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(On the Spine)

The impact of non-adsorbing macromolecules on RBC adhesion to EC

YANG YANG

2010

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**THE IMPACT OF NON-ADSORBING
MACROMOLECULES ON RED BLOOD CELL
ADHESION TO ENDOTHELIAL CELLS**

**YANG YANG
SCHOOL OF CHEMICAL AND BIOMEDICAL
ENGINEERING
2010**

THE IMPACT OF NON-ADSORBING MACROMOLECULES ON RED BLOOD CELL ADHESION TO ENDOTHELIAL CELLS

YANG YANG

School of Chemical and Biomedical Engineering

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List of Abbreviations

BBE	bovine brain extract
BSA	bovine serum albumin
CCD	charge-coupled device
Conc.	concentration
DEX	dextran
EC	endothelial cells
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescent-activated cell sorting
FBS	fetal bovine serum
Fg	fibrinogen
FITC	fluorescein isothiocyanate
FN	fibronectin
HBSS	Hanks' buffered saline solution
HCT	hematocrit
HUVEC	human umbilical vein endothelial cells
ICAM-1	Inter-Cellular Adhesion Molecule-1
Mw	molecular mass
NEM	N-ethyl maleimide
PEG	poly(ethylene glycol)
PSS	poly(styrene sulfonate)
PBS	phosphate-buffered saline
PS	phosphatidylserine
RBC	red blood cells
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	serum free medium
TIRF	total internal reflection fluorescence microscopy
TSP	thrombospondin
VCAM-1	vascular cell adhesion molecule-1
vWF	von Willebrand factor

Summary

Abnormal adhesion of red blood cells (RBC) to endothelial cells (EC) has been linked to several vascular diseases such as sickle cell anemia and diabetes mellitus. Plasma proteins have been identified to promote abnormal RBC-EC adhesion but the mechanism has not been fully understood. Aiming to provide a better understanding of the adhesion-promoting effects of plasma proteins, this thesis studies the hypothesis that non-adsorbing macromolecules can induce or promote RBC adhesion to EC by inducing depletion interaction. Furthermore, it is suggested that depletion interaction is an alternative mechanism for the adhesion promoting effects of non-adsorbing plasma proteins.

RBC adhesion to EC was studied with a parallel-plate flow chamber system. Dextran, a polyglucose that has been shown to be depleted from the RBC surface (i.e. a non-adsorbing macromolecule), was used in this thesis to mimic the effect of non-adsorbing plasma proteins. Fibrinogen, a plasma protein that has been identified to promote RBC-EC adhesion, was also employed and its effects on RBC-EC adhesion were compared to those of dextran. Dextran is shown to be a suitable macromolecule to mimic the adhesion-promoting effects of plasma proteins. Thus, it was used throughout the thesis to study the impact of non-adsorbing macromolecules on RBC adhesion to EC. Depletion interaction originating from non-adsorbing macromolecules is shown to induce adhesion of normal RBC to EC in buffer/albumin as well as in plasma-like media. In particular, fibrinogen is suggested to promote RBC-EC adhesion via depletion interaction. The physicochemical properties of the non-adsorbing macromolecules and the RBC surface properties are identified to be determinants of depletion interaction. The possible pathophysiological relevance of depletion interaction is studied by investigating the adhesion of RBC with enhanced PS exposure to EC as well as the adhesion of RBC to thrombin-activated EC in the presence of dextran. It is concluded that depletion interaction can induce or promote RBC-EC adhesion. Thus, this thesis provides a potential mechanism for the adhesion-promoting effects of non-adsorbing plasma proteins in pathological RBC-EC adhesion.

Chapter 1 Introduction

Abnormal RBC-EC adhesion

Normal red blood cells (RBC) normally do not adhere to endothelial cells (EC). However, abnormal adhesion of RBC to EC has been identified in diseases associated with vascular pathologies such as sickle cell disease [1-3], diabetes mellitus [4], malaria [5, 6], and thalassemia [7].

The consequences of abnormal RBC-EC adhesion have been manifested to lead to further vascular complications [8]. For example, the abnormal adhesion can up-regulate the gene expression of adhesion molecules in EC, leading to malfunction of the endothelium [9]. It can also impair blood flow, leading to the adhesion of other blood elements to the vessel wall [10, 11]. The resultant endothelium damage can lead to thrombosis [12, 13] and affect the coagulation system leading to inflammation [14]. Several methods have been clinically tested to prevent RBC-EC adhesion [15-17] with limited success [18, 19], indicating that a thorough understanding of the mechanism of abnormal RBC-EC adhesion has not been accomplished. This thesis aims to provide a better understanding of the underlying mechanism of RBC-EC adhesion in diseases associated with vascular complications [7, 20-22].

Table 1-1: The main experimental methods used for RBC-EC adhesion assay.

Diseases	Experimental methods	Reference
Sickle cell disease	Static assay	[2, 23]
	Parallel-plate flow chamber	[24]
	Micropipette	[25, 26]
	Ex vivo and in vivo	[27-29]
	Perfusion through umbilical vein	[30, 31]
Diabetes	Static assay	[4]

Experimental models of RBC-EC adhesion

Abnormal RBC-EC adhesion has been studied with miscellaneous experimental models as summarized in Table 1-1 [8]. To date, most significant findings related

to abnormal RBC-EC adhesion were identified with *in vitro* experimental methods, which have the advantage to provide a platform that is easy to control in order to specifically investigate the factors of interest that are responsible for abnormal RBC-EC adhesion.

It should be noted that different experimental models may lead to different conclusions. For example, increased sickle RBC adhesion to endothelium was first identified with a static assay, but attempts to confirm these results under flow conditions failed initially [32]. Furthermore, different experimental models may provide different results even if similar conclusions can be drawn. For example, both the static assay and the flow assay suggest distinct subpopulations of sickle RBC to be more adherent than others [8, 33]. Therefore, different experimental models may provide different perspectives for the problem of interest. Thus, it is necessary to briefly review the experimental models that have been employed in the study of RBC-EC adhesion so far.

Static assay

The static assay was the first experimental method that allowed the identification of abnormal RBC-EC adhesion [1, 3, 4]. In this assay, a static incubation of RBC and EC ranging from minutes to hours is followed by repeated washes to remove non-adherent RBC. Technically, it provides a simple and quick method, and it is also convenient for the detection of adhesion molecules by employing ELISA or other fluorescence methods [34]. However, there are also a few drawbacks: 1) the wash-off of the non-adherent RBC sometimes leads to inconsistent results due to differences of the experimental conditions, and it does not allow quantitative measurements of the adhesion strength; 2) EC cultured in microplates are usually covered by a multilayer of RBC during the static incubation, which creates an oxygen- and nutrient-deprived interface between RBC and EC, and thus affects the metabolism of the cells [8]; 3) the static assay lacks dynamic flow conditions, which may limit the physiological relevance of the results obtained.

Micropipette assay

The micropipette assay employs a micropipette to detach a single RBC from the endothelium by pipette suction in the normal or tangential direction [25], and

allows to quantify the adhesion energy of single cells. However, the micropipette assay also has a few drawbacks: 1) it is technically a demanding procedure, which requires many single cell operations to obtain results that can represent the whole population; 2) the manipulation of the attachment and detachment of single cells by pipette suction does not mimic the dynamic *in vivo* flow conditions. A modified micropipette assay (i.e. micropipette-flow) provides an alternative way to quantify the adhesion strength [26]. After a static incubation of RBC and EC, a flow is generated by the micropipette to detach the adherent RBC from EC and the shear stress applied represents the adhesion energy.

Parallel-plate flow chamber

The parallel-plate flow chamber is a widely used method that mimics *in vivo* flow conditions. It allows monitoring of the adhesion dynamics. Moreover, it has several other applications depending on the experimental needs and research interests. For example, it allows adhesion studies not only between RBC and EC cultured at the bottom of the flow chamber [24, 35, 36], but also between RBC and immobilized plasma proteins [37-39].

Two common assays often employed are the static-flow assay and the continuous-flow assay. In the static-flow assay [24, 40-42] cells are allowed to settle for a static incubation time before applying fluid shear force to remove non-adherent cells. The extent of the adhesion can then be quantified as the number of adherent RBC per unit area, while the adhesion strength can be evaluated by calculating the shear force required to detach the adherent cells. Compared to the micropipette assay, the parallel-plate flow chamber has the advantage that it allows to quantify the extent of the adherence and the adhesion strength for many individual cells (i.e. the whole population).

In the continuous-flow assay [24, 36, 37, 40, 41, 43, 44] the cultured endothelium or immobilized proteins are subject to a constant flow without the static incubation as described above. Cell suspensions are first perfused for a fixed time to allow adhesion under flow, followed by continuous perfusion with cell-free medium to remove non-adherent cells. This assay provides direct and dynamic information of adhesion under flow, and thus allows mimicking *in vivo* flow conditions. Different

applications of this assay have been utilized by modifying the experimental conditions. For example, by varying the flow rate (i.e. shear stress), it is possible to mimic the adhesion in different regions (e.g. microcapillary and artery) [40]; or by varying the hematocrit (i.e. volume fraction of RBC) or the RBC deformability, it is possible to study the impact of hemorheological properties of RBC on the adhesion under flow. Moreover, by analyzing the EC surface as well as their RNA after perfusion with RBC, it is also possible to gain insight on how blood flow (i.e. shear force) triggers the expression of adhesion molecules (i.e. mechanotransduction) and how their gene expression is regulated [9].

Perfusion through umbilical vein

The perfusion through umbilical veins is based on the perfusion of RBC suspensions through human umbilical cords. This technique was first employed to study the interaction of sickle RBC and the vessel wall [30]. These observations demonstrated an increased adhesiveness of sickle RBC. This method was also used to compare the effects of normal and sickle RBC perfusion on the release of prostacyclin from endothelial cells, which demonstrates that the perfusion of sickle RBC suspended in autologous plasma can increase the release of prostacyclin from umbilical cord veins [31]. Compared to the three assays mentioned above, the perfusion through umbilical veins has the advantage of employing an adhesion environment closer to the *in vivo* conditions, but it also has a few drawbacks. First of all, the umbilical cord is not a practical material that can be widely used. In addition, the flow conditions exerted on the endothelium are difficult to control due to uncertainties of the umbilical cord geometry [8].

Ex vivo and in vivo models

In *ex vivo* models, RBC suspensions are perfused through an isolated rat mesoappendix vasculature [45]. With intravital microscopic observations, this method allows to characterize the rheological (bulk viscosity) and hemodynamic (peripheral resistance in an *ex vivo* vascular preparation) properties of RBC [27]. However, since this method usually employs perfusion of human RBC through rat vessels, the results may only be of limited value due to possible trans-species effects.

In the *in vivo* model, mouse red blood cells containing human sickle hemoglobin were tested for their adhesiveness to endothelium in transgenic mice [28]. Under intravital microscopic observation, the increased adhesion of transgenic mice RBC to the endothelium was confirmed to occur *in vivo* and the adhesion sites were identified to depend on the diameter of the venules as well as the rheological properties of RBC [46]. This method has been one of the most successful models providing information of the adhesion dynamics similar to those as expected in patients with sickle cell disease.

Summary

As reviewed above, different experimental models have been designed for different experimental needs, focusing on different aspects. Although results obtained from these models are sometimes different, to date, several cellular factors and plasma factor have been identified as determinants of abnormal RBC-EC adhesion.

Cellular factors

Adhesion molecules

Table 1-2: Specific ligands, receptors and plasma ligands involved in RBC-EC adhesion.

RBC	RBC ligand	Plasma ligand	EC receptor	Reference
sickle	CD36	TSP	CD36	[23]
	CD47		HSPG	[44, 47]
	Sulfated glycolipid		$\alpha_v\beta_3$	
	Sulfated glycolipid	vWF	$\alpha_v\beta_3$	
	(unclear)		GP1b	
	$\alpha_4\beta_1$		VCAM-1	[34]
	(unclear)	-	P-selectin	[48]
diabetic	AGEs		RAGEs	[49]
malaria	Sequestrin		CD36	[50]
	Modified band 3		CD36	[51]
			Thrombospondin	[52]
			CD36	[53]
	PfEMP1		Thrombospondin	[54]
			Chondroitin sulfate A	[55]
			ICAM-1	[5]
			PECAM-1	[53, 56]
			P-selectin	[57]
	(unclear)		VCAM-1	[6]
	(unclear)		E-selectin	[6]
	(unclear)		$\alpha_v\beta_3$	[58]

One of the most well-defined cellular factors involved in abnormal RBC-EC adhesion are the ligands and receptors as summarized in Table 1-2. Note that the plasma ligands will be discussed separately in the following chapter. There can be various ligands and receptors even in the same disease, indicating the complexity of abnormal or pathological RBC-EC adhesion. Normal RBC usually do not bear these ligands and receptors, but RBC from patients with vascular diseases express them. Ligand-receptor interactions can be established directly between RBC and EC, or with the involvement of plasma ligands. Establishment of these interactions is usually very specific and can result in firm RBC-EC adhesion [59]. The

specificity of these interactions has not only allowed identifying them by using monoclonal antibodies, but has also provided the possibility of anti-adhesion therapy by antibody blockage [59, 60].

Adhesion molecules can act as ligands and specific receptors in RBC-EC adhesion as mentioned above, but they can also be non-receptor ones. Phosphatidylserine (PS) is a membrane lipid that under normal conditions is exclusively located on the inner leaflet of the RBC membrane [61]. However, in hemoglobinopathies or under oxidative stress states, PS is flip-flopped to the outer leaflet [62].

PS exposure has been suggested as a significant adhesion molecule in RBC-EC adhesion in sickle cell anemia [62-65], hereditary hydrocytosis [21], and chronic uremia [20]. An increased PS exposure provides RBC with an increased adhesiveness to EC or the sub endothelial matrix. The mechanism of PS in RBC-EC adhesion is not fully understood though the adherence mediated by it has been correlated to multiple specific receptor-mediated interactions [63, 64, 66]. PS is usually considered as a non-receptor adhesion molecule that is able to trigger receptor-mediated interactions [8]. For example when PS is expressed on RBC, Thrombospondin (TSP) has been suggested to act as a plasma ligand with $\alpha_v\beta_3$ being its EC receptor [63]. More recently, it has been suggested that EC express a receptor capable of recognizing PS on RBC [67].

As reviewed above, adhesion molecules dictate the ligand-receptor interactions between RBC and EC. One of the major advances in understanding the mechanism of adhesion molecules is the identification of signaling pathways. In a signaling pathway, adhesion molecules can be agonist or participant in a cascade of intercellular or intracellular signal transductions, which alter the membrane properties and finally increase the adhesiveness of cells. One relatively well-developed example is the TSP-CD47 signaling pathway [47, 68, 69]. As shown in Figure 1-1, the binding of solute plasma TSP to integrin-associated protein (IAP, CD47) activates an intracellular signaling pathway mediated by shear stress. With subsequent involvement of both G protein and tyrosine kinase(s), the pathway finally activates the specific interaction between $\alpha_4\beta_1$ on sickle RBC membranes and immobilized TSP on the subendothelial matrix.

Figure 1-1: Proposed sickle RBC adhesion pathway activated by plasma TSP [47]. When the plasma level of TSP is negligible, IAP on sickle RBC adheres to TSP on the sub endothelial matrix (left). When the plasma level of TSP is elevated during vaso-occlusion, IAP on sickle RBC is mainly occupied by plasma TSP (right). With the mediation of physiological shear stress, an intracellular pathway is activated involving G- and tyrosine kinase. This pathway finally triggers the binding of $\alpha_4\beta_1$ on sickle RBC to TSP at the sub endothelial matrix.

Another example for a signaling pathway involved in RBC-EC adhesion is the interaction between the basal cell adhesion molecule Lutheran (Lu/B-CAM) and laminin [70]. As shown in Figure 1-2, increased levels of circulating epinephrine activate β_2 -AR on the RBC membrane and then $G\alpha_s$. The following stimulation of adenylyl cyclase (AC) accelerates the conversion of ATP to cAMP, leading to the activation of protein kinase A (PKA). Through an unknown subsequent process, Lu/B-CAM finally becomes a specific receptor on sickle RBC for laminin, a protein located at the subendothelial matrix throughout most vascular beds. When an inhibitor to PKA is used, the sickle adhesion is markedly reduced, suggesting a strong dependence of the adhesion on PKA activation and thus the significance of this adhesion pathway in sickle RBC adhesion to the subendothelial matrix [38, 41]. Additionally, a similar adhesion pathway involving Lu/B-CAM has been suggested to control the increased RBC-EC adhesion in polycythemia vera (PV) [71].

Figure 1-2: Proposed involvement of Lu/B-CAM in sickle RBC [59]. An increased level of circulating epinephrine activates β_2 -AR on the RBC membrane and then $G\alpha_s$. The following stimulation of adenylyl cyclase (AC) accelerates the conversion of ATP to cAMP, leading to the activation of protein kinase A (PKA). Through an unknown subsequent process, Lu/B-CAM on RBC surface is finally activated becoming a specific receptor for laminin at sub endothelial matrix.

The heterogeneity of RBC adhesiveness

The above review demonstrates how adhesion molecules govern RBC-EC adhesion in disease. Other than these biochemical cellular factors, rheological and biophysical properties of RBC have also been identified to play an important role in RBC-EC adhesion. For example, density-separated RBC in sickle cell disease have been shown to exhibit distinct mechanical and rheological characteristics [45, 72] with the lighter fraction retaining higher deformability, whereas the denser fraction is more rigid [72]. This difference has been correlated to the heterogeneity of RBC adhesiveness to the endothelium [46].

With the micropipette assay, it has been demonstrated that deformable sickle RBC are far more adhesive to the endothelium as compared to rigid irreversibly sickled cells (ISC) [25]. This was also confirmed by a study employing an *ex vivo* method using intravital microscopy. In this study it was demonstrated that adhesion of sickle RBC to the endothelium depends on the density and thus deformability of the cells. It was shown that reticulocytes and young discocytes are the most adhesive subpopulation, followed by discocytes, irreversibly sickled cells, and finally unsicklable dense discocytes [27]. It should be noted that clinical studies have linked this heterogeneity of the RBC adhesiveness to the severity of sickle occlusion in patients [73].

The deformability of red blood cells affects the rheological properties and thus the flow behavior. Deformable RBC can obtain sufficient surface contact to the endothelium more easily than rigid RBC and are thus more likely to adhere to EC. After the adhesion of sickle RBC to EC was widely reported with *in vitro* models, it was demonstrated with both *ex vivo* and *in vivo* models that abnormal RBC-EC adhesion occurs mainly in post capillary venules [27, 28]. In particular, observations via intravital microscopy revealed that the extent of adhesion is inversely related to the venular diameter (s.a. Figure 1-3). This finding was subsequently confirmed with a mouse model [74]. Hence, it was concluded that adhesion *in vivo* preferably occurs at regions with low shear stress (~ 1 dynes/cm²).

Figure 1-3: Sickie RBC adhesion to EC as a function of venular diameter studied with *ex vivo* model using rat vasculature [27]..

Based on these observations, it has been suggested that adhesion of sickle RBC to the vessel wall involves several steps [27, 29, 75]. As illustrated in Figure 1-4A, initially deformable sickle RBC (i.e. reticulocytes and discocytes) adhere in the post capillary regions where the blood flow is slow, but this initial adhesion does not immediately induce vaso-occlusion. However, once the blood flow is impaired, as illustrated in Figure 1-4B, the rigid irreversibly sickled cells are subsequently trapped in the vessel wall, thereby inducing vaso-occlusion.

Figure 1-4: A two-step model proposed for the dynamic adhesion process of sickle RBC to vessel wall based on the heterogeneity of RBC adhesiveness [75]. See text for more details.

An extended multi-step model has also been suggested for other blood elements [10, 11, 76, 77]. Figure 1-5 illustrates the rolling of activated leukocytes along the

vessel wall, which is known to activate EC (step i) [78]. Compared to RBC adhesion, the recruitment of activated leukocytes on the EC surface can impair blood flow more severely in the post capillary venules whose size are about 20 μm . With similar size ($\sim 7 \mu\text{m}$), RBC adhere to EC usually in a flattened shape (due to their deformability) but leukocytes adhere to EC in a spherical shape (due to their rigidity). Hence, the adherence of leukocytes occupies larger areas of the luminal venule, impairing blood flow. The intermittent blood flow increases the transit time of RBC and thus enhanced sickling [10, 18], leading to interactions between sickle RBC and leukocyte (step iii). The subsequent adhesion of irreversibly sickled cells to the vessel wall may then cause vaso-occlusion (step iv).

Figure 1-5: A multi-step model proposing the involvement of leukocyte adhesion in vaso-occlusion [10]. See text for details.

The activation of endothelium

Changes to the endothelium can also lead to elevated RBC-EC adhesion. The endothelium not only comprises a physical barrier separating blood flow from tissue, but also governs various pathophysiological events by its high sensitivity to the local environment. During sickle occlusion, the endothelium deviates from its quiescent state and becomes activated [14, 79-81]. The activated endothelium may express adhesion molecules on the surface (e.g. VCAM and selectins) and thus become proadhesive. It may also express tissue factors and secrete von Willebrand factor (vWF) and acquire a procoagulant phenotype [82-84]. The proadhesive and procoagulant endothelium represents an activated coagulation system in sickle cell disease [14, 85]. Thrombin, an important regulator of the coagulation system, has been identified to be significantly increased during vaso-occlusion and has been suggested as a critical EC perturbant capable of activating the endothelium [86-88].

EC activated by thrombin have been found to exhibit increased adhesiveness for sickle RBC and even for normal RBC, but the detailed mechanism are only partly understood [89]. EC activated via thrombin can express adhesion molecules, such as ICAM-1 and P-selectin [90]. Specifically, P-selectin has been suggested as a significant mediator of thrombin-activated endothelial cell adhesion in that both normal and sickle RBC adhere to this receptor [48, 91]. Moreover, it has also been demonstrated that thrombin activation of EC induces EC contraction and thus exposure of the subendothelial matrix [92], and thus thrombospondin (TSP) and laminin, which have been suggested as specific receptors for sickle RBC [38, 63].

Plasma factors

Apart from the above-mentioned cellular factors, plasma, as the adhesion environment, is also known to be involved in abnormal RBC-EC adhesion [8, 33].

The adhesion-promoting effect of plasma

At the same time when sickle RBC were identified to have increased adhesiveness to EC, the adhesion-promoting effect of plasma was also suggested [93]. The adhesion of sickle RBC to the endothelium was found to be highly dependent on the media, with the adhesion rates in acute-phase plasma being the highest and those in isotonic buffer being the lowest. These adhesion-promoting effects of plasma were also confirmed with the micropipette technique [26]. In particular, it was found that both membrane changes and plasma factors are required for sickle RBC adhesion [25].

It has been recognized that the adhesion-promoting effects of plasma results from some proadhesive proteins. This was demonstrated by the fact that the adhesion-promoting effects of acute phase plasma from patients with sickle cell anemia was significantly reduced when it was deprived of proteins via cryo-precipitation [93], or if it was heated to 60°C for one hour denaturizing the proteins [26]. Moreover, when isotonic buffer with albumin was supplemented with cryo-precipitated proteins from acute-phase plasma, it caused a significantly increased adhesion of sickle RBC to EC [93]. It should also be noted that the onset of abnormal RBC-EC adhesion during sickle occlusion has been observed to be always accompanied by an elevated level of several plasma proteins [93, 94].

Table 1-3: Major pro-adhesive plasma proteins identified for RBC-EC adhesion

RBC	Receptor on RBC	Plasma proteins	Receptor on EC	References
Sickle	(unclear)	Fibrinogen (Fg)	(unclear)	[93]
	$\alpha_4\beta_1$	Fibronectin (FN)	(unclear)	[40, 95]
	CD36 CD47	Thrombospondin (TSP)	CD36 $\alpha_v\beta_3$	[23, 44, 47, 68]
	GPIb	Unusually large von willebrand factor (vWF)	$\alpha_v\beta_3$	[36, 96]
Diabetes	(unclear)	Fibrinogen (Fg)	(unclear)	[97]
Normal	(unclear)	Fibrinogen (Fg)	(unclear)	[97]
		Fibronectin (FN)		

Plasma ligands for receptor-mediated interactions

Table 1-3 summarizes proadhesive plasma proteins. As a likely mechanism for the proadhesive effect of plasma proteins, it has been postulated that they act as ligands cross-linking specific receptors on RBC and EC.

Figure 1-6: Comparison of plasma level of TSP between steady state and acute vaso-occlusive crisis in patients with sickle cell disease. Data rearranged from literature [94].

For example solute TSP has been suggested to act as a plasma ligand, cross-linking sickle RBC and EC. Receptors for this plasma ligand have been identified to be

CD36 on the RBC surface and $\alpha_v\beta_3$ on the EC surface [23, 96]. The plasma level of TSP is significantly higher during sickle occlusion as indicated in Figure 1-6 [94] and it was demonstrated that the elevated level of TSP correlates with the increased adhesiveness of sickle RBC to EC. Studies using antibody blockade revealed that the increased level of TSP in plasma during vaso-occlusion results from platelet activation [23, 44], which has also been correlated with increased RBC-EC adhesion [98-100].

Another important protein that has been suggested to act as a plasma ligand in RBC-EC adhesion is large vWF [36, 96]. Similar to TSP, vWF also has two forms with one being solute in plasma and the other one located at subendothelial matrix. Plasma vWF has a molecular mass (M_w) ranging from 850 kDa to a few thousand kDa, whereas vWF at the subendothelial matrix secreted by EC has even higher M_w of up to 20,000 kD [101]. Upon certain stimuli, the large vWF at subendothelial matrix can be released into the plasma. Studies detailing the involvement of platelet aggregation in sickle RBC adhesion to the endothelium also revealed that large vWF can act as a plasma ligand also promoting RBC EC adhesion. Briefly, via its tetrapeptide of Arg-Gly-Asp-Ser, vWF can bind to the GPIb complex on platelets thereby causing platelet aggregation. On the other hand, pre-incubation of sickle RBC with Arg-Gly-Asp-Ser significantly reduced sickle RBC adhesion in the presence of vWF [36], implying that GPIb on RBC is a specific receptor for vWF. The vWF receptor on the EC surface has been suggested to be $\alpha_v\beta_3$ as detected via monoclonal antibodies [96]. It should be noted that the interaction of large vWF and deformable sickle RBC has been suggested to be an initial step in vaso-occlusive crisis [102].

Fibrinogen

The level of fibrinogen in plasma has been found to be much higher in patients with sickle cell disease as compared to that in healthy donors, and it is further elevated during sickle occlusion [93, 94, 98]. This elevated level of fibrinogen has been correlated with an increased adhesion of sickle RBC to EC. As shown in Figure 1-7A [93] the adhesion has a strong dependence on the medium, with the adhesion in normal plasma plus fibrinogen being the highest, followed by normal plasma and then fibrinogenemic plasma (fibrinogen deprived). When this effect is

compared to that of plasma on sickle RBC-EC adhesion (Figure 1-7B), it can be seen that the adhesion in normal plasma plus fibrinogen resembles that in acute-phase plasma. These comparisons clearly suggest that fibrinogen is a modulator of sickle RBC adhesion to the endothelium. A similar proadhesive effect of fibrinogen has also been observed for RBC from patients with diabetes mellitus [12, 97].

Figure 1-7: (A) The impact of the addition of fibrinogen into normal plasma in sickle RBC adhesion; (B) The promoting effect of plasma from patients with sickle cell disease [93]

Although the adhesion-promoting effect of fibrinogen has been reported for pathological and even normal RBC [103], the mechanism remains unclear. Compared to TSP and vWF (Page 18 and 19), fibrinogen does not seem to be required for a specific signaling pathway. However, when normal plasma is supplemented with fibrinogen to an extent equivalent to that during vaso-occlusive crisis, it leads to significantly increased adhesion of sickle RBC to EC [93]. This suggests an additive effect of fibrinogen for the adhesion of sickle RBC to the endothelium. Moreover, unlike TSP and vWF, which act as ligands cross-linking receptors between RBC and EC, to date no specific receptors for fibrinogen have been identified on either the RBC or the EC surface regarding its adhesion-promoting effects [8, 33]. Therefore, it has been suggested that specific receptors might not be necessary for its adhesion-promoting effect [104]. This possibility is

supported by the finding that different from the case of TSP, neither sickle nor normal RBC adhere to purified immobilized fibrinogen in the presence of Ca^{2+} [23], which is usually involved in receptor-mediated interactions.

Intercellular forces

When considering cell adhesion, it is important to distinguish between specific and non-specific forces (Figure 1-8). Specific forces are regulated by the expression of receptors on the cell surface and their binding to specific receptors on the target cell. Non-specific forces usually include attractive van der Waals forces, repulsive electrostatic forces, and repulsive steric forces.

Figure 1-8: A schematic picture adopted from literatures to illustrate the intercellular forces [105].

Ligand-receptor interactions

In terms of cell-cell adhesion, there are a few important characteristics of ligand-receptor interactions that need to be emphasized: 1) ligand-receptor interactions are strong associations with the bonding energy ranging from a few $k_B T$ to a some ten $k_B T$ per bond [106]; 2) the formation of ligand-receptor interactions is very specific as outlined in the previous paragraph; 3) cell-cell distance and a correct domain orientation of the biomolecules involved are important factors for the establishment of ligand-receptor interactions [107]; 4) the formation of bonds is a dynamic process with the association rates outweighing the dissociation rates [108].

Van der Waals forces

Van der Waals forces (VDW) are electrodynamic interactions originating from the quantum mechanical dance of the electrons. At any moment, fluctuations of the electrons produce an attractive dipole-dipole interaction when two cells are close. However, VDW is weak between cells in biological media due to high water content [105].

Steric repulsion

The cell glycocalyx consists of many glycoproteins and glycolipids, which can be considered as an immobilized layer of macromolecules. When two cells are in close contact, the glycocalyx becomes compressed and thus an entropic repulsion of the macromolecules arises. Consequently, steric repulsion becomes significant when the cell-cell distance is smaller than the sum of glycocalyx thickness of the two cells.

Electrostatic forces

Figure 1-9: Sialic acids on oligosaccharide chains. The carboxyl group of sialic acids dissociates protons under physiological pH [109].

Electrostatic forces originate from the net surface charge of cells and are usually repulsive when cells are in close contact. Cell surfaces are usually negatively charged under physiological pH due to the presence of sialic acids on the membrane (e.g. [110]). For RBC, sialic acids are the terminal residues on oligosaccharide chains that are carried by glycophorin, a glycosylated membrane protein with its hydrophilic N-terminal located on the external surface of the RBC membrane. Glycophorin contains about 90% of the sialic acids of the RBC membrane [111]. The carboxyl group of sialic acid, as shown in Figure 1-9, dissociates at physiological pH, rendering the RBC surface negatively charged.

Summary

If the distance between adjacent cells or surfaces becomes smaller, electrostatic forces and steric repulsion build up. Once the cells are in close contact specific ligand-receptor interactions can be established. Consequently, repulsive and attractive non-specific forces control cell-cell distances, binding probabilities of ligand- receptor interactions [112], and thus cell adhesion.

Polymer depletion interaction

As reviewed above, the model of ligand-receptor interactions has been applied to explain the adhesion-promoting effects of TSP and vWF on RBC-EC adhesion. However, the results from fibrinogen seem to suggest an alternative model. As illustrated in Figure 1-8, another non-specific force that can also act on cell-cell interactions is polymer depletion interaction.

Polymer depletion interaction is an attractive non-specific entropic force. When cells are in a solution containing non-adsorbing macromolecules and if the entropy loss of the macromolecules near the cell surface is not balanced by an attractive adsorption energy (i.e. no specific binding or only limited non-specific adsorption of the macromolecule), the macromolecules are excluded from the proximity of cell surfaces. Thus, surrounding the cells is a depletion layer in which the concentration of the macromolecules is much lower than that in the bulk solution. This exclusion of macromolecules near the cell surface leads to an osmotic gradient and as two cells or surfaces approach, solvent is displaced from the depletion zone into the bulk phase leading to an attractive force.

By comparing the effects of fibrinogen on RBC-EC adhesion (Page 24) with the mechanism of depletion interaction mentioned above, it seems possible that fibrinogen induces RBC-EC adhesion via depletion interaction. This possibility is supported by the following findings: 1) the adhesion-promoting effect of fibrinogen has not been correlated to any receptors on RBC or EC, and it has been suggested that receptors are not necessary for the adhesion promoting effect of fibrinogen [104]; 2) depletion interaction has been suggested to be the underlying mechanism of RBC aggregation [113], which is also induced via fibrinogen,

thereby indirectly suggesting that fibrinogen might be depleted from the RBC surface.

Polymer depletion from RBC surface

The evidence for polymer depletion from the RBC surface has been provided by the measurement of the electrophoretic mobility (EPM) of RBC in polymer solutions (e.g. PEG and dextran) [114-117]. Adding polymers into a solution results in an increased viscosity and thus a reduced EPM as compared to that in polymer-free solution. Usually the EPM and the viscosity can be correlated by Smoluchowski's equation (see details in Page 45). For example, in Figure 1-10, the dashed line represents the ratios of the EPM in dextran solutions to the EPM in polymer-free solutions as predicted by Smoluchowski equation. However, the ratios calculated from experimental results indicate higher EPM in dextran solutions than those predicted by Smoluchowski's equation. This effect also shows a strong dependence on the molecular mass (M_w) of dextran. Suspending RBC in dextran 500 kDa results in much higher EPM values than those predicted by Smoluchowski's equation, while suspending RBC in dextran 10 kDa results only moderately increased EPM values, close to those predicted by Smoluchowski's equation.

Figure 1-10: Ratios of electrophoretic mobility (EPM) in dextran solutions to EPM in polymer-free solutions. The dash line represents the theoretical predicted ratios by Smoluchowski's equation, while the solid lines represent the ratios calculated from experimental results [113].

Figure 1-11: Schematic profile of the viscosity near the RBC surface. The x-axis represents the distance from the cell surface.

Smoluchowski's equation uses the bulk viscosity to compute the EPM. The higher EPM ratios in Figure 1-10 indicate a lower viscosity near the RBC surface as compared to the bulk viscosity and thus suggest polymer depletion from the cell surface. As illustrated in Figure 1-11, due to polymer depletion, there are less polymer segments in the proximity of the RBC surface, leading to a reduced localized viscosity, which enables the particle to move faster and thus to obtain a higher electrophoretic mobility. The thickness of the depletion layer is in the same range as the size of the depleted polymer [118, 119]. Therefore, with a higher molecular mass, dextran 500 kDa results in a larger depletion layer, leading to higher EPM ratios than dextran 10 kDa.

Although several polymers (e.g. dextran and PEG) have been demonstrated to be depleted from RBC surface by EPM measurement, direct evidence of protein depletion from the cell surface is still lacking. Plasma proteins usually have a net charge under physiologic conditions, for example, fibrinogen is negative. Thus, when using measurement of the EPM to study the possible depletion effect of plasma proteins on RBC, the impact of the electrostatic forces between the cell surface and the proteins on polymer adsorption or depletion needs to be considered. With this regard, the EPM of RBC in solutions containing an anionic polymer poly(styrene sulfonate, PSS) was studied [120]. It was demonstrated that the EPM of RBC was significantly increased as compared to control (the EPM of RBC in PBS) and the mechanism is suggested to be due to both PSS depletion and weak adsorption on the RBC surface. These results thus indicate that plasma proteins can be depleted from the RBC surface although there is weak adsorption.

Polymer adsorption on the RBC surface has been reported [121-124]. This adsorption may affect the depletion layer thickness as well as steric repulsion when cells are in close contact [125]. However, due to the fact that the data of polymer adsorption (e.g. dextran and fibrinogen) are subject to experimental artifacts and are quantitatively difficult to interpret [126], the effects of polymer adsorption in the depletion-mediated RBC interactions remain to be explored.

RBC aggregation induced by depletion interaction

When RBC are suspended in solutions containing polymers, they form aggregation. Because of their symmetrical biconcave shape, RBC usually form rouleaux, a 2-dimensional aggregate that resembles a stack of coins. However, when RBC are suspended in polymer-free isotonic buffer, they do not aggregate. Fibrinogen has been found to induce RBC aggregation [121, 127, 128]. Recent reports suggested that RBC aggregation is induced by depletion interaction [113, 129]. This in turn suggests that fibrinogen is also depleted from the RBC surface. However, it should be emphasized that direct evidence of fibrinogen depletion from the RBC surface has not been reported. Figure 1-12 illustrates schematically how depletion interaction induces RBC aggregation in polymer solutions. When RBC are suspended in solutions containing non-adsorbing macromolecules (Figure 1-12A), they are surrounded by a depletion layer. This exclusion of macromolecules near the cell surface leads to an osmotic gradient and as RBC approach each other, solvent is displaced from the depletion zone into the bulk phase leading to an attractive force and thus RBC aggregation or rouleaux formation (Figure 1-12B).

Figure 1-12: Schematic pictures showing how depletion interaction induces RBC aggregation. (a), RBC are surrounded by depletion layers when they are suspended in polymer solutions. Concentration of the polymers in the depletion layer is lower than the

bulk value. (b) When two cells approach each other, solvent is displaced from the cell-cell interface into the bulk and aggregation forms due to the attractive depletion interaction.

The energy of RBC-RBC interactions induced by depletion interaction has been theoretically predicted as shown in Figure 1-13 [129]. In this modeling, only depletion interaction and electrostatic repulsion are considered. This is because: 1) the RBC interaction energy usually reaches the minimum at a distance larger than twice of the cell glycocalyx. Hence, the steric repulsion can be neglected; 2) at such a cell-cell distance, van der Waal forces are relatively very small and are negligible. Figure 1-13 conveys the important messages that depletion interaction is a strong non-specific force that can induce cell-cell interactions. As demonstrated, depletion interaction can overcome electrostatic repulsion and lead to RBC-RBC interaction with an energy ranging from 0 to $8 \mu\text{J}/\text{m}^2$ (i.e. 0-2000 $k_B T$). These computed values of RBC-RBC interaction energy were found to be in the same range as the experimental data (Figure 1-13B). In addition, the theoretical dependence of the RBC-RBC interaction on polymer concentration and molecular mass is in good agreement with the experimental findings [124, 130-132]. In brief, RBC-RBC interaction depends on polymer concentration in a biphasic profile and increases with increasing molecular mass for a given type of polymer.

Figure 1-13: (A) The energy of RBC-RBC interaction as a function of polymer concentration. The energy is calculated as the sum of depletion interaction and electrostatic repulsion for different polymers as indicated. (B) Comparison between calculated (solid line) and experimental data (points) on the RBC-RBC interaction energy. [129].

RBC adhesion to artificial surfaces induced by depletion interaction

Depletion interaction has been shown to induce not only RBC aggregation, but also RBC adhesion to artificial surfaces. In a quasi-flow adhesion assay using a parallel-plate flow chamber, normal RBC were found to adhere to albumin-coated glass surfaces, when dextran with high Mw (> 70 kDa) was present (Figure 1-14) [133]. After allowing RBC suspended in dextran solutions to settle onto albumin coated glass surfaces for fixed times, some RBC remained adherent during rinsing (Figure 1-14A). This adherence was found to increase with the polymer concentration and polymer molecular mass (Figure 1-14B). This adherence was attributed to a reduced surface concentration of large polymers, and thus attractive depletion interaction.

Figure 1-14: Adherence of normal RBC to albumin-coated glass surfaces studied with a parallel-plate flow chamber. RBC were suspended in polymer-free solution or in dextran solutions. The cells were given a static period to settle prior to rinsing with a constant shear stress. (A) Adherence as a function of time during rinsing at 0.75 Pa. (B) Adherence as a function of the polymer concentration and molecular mass. Note that the adherence is presented as % of the cells settled at the chamber bottom before rinsing [133].

The suppression of membrane undulation also demonstrated that RBC adhesion to surfaces is induced by depletion interaction. Cell membranes are flexible and thermal excitation can lead to visible undulations at room temperature [134]. These undulations generate a repulsive pressure because of their entropic resistance to compression [106]. Figure 1-15 demonstrates that in the presence of non-adsorbing macromolecule (i.e. dextran 500 kDa), membrane undulation of RBC are significantly suppressed by depletion interaction [135], i.e. the vertical

displacement is 5.0 ± 1.7 nm in the dextran solution and 10.0 ± 5.2 nm in the polymer-free solution. This indicates a firm adhesion of RBC to the surface induced by depletion interaction.

Figure 1-15: Membrane undulations along the vertical direction for a cells suspended in (A) polymer-free solution and (B) in 5 mg/ml of dextran 500 kDa and (C) the respective undulations amplitude as a function of time [135].

Determinants of depletion interaction

Physicochemical properties of polymers

As outlined above, both RBC aggregation and RBC adhesion to albumin-coated glass surfaces can be induced by depletion interaction and in both cases a strong dependence on the polymer concentration and molecular mass can be observed. At constant molecular mass, an optimal polymer concentration exists maximizing the interaction energy of RBC. This can be seen for RBC aggregation (Figure 1-13A) and also for RBC adhesion to albumin-coated glass surfaces (Figure 1-16).

Figure 1-16: Adhesion energy of RBC to albumin-coated glass surface as a function of the polymer concentration. RBC were suspended in dextran 70 kDa (circle) or 500 kDa (square) at different concentrations.

Not only polymer concentration but also the molecular mass and thus polymer size play a role in depletion-mediated RBC interactions as shown in Figure 1-13A. The interaction energy between RBC increases with increasing molecular mass and thus polymer size. This dependence is also illustrated in Figure 1-14B and Figure 1-16, where the presence of dextran 500 kDa results in higher adherence of RBC to albumin-coated glass surface as compared to dextran 70 kDa.

In conclusion, the physicochemical properties of polymers, such as the molecular mass and the concentration, are determinants of depletion-mediated RBC interactions.

Cellular factors

Other than physicochemical properties of polymers, cellular factors are also known to play an important role in depletion-mediated RBC aggregation. This has been shown by the fact that in a defined media containing macromolecules, RBC from different populations aggregate to different extents. The intrinsic cellular properties of RBC that determine their abilities to form aggregation in a defined medium are termed RBC aggregability [113]. For example, RBC from different healthy donors often show differences in their aggregability in plasma [136, 137] or in polymer solutions (e.g. dextran [137] and polyvinylpyrrolidone 360 [138]). Apart from this

donor effect, RBC from the same donor but of different subpopulations have also been found to show differences in their aggregability [139]. For example, old RBC have been shown to have greater extent of aggregation than young RBC in autologous plasma [137]; and a two-fold greater aggregation was observed for old RBC than for young RBC in solutions containing dextran 70 kDa [132].

It has been suggested that these differences in RBC aggregability are due to the differences of RBC surface properties. Changes of RBC surface properties can lead to altered depletion interaction. For example, it has been suggested that as RBC become older in their life span, the thickness of glycocalyx is reduced, resulting in increased RBC aggregability [140]. This impact of the RBC glycocalyx thickness on the interaction energy in RBC aggregates has been theoretically elucidated in Figure 1-16 [140].

Figure 1-17: Impact of different thicknesses of RBC glycocalyx (δ) on the interaction energy of RBC aggregates in defined polymer solutions. The interaction energy was calculated based on a model that considers depletion interaction and electrostatic repulsion [140].

Apart from the thickness of the RBC glycocalyx, the surface charge is also playing a significant role in RBC aggregation in polymer solutions. For example RBC from healthy donors do not aggregate in dextran 40 kDa, while RBC from patients with sialic acid deficiency demonstrate a significantly increased aggregation in dextran 40 kDa [141]. This altered aggregability was ascribed to the significant

loss of sialic acid on the patient's RBC membrane, thereby reducing the electrostatic repulsion between cells in close contact. Other studies using neuraminidase to remove sialic acid from the RBC surface confirmed the significant impact of the surface charge on RBC-RBC interactions in polymer solutions [142-145].

In conclusion, the RBC surface properties, such as the thickness of glycocalyx and surface charge, are determinants of depletion-mediated RBC interactions.

Conclusion and Specific Aims

As reviewed above, plasma proteins can have a marked impact on abnormal RBC-EC adhesion. The central hypothesis of this thesis is that depletion interaction resulting from non-adsorbing macromolecules is an alternative mechanism for the proadhesive effects of plasma proteins. Specifically, fibrinogen is suggested to be a non-adsorbing macromolecule that promotes RBC-EC adhesion by inducing depletion interaction.

In order to substantiate this hypothesis, dextran, a polyglucose that has been shown to be depleted from RBC surface, is used to test whether non-adsorbing macromolecules can induce RBC-EC adhesion in buffer/albumin as well as in plasma. In order to study the effects of RBC surface properties on depletion-mediated RBC adhesion to EC, two enzyme treatments are applied to alter RBC surface properties. Neuraminidase is used to remove sialic acid from RBC membrane and thus to reduce the surface charge, while α -chymotrypsin is used to dissociate the RBC glycocalyx proteins.

Additionally, it is hypothesized that depletion interaction can promote RBC-EC adhesion under pathological conditions. To mimic these conditions, the adhesion of RBC with enhanced PS exposure is investigated in the presence of non-adsorbing macromolecules as well as the adhesion of RBC to thrombin-activated EC. These studies are believed to provide some insights for the proadhesive effects of the non-adsorbing macromolecules in plasma under pathological conditions.

Chapter 2 Materials and methods

Samples and solutions

Dextran solutions

Dextran with molecular weight of 40 kDa, 70 kDa, 500 kDa, 2 MDa (Sigma) were dissolved separately in the desired buffer or medium as stated in the Results section of each chapter.

Preparation of whole plasma or diluted plasma

Blood was drawn from healthy donors into plastic tubes with glycolytic inhibitor (5 mg sodium fluoride and 4 mg potassium oxalate, BD, Cat# 367921). Plasma was separated from whole blood by centrifugation at 1000 g for 10 minutes and stored at -20 degree. Upon usage, the frozen plasma was thawed. Dextran was first dissolved in HBSS (without Ca^{2+} and Mg^{2+}) and this DEX-HBSS was then mixed with whole plasma in a 1:1 volume ratio. For the preparation of diluted plasma with added fibrinogen (Fg), Fg was first dissolved in whole plasma and this Fg-plasma was then mixed with HBSS (without Ca^{2+} and Mg^{2+}) in 1:1 volume ratio. These two solutions are thus termed diluted plasma and the added concentration of dextran or fibrinogen are stated in the figures of the Results section (Chapter 5). For studies employing whole plasma, fibrinogen was added directly into the plasma without any further dilution.

Erythrocytes

Preparation of erythrocytes

With written consent, blood was drawn from the antecubital vein of healthy adult volunteers into plastic whole blood tubes with spray-coated with K_2EDTA (BD, Cat # 367835) at the university medical centre. The study was approved by the Institutional Review Board of Nanyang Technological University. Red blood cells were separated from whole blood by centrifugation at $1,000\times g$ for 10 min, with the plasma and buffy coat removed. The cells were then washed three times with Phosphate Buffered Saline (PBS without Ca^{2+} and Mg^{2+} , 0.01M phosphate buffer

and 0.154M sodium chloride, pH = 7.4) containing 0.2% bovine serum albumin (BSA, Sigma) and re-suspended in the desired solutions.

Neuraminidase

After repeated washings as described above, RBC were re-suspended in Tris-acetate buffer (40 mM Tris base, 40 mM acetic acid, 0.3 mg/ml BSA, PH 5.7) with the hematocrit adjusted to approximately 20%. Neuraminidase (Sigma) was added at the desired concentration, followed by an incubation at 37°C for 1 hour for the blood suspension. The enzyme treatment was terminated by three subsequent washings with cold (4°C) PBS containing 0.2% BSA. A control sample was prepared in parallel without any enzyme treatment.

α -chymotrypsin

After repeated washings as described above, RBC were re-suspended in PBS-Glucose buffer (0.1 g/dL glucose in PBS) with the hematocrit adjusted to approximately 40%. Concentrated α -chymotrypsin (Type II, Sigma) solution was prepared by dissolving 10 mg of the enzyme powder in 1 ml PBS-Glucose buffer before usage (i.e. 10 mg/ml). The concentrated enzyme solution was then added into the RBC suspension (HCT 40%) to obtain the desired concentration of α -chymotrypsin and incubated at 37 °C for 1 hour, followed by three washings with cold (4°C) PBS containing 0.2% BSA to terminate the enzyme treatment. A control sample was prepared in parallel without any enzyme treatment.

Phosphatidylserine exposure

PS exposure was obtained from normal RBC by calcium-ionophore treatment as described elsewhere [146]. In brief, RBC were washed three times with Hanks' buffered saline solution (HBSS without Ca^{2+} and Mg^{2+} , 0.4 g/L potassium chloride, 0.06 g/L potassium phosphate monobasic (anhydrous), 8 g/L sodium chloride, 0.04788 g/L sodium phosphate dibasic (anhydrous), 1 g/L glucose, Sigma) and were then re-suspended in the same solution with the HCT adjusted to approximately 30%. This RBC suspension was incubated with 5mM N-ethyl maleimide (NEM, Sigma) for 30 minutes at room temperature, followed by two washings with HBSS without Ca^{2+} and Mg^{2+} . The cells were then re-suspended in

the same solution with the HCT adjusted to approximately 16%. Subsequently, this suspension was incubated with 2mM CaCl_2 (Sigma) at 37°C for 3 minutes, followed by an incubation for 1 hour at 37°C with the calcium ionophore A23187 (Sigma) at the desired concentrations. The treatment was terminated by washing the cells once with HBSS containing 2.5mM EDTA (Bio-Rad) to remove Ca^{2+} , and two washings with HBSS containing 1% BSA. The control sample was prepared in parallel without any treatments (i.e. normal RBC).

Endothelial cells

Cell culture

Human umbilical vein endothelial cells (HUVEC, termed EC in the following text) were purchased from Lonza (UK). The culture medium consisted of 90vol% basal medium (MCDB131 containing 2 mM L-Glutamine, Sigma) and 10vol% FBS (PAC) with added sodium bicarbonate (1.5 g/L; Sigma), heparin (0.1 mg/ml; Sigma), bovine brain extract (BBE) (0.5 ml/L; Hammond Cell Tech), and penicillin/streptomycin (120 U/ml, Sigma). EC were cultured in tissue culture flasks at 37°C in a CO_2 (5%) incubator. Once the cells reached 70-80% confluence, they were sub-cultured into 35 mm petri dish (Greiner) pre-coated with gelatin (Sigma). EC were then cultured to 80% confluence for the adhesion assays. EC with passages from 10 to 20 were investigated. No significant differences in adhesion were observed.

EC activation by thrombin

The experimental method is similar as described elsewhere [89, 91]. In brief, EC cultured in 35 mm petri dishes were gently washed twice with serum free medium (SFM, MCDB 131 basal media, 2 mM L-glutamine, 1.5 g/dL sodium bicarbonate, 0.1 mg/ml heparin) to remove serum and secreted adhesive proteins during cell culture, after which SFM or dextran-SFM containing thrombin (Sigma) at the desired concentration was added. The dish was placed at room temperature for 5 minutes and then the medium was removed. These treated EC were subsequently used for adhesion assays.

Methods

RBC-EC adhesion assay

The parallel-plate flow chamber system

Figure 2-1: Setup of parallel-plate flow chamber system.

The parallel-plate flow chamber system is similar as described elsewhere [8, 24] with some minor modifications as illustrated in Figure 2-1. The chamber (Glycotech, USA) consists of an acrylic flow deck and a silicone rubber gasket with the cutout area forming the flow channel. Both the gasket and flow deck were placed into a 35mm petri dish cultured with confluent layers of endothelium. This flow chamber was then mounted on an inverted microscope (IX71, Olympus), with the inlet connected by silicone tubing to an electronic valve that controlled switching between reservoirs containing either RBC suspensions or rinsing solutions. The outlet of the chamber was connected to a syringe pump (Harvard PHD2000) that withdrew either RBC suspensions or rinsing solutions through the flow chamber at a selected volumetric flow rate Q . The microscope, valve, tubing and the two reservoirs were maintained at 37°C inside a thermostated enclosure.

Solutions

RBC were re-suspended in the desired solutions for the adhesion assays. This thesis mainly investigates the impact of different solutions on RBC-EC adhesion under different conditions. Therefore, in order for the readers to easily understand the results, the solutions used for the adhesion assays are stated at the beginning of Results section in the following chapters.

Static-flow assay

RBC suspensions were withdrawn into the flow chamber by the syringe pump, followed by a static incubation (i.e. settling) allowing RBC to settle onto the EC monolayer. The cells were then rinsed by employing a stepwise-increasing shear stress with the same solution (unless otherwise stated) as used for the static incubation but without cells (i.e. cell-free medium). It should be noted that different settling times between 2 and 30 minutes were used as detailed in the results section of the following chapters.

Continuous-flow assay

In the continuous-flow assay the EC monolayer was initially perfused with RBC suspensions for 10 minutes at the desired shear stress and then was rinsed with cell-free medium for another 10 minutes. Cell adhesion was then analyzed by determining the number of adherent RBC.

Wall shear stress in the flow chamber

The wall shear stress τ (dynes/cm²) can be calculated by:

$$\tau = \frac{6\eta Q}{a^2 b} \quad (0.1)$$

where η (poise) is the dynamic viscosity of the solution, Q (ml/sec) is the volumetric flow rate, a is the channel height (0.0254 cm) and b is the channel width (0.5 cm). In this thesis τ is finally presented as Pascal (Pa, 1 dynes/cm² = 0.1 Pa).

Quantification of the adherence

At the end of each rinse captures were taken at 20 random locations along the center of the flow area. The absolute number of adherent RBC was counted in each pictures, and the mean $\pm$ standard deviation (SD) was then converted to adherent cells per millimeter square (mm²). The actual area of a captured picture was obtained by:

$$\text{actual area of the capture} = \frac{\text{CCD length} \times \text{width}}{(\text{magnification})^2}, (\text{mm}^2) \quad (0.2)$$

in which the CCD (charge-coupled device) length is 8.8 mm and the width is 6.6 mm.

Statistics

The above calculation gives the absolute adherence as a function of the applied shear stress during rinsing for an individual experiment. The adhesion is finally presented as the mean $\pm$ SD from several individual experiments under the same conditions at each specific value of shear stress. Between the investigated samples, the Wilcoxon-Mann-Whitney U test, a non-parametric method for two unpaired samples, was performed on the adherence values.

Flow cytometry and fluorescence microscope*Labeling*

Annexin V is a specific antibody for PS on RBC membrane [65]. At a cell count of 10×10^6 /ml, treated RBC as well as normal RBC were separately re-suspended in 0.5 ml of HBSS containing 1.2 mM Ca²⁺. Thereafter 7 μ l of FITC-labeled annexin

V (Invitrogen) were added to the RBC suspensions (i.e. 5 μ l/0.5ml) and incubated in darkness at room temperature for 30 minutes. The cells were then washed once with HBSS containing 1.2 mM Ca^{2+} at 1500 g for 5 minutes, and were then re-suspended in 0.5 ml of the same solution for flow cytometric measurements or observation with a fluorescence microscope.

Setting on flow cytometer

The samples were analyzed on a Becton Dickinson FACScan flow cytometer. 50×10^3 events per sample were acquired. The light scatter and fluorescence channels were set at a logarithmic gain. The forward angle light scatter was E-1 with a threshold of 36. The background and gated zone was defined based on the fluorescence signal of the control. For the A23187-treated sample, only intact cells were gated for fluorescence analysis. The measurements were done in triplicate for each sample.

Electrophoretic mobility

Figure 2-2: Concept of electrophoretic mobility (EPM) and membrane zeta-potential. This schematic picture was adopted (from the manual of nano zetasizer, Malvern Instruments, UK) and then revised.

As illustrated in Figure 2-2, the net charge at the particle or cell surface affects the distribution of counter ions (opposite charge) in the surrounding interfacial region. In the inner region, the so-called stern layer, the ions are strongly bound to the particle or the cell surface. In the outer region, the so-called slipping plane, the

ions are less firmly bound and are able to diffuse. Within the slipping plane, the ions travel with the particle movement, thereby defining the so-called zeta potential.

When charged particles are placed in solutions containing electrolytes and if an electric field is applied, the particles will be attracted by the electrodes. The drag force resulting from the viscosity of the solution tends to oppose this movement. When an equilibrium is reached between these two forces, the particle will move at a constant velocity, which is termed the electrophoretic mobility (EPM). The zeta-potential (ζ) and the electrophoretic mobility (U_E) are related by the Smoluchowski equation:

$$U_E = \frac{\varepsilon \zeta}{\eta} \quad (0.3)$$

where ε is the dielectric constant and η is dynamic viscosity of the solution.

Measurement of the EPM

Normal RBC (i.e. control) or RBC treated with enzymes as described above were washed three times with Sucrose Buffer (10 mM ionic strength, PH 7.4) and then re-suspended in the same buffer at an RBC cell count of $5 \times 10^6/\text{ml}$. The measurement was conducted on a nano zetasizer (Malvern Instrument, UK) with the absolute ζ value calculated as mean $\pm$ standard deviation from three individual experiments (i.e. $n=3$). In Chapter 6, the ζ is finally presented as % of ζ loss as obtained by:

$$\% \text{ of } \zeta \text{ loss} = \left| \frac{\zeta_{\text{treated}} - \zeta_{\text{control}}}{\zeta_{\text{control}}} \right| \quad (0.4)$$

RBC deformability

EI is an indicator of cell deformability and has been used to characterize the deformability of RBC in hemolytic anemia [147] and sickle cell diseases [21]. EI can be measured by ekta-cytometer by applying a series of shear stress on cells passing through a slit monitored by laser. Ekta-cytometer determines the ellipsoidal diffraction image of the cell under shear, which gives the elongation index (EI) as:

$$EI = \frac{A - B}{A + B} \quad (0.5)$$

in which A is the length of major axis while B is length of the minor axis in the ellipsoidal diffraction image.

Measurement of the EI

Normal RBC (i.e. control) or those treated by enzymes were washed once with PBS containing 0.2% BSA, with the supernatant decanted. 6 µl of the packed RBC were collected from the bottom of the tube and were then re-suspended into 0.6 ml of the Medium Solution (RSD-P01, Sewon Meditech, Korea). The deformability of RBC was then measured with an ekta-cytometer (Rheoscan, Sewon Meditech, Korea). All experiments were done in duplicate.

SDS-PAGE gel electrophoresis

Ghost preparation

RBC ghosts were prepared according as described elsewhere [148, 149]. In brief, normal RBC (i.e. control) or those after enzyme treatments were washed once with PBS (without BSA), and the supernatant was then decanted. After centrifugation, 0.5 ml of the packed RBC were collected from the bottom of the tube and then mixed with equivalent amounts of PBS. Thereafter, 14 ml of a hypotonic buffer solution (NaH₂PO₄, 21 mOsm) were added resulting in a volume ratio of the total suspension to the packed RBC of about 30:1. This suspension was then centrifuged at 20,000 g for 40 minutes at 4°C and washed with the same hypotonic buffer (21 mOsm NaH₂PO₄). These procedures (i.e. centrifugation plus washing) were repeated till the ghost pellet at the bottom of the tube became white.

Gel electrophoresis

SDS-PAGE gel electrophoresis was performed on the RBC ghost prepared as described above. The method is essentially the same as described elsewhere [148, 150, 151]. In brief, 12% separating gel and 5% stacking gel were used. The experiments were run at 120V for 2 hours for the separation gel, followed by gel staining with Coomassie brilliant blue. After staining, the gel was captured on a 2D

proteomic imaging system (Perkin Elmer) and was also scanned at 280 nm for membrane protein analysis.

Miscellaneous techniques

RBC cell count

Unless otherwise stated, RBC cell count was fixed to $5 \times 10^6/\text{ml}$ by hemacytometer counting. This cell count gives a hematocrit (HCT) of approximately 0.05%.

Osmolarity measurement of solutions

Different macromolecules were added in isotonic buffer in order to study their effects on RBC-EC adhesion. To determine the osmolarity of the solutions, measurements were conducted in triplicate on a micro-osmometer (Model 3300, The Advanced Instruments). This measurement is based on freezing point depression (FPD) osmometry. The freezing point of a solution is depressed in direct relation to the amount of solute in solution and Osmometer measures the total molar concentration of the dissolved solids in a solution.

Size measurement of macromolecules

A series of dextran (polyglucose) with different molecular mass were used in this thesis and their molecular sizes were measured by dynamic light scattering (DLS). Dextran was dissolved in 0.22 μm -filtered DI water at a concentration of 1 g/L. The size measurements were conducted on nano zetasizer (Malvern, UK).

Density and viscosity of the solutions

In order to calculate the wall shear stress exerted on the EC monolayer, it is necessary to measure the density and viscosity of the solutions used. The density of the solutions was measured with a density meter (Anton Paar DMA35) at 37°C and the viscosity was measured by an automated micro capillary viscometer (Anton Paar AMVn) at 37°C.

Chapter 3 The theory of depletion interaction

With the aim to test whether depletion interaction originating from non-adsorbing macromolecules plays a role in RBC-EC adhesion, this chapter introduces the theory of depletion interaction. In particular, an equation is derived to theoretically calculate the energy of depletion interaction acting between RBC and EC, and the dependence of the adhesion on molecular mass (M_w) and concentration of the depleted polymer is analyzed. This chapter about theory is provided based on the studies on depletion-mediated RBC aggregation and RBC adhesion to artificial surfaces reviewed in the Introduction.

Depletion interaction

When cells are in a solution containing non-adsorbing macromolecules and if the entropy loss of the macromolecules near the cell surface is not balanced by adsorption energy (i.e. no specific binding or only limited non-specific adsorption of the macromolecule), the macromolecules are excluded from the proximity of cell surfaces. Thus, surrounding the cells is a depletion layer in which concentration of the macromolecules is much lower than that in the bulk solution. The resultant osmotic pressure leads to overlapping of depletion layers when cells approach each other in order to reduce the free energy of the whole system. Hence, an attractive force develops and cell-cell interactions occur.

Polymer concentration

The osmotic pressure (Π) acting between the contacting cells can be calculated from chemical potential of the solutions:

$$\Pi = -\frac{\mu - \mu_0}{v} \quad (0.6)$$

in which μ and μ_0 are the chemical potential of the solution when the bulk polymer concentration is at C^b and zero, respectively. v is the volume of the solution. Π can also be calculated from the bulk polymer concentration (C^b) by the equation below [129]:

$$\Pi = \frac{RT}{M} C^b + B_2 (C^b)^2$$

in which R , T , and M are the gas constant, absolute temperature, and the molecular mass (Mw) of the polymer. B_2 is the second virial coefficient of the polymer. When the bulk concentration C^b is low, the above equation can be further simplified as:

$$\Pi = \frac{RT}{M} C^b$$

It can be clearly seen that at a constant Mw, the osmotic pressure (Π) increases linearly with the bulk polymer concentration.

Penetration depth

Both RBC and EC surfaces consist of a soft layer of glycocalyx, which can be considered as an attached layer of macromolecules. As shown in Figure 3-1, the curve of C_3 represents the concentration profile of the attached macromolecules near a cell surface; while the curve of C_2 represents the concentration profile of the non-adsorbing macromolecules in solutions. δ denotes thickness of the attached layer of macromolecules (i.e. glycocalyx) with a penetration depth of p by the free non-adsorbing macromolecules in solutions.

Figure 3-1: Schematic picture of the polymer concentration profiles near a cell surface. x-axis represents the distance from the surface.

It can be seen that the concentration of non-adsorbing macromolecules gradually increases from zero near the cell surface to the value of bulk concentration. These actual concentration profiles can be simplified as a stepwise profile as illustrated in Figure 3-2.

Figure 3-2: Stepwise-approximation of the polymer concentration profiles near a cell surface. x-axis represents the distance from the surface.

It can be seen that the penetration of the non-adsorbing macromolecules into the cell glycocalyx renders the effective depletion layer thickness to be $(\Delta - p)$.

The energy of depletion interaction

Given a distance of d between two contacting cell surfaces, w_D is obtained by [129]:

$$W_D = -\Pi(2\Delta - d + \delta_1 - \rho_1 + \delta_2 - \rho_2) \quad (0.7)$$

where the thickness of glycocalyx (δ_1 and δ_2) and the polymer penetration (p_1 and p_2) for both cells are considered. The depletion layer thickness can be calculated via [152]:

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

and the parameter D can be obtained by:

$$D = \frac{2k_B T}{\Delta^2} \left(\frac{C^b N_a}{M} \right)^{\frac{2}{3}}$$

in which k_B and N_a are the Boltzmann constant and Avogadro's number. It can be seen that depletion layer thickness (Δ) is a function of the bulk concentration (C^b). Figure 3-3 demonstrates that for several dextran molecules with different Mw, the depletion layer thickness decreases rapidly when the bulk concentration is higher than 1 g/dL (10 mg/ml) but keeps almost unchanged when it is lower than 1 g/dL (10 mg/ml).

Figure 3-3: Calculated depletion layer thickness for dextran molecules with different Mw as a function of the bulk polymer concentration.

When the bulk concentration is low, Δ can be simplified as [153]:

$$\Delta = 1.4R_g$$

where R_g is the polymer's radius of gyration and can be estimated via:

$$R_g = A_{ec} \sqrt{M}$$

in which $A_{ec}=0.88 \text{ nm}\cdot\text{mol}^{0.5}\cdot\text{kg}^{-0.5}$ [154]. Combining the above equations, the energy of depletion interaction acting on two cells at close proximity (i.e., $d \approx \delta_1+\delta_2$) then becomes:

$$w_D = -\frac{RT}{M} C^b (1.4R_g - p)$$

or

$$w_D = -\frac{RT}{M} C^b (2.8A_{ec}\sqrt{M} - p_t) \quad (0.8)$$

where p_t represents the total penetration of polymer molecules into two contacting cell glycocalyx. From the equation (0.8) above, it can be seen that as polymer concentration increases, the energy of depletion interaction increases. Penetration of the polymer molecules into cell glycocalyx clearly reduces the energy of depletion interaction.

Conclusion

This chapter explains the theory of depletion interaction acting on cells. The energy of depletion interaction is determined by both the physicochemical properties of the non-adsorbing macromolecules and polymer penetration into the cell glycocalyx. In particular, at a constant molecular mass of the non-adsorbing macromolecules, the energy of depletion interaction increases with polymer concentrations. Polymer penetration into the cell glycocalyx reduces depletion interaction.

Chapter 4 The adhesion of normal RBC to EC in buffer/albumin containing non-adsorbing macromolecules

In this chapter, the impact of non-adsorbing macromolecules on RBC-EC adhesion in isotonic buffer containing albumin is studied. Dextran is a neutral polyglucose that has been shown to be depleted from the RBC surface (i.e. non-adsorbing macromolecules) [115, 117]. It is commonly used as a plasma expander without cyto-toxicity reported [155]. Additionally, since it has a wide range of molecular mass (Mw) with stable biochemical properties, dextran has been widely used to study the mechanism of RBC aggregation [123, 128, 131, 156-158], with depletion interaction suggested to be the underlying mechanism [129].

Results

The dependence of adhesion on polymer concentration and Mw

RBC were suspended in PBS containing 0.2% BSA (control) or in solutions containing dextran at the desired final concentration. The cells were given an 8-minute static incubation, followed by rinsing with stepwise-increasing shear stress with an interval of 3 minutes. Figure 4-1 demonstrates that in the control only 24 (± 10) cells adhere to EC at 0.01 Pa, and they are quickly removed once the shear stress exceeds 0.03 Pa. However, the presence of dextran 500 kDa leads to a significant increase of adhesion. Compared to the control, at 0.01 Pa the adhesion increases to two and eleven folds with dextran concentration being 5 mg/ml and 10 mg/ml, respectively. The adhesion strength also increases significantly. Even under higher shear stress (0.04 and 0.05 Pa), a significant number of RBC remain adherent in 10 mg/ml of dextran 500 kDa solution.

Figure 4-1: The adhesion of normal RBC to endothelium as a function of the applied shear stress during rinsing. RBC were suspended in polymer-free solution or solutions containing dextran 500 kDa (5 or 10 mg/ml), followed by a static incubation of 8 minutes prior to rinsing. Error bars are SD of the mean adherence values from 3 individual experiments for dextran solutions (i.e. $n=3$) and 6 individual experiments for control (i.e. $n=6$).

In Figure 4-2, a similar set of experiments demonstrate the effect of dextran 2 MDa on the adhesion of normal RBC to EC. Compared to that in the control, at 0.01 Pa the adhesion is increased by ten folds in 10 mg/ml of dextran 2 MDa. The adhesion remains significant even at 0.05 Pa, indicating a strong resistance to shear. However, at the lower dextran concentration of 5 mg/ml, the adhesion is increased to only two folds as compared to that in the control, and the cells were washed away quickly when the shear stress exceeded 0.03 Pa.

Figure 4-2: The adhesion of normal RBC to endothelium as a function of the applied shear stress during rinsing. RBC were suspended in polymer-free medium or solutions containing dextran 2 MDa (5 or 10 mg/ml), followed by a static incubation of 8 minutes prior to rinsing. Error bars are SD of the mean adherence values from 3 individual experiments for dextran solutions (i.e. n=3) and 6 individual experiments for control (i.e. n=6).

In Figure 4-3, dextran molecules with lower molecular mass were tested for their ability to induce RBC-EC adhesion. At a concentration of 10 mg/ml, dextran 70 kDa was found unable to induce adhesion strong enough to withstand the lowest shear stress of 0.01 Pa. The adhesion almost vanishes when the shear stress reaches 0.04 Pa, with no difference as compared to the control. Dextran 150 kDa significantly increases RBC-EC adhesion, e.g. at 0.01 Pa the adhesion is increased by 5 and 6 times with dextran concentration being 10 and 20 mg/ml, respectively. Compared to dextran 500 kDa in Figure 4-1 and dextran 2 MDa in Figure 4-2, at 10 mg/ml dextran 150 kDa induces lower adhesion, e.g. at 0.01 Pa the adhesion is 56% of that induced by 500 kDa and 55% of that induced by 2 MDa.

Figure 4-3: The adhesion of normal RBC to endothelium as a function of the applied shear stress during rinsing. RBC were suspended in polymer-free medium or dextran solutions, followed by a static incubation of 8 minutes prior to rinsing. Error bars are SD of the mean adherence values from 3 individual experiments for dextran solutions (i.e. n=3) and 6 individual experiments for control (i.e. n=6).

The effect of the presence of polymer during rinsing

In order to evaluate if the presence of polymer is required to maintain the adhesion during rinsing, the effects of rinsing with either dextran-containing or dextran-free solution (PBS containing 0.2% BSA) were compared. RBC were suspended in dextran solutions and co-incubated for 8-minutes before rinsing with either the same dextran solution or with dextran-free solution.

Figure 4-4 presents the effect of rinsing with or without dextran 500 kDa. The initial adherence at 0.01 Pa is almost the same regardless of the rinsing solutions. However, as the rinsing went on without dextran, the adhesion decreased significantly. From 0.02 Pa onwards, the number of adherent cells was only 48% (0.02 Pa), 58% (0.03 Pa), 55% (0.04 Pa) and 50% (0.05 Pa) of that after rinsing with dextran 500 kDa solutions.

Figure 4-4: The effect of the presence of dextran during rinsing on the adhesion of normal RBC to EC. RBC were suspended in 10 mg/ml of dextran 500 kDa and allowed for 8-minute incubation, after which the rinsing was applied with either the same dextran solution as that during incubation or with polymer-free medium. Error bars are SD of the mean adherence values from 3 individual experiments with Mann-Whitney U test performed between the investigated samples (i.e. n=3).

Figure 4-5 presents a similar set of experiments investigating the effect of dextran 2M kDa during rinsing on the adhesion. The initial adherence at 0.01 Pa is almost the same regardless of the rinsing solutions. However, as the rinsing went on

without dextran, the adhesion decreased significantly from 0.02 Pa onwards. The number of adherent cells was only 56% (0.02 Pa), 45% (0.03 Pa), 52% (0.04 Pa) and 57% (0.05 Pa) of that after rinsing with dextran 2M kDa solutions.

Figure 4-5: The effect of the presence of dextran during rinsing on the adhesion of normal RBC to EC. RBC were suspended in 10 mg/ml of dextran 2 MDa and allowed for 8-minute incubation, after which the rinsing was applied with either the same dextran solution as that during incubation or with polymer-free medium. Error bars are SD of the mean adherence values from 3 individual experiments with Mann-Whitney U test performed between the investigated samples (i.e. n=3).

The effect of RBC cell count

To evaluate whether the cell count of RBC plays a role in RBC-EC adhesion, RBC were suspended at a cell count ranging from $5 \times 10^6/\text{ml}$ to $50 \times 10^6/\text{ml}$ in either dextran 500 kDa (10 mg/ml) or in PBS containing 0.2% BSA (control). Both RBC aggregation and RBC-EC adhesion occurred during the 8-minute incubation prior to rinsing with a solution containing 10 mg/ml dextran of 500 kDa. However, RBC aggregates were observed to be less at lower cell count (e.g. $5 \times 10^6/\text{ml}$). Both RBC aggregates and individual cells were found to adhere to EC, but the former were rinsed away quickly as the shear stress increased. At higher shear stress, most of the remaining adherent cells were individual. In Figure 4-6A, at 0.02 Pa increasing the cell count from $5 \times 10^6/\text{ml}$ to $10 \times 10^6/\text{ml}$ led to an increase of 40% of the number of adherent cells, but further increasing the cell count to $50 \times 10^6/\text{ml}$ led to a decrease of 20% as compared to $5 \times 10^6/\text{ml}$. The overall adhesion in the

control suspensions is low as compared to that in dextran 500 kDa, with a slight effect of the different RBC cell counts as demonstrated in Figure 4-6B. In conclusion, the increase of RBC cell count does not necessarily lead to monotonically increasing RBC-EC adhesion.

Figure 4-6: The impact of RBC cell count on the adherence of normal RBC to EC as a function of the applied shear stress in (A) dextran 500 kDa at 10 mg/ml; (B) PBS containing 0.2% BSA. Error bars are SD of the mean adherence values from three individual experiments (i.e. n=3).

The coexistence of RBC aggregation and RBC-EC adhesion

In polymer solutions such as dextran and fibrinogen, RBC form rouleaux or aggregates [131]. However, such phenomenon is not found when they are suspended in polymer-free buffer [156]. Figure 4-7 demonstrates that as RBC cell count increases, there are more RBC aggregates in 10 mg/ml dextran 500 kDa. Figure 4-8 shows that during rinsing with low shear stress, both individual RBC and RBC aggregate adhere to EC in the presence of dextran of high Mw. Hence, both RBC aggregation and RBC-EC adhesion can occur in dextran solutions.

Figure 4-7: Examples of RBC aggregation during the 8-minute incubation. RBC cell count is $5 \times 10^6/\text{ml}$ in (A) polymer-free medium; (B) 10 mg/ml of dextran 500 kDa. In 10 mg/ml of dextran 500 kDa, RBC cell count is (C), $10 \times 10^6/\text{ml}$; (D), $50 \times 10^6/\text{ml}$.

Figure 4-8: An example of the adhesion dynamics during rinsing at 0.01 Pa in 10 mg/ml of dextran 500 kDa. (A), RBC aggregates adherent to EC; (B), individual RBC adherent to EC; (C), flowing RBC. The picture was captured by transmitted light microscope at the magnification of 20 X.

Discussion

The model of depletion interaction on RBC-EC adhesion

The results in this section clearly demonstrate that large dextran molecules (> 70 kDa) can induce adhesion of normal RBC to EC, capable of withstanding rinsing with low shear stress (e.g. 0.01-0.05 Pa). Considering that dextran is a neutral, uncharged polymer unable to develop attractive electrostatic interactions and that it has been repeatedly shown to be depleted from the RBC surface [159-161], these results suggest that the adhesion induced by dextran can be ascribed to polymer depletion interaction as illustrated in Figure 4-9. In the presence of non-adsorbing macromolecules, a depletion layer is built up near the cell surfaces. When RBC approach EC, overlapping of the depletion layers reduces the free energy of the system leading to cell-cell interaction.

Figure 4-9: Schematic picture showing how depletion interaction induces RBC-EC adhesion. The presence of non-adsorbing macromolecules induces depletion layers on both RBC and EC surfaces. Overlapping of the depletion layers is driven by osmotic pressure in order to reduce the free energy of the system, leading to attractive entropic force and thus RBC-EC adhesion.

When considering the adhesion due to the presence of dextran, another possibility might be that dextran may induce the expression of adhesion molecules on EC, and thus the adhesion could be ascribed to other interactions induced by the adhesion molecules. However, this possibility seems unlikely. As demonstrated (Figure 4-1 to Figure 4-3), the adhesion depends strongly on the molecular mass (M_w) and concentration of dextran: the number of adherent RBC as well as adhesion strength increase with increasing dextran concentrations. This is in good agreement with the theory that higher polymer concentration leads to higher osmotic pressure, higher energy of depletion interaction, and thus enhanced RBC-EC adhesion (Chapter 3).

The dependence of adhesion on polymer concentration and M_w

By employing Equation (0.8) (see Page 51 for details),

$$w_D = -\frac{RT}{M} C^b (2.8 A_{ec} \sqrt{M} - p_t)$$

Figure 4-10 presents the calculated energy of depletion interaction (w_D) at a constant bulk polymer concentration (C^b) of 10 mg/ml.

Figure 4-10: Theoretical dependence of depletion interaction energy on polymer molecular mass (M_w) for a constant bulk polymer concentrations of 10 mg/ml. The four curves represent four assumed total penetration depth of dextran into both RBC and EC glycocalyx: 0, 10, 20 and 30 nm, respectively.

For a hard surface ($p=0$), the depletion energy depends linearly on $C^b/M^{0.5}$ and thus decreases with increasing molecular mass (M). However, by assuming the total dextran penetration into both RBC and EC surfaces to be 10 nm, 20 nm and 30 nm, the minimal M_w for the onset of attractive depletion interaction energy are predicted to be 16.4 kDa, 66 kDa and 149 kDa, respectively. Above this minimal M_w , the depletion interaction energy increases until it reaches a maximal affinity at an optimal M_w . This optimal M_w is different with different penetration, e.g. it increases from about 100 kDa at $p=10$ nm to 600 kDa at $p=30$ nm. Further increasing M_w , beyond the optimal M_w , leads to decreasing depletion interaction energy asymptotically approaching zero.

The results obtained in this chapter are in good agreement with the above theory. Table 4-1 summarizes the number of RBC adherent at 0.01 Pa from the figures in this chapter. At a constant concentration of 10 mg/ml, a strong dependence of the adhesion on M_w of dextran can be seen. In brief, 70 kDa can not induce significant adhesion as compared to polymer-free solution, while 150 kDa significantly induces normal RBC adhesion to EC. 500 kDa further elevates the adhesion as compared to 150 kDa, but 2 MDa does not lead to a further significant increase of the adhesion. Hence, an optimal M_w to induce maximal adhesion of normal RBC

to EC seems to locate between 500 kDa and 2 MDa. These results, when compared to the theoretical curves in Figure 4-10, suggest that the total penetration of dextran molecules into both RBC and EC glycocalyx are in the range of 20-30 nm.

Table 4-1: RBC adherent at 0.01 Pa in different solutions summarized from the figures in this chapter.

solutions	adherent RBC number at 0.01 Pa (cells/mm ²)
polymer-free	24
70 kDa (10 mg/ml)	27
150 kDa (10 mg/ml)	149
500 kDa (10 mg/ml)	262
2 MDa (10 mg/ml)	275

The effect of dextran presence during rinsing

It is shown that the presence of dextran is required during rinsing to maintain the adhesion (Figure 4-4 and Figure 4-5). However, there was a remaining amount of RBC adherent even when dextran was absent during rinsing, indicating that the adhesion is not completely reversible. As for this remaining adherence between RBC and EC, it might be due to other interactions triggered by close cell-cell contact initiated by depletion interaction. For example, the subpopulation of senescent RBC may specifically bind to the endothelium since the phosphatidylserine (PS) exposure on their outer membrane has been correlated to their increased adhesiveness [67, 162].

RBC aggregation and RBC-EC adhesion

The coexistence of RBC aggregation and RBC-EC adhesion was observed in dextran solutions, which confirms that depletion interaction is indeed a non-specific entropic force universally operating on interfacial interactions regardless of cell types. However, RBC demonstrate a stronger tendency to aggregate among themselves than to adhere to EC, as indicated by the insignificant increase of RBC-EC adhesion along with increasing RBC cell count (Figure 4-6). The stronger tendency of RBC to aggregate indicates that in the same dextran solutions, depletion interaction has a more pronounced effect on RBC aggregation than on RBC-EC adhesion. This clearly reveals the significant impact of cell surface

properties on depletion-mediated cell-cell interactions, i.e. due to the fact that EC has a much thicker glycocalyx than RBC [163], the penetration of dextran molecules into EC glycocalyx may be of a greater extent than that into the RBC glycocalyx. Thus, the depletion interaction acting on RBC-EC is smaller than that between RBC. This is also supported by the fact that 70 kDa can hardly induce any RBC-EC adhesion (Figure 4-3), but it has been shown to induce RBC aggregation [122, 142].

Conclusion

In conclusion, dextran with Mw higher than 70 kDa induces adhesion of normal RBC to the endothelium capable of withstanding rinsing with low shear stress (e.g. 0.01-0.05 Pa). The adhesion exhibits a strong dependence on Mw and concentration of dextran and can be ascribed to depletion interaction. In brief, higher polymer concentration leads to higher osmotic pressure and thus enhanced adhesion. The total penetration of dextran molecules into cell glycocalyx is suggested to be 20-30 nm based on the experimental results, and an optimal Mw exists between 500 kDa and 2 MDa for the maximal adhesion. These findings suggest that depletion interaction could be an alternative mechanism for the adhesion-promoting effect of non-adsorbing proteins or macromolecules.

Chapter 5 The adhesion of normal RBC to EC in whole plasma or diluted plasma

Chapter 4 has demonstrated that a non-adsorbing macromolecule (i.e. dextran) in isotonic buffer containing albumin can induce the adhesion of normal RBC to endothelium capable of withstanding rinsing with shear stress up to 0.1 Pa. The adhesion is ascribed to an attractive depletion interaction induced by dextran. Hence, it is possible that the presence of non-adsorbing plasma proteins or macromolecules *in vivo* can induce depletion interaction promoting RBC-EC adhesion. In this chapter, dextran in plasma-like media (i.e. diluted plasma) is tested for its capability to induce RBC-EC adhesion and compared to the adhesion-promoting effect of fibrinogen.

Results

The adhesion in diluted plasma containing non-adsorbing macromolecules

In the adhesion studies using buffer/albumin (PBS containing 0.2% BSA), dextran was dissolved at the desired concentration; while in the adhesion studies using diluted plasma or plasma, the macromolecules (dextran and fibrinogen) were dissolved according to the protocol described in Page 39. RBC in the desired solution were given an 8-minute static incubation prior to rinsing with a step-wise increasing shear stress.

Figure 5-1: Adherence of normal RBC to endothelium as a function of the shear stress applied during rinsing. RBC were allowed to settle for 8 minutes before rinsing with stepwise-increasing shear stress. The adhesion in diluted plasma is compared to that in buffer/albumin, in the presence or absence of dextran 150 kDa (10 mg/ml). Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$).

Figure 5-1 compares the adhesion of normal RBC to the endothelium in diluted plasma to that in buffer/albumin, focusing on the effect of dextran 150 kDa. In the absence of dextran, the adhesion in diluted plasma or in buffer/albumin is almost the same (white bar and white stripe bar). However, the presence of dextran 150 kDa (10 mg/ml) in diluted plasma results in marked increase of the number of adherent cells to 4.8 times at 0.01 Pa and 25 times at 0.05 Pa. Additionally, the adhesion in diluted plasma with dextran exhibits a much stronger resistance to

shear as compared to dextran in buffer/albumin, although the initial adherence at 0.01 Pa is similar in both solutions. For example, no cells remain adherent in buffer/albumin at 0.05 Pa but in diluted plasma 50 (± 29) cells/mm² remain adherent. In conclusion, compared to its presence in buffer/albumin, dextran in diluted plasma leads to a significant increase of adhesion of normal RBC to EC.

Figure 5-2: Adherence of normal RBC to endothelium as a function of the shear stress applied during rinsing. RBC were allowed to settle for 8 minutes before rinsing with stepwise-increasing shear stress. The adhesion in diluted plasma is compared to that in buffer/albumin, in the presence or absence of dextran 500 kDa (5 mg/ml). Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$).

Figure 5-2 presents a similar set of experiments investigating the effect of dextran 500 kDa at 5 mg/ml on RBC-EC adhesion. When this polymer is present in buffer/albumin, it increases the initial adhesion at 0.01Pa to 2.2 folds (grey bar) as compared to buffer/albumin without dextran 500 kDa (white bar). However, when this polymer is present in diluted plasma (grey stripe bars), it significantly elevates the adhesion at 0.01 Pa to 13.1 times as compared to diluted plasma without dextran, and 7.1 times as compared to buffer/albumin with dextran 500 kDa. At 0.02 Pa, dextran 500 kDa in diluted plasma increases the adhesion to 41.5 times as compared to diluted plasma without dextran, and 12.7 times as compared to buffer/albumin with dextran 500 kDa. Furthermore, at 0.05 Pa the presence of dextran in diluted plasma leads to 201 (± 66) cells/mm² remaining adherent, but almost no RBC are adherent in other solutions. In conclusion, compared to its

presence in buffer/albumin, dextran in diluted plasma leads to significantly enhanced adhesion of normal RBC to EC.

Figure 5-3 presents a similar set of experiments studying dextran 500 kDa at a higher concentration of 10 mg/ml. When this polymer is present in either buffer/albumin or diluted plasma, it leads to much higher adhesion compared to its absence. But the presence in diluted plasma leads to even higher adhesion as compared to buffer/albumin with dextran 500 kDa, i.e. the adhesion is increased by a factor of 2.2 (0.01 Pa), 3.8 (0.02 Pa), and 7.5 (0.05 Pa). In conclusion, compared to its presence in buffer/albumin, dextran in diluted plasma leads to further enhanced adhesion of normal RBC to EC.

Figure 5-3: Adherence of normal RBC to endothelium as a function of the shear stress applied during rinsing. RBC were allowed to settle for 8 minutes before rinsing with stepwise-increasing shear stress. The adhesion in diluted plasma is compared to that in buffer/albumin, in the presence or absence of dextran 500 kDa (10 mg/ml). Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$).

Comparing Figure 5-2 and Figure 5-3, a clear dependence of the adhesion on the concentration of dextran can be seen. This dependence has been studied on the adhesion in buffer/albumin (Chapter 4), and it is observed again in the adhesion in diluted plasma. At a constant dextran molecular mass (M_w) of 500 kDa, the adhesion in diluted plasma containing 10 mg/ml of dextran is as high as 1.6 (0.01 Pa), 1.7 (0.02 Pa), and 1.9 (0.05 Pa) times as compared to that in 5 mg/ml of dextran.

Comparing Figure 5-1 and Figure 5-3, a dependence of the adhesion on M_w of dextran can be seen as well. This dependence has been studied on the adhesion in buffer/albumin (Chapter 4), and it is observed again for the adhesion in diluted plasma. At a constant concentration of 10 mg/ml, dextran 500 kDa in diluted plasma leads to as high as 4.4 (0.01 Pa), 6.7 (0.02 Pa), and 7.5 (0.05 Pa) times of adhesion as compared 150 kDa.

The adhesion in diluted plasma or whole plasma with increased fibrinogen

After the adhesion of normal RBC to EC was studied in diluted plasma with added dextran, it was also studied in diluted plasma with added fibrinogen. Figure 5-4 demonstrates that an elevated concentration of fibrinogen in diluted plasma significantly increases the adhesion of normal RBC to EC, e.g. an increase of 6 mg/ml doubles the number of adherent RBC at 0.01 Pa as compared to control; while an increase of 12 mg/ml elevates the adhesion to 3.8 (0.01 Pa), 3.4 (0.02 Pa), and 3.0 (0.05 Pa) times as compared to the control.

Figure 5-4: Adherence of normal RBC to endothelium as a function of the shear stress applied during rinsing. RBC were suspended in diluted plasma containing added fibrinogen at different concentrations and then allowed to settle for 8 minutes before rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. n=3).

The above results provide a direct comparison between dextran and fibrinogen in diluted plasma. In order to further mimic the adhesion *in vivo*, fibrinogen was directly added into plasma separated from whole blood without dilution. Figure 5-5 demonstrates that an elevated concentration of fibrinogen in whole plasma significantly increases the adhesion of normal RBC to EC, e.g. an increase of 6 mg/ml elevates the adhesion to 3.9 and 2.8 times at 0.01 and 0.02 Pa as compared to control, respectively; while an increase of 12 mg/ml elevates the adhesion to 7.1 (0.01 Pa), 7.6 (0.02 Pa), and 5.8 (0.05 Pa) times as compared to control.

Figure 5-5: Adherence of normal RBC to endothelium as a function of the shear stress applied during rinsing. RBC were suspended in plasma containing fibrinogen at different concentrations and then allowed to settle for 8 minutes before rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. n=3).

As demonstrated in Figure 5-4 and Figure 5-5, the increase of fibrinogen concentration in whole plasma, compared to that in diluted plasma, has a more pronounced effect on the increase of adhesion. For example, with 6 mg/ml increase of fibrinogen, the adhesion in whole plasma is 1.2 (0.01 Pa) and 1.8 (0.02 Pa) times of that in diluted plasma; while with 12 mg/ml increase of fibrinogen, the adhesion in whole plasma is 1.1 (0.01 Pa), 1.5 (0.02 Pa), and 2.8 (0.05 Pa) times of that in diluted plasma. Additionally, from both Figure 5-4 and Figure 5-5 it can be seen that higher concentrations of fibrinogen lead to a further increase of the number of adherent cells.

Discussion

The experimental results clearly lead to two conclusions. Firstly, an elevated level of non-adsorbing macromolecules (i.e. dextran) in diluted plasma induces enhanced adhesion of normal RBC to EC. This adhesion depends strongly on molecular mass and concentration of the macromolecules. By comparing this adhesion to that in buffer/albumin, it can be seen that the effect of non-adsorbing macromolecules on RBC-EC adhesion is even stronger. Considering that depletion interaction has been elucidated to be the mechanism of the adhesion induced by dextran in buffer/albumin (Chapter 4), it is reasonable to conclude that depletion interaction is the underlying mechanism of the increased adhesion of normal RBC to EC in plasma-like media (i.e. diluted plasma) supplemented with dextran.

Secondly, an elevated concentration of fibrinogen in plasma-like media (i.e. diluted plasma) or plasma induces enhanced adhesion of normal RBC to EC *in vitro*. These experimental findings confirm earlier reports [97, 103], and they corroborate the significant adhesion-promoting effect of fibrinogen on pathological RBC to EC in vascular diseases [93, 97]. Since the mechanism behind the adhesion-promoting effect of fibrinogen remains unclear, this chapter proposes depletion interaction to be the underlying mechanism. This is supported by comparing the effects of fibrinogen to that of dextran on RBC-EC adhesion.

Figure 5-6 demonstrates that in terms of number of adherent cells as well as adhesion strength against shear, the adhesion induced by the added dextran 150 kDa closely resembles that induced by the added fibrinogen. Theoretically, the energy of depletion interaction (w_D) induced by non-adsorbing macromolecules can be estimated for small polymer concentration by the equation below:

$$w_D = -\frac{RT}{M} C^b (1.4R_g - p) \quad (0.9)$$

in which R, T, and M are gas constant, absolute temperature, and molecular mass (Mw), respectively. It can be seen that for two macromolecules with similar physicochemical properties including Mw, molecular size (R_g), and bulk concentration (C^b , molar concentration), their energies of depletion interaction are similar provided that they have similar penetration (p) into cell glycocalyx.

Table 5-1: The comparisons between dextran and fibrinogen on their physicochemical properties. The concentrations of these two macromolecules used in this chapter are summarized to be the added values into diluted plasma or plasma. The concentration of fibrinogen *in vivo* during pathophysiological events is summarized from literatures [93, 98, 145, 164].

Media	Added dextran		Added fibrinogen		Fibrinogen <i>in vivo</i>	
	Diluted plasma		Diluted plasma	Whole plasma	Normal	Patients
Mw (kDa)	150	500		340		
R_g (nm)	11.5	19.5		10.7		
Conc. (mg/ml)	10	5 or 10	6 or 12	6 or 12	2-4.5	4-12

With the consideration that dextran 150 kDa (neutral) and fibrinogen (negatively charged) have similar molecular sizes, and that similar range of concentrations of these two polymers have been applied in this chapter (Table 5-1), the resemblance in adhesion (Figure 5-6) suggests that the same mechanism (i.e. depletion interaction) governs dextran 150 kDa-induced RBC adhesion and fibrinogen-induced adhesion. The discrepancies in adhesion could be due to: 1) the electrostatic interaction of fibrinogen with cell surfaces and thus a different penetration depth (p) from that of dextran; 2) the difference in the molar concentration of the polymers.

Figure 5-6: The comparisons on adhesion of normal RBC to EC between dextran 150 kDa and fibrinogen. The results are summarized from Figure 5-1, 5-4, and 5-5.

It should be noted that this chapter focuses on the effects of an increased level of non-adsorbing macromolecules or proteins in plasma (Table 5-1) on RBC-EC adhesion since during many pathological events (such as sickle cell disease and diabetes), increased plasma proteins and enhanced RBC-EC adhesion are manifested to be concurrent. In particular, this chapter suggests depletion interaction to be the mechanism for the adhesion-promoting effect of fibrinogen, although this protein has also been suggested to be specifically or non-specifically adsorbed on RBC surface [121, 165, 166]. This chapter further suggests that depletion interaction might have a stronger adhesion-promoting effect *in vivo*, since the effects of non-adsorbing macromolecules (i.e. dextran) on RBC-EC adhesion are more pronounced in diluted plasma than in buffer/albumin, and the RBC cell count used in this chapter is much lower than the *in vivo* value.

Conclusion

In conclusion, this chapter demonstrates that an increased level of non-adsorbing macromolecules in plasma-like media induces enhanced adhesion of normal RBC to EC, and the adhesion depends strongly on molecular mass and concentration of

the non-adsorbing macromolecules. Depletion interaction is suggested to be the underlying mechanism and an alternative mechanism has thus been provided for the adhesion-promoting effects of plasma proteins in vascular diseases. In particular, the adhesion-promoting effects of fibrinogen resemble those of dextran, thus, it is suggested that fibrinogen promotes RBC-EC adhesion via depletion interaction.

Chapter 6 The impact of RBC surface charge on RBC-EC adhesion

Sialic acid has been extensively correlated to many pathophysiological events, with diverse biochemical and biophysical effects reported in molecular and cellular interactions [167]. For example, sialic acid been found to be involved in senescent RBC sequestration, with its role postulated to mask the recognition sites of RBC undergoing phagocytosis by macrophages [168, 169]. Sialic acid has also been found to be involved in protein- (e.g. IgG and fibrinogen [143, 144]) or polymer- (e.g. dextran [127, 142, 170]) mediated RBC aggregation, with its role identified to be the determinant of electrostatic repulsion. Increased RBC aggregation and instability of blood flow have been found to be associated with sialic acid loss on RBC membranes in patients with polyagglutinability [141], acute myocardial infarction [171] and diabetes [172, 173].

The correlation of sialic acid to altered RBC-EC adhesion has also been reported. For example, experiencing physiological sialic acid loss during their life span [174], senescent RBC were found to be the most adhesive subpopulation to EC [175]. Additionally, abnormal RBC-EC adhesion has been correlated to the vascular complications in diabetic patients whose RBC have been identified to undergo sialic acid loss [4, 173]. Furthermore, some sialylated membrane proteins have been suggested to be involved in the P-selectin mediated RBC-EC adhesion in sickle cell disease [91]. Finally, increased plasma sialidase activity has been found in patients with acute myocardial infarction, and the resultant sialic acid loss on RBC membrane has been suggested to be responsible for the high RBC-EC adhesion index as well as altered blood flow in microcirculation [176, 177].

Reviewing the above diverse studies in RBC aggregation and adhesion, the role of sialic acid can be concluded as follows. Firstly, it has a masking or unmasking function that is linked to ligand-receptor interaction (e.g. in RBC phagocytosis and in P-selectin mediated sickle RBC adhesion). Secondly, it is a determinant of the electrostatic forces governing RBC interactions with other surfaces.

In the previous chapters, it has been demonstrated that depletion interaction can induce RBC-EC adhesion in buffer/albumin, which can withstand rinsing with low shear stress [178]. Moreover, it was concluded that (Chapter 5) an increased level of non-adsorbing macromolecules in plasma-like media or plasma induces depletion interaction, leading to significantly enhanced adhesion of normal RBC to EC. It was suggested that fibrinogen initiates depletion interaction. Taken into consideration that increased levels of proteins or macromolecules in plasma (e.g. fibrinogen) have been found in several pathophysiological conditions associated with both abnormal RBC-EC adhesion and sialic acid loss on RBC membrane, such as sickle cell disease [93, 98], diabetes [97, 103, 164] and myocardial infarction [171, 179], this chapter aims to identify the role of non-adsorbing macromolecules on the adhesion of desialylated RBC (i.e. RBC that experience sialic acid loss) to EC.

Results

RBC morphology

Desialylated RBC were obtained by treating normal RBC with neuraminidase, which is a widely used enzyme for specific sialic acid removal [142, 144, 175]. After the enzyme treatment, several methods were employed to characterize the morphological, mechanical, and physicochemical properties of RBC, followed by adhesion studies using a quasi-flow assay. The RBC morphology was found unchanged after neuraminidase treatment with concentrations ranging from 10 to 80 mU/ml. As shown in Figure 6-1, the biconcave shape of RBC was found to be retained.

Figure 6-1: RBC morphology observed on a transmitted light microscope. (A), normal RBC; RBC treated by neuraminidase at the concentration of (B), 10 mU/ml; (C), 20 mU/ml; (D), 40 mU/ml; (E), 60 mU/ml; (F), 80 mU/ml.

Zeta-potential measurement

Neuraminidase releases sialic acid from RBC membrane glycoproteins by cleaving the O-glycosidic linkage and reduces membrane surface charge [180, 181]. As a direct indicator of the effective surface charge, the zeta potential (ζ , mV) of RBC membrane was measured with a Nano Zetasizer (detailed protocol on Page 46, zeta-potential measurement). Figure 6-2 demonstrates the loss of ζ as a function of the concentrations of neuraminidase. It can be seen that the loss of ζ increases rapidly with the increasing neuraminidase concentrations. At 10 mU/ml of neuraminidase, the loss of ζ has reached 30%. The further increase of neuraminidase concentration results in a plateau of the % loss of ζ with a limit of 70%. The loss of ζ directly indicates the decrease of RBC surface charge, which is consistent with previous reports indicating that neuraminidase is an effective enzyme for sialic acid removal, and that RBC surface charge correlates with the amount of sialic acid [180]. The remaining surface charge demonstrates that a part of the sialylated components on RBC surface are resistant to neuraminidase hydrolysis [144].

Figure 6-2: Relative loss of ζ as a function of neuraminidase concentration. ζ of RBC membrane was measured in 5 mM ionic strength isotonic Sucrose Buffer at pH 7.4. Error bars are standard deviation of the mean values from three individual experiments (i.e. $n=3$).

RBC deformability measurement

As an indicator of RBC deformability, the elongation index of RBC was measured as a function of the applied shear stress from 0 to 20 Pa on a slit-flow ektacytometer (detailed protocol on Page 46). Figure 6-3 demonstrates the effect of neuraminidase treatment on the elongation index of RBC (i.e. RBC deformability). A series of neuraminidase concentrations were used (i.e. 20, 40, and 80 mU/ml) and the elongation index of RBC was found to decrease after the treatment as compared to the control (normal RBC), indicating the reduced RBC deformability after the treatments. However, the elongation index does not further decrease with the increasing neuraminidase concentrations, which implies that RBC deformability is not altered when the concentration of neuraminidase reaches 20-80 mU/ml. The identified loss of RBC deformability due to neuraminidase treatment in the current chapter is different from some previous reports [143, 144, 174], which is perhaps due to the difference of characterization methods applied, i.e. membrane filtration was used to determine RBC deformability in the literatures.

Figure 6-3 Elongation index as a function of applied shear stress on a slit-flow ektacytometer. The experiments were done in duplicate and shown here is a representative one.

SDS-PAGE gel electrophoresis

To determine whether RBC membrane structure was altered by neuraminidase treatment, i.e. whether the treatment additionally cleaved proteins other than sialic acid, SDS-PAGE gel electrophoresis was performed on ghosts prepared from both normal RBC and those treated by neuraminidase (detailed protocol on Page 47). Figure 6-4 demonstrates that in the Mw range of 37-250 kDa, most of the bands are in similar patterns between normal RBC and the ones treated by neuraminidase (e.g. 40 mU/ml and 80 mU/ml), indicating that most of the membrane proteins in this range of Mw stay unchanged. In the lower range of Mw, e.g. between 20-37 kDa, some small changes on the bands can be found in the treated samples, indicating that neuraminidase may induce some small shift of some membrane components to a lower range of Mw. Therefore, it can be concluded that neuraminidase is a specific enzyme for sialic acid removal with little impact on the membrane proteins.

Figure 6-4: Membrane protein analysis obtained with SDS-PAGE gel electrophoresis performed on ghosts prepared from both normal and neuraminidase-treated RBC. (A), normal RBC; RBC treated with neuraminidase at (B), 40 mU/ml and (C), 80 mU/ml. The bands are labeled with known proteins summarized from literatures [182, 183].

Alternatively, the gel was analyzed by scanning it at 280 nm on a 2D proteomic imaging system (Perkin Elmer, US). In Figure 6-5 and Figure 6-6, the peaks on the curves represent the membrane proteins of RBC at a certain Mw (i.e. each peak corresponds to a band on the gel). It was found that the peaks of both normal RBC and those treated by neuraminidase (e.g. 40 mU/ml and 60 mU/ml) overlap, indicating that most of the membrane proteins are unaltered by the treatments.

Figure 6-5: Scanning of the gel at 280 nm on a 2D proteomic imaging system (Perkin Elmer, US). The gel was from SDS-PAGE gel electrophoresis and peaks in the graph represent membrane proteins of RBC at a certain Mw. Normal RBC were treated by 40 mU/ml neuraminidase.

Figure 6-6: Scanning of the gel at 280 nm on a 2D proteomic imaging system (Perkin Elmer). The gel was from SDS-PAGE gel electrophoresis and peaks in the graph represent membrane proteins of RBC at a certain Mw. Normal RBC were treated by 60 mU/ml neuraminidase

The adhesion of desialylated RBC in polymer-free solution

After a detailed analysis of the effects of neuraminidase treatment on RBC membrane properties, the adhesion of desialylated RBC to EC was investigated

with a quasi-flow assay with an 8-minute static incubation prior to rinsing with stepwise-increasing shear stress. PBS containing 0.2% BSA was used as polymer-free solution, into which dextran was dissolved at the desired concentration. Normal RBC or desialylated RBC were re-suspended in either polymer-free solution or dextran solutions. Figure 6-7 compares the adhesion in polymer-free solution between normal RBC and desialylated RBC with different degrees of sialic acid loss (indicated by loss of zeta-potential (ζ)). It can be seen that a 30% reduction of ζ leads to a small increase of adhesion. However, a high reduction of ζ leads to significantly increased adhesion. For example, a 45% reduction of ζ elevates the adhesion to 2.2 folds at 0.01 Pa and 2.5 folds at 0.02 Pa; while a 60% reduction of ζ elevates the adhesion to 3.1 folds at both 0.01 Pa and 0.02 Pa. In conclusion, a decrease of sialic acid content and thus surface charge on RBC membrane results in increased adhesion of RBC to EC.

Figure 6-7: The adherence of normal and desialylated RBC in polymer-free solution as a function of the shear stress applied during rinsing. RBC were allowed settling for 8 minutes prior to rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$), except the one with 60% loss of ζ ($n=6$).

The adhesion of desialylated RBC in dextran solutions

After the adhesion of desialylated RBC to EC was investigated in polymer-free solution, it was studied in dextran solutions. Figure 6-8 compares the adhesion of desialylated RBC with 60% reduction of ζ in polymer-free solution to that in dextran 70 kDa. It can be seen that at the lowest applied shear stress of 0.01 Pa, the adhesion in both 5 mg/ml and 10 mg/ml of dextran 70 kDa are significantly increased, e.g. both demonstrate a 50% increase as compared to the one in polymer-free solution. However, the adherence in dextran 70 kDa in the subsequent rinsing of 0.02-0.05 Pa is slightly lower than that in polymer-free solution. This is explained by the observation that compared to those in polymer-free solution, much more RBC aggregates were found to adhere to the endothelium in dextran 70 kDa at low shear stress (e.g. 0.01 Pa). However, these aggregates were collectively rinsed away (i.e. the whole aggregate) with the increasing shear stress, without leaving any individual adherent RBC adherent, leading to a significant decrease of adhesion.

Figure 6-8: The adherence of desialylated RBC with 60% loss of ζ as a function of the shear stress applied during rinsing. RBC were suspended in either polymer-free solution or in dextran 70 kDa, followed by a settling of 8 minutes prior to rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$), except the one in polymer-free medium ($n=6$).

Figure 6-9 presents a similar set of experiments in polymer-free solution or in dextran 500 kDa. RBC with 60% reduction of ζ were allowed an 8-minute settling onto a confluent layer of EC prior to rinsing with a stepwise-increasing shear stress. The adhesion in dextran 500 kDa can be seen to be significantly increased as compared to that in polymer-free solution. In 5 mg/ml of dextran 500 kDa, the adhesion is increased to 1.7 folds at 0.01 Pa and remains above the adhesion in polymer-free solution with the increasing shear stress. At a higher dextran concentration of 10 mg/ml, the adhesion is increased to 4.4 folds as compared to that in polymer-free solution at 0.01 Pa. Additionally, the adherence in 10 mg/ml dextran solution exhibits strong resistance to shear stress during rinsing, indicated by the fact that 42% of the cells adherent at 0.01 Pa remain adherent at 0.05 Pa. In conclusion, at the same molecular mass, higher dextran concentration results in higher adhesion of desialylated RBC to EC.

Figure 6-9: The adherence of desialylated RBC with 60% loss of ζ as a function of the shear stress applied during rinsing. RBC were suspended in either polymer-free solution or in dextran 500 kDa, followed by a settling of 8 minutes prior to rinses with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$), except the one in polymer-free medium ($n=6$).

Furthermore, the comparison between Figure 6-8 and Figure 6-9 demonstrates a strong dependence of the adhesion on the molecular mass of dextran. At a concentration of 5 mg/ml, 500 kDa leads to slightly higher adhesion than 70 kDa, e.g. 128 cells/mm² vs. 114 cells/mm² at 0.01 Pa; while at 10 mg/ml, 500 kDa leads to significantly higher adhesion than 70 kDa, e.g. 327 cells/mm² vs. 115 cells/mm² at 0.01 Pa. In conclusion, at the same concentration, higher Mw of dextran results in higher adhesion of desialylated RBC to EC.

Figure 6-10: The adherence of desialylated RBC in 5 mg/ml of dextran 70 kDa as a function of the shear stress applied during rinsing. RBC with 45% or 60% loss of ζ were allowed settling for 8 minutes prior to rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$).

In addition, the adhesion of desialylated RBC with a lower reduction of ζ was investigated. Figure 6-10 demonstrates that with a 45% reduction of ζ , the presence of dextran 70 kDa at 5 gm/ml slightly increases the adhesion as compared to polymer-free solution at 0.01 Pa. However, at higher shear stress there is no significant difference of the adhesion between dextran solution and polymer-free solution. As described similarly in Figure 6-8, this is because the adherent aggregates were collectively rinsed away (i.e. the whole aggregate) with the increasing shear stress, without leaving any individual adherent RBC adherent and leading to a significant decrease of adhesion. Comparing the adhesion in the same dextran solutions (i.e. 5 mg/ml of 70 kDa) demonstrates that 60% reduction of ζ

results in a significant increase of adhesion, e.g. the increases are 93% at 0.01 Pa and 39% at 0.02 Pa.

Figure 6-11 presents a similar study on the adhesion in 5 mg/ml of dextran 500 kDa. With 45% reduction of ζ , the presence of 500 kDa at 5 gm/ml significantly increases the adhesion as compared to polymer-free solution, e.g. the increases are 2.3 folds and 1.7 folds at 0.01 Pa and 0.02 Pa, respectively. Comparing the adhesion in the same dextran solutions (i.e. 5 mg/ml of 500 kDa) demonstrates that 60% reduction of ζ results in a significant increase of adhesion, e.g. the increases are 35% at 0.01 Pa and 52% at 0.02 Pa.

Figure 6-11: The adherence of desialylated RBC in 5 mg/ml of dextran 500 kDa as a function of the shear stress applied during rinsing. RBC with 45% or 60% loss of ζ were allowed settling for 8 minutes prior to rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n=3$)

Discussion

Depletion interaction promotes adhesion of desialylated RBC to endothelium

Desialylation of RBC by neuraminidase is clearly shown to be capable of inducing RBC-EC adhesion (Figure 6-7), which increases with the decrease of sialic acid content on RBC membrane (as indicated by the loss of surface charge), although the adhesion in polymer-free solution does not withstand high shear stress. The presence of non-adsorbing macromolecules (i.e. dextran) significantly promotes desialylated RBC adhesion to EC. This adhesion-promoting effect can be ascribed to depletion interaction as elucidated in Chapter 4. Specifically, the strong dependences of the adhesion on polymer concentration and Mw identified in this chapter are consistent with the previous results.

Characterization of the properties of RBC membrane after neuraminidase treatment

In order to analyze the effect of reduced RBC surface charge (due to sialic acid removal) on RBC-EC adhesion, it is necessary to characterize the possible changes on RBC membrane after the neuraminidase treatment. Firstly, results of the zeta-potential measurement show that neuraminidase treatment (< 80 mU/ml) effectively reduces RBC surface charge, indicating that the electrostatic repulsion between RBC and EC is decreased. In addition, it is possible that the reduction of surface charge may lead to structural alteration of the RBC glycocalyx [184]. Secondly, results of the SDS-PAGE gel electrophoresis demonstrate that neuraminidase treatment (< 80 mU/ml) does not cleave RBC membrane components in the Mw range of 37-250 kDa, indicating the specificity of sialic acid removal by neuraminidase. Thirdly, results of the elongation index measurement demonstrate that RBC deformability is moderately reduced by neuraminidase treatment but is independent on the applied enzyme concentrations. This indicates that within the applied range of neuraminidase concentrations (< 80 mU/ml), RBC deformability does not affect the mechanical properties of RBC and thus the contact between RBC and EC. Taken together, results from these characterization methods suggest that neuraminidase treatment applied in the

current study mainly provides an opportunity to specifically investigate the effect of RBC surface charge on their adhesion to the endothelium.

Increased macromolecules in plasma may promote desialylated RBC adhesion

In this chapter, the reduction of RBC surface charge has been shown to have a significant impact on RBC-EC adhesion when non-adsorbing macromolecules are present. The significant increase of adhesion is most likely due to a decreased electrostatic repulsion between RBC and EC and thus an enhanced adhesion promoting effect of depletion interaction. This study thus suggests that adhesion-promoting effect of depletion interaction needs to be considered in diseases associated with sialic acid loss on RBC membrane and increased plasma protein levels as summarized in Table 6-1. The effects of the concurrence of these two factors on the adhesion provide a rational explanation for the finding that the loss of sialic acid alone is not a determinant of senescent RBC adhesion to EC [175]. Other than being a determinant of electrostatic forces, the loss of sialic acid has been suggested to unmask P-selectin on the endothelium for the specific bindings of sickle RBC [91]. Thus, the adhesion-promoting effect of an enhanced depletion interaction (due to elevated levels of plasma proteins [93]) on receptor-mediated RBC-EC interactions also needs to be considered.

Table 6-1 the loss of sialic acid content identified in pathophysiological events associated with increased RBC-EC adhesion or RBC aggregation.

pathophysiological events	old RBC vs. young RBC		RBC (patients) vs. RBC (normal)		ref.
	on whole RBC	on glycophorin	on whole RBC	on glycophorin	
RBC senescence	10-24%	-	-	-	[175, 185]
diabetes	-	-	-	80%	[173]
β -thalassemia	-	50%	-	25%	[186]
sialic acid deficiency	-	-	65%	-	[141]

Conclusion

In conclusion, the current chapter has shown that sialic acid loss can induce weak RBC-EC adhesion, and that depletion interaction resulting from non-adsorbing macromolecules can significantly promote desialylated RBC adhesion to the endothelium. The decrease of surface charge on RBC membrane due to sialic acid loss leads to a reduced electrostatic repulsion between RBC and EC, which enhances the adhesion-promoting effect of depletion interaction and thus RBC-EC adhesion. Considering that increased levels of plasma proteins and decreased sialic acid content on RBC membrane are often concurrent in diseases associated with enhanced RBC-EC adhesion, the current chapter suggests that depletion interaction can be a significant determinant of abnormal RBC-EC adhesion.

Chapter 7 Other Cellular Factors

The previous chapter has demonstrated that the RBC surface charge plays a significant role in RBC-EC adhesion when non-adsorbing macromolecules are present. It is suggested that reduced electrostatic repulsion can significantly enhance the RBC-EC adhesion mediated by depletion interaction. Apart from RBC surface charge, other cellular factors, such as RBC glycocalyx thickness, have also been identified to have an impact on depletion interaction acting between cells (Introduction, Page31). Thus, in this chapter α -chymotrypsin treatment is employed to specifically pinpoint the effects of some cellular factors on RBC-EC adhesion in the presence of non-adsorbing macromolecules.

Results

RBC membrane zeta-potential measurement

To characterize the effects of α -chymotrypsin treatment on RBC surface charge, the membrane zeta potential (ζ , mV) was obtained by measuring RBC electrophoretic mobility in sucrose-PBS buffer (5 mM ionic strength, pH= 7.4). Figure 7-1 demonstrates that the reduction of ζ potential increases linearly with the increasing concentration of α -chymotrypsin. However, in the range from 0 to 90 mg/dl, the ζ potential decreased only moderately (0-20%), indicating that the sialylated components on the RBC membrane were only partly cleaved. It can thus be concluded that compared to neuraminidase, α -chymotrypsin is a non-specific enzyme for sialic acid removal [187].

Figure 7-1: Relative reduction (%) of RBC membrane zeta-potential (ζ , mV) as a function of α -chymotrypsin concentration. ζ potential was obtained by measuring RBC electrophoretic mobility in Sucrose-PBS buffer (5 mM ionic strength, pH= 7.4). Error bars are the standard deviations of the mean values (ζ potential) from three individual measurements.

Deformability quantification by ekta-cytometer

As a direct indicator of RBC deformability, the elongation index was measured with an ekta-cytometer as a function of the applied shear stress from 0 to 20 Pa. Figure 7-2 demonstrates that the elongation index is decreased after the enzyme treatment. However, the elongation index does not further decrease with the increasing enzyme concentration, which implies that the loss of RBC deformability due to α -chymotrypsin treatment is independent on the enzyme concentrations applied (0-90 mg/dl).

Figure 7-2: Elongation index of RBC as a function of the applied shear stress. Normal RBC and RBC treated by α -chymotrypsin were measured with a slit-flow ekta-cytometer in duplicate and shown here is a representative experiment.

Membrane protein analysis by gel electrophoresis

To analyze the remaining membrane proteins on RBC after α -chymotrypsin treatment, SDS-PAGE gel electrophoresis was performed on membrane ghost prepared from both normal and treated RBC. Figure 7-3 demonstrates that after α -chymotrypsin treatment, most of the bands in the range of mid and high MW (37-250 kDa) vanished or became significantly blurred with only one exception (highlighted with an arrow). The most significant loss of bands is located in the Mw range of 75-250 kDa and this loss increases with the applied concentration of

α -chymotrypsin. In the range of low MW (< 37 kDa), many new bands emerge in the treated cells as compared to normal RBC. It is thus concluded that α -chymotrypsin treatment dramatically dissociates RBC membrane proteins in the range of mid and high MW (37-250 kDa), shifting them down to low MW region (< 37 kDa). The digestion of RBC membrane proteins by α -chymotrypsin increases as the enzyme concentration increases from 20 to 60 mg/dL. In addition, scanning of the gel at 280 nm demonstrates that a number of peaks are lost (Figure 7-4), also indicating that α -chymotrypsin is an effective enzyme to dissociate RBC membrane proteins.

Figure 7-3: Membrane protein analysis obtained with SDS-PAGE gel electrophoresis performed on (A) normal RBC and RBC treated with α -chymotrypsin at the concentration of (B) 20 mg/dl; (C) 40mg/dl; (D) 60 mg/dl.

Figure 7-4: Scanning of the gel at 280 nm on a 2D proteomic imaging system (Perkin Elmer, US). The gel was from SDS-PAGE gel electrophoresis and peaks in the graph represent membrane proteins of RBC at a certain Mw.

RBC-EC adhesion assay

The study first investigated the impact of α -chymotrypsin treatment on RBC-EC adhesion. RBC were re-suspended in polymer-free solution (PBS containing 0.2% BSA) and then allowed to settle for 8 minutes prior to rinsing with a stepwise-increasing shear stress. The adhesion of treated RBC was compared to that of normal RBC, with the reduction of RBC membrane zeta-potential employed as a reference value.

Table 7-1: The comparisons on the number of adherent cells between normal RBC and RBC treated with α -chymotrypsin. The treated RBC were classified according to their reduction of zeta-potential. The numbers of adherent cells are mean $\pm$ standard deviations from six individual experiments for normal RBC (i.e. $n=6$), while the numbers of adherent cells for the α -chymotrypsin-treated RBC are from a representative experiment.

	normal	α -chymotrypsin-treated		
zeta-potential reduction	0.0%	2.3%	8.5%	14.4%
number of adherent RBC (0.01 Pa)	24 ± 10	6 ± 7	22 ± 13	56 ± 30
number of adherent RBC (0.02 Pa)	9 ± 7	1 ± 3	4 ± 4	15 ± 7

Table 7-1 demonstrates that as the reduction of RBC membrane zeta-potential increases, the number of adherent RBC increases. Furthermore, when the reduction of RBC membrane zeta-potential is low (e.g. 2.3%), α -chymotrypsin treatment leads to less adherent RBC, indicating a decreased RBC adhesiveness to EC. When the reduction of RBC membrane zeta-potential is high (e.g. 14.4%), α -chymotrypsin treatment leads to more adherent RBC, indicating an increased RBC adhesiveness to EC.

After the effects of α -chymotrypsin treatment on RBC-EC adhesion were elucidated in polymer-free solution, the study moved on to investigate the adhesion in dextran solutions (10 mg/ml of dextran 500 kDa). Figure 7-5 compares the adhesion of the treated RBC to that of normal RBC. With 8.5% reduction of RBC membrane zeta-potential, the number of adherent cells of the treated RBC is always lower than that of the normal RBC with the increasing shear stress, indicating a reduced adhesiveness of RBC to EC due to α -chymotrypsin treatment. However, with 14.4% reduction of RBC membrane zeta-potential, the treated RBC

exhibits an increased adhesiveness to EC as compared to normal RBC, for example, at 0.01 Pa α -chymotrypsin treatment results in 1.4 times as many adherent cells as that of normal RBC.

Figure 7-5: Adherence of RBC as a function of the shear stress applied during rinsing. Normal RBC or RBC treated with α -chymotrypsin were suspended in 10 mg/ml dextran 500 kDa and allowed settling for 8 minutes. The cells were then rinsed with stepwise-increasing shear stress. Shown here is a representative experiment with the error bars being the standard deviations of the 20 captures taken at the end of each shear stress.

Discussion

This chapter applied α -chymotrypsin treatments to alter the RBC surface properties and thus to study the resultant RBC-EC adhesion. The characterization methods demonstrate that α -chymotrypsin treatments not only reduce RBC surface charge, but also decrease RBC deformability and dissociate glycocalyx proteins. However, the loss of RBC deformability due to α -chymotrypsin treatment is observed to be constant, which provides a platform to study the effects of reduced RBC surface charge and the dissociation of glycocalyx proteins on RBC-EC adhesion.

α -chymotrypsin treatments alter the adhesiveness of RBC to EC. This has been shown in both polymer-free solution (Table 7-1) and dextran solutions (Figure 7-5, 10 mg/ml of dextran 500 kDa). To elucidate the effects of α -chymotrypsin treatment on RBC adhesiveness to EC, a comparison between neuraminidase and α -chymotrypsin on their resultant RBC-EC adhesion is necessary.

Figure 6-7: The adherence of normal and desialylated RBC in polymer-free solution as a function of the shear stress applied during rinsing. RBC were allowed settling for 8 minutes prior to rinsing with stepwise-increasing shear stress. Error bars are standard deviations of the mean adherence values from three individual experiments (i.e. $n = 3$), except the one with 60% loss of ζ ($n = 6$).

Comparing Table 7-1 to Figure 6-7 (copied from Chapter 6) leads to the conclusion that reduced RBC surface charge due to α -chymotrypsin treatment is not the only factor that increases RBC adhesiveness to EC. This is because even a

30% reduction of RBC membrane zeta-potential due to neuraminidase treatment resulted in a significantly smaller increase of RBC-EC adhesion (Figure 6-7) as compared to that of α -chymotrypsin treatment. Note that neuraminidase is a specific enzyme for sialic acid removal. It can thus be concluded that the digestion of the glycocalyx proteins most likely increases the RBC adhesiveness to EC.

Furthermore, the altered RBC adhesiveness to EC due to α -chymotrypsin treatment can be classified into two cases. Treatments with lower concentration of α -chymotrypsin lead to reduced RBC adhesiveness to EC. This could be due to the reduced RBC deformability, which hinders RBC from establishing contact with EC. However when the applied concentration of α -chymotrypsin is higher, the further reduction of the RBC surface charge and further dissociation of the glycocalyx proteins are suggested to outweigh the reduction of RBC deformability, thus leading to increased RBC adhesiveness to EC.

The comparison between Table 7-1 and Figure 7-5 demonstrates that the presence of 10 mg/ml of dextran 500 kDa significantly enhances the adhesion of the treated RBC as compared to polymer-free solution. The adhesion-promoting effects of dextran are ascribed to depletion interaction as discussed in Chapters 4. In particular, it is suggested that the dissociation of RBC glycocalyx proteins due to α -chymotrypsin treatment may lead to reduced glycocalyx thickness, which results in less penetration of the dextran molecules into the RBC glycocalyx and thus enhanced depletion interaction. This is shown by the increase of the adhesion (dark grey bar vs. blank bar) in Figure 7-5 due to the α -chymotrypsin treatment with higher enzyme concentration.

The above discussion suggests that RBC adhesiveness is an important factor of RBC-EC adhesion. However, the changes to RBC glycocalyx proteins due to α -chymotrypsin treatments require further characterization methods in order to identify the specific membrane components that determine RBC adhesiveness. These studies are beyond the current scope of this thesis and indicate a direction of future studies.

Conclusion

In conclusion, α -chymotrypsin treatments reduce RBC surface charge and deformability and dissociate RBC glycocalyx proteins. These interplaying factors determine the RBC adhesiveness to EC. Depletion interaction resulting from non-adsorbing macromolecules promotes the adhesion of RBC treated with α -chymotrypsin. The dissociation of RBC glycocalyx proteins due to α -chymotrypsin treatment is suggested to reduce polymer penetration into cell glycocalyx and thus enhance depletion interaction.

Chapter 8 The effect of non-adsorbing macromolecules on the adhesion of RBC with PS exposure to EC

Chapter 4 has demonstrated that depletion interaction resulting from non-adsorbing macromolecules can induce adhesion of normal RBC to the endothelium in buffer/albumin, while Chapter 5 has further demonstrated that an elevated level of non-adsorbing macromolecules in plasma-like media or plasma can induce depletion interaction leading to enhanced adhesion of normal RBC to endothelium. These results thus strongly suggest that depletion interaction can be a potential mechanism for the adhesion-promoting effects of non-adsorbing macromolecules or proteins in plasma during pathological conditions.

Aiming to provide some insights to the mechanism of pathological RBC-EC adhesion, RBC with enhanced PS exposure are investigated regarding their adhesiveness to the endothelium. PS exposure has been continuously suggested to be a significant adhesion molecule in RBC-EC adhesion in sickle cell anemia [63, 64], hereditary hydrocytosis [21], and chronic uremia [20] (also see in Page 11). In this chapter, PS exposure was obtained by treating normal RBC with calcium ionophore A23187, a method that is widely used to specifically investigate the significance of PS in abnormal RBC adhesion to the endothelium [62, 64, 146]. It is hypothesized that non-adsorbing macromolecules can promote the adhesion of RBC with increased PS exposure to EC. Dextran is again chosen to mimic the impact of non-adsorbing plasma proteins.

Results

RBC morphology observed by transmitted microscope

As demonstrated in Figure 8-1, Normal RBC have a biconcave (or so-called discocyte) shape with a size of approximately 7-8 μm (Figure 8-1A), whereas RBC treated with A23187, a calcium ionophore which can induce PS exposure on normal RBC, become smaller ($\sim 5\mu\text{m}$) and more spherical (Figure 8-1, B-D).

Figure 8-1 RBC morphology observed on transmitted light microscope. (A), normal RBC. RBC treated with A23187 at (B), 4 μM ; (C), 8 μM ; (D), 16 μM .

PS labeling observed by fluorescence microscope

Annexin V has been widely used in detecting cell apoptosis and it binds specifically to PS on the RBC membrane [65]. In order to see whether PS has been successfully flip-flopped to the outer membrane, RBC were labeled with FITC-labeled annexin V. As detected via fluorescence microscopy, normal RBC did not show any fluorescence signal (data not shown), indicating that they do not bind annexin V, indicating no PS exposure on the outer RBC membrane. However, in

Figure 8-2 strong fluorescence signal can be detected from RBC treated with A23187, indicating PS exposure on the outer RBC membrane.

Figure 8-2: (A) The morphology of RBC treated with A23187 observed under transmitted light microscope. (B) RBC with 90% of their population exhibiting PS exposure detected by FITC-annexin V labeling under fluorescence microscope.

PS exposure measured by flow cytometry

To quantify the percentage of RBC exhibiting PS exposure (PS-RBC), the cells were labeled with FITC-annexin V and then measured by flow cytometry. The dotplots presented in Figure 8-3 demonstrate a decreased size of RBC after the treatment with A23187, which is consistent with the microscopic observation in Figure 8-1. As can be seen, there is a slight shift of FSC-H (i.e. cell size) in Figure 8-3B (91.41% PS) compared to that in Figure 8-3A (normal RBC) and the “tail” of

SSC-H (i.e. cell granularity and density) in Figure 8-3B indicates that there are many fragments after the A23187 treatment.

Figure 8-3: Flow cytometric quantification of the phosphatidylserine (PS) exposure obtained by A23187 treatment on normal RBC. 50×10^3 events per sample were acquired. The light scatter and fluorescence channels were set at a logarithmic gain. The forward angle light scatter was E-1 with a threshold of 36. M1 gate and background was defined by control (normal RBC). The fluorescence of the erythrocyte bearing PS exposure was collected in the M1 gated zone. (A), dotplot of the control (normal RBC); (B), dotplot of the A23187-treated RBC; (C), histogram of the control; (D), histogram of the A23187-treated RBC.

For the analysis of fluorescence intensity, only intact cells were included. In accordance to Figure 8-3A, the histogram of Figure 8-3C (normal RBC) indicates a tiny subpopulation of RBC with PS exposure undergoing apoptosis [162]. In accordance to Figure 8-3B, the histogram of Figure 8-3D demonstrates an enhanced PS exposure (91.41%) obtained from the A23187 treatment. In this study, only RBC with 90% of their population exhibiting PS exposure (i.e. PS-RBC, 90%) were used for the subsequent adhesion assay.

PS exposure induces RBC-EC adhesion

The adhesion studies were started by investigating the effects of PS exposure on RBC-EC adhesion. Normal RBC or PS-RBC (90%) were suspended in polymer-free solution (HBSS containing 0.2% BSA and 1.2 mM Ca^{2+}), after which they were allowed a settling for 2 minutes onto confluent layers of EC. Thereafter the endothelium was rinsed with stepwise-increasing shear stress and the adherent cells were counted.

Figure 8-4: Adherence of normal RBC (control) and PS-RBC (90%) to EC as a function of the shear stress during rinsing. RBC were suspended in HBSS containing 1.2 mM Ca^{2+} and then allowed to settle for 2 minutes before rinsing with a stepwise-increasing shear. Error bars are standard deviations (SD) of the mean adherence values from 4 individual experiments (i.e. $n=4$).

Figure 8-4 demonstrates that normal RBC hardly adhere to EC. In contrast, PS-RBC demonstrate significantly higher adhesion efficiency, e.g. at the lowest applied shear stress of 0.02 Pa, 128 (± 53) cells/mm² PS-RBC adhere to EC with more than half of the cells remaining adherent at 0.12 Pa.

The impact of dextran concentration and Mw on PS mediated RBC-EC adhesion

After the adhesion was studied in polymer-free solution, it was studied in solutions containing non-adsorbing macromolecules. Figure 8-5 demonstrates the impact of dextran 2 MDa at different concentrations on the adhesion of PS-RBC to EC. Cells were allowed to settle for 2 minutes prior to rinsing with stepwise-increasing shear stress from 0.02 to 0.10 Pa.

Figure 8-5: Impact of dextran 2 MDa on the adhesion of PS-RBC (90%) to EC. PS-RBC (90%) were suspended in either polymer-free solution or dextran solutions. The cells were then allowed to settle for 2 minutes onto EC monolayers before rinsing with stepwise-increasing shear. Error bars are standard deviations (SD) of the mean adherence values from three individual experiments. Wilcoxon-Mann-Whitney U test was performed on the adherence values at 0.02 Pa between the investigated solutions.

The presence of dextran 2 MDa significantly increases the adhesion efficiency. In 5 mg/ml of 2MDa, about 470 cells/mm² are adherent after applying a shear stress of 0.02 Pa, leading to 3.7 folds of that in polymer-free solution; and 43% of these cells remain adherent at 0.1 Pa, leading to 2.6 folds of that in polymer-free solution at the same shear stress. In 10 mg/ml of 2 MDa, the adhesion further increases, with 549 cells/mm² adherent at 0.02 Pa and 298 cells/mm² at 0.1 Pa, leading to 4.3 folds and 3.9 folds of that in polymer-free solution at the same shear stress.

Figure 8-6 presents a similar set of experiments illustrating the impact of dextran with the same concentration but different molecular mass and thus polymer size (Table 8-1) on the adhesion of PS-RBC to EC. PS-RBC (90%) were suspended in solutions containing dextran 40 kDa, 70 kDa or 500 kDa at a fixed concentration of 5 mg/ml.

Figure 8-6: Dependence of PS-RBC (90%) adhesion to EC on the molecular mass of dextran at a constant polymer concentration (5 mg/ml). The cells were allowed to settle for 2 minutes onto EC monolayers before rinsing with stepwise-increasing shear. Error bars are standard deviations (SD) of the mean adherence values from three individual experiments. Wilcoxon-Mann-Whitney U test was performed on the adherence values at 0.02 Pa between the investigated solutions.

Having the smallest polymer size, dextran 40 kDa results in similar extent of adhesion as the polymer-free solution does (refer to Figure 8-5). When the shear increases to 0.1 Pa, more than 80% of the adherent cells that were initially adherent at 0.02 Pa are removed. In contrast, the number of adherent cells and the adhesion strength increased significantly in the presence of either dextran 70 kDa or dextran 500 kDa. In dextran 70 kDa, 380 cells/mm² remain adherent after applying the initial shear stress of 0.02 Pa, leading to about three folds of that in 40 kDa, and 70% of these cells remain adherent at 0.1 Pa. In dextran 500 kDa 480 cells/mm² are adherent at the initial shear stress of 0.02 Pa, and about 73% of these cells remain adherent at 0.1 Pa.

The above data clearly demonstrates that dextran enhances endothelial adhesion of PS-RBC with a marked dependence on the molecular mass and polymer concentration. However, since the molecular mass and the polymer concentration may lead to variations in solution viscosities (Table 8-1), the number of RBC settled onto EC and thus established contact with EC may also vary in different solutions even at the same settling time (Table 8-2).

Table 8-1: Physicochemical properties of the dextran molecules used in this chapter.

	molecular mass (Mw), kDa	Mw /Mn	radius of gyration, nm	viscosity at 5 mg/ml (10 mg/ml), mPa·s
DEX 40	35-45	1.6	5.1	0.80
DEX 70	74.0	1.5	7.8	0.82
DEX 500	519	2.2	17.7	0.87
DEX 2000	~2000	-	38.5	0.94 (1.24)

Molecular mass data and weight-average (Mw) to number-average (Mn) molecular mass ratios (Mw/Mn) are given as supplied by the vendors.

Table 8-2: The number of RBC settled onto EC monolayer in the 2-minute settling time

solutions	number of RBC settled ($\pm$ SD)
HBSS	1470 $\pm$ 329
DEX 40 kDa (5 mg/ml)	1463 $\pm$ 153
DEX 70 kDa (5 mg/ml)	1393 $\pm$ 18
DEX 500 kDa (5 mg/ml)	1367 $\pm$ 70
DEX 2 MDa (5 mg/ml)	1308 $\pm$ 248
DEX 2 MDa (10 mg/ml)	737 $\pm$ 111

3 pictures were taken at random spots right before starting the rinse and the numbers of RBC settled are presented as mean $\pm$ SD of the three pictures counted.

Therefore, the number of adherent RBC is also dependent on the number of RBC that have established contact with EC. This factor has to be taken into consideration when comparing the absolute adherence as presented above (Figure 8-5 and Figure 8-6). Hence, for a more accurate comparison on the adhesion within the same settling time but in solutions with different viscosities, we present the relative adherence, which converts the absolute adherent RBC number to percentage of the number of RBC settled onto EC monolayer within the given settling times. Figure 8-7 summarizes this relative percentage in both polymer-free solution and solutions containing dextran. At a constant concentration of 5 mg/ml, increasing the molecular mass (Mw) from 40 kDa to 2 MDa reveals a pronounced impact of dextran on the adhesion efficiency. At 0.02 Pa the relative adherence increases from 8% in 40 kDa to 37% in 2 MDa. A plateau seems to locate between 500 kDa and 2 MDa. Moreover, at 0.10 Pa the adhesion reveals a biphasic adherence–Mw relation with a peak of 26% at 500 kDa. Employing 10 mg/ml of dextran 2MDa further increases the adhesion, i.e. 77% of the cells settled are adherent at 0.02 Pa and 42% remain adherent at 0.1 Pa, which correspond to almost 9 times and 8 times as many cells as compared to in the polymer-free solution.

Figure 8-7: Relative adherence of PS-RBC (90%) to EC at 0.02 Pa and 0.1 Pa in percent of cells settled within 2 minutes. Error bars are standard deviations (SD) of the mean adherence values from three individual experiments.

The impact of dextran on PS-RBC adhesion to EC with different contact times

As demonstrated above, the presence of dextran has a pronounced effect on the adhesion of PS-RBC to EC. Figure 8-8 demonstrates the effect of longer settling times for PS-RBC suspended in either polymer-free solution or the solution containing 10 mg/ml of dextran 2 MDa. Regardless of the applied shear stress, the adhesion is always higher in the presence of dextran 2 MDa. However, the difference between these two solutions is more significant at a shorter settling time of two minutes (Figure 8-9). Thus, it can be concluded that the presence of dextran leads to a significant increase of the attachment rate but with increasing contact times this effect becomes less visible.

Figure 8-8: Comparison of PS-RBC (90%) adhesion to EC in polymer-free medium and in dextran 2 MDa (10 mg/ml) after 10 minutes' settling time. Error bars are standard deviations (SD) of the mean adherence values from three individual experiments.

Figure 8-9: Comparison of PS-RBC (90%) adhesion to EC in polymer-free medium and in dextran 2 MDa (10 mg/ml) after 2 minutes' settling time. Error bars are standard deviations (SD) of the mean adherence values from four individual experiments.

The impact of dextran on PS-RBC adhesion to EC under continuous flow

This effect of dextran on the adhesion of RBC to EC can also be observed under continuous flow conditions. EC monolayers were perfused with RBC suspensions at constant shear stresses of 0.02 Pa, 0.03 Pa or 0.04 Pa for 10 minutes, followed by 10 minutes' rinsing with cell-free medium at the same shear stresses.

Figure 8-10 compares the adhesion of normal RBC and PS-RBC (90%) in polymer-free solution to that in 10 mg/ml 2 MDa dextran. Normal RBC demonstrate no adhesion in polymer-free solution, whereas the presence of dextran results in a minor adherence at shear stresses of 0.02 and 0.03 Pa. Increasing the shear to 0.04 Pa completely prevents the adhesion of normal RBC. On the other hand for PS-RBC, they exhibit strong adhesiveness to EC. Even in polymer-free solution, PS-RBC can adhere at 0.04 Pa. The presence of dextran further elevates the adherence of PS-RBC. Compared to that in polymer-free solution, the adherence in dextran solutions is about three folds more at 0.02, five folds more at 0.03 Pa and seven folds more at 0.04 Pa. As a result, it is concluded that the presence of dextran significantly enhances PS-RBC adhesion to EC even without settling time but under constant flow.

Figure 8-10: Impact of the suspending medium on PS-RBC (90%) adhesion to EC under continuous flow. EC monolayers were perfused with RBC suspension for 10 minutes, followed by another 10 minutes' rinsing with cell-free medium. Error bars are standard deviations (SD) of the mean adherence values from three individual experiments.

Ca²⁺ dependence of PS-RBC adhesion in dextran solutions

It has been reported that PS mediated RBC-EC adhesion is Ca²⁺ dependent [32, 66]. To determine whether this Ca²⁺ dependence also exists in dextran solutions, the adhesion of PS-RBC (90%) in 5 mg/ml of dextran 500 kDa was compared between the presence and absence of Ca²⁺. Figure 8-11 demonstrates that the presence of Ca²⁺ is required for PS-RBC adhesion in dextran solutions. At the initial rinsing of 0.02 Pa after 2 minutes' settling, a 50% reduction of PS-RBC adhesion can be seen due to the absence of Ca²⁺, and the remaining adhesion decreases quickly with increasing shear stress and finally vanishes at 0.08 Pa. Comparing Figure 8-11 to Figure 8-4 demonstrates that PS-RBC adhesion in dextran solution without Ca²⁺ is lower than the adhesion in polymer-free solution, indicating that Ca²⁺ is essential for PS-RBC adhesion and that the adhesion-promoting effects of dextran is additive.

Figure 8-11: The effect of Ca^{2+} on PS-RBC (90%) to EC in dextran solutions. RBC were suspended in dextran 500 kDa (5 mg/ml) with or without 1.2 mM Ca^{2+} . 2 minutes' settling was given prior to rinsing with the same solutions as that during settling. Shown here is a representative experiment and the error bars are standard deviations (SD) of the mean adherence values counted from the 20 captures for each shear stress.

Discussion

Non-adsorbing macromolecules promote PS-RBC adhesion to EC

This chapter confirms the significant role of PS in RBC-EC adhesion, which is consistent with other reports investigating sickle RBC adhesion to the endothelium [63, 64, 188]. Furthermore, this chapter clearly demonstrates the significant promoting effect of dextran (> 70 kDa) on PS-RBC adhesion to EC, especially when the contact time between RBC and EC is short (e.g. in the quasi-flow assay with 2 minutes' settling time and in the continuous-flow assay). This proadhesive effect can be ascribed to depletion interaction, which is capable of promoting close cell-cell contact (Chapter 4). The dependence of the PS-RBC adhesion to EC on the concentration and Mw of dextran is in agreement with the theoretical predictions (Chapter 3) as well as the experimental results (Chapter 4) in the previous parts of this thesis.

Figure 8-12: Schematic picture on how macromolecular depletion interaction brings adjacent cells into close contact. Macromolecular depletion occurs at the cell surface if the loss of entropy for the macromolecule at the interface is not compensated by an attractive interaction. As two surfaces approach, overlapping of the depletion layers is driven by osmotic pressure to reduce the Free Energy of the system, promoting cell-cell interaction.

Figure 8-12 schematically illustrates how depletion interaction may promote close contact and thus cell-cell interactions. In order to calculate the energy of depletion interaction acting between RBC and EC, it is assumed that cell-cell distance d is approximately equal to the sum of the glycocalyx thickness (Figure 8-12).

Figure 8-13: Theoretical dependence of the energy of depletion interaction on the penetration (p_t) of the polymer into the two glycocalyxes. The energies were calculated for the dextran solutions employed in this section. The gray area indicates the estimated total penetration as suggested by the experimental data of this thesis (see text for details).

In Figure 8-13 the change of the free energy per area due to depletion interaction for the dextran solutions used in this chapter was calculated (s.a. equation (0.8) in Chapter 3 (Page 51)) as a function of the total penetration of dextran into both RBC and EC glycocalyx. A minimal Mw is required to initiate an attractive depletion force, since polymer penetration into cell glycocalyx reduces depletion interaction (Chapter 3). This theoretical prediction (line a in Figure 8-13) is confirmed by the experimental result in Figure 8-7 showing that dextran 40 kDa does not induce PS-RBC adhesion to EC. Furthermore, the dependence of the adhesion on Mw of dextran in Figure 8-7 indicates that the promoting effect increases from 70 kDa to 500 kDa and reaches a peak somewhere between 500 kDa and 2 MDa. This suggests a total penetration (p_t) to be 15-20 nm, i.e. $b < c \approx d < e$ in Figure 8-13. This range is consistent with estimates of the glycocalyx thickness between 5 to 20 nm [112]. Therefore, Figure 8-13 suggests that the energy of depletion interaction acting on RBC and EC is approximately 1 to 2 $\mu\text{J}/\text{m}^2$ or 250 to 500 $k_B T/\mu\text{m}^2$, which is in the same range as experimentally measured adhesion affinities of RBC to artificial surfaces or RBC-RBC aggregation in polymer solutions [156, 189].

Depletion interaction facilitates receptor-mediated interactions

The loss of free energy predicted by Figure 8-14 provides a theoretical estimation of how strong the adhesion-promoting effect of depletion interaction can be. This promoting effect has been experimentally shown to be very efficient in PS-RBC adhesion to EC when the contact time is short (Figure 8-7 and Figure 8-10). It is predicted that depletion interaction effectively facilitates the specific ligand-receptor interactions, since PS-RBC adhesion has been reported to involve multiple cell surface receptors and plasma ligands, such as PSR on EC [67] and TSP in the sub endothelial cell matrix [63] or in plasma [37]. To confirm this prediction, this chapter tested the Ca^{2+} dependence of PS-RBC adhesion in dextran solutions. The significant reduction of PS-RBC adhesion in the absence of Ca^{2+} reveals the participation of specific receptor-mediated interactions in PS-RBC adhesion to EC, in particular, the specific bindings in which TSP is involved, since Ca^{2+} regulates the binding domain of TSP (i.e. transforms it from non-adhesive formation to adhesive formation for the specific binding of sickle RBC or PS-RBC) [63, 190]. The results of this chapter thus suggest a novel adhesion pathway that depletion interaction can facilitate specific receptor bindings between RBC and EC by promoting close cell-cell contact, as illustrated in Figure 8-14.

Figure 8-14: Schematic picture of how macromolecular depletion interaction brings adjacent cells into intimate contact. (I) Macromolecules are depleted from both RBC and EC surfaces; (II) And then attraction develops when depletion layers overlap; (III) The resultant intimate cell-cell contact thus facilitates receptor-mediated interactions to take place.

Conclusion

In conclusion, the current chapter demonstrates that depletion interaction resulting from dextran of high Mw (>70 kDa) can significantly promote PS-RBC adhesion to EC. The adhesion exhibits a strong dependence on the concentration and Mw of dextran, which is in agreement with the findings in Chapter 4 and 5. Furthermore, the focus on the impact of cell-cell contact time reveals that depletion interaction can facilitate PS-mediated RBC-EC adhesion. This effect can be ascribed to an intimate cell-cell contact promoted by depletion interaction and thus an increased probability of specific receptor bindings. Thus, depletion interaction is suggested to be an underlying mechanism of the adhesion-promoting effects of non-adsorbing proteins in pathological RBC-EC adhesion.

Chapter 9 The effect of non-adsorbing macromolecules on the adhesion of normal RBC to thrombin-activated EC

The effect of thrombin on endothelium in sickle cell disease has been reviewed in the Introduction (Page 16). Clinically it has been observed in sickle occlusion that not only the level of thrombin is elevated in plasma, but also the proadhesive plasma proteins such as TSP and fibrinogen [93, 94]. The increased EC perturbant (i.e. thrombin) and plasma proteins finally are in concurrence with the pathological RBC-EC adhesion. This concurrence involves many interplaying factors forming a vicious cycle enhancing vaso-occlusion [14]. Most of the previous studies on the pathological RBC-EC adhesion during this concurrence in vaso-occlusion have been focusing on the specific receptors on cell surfaces and the adhesion pathways involved [10, 14], but limited effort has been made to elucidate the adhesion-promoting effect of plasma proteins. In this chapter, depletion interaction resulting from non-adsorbing macromolecules is investigated regarding its role in the adhesion of RBC to thrombin-activated endothelial cells.

Results

Thrombin induces adhesion of normal RBC to EC

The study first investigates the effect of thrombin on RBC-EC adhesion. Serum free medium (SFM) was used as polymer-free medium based on other studies which investigated the effects of thrombin on RBC-EC adhesion [48, 91]. Dextran 40 kDa or 2 MDa were dissolved in SFM at the desired concentrations. Confluent EC monolayers were pretreated with 0.01 U/ml of thrombin in dextran-free SFM for 5 minutes, followed by co-incubation with normal RBC suspended in the same medium for 15 minutes before rinsing with cell-free medium. The settling time was chosen based on a previous study, which reported a time dependence of the expression of adhesion molecules on endothelium activated by thrombin [90].

Figure 9-1: Adherence of RBC to the control (normal) or thrombin-treated EC as a function of the applied shear stress in SFM. EC were pretreated with 0.01 U/ml of thrombin for 5 minutes, followed by co-incubation with RBC in the presence of thrombin under stasis for 15 minutes. Error bars are standard deviation (SD) of the mean adherence values from three individual experiments.

Figure 9-1 demonstrates that only a few RBC (21 ± 11 cells/mm²) adhere to normal untreated EC at the lowest applied shear stress of 0.01 Pa. However, EC treated with 0.01 U/ml of thrombin exhibit a substantially increased adhesion, with 5 times as many RBC adherent at 0.01 Pa and 7 times at 0.02 Pa. Thus, it can be concluded that thrombin can induce adhesion of normal RBC to EC, which is in agreement with previous reports [89, 91].

Dextran of high Mw induces adhesion of normal RBC to EC

After 15 minutes of co-incubation with normal RBC in dextran-SFM, the EC monolayers were rinsed with cell-free medium containing the same dextran molecules. Figure 9-2 demonstrates that even at the initial shear stress of 0.02 Pa, the adhesion in dextran 40 kDa is minor. However, the adhesion in dextran 2 MDa is tremendously increased, with 213 (± 58) cells/mm² adherent at 0.02 Pa and 24% of these cells still remaining adherent even at 0.1 Pa. Hence, with the quasi-flow assay applying 15 minutes' incubation, dextran of high Mw can effectively induce adhesion of normal RBC to EC.

Figure 9-2: Adherence of normal RBC to the control (normal EC) as a function of the applied shear stress in dextran-SFM. EC was co-incubated with RBC in either dextran 40 kDa or 2 MDa at the concentration of 10 mg/ml under stasis for 15 minutes, followed by rinsing with stepwise-increasing shear stress. Error bars are standard deviation (SD) of the mean adherence values from three individual experiments.

The adhesion enhances with increased thrombin concentration in dextran solutions

With the above studies performed separately on thrombin and dextran, the effort was continued to investigate the impact of dextran on thrombin-activated endothelial adhesion of normal RBC. EC monolayers were pretreated with thrombin in different concentrations ranging from 0.005 to 0.1 U/ml as described above. The adhesion assay was conducted in either dextran-free SFM or SFM containing 10 mg/ml of dextran 2 MDa.

Figure 9-3: Adherence of RBC to thrombin treated EC as a function of the applied shear stress in: (left) SFM; (right) dextran 2 MDa at 10 mg/ml. Thrombin concentrations were applied in a range of 0.005-0.1 U/ml. Error bars are standard deviation (SD) of the mean adherence values from three individual experiments.

Figure 9-3 (the left part) demonstrates that the overall adhesion in dextran-free SFM is low, i.e. less than 50 cells/mm² are adherent at 0.02 Pa after a 5-minute rinsing, and there are no significant differences in the adhesion among different thrombin concentrations as well. However, Figure 9-3 (the right part) demonstrates that in dextran 2 MDa, the adhesion is dramatically increased regardless of the thrombin concentrations, and it exhibits a strong resistance to shear compared to that in dextran-free SFM. At 0.1 Pa, the adhesion in dextran-free SFM is zero, whereas at least 50 cells/mm² remain adherent in the presence of dextran. In addition, the adhesion in dextran-SFM clearly exhibits a dependence on the concentrations of thrombin, e.g. 243 ($\pm$ 59), 300 ($\pm$ 15), 383 ($\pm$ 103) and 425 ($\pm$ 108) cells/mm² are adherent at 0.02 Pa after a 5-minute rinsing for 0.005, 0.01, 0.05 and 0.1 U/ml of thrombin, respectively. Thus, dextran of high Mw promotes thrombin-activated endothelial adhesion of normal RBC and the adherence increases with increasing thrombin concentrations.

The adhesion increases with prolonged thrombin treatment in dextran solutions

The expression of adhesion molecules on the EC surface has been reported to be time dependent on thrombin treatment [90], thus this study investigates whether thrombin-activated endothelial adhesion is also time dependent in dextran solutions. The experimental procedures are similar as above. 15 minutes and 30 minutes of co-incubation were employed. Two thrombin concentrations were studied, 0.01 U/ml and 0.05 U/ml.

Figure 9-4: Time dependence of the adhesion on thrombin treatment. EC was co-incubated with RBC in 0.01 U/ml of thrombin for either 15 minutes or 30 minutes prior to flow in (left) SFM; (right) dextran 2 MDa at 10 mg/ml. Error bars are standard deviation (SD) of the mean adherence values from three individual experiments.

Figure 9-4 (the left part) demonstrates that the adhesion in SFM is always low for both 15 and 30 minutes' thrombin treatment, i.e., less than 50 cells/mm² are adherent at 0.02 Pa after a 5-minute rinsing. Moreover, no significant difference can be detected for different co-incubation times. However, on the right part of Figure 9-4, it is demonstrated that the adhesion is much higher in dextran-SFM and a significant increase can be seen for the 30-minute co-incubation as compared to the 15-minute one. Besides leading to more adherent RBC, prolonged co-incubation time also seems to strengthen the adhesion between RBC and EC.

Compared to 15 minutes, 30 minutes results in 1.68 times as many cells adherent at 0.05 Pa and 1.92 times as many at 0.1 Pa.

Figure 9-5 presents another similar set of experiments using 0.05 U/ml of thrombin. The adhesion in dextran-SFM is much higher than that in SFM and also demonstrates a time dependence on thrombin treatment. Thus, it can be concluded that dextran of high Mw promotes thrombin-activated endothelial adhesion of normal RBC and the adhesion increases with prolonged thrombin treatment.

Figure 9-5: Time dependence of the adhesion on thrombin treatment. EC was co-incubated with RBC in 0.05 U/ml of thrombin for either 15 minutes or 30 minutes prior to flow in (left) SFM; (right) dextran 2 MDa at 10 mg/ml. Error bars are standard deviation (SD) of the mean adherence values from three individual experiments.

Discussion

This chapter applies a quasi-flow assay to illustrate the effect of a non-adsorbing macromolecule, dextran, on thrombin-activated endothelial adhesion of normal RBC. The results demonstrate in brief that in the presence of 10 mg/ml of dextran 2 MDa, the adhesion increases with increasing thrombin concentrations from 0.005 to 0.1 U/ml. The adhesion also increases with prolonged thrombin treatments from 15 to 30 minutes. With the previous work showing that adhesion of normal RBC to EC can be induced by depletion interaction generated by large dextran ($M_w > 70$ kDa) [178], the current chapter demonstrates that dextran-induced depletion interaction promotes thrombin-activated endothelial adhesion of normal RBC.

Figure 9-6: The vicious cycle involving inflammation and vaso-occlusion in sickle disease. Note that from both micro and macro views, the concurrence of elevated EC perturbants (e.g. thrombin), inflammatory proteins (e.g. fibrinogen) and increased pathological RBC-EC adhesion can be seen.

As illustrated in Figure 9-6, the level of thrombin in plasma has been found to be increased during vaso-occlusion [86], leading to activated endothelium [88, 191]. EC activated by thrombin have been found to express adhesion molecules on their surfaces (e.g. ICAM-1 and P-selectin [90]). At the same time, the levels of proadhesive proteins are also dramatically elevated during vaso-occlusion (e.g. TSP [94], vWF [101], and fibrinogen [98]). Some of these proadhesive proteins may bridge between RBC and EC as ligands (e.g. TSP and vWF, Page 18) cross-linking the specific receptors; while some others (e.g. fibrinogen and other non-

adsorbing macromolecules) may induce depletion interaction promoting intimate cell-cell contact and thus facilitating receptor-mediated interactions. Multiple specific receptor-mediated interactions contribute to increased pathological RBC-EC adhesion, which has been closely correlated to the severity of vaso-occlusion [3]. In this cycle (blue dashed cycle in Figure 9-6), depletion interaction is suggested to be a significant promoter of pathological RBC-EC adhesion. This is based on the findings in Chapter 5 that an increased level of non-adsorbing macromolecules in plasma can induce enhanced depletion interaction, and in Chapter 8 that depletion interaction can increase the binding probabilities of receptor-mediated interactions by promoting close cell-cell contact. In this chapter, the promoting effect of depletion interaction on receptor-mediated bindings is demonstrated by the fact that normal RBC have been suggested to bear receptors for P-selectin [91]. This is also supported by the observation that the adhesion in dextran solutions increases with prolonged thrombin treatments (Figure 9-4 and Figure 9-5), since the expression of adhesion molecules on EC surface induced by thrombin (such as ICAM-1 and P-selectin) has been identified to be time dependent [14, 90].

It should also be noted that that it has been suggested that sickle cell disease is an inflammation (green dashed circle in Figure 9-6) in which the coagulation system is always activated [14]. This is also reflected by increased levels of both fibrinogen and thrombin in plasma. In concurrence with these increases is the enhanced pathological RBC-EC adhesion, which initiates a multi-step vaso-occlusion (Page 19), the consequences of which include impaired blood flow, reduced oxygen transport, and enhanced sickling of RBC [14]. All these lead to tissue ischemia and injury, which finally cause inflammation and activates the coagulation system, closing a vicious cycle as illustrated in Figure 9-6.

Conclusion

In conclusion, depletion interaction resulting from non-adsorbing macromolecules can significantly promote the adhesion of normal RBC to thrombin-activated EC. The adhesion in dextran solutions is found to increase with increasing thrombin concentrations, as well as with prolonged thrombin treatments on EC. These experimental findings resemble that in vaso-occlusion during which increased

proadhesive proteins, elevated level of thrombin, and enhanced pathological RBC-EC adhesion are identified to be concurrent. Therefore, this chapter demonstrates a potential pathophysiological relevance of depletion interaction and suggests it to be the underlying mechanism of the adhesion-promoting effect of non-adsorbing plasma proteins in sickle occlusion. This model of depletion interaction is believed to be beneficial for a better understanding of the pathology of vaso-occlusion as well as other diseases associated with similar vascular disorders [97].

Chapter 10 Overall conclusions and future studies

This thesis studies the impact of non-adsorbing macromolecules on RBC adhesion to EC. Dextran, a polyglucose that has been shown to be depleted from the RBC surface, was used to mimic the effects of non-adsorbing proteins or macromolecules on RBC-EC adhesion.

It is demonstrated that dextran with high molecular mass (> 70 kDa) can induce adhesion of normal RBC to EC in buffer/albumin. The adhesion can withstand rinsing with low shear stress (e.g. 0.01-0.05 Pa) and depends strongly on polymer concentration and molecular mass (M_w). Depletion interaction is suggested to be the underlying mechanism and it is concluded that the physicochemical properties of the non-adsorbing macromolecules are determinants of RBC-EC adhesion mediated by depletion interaction. In brief, at a constant M_w , higher polymer concentration results in higher osmotic pressure and thus enhanced RBC-EC adhesion. At a constant polymer concentration, higher M_w leads to increased RBC-EC adhesion. An optimal M_w was experimentally found to be between 500 kDa and 2 MDa maximizing the adhesion, which reveals a biphasic dependence of the adhesion on polymer M_w . This is in agreement with the depletion model, which suggests that the energy of depletion interaction is a biphasic function of the polymer M_w if we assume a total penetration of the dextran molecules into RBC and EC glycocalyx of 20-30 nm. Future studies should try to indentify the biochemical or biophysical properties of the EC surface, which might provide a better understanding on how polymer penetration into the EC surface affects the interactions between non-adsorbing macromolecules and cell membranes. Such studies should further improve the current understanding of depletion interaction.

In addition, it is demonstrated that the RBC surface properties are also determinants of RBC-EC adhesion mediated by depletion interaction. Sialic acid loss from the RBC membrane due to neuraminidase treatment (i.e. desialylation) was found to induce weak RBC-EC adhesion. Depletion interaction resulting from non-adsorbing macromolecules was found to significantly promote the adhesion of desialylated RBC to the endothelium. The decrease of RBC surface charge due to

sialic acid loss is suggested to reduce the electrostatic repulsion between RBC and EC, which enhances the adhesion-promoting effect of depletion interaction and thus RBC-EC adhesion. α -chymotrypsin treatment not only reduces RBC surface charge, but also RBC deformability and results in the dissociation of glycocalyx proteins. This leads to altered RBC adhesiveness to EC and thus a different extent of adhesion mediated by depletion interaction, thus confirming the conclusion that the RBC surface properties have a marked impact on depletion interaction. In particular, the dissociation of RBC glycoproteins may lead to a reduced RBC glycocalyx thickness, which reduces the polymer penetration and thus enhances depletion interaction.

Depletion interaction resulting from non-adsorbing macromolecules was found to significantly promote adhesion of RBC with PS exposure to EC. It is suggested that a closer cell-cell contact is induced by depletion interaction, leading to increased binding probabilities of receptor-mediated interactions and thus facilitating the PS-mediated RBC adhesion to EC. Depletion interaction was also found to significantly promote RBC adhesion to thrombin-activated EC. These experimental findings demonstrate the potential pathophysiological relevance of depletion interaction and suggest that macromolecular depletion could be the underlying mechanism of the adhesion-promoting effects of non-adsorbing plasma proteins in sickle occlusion. Future studies should be carried out to further identify the significance of depletion interaction in the pathophysiology of diseases associated with vascular complications. For example, the role of non-adsorbing macromolecules on the adhesion of RBC from patients (e.g. sickle cell anemia or diabetes) needs to be investigated. Other than this, it would also be of interests to investigate the adhesiveness of EC originating from other tissues or organs, such as microvascular or pulmonary EC (Note that this thesis employed endothelial cell lines from human umbilical vein).

An increased level of dextran in plasma-like media was also observed to induce enhanced adhesion of normal RBC to EC. The adhesion also depends strongly on molecular mass and concentration of dextran. Thus, it is concluded that an increased level of non-adsorbing macromolecules in plasma can induce depletion interaction promoting RBC-EC adhesion. Furthermore, the adhesion-promoting

effects of fibrinogen resemble those of dextran, thus, it is suggested that fibrinogen is a non-adsorbing plasma protein inducing or promoting RBC-EC adhesion via depletion interaction. This conclusion further reveals that depletion interaction can be induced by increased levels of non-adsorbing proteins in plasma, and may also promote other types of cell-cell interactions [192], such as leukocyte-endothelial adhesion [11, 193-195], RBC-macrophage interaction [162] and so on. Future studies along these directions may provide a better understanding of the role of non-adsorbing proteins in the pathophysiology of vascular diseases associated with abnormal cell-cell interactions.

Lastly, a binary mixture of large polymers and small polymers has been reported to be feasible to control depletion interaction [158]. Hence, it might be possible to use some small polymers to reduce the adhesion-promoting effects of depletion interaction resulting from non-adsorbing macromolecules. This may lead to possible novel therapies in the treatments of sickle occlusion and other vascular complications.

To sum up, this thesis has shown that depletion interaction originating from non-adsorbing macromolecules can induce or promote RBC-EC adhesion. This provides a novel mechanism for the adhesion-promoting effects of plasma proteins in vascular diseases. The role of non-adsorbing macromolecules and thus the effects of depletion interaction are suggested to be significant and deserve more attention in cell-cell interactions.

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