SPECIFIC BINDING OF RED BLOOD CELLS TO ENDOTHELIAL CELLS IS REGULATED BY NON- ADSORBING MACROMOLECULES.

Yang Yang, Stephanie Koo, Cheryl Shuyi Lin and Björn Neu
Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore

Running title: Regulation of red blood cell adhesion
Address correspondence to: Dr. Björn Neu, Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 639798, Fax: (65) 6791 1761; E-mail: neu@ntu.edu.sg

Abnormal adhesion of red blood cells to the endothelium has been linked to the pathophysiology of several diseases associated with vascular disorders. Various biochemical changes, including phosphatidylserine exposure on the outer membrane of red blood cells as well as plasma protein levels have been identified as being likely to play a key role, but the detailed interplay between plasma factors and cellular factors remains unknown. It has been proposed that the adhesion-promoting effect of plasma proteins originates from ligand interaction but evidence substantiating this assumption is often missing. In this work, we identified an alternative pathway by demonstrating that non-adsorbing macromolecules can also have a marked impact on the adhesion efficiency of red blood cells with enhanced phosphatidylserine exposure to endothelial cells. It is concluded that this adhesion-promoting effect originates from macromolecular depletion interaction and thereby presents an alternative mechanism by which plasma proteins could regulate cell-cell interactions. These findings should thus be of potential value for a detailed understanding of the pathophysiology of diseases associated with vascular complications and might be applicable to a wide range of cell-cell interactions in plasma or plasma-like media.

INTRODUCTION

The adhesion of red blood cells (RBC) to endothelial cells (EC) is usually insignificant. However, increased RBC adhesion to endothelial cells has been observed in various clinical states such as sickle cell disease, β-thalassemia, or diabetes mellitus and the degree of RBC-EC adhesiveness has been linked to the vascular complications associated with these diseases (1-5). There is now general agreement that various cell surface alterations control the increased RBC adhesiveness in such disease states. For example, an enhanced phosphatidylserine (PS) exposure on the outer leaflet of the RBC membrane has been linked to abnormal RBC-EC adhesion in sickle cell disease, hereditary hydropsysis, and chronic uremia (6-8). Usually this anionic phospholipid is located exclusively on the inner leaflet of the RBC membrane, but is trans-located to the outer leaflet in hemoglobinopathies and oxidative stress states (9). The role of PS in adhesion has not been fully understood but RBC with enhanced PS exposure have been identified to
establish specific interactions with EC or the endothelial matrix via several receptors including TSP, $\alpha_v\beta_3$, CD36, and PS receptor (PSR) (6,10-11).

Moreover, several plasma factors have been identified to be involved in abnormal RBC adhesion to EC. For example, fibrinogen enhances the adhesion of pathological RBC to EC (12-13), which is consistent with the observation that the onset of vaso-occlusive crisis in sickle cell disease is always accompanied by a temporally elevated level of this acute-phase protein (12,14). However, the underlying mechanisms behind the increased adhesion efficiency in the presence of this acute-phase protein remain obscure. It has been suggested that fibrinogen acts as a ligand, cross-linking receptors on adjacent cells, but to date the identification of binding sites or receptors that could confirm this hypothesis have not been reported (15).

On the other hand, various non-specific forces such as van der Waals interaction, electrostatic repulsion, steric interaction, and membrane undulations are also known to play an important role in cell adhesion. Another non-specific force, which has not received much attention in the area of cell-cell interaction, is depletion interaction. Depletion interaction is a result of a lower localized protein or polymer concentration near the cell surface as compared to the suspending medium (16). This exclusion of macromolecules near the cell surface leads to an osmotic gradient. As two cells or surfaces approach, solvent is displaced from the depletion zone into the bulk phase leading to an attractive force (16-19). In the past, it has been demonstrated that depletion interaction is most likely the driving force behind the reversible aggregation of RBC (20-21) and more recent works have shown that depletion interaction can also induce weak adhesion of normal RBC to EC or glass surfaces (22-23). However, depletion interaction as a potential mechanism inducing adhesion of abnormal or pathological RBC to the endothelium has not yet been considered.

In this study, we investigated if macromolecular depletion can promote adhesion of RBC with enhanced PS exposure to EC and, thus, if polymer depletion might be an alternative mechanism for the adhesion-promoting effects of plasma proteins such as fibrinogen. RBC with enhanced PS exposure were obtained by the treatment of normal RBC with the calcium-ionophore A23187 (6,24-25) and suspended in solutions containing dextran to mimic the impact of non-adsorbing macromolecules. Dextran is a neutral poly-glucose that has been shown to be depleted from the RBC surface (26-27) and that is frequently used for in vitro hemorheological studies (28); adhesion efficiency was quantified with a flow adhesion assay (29).

**MATERIALS AND METHODS**

*Red blood cell preparation*

Blood was drawn into EDTA (1.5 mg/ml) from the antecubital vein of healthy adult volunteers. RBC were separated from whole blood by centrifugation (1,000g, 10 min) and then washed thrice with HBSS without Ca$^{2+}$ and Mg$^{2+}$, Sigma) containing 0.2% bovine serum albumin (BSA, Sigma). Expression of PS on the exterior RBC membrane surface was obtained by treating normal RBC with the calcium-ionophore A23187 (Sigma) as described elsewhere (30). In brief, RBC were incubated with N-ethyl
maleimide (Sigma) for 30 minutes at room temperature at a hematocrit of 30%, followed by two washings with HBSS containing 0.2% BSA. The cells were then re-suspended at a hematocrit of 16% in HBSS containing 2 mM CaCl2 and incubated for three minutes at 37°C. Subsequently, the calcium ionophore A23187 was added at the desired concentrations. After an incubation of one hour at 37°C, the treatment was terminated by washing the cells once with HBSS containing 2.5 mM EDTA (Bio-Rad) and then twice with HBSS containing 1% BSA. Control cells were prepared in parallel without any treatments. To investigate the PS dependence of the adhesion, RBC with enhanced PS exposure were incubated with annexin V as reported previously (6,8,11,31). In brief, RBC with enhanced PS exposure were incubated with 8.8 µg/ml annexin V at 37°C for 30 minutes on a mixer at 400 rpm prior to the adhesion assay.

**Flowcytometry**

To quantify the percentage of cells with enhanced PS exposure, RBC were incubated for 30 minutes in HBSS containing 1.2 mM Ca2+ and fluorescein isothiocyanate labeled annexin V (FITC-annexin V; Invitrogen) at room temperature, following which the cells were washed and re-suspended in the same buffer at a cell count of 10^6/ml. RBC samples were analyzed with a flowcytometer (Becton Dickinson FACScan) using logarithmic gain for the light scatter and fluorescence channels. The background and gated zone were defined from the control sample (i.e. normal RBC). Only intact cells were collected for the analysis of the fluorescence intensities with the cell fragments excluded from the acquisition region (Figure 1a, b). The shift on the FSC-H axis as well as microscopic observations (data not shown) reveals that after the ionophore treatment the cells are smaller. Figure 1c shows the fluorescence intensity distribution of the control sample, i.e. untreated RBC. Approximately 0.5% of the cells are located in the gated zone, indicating a small subpopulation of PS-exposing-RBC (32). Figure 1d shows the fluorescence intensity distribution of cells treated with the calcium-ionophore A23187. Approximately 90% of the cells are located in the gated zone, indicating an enhanced exposure of PS on the outer leaflet of the treated RBC membrane. Throughout this study, the concentration of the ionophore A23187 was adjusted in order to obtain 90 % RBC populations with PS exposure.

**Endothelial cells**

Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza, UK. The culture medium consisted of 90% of Ham’s F-12K with 2 mM L-Glutamine (Sigma), 1.5 g/L sodium bicarbonate (Sigma), 0.1 mg/ml heparin (Sigma), 0.2vol% bovine brain extract (Hammond Cell Tech), 120 U/ml penicillin/streptomycin (Sigma) and 10% fetal bovine serum (FBS). HUVEC were placed in tissue culture flasks pre-coated with gelatin and cultured at 37°C in a CO2 (5%) incubator. Once the cells reached 80% confluence, the cells were sub-cultured in 35 mm Petri-dishes (Greiner) pre-coated with collagen from fish skin (Sigma) and grown to confluence.

**Flow chamber adhesion assay**

The flow system consisted of an acrylic flow deck and a silicone rubber gasket (Glycotech) with the cutout area of the gasket forming the flow channel. Both the gasket and flow deck were placed into 35mm Petri-dishes coated with confluent layers of HUVEC. The flow chamber was then placed on an inverted
microscope. The inlet of the chamber was connected by silicone tubing to a miniature low displacement electronic valve that allowed switching between reservoirs containing either RBC suspensions or cell-free medium. The outlet of the chamber was connected to a syringe pump (Harvard Apparatus Co., Millis, MA) that drew either RBC suspension or rinsing solution (i.e., cell-free medium) through the flow chamber at a selected volumetric flow rate $Q$. The wall shear stress $\tau$ was calculated via $\tau = \frac{6\mu Q}{a^2 b}$, where $\mu$ is the dynamic viscosity of the solution, $a$ the channel height (0.254 mm), and $b$ the channel width (2.5 mm). The microscope, valve, inflow tubing, and the reservoirs for the two fluids were maintained at 37°C via a thermostated enclosure.

**Experimental protocol**

RBC were re-suspended at a final concentration of $10 \cdot 10^6$ cells/ml in either polymer-free solution (i.e. HBSS with 0.2% BSA) or in HBSS containing the desired concentration of dextran 40 kDa, dextran 70 kDa, dextran 500 kDa or dextran 2 MDa (Sigma Aldrich, Singapore). The chamber was initially filled with RBC suspension and the RBC were allowed to settle onto the EC surface without flow for two minutes. The chamber was then rinsed with stepwise-increasing flow rates corresponding to shear stresses between 0.02 Pa and 0.12 Pa. Adhesive cells were determined at the end of each rinsing flow rate at 20 random locations at the center of the flow channel and the absolute number of cells attached per square millimeter was calculated. In an individual experiment, the adherence at each shear stress was calculated as mean ± standard deviation of the measurements at the 20 random locations; the average adherence was determined as mean ± standard deviation of at least three individual experiments repeated under the same conditions. Wilcoxon-Mann-Whitney U test, a non-parametric method for two unpaired samples, was performed on the adherence values of different samples. In another set of experiments, a continuous-flow assay was employed: the EC monolayer was perfused with RBC suspensions for 10 minutes at the desired shear stress and then rinsed with cell-free medium for another 10 minutes. Cell adhesion was then analyzed by determining the number of adherent RBC from 20 random locations as described above.

**Miscellaneous techniques**

The density of the solutions was measured by a density meter (Anton Paar DMA35) and the dynamic viscosities by a viscometer (Anton Paar AMVn) at 37°C; the polymer-free solution had a viscosity of 0.73 mPa·s. The radius of gyration of each dextran molecular weight fraction was determined via dynamic light scattering (Malvern Zetasizer Nano ZS).

**RESULTS**

Initial efforts were directed towards comparing the binding efficiency of RBC with enhanced PS exposure to EC to that of normal RBC under static conditions. For this purpose RBC with and without enhanced PS exposure were suspended in polymer free media and allowed to settle for 2 minutes onto confluent layers of EC. Thereafter a stepwise increase of shear stress was applied and the adherent cells were counted. Normal cells (i.e., cells without enhanced PS exposure) did not show any significant adherence to EC (Figure 2): on average, less than one cell/mm$^2$ was able to withstand a shear stress of 0.2 Pa and no cells
remained adherent at higher shear stresses. In contrast, RBC with enhanced PS exposure demonstrated significantly higher adhesion at the lowest applied shear stress of 0.02 Pa (i.e., 128±53 cells/mm²) and more than half of these adherent cells remained at a shear stress of 0.12 Pa.

Having confirmed the adhesion promoting effect of enhanced PS exposure on the outer leaflet of RBC, studies were conducted to determine the effects of dextran on RBC-EC adhesion efficiency. Figure 3 shows the adhesion of PS-exposing RBC suspended in solutions containing 2 MDa dextran. Cells were again allowed to settle for 2 minutes followed by rinsing with stepwise increases of shear stress. Adding dextran 2 MDa at concentrations of either 5 mg/ml or 10 mg/ml significantly increased the adhesion efficiency compared to the polymer-free medium: 1) at 5 mg/ml about 470 cells/mm² remained adherent after applying a shear stress of 0.02 Pa and a shear stress of 0.12 Pa still left almost 200 cells/mm² adherent; 2) 10 mg/ml further increased this effect such that at 0.02 Pa the number of adherent cells increased to almost 600 cells/mm² and at 0.12 Pa more than three times as many cells were adherent compared to the polymer-free suspension.

Figure 4 presents a similar set of experiments illustrating the impact of dextran molecular mass and thus polymer size (Table 1) on RBC-EC adhesion efficiency. RBC with enhanced PS exposure were suspended in solutions containing dextran with a molecular mass of 40 kDa, 70 kDa or 500 kDa at a concentration of 5 mg/ml. Clearly, the addition of 40kDa dextran does not have any adhesion promoting effect: at the smallest applied shear stress about the same number of cells as in the polymer-free suspension, remained adherent and increasing the shear stress to 0.12 Pa removed most of the adherent cells. In contrast, the presence of either dextran 70 kDa or dextran 500 kDa has a similar adhesion promoting effect on the adhesion efficiencies as observed for dextran 2 MDa (Figure 3). Comparing the adhesion efficiencies at 5.0 mg/ml (Figure 3a, Figure 4a-c) further suggests a biphasic dependence of the adhesion efficiencies on the molecular mass, with dextran 500 kDa having the strongest impact on the adhesion efficiency.

The above data clearly demonstrate that dextran induces adhesion of PS-exposing RBC to EC with a marked dependence on molecular mass and polymer concentration. However, since the suspension viscosities also varied somewhat (Table 1), the number of RBC settled within the two-minute period of sedimentation also slightly varied. Thus, when considering the number of cells settled within the stipulated times the effect of large dextran molecules on the adhesion efficiency can be expected to be even more significant as demonstrated in Figure 3 and Figure 4. Figure 5 summarizes the relative adherence (i.e. percent of settled cells that remained adherent) in polymer-free solutions or solutions containing dextran after applying 0.02 Pa and 0.10 Pa. Increasing the molecular mass from 40 kDa to 2 MDa at constant polymer concentration confirms a pronounced impact of the dextran molecular mass on the adhesion efficiency. At 0.02 Pa, the relative adherence increased from 8% in 40 kDa to 37% in 2 MDa. Moreover, increasing the shear stress to 0.10 Pa clearly underlines the biphasic, bell shaped response to the molecular mass. Most remarkably, increasing the dextran 2 MDa concentration to 10 mg/ml lead to a binding rate
of 75% after applying 0.02 Pa and still leaves 41% adherent even after applying a shear stress of 0.1 Pa.

The results presented in Figures 3 to 5 clearly indicate that the adhesion of PS-exposing RBC to EC can be regulated by dextran with a molecular mass higher than 70 kDa, when the initial adhesion process occurs under static conditions. We have also evaluated the impact of these polymers when an RBC suspension is perfused through the chamber at constant shear stress. Normal RBC or RBC with enhanced PS-exposure were suspended in polymer-free solution or in solutions containing 10 mg/ml of dextran 2 MDa. The EC monolayer was then perfused with the RBC suspensions for 10 minutes at constant stresses of 0.02 Pa, 0.03 Pa, or 0.04 Pa, followed by a 10-minute rinse at the same shear stress with cell-free medium. Figure 6 compares the adhesion of normal RBC and RBC with enhanced PS exposure in polymer-free medium with the adhesion in 2 MDa dextran at a concentration of 10 mg/ml. Whereas normal RBC demonstrate no adhesion in polymer-free medium, the presence of dextran results in minor adherence of normal RBC at shear stresses of 0.02 Pa (14±4 cells/mm²) and 0.03 Pa (7±1 cells/mm²). Increasing the shear stress to 0.04 Pa completely prevents the adhesion of normal RBC. However, for RBC with enhanced PS exposure there is a marked difference. In polymer-free solution 61±15 cells/mm² (0.02 Pa) and 16±5 cells/mm² (0.03 Pa) were adherent and some cells even adhere to EC at 0.04 Pa. The presence of dextran further elevates RBC adherence with about three times more cells adherent at 0.02 Pa, five times more at 0.03 Pa and 7 times more at 0.04 Pa.

The above results clearly indicate that the adhesion of PS-exposing RBC to EC can be modified by dextran under static conditions and under constant flow conditions. Lastly, we tested the impact of blocking PS with annexin V on the adhesion of PS exposing RBC to EC in the presence and absence of dextran. Figure 7a shows the adhesion of PS-exposing RBC with and without blocking PS via annexin V in polymer free solutions. Cells were again allowed to settle for 2 minutes followed by rinsing with stepwise increases of the shear stress. Blocking phosphatidylinerine with annexin V results in a significant reduction of the adhesion efficiency: At 0.02 Pa, the reduction is 42% and at 0.12 Pa 72%, which is consistent with earlier reports (6,8,11,31). In Figure 7b similar experiment in the presence of 5 mg/ml dextran 500 kDa, reveal an even more pronounced effect of annexin V blocking on the adhesion efficiency: at 0.02 Pa, the number of adherent cells was reduced by 79% and at 0.12 Pa by 86%. These results thus confirm earlier reports regarding the significant involvement of PS in abnormal RBC-EC interactions (31) and indicate that this interaction is enhanced in the presence of dextran.

**DISCUSSION**

An enhanced PS exposure on the outer leaflet of the RBC membrane has been linked to an increased adhesiveness of pathological RBC to the endothelium (6,11,15,31). It has been demonstrated that PS is a major component of this interaction in that PS can directly interact with EC features such as CD-36 and PS receptor (PS-R) (6,10,15,31), or bind to EC via plasma ligands such as TSP and lactadherin (33-34). This involvement of PS is also demonstrated in Figure 7, which shows a significant reduction of the adhesion efficiency due to annexin V.
blocking of the exposed PS in polymer free and in polymer containing suspensions. However, it should be pointed out that past reports also indicated that annexin V blocking could not completely prevent binding of PS-exposing RBC to EC indicating the presence of multiple adhesion pathways (31). It has been suggested that due to the calcium loading (i.e. calcium-A23187 treatment) RBC dehydration and expression of adhesive segments of band 3, might also influence the adhesion but the detailed underlying mechanism has not been fully clarified (31,35).

Past studies aimed at identifying plasma proteins involved in abnormal RBC-EC adhesion were usually limited to identifying cell receptors, in that they only considered plasma proteins as ligands cross-linking adjacent cells. This study tested the impact of non-adsorbing macromolecules on the adhesion of PS-exposing RBC to EC. The results presented demonstrate that dextran can have a marked impact on the adhesion efficiency of PS-exposing RBC to EC: dextran with a molecular mass of 70 kDa or more leads to a significant increase in the number of adherent cells as well as in the adhesion strength. Considering that dextran is a neutral, uncharged polymer without the ability to develop attractive electrostatic interactions and that it has been repeatedly demonstrated that dextran is depleted from the RBC surface (21,26-27), it can be concluded that the presence of depleted macromolecules can be a significant factor for the adhesion of RBC with an enhanced PS exposure to EC.

The proposed mechanism is illustrated in Figure 8. If a surface is in contact with a solution containing macromolecules and an attractive force does not balance the loss of configurational entropy of these molecules, a depletion layer builds up near the surface. Within this layer, the polymer concentration is lower than in the bulk phase. This exclusion of macromolecules near the cell surface results in an osmotic gradient and as two cells approach, solvent is displaced from the depletion zone into the bulk phase leading to an attractive entropic force that tends to minimize the polymer-reduced space between the cells (grey arrow in Figure 8) thereby inducing specific cell-cell interactions.

To estimate the depletion attraction between two approaching surfaces one has to multiply the change in the volume of the depletion zone by the osmotic pressure drop. The main difficulty when calculating such energies between RBC and EC is that both cells have a glycocalyx, which can be penetrated by the depleted polymers thereby reducing the effective size of the depletion layer (19). Thus for two adjacent cells with soft surfaces at a separation distance $d$, a combined glycocalyx thickness $\delta$, a depletion layer thickness $\Delta$, and a combined penetration $p$, of the polymers into the adjacent glycocalyces, the reduction in the free energy per area due to depletion interaction is approximately given by:

$$\Delta F_d = -\Pi(2\Delta - d + \delta - p),$$

with $\Pi$ being the osmotic pressure of the polymer solution (20). The depletion layer thickness ($\Delta$) depends on the polymer properties as well as the polymer concentration and can be calculated via (18):

$$\Delta = -\frac{\Pi}{D} + \sqrt{\left(\frac{\Pi}{D}\right)^2 + \Delta_0^2}$$

with the parameter $D$ being a function of the bulk polymer concentration $c$:
\[ D = \frac{4k_BT}{\Delta_0^2} \left( \frac{cN_a}{M} \right)^{\frac{2}{3}} \]

where \( k_B \) and \( N_A \) are the Boltzmann constant and Avogadro's number, \( M \) is the polymer molecular mass and \( \Delta_0 \) is the depletion thickness for vanishing polymer concentration and is equal to \( 1.4\cdot R_g \). For two cells in close contact, the cell separation \( d \) can be approximated as being roughly equal to the sum of the two glycocalyces (Figure 8). Therefore, the loss in free energy due to depletion interaction only depends on the thickness of the depletion layer \( \Delta \) and the penetration \( p_t \).

Figure 9 shows the change of the free energy in various suspensions for polymer glycocalyx penetrations of up to 50 nm. Our experimental results (e.g., Figure 5) indicate that dextran 40 kDa does not induce adhesion, whereas increasing the molecular mass results in a steady increase of the adhesion efficiency until it reaches a maximum around a molecular mass of 500 kDa. Comparing these observations to the theoretical results in Figure 9 suggests that the penetration should be between 15 nm to 20 nm (i.e., \( a < b < c \geq d \) in Figure 9). This range is consistent with estimates of the glycocalyx thicknesses for RBC and EC of between 5 to 20 nm (36). Moreover, this comparison also suggests that the loss in free energy due to depletion interaction should be in the order of a few \( \mu J/m^2 \), which is in the same range as experimentally measured adhesion affinities of RBC to artificial surfaces or to RBC in polymer solutions (22,37). Even though these calculations of free energy can only be seen as an estimate, they clearly underline the merit of our approach.

Therefore, the current study suggests that depletion interaction can regulate RBC adhesion to EC by reducing the free energy between these cells, thereby increasing the binding rate or adhesion efficiency. The presented data thus also suggests that macromolecular depletion could be a potential mechanism for the effects of an increased level of certain plasma proteins (e.g. fibrinogen) on abnormal RBC-EC adhesion. It should be noted that in a recent study we were able to demonstrated that macromolecular depletion can also induce weak adhesion of normal human RBC to EC (23). This observation is also confirmed by the experimental results presented in Figure 6, which clearly shows that normal RBC, i.e. without ionophore treatment, can adhere to EC in the presence of dextran, whereas no adherence is observed in polymer free solution. This in turn reveals that macromolecular depletion interaction between RBC and EC is not limited to PS exposing RBC, but should be applicable to a wide range of receptor mediated RBC or other cell interactions.

In conclusion, this study implies that macromolecular depletion can promote specific binding of RBC to EC and might thus play a significant role for the abnormal adhesion of RBC in pathological conditions such as sickle cell disease or diabetes mellitus. However, there are still many questions, which need to be addressed for a detailed understanding of the significance of the depletion mechanism. On a microscopic scale, answers to these questions might lead to the identification of new cellular and plasma factors regulating RBC adhesion (i.e. protein depletion), and on a macroscopic scale, they could lead to a better understanding of factors regulating the functioning of the vascular system. This should not only be of potential
value for a detailed understanding of the patho-physiology of diseases associated with abnormal RBC-EC interactions, but might also be applicable to other cell-cell interactions such as leukocyte-endothelium adhesion, which has been correlated to abnormal RBC-EC adhesion (38).

Further, recent reports have indicated that depletion interaction is influenced by the concentration of large non-adsorbing macromolecules as well as by cellular properties and the concentrations of smaller polymers, which may reduce or inhibit depletion interaction (39-40). Therefore, future research will not only need to identify relevant large non-adsorbing plasma proteins but will also need to consider cellular properties as well as other plasma constituents. Such new information may provide a better understanding on how depletion forces affect the stability of in vivo blood flow and thus new strategies to regulate in vivo and in vitro cell-cell interactions.
REFERENCES


FOOTNOTES
* We would like to thank Prof. Dr. Meiselman for critically reading this manuscript and his valuable comments. This work was supported by grants from the Ministry of Education (Singapore) and from A*Star (Singapore, BMRC grant 05/1/22/19/382).

The abbreviations used are: RBC, red blood cells; EC, endothelial cells; PS, phosphatidylserine; HBSS, Hanks’ buffered saline solution; HUVEC, human umbilical vein endothelial cells; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.
FIGURE LEGENDS

Figure 1  Flow cyometric dotplot and quantification of phosphatidylserine exposure of RBC (a, c) w/o treatment and (b, d) after treatment with the calcium ionophore A23187 (see text for details).

Figure 2  Adherence of normal RBC and RBC with enhanced PS exposure (PS-RBC) to EC as a function of the applied shear stress. Normal RBC and RBC with 90% of the cells showing an enhanced PS exposure were suspened in HBSS containing 0.2% BSA and then allowed to settle for 2 minutes before rinsing with stepwise-increasing shear stress. Error bars are standard deviation (s.d.) of the mean adherence values from four individual experiments.

Figure 3  Impact of dextran 2 MDa on the adhesion of RBC with enhanced PS exposure. RBC with 90% of the cells showing an enhanced PS exposure were suspended in polymer-free solution (grey dashed line) or in solutions containing (a) 5 mg/ml or (b) 10 mg/ml dextran 2 MDa. The cells were allowed to settle for 2 minutes onto EC monolayers before rinsing with stepwise-increasing shear stress. Error bars are standard deviation (s.d.) of the mean adherence values from three individual experiments.

Figure 4  Dependence of the adhesion of PS exposing RBC to EC on the molecular mass of dextran. RBC with 90% of the cells showing an enhanced PS exposure were suspended in polymer-free solution (grey dashed line) or in solutions containing 5 mg/ml of (a) dextran 40 kDa, (b) dextran 70 kDa, and (c) dextran 500 kDa. The cells were allowed to settle for 2 minutes onto EC monolayers before rinsing with stepwise-increasing shear stress. Error bars are standard deviation (s.d.) of the mean adherence values from three individual experiments.

Figure 5  Relative adherence of PS exposing RBC to EC at 0.02 Pa and 0.1 Pa in percent of cells settled onto EC monolayers within 2 minutes. Error bars are standard deviation (s.d.) of the mean adherence values from three individual experiments.

Figure 6  Impact of the suspending medium on the adhesion of PS exposing RBC to EC under continuous flow. Normal RBC and RBC with 90% of the cells showing an enhanced PS exposure were suspended in polymer-free solution (light grey) or in solutions containing 10 mg/ml dextran 2 MDa (dark grey). EC monolayers were perfused with RBC suspension for 10 minutes, followed by another 10 minutes’ rinsing with cell-free medium. Error bars are standard deviation (s.d.) of the mean adherence values from three individual experiments. Wilcoxon-Mann-Whitney U test was performed on the adherence values of PS exposing RBC between polymer-free solution and 10 mg/ml dextran 2 MDa.

Figure 7  Impact of blocking PS with annexin V on the adhesion of PS exposing RBC to EC. RBC with 90% of the cells showing an enhanced PS exposure were incubated with
8.8 µg/ml annexin V for 30 minutes at 37°C and suspended in (a) polymer-free solution or in solutions containing (a) 5 mg/ml dextran 500 kDa. The cells were allowed to settle for 2 minutes onto EC monolayers before rinsing with stepwise-increasing shear stress. Error bars are standard deviation (s.d.) of the mean adherence values from three individual experiments.

Figure 8  Schematic picture on how macromolecular depletion interaction brings adjacent cells in close contact. Macromolecular depletion occurs in the region between solid and liquid phases if the decrease of the configurational entropy at the interface is not compensated by an attractive interaction. As the two surfaces approach, solvent is displaced from the depletion zone into the bulk phase, reducing the free energy of the system, thereby promoting close cell-cell contacts and thus facilitating receptor-mediated interactions (Δ, depletion layer thickness; d, cell-cell separation; δ, glycocalyx thickness).

Figure 9  Theoretical dependence of the depletion energy on the penetration (p_t) of the polymer into the two glycocalyces. The energies were calculated for the dextran solutions employed in this study. The gray area indicates the estimated total penetration as suggested by the experimental data (see text for details).
Table 1: Physicochemical properties of dextrans

<table>
<thead>
<tr>
<th>Molecular Mass</th>
<th>Mw/Mn</th>
<th>Radius of Gyration</th>
<th>Viscosity at 0.5g/dl (1.0g/dl), mPa·s</th>
</tr>
</thead>
<tbody>
<tr>
<td>dextran 40</td>
<td>35-45</td>
<td>1.6</td>
<td>5.1</td>
</tr>
<tr>
<td>dextran 70</td>
<td>74.0</td>
<td>1.5</td>
<td>7.8</td>
</tr>
<tr>
<td>dextran 500</td>
<td>519</td>
<td>2.2</td>
<td>17.7</td>
</tr>
<tr>
<td>dextran 2000</td>
<td>~2000</td>
<td>-</td>
<td>38.5</td>
</tr>
</tbody>
</table>

#Poly-dispersity indices (Mw/Mn) are given as supplied by the vendors.
Figure 1
Figure 2
Figure 3

(a) dextran 2 MDa 5 mg/mL

(b) dextran 2 MDa 10 mg/mL
Figure 4

- **a)** dextran 40 kDa 5 mg/mL
- **b)** dextran 70 kDa 5 mg/mL
- **c)** dextran 500 kDa 5 mg/mL
Figure 5
Figure 6
Figure 7: 

(a) in polymer-free solution
- annexin V (0 µg/ml)
- annexin V (8.8 µg/ml)

(b) in dextran 500 kDa (5 mg/ml)
- annexin V (0 µg/ml)
- annexin V (8.8 µg/ml)
Figure 8
Figure 9

\[
\Delta F_d \text{ (µJ/m}^2\text{)}
\]

- a. 40 kDa (5 mg/ml)
- b. 70 kDa (5 mg/ml)
- c. 500 MDa (5 mg/ml)
- d. 2 MDa (5 mg/ml)
- e. 2 MDa (10 mg/ml)
Specific binding of red blood cells to endothelial cells is regulated by non-adsorbing macromolecules
Yang Yang, Stephanie Koo, Cheryl Shuyi Lin and Bjoern Neu

J. Biol. Chem. published online October 15, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.116608

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2010/10/15/jbc.M110.116608.full.html#ref-list-1