begin{aligned} \psi_1(x) = \frac{1}{\kappa \epsilon \epsilon_0} [& \frac{\cosh(\kappa x)}{\sinh(\kappa d)} \int_0^d \rho_{fix}(x) \cosh(\kappa(x-d)) dx \\ & + \int_0^x \rho_{fix}(\xi) \sinh(\kappa(\xi-x)) d\xi] \end{aligned} \quad (2.5-12)$$

while the potential distribution for the other half will be the mirror image of the equation above.

2.5.3 *Electrostatic interaction energy*

Electrostatic free energy is the energy to increase the fixed charge by $d\tilde{\rho}$ in the condition where the potential $\tilde{\psi}$ exists as a result of pre-existing stable distributed charge $\tilde{\rho}$. The electrostatic free energy is evaluated by assuming a stepwise isothermal charging process. The free energy of two cells is found to be [116]:

$$E(x) = \int_0^{2d} \int_0^{\tilde{\rho}} \psi(x) d\tilde{\rho} dx \quad (2.5-13)$$

The charge density at each interval can be expressed as a function of final charge density in the form of:

$$\tilde{\rho} = k\rho \quad (2.5-14)$$

where the coefficient k varies from 0 to 1. If the potential is related to the charge by a linear differential equation, then the potential at each interval can be related to the final potential with the same manner:

$$\tilde{\psi}(\tilde{\rho}) = k\psi(\rho) \quad (2.5-15)$$

Substituting the two latter equations into (2.5-13), gives the following equation for electrostatic free energy:

$$E = \frac{1}{2} \int_0^{2d} \psi(x) \rho(x) dx \quad (2.5-16)$$

The electrostatic interaction energy of two cells can be found by calculating the free energy of two cells at the distance of d and deducting the free energy of two single cells when the distance between them goes to infinity ($d \rightarrow \infty$), i.e.:

$$W_E = E(d) - E(\infty) \quad (2.5-17)$$

It should be noted that the charge density of each cell can be calculated by dividing the surface charge density to glycocalyx thickness:

$$\rho_{fix} = \frac{\sigma}{\delta} \quad (2.5-18)$$

2.6 Electrophoretic Mobility

When there is a relative motion between the charged particles and surrounding electrolyte, there will be some electro-kinetic phenomena. This includes electro-osmosis, electrophoresis, sedimentation current and streaming potential. Two different driving forces may exist at the same time in the system: pressure gradient and potential gradient. In calculating the electrophoretic mobility, one needs to find the electro-osmotic velocity far away from the surface $u_e(\infty)$ by solving the appropriate Navier-Stokes equation with the electric field E as the driving force. An alternative but equal approach is to find the streaming current of a pressure driven flow along a flat surface and use it to calculate the electrophoretic mobility. In this approach the hydraulic and electrostatic terms are separated which makes it more convenient.

According to Donath and Voigt [117, 118], electric current can be produced by both electric potential and pressure difference which force free ions to move:

$$I = L_{11}\Delta\Psi + L_{12}\Delta P \quad (2.6-1)$$

The terms L_{11} and L_{12} are the electrical conductivity and streaming current respectively. On the other hand, the volume flow can also be produced by means of pressure gradient or exerting an electric field to the electrolyte. Hence the volume flow also can be written in the form of:

$$J = L_{21}\Delta\Psi + L_{22}\Delta P \quad (2.6-2)$$

where L_{21} and L_{22} are electro-osmotic flow and hydraulic conductivity respectively.

Onsager's reciprocity principle provides that:

$$L_{21} = L_{12} \quad (2.6-3)$$

If $\Delta\psi = 0$ in equation (2.6-1), I will be the streaming current and if $\Delta P = 0$ in equation (2.6-2), J will be the electro-osmotic flow. It is convenient to pose the assumption considering that the fluid flows due to pressure or potential gradient in a channel of thickness $2d$. The glycocalyx structure with thickness δ is attached to the channel walls and the coordinate system is fixed to the upper surface with direction of x normal to it. The schematic representation of the flow channel is illustrated in Figure 2-5. It is assumed that d is much larger than δ , Δ and κ^{-1} which are glycocalyx thickness, depletion layer thickness and Debye length respectively.

Figure 2-5 Schematic structure of the flow channel with thickness $2d$; attached to the wall is a glycocalyx-like structure with thickness δ where $\delta \ll 2d$. Fluid flows in the Y - direction due to pressure or potential gradient across the length of the channel. Velocity profile in the Y -direction is a function of distance from channel walls

The streaming current per unit of width (w) as a function of mobile charge density $\rho_m(x)$ can be calculated as:

$$I = \frac{dQ}{dt} = \int_0^{2d} \frac{\rho_m(x) dA dl}{dt} = \int_0^{2d} \rho_m(x) w u(x) dx \Rightarrow \quad (2.6-4)$$

$$\frac{I}{w} = \int_0^{2d} \rho_m(x) u(x) dx$$

On the other hand, the electro-osmotic flow per unit of width (w) is:

$$J = u_e A = \int_0^{2d} u_e w \cdot dx \Rightarrow \frac{J}{w} = 2du_e \quad (2.6-5)$$

The velocity of electro-osmotic flow is the velocity of flow far away from the surface where the velocity is constant.

From the equations (2.6-1) to (2.6-5) one can deduce:

$$\frac{J}{\Delta\psi} = \frac{I}{\Delta P} \Rightarrow \frac{2du_e}{\Delta\psi} = \frac{\int_0^{2d} \rho_m(x) u(x) dx}{\Delta P}. \quad (2.6-6)$$

The electrophoretic mobility is defined as the electro-osmotic velocity in the units of applied field or:

$$b = \frac{u_e}{E} = \frac{-u_e \Delta L}{\Delta\psi}. \quad (2.6-7)$$

The equation above is derived by taking into account that the electric field is the gradient of potential ($E = -\nabla\psi$) and L is the length in the direction of the flow.

By considering the equations (2.6-6) and (2.6-7), the electrophoretic mobility is found as:

$$b = \frac{-\Delta L \int_0^{2d} \rho_m(x) u(x) dx}{2\Delta P d}. \quad (2.6-8)$$

So in order to calculate the electrophoretic mobility, one needs to find mobile charge density distribution and the velocity profile of a pressure driven flow.

Mobile charge density can be obtained via equation (2.5-4). Substituting equation (2.5-4) in the mobility equation (2.6-8) yields to:

$$b = \frac{\Delta L \varepsilon \varepsilon_0 \kappa^2 \int_0^{2d} \psi(x) u(x) dx}{2\Delta P d}. \quad (2.6-9)$$

2.6.1 Electric potential

To find the electric potential one needs to solve the Poisson-Boltzmann equation (2.5-9) with the appropriate boundary conditions of the problem (2.5-11). The potential function (2.5-12) for the case where d is much larger than δ, Δ and κ^{-1} would be [114]:

$$\begin{aligned} \psi(x) = \frac{1}{\kappa \varepsilon \varepsilon_0} & \left(\cosh(\kappa x) \int_0^\infty \rho_{fix}(x) e^{-\kappa \xi} d\xi \right. \\ & \left. + \int_0^x \rho_{fix}(x) \sinh(\kappa(\xi - x)) d\xi \right) \end{aligned} \quad (2.6-10)$$

which results to the following equation when the constant fixed charge density of ρ_u is assumed:

$$\psi(x) = \begin{cases} \frac{\rho_u}{\kappa^2 \varepsilon \varepsilon_0} (1 - \cosh(\kappa x) e^{-\kappa \delta}) & x \leq \delta \\ \frac{\rho_u}{\kappa^2 \varepsilon \varepsilon_0} (\cosh(\kappa(\delta - x)) - \cosh(\kappa x) e^{-\kappa \delta}) & x > \delta \end{cases} \quad (2.6-11)$$

2.6.2 Velocity profile

Next we are going to formulate the velocity profile of our special problem. The velocity profile is found using Navier-Stokes equation. For steady state one dimensional flow along y direction for a fluid with viscosity $\mu(x)$, Navier-Stokes equation is:

$$\frac{d}{dx} \left(\mu(x) \frac{du(x)}{dx} \right) = \frac{\Delta P}{l}. \quad (2.6-12)$$

Inside the glycocalyx the viscosity is assumed to be the same as bulk phase (μ_b). The small segments of attached polymer cause friction inside the glycocalyx layer. So the Navier-Stokes equation is written as:

$$\begin{aligned} \frac{d}{dx} \left(\mu(x) \frac{du(x)}{dx} \right) &= \frac{\Delta P}{l} & x \geq \delta \\ \mu_b \frac{d^2 u(x)}{dx^2} - a^2 \mu_b u(x) &= \frac{\Delta P}{l} & x \leq \delta \end{aligned} \quad (2.6-13)$$

The parameter a is the friction factor of the attached polymer, i.e. glycocalyx. The boundary conditions for equation (2.6-13) are considered as follows:

$$u(x)|_{x=\delta^+} = u(x)|_{x=\delta^-} \quad (1)$$

$$\mu(x) \frac{du(x)}{dx} \Big|_{x=\delta^+} = \mu_b \frac{du(x)}{dx} \Big|_{x=\delta^-} \quad (2) \quad (2.6-14)$$

$$\frac{du(x)}{dx} \Big|_{x=d} = 0 \quad (3)$$

$$u(x)|_{x=0} = 0 \quad (4)$$

The solution to equation (2.6-13) with these boundary conditions, assuming that d is much larger than δ will be [114]:

$$u(x) = \begin{cases} -\frac{\Delta P d}{l a \mu_b} \frac{\sinh(ax)}{\cosh(a\delta)} & x \leq \delta \\ u(\delta) - \frac{\Delta P d}{l} \int_{\delta}^x \frac{d\xi}{\mu(\xi)} & x \geq \delta \end{cases} \quad (2.6-15)$$

When there are free non-adsorbing polymers in the solution, a depleted region near the cell surface appears. The viscosity changes inside the depletion layer from μ_{δ} to μ_b . The friction coefficient inside the glycocalyx structure is a function of both glycocalyx polymer segments and penetrated free polymers into glycocalyx. Hence when there is polymer penetration into the glycocalyx, friction coefficient will be [119]:

$$a_p^2 = a^2 + \frac{f}{\mu_b} \quad (2.6-16)$$

The friction coefficient of glycocalyx is assumed to remain intact with and without free polymer existence. f is the effective stokes friction of the free polymer which is characterized by the average polymer segment density N_v and hydrodynamic radius of the dextran segments, r_{seg} [120]:

$$f = 6\pi N_v \mu_b r_{seg} \quad (2.6-17)$$

The segment density N_v is given by the segment molecular weight and the polymer concentration within the glycocalyx. The velocity profile of equation (2.6-15) for the polymer-free solution is:

$$u_0(x) = \begin{cases} -\frac{\Delta P d}{l a \mu_b} \frac{\sinh(ax)}{\cosh(a\delta)} & x \leq \delta \\ -\frac{\Delta P d}{l \mu_b} \left(\frac{\tanh(a\delta)}{a} + x - \delta \right) & x \geq \delta \end{cases} \quad (2.6-18)$$

and for the case where free polymer exists in the system, the velocity profile is equal to:

$$u_p(x) = \begin{cases} -\frac{\Delta P d \sinh(a_p x)}{l a \mu_b \cosh(a_p \delta)} & x \leq \delta \\ -\frac{\Delta P d}{l} \left(\frac{\tanh(a_p \delta)}{a_p \mu_b} + \int_{\delta}^x \frac{d\xi}{\mu(\xi)} \right) & x \geq \delta \end{cases} \quad (2.6-19)$$

The viscosity of the solution can be estimated by using Huggins expression [121]:

$$\mu_{sp}(x) = [\eta]c(x) + k'[\eta]^2 c(x)^2 \quad (2.6-20)$$

where $\mu_{sp}(x)$ is the specific viscosity and is related to the viscosity of polymer solution and solvent by $\mu_{sp}(x) = \frac{\mu(x) - \mu_0}{\mu_0}$. Equation (2.6-20) then becomes:

$$\mu(x) = \mu_0(1 + [\eta]c(x) + k'[\eta]^2 c(x)^2) \quad (2.6-21)$$

In the equations above, $[\eta]$ is the intrinsic viscosity of dextran, i.e. the measure of contribution of dextran in the viscosity of the sample. k' is the Huggins constant, μ_0 is the viscosity of the solvent and $c(x)$ is the polymer concentration profile near the cell surface. The concentration profile of the free polymer in the depleted region for the penetration depth of p is approximated by [4, 122, 123]:

$$c(x) = c_b \tanh^2\left(\frac{x - (\delta - p)}{\Delta}\right) \quad (2.6-22)$$

where c_b is the bulk polymer concentration, δ is the glycocalyx thickness and Δ is the thickness of polymer depleted region.

2.6.3 *Electrophoretic mobility ratio*

Having found the equations for velocity profile and potential distribution, one can now calculate the mobility from equation (2.6-9). As a result, the following expression for the ratio of mobility for the particles in a polymer-free solution b_0 and in a solution containing polymer b_p is achieved:

$$\frac{b_0}{b_p} = \frac{\int_0^\infty \psi(x)u_0(x)dx}{\int_0^\infty \psi(x)u_p(x)dx}. \quad (2.6-23)$$

2.7 Concluding Remarks

In this chapter the required theoretical background and the mathematical insight of the major forces involved in the cell-cell interaction were described. The total interaction energy between cells can be found by summing up all the positive and negative interaction energies where positive and negative values indicate repulsion and attraction, respectively.

Among the reviewed analysis provided, the theory of electrophoretic mobility will be used in chapter 3 to compare the theoretical values of mobility ratio with the experimental results which can predict whether or not depletion layer exists around the red blood cell in polymer solutions.

The electrostatic, steric and depletion energies which are illustrated in this chapter will be effectively applied in chapters 4 and 5 to provide a theoretical framework for calculating adhesion energies between a system of two cells (doublets) and determine the impact of cell and suspending phase properties on the cell-cell affinities.

Chapter 3 Can Depletion Play a Role? Experimental Approach

3.1 Introduction

Red cell aggregation is mediated by macromolecules like polymers or proteins; therefore the interaction between cells and surrounding macromolecules is of great importance in studying aggregation. Effects of polymer size on the red cell aggregation has been studied for dextran fractions between 40-500 kDa using optical, ultrasound and viscometric methods [41, 124, 125]. These investigations indicate the increasing trend of aggregate formation with increasing molecular weight of dextran. Dextran has been used vastly for this purpose due to its existence in different sizes. However, a non-monotonous dependence of aggregate formation to polymer-size has been recently observed for high molecular weight dextran ($M > 500kDa$) [126]. In fact, these recent observations suggest an optimum dextran molecular mass of 200-500kDa for inducing aggregation.

Depletion model has been introduced as a likely mechanism of reversible erythrocyte aggregation in plasma and polymer solutions [4, 127]. So far this method is consistent with the reported data for dextran with molecular mass of less than 500kDa [4, 123]. Yet it should be examined how this model can predict the aggregation behavior with higher molecular weight dextran.

In this chapter the effect of high molecular weight dextran on the induction of depletion layer will be examined. The polymer-depleted layer around the cell surface will be studied both theoretically and experimentally for dextran fractions of 40kDa-28MDa followed by a comparison of the results.

One of the methods that can measure and validate the polymer depletion layers around the cell surface is electrophoresis. This method- which is pioneered by Baumler, Donath and co-workers [39, 114, 127]- is considered to be a great tool for studying the conditions near the cell surface. It has been shown that the red blood cells in the solutions containing soluble polymers have marked higher mobility than predicted values [40, 72, 128]. This happens because of the lower viscosity near the cell surface as a result of depletion effect [114].

When a charged particle in solution is exposed to an electric field, it moves. The electrostatic attractive forces between the particle and counter-ions in the solution cause the fluid in proximity to the cell surface to move with the particle. This proximity is defined by slipping plane which is in distance of Debye length from the particle's surface. Hence the mobility is sensitive to the viscosity of the slipping plane. Now if the depletion layer becomes greater than the Debye length, the slipping plane will be located in the depleted region in which the viscosity is lower than the bulk phase. So the effect of bulk viscosity on the mobility is reduced. By changing the ionic strength of samples, Debye length changes and provides the possibility to estimate the depletion layer thickness [114, 129]. As long as the depletion layer is considerably larger than Debye length, the actual mobility will be higher than the predicted mobility value based on bulk viscosity. By increasing the Debye length, i.e. decreasing ionic strength of the sample, the influence of bulk viscosity on the mobility increases and when it becomes significantly greater than the depletion layer thickness, only the bulk phase viscosity influences the measured mobility values.

The present study is designed to examine the extent of induced depletion effect by high molecular mass dextrans.

3.2 Material and Method

3.2.1 *Sample preparation*

Blood was drawn from the antecubital vein of healthy adult volunteers into EDTA (1.5 mg/mL) and all measurements were performed within 6 hours after venesection. RBC was separated from whole blood by 2000g centrifugation for 10 minutes and the plasma and buffy coat removed. The RBC were washed twice in isotonic PBS (0.01M phosphate, 295 mOsm/kg, pH 7.4), then fixed by gentle re-suspension at low hematocrit in PBS containing 2% high-purity glutaraldehyde (Sigma Chemical, St. Louis, MO). Following fixation the cells were washed thrice in PBS and stored at room temperature until use. Dextran fractions with the molecular masses of 40, 70, 500 kDa and 2MDa were obtained from Sigma Chemical, TDB Consultancu AB, Uppsala, Sweden, and Amersham Pharmacia biotech, Uppsala, Sweden, and used as received. The 28 MDa fraction was prepared by dialysis of a commercial dextran with a nominal molecular mass of 22 MDa following which its molecular weight was determined by viscometry. The dextran fractions were dissolved at the desired concentrations in 5 and 15 mM ionic strength buffered saline (1mM phosphate plus appropriate amount of NaCl, pH 6.9) and the fixed RBC were suspended in the dextran solutions at a hematocrit of about 1%.

3.2.2 *Miscellaneous techniques*

RBC suspension hematocrit were determined by an electric hematology analyzer (Micros, ABX Diagnostics, Irvine, CA), solution osmolalities by a freezing-point osmometer (model 5004, Precision Systems, Natick, PA), and pH by an Orion model 410A system (Orion Research, Boston, MA). The viscosity of all suspending media was measured at 25°C by capillary viscometry (Plasma Viscometer II, Coulter

Electronics, Luton, UK). The same equipment was also used to determine the intrinsic viscosity of various polymers by extrapolation of their specific viscosity/concentration ratios to zero concentration [130]. The presence of RBC aggregation was observed microscopically using low hematocrit wet mount of cells suspended in the appropriate medium. RBC electrophoretic measurements at 25°C were carried out using a Malvern ZetaSizer Nano Z automated EPM system (Malvern, Malvern, UK).

3.3 Theoretical Considerations

3.3.1 Viscosity-corrected mobility

To compare the electrophoretic mobility of the cells in different mediums, it is useful to define a parameter which is independent of the medium viscosity. This parameter is called viscosity-corrected mobility and is found by

$$\tilde{b}_p = \frac{\mu_p}{\mu_0} b_p \quad (3.3-1)$$

Subscript p and 0 refer to solution with and without polymer, respectively. b_p is the measured mobility and $\tilde{b}_p$ is the “corrected” mobility for any difference of suspending phase viscosity. So if there is no depletion with the cells suspending in mediums of different viscosity, the viscosity-corrected mobility will be constant and equal to the mobility in polymer free solution b_0 . However, because of the presence of a polymer depleted layer and lower viscosity in the Debye-Hückel region, the corrected mobility becomes greater than b_0 . This provides an indirect measure of depletion layer thickness. Thus the ratio of viscosity-corrected mobility to polymer-free mobility ($\frac{\tilde{b}_p}{b_0}$) will be used from now on in this chapter. When the effect of depletion layer on the viscosity of slipping plane is negligible, this ratio tends to 1. Otherwise it will be higher than 1 because of lower viscosity in the Debye-Hückel region.

To have a quantitative understanding of the experimental data, the theoretical mobility ratios are calculated using equation (2.6-23) with potential function of equation (2.6-11), velocity profiles of equations (2.6-18) and (2.6-19).

Table 3-1 gives the values of intrinsic viscosities for various fractions of dextran. Values of $1nm^{-1}$ [119, 131, 132] and 0.4 [133] are chosen for a and k' respectively. The viscosity of polymer-free solution (μ_0) is measured to be $0.89 mPa.s$ and the hydrodynamic radius of dextran monomer segment r_{seg} is taken as $6.5 \text{ \AA}$ [134]. Furthermore, in these calculations it is assumed that there is no penetration of free polymer into the glycocalyx, i.e. $p = 0$.

Table 3-1 Intrinsic viscosities for various fractions of dextran

Molecular Mass of Dextran (Da)	$[\eta] (\frac{dl}{gr})$
40×10^3	0.17
70×10^3	0.22
500×10^3	0.46
2×10^6	0.57
28×10^6	0.94

3.4 Results

3.4.1 Depletion layer thickness versus Debye-Hückel length

The depletion layer thickness for dextrans of different molecular mass and concentration are calculated using equations (2.2-1) to (2.2-3) with the physiochemical parameters summarized in Table 3-2. The parameters B and R_g have been estimated by Neu and Meiselman [4] for dextran 40 to 500 kDa, and power fitting has been used to estimate these values for dextran 2 and 28MDa. The result is shown in Figure 3-1.

Figure 3-1 Depletion layer thickness as a function of polymer mass and concentration (from [135] with permission)

Table 3-2 Physiochemical properties of dextran

Molecular Mass of Dextran (Da)	$B \text{ (} m^{-5} s^{-2} kg^{-1} \text{)}$	$R_g \text{ (nm)}$
40×10^3	2.5	5.56
70×10^3	1.69	7.36
500×10^3	0.43	19.7
2×10^6	0.16	39.4
28×10^6	0.025	147

As illustrated in the Figure 3-1, depletion layer thickness increases dramatically with increasing polymer weight ranging from 7nm for dextran 40kDa to 200 nm for dextran 28MDa. By increasing polymer concentration, the depletion layer thickness decreases slightly. This decrease is negligible for the concentration range of our studies for dextrans 40kDa to 2MDa. However, for dextran 28MDa, the decreasing trend after 0.5g/dl is significant.

The strong dependence of depletion layer thickness on the molecular mass of dextran, results in a great difference in the concentration of free polymer close to the cell surface. Having calculated the depletion layer thickness, one can find the polymer concentration profile near the cell surface using equation (2.6-22). Figure 3-2 shows the profile concentration profile near the cell surface for bulk polymer concentration of 0.9g/dl.

Figure 3-2 Free polymer concentration profile for different fractions of dextran at bulk concentration of 0.9g/dl (from [135] with permission)

From these calculations, a pronounced difference is observed for different dextran fractions; dextran 40 kDa reaches to the bulk concentration in around 20nm distance from the cell surface while dextran 28MDa reaches to only 25% of the bulk concentration in the distance of 50 nm. This difference causes various magnitudes of viscosity near the cell surface. If full penetration is considered, one can calculate the concentration of polymer at glycocalyx edge for different polymer molecular weight and bulk concentration. This is illustrated in Figure 3-3. The dashed line is used for the situation in which no depletion effects exist near the cell surface, so the concentration of polymer at glycocalyx edge would be the same as bulk phase. But when depletion exists, the concentration of polymer at glycocalyx edge is lower than bulk phase: about 40% of bulk concentration for dextran 40kDa and less than 0.1% for dextran 28MDa.

Figure 3-3 Calculated polymer concentration at the edge of glycocalyx as a function of bulk polymer concentration for different dextran fractions (from [135] with permission)

Since the electrophoretic mobility is a function of the viscosity in the Debye-Hückel region, it is useful to calculate the Debye-Hückel length for both ionic strengths. The Debye length is calculated by means of equation (2.5-6) and is equal to $2.5nm$ and $4.3nm$ for $I = 15mM$ and $5mM$, respectively, which are both less than the calculated depletion layer thicknesses presented in Figure 3-1. This implies that the slip plane is situated inside the depleted region. Consequently, because of lower viscosity in depleted region, mobilities in these polymer solutions are expected to be higher than those predicted by Smoluchowski equation.

3.4.2 *Experimental and theoretical mobility ratios*

The absolute value of electrophoretic mobility for cells suspended in $15mM$ buffered saline at $25^{\circ}C$ is $2.75 \pm 0.05 \times 10^{-8} m^2 V^{-1} s^{-1}$ (mean $\pm$ standard deviation). Figure 3-4 shows the electrophoretic mobility of cells suspended in $0.9g/dl$ dextran of different molecular mass in $15mM$ saline solutions as well as the viscosity of each sample.

Figure 3-4 Measured electrophoretic mobility values (left axis) and sample viscosity (right axis) as a function of Dextran molecular mass (from [135] with permission)

A clear trend is observed for the electrophoretic mobility: by increasing the molecular mass, mobility decreases steadily. Dextran 28MDa has the maximum decreases of about 25% of control polymer-free solution. The bulk viscosity of surrounding medium increases with increasing molecular mass of dextran as illustrated in the Figure 3-4. According to well-known Smoluchowski equation (3.4-1), the electrophoretic mobility is related inversely to the medium viscosity. Hence, the decreasing trend for mobility values with increasing molecular mass of dextran is expected. However, the magnitude of decrease in mobility is not in accordance with the magnitude of increase in viscosity. The increase in viscosity is much sharper than the decay of mobility; for instance, the viscosity of dextran 28 MDa increases by about 250% relative to control solution while the decrease in mobility is about 25%.

$$b = \frac{\varepsilon \zeta}{\mu}. \quad (3.4-1)$$

This marked discrepancy between the magnitude of viscosity increase and mobility decrease indicates a reduction of viscosity inside the Debye-Hückel atmosphere suggesting the polymer depletion.

In order to evaluate depletion effect for high molecular weight dextrans, RBC mobility is measured in 5 and 15 mM saline solution containing dextran with various concentrations and molecular weights. The experimental mobility ratios are shown in Figure 3-5 in symbols for saline concentration of 15mM (a) and 5mM (b). The experimental data clearly demonstrate that for all dextran fractions, the mobility ratio steadily increases with polymer concentration. Also for each concentration the ratio increases by increasing molecular mass of dextran. While for smaller polymers, the increase in mobility is less pronounced, the rate of increase for larger polymers is higher. For instance, the increase of about 240% is observed for dextran 28MDa in ionic strength of 5mM whereas for dextran 40kDa this increase is only about 110%. These experimental values strongly imply the existence of polymer depletion layer near the cell surface. The theoretical mobility ratios are calculated and shown in Figure 3-5 by solid lines.

Figure 3-5 Experimental (symbols) and theoretical (solid lines) of mobility ratio as a function of polymer size and concentration for (a) $I = 15 \text{ mM}$ and (b) $I = 5 \text{ mM}$ (from [135] with permission)

The theoretical and experimental results both show similar trend in the viscosity-corrected mobility ratios: increase steadily with increasing molecular weight and concentration. In ionic strength of 15mM, theoretical values for dextran 40 and 70kDa are in good agreement with experimental results (Figure 3-5-a). By increasing molecular mass and concentration, the theoretical mobility ratios become higher than experimental values. This discrepancy reaches to maximum for 0.9g/dl of dextran 28MDa where the theoretical mobility ratio of 2.6 is about 40% higher than measured value of 1.8. For ionic strength of 5mM (Figure 3-5-b), the agreement between theoretical and experimental data is much more significant. At this ionic strength the theoretical mobility ratio for 0.9g/dl dextran 28MDa is only 10% higher than measured value. This difference in magnitude further reduces to less than 3% for other fractions.

3.5 Discussion

Comparison between the experimental and theoretical data of a wide range of dextran fractions from 40kDa to 28MDa strongly supports existence of polymer depleted layer around the cell surface. Nevertheless, the difference between theoretical and experimental results in Figure 3-5 requires further attention.

First it should be noted that the cell mobility depends not only on the bulk polymer size and concentration, but also to some extent on the polydispersity of the polymer. For polydisperse high molecular mass fractions of dextran (2MDa and 28MDa) which contains smaller polymer molecules, the concentration of smaller polymers in the depletion layer is higher than larger molecules by several orders of magnitude. Existence of smaller molecules in the depletion layer, results in an increase in the viscosity of this region. For smaller polymers (40 kDa and 70kDa) this polydispersity is less significant. Note that the dextrans used in this study were commercial dextrans and were not specially processed for a narrow molecular weight distribution. This is one reason for the higher theoretical predictions of mobility ratio than experimentally obtained values (Figure 3-5).

Next, the slight difference between theoretical and experimental values might arise from the assumption that depletion layer around the moving particle is formed instantaneously. In reality depletion layer is formed in a characteristic time the polymer needs to diffuse over a distance equal to the thickness of the depletion layer. However, the effects of time on the formation of depletion layers are still poorly understood [136].

Then, this difference between theoretical and experimental data might arise from the assumption that we made in calculating the electrophoretic mobility in the presence of

polymer-depleted region. The fluid has been assumed as a continuum phase and Navier-Stokes equation has been solved to calculate the mobilities. However, for our problem, continuum assumption might not be fully correct since the size of the polymers can be in the same order of magnitude or larger than the depletion layer thickness and the characteristic length scale of velocity change in the moving fluid is comparable with the inhomogeneities of the fluid in the depletion layer. This can describe the increasing discrepancy between theoretical and experimental results by increasing molecular weight of the polymer.

Finally, it should be noted that in the plotted theoretical data, it is assumed that there is no penetration of free polymer into the glycocalyx. This assumption seems reasonable for the polymer concentration range used herein (i.e. up to 0.9 g/dl) [4]. However, penetration of smaller polymers into the glycocalyx is strongly suggested [4]. The calculations are performed again assuming full penetration into the glycocalyx. The reduction in theoretical mobilities ranged from less than 1% for the 28MDa dextran to about 7% for dextran 40kDa. This 1-7% reduction in theoretical data makes it difficult to distinguish between polymer penetration and no penetration.

In conclusion, the overall agreement between the experimental results and theoretical data is clear and strongly suggests a depletion layer around the cell surface which is more dominant for higher molecular weight dextrans.

Chapter 4 Impact of Cellular Properties on Red Cell-Cell Affinity in Plasma-Like Medium- Theoretical Approach Considering Electrostatic and Depletion Interactions with Uniform Glycocalyx Structure

4.1 Introduction

The alterations in the extent of aggregation are defined by cellular parameters or alteration in plasma proteins [123]. However, the factors determining altered aggregation in diseased states are not clearly known. In fact, no cellular factor has been clearly identified for this phenomenon yet [5]. This lack of information is mainly due to uncertainty regarding the specific mechanism involved in the aggregation process and the inability to establish cause-effect relations. Hence providing a theoretical framework for describing the effects of different cellular and polymeric parameters on the aggregation will be of great advantage.

Alterations in the surface charge densities, glycocalyx structure, free polymer properties and concentration, as well as variations in interactions between the glycocalyx and free polymers, all can be reasons for the changes of interaction energy between erythrocytes in the pathological conditions.

So, in the following two chapters it is aimed to provide a theoretical model based on the depletion mechanism. The adhesion energy between cells will be calculated by considering repulsive and attractive forces which are involved during the cell-cell interactions. The effect of various cellular and medium parameters on the RBC affinities in the polymer solutions will be studied and, where possible, compared to available experimental data.

In this chapter, we start with a simplified model in which a simple glycocalyx structure is used and only depletion and electrostatic interactions are taken into account.

Next chapter deals with a more comprehensive model by considering other profiles for glycocalyx structure and also considers the steric interactions in addition to depletion and electrostatic energies.

4.2 Model Description

4.2.1 Assumptions

Let's assume a system of two cells that are brought close together by means of shear flow, Brownian motion, sedimentation, or any other way. Cells are close enough so that short-ranged forces can act on them. As a simple model, it is assumed that only electrostatic repulsive and depletion attractive energies exist in the system. Both cells are identical with an attached glycocalyx layer of thickness δ and surface charge density of σ . As another assumption and for sake of simplicity, the polymer segments in the glycocalyx are considered to have a uniform distribution. Assuming a uniform distribution of charge on each segment leads to uniform profile of charge density throughout the glycocalyx thickness. Simple linear profile has been chosen for the free polymer in the depletion region which starts from 0 at $x=\delta-p$ and reaches to bulk concentration φ_b at $x = \Delta$. More accurate profiles can be chosen for free polymer concentration near the cell surface, i.e. Guassian distribution predicted by Flory [137] or the profile of (2.6-22) introduced by de Gennes [122]. However, in these cases problems will arise in integration and obtaining analytical expressions for the penetration depth [69]. The schematic illustration of this model is shown in Figure 4-1.

Figure 4-1 Schematic illustration of the concentration profiles for a system of two identical cells; uniform concentration profile $\bar{\varphi}_a$ has been chosen for the glycocalyx while linear profile $\varphi_f(x) = \varphi_b \frac{x+p-\delta}{\Delta}$ has been selected for the free polymer- Cells are located at a distance $2d$. Subscripts a, b and f relate to the attached, bulk and free polymer respectively.

4.2.2 Polymer penetration into the glycocalyx

The polymer penetration into the surface is described in section 2.3. Free polymers penetrate into the glycocalyx until the local osmotic pressure in the penetration zone is balanced with the bulk osmotic pressure. The penetration depth is calculated using equations (2.2-3), (2.3-1), and (2.3-2).

The penetration depth for the profiles shown in Figure 4-1 is found to be:

$$p_u = \frac{\left(\frac{c_b}{M}RT + Bc_b^2\right)v_1\Delta}{kT\varphi_b\varphi_a^*} \quad (4.2-1)$$

where $\varphi_a^* = (1 - 2\chi)\bar{\varphi}_a$ and subscript u indicates the uniform distribution. In this approach, δ is assumed to be constant and independent of bulk polymer concentration. In a more realistic model, the collapse of glycocalyx under the osmotic pressure of bulk polymer can also be considered [106]. However, it is difficult to accurately apply this model for RBCs in polymer or a protein solution, since too little is known about the physiochemical properties of the glycocalyx, and in particular, about the interaction between the glycocalyx and different polymers or proteins.

4.2.3 *Physiochemical properties of cells and suspensions*

Published experimental data for polymer-induced aggregation have mainly used dextran which is a water soluble neutral polymer [36, 138-140]. Thus, in order to provide a comparison between the theoretical model and experimental data, dextran is also used in the current model as the bulk free polymer.

This polymer exists in various molecular weights and is composed of a long chain of glucose segments attached together by 1:6 alpha links with some 1:3 and 1:4 links. The physiochemical properties of dextran have been estimated from various reports by Neu and Meiselman [4]. The radius of gyration R_g of dextran is calculated by [141]:

$$R_g = 0.8802M^{0.5} (nm) \quad (4.2-2)$$

and the second virial coefficient B is set to be

$$B = 33M^{-0.7} (m^5 s^{-2} kg^{-1}) \quad (4.2-3)$$

by fitting a power function to the estimated data for dextran 40-500kDa [4]. In these equations M is the molecular mass of dextran. Table 4-1 shows the estimated values for second virial coefficient and radius of gyration for different dextran fractions.

The surface charge density of red blood cells is assumed to be $0.036 \text{ C}/m^2$ [131, 142]. The Debye parameter is calculated by means of equation (2.5-6). Using this equation, the corresponding Debye length for isotonic phosphate buffered saline solution would be equal to 0.76 nm.

Table 4-1 Estimated values for radius of gyration and second virial coefficient for different molecular weight dextrans

MW (kDa)	40	70	150	250	500	1000	1500
$B(m^5 s^{-2} kg^{-1})$	2.49501	1.68634	0.98912	0.69176	0.42583	0.26213	0.19736
R_g (nm)	5.56	7.36	10.8	13.9	19.7	27.8	34.1
MW (kDa)	2000	5000	10000	15000	20000	25000	28000
$B(m^5 s^{-2} kg^{-1})$	0.16136	0.08496	0.05230	0.03938	0.03219	0.02754	0.02544
R_g (nm)	39.3	62.2	87.9	108	124	139	147

4.2.4 Total interaction energies

4.2.4.1 Electrostatic energy

The electrostatic interaction energy is calculated using equation (2.5-17) by substituting the corresponding potential function $\psi(x)$ for uniform charge density distribution in the glycocalyx. The electric potential as a function of distance from the cell surface can be found using equation (2.5-12) for left half space. The charge distribution is shown in Figure 4-2. As it is illustrated in the figure, for cell-cell distance smaller than 2δ , in the section between $2d - \delta$ and d , the charge density will be twice of the cell's charge density. This happens because of the overlap of charged layers when the distance between them is smaller than twice of glycocalyx thickness.

Figure 4-2 Charge distribution for different cell-cell distances: (a) $2d > 2\delta$, (b) $2d < 2\delta$. In the $[2d - \delta, \delta]$ region, the charge density is twice of the cell's charge density due to the overlap of charged layers

For the case in which the distance between cells is greater than 2δ , the electrostatic potential for half of the system is:

$$\psi_1(x) = \frac{1}{\kappa \epsilon \epsilon_0} \left[\frac{\cosh(\kappa x)}{\sinh(\kappa d)} \int_0^\delta \rho_u \cosh(\kappa(x-d)) dx \right. \\ \left. + \begin{cases} \int_0^x \rho_u \sinh(\kappa(\xi-x)) d\xi & x < \delta \\ \int_0^\delta \rho_u \sinh(\kappa(\xi-x)) d\xi & x > \delta \end{cases} \right] \quad (4.2-4)$$

while for the case where the distance between cells is smaller than 2δ , it will be:

$$\begin{aligned}
\psi_1(x) &= \frac{1}{\kappa \varepsilon \varepsilon_0} \left[\frac{\cosh(\kappa x)}{\sinh(\kappa d)} \left(\int_0^{2d-\delta} \rho_u \cosh(\kappa(x-d)) dx + \int_{2d-\delta}^d 2\rho_u \cosh(\kappa(x-d)) dx \right) \right. \\
&\quad \left. + \begin{cases} \int_0^x \rho_u \sinh(\kappa(\xi-x)) d\xi & x < 2d-\delta \\ \int_0^{2d-\delta} \rho_u \sinh(\kappa(\xi-x)) d\xi + \int_{2d-\delta}^x 2\rho_u \sinh(\kappa(\xi-x)) d\xi & x > 2d-\delta \end{cases} \right] \quad (4.2-5)
\end{aligned}$$

It should be noted that equations (4.2-4) and (4.2-5) give the potential for left half space of the system and because of symmetry the potential of the right half space is the mirror image of abovementioned potentials.

Using these potentials, the electrostatic interaction energy is calculated to be:

$$\begin{aligned}
W_E(d) &= \frac{\rho_u^2}{\kappa^3 \varepsilon \varepsilon_0} \begin{cases} \sinh(\kappa \delta) (e^{\kappa \delta - 2\kappa d} - e^{-2\kappa d}) & d \geq \delta \\ (2\kappa \delta - 2\kappa d) - (e^{-\kappa \delta} + 1) \sinh(\kappa \delta - 2\kappa d) - \sinh(\kappa \delta) e^{-2\kappa d} & d < \delta \end{cases} \quad (4.2-6)
\end{aligned}$$

4.2.4.2 Depletion energy

The depletion energy can be found using equation (2.2-8) for $d \leq \Delta + \delta - p$ where d is half of the distance between cells. For $d > \Delta + \delta - p$, depletion interaction energy is zero.

$$W_D(d) = \begin{cases} -2\Pi(\Delta + \delta - p - d) & d \leq \Delta + \delta - p \\ 0 & d > \Delta + \delta - p \end{cases} \quad (4.2-7)$$

4.2.4.3 Total interaction energy

Total interaction energy is obtained by summing up the depletion and electrostatic energies (equations (4.2-6) and (4.2-7)):

$$W_T(d) = W_D(d) + W_E(d) \quad (4.2-8)$$

4.3 Results

4.3.1 Initial considerations

Total interaction energy is calculated for concentrations from 0.25 g/dl to 4 g/dl of different molecular weight dextrans and the result is shown in Figure 4-3-a and corresponding cell-cell separation distance is presented in Figure 4-3-b.

Figure 4-3 (a) Calculated dependence of interaction energy on molecular mass and concentration of dextran, (b) Cell-cell distance as a function of polymer weight and concentration (from [143] with permission).

The cell-cell distance is calculated by finding the distance which has the minimum value of interaction energy for each polymer mass and concentration. In these considerations the glycocalyx volume fraction is set equal to zero. The value of zero represents an ideal soft glycocalyx in which free polymer can penetrate freely, i.e. $p = \delta$. When the volume fraction is equal to one, the glycocalyx acts as a hard surface which does not permit any penetration of macromolecules or $p = 0$.

As illustrated in Figure 4-3, the attractive interaction energy is observed beyond a minimal molecular mass. After this minimal mass, the adhesion energy increases until a certain molecular weight and by increasing molecular weight any further, the energy declines toward zero. The minimal molecular mass for the polymer concentration

of 0.25g/dl is about 40kDa. This molecular mass increases to about 60kDa in high polymer concentration of 4g/dl . The molecular mass in which the maximal adhesion energy is observed is about 200kDa which depends, to some extent, on the bulk polymer concentration. If concentration is increased from 0.25g/dl to 4g/dl , the molecular mass shifts from 175kDa to 230 kDa approximately. The total interaction energy also depends strongly on the bulk polymer concentration. For the given range, by increasing the concentration from 0.25g/dl to 4g/dl , the interaction energy changes from $-0.6\text{ }\mu\text{J/m}^2$ to $-7.8\text{ }\mu\text{J/m}^2$.

Figure 4-3-b suggests a strong dependence of cell-cell distance on the bulk polymer mass and concentration. For the given range, doubling the concentration results in 1nm reduction of cell-cell separation distance which is independent of the dextran molecular mass. It is also clear from the Figure 4-3-b that the cell-cell distance is always greater than twice of glycocalyx thickness (10nm) for the given range which justifies that the forces other than electrostatic and depletion can be neglected.

4.3.2 Impact of glycocalyx volume fraction on cell-cell affinity

The impact of glycocalyx volume fraction on the adhesion energy between cells is shown in Figure 4-4-a, while Figure 4-4-b represents the penetration depth for each polymer mass and glycocalyx volume fraction. The bulk polymer concentration is set equal to 1g/dl and glycocalyx thickness is 5nm . The glycocalyx volume fraction is varied from 0 to 1, where the values zero and 1 represent the perfect soft and hard surface, respectively. Again like previous section, in each volume fraction, attractive interaction is observed above a minimal molecular mass. Above this minimal molecular mass the adhesion energy increases followed by an asymptotic decline toward zero. The threshold of this minimal molecular mass decreases with increasing

volume fraction or decreasing glycocalyx penetrability. The threshold for volume fraction zero is 40kDa while increasing the volume fraction to 0.005, gives a threshold of 12kDa.

Figure 4-4 (a) Interaction energy as a function of molecular mass and glycocalyx volume fraction, (b) Calculated penetration depth as a function of polymer molecular mass and glycocalyx volume fraction. Bulk polymer concentration is considered constant and equal to 1 gr/dl and glycocalyx thickness is 5 nm (from [143] with permission)

The minimal interaction energy (maximal adhesion energy) and the molecular mass in which the minimal interaction energy occurs depend strongly on the glycocalyx volume fraction; increasing the volume fraction leads to an increase in maximal adhesion energy between cells and a decrease in dextran molecular mass in which the interaction energy is minimal. For instance increasing the volume fraction from zero to 0.05 causes a more than two fold increase in the maximal interaction energy from $-2.8 \mu\text{J}/\text{m}^2$ to $-6.1 \mu\text{J}/\text{m}^2$, with its position shifting from 170kDa to 44kDa.

4.3.3 Impact of glycocalyx thickness on cell-cell affinity

The effect of glycocalyx thickness on the adhesion energy is shown in Figure 4-5. The bulk polymer concentration is held constant at 1 g/dl . The figure clearly illustrate that by doubling the glycocalyx thickness (from 5 to 10, or 10 to 20 nm), the peak energy decreases by about 50%. By increasing the glycocalyx thickness, the threshold for the onset of attraction shifts towards higher molecular mass. The reason is that for smaller molecular mass, the depletion zone will be within the glycocalyx thickness and the depletion effect is compensated by the glycocalyx structure.

Figure 4-5 Interaction energy as a function of molecular mass and glycocalyx thickness (from [143] with permission)

4.3.4 Red blood cell affinity

The calculations in previous sections demonstrated the strong dependence of the cell-cell affinity on the cellular properties in the dextran solutions. To have an overall understanding about the effect of polymer size and concentration on the cell-cell affinity, landscapes of Figure 4-6 to Figure 4-8 have been calculated. These landscapes present the dependence of adhesion energy between cells on the polymer size and concentration for glycocalyx thickness of 6 nm (Figure 4-6), 5 nm (Figure 4-7) and 4 nm (Figure 4-8). Horizontal and vertical axes represent the polymer

concentration and molecular mass respectively while the curves are lines of constant interaction energy in $\mu J/m^2$. The limiting case of soft surface ($\varphi_a = 0$) is considered in all the landscapes.

Figure 4-6 Lines of constant total interaction energy ($\mu J/m^2$) for glycocalyx thickness of $6nm$ $\varphi_a = 0$, $\sigma = 0.036 C/m^2$; Minimum energy occurs at molecular mass of 312 kDa and concentration of 3.16 g/dl.

Figure 4-7 Lines of constant total interaction energy ($\mu J/m^2$) for glycocalyx thickness of $5nm$ $\varphi_a = 0$, $\sigma = 0.036 C/m^2$; Minimum energy occurs at molecular mass of 216 kDa and concentration of 3.6 g/dl.

Figure 4-8 Lines of constant total interaction energy ($\mu\text{J}/\text{m}^2$) for glycocalyx thickness of 4nm $\phi_a = 0$, $\sigma = 0.036\text{ C}/\text{m}^2$; Minimum energy occurs at molecular mass of 149 kDa and concentration of $4.11\text{ g}/\text{dl}$.

In the given range, both $W_T - c_b$ and $W_T - M$ relations are bell-shaped and concave toward the center. This results in a central minimum of interaction energy. The magnitude and position of this minimal energy varies dramatically with changing glycocalyx thickness. For $\delta = 6\text{nm}$, the minimal energy is $-6.09\text{ }\mu\text{J}/\text{m}^2$ at $3.16\text{ g}/\text{dl}$ and 312 kDa . For $\delta = 5\text{nm}$, the concentration of minimal energy increases to $3.6\text{ g}/\text{dl}$ and the molecular mass of dextran decreases to reach 216 kDa with the energy magnitude of $-7.91\text{ }\mu\text{J}/\text{m}^2$. By decreasing glycocalyx thickness further to $\delta = 4\text{nm}$, the position of minimal energy shifts to $4.11\text{ g}/\text{dl}$ and 149 kDa with the magnitude of $-10.79\text{ }\mu\text{J}/\text{m}^2$.

4.4 Discussion

So far, it is not possible to quantitatively compare the theoretical calculated energies and the extent of aggregation obtained experimentally, yet a relation between these two can be found. Neu et al [126] have reported the maximum aggregation for molecular mass of about 500kDa which is almost in the same range as predicted theoretically in Figure 4-3-a. It is also known that the maximal aggregation occurs in the range of $3 - 4g/dl$ with the threshold of 40kDa to 70ka for observation of attractive energy [41]. These experimental observations are in agreement with the model, provided that the glycocalyx volume fraction becomes less than 10^{-3} for the glycocalyx thickness of 5nm.

One drawback of the current model might arise from its simplicity. Other factors should be included into the model to provide better estimation of the experimental results. For example, it might be necessary to focus on more realistic models of the RBC glycocalyx (e.g., structure). Charge distribution within the glycocalyx also needs to be considered since it has a significant impact on the electrostatic interactions between adjacent surfaces [144]. These parameters will be discussed and inserted into the model in the next chapter.

Chapter 5 Impact of Cellular Properties on Red Cell-Cell Affinity in Plasma-Like Medium- Theoretical Approach Considering Electrostatic, Depletion and Steric Interactions with Various Glycocalyx Structures

5.1 Introduction

In our previous model of cell-cell aggregation (Chapter 4), only two kinds of forces were considered: electrostatic repulsion and depletion attraction. Furthermore, the distribution of the glycocalyx segments was assumed to be uniform. Although all these simplifications had been made, still the model was capable of explaining the extent of aggregation.

In this chapter, we are going to consider the effects of more striking factors to enhance the previous model. Glycocalyx structure, various potential distributions as well as steric interactions will be taken into account in order to approach to a more realistic model.

5.2 Model Description

5.2.1 Glycocalyx Structure

Glycocalyx is the extracellular polymeric structure of the cell membrane consisting of polysaccharide chains which form a layer with thickness δ on the outer surface of the lipid bilayer. When cells are in close proximity, these layers interact with each other. Therefore, the structure of this attached layer and distribution of polymer segments in it is of great importance.

The red cell membrane is composed of 49.2% (w/w) protein, 43.6% (w/w) lipids and the remaining 7.2% of carbohydrates [145]. The glycocalyx structure is composed of glycolipids and glycoprotein. Glycolipids located mostly on the extracellular face of membrane, are consisting about 10% of total $5 \times 10^{-10}mg$ of membrane lipid.

The membrane proteins are divided into three main categories (Figure 5-1):

1. Cytoskeletal proteins, like spectrin, protein 4.1 and actin, which form a cytoskeletal network in the interior region under the lipid bilayer;
2. Integral proteins, which are embedded in and passing through the lipid bilayer;
3. Anchor proteins, like ankyrin and protein 4.2, which interconnect the other two kinds of proteins.

Figure 5-1- Schematic representation of red cell membrane [146]

Among all these different proteins, integral proteins, like band 3 and glycophorins, are categorized as glycoproteins which contribute to formation of glycocalyx with their attached carbohydrate chains. Table 5-1 shows the number of different glycoproteins per cell.

Table 5-1 Name and number of glycoproteins per cell [147]

Protein name	Number per cell x 10 ³
Band 3	1200
GPA	1000
GPC	143
GPB	150
GPD	82

The carbohydrate of the cell membrane is distributed in glycolipids and glycoproteins. This amount is approximately $8.257 \times 10^{-14} gr$ per cell. Marchant *et al* in their patent have proposed that the coating glycocalyx can be modeled by a neutral oligosaccharide chain having a molecular weight less than 7kDa [148]. Dextran is one of their suggestions for the membrane-attached polymer. We rely on this assumption for estimating the cell-attached polymer concentration. Dextran chain is estimated by

a number of glucose segments. Since the mass of one mole of glucose is 180.16 gr, totally 2.759×10^8 glucose segments are predicted per red blood cell. The surface area of normal red cells with a biconcave disk shape is about $140 \mu\text{m}^2$ [146], leading to approximately 1.97×10^{18} segments per square meter of the cell surface area.

For the distribution of these segments along the glycocalyx thickness, three different profiles are going to be considered:

- Uniform distribution: $\rho_a(x) = \bar{\rho}_a$
- Pseudo-tail distribution: $\rho_a(x) = \bar{\rho}_a \left(1 + \frac{2x}{\delta} - \frac{3x^2}{\delta^2}\right)$
- Linear distribution: $\rho_a(x) = 2\bar{\rho}_a \left(1 - \frac{x}{\delta}\right)$

Where the subscript a indicates attached layer, $\bar{\rho}_a$ and δ are the average segment density and the glycocalyx length, respectively. x is the direction normal to cell surface and the origin of coordinate system is set on the cell surface as illustrated in Figure 5-2. Figure 5-3 shows the schematic shape of the abovementioned profiles.

Figure 5-2 Schematic representation of glycocalyx structure composing of small glucose segments; x-axis is normal to the cell surface with its origin located at outer layer of the lipid bilayer

A uniform segment distribution should be a proper assumption for terminally attached low molecular mass polymers [149] and this corresponds to the model first developed by Fischer [149] and by Ottewill and Walker [35]. Yet, by increasing the molecular mass of the attached polymers, uniform distribution becomes less applicable. For

higher molecular mass attached chains, (say $M > 2\text{kDa}$), pseudo-tail distribution presented by Vincent *et al* [149] should be a more reasonable model, which is a simplified mathematical form of the skewed Gaussian form derived by Hesselink [95]. Since the molecular mass of carbohydrate chains on the cell membrane are different and in the range of around 1.3kDa for glycoporphins (inferred from [147]) and about 3-8 kDa for band-3 proteins [150] both distributions have been considered. A linear profile has also been considered for this comparison [69].

Figure 5-3 Segment distribution as a function of distance from the cell surface for uniform, linear and pseudo-tail profiles

It is also assumed that the surface charges are distributed homogenously on each segment. Hence, any arbitrary segments distribution results in the same distribution of charges in the glycocalyx.

5.2.2 Glycocalyx volume fraction and steric interactions

Glycocalyx structure is modeled by glucose segments and three different segment density functions are chosen in accordance with section 5.2.1. The assumptions are as follows:

- 1- The total number of segments in the glycocalyx is the same and only the distribution along the glycocalyx thickness is different
- 2- The segment distribution remains intact during steric interactions
- 3- The cells are similar with attached layer of thickness δ and Flory interaction parameter of χ .

Due to the high electrostatic and steric repulsion, it is assumed that cells never reach to the “interpenetration+ compression” domain. The assumption validity will be examined in the result section. In this case, one only needs to consider the last term of R in equation (2.4-2).

5.2.2.1 Uniform segment distribution

In this case the segments are distributed uniformly all over the thickness δ , so the average segment density is equal to:

$$\rho_a(x) = \bar{\rho}_a = \frac{\sigma_a}{\delta} \quad (5.2-1)$$

where σ_a is the number of segments per m^2 of the cell surface. The uniform segment distribution is shown schematically in Figure 5-4.

Figure 5-4 Schematic representation of uniform segment distribution- in the penetration zone ($2d - \delta$ to δ) the segment density of two cells, are added together.

For the interpenetration zone, only the last term of equation (2.4-2) have to be calculated:

$$\int_0^\delta A \bar{\rho}_a dx = 1 \Rightarrow A = \frac{1}{\bar{\rho}_a \delta} \Rightarrow \hat{\rho}_a = \frac{1}{\delta} \quad (5.2-2)$$

$$R_i = \int_0^d \hat{\rho}_a \hat{\rho}'_a dx = \int_{d-\delta}^\delta \frac{1}{\delta^2} dx = \frac{2\delta - d}{\delta^2} \quad (5.2-3)$$

5.2.2.2 Linear segment distribution

For linear segment distributions in the form of $\rho_a(x) = ax + b$ and $\rho'_a(x) = -a(x - d) + b$, values of A , normalized segment density and R_i are as follows:

$$\begin{aligned} \int_0^\delta A(ax + b) dx = 1 &\Rightarrow A = \frac{1}{\frac{a\delta^2}{2} + b\delta} \Rightarrow \hat{\rho}_a = \frac{ax + b}{\frac{a\delta^2}{2} + b\delta} \\ \int_{d-\delta}^d A(-a(x - d) + b) dx = 1 &\Rightarrow A = \frac{1}{\frac{a\delta^2}{2} + b\delta} \Rightarrow \hat{\rho}'_a \\ &= \frac{-a(x - d) + b}{\frac{a\delta^2}{2} + b\delta} \end{aligned} \quad (5.2-4)$$

$$R_i = \int_0^d \hat{\rho}_a \hat{\rho}'_a dx = \int_{d-\delta}^\delta \frac{(ax + b)(b - a(x - d))}{(\frac{a\delta^2}{2} + b\delta)^2} dx \quad (5.2-5)$$

Where a and b are $\frac{-2\sigma_a}{\delta}$ and $2\sigma_a$ respectively.

5.2.2.3 Pseudo-tail segment distribution

Segment profiles for pseudo-tail distributions follow the functions

$\rho_a = ax^2 + bx + c$ and $\rho'_a = a(x - d)^2 - b(x - d) + c$ where a , b and c are $\frac{-3\sigma_a}{\delta^2}$, $\frac{2\sigma_a}{\delta}$ and σ_a , respectively. Values of A , normalized segment density and R_i will be:

$$\int_0^\delta A(ax^2 + bx + c) dx = 1 \Rightarrow A = \frac{1}{\frac{a\delta^3}{3} + \frac{b\delta^2}{2} + c\delta}$$

$$\Rightarrow \hat{\rho}_d = \frac{ax^2 + bx + c}{\frac{a\delta^3}{3} + \frac{b\delta^2}{2} + c\delta} \quad (5.2-6)$$

$$\int_{d-\delta}^d A(a(x-d)^2 - b(x-d) + c) dx = 1 \Rightarrow A = \frac{1}{\frac{a\delta^3}{3} + \frac{b\delta^2}{2} + c\delta}$$

$$\Rightarrow \hat{\rho}'_d = \frac{a(x-d)^2 - b(x-d) + c}{\frac{a\delta^3}{3} + \frac{b\delta^2}{2} + c\delta}$$

$$R_i = \int_0^d \hat{\rho}_d \hat{\rho}'_d dx \quad (5.2-7)$$

$$\Rightarrow R_i = \int_{d-\delta}^\delta \frac{(ax^2 + bx + c)(a(x-d)^2 - b(x-d) + c)}{(\frac{a\delta^3}{3} + \frac{b\delta^2}{2} + c\delta)^2} dx$$

By substituting the values of R_i (equations (5.2-3), (5.2-5) and (5.2-7)) in equation (2.4-1), the steric mixing energy per unit area of cell surface for each distribution is obtained.

5.2.3 Penetration depth calculation

Using equations (2.2-3), (2.3-1) and (2.3-2), one can calculate the polymer penetration depth into the glycocalyx.

A linear distribution in the form of $\varphi_f = \frac{\varphi_b}{\Delta}(\delta - p + \Delta)$ is considered for free polymer inside the depletion layer as depicted in Figure 5-5. φ_b is the bulk polymer volume fraction, p is the penetration depth and Δ is the depletion layer thickness.

Figure 5-5 Free and attached polymer profiles, subscripts a, b and f are related to the attached, bulk and free polymers, respectively.

To find the volume fraction of free polymer, i.e. the volume occupied by polymer molecules in the solution, we need to know the concentration and density of the polymer. Bulk volume fraction then can be found by dividing bulk polymer concentration c_b to polymer density ρ :

$$\varphi_b = \frac{c_b}{\rho} \quad (5.2-8)$$

It is assumed that each mole of dextran is composed of n mole of glucose segments. So $M_{Dex} = nM_{glucose}$ and $v_{Dex} = nv_{glucose}$ in which M and v are molar mass and volume respectively. The density of dextran is estimated to be:

$$\rho_{Dex} = \frac{M_{Dex}}{v_{Dex}} = \frac{nM_{glucose}}{nv_{glucose}} = \rho_{glucose} = 1.54 \text{ gr/cm}^3 \quad (5.2-9)$$

Volume fraction of the attached layer is calculated by the following equation:

$$\bar{\varphi}_a = \frac{M_{glucose}}{\delta N_A \rho_{glucose}} v \cdot i \quad (5.2-10)$$

where N_A , δ , v and i are Avogadro's number, glycocalyx thickness, the number of chains per unit area and the number of segments per chain, respectively. Attached layer volume fraction obeys the same uniform, linear and pseudo-tail distributions. i.e.:

$$\begin{aligned}
\varphi_a(x)|_{uniform} &= \bar{\varphi}_a \\
\varphi_a(x)|_{linear} &= 2\bar{\varphi}_a(1 - \frac{x}{\delta}) \\
\varphi_a(x)|_{psudotail} &= \bar{\varphi}_a(1 + \frac{2x}{\delta} - \frac{3x^2}{\delta^2})
\end{aligned} \tag{5.2-11}$$

Solving equation (2.3-2), gives the following results for penetration depth:

Uniform distribution:

$$p_u = \frac{\left(\frac{c_b}{M}RT + Bc_b^2\right)v_1\Delta}{kT(1 - 2\chi)\bar{\varphi}_a\varphi_b} \tag{5.2-12}$$

Linear distribution:

$$p_L = \sqrt{\frac{\left(\frac{c_b}{M}RT + Bc_b^2\right)v_1\Delta\delta}{kT(1 - 2\chi)\bar{\varphi}_a\varphi_b}} \tag{5.2-13}$$

Pseudo-tail distribution:

$$-p_P^3 + 2\delta p_P - \frac{\left(\frac{c_b}{M}RT + Bc_b^2\right)v_1\Delta\delta^2}{kT(1 - 2\chi)\bar{\varphi}_a\varphi_b} = 0 \tag{5.2-14}$$

Equation (5.2-14) is a third order polynomial solved by MATLAB, and among the three roots the real *and* positive answer is chosen as the penetration depth.

5.2.4 *Electrostatic energy*

Theory of electrostatic interactions is described in section 2.5. The electrostatic potential and electrostatic energy are found using equations (2.5-12), (2.5-16) and (2.5-17). In equation (2.5-12), the potential is for half space ($0 < x < d$) and due to the symmetry of the structure, the potential of second half space ($d < x < 2d$) is

simply a mirror image of the first one. It should be noted that the full space potential is used in equation (2.5-16). Electrostatic potential and energy can be found only when the charge density distribution function of glycocalyx ($\rho_{fix}(x)$) is known. In Chapter 4, the uniform charge distribution was used. In the solutions of high ionic strength, e.g., plasma, charge distribution can have a greater effect on electrostatic repulsion between cells than the charge density itself. So assumption of uniform charge distribution can be of huge simplification. With regard to the glycocalyx structure described in section 5.2.1, linear and pseudo-tail in addition to uniform distribution are going to be used for calculating electrostatic repulsion between cells.

5.2.4.1 Linear charge distribution profile:

For $2d > 2\delta$

$$\rho_l(x) = \begin{cases} -\frac{2\sigma}{\delta^2}x + \frac{2\sigma}{\delta} & 0 < x < \delta \\ 0 & \delta < x < 2d - \delta \\ \frac{2\sigma}{\delta^2}(x - 2d) + \frac{2\sigma}{\delta} & 2d - \delta < x < 2d \end{cases}$$

(5.2-15)

For $\delta < 2d < 2\delta$

$$\rho_l(x) = \begin{cases} -\frac{2\sigma}{\delta^2}x + \frac{2\sigma}{\delta} & 0 < x < 2d - \delta \\ \frac{4\sigma}{\delta} - \frac{4\sigma}{\delta^2}d & 2d - \delta < x < \delta \\ \frac{2\sigma}{\delta^2}(x - 2d) + \frac{2\sigma}{\delta} & \delta < x < 2d \end{cases}$$

5.2.4.2 Pseudo-tail charge distribution profile:

For $2d > 2\delta$

$$\rho_p(x) = \begin{cases} -\frac{3\sigma}{\delta^3}x^2 + \frac{2\sigma}{\delta^2}x + \frac{\sigma}{\delta} & 0 < x < \delta \\ 0 & \delta < x < 2d - \delta \\ -\frac{3\sigma}{\delta^3}(x - 2d)^2 - \frac{2\sigma}{\delta^2}(x - 2d) + \frac{\sigma}{\delta} & 2d - \delta < x < 2d \end{cases} \quad (5.2-16)$$

For $\delta < 2d < 2\delta$

$$\rho_p(x) = \begin{cases} -\frac{3\sigma}{\delta^3}x^2 + \frac{2\sigma}{\delta^2}x + \frac{\sigma}{\delta} & 0 < x < 2d - \delta \\ -\frac{6\sigma}{\delta^3}x^2 + \frac{2\sigma}{\delta} - \frac{12\sigma}{\delta^3}d^2 + \frac{4\sigma}{\delta^2}d + \frac{12\sigma}{\delta^3}dx & 2d - \delta < x < \delta \\ -\frac{3\sigma}{\delta^3}(x - 2d)^2 - \frac{2\sigma}{\delta^2}(x - 2d) + \frac{\sigma}{\delta} & \delta < x < 2d \end{cases}$$

To find the solutions of equations (2.5-12), (2.5-16) and (2.5-17) with charge distribution functions of $\rho_p(x)$ and $\rho_l(x)$, numerical trapezoidal integration method can be adopted. In this method of integration, the entire domain is divided to very thin trapezoids and summation of surface areas of all these trapezoids gives the solution of the integral (Figure 5-6).

Figure 5-6 Trapezoidal integration; the integral is estimated by the sum of the area of the thin trapezoids covering the surface area under the curve $f(x)$

If we divide the distance by N equal sections from a to b , we will have:

$$\begin{aligned} \int_a^b f(x)dx = & \frac{f(a) + f\left(a + \frac{b-a}{N}\right)}{2} \left(\frac{b-a}{N}\right) + \frac{f\left(a + \frac{b-a}{N}\right) + f\left(a + 2\frac{b-a}{N}\right)}{2} \left(\frac{b-a}{N}\right) \\ & + \dots + \frac{f\left(a + (N-1)\frac{b-a}{N}\right) + f(b)}{2} \left(\frac{b-a}{N}\right) \end{aligned} \quad (5.2-17)$$

$$\Rightarrow \int_a^b f(x)dx = \left(\frac{b-a}{N}\right) \left[\left(\frac{f(a) + f(b)}{2}\right) + \sum_{i=1}^{N-1} f\left(a + i\frac{b-a}{N}\right) \right]$$

This integration has been performed using MATLAB. To have a more accurate integration, it is important to increase the number of sections. In our code, the thickness of each segment is set to be equal to $5 \times 10^{-11} m$. So, for cell-cell distance equal to 5 nm and 15nm, totally 100 and 300 segments have been used, respectively.

5.3 Results

5.3.1 Electrostatic interactions

5.3.1.1 Impact of charge distribution on the electrostatic potential

The calculated electrostatic potential for each segment distribution is depicted in Figure 5-7. The values for glycocalyx thickness, surface charge density and ionic strength are set to be 5nm , 0.036 C/m^2 [120, 131, 142] and 150mM , respectively. The potential has been shown for three cell-cell distances of $7\text{ nm}(< 2\delta)$, $10\text{ nm}(= 2\delta)$ and $15\text{ nm}(> 2\delta)$.

Figure 5-7 Electrostatic potential as a function of cell-cell distance for various charge distributions; three cell-cell distance have been considered herein: $2d < 2\delta$ (7nm), $2d = 2\delta$ (10nm) and $2d > 2\delta$ (15nm)

In any cell-cell distance higher than 2δ , the electrostatic potential starts with the same magnitude on the cell surfaces and approaches to zero value with increasing the cell-cell distance. Hence, for cell-cell distance of 15nm (i.e. 3δ) the potential at the midsection is roughly zero. Uniform distribution produces the maximum potential

while linear distribution has the minimum value among them. For the cell-cell distance of less than 2δ higher electrostatic potential is observed due to accumulation of surface charges in the $[2d - \delta, \delta]$ region. The electrostatic potential has a greater value for uniform distribution and a lower value for linear distribution.

5.3.1.2 Impact of ionic strength on the electrostatic potential

The impact of ionic strength on the electrostatic potential is shown in Figure 5-8 for glycocalyx thickness of 5nm , surface charge density of 0.036 C/m^2 and ionic strength of 50, 100 and 150 mM. Ionic strength is inversely proportional to the Debye length (κ^{-1}) according to equation (2.5-6) while Debye length is directly proportional to the electrostatic potential (eq. (2.5-12)). It can be concluded that increasing ionic strength results in a considerable decrement in the electrostatic potential.

Figure 5-8 Impact of various ionic strengths on the electrostatic potential distribution for cell-cell distance of 15 nm

5.3.1.3 Impact of ionic strength and charge distribution on the electrostatic energy

The impact of different ionic strengths and charge distributions on the electrostatic interaction energy has been calculated and depicted in Figure 5-9.

Figure 5-9 Electrostatic energy as a function of cell-cell distance for various ionic strengths and charge distributions for cell-cell distances less than 15nm

As shown in Figure 5-9, the electrostatic interaction energy for uniform distribution is higher than the other distributions. For instance at cell-cell distance of 12nm, the electrostatic energy for uniform distribution is about 4.16 and 10.4 fold higher than that of pseudo-tail and linear distributions, respectively.

It is possible to concentrate all the glycocalyx charges at a certain point in a way that the effect of the accumulated charges remains equivalent to the distributed charges at any arbitrary point between $[\delta, 2d - \delta]$. For uniform, linear and pseudo-tail distributions, the equivalent point charges are placed at $x = \delta/2, \delta/3$ and $5\delta/12$, respectively. Hence, since the distance between accumulated charges of two cells in the uniform distribution is less than the other types of charge distribution, greater

interaction energy is expected. With the same reasoning, linear distribution has the lowest interaction energy and pseudo-tail is in between.

By increasing the ionic strength from 50mM to 150mM, a decrement of interaction energy is observed. Therefore, it is predicted the extent of aggregation increases for higher magnitudes of ionic strength.

5.3.2 Steric interactions

5.3.2.1 Impact of glycocalyx segment distribution on the steric interactions

The number of glucose segments in the glycocalyx per m^2 of surface area is calculated in section 5.2.1 to be around 1.97×10^{18} segment. In order to see the effect of number of glycocalyx segments in steric energy we use a vast range of v , i.e., from 0.1×10^{18} to 3.5×10^{18} . The average glycocalyx volume fraction then is found for each segment density by use of equation (5.2-10). This gives the range of [0.004 0.1360] for the average volume fraction of glycocalyx and the volume fraction for the estimated number of glucose segments of 1.97×10^{18} will be 0.077. Values of v_s and v_1 in equation (2.4-1) is found by dividing the molar volume of glucose and water by Avogadro number respectively, i.e.:

$$\begin{aligned}
 v_s &= \frac{v_{glucose}}{N_A} = \frac{M_{glucose}}{\rho_{glucose} N_A} = \left(\frac{180.13}{1.54 \times 6.02 \times 10^{23}} \right) \times 10^{-6} \\
 &= 1.9435 \times 10^{-28} \quad (m^3) \\
 v_1 &= \frac{v_{water}}{N_A} = \frac{M_{water}}{\rho_{water} N_A} = \left(\frac{18}{1 \times 6.02 \times 10^{23}} \right) \times 10^{-6} \\
 &= 2.99 \times 10^{-29} \quad (m^3)
 \end{aligned} \tag{5.3-1}$$

Figure 5-10 shows the effect of various segment distributions on the steric interactions as a function of cell-cell distance.

Figure 5-10 Steric energy as a function of cell-cell distance for various distributions

Uniform distribution has the highest steric energy, linear segment distribution has the lowest and pseudo-tail distribution lies in between. This figure demonstrates that if the cell-cell distance becomes less than 2δ then the repulsion due to steric interaction is dominant and must be taken into account in calculating total interaction energy.

5.3.2.2 Impact of glycocalyx volume fraction on the steric interactions

The effect of glycocalyx volume fraction and Flory interaction parameter on the steric energy for the cells at the separation distance of 9.5 nm is depicted in Figure 5-11.

Figure 5-11 Steric energy as a function of glycocalyx volume fraction; impact of various segment distributions and Flory interaction parameters on the steric interaction is presented for cell-cell distance of 9.5 nm

In general, increasing the volume fraction increases the steric interaction between adjacent glycocalyxes. At low volume fraction of 0.004, steric interaction is almost negligible while by increasing the volume fraction, it increases monotonically. Steric interaction for uniform distribution increases sharply by increasing volume fraction, suggesting that cell-cell distance cannot be less than 2δ due to their substantial repulsion. However, for linear and pseudo-tail segment distributions, the slope is less steep.

The effect of Flory interaction parameter has also been shown in Figure 5-11. Increasing χ leads to the lower interaction energies between cells. As an example, the steric interaction energy with the Flory interaction parameter of 0.48 and volume fraction of 0.077 is about $0.106 \mu\text{J}/\text{m}^2$ for linear and $0.394 \mu\text{J}/\text{m}^2$ for pseudo-tail

distributions, respectively, while these values with Flory parameter of 0.4 will be 0.53 and $1.97 \mu\text{J}/\text{m}^2$, respectively.

5.3.3 *Penetration depth*

The penetration depth is not only a function of bulk polymer size and concentration, but also of attached polymer segment density. Physically it is predicted that the smaller molecules are able to penetrate more into the glycocalyx and the length of penetration increases with increasing bulk polymer concentration. By increasing the concentration of attached layer, the hindrance to further penetration elevates which results in lower penetration depths.

The penetration depths calculated for 0 to $8\text{g}/\text{dl}$ of dextran 500kDa via equations (5.2-12) to (5.2-14) are illustrated in Figure 5-12 for volume fraction of 0.077 (a) and 0.004 (b). For larger glycocalyx volume fraction (Figure 5-12-a), the penetration is almost independent of bulk polymer concentration, with full penetration for pseudo-tail distribution, $\sim 0.17 \text{ nm}$ for uniform and $\sim 0.9 \text{ nm}$ for linear distribution.

(a)

(b)

Figure 5-12 Penetration depth vs. polymer concentration; impact of segment distribution for 500kDa dextran, (a) $\phi_a = 0.077$, (b) $\phi_a = 0.004$

On the other hand, for lower glycocalyx volume fraction of 0.004 (Figure 5-12-b), penetrations are higher in magnitude. Still pseudo-tail distribution suggests a full penetration while those of uniform and linear distributions show bell-shaped dependence to the polymer concentration. Again, uniform distribution has the lowest penetration following by linear distribution. For both of them the maximum penetration occurs at $3 - 4 \text{ g/dl}$ of polymer concentration with the values of 3.3 nm for uniform and 4.1 nm for linear distributions.

Figure 5-13 shows the dependence of penetration depth to the molecular mass for 1 g/dl dextran 40 to 800 kDa. Again full penetration is observed for pseudo-tail distribution while for the other two distributions, penetration decreases steadily by increasing molecular mass of dextran.

Figure 5-13 Penetration depth vs. polymer mass for $\phi_a = 0.004$ and $c = 1 \text{ g/dl}$

The effect of glycocalyx volume fraction on the penetration depth is depicted in Figure 5-14. The figure demonstrates that the penetration depth for pseudo-tail distribution is full, i.e. 5nm, and independent of volume fraction for the given range. For uniform and linear distributions, penetration decreases sharply with increasing volume fraction from 0.004 to 0.01 following by less steep slope till volume fraction of 0.04. After that there is a gradual fall in penetration toward zero for uniform and ~ 0.5 nm for linear distribution.

Figure 5-14 Penetration depth vs. Volume fraction; Impact of segment distribution for $M = 500kDa$ and $c = 1g/dl$

5.3.4 Depletion interactions

The depletion interaction energies as a function of cell-cell distances for 1g/dl dextran 500kDa and glycocalyx volume fraction of $\varphi_a = 0.004$ and $\varphi_a = 0.077$ are presented in Figure 5-15. The figure illustrates that the depletion interactions for pseudo-tail distribution is the same for both volume fractions, whereas for uniform and linear distributions the magnitude of depletion energy raises with increasing volume fraction. This phenomenon can be described as follows; by decreasing volume fraction, the penetration of free polymer into the glycocalyx increases and more glycocalyx segments contribute to the osmotic pressure inside the polymer-depleted layer. Hence, the difference of osmotic pressure of polymer-depleted layer and bulk is lower than the situation where no or little penetration is available, which consequently leads to lower magnitudes of depletion force.

Figure 5-15 indicates about 1.128 and 1.145 fold increment in depletion energy by changing the volume fraction from 0.004 to 0.077 for uniform and linear distributions, respectively.

Figure 5-15 Depletion energy as a function of cell-cell distance for 1g/dl of 500kDa dextran (a) $\phi_a = 0.004$ and (b) $\phi_a = 0.077$

5.3.5 Total interaction energy

Having found all the interaction energies, one can calculate the total interaction energy between cells by superposing all the energies, i.e.

$$W_T = W_E + W_D + W_S \quad (5.3-2)$$

The effect of cell-cell distance on interaction energy for Dextran 500kDa is shown in Figure 5-16. The bulk polymer concentration is held constant at 1g/dl and the energy-distance graphs are plotted for various segment distributions and Flory interaction parameters.

Figure 5-16 Interaction energy vs. cell-cell distance for 1g/dl of dextran 500kDa; the impact of Flory interaction parameter (0.4 and 0.48) and glyocalyx structure on the interaction energy is presented.

The figure shows that at a certain distance, the interaction energy reaches to its minimum and the cells become stable at this distance. This cell-cell distance is about 14.2, 13.2 and 12.5 nm for uniform, pseudo-tail and linear distributions, respectively.

Increasing the value of Flory interaction parameter, χ , results in lower magnitudes of interaction energy, but the minimum energy distance for each distribution remains intact. For pseudo-tail distribution no dependence is observed for two utilized Flory parameters.

The minimum energy of the energy-distance curves calculated for each molecular weight and concentration are plotted in Figure 5-17, Figure 5-18 and Figure 5-19 for uniform, linear and pseudo-tail distributions, respectively. Polymer concentrations from 0 to 10 g/dl with molecular mass of 40, 70, 250 and 500kDa have been used for acquiring these graphs. Figure 5-17-a, Figure 5-17-b, Figure 5-18-a and Figure 5-18-b present the effect of Flory interaction parameter on the interaction energy for the soft surfaces, i.e. $\phi_a = 0.004$. These figures indicate that increasing Flory interaction parameter from 0.4 to 0.48 can bring about the compression of the graphs for the samples containing higher molecular weight dextrans ($>250kDa$), i.e. for smaller values of χ , aggregation exists for a broader range of polymer concentration. For instance in uniform distribution, the polymer concentration in which interaction energy reaches zero is $7g/dl$ for $\chi = 0.4$, while this amount is $6.5g/dl$ for $\chi = 0.48$ for dextran 250 kDa. This variation is more pronounced for dextran 500kDa in which uniform distribution shifts from $12.5g/dl$ for $\chi = 0.4$ to $7g/dl$ for $\chi = 0.48$. It is also worth noting that the reduction of adhesion energy by increasing the Flory parameter is observed. This reduction is slight for dextran 250 and prominent for dextran 500kDa.

In fact, in the equations (5.2-12) to (5.2-14) used to determine the penetration depth, the term $(1 - 2\chi)$ in the denominator plays an important role such that increasing the value of χ leads to the increment of penetration depth and consequently decreases the magnitude of both depletion and total interaction energy.

Figure 5-17 Interaction energy as a function of polymer concentration for uniform distribution for dextran 40, 70, 250 and 500kDa (a) $\chi = 0.4$, $\phi_a = 0.004$, (b) $\chi = 0.48$, $\phi_a = 0.004$, (c) $\chi = 0.4$, $\phi_a = 0.077$ (d) Cell-cell distance at which minimum interaction energy is found for $\chi = 0.4$, $\phi_a = 0.077$

Figure 5-18 Interaction energy as a function of polymer concentration for linear distribution for dextran 40, 70, 250 and 500kDa (a) $\chi = 0.4$, $\phi_a = 0.004$, (b) $\chi = 0.48$, $\phi_a = 0.004$, (c) $\chi = 0.4$, $\phi_a = 0.077$ (d) Cell-cell distance at which minimum interaction energy is found for $\chi = 0.4$, $\phi_a = 0.077$

Figure 5-17-c and Figure 5-18-c depict the total interaction energy for the case when the calculated volume fraction of 0.077 is used, i.e. almost hard surface. The slight penetration in this case creates extremely high depletion energy and hence strong attraction. Although there is a strong attractive force, the cell-cell distance remains greater than 9nm (Figure 5-17-d and Figure 5-18-d). It implies that our assumption that cells never reach the interpenetration plus compression domain is reasonable.

Figure 5-19 shows the interaction energy for pseudo-tail distribution. For this distribution total energy is independent of the glycocalyx volume fraction in the range of [0.004, 0.077], and the Flory interaction parameter between [0.4 0.48]. The cell-cell distance in this case hardly reaches 10 nm, so steric interaction can be neglected.

Comparisons between interaction energy for different distributions, Flory parameter of 0.48 and volume fraction of 0.004 (Figure 5-17-b, Figure 5-18-b and Figure 5-19-a) indicate that linear distribution have the highest magnitudes of adhesion energy followed by pseudo-tail and uniform distributions. The concentration range at which the adhesion energy exists also decreases with the same order. For instance, for dextran 70kDa, largest polymer concentration for observing adhesion is about 6.5g/dl for linear, 5.5g/dl for pseudo-tail and 4.5g/dl for uniform distribution. By comparing these three figures it can be noticed that the increment in molecular mass can elevate the polymer concentration range in which adhesion exists. Increasing molecular mass also affects the adhesion energy: by increasing the molecular mass from 40 kDa to 250 kDa, the adhesion energy increases. However, by increasing molecular mass of dextran further to 500kDa, a decrease in adhesion energy is observed.

(a)

(b)

Figure 5-19 (a) Interaction energy as a function of polymer concentration for pseudo-tail distribution for $\chi = 0.48$, $\varphi_a = 0.077$ and dextran 40, 70, 250 and 500 kDa, (b) Cell-cell distance at which minimum interaction energy is found

5.3.6 Validation of the results with experimental data

Now that the theoretical framework is built, it is important to see how this model works with experimental data. To validate the model, three sets of experimental data have been chosen. Buxbaum [151] has calculated the surface affinities of red cells by means of micropipette aspiration technique for dextran 70kDa and 150 kDa. On the other hand, Chien *et al* measured the light reflection density of the samples containing 45% hematocrit in different suspending medium. Dextran 80, 150 and 500kDa are used as the aggregating agents and three ionic strength of 50, 100 and 150 mM have been considered [152]. The extent of aggregation is quantified with use of a parameter called *Reflectometric Aggregation Index* (RAI) calculated as:

$$RAI_i = 1 - \frac{R_i}{R_{200}} \quad (5.3-3)$$

in which R_i is the reflectometer reading at a shear rate of $i \text{ s}^{-1}$.

For the last set, experimental data by Neu *et al* [126] for high molecular mass dextrans (500kDa, 2MDa and 28MDa) have been chosen for comparison with theoretical model.

5.3.6.1 Buxbaum's data

Figure 5-20 demonstrates the experimental and theoretical interaction energies for dextran 70kDa (a) and 150kDa (b). The magnitudes of surface charge density and glycocalyx thickness are chosen to be 0.036 C/m^2 and 5 nm respectively. The quadratic fitting method is adopted for drawing the interaction energy curve of experimental data as depicted by dashed lines in Figure 5-20. It can be noticed that the experimental data curve lies between the uniform and pseudo-tail curves for dextran 70kDa. However, for dextran 150kDa, although linear distribution resembles more

closely to the experimental result, none of the above-discussed models are able to predict the experimental behavior accurately.

(a)

(b)

Figure 5-20 Calculated results (solid lines), experimental data by Buxbaum *et al.* (points) and quadratic fitting of experimental result (dashed line) for (a) dextran 70kDa and (b) dextran 150kDa

In order to fit our model to the experimental data, two fitting parameters have been chosen:

- 1- Surface charge density of 0.02 C/m^2 and 0.036 C/m^2 ; both of which are considered as erythrocytes surface charge density in literature [131, 153].
- 2- Glycocalyx thickness of 4, 5 and 6 nm; this arises from the fact that glycocalyx structure might collapse under the effect of osmotic pressure [106]

Glycocalyx volume fraction is set to be 0.004. This parameter cannot be used as a fitting parameter since a slight increase of its magnitude causes unrealistic high interaction energies. Furthermore as discussed in page 97, Flory parameter seems to affect the interaction energy only for high molecular mass dextrans ($>250\text{kDa}$). Hence for the molecular mass of our interest (70 and 150 kDa), interaction energy is assumed to be independent of Flory parameter.

The fitting graphs for dextran 70kDa and 150kDa are depicted in Figure 5-21 and Figure 5-22 for various distributions: (a) uniform, (b) linear and (c) pseudo-tail. In order to compare the graphs, three basic comparison parameters have been chosen. These parameters are: the magnitude of minimum energy, the concentration of minimum energy and the concentration in which the adhesion energy becomes zero, i.e. upper threshold concentration. These parameters have been summarized in Table 5-2 to Table 5-7. The error magnitude for each of these parameters is calculated using the equation $\frac{X_{exp} - X_{The}}{X_{exp}} \times 100$ where X_{exp} and X_{The} are experimental and theoretical values of fitting parameters, respectively. The average error for each case is presented in the last row of tables for comparison.

(a)

(b)

(c)

Figure 5-21 Fitted data for dextran 70kDa; experimental data (dashed line), $\sigma = 0.02C/m^2$ (dotted line), $\sigma = 0.036C/m^2$ (solid line), $\delta = 4nm$ (blue line), $\delta = 5nm$ (green line), $\delta = 6nm$ (red line), (a) Uniform, (b) Linear, (c) Pseudo-tail

(a)

(b)

(c)

Figure 5-22 Fitted data for dextran 150kDa; experimental data (dashed line), $\sigma = 0.02 \text{ C/m}^2$ (dotted line), $\sigma = 0.036 \text{ C/m}^2$ (solid line), $\delta = 4 \text{ nm}$ (blue line), $\delta = 5 \text{ nm}$ (green line), $\delta = 6 \text{ nm}$ (red line), (a) Uniform, (b) Linear, (c) Pseudo-tail

Table 5-2 Comparison with experimental data, Dextran 70kDa, Uniform

	Experimental data	$\delta = 4nm$		$\delta = 5nm$		$\delta = 6nm$	
		$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2
Min Energy	5.57	12.42	8.54	6.3	4.1	2.97	1.45
% error		122.98%	53.32%	13.11%	26.39%	46.68%	73.97%
Min energy concentration	2.67	4.7	3.5	3.3	2.7	2.7	1.7
% error		76.03%	31.09%	23.60%	1.12%	1.12%	36.33%
Upper threshold concentration	4.93	7.5	6.3	5.7	4.5	4	3
% error		52.13%	27.79%	15.62%	8.72%	18.86%	39.15%
Average % error		83.71%	37.40%	17.44%	12.08%	22.22%	49.82%

Table 5-3 Comparison with experimental data, Dextran 70kDa, Linear

	Experimental data	$\delta = 4nm$		$\delta = 5nm$		$\delta = 6nm$	
		$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2
Min Energy	5.57	20.81	14.7	13.65	9.24	8.27	5.1
% error		273.61%	163.91%	145.06%	65.89%	48.47%	8.44%
Min energy concentration	2.67	5.5	4.7	4.5	3.7	3.7	3
% error		105.99%	76.03%	68.54%	38.58%	38.58%	12.36%
Upper threshold concentration	4.93	10.8	8	8.2	6.5	6.5	5
% error		119.07%	62.27%	66.33%	31.85%	31.85%	1.42%
Average % error		166.22%	100.74%	93.31%	45.44%	39.63%	7.41%

Table 5-4 Comparison with experimental data, Dextran 70kDa, Pseudo-tail

	Experimental data	$\delta = 4nm$		$\delta = 5nm$		$\delta = 6nm$	
		$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2
Min Energy	5.57	16.95	11.91	10.34	6.84	5.57	3.31
% error		204.31%	113.82%	85.64%	22.80%	0.00%	40.57%
Min energy concentration	2.67	5	4.3	4	3.3	3.3	2.5
% error		87.27%	61.05%	49.81%	23.60%	23.60%	6.37%
Upper threshold concentration	4.93	8.9	7.3	7	5.5	5.3	4.3
% error		80.53%	48.07%	41.99%	11.56%	7.51%	12.78%
Average % error		124.03%	74.32%	59.15%	19.32%	10.37%	19.91%

Table 5-5 Comparison with experimental data, Dextran 150kDa, Uniform

	Experimental data	$\delta = 4nm$		$\delta = 5nm$		$\delta = 6nm$	
		$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2
Min Energy	25.42	13.19	10.86	9.25	7.6	6.44	5.08
% error		48.11%	57.28%	63.61%	70.10%	74.67%	80.02%
Min energy concentration	4.18	4.5	4	4	3.5	3.5	3
% error		7.66%	4.31%	4.31%	16.27%	16.27%	28.23%
Upper threshold concentration	8.23	8.23	7.3	7	6	5.7	5
% error		0.00%	11.30%	14.95%	27.10%	30.74%	39.25%
Average % error		18.59%	24.29%	27.62%	37.82%	40.56%	49.16%

Table 5-6 Comparison with experimental data, Dextran 150kDa, Linear

	Experimental data	$\delta = 4nm$		$\delta = 5nm$		$\delta = 6nm$	
		$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2
Min Energy	25.42	18.1	14.7	13.84	11.22	10.42	8.35
% error		28.80%	42.17%	45.55%	55.86%	59.01%	67.15%
Min energy concentration	4.18	5.5	4.7	5	4.3	4.3	3.7
% error		31.58%	12.44%	19.62%	2.87%	2.87%	11.48%
Upper threshold concentration	8.23	10.6	8.7	8.5	7.5	7.5	6.3
% error		28.80%	5.71%	3.28%	8.87%	8.87%	23.45%
Average % error		29.72%	20.11%	22.82%	22.53%	23.58%	34.03%

Table 5-7 Comparison with experimental data, Dextran 150kDa, Pseudo-tail

	Experimental data	$\delta = 4nm$		$\delta = 5nm$		$\delta = 6nm$	
		$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2	$\sigma=0.02$ C/m^2	$\sigma=0.036$ C/m^2
Min Energy	25.42	15.84	13.06	11.94	9.67	8.76	6.95
% error		37.69%	48.62%	53.03%	61.96%	65.54%	72.66%
Min energy concentration	4.18	5.3	4.5	4.5	3.7	3.7	3.3
% error		26.79%	7.66%	7.66%	11.48%	11.48%	21.05%
Upper threshold concentration	8.23	9.75	8.12	8	7	6.5	5.7
% error		18.47%	1.34%	2.79%	14.95%	21.02%	30.74%
Average % error		27.65%	19.21%	21.16%	29.46%	32.68%	41.48%

By comparing the average error values of each table, it is observed that for dextran 70kDa and $\sigma = 0.036C/m^2$, the linear distribution with $\delta = 6nm$ is more consistent with the experimental results by only 7.41% error, while uniform and pseudo-tail distribution with $\delta = 5nm$ and respective error of 12.08% and 19.32% are in the next positions. Moreover, for dextran 150kDa, pseudo-tail distribution with $\delta = 4nm$ can describe the concentration of minimum energy and upper threshold concentration with low error magnitudes of 7.66% and 1.34% respectively. However, for this case the magnitude of interaction energy is about half of the experimental value which gives the total error of $\sim 19\%$.

Since the magnitude of surface charge density has not specified in the Buxbaum paper, we can also see how it works if the charge density is reduced to $\sigma = 0.02C/m^2$. In this case for dextran 70kDa, pseudo-tail distribution with $\delta = 6nm$ is the closest curve to the experimental result with the error of 10.37%, followed by uniform distribution of $\delta = 5nm$. For dextran 150kDa, uniform distribution with $\delta = 4nm$ is the closest fit by approximate error of 18%. These results are summarized in Table 5-8.

Table 5-8 Summary of comparison

	Dextran Mass	Uniform	Linear	Pseudo-tail
$\sigma = 0.036C/m^2$	70kDa	$\delta = 5nm$	$\delta = 6nm$	$\delta = 5nm$
		12.08%	7.4%	19.32%
	150kDa	$\delta = 4nm$	$\delta = 4nm$	$\delta = 4nm$
		24.29%	20.11%	19.21%
$\sigma = 0.02C/m^2$	70kDa	$\delta = 5nm$	$\delta = 6nm$	$\delta = 6nm$
		17.44%	39.63%	10.37%
	150kDa	$\delta = 4nm$	$\delta = 5nm$	$\delta = 5nm$
		18.59%	22.82%	21.16%

5.3.6.2 Chien's data

Aggregation index of cells suspending in Dextran solutions of various molecular mass and concentration has been measured for different ionic strength by Chien et al. [152] for $I = 50, 100$ and 150 mM and dextran $40\text{kDa} - 500\text{kDa}$.

Figure 5-23, Figure 5-24 and Figure 5-25, parts (a), (b) and (c), depict the calculated aggregation energies for dextran 80, 150 and 500 kDa, respectively, for different ionic strengths and glycocalyx segment distributions, and part (d) of these figures represent the experimental results by Chien *et al.*

(a)

(b)

(c)

(d)

Figure 5-23 Calculated and experimental results for aggregation energy for samples containing dextran 80kDa. (a) Uniform, (b) Linear, (c) Pseudo-tail and (d) experimental results by Chien *et al* [152] with permission

(a)

(b)

(c)

(d)

Figure 5-24 Calculated and experimental results for aggregation energy for samples containing dextran 150kDa. (a) Uniform, (b) Linear, (c) Pseudo-tail and (d) experimental results by Chien *et al* [152] with permission

(a)

(b)

(c)

(d)

Figure 5-25 Calculated and experimental results for aggregation energy for samples containing dextran 500kDa. (a) Uniform, (b) Linear, (c) Pseudo-tail and (d) experimental results by Chien *et al* [152] with permission

The aggregation index is a non-dimensional parameter and cannot be compared with the interaction energy quantitatively. However, qualitatively both aggregation index and interaction energy can predict the aggregation behavior. Thereby, the concentrations in which aggregate formation starts, reaches to the maximum value and finishes can be compared in the graphs of Figure 5-23, Figure 5-24 and Figure 5-25. Two comparison parameters have been selected:

- 1- Upper threshold concentration for aggregate formation, C_U
- 2- Concentration in which minimum energy is observed, C_{max}

Due to lack of data points in experimental graphs (part (d) of Figure 5-23, Figure 5-24 and Figure 5-25), the solid trend lines have been used for comparison. The comparison parameters are summarized in Table 5-9, Table 5-10 and Table 5-11. The calculated numbers in the following tables are merely rough estimations for the discussed parameters.

Table 5-9 Comparison parameters for dextran 80kDa

	50mM		100mM		150mM	
	C_U	C_{Max}	C_U	C_{Max}	C_U	C_{Max}
Experimental	3.5	2	6.5	4	8.4	4
Uni.	-	-	4	2.5	4.7	3
Error%	-	-	38.5%	37.5%	44%	25%
Average error%	-		38%		34.5%	
Lin.	3.5	2.3	5.7	3.5	6.7	3.8
Error%	0%	15%	12.3%	12.5%	20.2%	5%
Average error%	7.5%		12.4%		12.6%	
Pseud.	2.8	1.8	5	3	6	3.5
Error%	20%	10%	23.1%	25%	28.6%	12.5%
Average error%	15%		24%		20.5%	

Table 5-10 Comparison parameters for dextran 150kDa

	50mM		100mM		150mM	
	C_U	C_{Max}	C_U	C_{Max}	C_U	C_{Max}
Experimental	5.1	2.8	7.1	5.1	10.2	6
Uni.	3.85	2.5	5.2	3	6	3.3
Error%	24.5%	10.7%	26.7%	41.2%	41.2%	45%
Average error%	17.6%		33.9%		43.1%	
Lin.	4.9	3	6.7	4	7.5	4.3
Error%	3.9%	7.1%	5.6%	21.6%	26.5%	28.3%
Average error%	5.5%		13.6%		27.4%	
Pseud.	4.4	2.7	6.2	3.5	7	3.8
Error%	13.7%	3.8%	12.7%	31.4%	31.4%	36.7%
Average error%	8.6%		22%		34%	

Table 5-11 Comparison parameters for dextran 500kDa

	50mM		100mM		150mM	
	C_U	C_{Max}	C_U	C_{Max}	C_U	C_{Max}
Experimental	5.9	4	8.2	5.5	10.5	6
Uni.	5.2	3.3	6.5	3.5	7	3.7
Error%	11.7%	17.5%	20.7%	36.4%	33.3%	38.3%
Average error%	14.7%		28.5%		35.8%	
Lin.	6	3.3	7.5	4	8.3	4.3
Error%	1.7%	17.5%	8.5%	27.3%	20.9%	28.3%
Average error%	9.6%		17.9%		24.6%	
Pseud.	5.6	3.3	7	3.7	7.7	4
Error%	5.1%	17.5%	14.6%	32.7%	26.7%	33.3%
Average error%	11.3%		23.7%		30%	

The shaded cells in the above tables refer to the lowest error values. It is worth mentioning that linear glyocalyx distribution has the least error at the three different ionic strengths and dextran molecular masses. Also the calculated errors are directly proportional to the ionic strength, which means that the model is more reliable for lower ionic strengths. After the linear distribution, the pseudo-tail distribution is the more reliable and accurate model, and consequently the accuracy of uniform glyocalyx distribution is the least.

5.3.6.3 *Neu's data*

The previous data sets dealt with dextrans with molecular mass of at most 500kDa. In order to see how our model describes aggregation behavior induced by larger molecular weight dextrans, we will now take a look at the available experimental data published in the paper by Neu et al, 2008 [126]. In this experimental approach dextran fractions of 500kDa, 2MDa and 28MDa are added to phosphate buffered saline at desired concentrations ($<1\text{g/dl}$) followed by adding washed red blood cells by hematocrit of $40\pm0.3\%$.

Samples are first sheared at 600 s^{-1} to break all pre-existing aggregates. After that the shear rate is reduced abruptly to 3 s^{-1} and transmitted light through the sample is measured. Figure 5-26-a illustrates the best fitted third-order polynomials to measured aggregation index for each case, while Figure 5-26-b presents the calculated interaction energy for the abovementioned molecular mass dextrans and various segment distributions using $\delta = 5\text{nm}$ and $\sigma = 0.036C/m^2$.

(a)

(b)

Figure 5-26 (a) Experimental Values of aggregation index for high molecular mass dextran 500kDa, 2MDa and 28 MDa by Neu *et al* [126], (b) Calculated interaction energy for the same molecular mass dextrans

Again, it is not possible to compare two graphs quantitatively and only qualitative comparison can be made. It can be seen that both experimental and theoretical data describe the same behavior for the aggregation phenomena with 500kDa dextran having the highest adhesion energy and aggregation index, and 28MDa dextran showing the lowest adhesion energy and aggregation index. Only a little difference can be observed in adhesion energy for different glycocalyx structures in the given range of dextran molecular mass and concentration. Aggregation increases steadily with concentration in both theoretical and experimental graphs. While the rate of increase is highest for 500 kDa dextran, only slight increase can be observed for Dextran 28MDa.

The reason behind decreasing the aggregation by increasing molecular mass of the dextran might be due to lower number of polymer molecules for high molecular mass dextran for the same polymer concentration in the sample. For example for 0.5 g/dl polymer concentration, the number of molecules of dextran 500kDa is about 300 times more than that of dextran 28MDa.

Overall, this model is qualitatively in agreement with the experimental data for large molecular mass dextrans.

5.4 Discussion

In this chapter a more comprehensive model was presented to enhance the discussed model in Chapter 4 by considering the glycocalyx structure and steric interaction between cells.

The results of this chapter imply that in the normal conditions the steric interactions can be neglected due to the high electrostatic repulsion which causes the cell-cell distance to be greater than 2δ . However, it should be noted that for the case of altered surface charge density after neuraminidase treatments, the steric interactions can act as the major source of repulsion.

For uniform and linear segment distributions, assuming the calculated number of glycocalyx segments (i.e. 1.97×10^{18}), the surface acts like a hard surface and the total interaction energies are unrealistically high. This phenomenon can be addressed to the little penetration of free polymers into the glycocalyx layer. Only if we assume that there is a full penetration ($p = \delta$), the interaction energies will approach to the experimental measurements. For pseudo-tail segment distribution, the penetration is full for the whole range of glycocalyx volume fraction considered herein; hence the calculated interaction energy remains in the same order of magnitude of the experimental results.

A bell-shaped dependence of the adhesion energy to the polymer concentration is observed in the considered glycocalyx segment distributions, which means that by increasing the polymer concentration, aggregation energy reaches to its maximum value and then by further increment of the concentration, it falls off.

Comparison of the model with Buxbaum data (Table 5-8) suggests that by increasing the molecular weight from 70kDa to 150 kDa, the glycocalyx thickness decreases from 6nm to 4nm. This might happen due to the higher osmotic pressure produced by larger dextrans which may result in collapsing the glycocalyx structure.

Among these models, by the assumption of full penetration, linear distribution renders a closer approximation for experimental data for the cell-cell affinity in various ionic strengths. The pseudo-tail and uniform distributions are the next accurate models, respectively. However, if the exact values of attached segment density is needed to be used, still pseudo-tail distribution can predict the experimental data to some extent, whereas uniform and linear distributions are not accurate anymore.

The differences in the theoretical and experimental data might arise from our simplifying assumption about the glycocalyx length and structure which is in reality very complex. It is considered that the glycocalyx length and segment distribution remain intact during the cell-cell interaction. In real conditions, glycocalyx structure might change due to the surrounding ionic strength and the osmotic pressure caused by the polymer depletion. Hence, in the future models, the changes of glycocalyx structure can also be taken into account. Also, it should be noted that assuming a constant charge distribution in the glycocalyx is an oversimplifying assumption since glycocalyx can be considered as a responsive system and its structure and hence charge distribution can change during electrostatic interactions.

The effects of RBC deformability and membrane undulations have not been considered in the model of this chapter. While the latter is expected to have only a slight effect on RBC aggregation, it is known that RBC with less amount of deformability exhibit lower extents of aggregation [36].

Chapter 6 Conclusion and Future Directions

This dissertation aims to investigate the impact of cellular and medium properties on the depletion-mediated red cell aggregation. The first two chapters provide the background and theoretical foundations for this purpose.

In chapter 3, the theoretical and experimental values of viscosity-corrected mobilities for cells suspended in various dextran fractions were compared. The results of this chapter demonstrate a strong correlation between the experimental electrophoretic mobility values and those calculated theoretically by considering a polymer-depleted layer around the cell surface. Chapter 3 strongly supports the existence of a depletion layer around the cell surface in the polymer solutions.

In chapter 4 a simplified model was presented to calculate the adhesion energy between cells in dextran solutions based on electrostatic and depletion forces. The segment density as well as charge distribution inside the glycocalyx was assumed to be constant. The impact of various molecular mass and concentration of dextran on the cell-cell affinity was also investigated. Furthermore the effects of cellular factors like glycocalyx thickness and volume fraction were also studied. At the end of chapter 4, energy landscapes were presented for different dextran sizes and concentrations.

Chapter 5 dealt with more comprehensive model to predict the adhesion energy between cells. In this model, different charge and segment distributions in the glycocalyx as well as steric interactions were considered. Also, the penetration depth of free polymer into the glycocalyx structure was calculated. The theoretical values of adhesion energies were compared with three sets of experimental data. However, since most of the experimental data dealt with measurement of aggregation index

instead of adhesion energy, quantitative comparison of adhesion energies was impossible and only qualitative comparison could be made.

In overview, the findings of the last three chapters provide new insights into polymer-RBC interactions and a rational explanation of the observed red cell aggregation behavior in polymer solutions by means of depletion interactions.

One of the significant applications of this model is in identification of the cellular or physiochemical properties of RBCs that determine aggregate formation in pathological conditions like diabetes mellitus, renal disease and myocardial infarction [10]. This will provide a better understanding of pathology of such diseases and allow promising development of possible therapeutic agents.

It should be noted that this study dealt with neutral polymers like dextran. Since the proteins which induce aggregation *in-vivo*, *i.e.* fibrinogen, are charged, one may extend this study by considering charged polymers in the model. Also, deformation and undulation of the membrane as well as possible collapse/reconfiguration of glycocalyx structure as a result of bulk osmotic pressure/electrostatic interactions may be considered to provide a more comprehensive model. To have an accurate model, these factors should also be taken into account in the future.

Further efforts should be directed to utilize this model for investigating the current unresolved aspects of human RBC aggregation like lower extent of red cell aggregation in neonatal, both in plasma and polymer solutions [37, 154], or the wide range of RBC aggregability for different donors.

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