Role of non-adsorbing macromolecules in cell-cell interaction and cell-substrate adhesion

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**ROLE OF NON-ADSORBING MACROMOLECULES IN
CELL-CELL INTERACTION AND CELL-SUBSTRATE
ADHESION**

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SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

2015

**ROLE OF NON-ADSORBING MACROMOLECULES IN
CELL-CELL INTERACTION AND CELL-SUBSTRATE
ADHESION**

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Summary

The realization that the depletion interaction plays an important role in cell-cell interaction and cell-substrate interaction has become manifest only within recent decades. Especially in explaining the red blood cell aggregation induced by non-adsorbing macromolecules. Besides, there are a handful of studies that demonstrated the bacterial phase transition due to the depletion effect of extracellular polymeric substances.

This study aimed to widen the applicability of the depletion hypothesis in cell-polymer system, since the depletion interaction is generic. In part of this study, the depletion interaction in bacterial phase separation as well as bacteria adhesion to surfaces was examined. The adhesion energy between the adherent bacteria and the surface in various polymer solutions were measured. The depleted effect near the bacteria surface was also evaluated by means of electrophoresis. The results indicated that the polymers deplete from the bacteria and induces the potential to aggregate and adhere. Furthermore, the increased adhesion of tumor cells to plasma protein-coated surface had been obtained as well. These results agreed with the concept of polymer depletion hypothesis. They served as evidence suggesting that depletion interaction play an important role in these cell-polymer systems.

The impact of non-adsorbing macromolecules on red blood cell in diabetes mellitus had been detected as well. The adhesion of diabetic red blood cells to endothelial cells under various flow rates had been measured. The cellular properties, such as cell deformability, aggregation behaviour and zeta potential, had been characterized. These data revealed important information of abnormal adhesion in vascular diseases.

Our findings provide good support to the feasibility of the depletion hypothesis and may help in developing the application of depletion interaction, such as new therapeutic agents to vascular disorders.

1. Introduction

It had been found that flocculation is very often observed in the colloidal dispersions in the presence of polymers.[1] This instability enhanced by polymer is also found in cells-polymer or bacteria-polymer mixtures.[2-10] This subject has gained an increasingly attention and two almost mutually exclusive theories were established: bridging or depletion model.

In the bridging model, particles or cells are proposed to aggregate due to the adsorption of polymers upon the particle surface. The large macromolecules serve as cross-linking “bridge” on neighboring particles[11]. In depletion model, on the contrary, it is proposed that the polymer segments concentration near the particles surface are lower than the bulk phase. The solvent displacement into the bulk phase leads to an osmotic gradient and results in aggregation between neighboring cells. This attractive force is considered as depletion interaction [12-13].

A few studies supporting the bridging model have been reported. They favored that either nonspecific or specific binding of polymer enhances the aggregation in colloidal system.[14-16] However, the explanation of these results of polymer bridging should be taken great care since the determination of polymer adsorbing on particles or cells is difficult to interpret due to the various technical artifacts.[17] Moreover, some converse results were reported that attaching a “pro-aggregant” neutral polymer onto red blood cell surface reduced or even prevented the aggregation.[18]

On the other hand, in recent decades, more and more studies have shown evidences in good agreement with the depletion model[10,19-20]. They proposed if a surface is in contact with a polymer solution and the equilibrium between the loss of configurational entropy of the polymer and the adsorption energy doesn't achieve, polymer concentration near the particle surface becomes lower compared to the bulk phase. Therefore, cell adhesion is promoted by the solvent exclusion between adjacent cells due to osmotic pressure, which is called the depletion interaction. The zone where polymers are displaced into the bulk phase is known as the depletion layer. The hypothesis of depletion is discussed in the following content.

As a result, more information related to these two models is required to distinguish their role in cell-cell aggregation and cell-surface adhesion.

1.1 Motivations

Although current understanding of depletion-mediated aggregation or adhesion has been demonstrated in good agreement with experimental examination, more considerations are required in detailed aspects. One of them is the applicability of the hypothesis since it is based on simple ideal assumption and only applied on limited types of cell and bacteria. Depletion interaction has been proved to play an important role in bacterial aggregation.[19-21] However, they focused on the phase transition behaviour of the bacteria while the effect of different membrane components and structure of bacteria on depletion interaction has not been well elucidated.

Furthermore, the application of depletion model in abnormal cells under clinical

states is expected in the future research. For example, although elevated red blood cell (RBC) adhesion to endothelial cell (EC) has been detected in various pathogenic states such as β -thalassemia or diabetes mellitus and the degree of RBC-EC adhesiveness has been linked to the vascular complications associated with such diseases[22-24], the detailed underlying mechanism of altered RBC interactions in disease remains obscure. Moreover, it has been suggested that the addition of non-adhesive proteins in the cell suspension “activates” the human breast carcinoma cell adhesion with low amount of adhesion protein.[25] The migration of tumor cells was also found to be related with the extracellular fibrinogen, which underlying mechanism is still unclear.[26] These findings indicate the polymer depletion interaction may underlie the mechanism to modulate migration or adhesion behaviour of tumor cells as well.

1.2 Objectives

The objective of this dissertation aimed to testify the applicability of the depletion hypothesis and reveal the underlying mechanism to regulate the aggregation or adhesion behaviour in various cell-polymer systems. Different cells or bacteria have been considered in the depletion-mediated interaction in this dissertation. The adhesion of abnormal RBC in diseases as well as their cellular properties variations has been examined under the influence of polymers. The polymer-induced interaction in bacteria aggregation and adhesion has also been detected. The results are supposed to serve as evidence in support of the depletion interaction and these

new insights are expected to lead to new diagnostic or therapeutic tool to improve the clinical states related to polymer-induced cell aggregation or adhesion..

1.3 Structure of the dissertation

This dissertation is composed of six chapters. The basic concepts of depletion interaction and the background knowledge for this study have been introduced in chapter 2. The considerations and experimental examination of this model, the potential effect on diabetes RBC adhesion, tumor cell metastasis and phase separation and biofilm formation of bacteria are reviewed.

In chapter 3, the impact of polymer depletion effect on diabetic red blood cell in static flow and continuous flow states has been determined by parallel-plate flow chamber system. The alterations of cellular properties in diabetic red blood cell have been measured as well.

In chapter 4, the depletion-induced bacterial phase separation and adhesion to solid surface have been investigated. Using electrophoresis, the depletion layer has been detected.

Chapter 5 has tested the applicability of depletion interaction in tumor cells adhesion by measuring the adherent efficiency of breast carcinoma cells and cervical cancer cells in the presence of non-adsorbing polymers.

At the last chapter 6, a summary of the dissertation has been presented and the perspective future studies have been shown.

2. Literature review

2.1 Depletion Hypothesis

The depletion theory was first introduced by Asakura and Oosawa in 1954.[27] The theory argued that if a surface of particle is in contact with a polymer solution and the loss of configurational entropy of the polymers is not balanced by adsorption energy, the polymer segment concentration is comparative lower than the bulk solution. A polymer-depleted layer develops next to the surface. When the particles approach to each other, these layers overlap. The exclusion of polymers from the overlapped zone to the bulk solution, which results from the effect of osmotic gradient, promotes the adjacent particles to aggregate. The depletion zone between these two particles is minimized and the free energy of the system is lowered owing to this attractive interaction. [2-3].

Figure 2.1 Schematic diagram of the depletion-induced attraction. Two colloidal particles (or cells) are in contact with non-adsorbing polymers (presented in red) in the solution: a. Since the loss of configurational entropy of the polymer is not balanced by adsorption energy, depletion layer occurs near the particle surface where the concentration of polymers is lower than the bulk phase; b. When the particles approach to each other, depletion zones overlap, resulting in polymers exclusion into the bulk phase (blue arrow); c. Particles aggregate due to osmotic pressure difference between the depletion layer and the bulk phase.

2.1.1 Depletion layer

The occurrence of the depletion layer near the surface was concluded from the weak reversible flocculation of colloid due to the presence of non-adsorbing polymers.[28] Allain *et al.* first optically observed the existence of a depletion layer in polymer solutions by determining the solute concentration of polystyrene closed to a non-adsorbing wall by the evanescent wave (EW) technique.[29] Numerous efforts have been made in order to quantify the depletion layer thickness or calculate the concentration profile near the surfaces.[30]

An approach has been derived to analyze the depletion layer thickness.[31] This theory was established based on the equilibrium between the elastic free energy and the osmotic pressure of the bulk polymer solution near the surface. It developed the depletion layer thickness (Δ) as a function of polymer molar mass (M), bulk solution concentration (c_b) and solvency parameter:[31]

$$\Delta = -\frac{1}{2} \frac{\Pi}{D} + \frac{1}{2} \sqrt{\left(\frac{\Pi}{D}\right)^2 + 4\Delta_0^2}. \quad (2.1)$$

where Δ_0 refers to the depletion layer thickness for vanishing polymer concentration and is equal to 1.4 times of the polymer radius of gyration[31]. D is a function of suspending phase concentration c_b [31]

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

where N_a and k_b are the Avogadro's number and Boltzmann constant, T and M are the temperature and molecular weight of the polymer, respectively. The osmotic

pressure (Π) can be calculated by applying a virial equation neglecting all coefficients higher than the second order (B_2)[31]

$$\Pi = \frac{RT}{M} c_b + B_2 c_b^2. \quad (2.3)$$

where R is the gas constant. Using equations (2.1)-(2.3), the relation associated with depletion interaction energy, polymer concentration and depletion layer thickness can be found.[31]

Based on this approach, when the concentration is relatively low, the depletion interaction energy increases with the concentration because the attractive force caused by osmotic pressure predominates the interaction. However, the depletion layer thickness decreases considerably when further increasing the concentration.[31] Therefore, the attractive interaction becomes weaker and thus the adhesion energy declines eventually.[32] **Figure 2.2** presents the schematic diagram of the polymer concentration profile near a soft surface (*e.g.*, cell glycocalyx) where polymers are depleted. The depletion layer thickness, penetration depth and surface thickness are denoted as: Δ , p , and δ , respectively.

Figure 2.2 Schematic illustration of depletion layer near a soft surface. Polymers are presented in grey. The concentrations of fixed surface polymers and free polymers are shown in solid line and dash line respectively. The depletion layer thickness, penetration depth of free polymers into the soft surface, and surface thickness are denoted as: Δ , p , and δ , respectively.

2.1.2 Depletion interaction energy

Before the examination of the energy of depletion interaction, it is necessary to distinguish the consideration between the “hard” and “soft” surface. Surfaces that are smooth and no penetration of polymers into it are considered as “hard” surfaces, while “soft” surfaces allow free polymers penetrate into the surface partly or entirely[33]

The depletion interaction energy (w_D) between two adjacent surfaces could be calculated as:[31-32]

$$w_D = -2\Pi \left(\Delta - \frac{d}{2} + \delta - p \right). \quad (2.4)$$

where the osmotic pressure is denoted as Π , the separation distance is denoted as d , p and δ are the penetration depth of polymers and the thickness of the two neighbouring surface layers, respectively. The depletion interaction energy is usually in the order of a few $\mu\text{J}/\text{m}^2$. According to the calculation, the depletion interaction doesn't change monotonically as the increase of polymer molecular mass or bulk solution concentration.

2.1.3 Evidence supporting depletion hypothesis

It has been known for a long time that red blood cells are inclined to flocculate in high concentration proteins mixtures[34]. Moreover, colloid dispersions, like aqueous latex[35] and oil droplets[36], were reported to have a tendency to aggregate (*i.e.*, enhanced creaming) in polymer solution. However, it is not until 1954 when Asakura and Oosawa had first given a theoretical model on the depletion interaction due to the exclusion of macromolecules near the interface.[27] Detecting the occurrence of a depletion layer is direct and convincing evidence for the depletion model. However, it is very difficult to optically observe these layers because of their very small scale. According to Vincent's model[31], depletion layers are in the same size scale of the depleted polymers which are only a few nanometers. Nevertheless,

researchers found some convincing evidence in support of the depletion hypothesis.

2.1.3.1 Determining depletion layers via electrophoresis

Electrophoresis has been developed to inspect the depletion layer near biological interfaces indirectly caused by depletion effects in the presence of non-adsorbing polymers. Quite a few studies had reported that unexpectedly high viscosity-corrected mobility of particles, liposomes or cells in neutral polymer solutions.[2,6,9,37]. It has been suggested that this discrepancy was because the local concentration of polymer is less compared to the bulk phase.[38-40] The electrophoretic mobility is determined by the viscosity inside the electrical double layer. Thus, once the electrical double layer is overwhelmed by the depletion layer thickness, the effective viscosity near the surface is lower compared to the suspending phase. Subsequently, the influence of suspending phase viscosity on mobility is reduced. Donath *et al.* had also developed a model to measure the depletion layer thickness by changing the ionic strength of the solutions.[41]

2.1.3.2 Quantitative comparison of the attractive strength

Among the studies of depletion interaction, the macromolecular effect on RBC aggregation is one of most high-profile issues. Quite a few reports have described the effect of polymers on RBC aggregation using various methods like atomic force microscopy, micropipette techniques and ultrasound backscattering methods.[7,11,42-45] Neu and Meiselman have developed a theoretical model to

calculate the depletion effect of polymers on RBC aggregation regarding the polymer molecular weight and bulk concentration of polymers, which allowed comparisons between those previous experimental results and the suggested model.[32] They quantitatively compared the theoretical interaction energy calculated via their model with the cell-cell surface affinities for normal RBC determined by employing micropipette techniques[7], and found the calculated results were in great consistency with the literature data. Moreover, Steffen and coworkers measured the adhesion force between human RBC at the single cell level by using atomic force microscopy-based spectroscopy (**figure 2.3**).[45] They compared the measured data with the theoretical results calculated by Neu's model and also found great agreement between them. This agreement between the experimental results and theoretically predicted data in terms of depletion interaction serve as strong evidence that the attractive interaction between RBC is governed by depletion effect.

Figure 2.3 Comparison between measured adhesion force and calculated values. (a) Adhesion force of human RBC in different dextran solutions, which was measured by atomic force microscopy-based spectroscopy. (b) Comparison between the measured interaction energy and calculated results. The solid curve shows the predicted results calculated from the depletion model[32]. From [45] with permission.

2.1.3.3 Phase separation studies

As mentioned earlier, direct observation or measurement of depletion layer or interaction is limited. Thus, some studies put their focus on the dispersion stability affected by polymers to reveal the depletion effect. Phase transition is expected in mixture of colloid and non-adsorbing polymers, since the colloid or particles in the mixture are tend to form a reversible flocculation due to the depletion interaction.

Sieglauff first described that addition of polystyrene (macromolecules) to microgel (colloid) lead to a phase separation.[46] In that study, Sieglauff also theoretically demonstrated this phase separation was resulted from polymer depletion attraction and extended the application range of the depletion concept established by Asakura and Oosawa[27]. Later, many subsequent works were aimed to give experimental evidence to that demonstration. For example, it had been reported that adding polyethylene oxide into a suspension of stabilized particles lead to a reversible flocculation when the concentration of particle is above a threshold in the mixture. [47-49] This founding was in great support of the depletion hypothesis since the flocculation formed by bridging crosslink is expected to be irreversible.[50] Moreover, De Hek *et al.* [51-52] have compared the theoretical predicted results with the experimental data by mixing silica dispersions with polystyrene in cyclohexane and demonstrated the “phase separation threshold concentration” could be explained by depletion-induced attraction in the colloid system due to the presence of polymers.

Like colloid particles, bacteria also display attractive tendency in macromolecule

solutions.[20,53-55] Schwarz-Linek *et al.*[21] found that in the suspension of sodium polystyrene sulfonate (NaPSS), de-flagellated *Escherichia coli* (AB1157) were inclined to aggregate and phase separate out of the suspension. They also carried out Monte Carlo simulations based on Asakura and Oosawa model[27] in the parameters matching their experiment and found satisfactory agreement with their experimental results. Moreover, Dorken *et al.* [19] also reported that *S. meliloti* aggregate in the cultures when the production of exopolysaccharide by the bacteria was over a “limiting polymer concentration”. They suggested it was the depletion effect rather than the bridging interaction that induced the aggregation since the “limiting polymer concentration” decrease as increase the cell concentration in the culture.

2.2 The role of macromolecules in cell-cell and cell-surface interaction

Although current understanding of depletion-mediated aggregation or adhesion is demonstrated in good agreement with experimental examination, more considerations are required in detailed aspects. One of them is the application of depletion model to abnormal cells in clinical states. The manner, kinetics and mechanism of depletion effect of normal RBC have been illustrated in a good few studies. However, information associated with the depletion interaction, which is responsible for the abnormal aggregation or adhesion in pathological conditions, is deficient. Furthermore, the applicability of depletion hypothesis needs to be testified in other cell-polymer mixture, since depletion interaction is suggested to be generic.[56]

2.2.1 Increased red blood cell adhesion to endothelial cells in diabetes mellitus

Normally, the adhesion between red blood cell and endothelium is negligible. The RBCs disperse in the healthy blood stream without forming the aggregation or adhesion. However, the cellular and rheological properties of RBCs are changed due to the biochemical and functional alteration of blood in various pathological conditions. The abnormal adhesion between RBCs and endothelial cells has been observed in some vascular disorders, including diabetes mellitus, β -thalassemia and occlusion.[22-24,57-60] In diabetes, cellular properties of RBCs are changed due to the hyperglycemia (increased blood glucose level). Some of these alterations are given below.

2.2.1.1 Structural alterations of RBC

Membrane lipids: The membrane of RBC is composed of a lipid bilayer and a cytoskeletal network. The lipid bilayer separates the cytoplasm from extracellular environment. The lipid bilayer consists of various types of phospholipids, such as phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine (PE), which distribute asymmetrically in the bilayer. The bilayer is flexible and robust because of the existence of cholesterol, which is dispersed equally all over the lipid bilayer.[61-62] In diabetes, the amount of cholesterol and phospholipids in the membrane increase while the latter one increases up to 4 times more than cholesterol. This reduced cholesterol to phospholipids ratio leads to an enhanced rigidity and

impaired deformability of RBC in diabetes.[63]

Membrane proteins: The cytoskeletal meshwork under the lipid bilayer, which is flexible and compressible, is attributed to the strength for RBC to survive under various external forces.[64-65] Spectrin and actin are the main constituents in the network. Together with some other proteins like protein 4.1 and ankyrin, they determine the viscoelastic properties of the RBC membrane.[66] In diabetes, some of these proteins in the cytoskeletal network have been found heavily glycosylated, namely the beta-spectrin, ankyrin and protein 4.1.[67]

Enzymes and ionic balance: Elevated concentrations of intracellular sodium and serum potassium have been observed in diabetic RBC due to the notable decline of Na^+/K^+ -ATPase activities.[68] It has been argued that the decreased Na^+/K^+ -ATPase level may play an critical role in the disturbance of intracellular ionic balance and subsequently accelerating the cellular ageing.[69]

2.2.1.2 Red blood cell deformability

The high deformability and robust membrane of RBCs allow them to squeeze in and go through the circulatory system where there are narrow capillaries and high shear flows. The extent of deformation under applied shear stress is considered as a parameter to estimate the deformability of RBC.

Some prominent methods have been used to measure the RBC deformability. The laser-assisted optical rotational cell analyzer (LORCA) detects the optical diffraction pattern of RBC to measure the cell elongation.[70-71] RBCs deform under different

shear stresses between two cylinders. The change of shape, from circular to elliptic, is presented by the laser beam diffraction pattern. The deformability of RBC is assessed by calculating the elongation index (EI). An increased EI at any given shear stress indicates greater deformability.[72-73] Passage time of RBC in different filter membranes has been applied to evaluate the RBC deformability as well.[74-75] Under applied pressure, RBC suspension flows through the microporous membrane while the passage time is recorded. The deformability is inversely proportional to the filtration time. It is a low-cost and rapid technique which has a good correlation in optimized conditions like hematocrit less than 10% and pressure below 10 Pa.[76]

The measurement by abovementioned methods show similar findings that RBC deformability reduces in diabetic state and it is associated with the severity of the disease.[67,77-79] The specific structural alteration of RBC is responsible for the impaired deformability. The oxidative pressure resulted from elevated extracellular glucose level causes the impairment of proteins in RBC membrane. The damage is significant even though exposing in a short time.[80] The heavy glycosylation of membrane proteins in diabetic RBC enhances the index of rigidity of membrane.[81] The changed phospholipid to cholesterol ratio also leads to the deformability reduction. [63] These variations in diabetic RBC are responsible for the change of viscoelastic properties of membrane and determine the deformability impairment in diabetic condition.

2.2.1.3 Electrokinetic properties of red blood cell

The glycosylated proteins on the membrane determine the negative surface charge of RBC. The heavily charged carboxyl groups of sialic acid play the central role in the surface charge distribution.[82-83] The negative charge on the membrane contributes to the haemorheological and haemodynamic properties of RBC. Zeta potential is an important parameter to estimate the electrical charge on cell surface. The variation in zeta potential reveals the electrokinetic properties of the RBC membrane in different states.

Laser Doppler electrophoresis is a conventional tool to measure the zeta potential. The charged cells suspend in medium migrate in an applied electric field. The Doppler shift caused by the scattering light is responsible for the electrophoretic mobility of the cells. Therefore, the zeta potential is calculated based on the Henry Equation[84]:

$$U_E = \frac{2\varepsilon\zeta f(ka)}{3\eta}. \quad (2.5)$$

where U_E , the electrophoretic mobility, ε , dielectric constant, ζ , zeta potential, η , the viscosity of bulk solution and $f(ka)$, the Henry Function which related to the ratio of particle radius to double layer thickness and generally has the value of either 1.5 (polar media) or 1.0 (non-polar media).

The decreased zeta potential has been observed in diabetic RBC and particularly in those with cardiovascular diseases states, which is attributed to the reduction of glycophorin sialic acid content.[84-85] The decreased sialylated glycoproteins

(*i.e.*, decreased zeta potential) is thus expected to associate with the abnormal cell-cell aggregation as well as the adhesion of RBC to endothelium.

2.2.1.4 Variation of plasma components

The extracellular plasmas also change in diabetes. Elevated levels of fibrinogen amount and plasma concentration have been observed in insulin-resistant type-2 diabetes.[86-87] Fibrinogen is an acute-phased protein which has been suggested to be regulated by the increased concentration of glucagon.[88] It enhanced the aggregation in various aspects, including aggregate size and blood viscosity.[89-90] It has been measured that the adhesion of normal RBC in normal plasma (fibrinogen deficient) with added fibrinogen is as great as in the acute phase plasma of diabetes.[91-92] These findings clearly proposed that fibrinogen have an important role in governing diabetic RBC adhesion.

2.2.2 Depletion-induced attraction in bacteria

Bacteria display properties in aggregation and adhesion which are much alike to those of non-biological colloidal particles. Especially when they are mixed with polymeric substances like exopolysaccharides and proteins, the mixture forms a dispersion of “like-charged” colloids (bacteria) and polymers (polymeric substances). Quite a few strains of bacteria produce polymers with large molecular mass into their extracellular microenvironment.[93] These macromolecules are known as extracellular polymeric substance (EPS) including proteins, polysaccharides and

nuclei acids, which differ in molecular weight and structure.[94] These substances have important role in various aspects, such as inducing bacteria aggregation and governing initial adhesion on surfaces as well as developing the formation of biofilm.[93,95]

2.2.2.1 Bacteria aggregation and adhesion

The bacteria not only grow in separated cells, but also cluster together under certain physiological conditions. They also colonize various surfaces and develop into the formation of biofilm. This aggregation as well as adhesion is regulated by the chemical and structural properties of bacteria surface, such as van der Waals forces and electrostatic forces. Especially the electrostatic interaction is considered to determine the stability of bacteria dispersion.[96] The secreted EPS is another main factor to govern the bacterial aggregation. For example, enhanced biological flocculation has been observed in the growth suspension of *S.meliloti* strains, which secreted succinoglycan into the medium. However, a succinoglycan-deficient mutant species remained suspended without obvious flocculation during the incubation. Furthermore, in the addition of succinoglycan, which was purified from the wild type growth medium, the suspension of mutant species aggregated and phase separated from the bulk medium.[97-99] Similar results have been found in other bacteria species as well, such as *Agrobacterium* species, *Rhizobium leguminosarum* and so on.[54,100-101]

2.2.2.2 Underlying principles of polymer-induced aggregation and adhesion

In the view of colloid science, the bacteria-polymer suspension can be considered as a dispersion of like-charged colloids and polymers. Therefore, several mechanisms may underlie the polymer-induced aggregation and adhesion. First of them is the polymer bridging. When polymers adsorb to the bacteria surface due to the heterogeneous charged. The polymers act as a cross-linking to bridge the adjacent cells.[102] This mechanism depends on the Coulomb forces without the screen of the ions in the aqueous phase. Therefore, it is only applicable in low salt concentrations. In addition, salt bridge also occurs between negative bacteria and polymers in the presence of polyvalent cations, which leading to aggregation.[103]

On the other hand, depletion interaction dominates the aggregation in the absence of polyvalent cations and in high salt concentration solutions. The detail principles of depletion attraction have been described in above section (section 1.1). The non-adsorbing polymers depleted from the bacteria surface in order to balance the entropy loss in the system (*i.e.*, dispersion). The displacement of polymers from the depleted zone to the bulk solution results in an unbalanced osmotic pressure, which promote the aggregation.

Although depletion interaction has been well studied in neutral colloid-polymer system[27], it has still not been well exploited in the studies on polymer-induced bacteria aggregation and adhesion, especially in determine the effect on biofilm formation. There are only a handful of literatures that suggest depletion effect plays

an important role in bacteria aggregation.[19-21,104-105]

2.2.3 Impact of polymer on tumor cells metastasis

2.2.3.1 Tumor cell metastasis

The development of cancer cells from benign and steady to malignant and metastatic is the most terrifying condition in cancer, which leads to a poor therapeutic outcome or even death. Cancer metastasis is a cascade that consists of successive and interconnecting processes (**figure 2.4**).[106-107] Briefly, tumor cells grow in primary neoplasm. They are affected by various cell-biological changes, shed from original sites and entry into the nearby vascular or lymphatic system. Most circulating tumor cells can't survive long before they reach a proper adhere site. Once they are survived and arrest an appropriate organ by attaching to the vessel wall, extravasation happens. The tumors cells invade into the adherent tissue and finally develop a secondary neoplasm. One critical step in the procession of metastasis is the adhesion to the new site of a distant tissue.

Figure 2.4 Schematic diagram of cancer metastasis. (1) Tumor cells grow progressively and become invasive. (2) Tumor cells detach from primary tumor and invade into the vessels, entry into the circulation system. (3) The circulating tumor cells adhere to the vessel wall. (4) Extravasation occurs. Tumor cells invade into the tissue and develop a secondary tumor.

2.2.3.2 The role of fibrinogen in tumor cell metastasis

A specific association between human cancer and coagulation factors was demonstrated over a century ago.[108] It has been demonstrated that the fibrinogen or fibrin deposition in hemostasis contributes to the potential of tumor cell metastasis.[109] Quite a few hemostatic disorders were observed in cancer states, such as disseminated intravascular coagulation and hemorrhagic events.[110-111] Active procoagulant events were observed in various types of tumor cells, which

significantly activate the hemostatic interactions.[111-112]

The derivatives of fibrinogen have been found in the tumor-host cell interface of many tumors in humans and experimental animals.[113-114] Fibrin is rich in the stroma of different kind of tumors. However, in breast cancers, fibrinogen, instead of fibrin, are found in the stroma. The fibrin or fibrinogen deposition act as a frame that allow the binding of various kinds of growth factors and enzymes, so that it regulates the adhesion, development and metastasis in tumor cell progression. Soluble fibrinogen also induces the potential of metastasis by serving as a cross-linking site since it is able to interact with various integrin (*e.g.*, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$)[115-116] as well as matrix components. It was suggested that fibrin(ogen) promotes the cancer metastasis by sustaining the adhesion and survival of the single tumor cell emboli, while it shows no significant effect on the proliferation of established metastases.[109] The underlying principles have not been well elucidated.

3. The role of non-adsorbing macromolecules in diabetic RBC adhesion to endothelial cells

3.1 Introduction

It has been suggested in recent research that macromolecular depletion interaction could serve as generic mechanism behind the induced adhesion caused by some plasma proteins, which originates from the depletion of non-adsorbing macromolecules from cell-cell interfaces. The central hypothesis of this chapter is that depletion interaction induced by non-adsorbing macromolecules is a determinant of the known increases of RBC adhesiveness in pathological conditions (*e.g.*, diabetes mellitus)

Diabetes mellitus is characterized by several physiological anomalies leading to wide range of problems including severe vascular and circulatory complications such as macrovascular diseases (*e.g.*, stroke, transient ischaemic attacks, myocardial infarction, limb ischaemia and gangrene) and microvascular diseases (*e.g.*, retinal artery occlusion, nephropathy, neuropathy). Increased adhesion of erythrocyte on endothelium has been reported in diabetes mellitus. The degree of RBC-EC adhesiveness has found to have a strong association with the severity of the vascular problems related with these diseases[117]. Even though it has been known for some time that structural changes of RBC as well as plasma proteins are involved in abnormal RBC-EC adhesion,[118-119] the detailed underlying

mechanism of altered RBC interactions in disease remains obscure.

This chapter aims to investigate if polymer depletion interaction can promote the adhesion of diabetic red blood cells to endothelial cells and thus whether polymer depletion might be an alternative mechanism behind the increased adhesiveness of pathological RBC in the presence of plasma proteins. For this purpose, normal and diabetic RBC will be suspended in solutions containing dextran to imitate the effect of non-adsorbing polymers. Adhesion efficiency will then be quantified with a flow chamber system.[120] In addition, RBC adhesion will be measured in non-adsorbing polymer and in polymer free solution to compare the effects of plasma proteins on the adhesiveness of pathological RBC to that of non-adsorbing macromolecules.

Furthermore, the cellular properties alterations of diabetic RBC have been determined. The cellular properties of RBC, such as RBC deformability, aggregation potential and surface charge, affect the rheological properties and thus the flow behaviour. It is known that these changes are response to the abnormalities in diseases associated with vascular complications.[59]

These studies should therefore help to identify possible structural differences of the glycocalyx or surface properties of pathological RBC that could be linked to abnormal RBC adhesiveness in the presence of non-adsorbing macromolecules.[121]

3.2 Materials and methods

3.2.1 Diabetic red blood cell preparation

Venous blood samples were obtained from the volunteers patients who were recruited from those seen and treated at the Diabetes and Endocrine Centre of Tan Tock Seng Hospital. Past research has correlated glycemic control assessed by glycosylated hemoglobin (HbA_{1C}) to the extent of RBC-EC adhesion.[57-58] 20 type- II patients with poor metabolic control and 10 with an ideal glycemic control were recruited. The health status of samples were judged by HbA_{1C} level over the past 6 month, with HbA_{1C} >10% considered poor glycemic control and HbA_{1C} <7% considered good glycemic control.

The whole blood samples were drawn into ethylenediaminetetraacetic acid (EDTA 1.5mg/ml) from the volunteers. RBCs were separated from whole blood by centrifuging at 1000 g for 5 min, washed three times with phosphate-buffered saline solution (1 ×PBS, pH 7.2) and then re-suspended at about 0.1% haematocrit (HCT) in either polymer free DMEM or DMEM containing various polymers. Medium contained 0.2% bovine serum albumin (BSA) which maintains the shape of RBCs.

3.2.2 Human umbilical vein endothelial cells preparation

The commercial Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (UK). Growth medium consisted of 90% DMEM, 10% fetal bovine serum (FBS, Life Technologies) and 1% Penicillin/Streptomycin solution

(Penicilin 10,000 units per ml and Streptomycin 10,000 µg/ml, Life Technologies).

Endothelial cells (EC) were grown in tissue culture flasks in an incubator with 5% CO₂ at 37°C. After they reached 70 to 80% confluence, they were sub-cultured onto 35 mm petri dish (Greiner) pre-coated with gelatin (Sigma). They were then incubated until 80% confluence for the adhesion flow plate.

3.2.3 Dextran solutions

A variety of macromolecules are present in the blood stream, such as albumin, immunoglobulins and clotting protein such as fibrinogen. Among the blood plasma proteins, fibrinogen has been identified to be associated with the aggregation of blood cells in 1960's.[124] Since mid-1940s, studies began to use the poly-glucose dextran as a blood or plasma substitute.[129] Dextran molecules have been widely used to investigate cell aggregation and adhesion and proved to be an ideal model polymer. Dextran is a complex branched glucan composed of chains of varying lengths. Like fibrinogen, they are long-chained structure. But unlike the common plasma proteins, dextran is a neutral poly-glucose. It is the electric neutrality and the wide range of molecular weight, excellent water solubility, low toxicity[42-43], which make it ideal to study the effect of macromolecule to the cells. It is also proved to deplete from the RBC surface[12,122]. It is frequently used for *in vitro* hemorheological study to mimic the extracellular macromolecule.[123] Dextran with molecular weight of 10, 40, 70 and 500 kDa (SigmaAldrich, Singapore) were dissolved into desired saline

and medium as the description in the text. The molecular sizes of dextran were detected by dynamic light scattering. They were dissolved in 0.22 μm -filtered DI water at a concentration of 1 g/l and measured by the Nano Zetasizer (Malvern Instruments, UK).

3.2.4 Flow chamber adhesion assay

The flow chamber system consists of an acrylic flow deck and a silicone rubber gasket (Glyco Tech Inc., USA). A slit was cut out in the gasket. In the experiment, the gasket sticks tightly on the flow deck and both of them are put into a 35 mm Petri dish where endothelial cells grow. Therefore, the slit becomes a flow channel which allows observation of adhesion between the endothelial cells and RBC. The cell adhesion inside the flow chamber is monitored by an inverted microscope. A programmable syringe pump (LEGATO 270, KD Scientific, Inc., USA) is connected to the outlet of the flow chamber, which provides desired flow rate to the flow system. The inlet of the chamber is connected to different solution pools so that the RBC suspension or rinse solution is allowed to flow through the channel at various flow rates controlled by the syringe pump.[124] **Figure 3.1** shows Schematic diagram of the parallel plate flow chamber. During the experiment, the solutions are drawn by the syringe pump at an applied volumetric flow rate Q . The shear stress inside the channel τ can be determined by:

$$\tau = \frac{6\eta Q}{a^2 b}. \quad (3.1)$$

where η is the dynamic viscosity of the solution, a is the channel height (0.254

mm), and b is the channel width (2.5 mm). The temperature of the whole setup is kept at 37°C.

Figure 3.1 Schematic diagram of the parallel plate flow chamber. (a): The flow chamber system consists of an acrylic flow deck and a silicone rubber gasket. The flow deck adhered by the gasket is place into the petri dish which coated with confluent endothelial cells. (b): Cross section of the mounted flow chamber along the flow channel. The cutout area in the gasket (green) forms a flow channel, allowing the perfusion of solutions on EC layer (blue).

RBCs were suspended in polymer free medium (*i.e.* DMEM) and different polymer medium with a final concentration of $5 \times 10^6 \text{ ml}^{-1}$. Dextran 70 kDa and 500 kDa were dissolved in DMEM in the concentration of 0.5 g/dl as the polymer medium. Two types of assay were carried out:

- (1) *Static flow assay*: RBCs were suspended in polymer free medium at final concentration. The suspension was perfused by the pump at a shear stress of 0.03 Pa for 2 minutes and settled on the EC surface without flow for 5 minutes. Then the chamber was rinsed with cell free medium at shear stress of 0.03 Pa for 3 minutes in order to remove the non-adherent cells. Images of adherent cells were captured at 20 random locations. This step of rinse and image capture was repeated at different shear stress, namely 0.06, 0.09, 0.12 and 0.15 Pa. The absolute number of cells attached per square millimeter was measured. The same procedure was performed with RBC suspended in medium containing dextran 70 kDa or 500 kDa respectively.
- (2) *Continuous flow assay*: RBCs were suspended in polymer free medium at final concentration. RBCs suspension was perfused by the syringe pump at a shear stress of 0.03 Pa for 10 minutes. Then the chamber was rinsed by cell free medium at a shear stress of 0.03 Pa for 10 minutes. Images of attached cells were captured at 20 random locations. The same procedure was performed with different shear stress 0.06 and 0.09 Pa using different EC dishes. This same experiment was carried out with RBC suspended in

dextran 500 kDa.

3.2.5 RBC deformability measurements

RBC deformability affects the rheological properties and thus the flow behaviour of blood. Even more important the study under consideration is that more deformable RBC can obtain sufficient surface contact to the endothelium more easily than rigid RBC and are thus more likely to adhere to EC and it is known that the RBC deformability is abnormal in diseases associated with vascular complications.

RBC deformability was detected by the laser-assisted optical rotational cell analyzer (LORCA, Lorrca Maxsis, Mechatronics Instruments B.V., Netherland), which the principle and methodology have been illustrated in some literatures. [70-71] Briefly, the RBC deformability (as well as the aggregation) measurement system consists of two concentric cylinders. A thin layer of red blood cell suspension is sheared between these two cylinders while the outer cylinder can rotate with desired speed. Deformation of RBC, or elongation, is caused by the rotation. The extent of deformation under different shear rate is presented by the laser beam diffraction pattern. The signal of the diffraction pattern is recorded by a camera and analyzed via the computer. **Figure 3.2** shows the schematic diagram of the measurement system.

Figure 3.2 Schematic diagram of the laser-assisted optical rotational cell analyzer (LORCA). Red blood cell suspension is sheared between two cylinders while the outer cup rotates. The laser beam diffraction pattern of RBC is detected by the sensor (blue) and analyzed by the computer.

All measurements were performed on RBC suspended in isotonic, viscous polymer solution (polyvinylpyrrolidone, PVP, Mechatronics Instruments B.V., Netherland). An aliquot of 25 μl EDTA-anticoagulated blood was mixed with 5 ml PVP homogeneously, which had a final viscosity of 31 Pa and osmolality of 300 mosmol/kgH₂O. After the mixed solution was inserted into the cup of ektacytometer, elongation indices at 37°C were measured at the shear stress level between 0.3 to 30 Pascal (Pa) for each sample. The detail mechanism has been described in other studies.[125-126] The deformability of RBC was assessed by calculation the elongation index (EI) which is calculated as: $EI = (L-W)/(L+W)$, where L and W represent the length and width of the diffraction pattern, respectively. An increased EI

at any given shear stress indicates greater deformability.

3.2.6 RBC aggregation measurements

Optical rheoscopy by light transmission and backscattering is commonly used to monitor the aggregation process. The application of the LORCA for measurement of RBC aggregation has been described in detail in some previous studies.[125,127] During the measurement, the aggregate behaviour of red blood cell is detected under different shear stress by recording the corresponding laser beam diffraction pattern. The detection and result analysis are controlled by the computer. The syllectogram obtained by plotting laser backscatter intensity (I_{sc}) versus time (**figure 3.3**) is used to study the aggregation. Shortly, in the beginning of measurement, RBCs are sheared under a high shear rate until they are completely disaggregated, which is shown by a stable level of scatter intensity. Then the rotator stop abruptly and RBCs which were elongated recover back to its normal shape randomly. An upstroke of scatter intensity is observed because of the reformation of cells. However, the scatter intensity starts to decrease when RBC begin to aggregate. It decreases as more and more RBCs aggregate until the formation of RBC rouleaux 3D-network.

Parameters obtained from the subsequent the syllectogram are used to interpret the aggregation of RBC (**figure 3.3**). The scatter intensity range between the peak ($I_{sc\ top}$) and the lowest value (I_{sc0}) is defined as the amplitude (AMP) to show the extent of aggregation. Aggregation kinetics is presented by the time that for the intensity to

decrease from the peak to the half of the amplitude. The aggregation index (AI) is used to combine the extent and kinetics of aggregation.

Figure 3.3 Sylllectogram of erythrocytes aggregation. It is plotted on a logarithmic time scale. Three main parameters: AMP, $t_{1/2}$ and AI, are illustrated in the text.

1 ml of whole blood sample was incubated with 10 ml of air (1:10) in a tube on a roller-bank for 15 minutes. The pretreated sample was inserted into the cup and the scatter intensity was measured.

3.2.7 Zeta potential measurement

Zeta potential is an important parameter to estimate the electrical charge on cell surface. The variation in zeta potential reveals the electrokinetic properties of the RBC membrane in different states. The decreased zeta potential has been observed

in diabetic RBC and particularly in those with cardiovascular diseases states, which is attributed to the reduction of glycophorin sialic acid content.[84-85] The decreased sialylated glycoproteins (*i.e.*, decreased zeta potential) is thus expected to associate with the abnormal cell-cell aggregation as well as the adhesion of RBC to endothelium.

Electrophoresis has been introduced to study the depletion layer indirectly caused by depletion effects in the presence of non-adsorbing polymers.[128] The electrical double layer of the charged particles and the hydrodynamic flow near the interface between the bulk and particle determine the electric properties (*e.g.*, electrophoretic mobility) of charged particles or cells. When the solution concentration changes, the thickness of depletion layer and thereby the EPM changes accordingly. This method has been applied to characterize the depletion layers in some prior reports.[37,41,129] A Zetasizer-Nano (Malvern Instruments, UK) was applied to determine the bacterial EPM.

Zeta potential (ζ) is determined as the electric potential at boundary between the electrical double layer and the bulk liquid in colloidal system. Electrical double layer appear on each particle when it is contact to the liquid phase. It consists of two layers: an inner region (Stern layer), where ions firmly adsorb on the particle surface due to chemical force, and an outer (diffuse) region where ions are attracted by the inner region but less associated with the particle. In the diffuse region, ions move together with the particle as a single entity within a notional boundary, while those won't move

beyond this boundary. This boundary is referred as the slipping plane and the electric potential at this plane is defined as zeta potential (**Figure 3.4**).

Figure 3.4 Schematic diagram of electrical double layer. The electrical double layer composes of two regions. (1) Stern layer (purple): ions absorb firmly on the particle surface. (2) Diffuse layer (green): ions are less firmly associated with the stern layer due to Coulomb force. Ions within the slipping plane move together with the particle as a unity. The electrical potential at the boundary of the slipping plane is the zeta potential.

When a charged particle, like RBC in our study, is under a constant electric field, it moves with constant velocity towards the positive electrode once the electric field attractive force is equal to the viscous force. The zeta potential is correlated to the

potential stability of the colloid dispersion. It is considered that particles with low zeta potential are easy to collide and aggregate. If particles have a large zeta potential of the same sign, they are supposed to repel each other and prevent from aggregation or gathering.

The relationship between electrophoretic mobility and zeta potential is described by Henry equation:

$$U_E = \frac{2\varepsilon\zeta f(ka)}{3\eta}. \quad (3.2)$$

where U_E , the electrophoretic mobility, ε , dielectric constant, ζ , zeta potential, η , the viscosity of bulk solution and $f(ka)$, the Henry Function which related to the ratio of particle radius to double layer thickness and generally has the value of either 1.5 (polar media) or 1.0 (non-polar media). The depletion layer in different molecular weight dextran solution was investigated by electrophoresis mobility experiment.

For measuring zeta potential of diabetic RBC, detail description was reported by other investigators[128]. In brief, 3 μ l RBC sample pellet was suspended either in isotonic PBS (5 mM) or solutions of dextran in PBS. The isotonic PBS solution is composed of 0.03 M sucrose and 0.5 g/ml bovine serum albumin. The solution was added into the cuvette for measurement. 3 measurements were made for each sample.

3.3 Results

3.3.1 Adhesion of normal and diabetic RBC to endothelial cells under static conditions at various applied shear stress

Initial effort was to compare the adhesiveness of diabetic RBC on endothelial cells (ECs) with that of normal RBC under static conditions. For this purpose, normal and diabetic RBC suspended in polymer free medium (DMEM), respectively, were perfused through the EC flow chamber. RBCs were settled for 2 minutes on the confluent monolayer of endothelial cells. Thereafter shear stress increased stepwise in order to see the adhesive strength of RBC on EC layer. Adherent cells were recorded under different shear stress (**figure 3.5**). No significant adhesion on EC layer was observed in normal RBC. Under the lowest shear stress, less than one cell per millimeter square was adhered on EC layer and nearly no cell was able to withstand the flow under higher shear stress. On the contrary, high adhesion was observed in diabetic RBC under the lowest shear stress 0.03 Pa, which 92 ± 21 cell/mm² were adhered on EC layer on average. Although less cells remained adherent with increasing applied shear stress, more than half of the cells (*i.e.* 57 ± 16 cell/mm²) were still adhered under the shear stress of 0.15 Pa.

Figure 3.5 Adhesion of normal and diabetic RBC to endothelial cells under static conditions at different applied shear stress. Data shown as mean $\pm$ SD. The numbers of normal and diabetic RBCs are 10 and 15 respectively.

3.3.2 Impact of dextran on the adhesion efficiency of diabetic RBC to endothelial cells

Having demonstrated the increased RBC adhesion on endothelial cells in diabetes mellitus, the impact of dextran on the adhesion efficiency was studied. Diabetic RBCs were mixed with 0.5 g/dl dextran 500 kDa solution. Cell-polymer solution was perfused through the EC-coated flow channel and the cells were allowed to settle for 2 minutes. Adherent cells were counted under stepwise increased shear stress. **Figure 3.6** shows the adherent cells on EC in the suspension with and without the addition of

dextran 500 kDa. Adhesion efficiency was greatly induced in the solution containing dextran 500 kDa. In dextran suspension, the number of adherent cells was 181 cells/mm² at the shear stress of 0.03 Pa, while it was 92 cells/mm² in polymer free medium. Under the shear stress of 0.15 Pa, 58 cells/mm² adherent cells were left after the flow in polymer free medium. However, 115 cells/mm² were remained adherent on EC in dextran suspension, almost 2 times of the number in polymer free medium.

Figure 3.6 Impact of 500 kDa dextran on the adhesion of diabetic RBC to endothelial cells. Data shown as mean $\pm$ SD. The numbers of normal and diabetic RBCs are 10 and 15 respectively.

3.3.3 Effect of dextran with different molecular weight on adhesion efficiency of diabetic RBC to endothelial cells

The effect of dextran with different molecular weight on diabetic adhesion was examined by similar set of experiments mentioned in previous section. The results were shown in **Figure 3.7**. Diabetic cells were mixed in either dextran free medium or solutions with different molecular weight of dextran. The dextran solutions contained 0.5 g/dl dextran 70 or 500 kDa respectively. Similar adhesion promotion was observed in dextran 70 kDa, as the addition of dextran 70 kDa induced the adhesion of diabetic RBC on EC as well. However, dextran 500 kDa had the strongest effect on the adhesion enhancement. The results suggested the association between dextran induced adhesion of diabetic RBC with the molecular weight of polymer.

Figure 3.7 The effect of dextran with different molecular weight on adhesion efficiency of diabetic RBC to endothelial cells. Data shown as mean $\pm$ SD. The numbers of normal and diabetic RBCs are 10 and 15 respectively.

3.3.4 Impact of dextran on adhesion efficiency of diabetic RBC to endothelial cells under continuous flow

The adhesion efficiency of RBC on endothelial cell layer in the presence of dextran was also studied by perfusing RBC through the EC flow chamber at constant applied shear stress. Normal RBCs or diabetic RBCs were suspended either in dextran free medium or in the concentration of 0.5 g/dl dextran 500 kDa solutions. The suspension was perfused for 10 minutes under constant shear stress of 0.03, 0.06 and 0.09 Pa respectively. Then the chamber was rinsed by cell free medium at the same shear stress for 10 minutes as well. **Figure 3.8** compares the adhesion of normal RBC and diabetic RBC in polymer free medium with the adhesion in dextran solution at the concentration of 0.5 g/ml. Nearly no adhesion of normal RBC was observed in polymer free medium at the lowest shear stress of 0.03 Pa. For diabetic RBC, however, significant adhesion was found. The number of adherent cells was 28 ± 9 cells/mm² at the constant shear stress of 0.03 Pa and it dropped to 7 ± 3 cells/mm² as the shear stress rose up to 0.09 Pa. The adhesion was much stronger in the addition of dextran 500 kDa into diabetic RBC suspension. There were 41 ± 9 cells/mm² remained adherent at the shear stress of 0.03 Pa. Even at the shear stress of 0.09 Pa, 28 ± 6 cells/mm² were still adherent on the EC layer, which is more than 4 times of the adherent number in polymer free medium at 0.09 Pa.

Figure 3.8 Impact of 500 kDa dextran on the adhesion of Diabetic RBC to endothelial cells under continuous flow. Data shown as mean $\pm$ SD. The numbers of normal and diabetic RBCs are 10 and 15 respectively.

3.3.5 Determination of deformability of diabetic RBC

The deformability of diabetic RBC has been detected. The shape of erythrocyte changes under an applied stress. The elongation of the shape has been referred as the deformability of RBC. It serves as an important property to estimate the ability of the erythrocyte to maintain its shape in microcirculation. Elongation index (EI) has been applied to describe the extent of RBC deformability, which is defined as: $EI = (L-W)/(L+W)$, where L and W represent the length and width of the diffraction pattern, respectively. An increased EI at any given shear stress indicates greater deformability. Normal and diabetic RBCs were suspended in an isotonic polyvinylpyrrolidone (PVP, 300 kDa) solution. Elongation indices were measured by applying a stepwise increase

shear stress at 37°C. **Figure 3.9** shows the elongation index versus shear stress curve from diabetic and normal erythrocytes. Overall, the EI of normal and diabetic cells increases as the shear stress become higher and it reaches a limit which is considered as the maximal elongation index. However, generally, the EI of diabetic erythrocytes were significantly lower than the normal ones especially under high shear stress ($p<0.05$). The difference between them rose with the increase of applied shear stress. EI were at the same level under low shear stress (*i.e.*, 0.3 to 1.69 Pa). When the cells were under high shear stress, higher EI were observed in normal RBC. It was 0.48 ± 0.03 for normal RBC while it was 0.30 ± 0.05 for diabetic RBC at shear stress of 30 Pa.

Figure 3.9 Elongation indices (EI) of RBC in diabetes and normal condition. 6 independent experiments were repeated. The data were shown by mean $\pm$ standard deviation (the numbers of normal and diabetic RBCs are 10 and 15 respectively). The results were analyzed by nonparametric Wilcoxon-Mann-Whitney U test: $*p<0.05$.

3.3.6 Determination of aggregation of diabetic RBC

The LORCA laser diffraction system was also applied to estimate the aggregation of diabetic RBC. RBCs were inserted into the rotational cup and the backscatter intensity was measured. The laser backscatter intensity had been determined as a function of time. In the beginning of measurement, RBCs are sheared under a high shear rate until they are completely disaggregated. Then the rotator stop abruptly and RBCs which were elongated recover back to its normal shape randomly. The measurement process was completely detected and controlled by computer. This backscatter intensity versus time graph refers as a syllectogram. The interpretation of the syllectogram reveals much useful information related to the aggregation behaviour of RBC, including: (1) the scatter intensity range between the peak ($I_{sc\ top}$) and the lowest value ($I_{sc\ 0}$) is defined as the amplitude (AMP) to show the extent of aggregation; (2) aggregation kinetics is represented by the time that elapsed until the peak intensity is decreased to the half AMP; (3) the aggregation index (AI) is used to combine the extent and kinetics of aggregation.

The aggregation behaviour of normal (control) and diabetic RBC was shown in **figure 3.10**. The measured parameters were summarized in **Table 3.1**. According to the results, the extent of aggregation (AMP) was significantly lower for diabetic RBC than for normal ones ($P < 0.01$). However, it took much less time for diabetic RBC to aggregate, since $t_{1/2}$ of them was about 1.05 seconds while it was 3.27 seconds for normal RBC. Overall, the aggregation index of diabetic RBC is much higher than it of

normal ones, which suggested diabetic RBCs are more inclined to aggregate. The results were analyzed by nonparametric Wilcoxon-Mann-Whitney U test.

Figure 3.10 Aggregation of normal and diabetic RBCs. The time shows in a log scale.

Table 3.1 Alteration in RBC aggregation of diabetic patients. Data are mean $\pm$ standard error. Difference between two groups was tested by Wilcoxon-Mann-Whitney U test: $**p < 0.01$.

	DBT	Control
	(n=15)	(n=10)
AMP (au)	15.1 $\pm$ 2.6	24.2 $\pm$ 0.6**
t_{1/2} (s)	1.05 $\pm$ 0.21	3.27 $\pm$ 0.44**
AI (%)	78.2 $\pm$ 0.6	53.2 $\pm$ 5.1**

3.3.7 Determination of zeta potential of diabetic RBC

It is confirmed that the deformability and aggregation behaviour of diabetic RBC differ greatly from normal ones. The electrostatic alteration of diabetic erythrocytes is going to determine in this section, which affect the stabilization of RBC. Normal and diabetic RBCs were suspended either in isotonic PBS or PBS containing various concentrations of dextrans. The isotonic PBS solution is composed of 0.03 M sucrose and 0.5 g/ml bovine serum albumin. Solutions were added into the cuvette for measurement. 3 measurements were made for each sample.

Figure 3.11 shows the alteration of zeta potential of diabetic and normal RBC. It can be seen that the zeta potential of diabetic RBC is generally lower than the normal ones. In PBS, the zeta potential of normal RBC was -17.37 ± 1.01 mV (mean $\pm$ standard deviation, $n=6$) in average, while it was -13.48 ± 3.28 mV in diabetic RBC and significantly lower than in normal ones ($p < 0.05$). In the presence of non-adsorbing dextran, zeta potential increased for both RBCs. For diabetic RBC, zeta potential of diabetic RBC was 16.58 ± 1.81 mV in dextran 70 kDa solution, while it rose to -19.42 ± 1.30 mV in dextran 500 kDa solution. The discrepancy of zeta potential also increased in larger molecular weight dextran solution. For example, zeta potential of normal RBCs was -31.77 ± 1.20 mV, more than 1.6 times as high as it of diabetic RBCs. These results indicated a decrease of surface charge of diabetic RBC, which increase the potential of erythrocytes aggregation.

Figure 3.11 Difference of zeta potential between normal and diabetic RBC. The means of zeta potential $\pm$ standard deviation were presented. 6 independent experiments were repeated for one sample. The difference to the control (PBS) was analyzed by Wilcoxon-Mann-Whitney U test: $*p < 0.05$, $**p < 0.01$.

3.4 Discussion

Elevated erythrocyte adhesion to endothelial cells not only influence the blood flow properties, but also response to the cellular function abnormalities, which has been detected in a board range of vascular and circulatory disorders including diabetes mellitus.[59] In this chapter, the impact of dextran on the adhesion between diabetic RBC and endothelial cells has been examined. The results clearly demonstrated that diabetic RBCs were greatly promoted to adhere on endothelial cells in the presence of large dextran polymer. This elevated adhesion was observed in static condition as well as in continuous flow. The polymer used here, dextran, has been used in quite a few studies and demonstrated that it is depleted from erythrocyte surface.[12-13,122] The electrostatic properties remain unchanged in the addition of dextran to the solution as well, since dextran is an uncharged poly-glucose. Thus, it can be concluded that the depletion interaction due to the non-adsorbing dextran are likely the driven force of enhanced RBC adherence to endothelial cells.

In diabetes mellitus, erythrocytes expose to a high glucose extracellular environment. The membrane proteins and lipid are damaged by the subsequent increased oxidative stress[130] The increased glycosylation-derived internal viscosity may also result in the impairment of RBC deformability.[81] The surface sialic acids are the main contributor to the RBC surface charge. RBC aggregation is also sensitive to the alteration in surface acid level.[59] A decrease of sialic acid content had been reported in diabetic RBC.[131] Changes of these structural and chemical properties

have a great impact on the mechanical characteristics and function of the erythrocytes.

Some of the alterations are the deformability reduction, aggregation enhancement and the reduced zeta potential of RBC in diabetic state.

It is not until these recent decades that the cell specific factors are considered to play an important role in governing erythrocyte aggregation. There is less attention focused on the depletion model to explain the intrinsic mechanism of RBC aggregation in diabetes mellitus. The information investigated in this chapter should help give deeper insights to the depletion-induced aggregation mechanism.

In conclusion, these findings provide useful information to develop important new knowledge regarding the mechanisms that control RBC adhesive interaction in diabetes. In addition, interpretation on the role of polymer-induced depletion interaction in abnormal cell adhesion also leads to a better detailed understanding of the patho-physiology of diseases associated with vascular complications and should thus pave the way for the rationale development of new therapeutic agents to alleviate vascular complications and to new or improved diagnostic tools and hence improve patient care.

4. The role of macromolecular depletion in *E.coli* phase separation and adhesion

4.1 Introduction

Polymeric substances around bacteria cells play an important role in bacterial adhesion to inanimate surfaces or during cell-cell adhesion,[132-136] which have been recognized to be the initial interaction during biofilm formation. These extracellular polymeric substances have been demonstrated to provide a cohesive and adhesive strength that induces the aggregation of microorganisms as well as the adhesion to various surfaces.[137-138] However, their underlying mechanisms to regulate the aggregation or adhesion process remain obscure. Thus, understanding of the attractive or repulsive force resulted from the polymeric substances is crucial to obtain detailed insight on how bacteria interact with each other or with abiotic surface.

The principles to explain the mechanism of depletion effect of colloidal suspension have been demonstrated in some literatures. Burns *et al.*[139] and Anderson *et al.*[140] have suggested a mechanism of depletion aggregation of particle flocs, who found that the structure and growth kinetics of the colloids were dependent on the molecular weight as well as the concentration of polymer in the suspension. Other prior studies have reported that several bacterial strains tend to aggregate more easily in the solution containing non-adsorbing polymers because of the depletion interaction,[19-20,105] resulting in fast phase separation of the

bacteria-polymer suspension. These previous studies focused on the inter-particle or intercellular interaction under the polymeric depletion effect. On the other hand, the adhesion between bacteria and abiotic surface in the presence of non-adsorbing polymer has not been fully understood and remains to be elucidated. The studies mentioned above can be applied to understanding the attractive force between motile *E. coli* as well as adhesive strength to surfaces.

In this chapter, the phase separation behaviour of *E. coli* was examined by suspending bacteria in various dextran solutions. The bacterial aggregation was assessed by monitoring sedimentation. *E. coli* were mixed with either polymer-free PBS or PBS containing different dextran. The bacteria aggregation in different concentrations of dextran was measured as well. Polyglucose dextran is used as a model of EPS, which is a neutral, water-soluble and non-adsorbing polymer of anhydroglucose. We found that the phase separation of *E. coli* is greatly induced by the addition of dextran. This enhancement showed a dependence on not only the molecular weight of dextran but also the concentration of polymer solution.

Depletion interaction has been found to operate between a bacterium/cell and a surface,[141-142] which may serve as a potential mechanism to the formation of biofilm. We aim to examine the role of polymers in the bacterial adhesion on albumin-coated surface and to understand the relation between polymer molecular weight and depletion attraction in bacteria suspension. Unlike the experiment of phase separation, the concentration of bacteria is much lower in order to observe the

sedimentation of single cell. It also allowed us to calculate the adhesion energy by measuring the contact area of bacteria on the surface.

Optical microscopy is a conventional method to study the cell adhesion by examining the adhesive behaviour of the single cell to a treated coverslip. In this study, the adhesion of *E. coli* on coverslip coated with bovine serum albumin (BSA) in the presence of non-adsorbing molecules has been measured. We study the deformation of the adherent bacteria to characterize the adhesion strength using interference reflection microscopy (IRM), which is a capable method allowing visual characterization of particle-interface interaction in real time.[143] In addition, we show the effect of polymer concentration and molecular weight on the bacteria aggregation and adhesion providing evidence to the existence of depletion zone near the bacteria. This study can provide further insight on the depletion theory in bacteria-surface interaction, and inspire new approaches to control bacterial biofilm formation by adjusting extracellular macromolecular environment.

There is no doubt that the structural and chemical properties of bacteria surface play a dominant role in cell-cell interaction including aggregation and adhesion.[137,141,144-145] Particle electrophoresis is a conventional tool to examine the surface electrostatic properties of bacteria. Under an electric field, the electrical double layer of the charged particles and the hydrodynamic flow near the interface between the bulk and particle determine the electric properties (*e.g.* electrophoretic mobility) of charged particles or cells. Therefore, the change of

viscosity within the electrical double layer affects the electrophoretic mobility of particles greatly. The electrophoretic mobility (EPM) is usually obtained by Helmholtz-Smoluchowski equation. However, more and more studies show the limitation of its application range. For example, EPM of liposomes and red blood cells was much higher in the presence of polymers than the theoretical mobility predicted by the classical Smoluchowski equation.[41,146] This discrepancy was found to be related to the existence of polymer-depleted zone, where local concentration of polymer is less compared to the bulk phase.[38-39] It is suggested that when the polymer-depleted zone (*i.e.* depletion layer) becomes thicker than the electrical double layer, the viscosity within is lower than in bulk phase due to the displacement of polymer segments. Hence, the mobility of the particles reduces. Therefore, detecting the change of electrophoretic mobility provides a method to investigate the existence of depletion layer as well as to estimate its thickness.

In this chapter, electrophoresis has been introduced to study the depletion layer indirectly caused by depletion effects in the presence of non-adsorbing polymers.[128] Although electrophoresis of liposomes and red blood cells have been used to evaluate their depletion effects, the electrophoretic mobility of bacteria has not been well characterized to understand their interactions in the presence of non-adsorbing polymers. It is more difficult to interpret the surface electrokinetic properties of bacteria because of the complex bacteria surface. We studied how polymer affect the EPM by using the method which was developed by Donath *et al.*[147]. They

considered the bacteria as a particle covered by a fixed charged layer which is penetrable for small ions but impenetrable for the polymer. *E. coli* were suspended in various dextran solutions and their electrophoretic mobility was measured. Based on the experimental results, the effective viscosity near the bacteria during electrophoresis was determined.

4.2 Materials and methods

4.2.1 Bacterial strain preparation

The non-pathogenic *E. coli* strain DH10B was grown in Luria Broth medium (tryptone 10.0 L^{-1} , yeast extract 5.0 L^{-1} and NaCl 5.0 L^{-1} , adjusted to pH 7.2) using an orbital shaker at 37°C and 200 rpm. Cells were harvested at mid-to-late exponential phase by centrifugation at 2700 g for 10 minutes (Eppendorf 5810R, Germany), and then washed three times with phosphate-buffered saline solution ($1 \times \text{PBS}$, pH 7.2) to remove residual medium for further experiments.

4.2.2 Bacterial sedimentation and aggregation

The bacterial aggregation was assessed by monitoring sedimentation. *E. coli* were resuspended at 1% in either dextran-free PBS or dextran-PBS solutions. Dextran with different molecular weights of 10, 70 and 500 kDa were dissolved in PBS at specific final concentrations, 0.5, 1, 2, 4 and 8 g/dL, respectively. The cells in different solutions were incubated in cuvettes with Parafilm covered for 24 h at 37°C without shaking. Images of sedimentation were taken by a digital camera. After the polymer

solution was added to the cuvette and incubated for 90 min, the optical density of the upper solution phase in the cuvette was measured at wavelength 600 nm (OD_{600}) by a spectrophotometer (Shimadzu UV-2450, Japan). Percentage of aggregation was calculated as[20]

$$\text{percentage aggregation} = 1 - \left(\frac{OD_{90m}}{OD_{0h}} \right). \quad (4.1)$$

where OD_{0h} is the optical density at 600 nm immediately after adding polymer and OD_{90m} is the optical density after 90 minutes.

4.2.3 Coverslip preparation

Albumin-coated coverslips were used as the adhesion substratum. Round glass coverslips (25 mm in diameter) were soaked in concentrated nitric acid (65%, Sigma Aldrich) overnight. They were washed by double-distilled water thoroughly to remove the residue acid, and dried at 50°C for 24 h. Coverslips were then sonicated in anhydrous acetone for 5 min, washed with anhydrous acetone twice, soaked in a 4% solution of 3-aminopropyl-triethoxy-silane (APTES) in anhydrous acetone for 10 min, and finally dried at room temperature (24°C). The coating of APTES allows various materials, such as proteins or polymers, to be immobilized on the coverslip.[148] These aminated coverslips were kept in a paraflim-sealed petri dish in store. Before the adhesion experiment, the prepared APTES-coated coverslips were soaked in 2% (w/v) bovine serum albumin (BSA) solution, incubated at room temperature for 2 hours and rinsed with PBS to clear away excess BSA. The BSA coating is able to effectively reduce non-specific binding between cells and the glass substrate.[149]

4.2.4 Interference reflection microscopy

Interference reflection microscopy (IRM) is an optical microscopy technique to investigate cell or bacterial adhesion, which allows real-time observation of the adhesion between *E. coli* and the substrate. Combined with a two-boundary model, IRM has been applied to calculate the cell-surface adhesion energy.[150] An inverted microscope (IX71, Olympus, Melville, NY, USA) was modified for the IRM experiment. The pictures were recorded via a Complementary metal–oxide–semiconductor (CMOS) camera (Infinity 1, Lumenera Corporation, Canada). IRM is popular due to several reasons: (1) It doesn't require the cell to be labeled. (2) The setup is easy and cheap, which can be achieved on the conventional inverted fluorescence microscope with additional components. (3) It could provide us with some qualitative and quantitative information about cell adhesion as well as the undulations of the cell.

In IRM, cells attach on the coverslip (*e.g.*, glass). The refractive indices vary in the cell membrane, cytosol, medium and the coverslip. Under epi-illumination, the light reflected at the interface where the refractive indices are different, forming the image base on the refractive intensity. The light intensity becomes higher as the greater the difference between refractive indices is, meaning the darker pixel in the image. The Fresnel reflection coefficient (r) at the boundary between media with refractive indices n_1 and n_2 is:[151]

$$r_{12} = \frac{n_1 - n_2}{n_1 + n_2}. \quad (4.2)$$

The reflectivity R , or the ratio between the intensity of the reflected light I_r and the intensity of the incident light I_o , can be calculated as:[151]

$$R = \frac{I_r}{I_o} = \left[\frac{n_1 - n_2}{n_1 + n_2} \right]^2 = r_{12}. \quad (4.3)$$

When cells are grown or attached on a coverslip and observed by epi-illumination through an oil-immersion objective, the first reflection will occur at the transition from coverslip to culture medium (A: R_{g-m} in **Figure 4.1**). According to the equation 4.3 and the n values, the reflection is relatively strong. In other words, the background will be relatively clear. The presence of a cell on the coverslip can then modify this background intensity.

Figure 4.1 Schematic diagram illustrating the image formation by Interference Reflection Microscopy (IRM). The light was reflected by various interfaces with difference refractive index: A. by the coverslip surface; B by the membrane which is not close (adhere) to the coverslip; C by the membrane which is close to the coverslip. When the membrane is in close contact to the coverslip, the interference is strong resulting in high intensity. On the contrast, if the membrane is not close to the coverslip, the interference is weaker and the subsequent intensity is lower.

At sites where the cell membrane is in immediate contact to the glass surface, the reflectivity will be determined by the coverslip-cell transition (C: R_{g-c}) The higher refractive index of the membrane ($n \geq 1.4$)[152-153] results in a low reflectivity. In other words, a site of direct apposition of the cell membrane to the coverslip results in darker pixel than the cell-free background.

Alternatively, when the cell is apart from the coverslip by culture medium, reflection will occur at the interface between coverslip-medium (A: R_{g-m}) and medium-membrane (B: R_{m-c}) interfaces. If the thickness of the medium film is of the order of magnitude of the wavelength used, both reflected beams can interfere. The optical path difference, Δ , between the two reflections is derived by the cosine law[154]: $\Delta = 2n_m d \cos \theta$, where n_m is the refractive index of the medium, d is the distance between the interfaces, and θ is the angle of refraction in the medium. A dark fringe will be seen when $\Delta = N\lambda$, a bright one when $\Delta = (N + \frac{1}{2})\lambda$. The integral number N is the order of interference. With monochromatic incident light, these interferences result in bright and dark zones compared to the background. In other words, the proximity between the cell and the substrate is able to obtain by measuring by the intensity of monochromatic light reflected at the interface between cell and substrate[151]

The IRM experiment was carried out on an Olympus IX71 inverted microscope equipped with a Zeiss Neofluoar 63x/1.25 oil immersion objective, which an additional magnification device is installed. The 546.1 nm monochromatic light was as

the light source, which was filtered out by a band-pass filter ($\Delta\lambda = 5 \text{ nm}$, 85%, Omega, USA) from a 100 W high pressure mercury arc lamp. The contrast in IRM is enhanced by suppressing stray light with the antiflex technique.[142] The incoming light is plane-polarized by polarizer p before being reflected into the objective, and the analyzer a behind the objective is crossed to cut off all reflections occurring within the microscope body and the objective. The light beam that forms the image then passes through a quartz plate, which is a rotatable plane-parallel quartz plate ($\lambda/4$) mounted on the antiflex objective, on its way to and back from the object. The reflected light becomes circularly polarized and passes through the analyzer[153]. The interference pictures of adherent cells were captured by the CMOS camera controlled by the software Infinity Analyze and Capture (Version 6.5.2 A, Lumenera Corporation, Canada). A customized version of the image analysis software package “NIH image J” (National Institute of Health, Bethesda, MD) was used to analyze the images.

4.2.5 Bacterial adhesion observation

In bacterial adhesion experiment, the coverslips were set in a rounded metallic chamber and fitted on the platform of the inverted microscope equipped with a camera. During the experiment, the chamber was filled with 1% *E. coli* suspension in dextran-free PBS and dextran-PBS solutions respectively, as prepared in the previous section. An inverted microscope (IX71, Olympus, Melville, NY, USA) was used for IRM experiment. Images were recorded after 15 minutes when cells were settled by

the camera. The experiment was carried out at room temperature.

After the bacteria were settled, the adhesion between *E. coli* and coated surface was examined with two microscopic methods. The projected area of the adhered bacteria was recorded under phase contrast microscopy, while the contact area was measured under interference reflection microscopy (IRM) (**Figure 4.2**). All images were taken with a 63 $\times$ oil immersion objective lens. The acquired images were both analyzed by ImageJ software. All the IRM images were taken at 8 bit and saved as TIFF format. The IRM images were processed and quantitatively analyzed as described previously.[155]

Figure 4.2 Schematic diagram of a cell adhered on a non-deformable planar surface , where a is the radius of contact zone, R is the radius of the original sphere and θ is the contact angle of the capsule.

4.2.6 Calculation of adhesion energy

A truncated sphere model has been developed to calculate adhesion energy based on the cell-substrate contact area and the cell volume.[156] The adhesion between *E. coli* and coated surface was examined under quantitative IRM and phase contrast microscopy. The area measured from the phase contrast microscope was defined as projected area (P , **figure 4.2**), while the one measured from IRM was defined as contact area (I , **figure 4.2**).

The applicability to this theory depends on the following assumptions:[157] (1) Only film stretching apply on the cell with thin and soft membrane, forces from shearing or bending are not allowed. (2) The capsule volume remains unchanged under any deformation. (3) No deformation occurs on the surface during the adhesion. (4) The cell membrane doesn't influenced by plastic or viscoelastic behaviour. (5) The contact area of cell membrane is stress-free. (6) Considerations like steric interaction of fluid membranes[158] or pressurized blisters due to ununiformed charge distribution[159].

The truncated spheroidal model was able to correlate the adhesion energy with the ratio between the radii of contact area to capsule radius.[156-157,160-161] Briefly, when a spheroidal capsule (*e.g.*, RBC) adheres on a non-deformable planar surface, the equilibrium geometry of the capsule is modeled as a truncated spheroidal with radius R , and the contact zone radius a . All the length dimensions of the cell are determined in units of the radius of the cell (R_0) which is in full-blown condition

without any stress. Therefore, the superficial area (A_0) and the volume (V_0) of the cell are referred as: $A_0 = 4\pi R^2$ and $V_0 = 4\pi R^3/3$, respectively. The radius of the contact area is a , and the contact angle is θ (**figure 4.2**), which is usually larger than 30° . The degree of deformation α is defined as $\alpha = a/R = \sin \theta$ (Fig. 3.3). The capsule membrane is assumed under uniform equi-biaxial pressure with membrane stress of $\sigma = C\varepsilon$, where ε is the elastic strain and C is the extensional rigidity of the membrane defined as $Eh/(1 - \nu)$ with elastic modulus E , Poisson's ratio ν and the membrane thickness h . In the absence of external influence, when the cell adheres on the surface, the surface tension stretch the cell to compliant with the attached substrate to enlarge the contact area until the equilibrium between the thermodynamic force G (in $\text{J}\cdot\text{m}^{-2}$) and interfacial adhesion energy W (in $\text{J}\cdot\text{m}^{-2}$) is achieved. The thermodynamic force G is calculated by an energy balance:[157]

$$G = (1 - \cos \theta)C\varepsilon + C\varepsilon^2. \quad (4.4)$$

The new geometry of the adherent cell is formed and the volume (V) and superficial area (A , without the contact circle) could be calculated as:[157]

$$V = \frac{2\pi R^3}{3} \left[1 + \left(1 + \frac{\alpha^2}{2} \right) (1 - \alpha^2)^{\frac{1}{2}} \right]. \quad (4.5)$$

$$A = 2\pi R^2 \left[1 + (1 - \alpha^2)^{\frac{1}{2}} \right]. \quad (4.6)$$

respectively. As $V=V_0=4\pi R^3/3$, where V_0 is the initial volume of a capsule, it can be shown that:

$$R = \frac{1}{16} \left[a^4 + \frac{a^8}{f(a)} + f(a) \right]. \quad (4.7)$$

where

$$f(a) = \left(2048 + 128a^6 + a^{12} + 16\sqrt{16384 + 2048a^6 + 80a^{12} + a^{12}} \right)^{1/3}. \quad (4.8)$$

Since only stretching energy is considered and bending is ignored in the model, the average biaxial strain is:

$$\varepsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{\frac{1}{2}}}{4/R^2 - \alpha^2} - 1 \right]. \quad (4.9)$$

Considering that the projected area and contact area are proportional to R^2 and a^2 respectively ($P \propto R^2$, $I \propto a^2$), a/R can be determined by measuring the contact zone (IRM) and projected area (phase-contrast microscopy). As the proportionality constant C is determined by three constants (elastic modulus E , Poisson's ratio ν and the membrane thickness h), an substitute parameter to evaluate the adhesion energy can be defined as W/C . By solving equations (4.4) to (4.9), the relation between the degree of deformation a/R and the effective adhesion parameter W/C can be calculated.

4.2.7 Electrophoretic mobility

The electrophoretic mobility (EPM) is usually obtained by Helmholtz-Smoluchowski equation. However, more and more studies show the limitation of its application range. For example, EPM of liposomes and red blood cells was found to be significantly higher in the presence of polymers than the theoretical mobility predicted by the classical Smoluchowski equation.[41,146] This discrepancy

was found to be related to the existence of polymer-depleted zone, where the local concentration of polymer is less compared to the bulk phase.[38-39] Although electrophoresis of liposomes and red blood cells have been used to evaluate their depletion effects, the electrophoretic mobility of bacteria has not been well characterized to understand their interactions in the presence of non-adsorbing polymers. It is more difficult to interpret the surface electrokinetic properties of bacteria because of the complex bacteria surface. We studied how polymer affect the EPM by using the method which was developed by Donath *et al.*[147] which considered the bacteria as a particle covered by a fixed charged layer which is penetrable for small ions but impenetrable for the polymer.

By comparing the corresponding mobility in various polymer solutions, the depletion effect can be evaluated. The mobility (b) is corrected by the viscosity in bulk phase (η) and thus the ratio is calculated as:[122]

$$k = \frac{\eta_p \cdot b_p}{\eta_0 \cdot b_0}. \quad (4.10)$$

where p is denoted as polymer solutions while 0 is denoted as polymer-free PBS. b_p is the measured mobility and η_p is the measured bulk viscosity. Since the zeta potential is determined by the vesicle surface charge and the ionic strength of solution and dextran is a kind of neural macromolecular, the addition of dextran makes no effect to the zeta potential. Therefore, according to equation (4.10), for a given bacteria without depletion interaction, the electrophoretic mobility is expected to unchanged, meaning the ratio k is supposed to be constant and equal to 1. However, in

the presence of depletion, polymers are depleted from the membrane surface, which lead to the decrease of viscosity near the cell surface. The lower viscosity within the depletion layer affects cell mobility.[37] Thus, mobility is higher than expected level.

Electrophoretic mobility was detected by a zeta potential analyzer (Zetasizer Nano Z, Malvern Instruments, UK). Harvested bacteria were added into disposable folded capillary cells and resuspended in either PBS or PBS containing different dextrans (pH 7.2, 5 mM) at the concentration of 1%. The measurements were carried out at 25 °C and repeated three times.

4.2 Results

4.2.1 Growth curve of *E. coli* strain DH10B

The nonpathogenic *E. coli* strain DH10B was used to determine bacterial aggregation and adhesion because of its high growth rate and fully sequenced genome.[162] *E.coli* is intensively used in studying bacterial aggregation and adhesion, such as the formation of biofilm and sedimentation, taking advantage of its useful properties like high transformation efficiency and the ability to take up and stably maintain large plasmids. **Figure 4.3** shows the increase in optical density over time for *E. coli* DH10B, which follows a typical growth curve with three distinct growth phases: a lag phase 0-2 h, an exponential phase 2-8 h, and a stationary phase after 10 h.

Figure 4.3 Increase in optical density over time for *E. coli* DH10B.

4.2.2 Phase separation of *E. coli* in dextran solutions with different molecular weight

E. coli was suspended in dextran-free PBS and dextran solutions with various molecular weights, namely 10, 70 and 500 kDa. Cuvettes containing bacteria and dextran or dextran-free solutions were monitored by time-lapsed imaging. **Figure 4.4** shows the *E. coli* DH10B suspended in dextran solutions with different molecular weights but under the same concentration of 2 g/dL. In the presence of dextran 10 kDa, there was no obvious aggregation even after 90 minutes incubation, while the supernatant solution remained turbid with certain bacterial aggregates appeared at the bottom after 24 h. In contrast, the bacterial suspension became unstable shortly in

dextran 70 kDa and 500 kDa solutions and the most bacteria were found to settle down at the cuvette bottom much faster compared to those in PBS or dextran 10 kDa. Within 24 h, the supernatant became very clear in the dextran solution with higher molecular weight.

Figure 4.4 Changes in the *E. coli* DH10B suspensions in PBS-dextran solutions with different molecular weight under the same concentration of 2 g/dL. The dextran molecular weight changes from 10 kDa, 70 kDa to 500 kDa (left to right) with PBS solution used as control. Time of change from 0 to 24 h (top to bottom).

4.2.3 Phase separation of *E.coli* in different concentrations of dextran solutions

The effect of different concentrations of dextran on the phase separation of bacterial suspension was also investigated. *E. coli* were dispersed in dextran-free PBS and dextran solutions with different concentrations ranging from 0.5 g/dL to 8 g/dL.

Figure 4.5 showed the phase separation of *E. coli* DH10B in PBS and dextran 70 kDa solutions with different concentrations. Initially, all bacterial suspensions appeared turbid. Then sedimentation and phase separation were observed gradually depending on the time and dextran concentration. In dextran-free PBS, the bacteria suspension remained turbid with some slight sedimentation at 24 h. In contrast, the presence of dextran 70 kDa resulted in clearer supernatant and notable bacterial sedimentation at the bottom of the cuvettes. Under extremely low and high concentrations of 0.5 g/dL and 8 g/dL, the solutions remained quite turbid even after 90 min, while under mediocre concentration levels of 1, 2 and 4 g/dL, the solutions became much clearer as settling time increased and aggregated bacteria were found at the bottom of cuvettes after 90 min. It was notable that even though the highest dextran 70 kDa concentration (8 g/dL) did not induce obvious bacterial sedimentation in the first a few hours, the eventual long-term sedimentation effect was still significant at 24 h.

Figure 4.5 Changes in the *E. coli* DH10B suspensions in PBS and PBS-dextran (Mw = 70 kDa) solutions. The concentration of dextran 70 kDa polymer increasing from 0 (PBS), 0.5, 1, 2, 4 to 8 g/dL (left to right). Time of change from 0 to 24 h (top to bottom).

4.2.4 Effect on bacterial phase separation in the presence of dextran

Figure 4.6 shows the quantitative percentage aggregation of bacteria in different solutions after incubation of 90 min. The percentage of aggregation in PBS was very low compared to those in dextran solutions. It indicated that the presence of dextran considerably promoted bacteria aggregation. Under lower dextran concentration levels, the aggregation rate increased with the dextran concentration. However, after

the concentration reaches a critical value, the aggregation rate started to reduce when further increasing the dextran concentration. The result also showed that the aggregation rate in the large-molecule Dextran 70 kDa and 500 kDa dextran solutions was much higher than in small-molecule dextran 10 kDa in lower concentration range (0.5 to 2 g/dL, Wilcoxon-Mann-Whitney U test, $p < 0.05$). However aggregation rate dropped rapidly when increasing dextran 500 kDa concentration. The results suggested that bacterial aggregation depends on both dextran molecular weight and concentration, which was consistent with previous studies on the bacterial aggregation in other types of polymer solution.[20-21] Similar phenomena were also reported in the studies using microparticles and mammalian cells.[142,163]

Figure 4.6 Percentage aggregation of *E. coli* in PBS and different dextran solutions after incubation of 90 min. Data shown as mean $\pm$ SD (n=6).

4.2.5 Bacterial adhesion in polymer solutions

The adhesion strength between *E. coli* and a hard surface was investigated by IRM microscopy. *E. coli* was suspended in dextran-free PBS and different dextran solutions with concentrations ranging from 0.5 g/dL to 8 g/dL. After allowing *E. coli* to settle onto the APTES-BSA coated surface for 15 min, images were recorded under both IRM mode and phase contrast mode (**Figure 4.7**), where the plane of focus was adjusted at the top surface of the substrate. In dextran-free solution, the blurred image indicated that the bacteria were suspended in PBS near the substrate (**Figure 4.7a**), while no direct contact or adhesion was observed in IRM mode (**Figure 4.7b**). Meanwhile, in 70 kDa dextran (2g/dL) solution, the rim of bacteria in phase contrast mode appeared much clearer (**Figure 4.7c**), and the corresponding IRM image exhibited dark areas indicating close contact between the bacteria and the substrate (**Figure 4.7d**). These results revealed that *E. coli* were hardly able to adhere onto the BSA-coated glass substrate in dextran-free solution, while addition of dextran induced strong bacterial adhesion.

Figure 4.7 Impact of dextran on *E. coli* adhesion characterized by microscopy. Cells were suspended in dextran-free PBS (a and b) and 70 kDa dextran solution 2 g/dL (c and d). The microscope was focused on the upper surface of coverslip. In phase contrast mode, the cell images in PBS were blurred because they were out of the focal plane. The dark regions in IRM mode showed the area of contact between the bacteria and the substrate. Scale bar: 2 μm .

The deformation rate, a/R , was used to characterize the adhesion strength between *E. coli* and the planar substrate. **Figure 4.8a** demonstrates the effects of dextran concentration and molecular weight on the deformation rate of *E. coli*. This parameter was equal to 0 in dextran-free and the smallest molecule (10 kDa) dextran solutions, because there was no observed adhesion. In the other mediocre (40 kDa and 70 kDa)

and large (500 kDa) molecule dextran solutions, a nonlinear dependence of deformation rate on the dextran concentration was found. Under lower concentration, the deformation rate steadily increased until it reached the maximum of 0.461 in 40 kDa dextran, 0.531 in 70 kDa dextran and 0.508 in 500 kDa dextran, respectively. Further increasing the concentration resulted in decline of the deformation rate. Particularly, the bacterial deformation rate reduced dramatically in 500 kDa dextran solution even under lower concentration range (2 g/dL), while for mediocre size dextran solutions the transition occurred at much higher concentration range (4~8 g/dL).

Figure 4.8 The change of bacterial deformation rate a/R (a) and contact area (b) under various dextran molecular weights (10 kDa, 40 kDa, 70 kDa, 500 kDa) and concentrations (0 to 8 g/dL). Data shown as mean $\pm$ SD (n=10).

4.2.6 Adhesion energy of bacteria

The capsule-substrate adhesion model to estimate the adhesion energy had been employed to quantitatively correlate the adhesion energy with the contact area and vesicle volume.[156,160] The relation between adhesion energy (W/C) and dextran concentrations is presented in **Figure 4.9**. Adhesion energy in 10 kDa dextran solution was zero since there was no adhesion. A nonlinear relation was obtained in three moderate and large molecular-size dextran solutions, which showed a similar trend as the adhesion area and deformation rate. The results indicated that adhesion energy was promoted with moderate to large size dextran, but the enhancement was not monotonically related to the increase of dextran concentration.

Figure 4.9 Change of adhesion energy (W/C) under various dextran molecular weights and concentrations. *E. coli* were dispersed in dextran-free PBS and dextran solutions (10 kDa, 40 kDa, 70 kDa, 500 kDa). Data shown as mean $\pm$ SD (n=10).

4.2.7 Electrophoretic mobility of bacteria in the presence of non-adsorbing polymer

We investigated the depletion layer by measuring the electrophoretic mobility of the bacteria in different dextran solutions of various molecular weights. It is known that the major composition of the bacterial outer membrane, such as proteins and lipopolysaccharides, may change in different growth phases and hence affect their surface charge.[20,164] Therefore all the tested *E. coli* in this study were harvested during late exponential phase in order to ensure the cell membrane had the same surface charge. The bacteria were suspended in different dextran solutions with concentrations ranging from 0.5 to 8 g/dL. Based on Helmholtz-Smoluchowski relation, the theoretical electrophoretic mobility b in dextran-PBS solutions is inversely proportional to the dynamic viscosity of the bulk solution η [37,122]

$$b_{DEX} = \frac{\eta_{PBS}}{\eta_{DEX}} b_{PBS}. \quad (4.11)$$

where the subscripts “DEX” and “PBS” represent the parameters for dextran solutions and dextran-free PBS, respectively.

As predicted, the theoretical electrophoretic mobility of *E. coli* in dextran solutions decreased with increasing polymer concentration (solid curves in **Figure 4.10**) due to the increased viscosity in the bulk phase (**Figure 4.11**). However, the experimentally measured electrophoretic mobility was generally higher than the theoretical prediction (**Figure 4.10**). The deviation from the theoretical value became even greater for dextran with larger molecular weight. Considering dextran is a neutral

polymer which does not affect dielectric constant of medium and interface potential.[37,165-168], this remarkable discrepancy between theoretical (b_{DEX}) and experimental ($\tilde{b}_{DEX}$) mobility suggested that the effective viscosity ($\tilde{\eta}_{DEX}$) near the bacterial surface reduced to less than the bulk viscosity according to equation (4.1), which further implied the depletion of dextran molecules near the bacterial surface. In prior studies, the EPM of human red blood cells and liposomes was also found to decrease under higher polymer concentration or molecular weight in dextran or polyethylene glycol (PEG) solution.[41,122]

Figure 4.10 Experimental and theoretical mobility of *E.coli* under different bulk viscosity in dextran solutions with various molecular weights: (A) 10 kDa, (B) 40 kDa, (C) 70 kDa, (D) 500 kDa. Experimental data shown as mean $\pm$ SD.

Figure 4.11 The change of viscosity of the dextran-PBS solution under different polymer concentrations.

4.2.8 Viscosity-corrected electrophoretic mobility of bacteria

In order to estimate the depletion effect in various dextran solutions, the bacterial EPM mobility was compared under different concentrations using viscosity-corrected mobility ratio[169]

$$k = \frac{\eta_{DEX} \tilde{b}_{DEX}}{\eta_{PBS} b_{PBS}}. \quad (4.12)$$

where $\tilde{b}_{DEX}$ refers to the observed electrophoretic mobility. The change of viscosity-corrected mobility ratio in different solutions (**Figure 4.12**) indicated the depletion effect revealed by the bacterial electrophoretic mobility. According to

Helmholtz-Smoluchowski relation, the viscosity at the bacterial surface remains the same to that in the bulk phase ($\eta_0 = \eta_{DEX}$), meaning the ratio k is equal to 1 regardless of the bulk viscosity. However, the experimental results exhibited steady increase of the mobility ratio with concentration and molecular weight. The results indicated considerable reduction of local viscosity at the interaction surface, suggesting the depletion effect of the polymer solution. It was noted that k in 10 kDa dextran solution was slightly greater than 1 but it hardly change with concentration, indicating very weak depletion interaction.

Figure 4.12 The change of viscosity-corrected electrophoretic mobility ratios under various dextran concentrations and molecular weights (10 kDa, 40 kDa, 70 kDa and 500 kDa). The ratio k is equal to 1 in PBS (control).

4.2.9 Calculation of effective viscosity near bacteria surface

The measured electrophoretic mobility also suggested the existence of depletion zone near the bacterial surface from another point of view. Krabi and Donath *et al.* had developed a theory to explain the depletion effect with non-adsorbing polymers in electrophoresis.[38-39,41,146] The concentration profile of non-adsorbing polymer within the interaction surface layer (*e.g.*, glycocalyx) is represented by[146]

$$c(x) = c_b \tanh^2\left(\frac{x}{\Delta}\right). \quad (4.13)$$

The density of molecule segments is assumed to remain constant at the boundary of glycocalyx. If the depletion layer is large than the Debye-Hückel length and the charged layer (*e.g.*, glcocalyx) is assumed non-penetrable for electrolyte flow, the ratio of the EPM in polymer-free solution b_{PBS} and EPM in solution containing polymer b_{DEX} is given by[41]

$$\frac{b_{PBS}}{b_{DEX}} = \frac{\eta_0}{\eta_{PBS}} \left[1 + \left(1 - \frac{\eta_0}{\eta_{DEX}} \right) \frac{1}{\kappa\Delta} \right]. \quad (4.14)$$

where the subscripts “DEX” and “PBS” represent the parameters for dextran solutions and dextran-free PBS, respectively. η_0 is the viscosity at the depletion interaction surface and $\frac{1}{\kappa}$ represents the Debye-Hückel length. From equations (2.1) – (2.3), theoretical depletion layer thicknesses could be obtained based on the various polymer weight and concentration used in our study. Therefore, the effective viscosity (η_0) was analyzed. The summary of results in different polymer solutions is shown in **Table 4.1**.

Table 4.1 Effective viscosity of bacteria in the presence of different dextran

c_b (g/dL)	40 kDa ^a		70 kDa ^b		500 kDa ^c	
	η_0	η_{DEX}	η_0	η_{DEX}	η_0	η_{DEX}
	mPa•s	mPa•s	mPa•s	mPa•s	mPa•s	mPa•s
0.5	0.79	1.24	0.78	1.27	0.90	1.41
1	0.85	1.28	0.81	1.41	0.92	1.66
2	0.97	1.55	0.83	1.73	1.06	2.57
4	0.94	2.03	0.91	2.70	1.00	5.15
8	1.14	3.70	1.11	5.19	1.86	14.56

^{a ~ c} The gyration radius of dextran are: *a.* 5.57 nm; *b.* 7.36 nm; *c.* 19.7nm.[32]

The calculated viscosities at the depletion interaction surface were much smaller than it in the bulk phase. This viscosity discrepancy became larger with increasing the concentration as well as molecular weight of polymer. At the concentration of 0.5 g/dl, the bulk viscosity is about 1.5 times of the viscosity near the surface. When the concentration increased to a high level, for example at 8 g/dl, the bulk viscosity was more than 3 times as high as the viscosity near the surface in dextran 40 kDa, while it was more than 5 times in dextran 70 kDa and about 8 times in dextran 500 kDa. In the case of 10 kDa, the calculated depletion layer thickness ($\Delta=3.9$ nm) while Debye-Hückel length ($1/\kappa$) is 4.16 nm in PBS solution. The equation 4.14 is not suitable for 10 kDa since the depletion layer is overwhelmed by the electrical double layer.

4.3 Discussion

We used dextran as a non-adsorbing polymer to induce phase transition of *E. coli*. The results have confirmed that when *E. coli* mix with dextran, they tend to aggregate in the stationary growth suspension and thus phase separation occurs. The aggregation also depends on the molecular weight as well as concentration of polymer. Based on the results, if the dextran reaches a certain critical concentration, bacteria aggregation is triggered and it increases as the dextran concentration increases. This “crowding effect” is similar to the induced flocculation of inert colloids in the non-adsorbing polymer suspension, which is governed by the depletion mechanism. The change of phase threshold depended on dextran of different molecular weight follows a similar tendency which is consistent with depletion.[104] These findings suggest that depletion interaction plays a dominant role on inducing bacteria phase separation.

The enhancement of aggregation by dextran varies in different molecular weight. According to **figure 4.6**, the level of aggregation in the larger dextran (*i.e* dextran 70 and 500 kDa) solution is more profound than it in small molecular weight (*i.e* dextran 10 kDa). Moreover, although the level in dextran 70 kDa and 500 kDa changed similarly at low concentration, an early decrease of aggregation was observed in dextran 500 kDa solution at high concentration. This observation could probably explained by the properties of dextran polymer and the consideration of depletion model. It is going to be further described in the next chapter.

As shown above, the depletion interaction for *E. coli* in the presence of non-adsorbing dextran solution was characterized by different methods, including aggregation screening in suspension, evaluation of adhesion strength using microscopy and electrophoretic mobility. The results indicated that dextran of large molecular weight can enhance the aggregation of *E. coli* in suspension and promote the bacterial adhesion on solid substrate.

According to the depletion model, when the concentration is relatively low, the depletion interaction energy increases with the concentration because the attractive force caused by osmotic pressure predominates the interaction. However, the depletion layer thickness decreases considerably when further increasing the concentration.[31] Therefore, the attractive interaction becomes weaker and thus the adhesion energy declines eventually.[32] The results in bacterial aggregation test (**Figure 4.6**) and adhesion test (**Figure 4.9**) showed good agreement with the above prediction by the theory of polymer depletion.

The kinetics of bacterial phase separation and adhesion on the albumin-coated surface was dependent both on the concentration and the molecular weight of dextran. As shown in the results, the smallest dextran molecules (10 kDa, radius of gyration $R_g = 2.8$ nm) induced no effect on phase separation or adhesion. It implied that 10 kDa molecules were too small to establish an effective depletion zone near the bacterial surface, because the depletion layer was too thin that it could not even extend beyond the layer of glycocalyx. Thus the repulsion due to steric and

electrostatic interaction was predominant over the attractive depletion effect by the thin depletion zone. However, for larger dextran molecules, the induced depletion layer was thick enough to induce strong attraction force against the steric repulsion, leading to cell aggregation and adhesion. However, in super large molecule (500 kDa) dextran solution, the depletion effect on bacteria was suppressed under high concentration levels due to the significantly increased viscosity that retarded bacterial aggregation and adhesion.

Comparing the predicted theoretical value and observed experimental data of a range of dextran polymers from 10 to 500 kDa, the discrepancy clearly indicates the presence of polymer depleted layer near the *E. coli* surface. It has clearly confirmed that in non-adsorbing polymer solution, electrophoretic mobility of bacteria enhances by the reduced viscosity near the bacteria surface, which is resulted from the polymer segments displacement near the surface due to the depletion interaction. However, the interpretation to the difference between experimental data and predicted values requires more precious consideration.

It should be noted that the calculation is a simplification considering small electrical double layer and neglectable electrolyte flow during depletion interaction. Since the bacteria surface is chemically and structurally complicated, more comprehensive consideration of electrophoretic mobility in the presence of non-adsorbing polymer should take into account the permeability of ions and the penetration of polymer on the bacteria surface. For example, the assumption of the

non-adsorbing dextran may seem reasonable in this study. However, in nature, extracellular polymers are able to bind to the bacteria or penetrate into the outer membrane in some extent. The electrolyte flow is also not neglectable in many situations. Further works about the electric properties of bacteria in the presence of non-adsorbing polymer are expected.

The displacement time of polymer near the surface should be examined as well. From the adhesion results mentioned by section 4, it showed that it likely took more time for large polymer to deplete from the interaction interface. There was a reported argued that dextran tend to form a 3D network structure after it reaches a “critical concentration”.[170]. The dynamics of formation of depletion layer remains poorly elucidated.

Lastly, the commercial dextran usually contains a range of molecular weight polymers. For example, the dextran 70 kDa used in this chapter contains a range of molecular weight dextran from 68 000 to 75 000 Da. The polydispersity of these dextrans with different molecular weight probably leads to a higher experimental result than the predicted data.

Nevertheless, in conclusion, the overall difference between the observed results and predicted data undoubtedly demonstrated the occurrence of polymer-depleted layer around the bacteria surface in the non-adsorbing polymer solution.

5. Impact of non-adsorbing macromolecules on tumor cells adhesion

5.1 Introduction

Adhesion between cells and extracellular matrix (ECM) components is of great importance in various biological activities including cell differentiation, adhesion and repair. The ECM also regulates the tumor development, proliferation, migration and metastasis.[171-174] The association between tumor cells and the surrounded ECM always catches the attention in intensive researches. ECM consists of numerous types of molecules, including collagen, elastin, fibronectin (FB), fibrinogen (FBG) and different proteoglycans. Many of these components have been demonstrated their regulation effect on cell adhesion.[175-177] These regulations are considered highly specific by the interaction between adhesion molecules and surface receptors. For example, it has been reported that cell proliferation is regulated by the availability of growth factors, which bind to FBG, and cytokines in tumor ECM.[178-179] Thus the proliferation is controlled by the degradation of this matrix to release the growth factors. FBG also provides important binding regions, like heparin binding domains and ICAM-1 binding sites, for cell adhesions.[180-181] Simpson-Haidaris and Rybarczyk have found that the migration of breast tumor cells, MDA-MB-231, was inhibit in the addition of free FBG in culture medium.[26] Briefly, a wound was made on the confluent cell layer by scraping an empty area. FBG was added right after the

wound was made (at the time of wounding). Tumor cells in the control group were incubated with FBG for 24 hours before wounding. The results showed that cells without pre-incubated with FBG were much less in the wounded area after 18 hours of incubation. They considered that molecule signaling events related to integrin engagement or actin cytoskeleton rearrangement were likely the underlying mechanism.

However, we proposed an alternative pathway to explain the inhibited migration by demonstrating that the adhesion of tumor cells is regulated by nonspecific depletion interaction. In this chapter, the adhesion efficiency of two tumor cell lines, MDB-MB-231 breast cancer cells and HeLa cervical cancer cells, was detected in the presence of macromolecules. Adherent tumor cells to plasma proteins coated surface were measured in solutions containing dextran in order to mimic the influence of free macromolecules in plasma. This study intends to investigate a potential mechanism to explain cell-surface interaction as well as to extent the possible significance of depletion interaction.

5.2 Materials and methods

5.2.1 Tumor cells preparation

Human breast carcinoma cells (MDA-MB-231) are known as “triple negative” cell line, which they don’t have estrogen receptors, progesterone receptors and high expression of Her2 protein.[182] It is an invasive and moderately metastatic breast cell line without the ability to produce detectable fibrinogen. A different tumor cell line, HeLa cervical cancer cells, was used as a compared group to the MDA-MB-231.

Cells were incubated in 24-well culture plate in 5% CO₂ at 37°C. The growth medium is composed of 90% DMEM with 10% FBS and 1% Penicilin/Streptomycin.

5.2.2 Tumor cell adhesion assay

Tumor cells were grown in 75 cm² tissue culture flasks. Adherent cells were stained by Hoechst 33342 which prefers DNA sequences rich in adenine and thymine. Hoechst 33342 allows staining in live cells and binds to the minor groove of double-stranded DNA.[183] Before the adhesion experiment, cells were washed by PBS. Complete growth medium (90% DMEM, 10% FBS and 1% Penicilin/Streptomycin) containing 500 ng/ml Hoechst 33342 was added into the flasks and cells with staining medium were incubated at 37°C for 30 minutes. After the staining, cells were washed twice with PBS to remove the residue medium and detached by 0.25% trypsin/EDTA. The cell suspension was centrifuged at 220g for 5

minutes and the cell pellets were re-suspended ($2 \times 10^5 \text{ ml}^{-1}$) in DMEM.

Tumor cell adhesion was assayed in 24-well plastic tissue culture plates. The plate was coated overnight at 4°C with different concentrations of a 0.5 ml volume of human plasma fibronectin (FB) in PBS. The wells were blocked with 5% BSA for 30 minutes at 37°C and washed twice with PBS before the experiment. 500 μl of cell suspension ($2 \times 10^5 \text{ ml}^{-1}$) was added to each well. In polymer effect experiment, polymer (*i.e.* dextran 70 kDa) solutions dissolved in DMEM with various concentrations was added into the well together with cell suspension. Dextran solutions have the final concentrations ranging from 0.5 g/dl to 8 g/ml. The adhesion assays were incubated for 1 hour at 37°C in 5% CO_2 . And then, suspension was aspirated from the wells and the plates were carefully washed twice with PBS to remove the unattached cells. The assays were observed under fluorescence microcopy with excitation wavelength of 351 nm and emission wavelength of 450 nm. Adhesive cells were determined at 3 random locations at the center of each well and the absolute number of cells attached per square millimeter was calculated. Every adhesion assay experiment was carried out three times repeatedly.

5.3 Results

5.3.1 Tumor cells adhesion on fibronectin-coated surface

In order to detect whether MDA-231 and Hela cells bound to fibronectin, 24-well culture plates were coated with a serious concentration of fibronectin. **Figure 5.1** shows the adherent cells on different concentrations of fibronectin. The wells were blocked with 5% BSA and only several cells were found adhered on the surface, which in average, 2 ± 1 cells/mm⁻² for MDA-MB-231 and 1 ± 1 cells/mm⁻² for HeLa cells, respectively. The efficiency of adhesion increased as the concentration of fibronectin increased. Most cells attached on the well surface in the highest concentration 1 μ g/ml of fibronectin, which were 306 ± 9 cells/mm⁻² for MDA-MB-231 and 347 ± 10 cells/mm⁻² for HeLa cells, respectively.

Figure 5.1 Adhesion of MDA-MB-231 and HeLa cells in different concentrations of fibronectin. The data were shown by mean $\pm$ standard deviation ($n=6$). The results were analyzed by nonparametric Wilcoxon-Mann-Whitney U test. Detail was explained in the text.

5.2.2 Tumor cells adhesion in the presence of dextran

Since the adhesion of tumor cells on fibronectin-coated surface was demonstrated, the following efforts were directed toward measuring the impact of dextran on the tumor cells adhesion. According to the results of previous section, the number of adherent cells in the 1 µg/ml concentration of fibronectin were significantly more than it in 0.1 µg/ml (Wilcoxon-Mann-Whitney U test, both of the results of MDA-MB-231 and HeLa cells: $p<0.01$). Thus, the plates were coated with 0.1 µg/ml fibronectin and

blocked with 5% BSA. Tumor cells were dispersed in different concentrations of dextran 70 kDa solutions and added in the wells. **Figure 5.2** shows the adhesion of MDA-MB-231 and HeLa cells on fibronectin (0.1 μ g/ml) plates in different concentrations of dextran 70 kDa. The adhesion efficiency of both cell lines presented similar tendency and indicated a biphasic relation with the dextran concentration. In low concentration, more cells were adherent as the increase of dextran. Once the number of adherent cells reached the peak at 4 g/dl dextran with 272 ± 10 cells/mm⁻² for MDA-MB-231 and 177 ± 5 cells/mm⁻² for HeLa cells respectively, it started to decreased. There were 157 ± 14 cells/mm⁻² for MDA-MB-231 and 89 ± 11 cells/mm⁻² for HeLa cells at 8 g/dl dextran, which were about 58% and 50% of the highest adherent number.

Figure 5.2 Adhesion of MDA-MB-231 and HeLa cells on fibronectin (0.1 μ g/ml) plates in different concentrations of dextran 70 kDa. The data were shown by mean $\pm$ standard deviation (n=6). The results were analyzed by nonparametric Wilcoxon-Mann-Whitney U test. Detail was explained in the text.

The summary of the adhesion efficiency of MDA-MB-231 cancer cells was presented in **figure 5.3** (HeLa cells not shown). Very little adherent cells were observed in the wells which were coated without fibronectin and only blocked with BSA (control). Similar results were obtained in those coated with 0.1 µg/ml fibronectin. However, the adhesion was enhanced significantly in addition of dextran 70 kDa ($p<0.01$). The number of adherent cells was 272 ± 10 cells/mm² in 4 g/dl dextran solution on 0.1 µg/ml fibronectin-coated wells, which was nearly as many as it on 1µg/ml fibronectin-coated wells without dextran solution. These results (**figure 5.2 and 5.3**) clearly demonstrated that the adhesion of tumor cells is induced in the presence of dextran and it shows a biphasic dependence on dextran concentration.

Figure 5.3 Effect of the addition of dextran on MDA-MB-231 cell adhesion to fibronectin-coated surface. (a): Images of adherent cells in fibronectin-coated surface with or without dextran 70 kDa. Only results of MDA-MB-231 cells were presented. (b): Number of adherent cells in fibronectin-coated surface with or without dextran 70 kDa. No fibronectin was coated on control wells. The results were shown in mean adherent cells per square millimeter $\pm$ standard deviation. Wilcoxon-Mann-Whitney U test was performed to compare the difference, which ** was referred to $p < 0.01$.

5.3 Discussion

In this study, the impact of non-adsorbing molecules on tumor cells was examined. The results presented above strongly indicated that the adhesion of MDA-MB-231 breast cancer cells and HeLa cervical cancer cells was enhanced in the presence of dextran 70 kDa. Cells didn't adhere on the well surface when the amount of fibronectin coated on the well surface is too low (*i.e.* 0.1 $\mu\text{g/ml}$). However, when the non-adsorbing dextran was added in the suspension, adhesion efficiency increased significantly. The adhesion was also found depend on the dextran concentration, which showed a biphasic relation. This biphasic relation could be explained by the consideration of depletion hypothesis: when the concentration is relatively low, the depletion interaction energy increases with the concentration because the attractive force caused by osmotic pressure predominates the interaction. However, the depletion layer thickness decreases considerably when further increasing the concentration. The attractive interaction becomes weaker thereafter and the adhesion energy declines eventually. Thus, the results presented suggested that the polymer depletion effect serves a potential mechanism to regulate the adhesion of tumor cells on plasma protein fibronectin.

However, some questions remained to be answered to have a deeper understanding of the role of depletion interaction on tumor cell interaction. The adhesion of tumor cells on other cells (*e.g.*, endothelial cells) is a continuum of sequential events which involve various factors including cellular properties, regulators and support from

extracellular matrix and so on. Therefore, in order to have a more precious description of depletion on tumor cell adhesion, other factors should be included to. For example, the effect of variation of cellular properties on depletion-induced interaction should be taken into consideration. From **figure 5.2**, generally, more MDA-MB-231 cells were adherent than HeLa cells as the increase of dextran concentration. The difference of cell membrane could be the reason to explain this discrepancy, since depletion interaction is depend on the surface characteristics. These studies are expected in our future research.

There were numerous studies to identify specific binding between cellular receptors and ligands (*e.g.* plasma proteins, growth factors) which regulate the adhesion of tumor cells. In this chapter, the effect of a non-specific force of depletion interaction on tumor cells adhesion was considered. Our findings confirm the induced adhesion of tumor cells in the presence of dextran. It reveals that depletion interaction play an important role in tumor adhesion and should show insight of studying the interaction of tumor cell migration, proliferation and metastasis.

6. Conclusion and future directions

In this dissertation, the cell-cell and cell-substrate interaction regulated by non-adsorbing macromolecules has been investigated. Chapter 2 provides the background introduction and methodology of investigation for this purpose.

The diabetic red blood cell adhesion to endothelial cells in the presence of dextran fractions was investigated. The results indicated that red blood cells in diabetes are much easier to adhere on endothelial cells. This adherent potential increased significantly in the solutions containing non-adsorbing polymer dextran. The cellular properties of diabetic red blood cell were also measured. In diabetes, the deformability of erythrocyte is much lower than the normal ones. They are easier to aggregate and have lower zeta potential. The inspection of these results reveals that the elevated diabetic erythrocyte adhesion to endothelial cells is regulated by depletion interaction of dextran. The depletion induced adhesion is associated with the cellular properties as well.

The bacteria phase separation as well as bacteria adhesion in dextran solution have also been inspected. Fast phase separation was triggered in bacteria suspension by adding large dextran (*e.g.*, larger than 10 kDa). The results showed a biphasic dependence of the kinetics of phase separation to molecular weight and concentration.

Similarly, the adhesion energy of bacteria to the albumin-coated surface indicated a biphasic relation to the polymer size and concentration. The existence of depletion layer near the bacteria in dextran solution has been detected by electrophoresis. These results showed good agreement to the consideration of depletion hypothesis so that they strongly suggested that the bacteria aggregation and adhesion are governed by the depletion interaction.

In addition, cell adhesion assay has been applied to study tumor cell adhesion promoted by non-adsorbing macromolecules. Nearly no MDA-MB-231 breast cancer cells and HeLa cervical cancer cells were adherent to the surface which coated with low amount of fibronectin (*e.g.*, 0.1 $\mu\text{g/ml}$) and blocked with bovine serum albumin. However, the adhesion was greatly promoted by adding dextran fractions. Biphasic dependence of the adhesion on dextran solutions was observed as well. These findings indicated that depletion interaction acts as an underlying mechanism to govern the adhesion of tumor cells in the presence of non-adsorbing polymer.

Depletion hypothesis suggests that the presence of non-adsorbing polymers in colloidal dispersion leads to the attraction between colloidal particles. The results in this study proved that the depletion interaction serves as generic mechanism behind the induced aggregation and adhesion in the abovementioned cell-polymer systems. However, the difference between the depletion effect of dextran on bacteria and on RBCs should take careful consideration. Dextran 500 kDa has a more profound effect than 70 kDa to induce the adhesion of RBC on EC. On the contrary, in

bacteria adhesion, 70 kDa is more effective than 500 kDa, especially in high concentration. This difference may originate from the different membrane structure and components. The depletion layer is determined not only by the solution and polymer properties, but also by the permeability of the membrane as well as the size of peripheral proteins and polysaccharides.

Overall, findings in the study provide a rational explanation to the cell-cell and cell-substrate interaction in non-adsorbing polymer solution by means of depletion interaction. They clearly suggest the applicability of depletion interaction in cell-polymer system and offer new insights to the application of depletion effect to different fields.

Noted that dextran has been used extensively in the depletion study. In the future research, more studies are expected in the direction about de depletion effect of other macromolecules in clinical state and in nature, such as the human plasma proteins (*e.g.*, fibrin, fibrinogen) and extracellular polymeric substances near bacteria. It is likely that depletion interaction may act as one of the driven force in other conditions. Fibrinogen, a kind of negative charge plasma proteins in the blood, has been proved that it enhanced the abnormal adhesion between RBC and EC due to the depletion effect.[163] It suggests the non-neutral polymers is potential to cause the depletion interaction. More detailed research is expected in order to distinguish the electrostatic force and the depletion effect. Moreover, it also reveals the potential role of depletion effect result from fibrinogen in tumor cell metastasis. The

coagulation factors, including fibrinogen or fibrin, are well associated with the development of tumor metastasis.

Another one of the prospective applications is to develop new therapy to abnormal adhesion or aggregation in vascular diseases, such as diabetes mellitus, β -thalassemia and occlusion.[59,184] Understandings of the depletion effect on vascular complications should lead to new diagnostic methods and development of possible therapeutic agents.

Appendix

A. Abbreviations

APTES	3-Aminopropyl-Triethoxy-Silane
BSA	Bovine Serum Albumin
Da	Dalton
DEX	Dextran
DMEM	Dulbecco's Modified Eagle Medium
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EI	elongation index
EPM	Electrophoretic Mobility
EPS	extracellular polymeric substances
EW	evanescent wave
FB	fibronectin
FBG	fibrinogen
FBS	fetal bovine serum
HbA _{1C}	Glycosylated Hemoglobin
HCT	haematocrit
HUVEC	Human Umbilical vein Endothelial Cells

IRM	Interference Reflection Microscopy
LB	Luria Broth
LORCA	Laser-Assisted Optical Rotational Cell Analyzer
NaPSS	sodium polystyrene sulfonate
Pa	Pascal
PBS	phosphate-buffered saline solution
PEG	Poly(ethylene glycol)
PVP	polyvinylpyrrolidone
RBC	Red Blood Cell

B. Refractive index

Isotonic dextran concentration (g/dl)	10 kDa	40 kDa	70 kDa	500 kDa
0	1.3346			
0.5	1.3352	1.3352	1.3353	1.3353
1	1.3358	1.3358	1.3359	1.3359
2	1.3371	1.337	1.3372	1.3373
4	1.3397	1.3393	1.3396	1.3398
8	1.3447	1.3441	1.3452	1.3442

C. Viscosity

dextran concentration (g/dl)	Dynamic viscosity (Pa)			
	10 kDa	40 kDa	70 kDa	500 kDa
0	0.89			
0.5	1.17745	1.23767	1.27305	1.41137
1	1.23416	1.27913	1.41427	1.65606
2	1.35629	1.55074	1.73582	2.55672
4	1.45172	2.0281	2.70177	5.15311
8	2.20667	3.70336	5.18602	14.56326

D. Physicochemical properties of dextran

Molecular weight (kDa)	10	40	70	500
B_2 ($\text{m}^5\text{s}^{-2}\text{kg}^{-1}$)	6.4	2.5	1.69	0.43
R_g (nm)	2.78	5.57	7.36	19.7

E. Miscellaneous techniques

The pH values of all solutions were measured by a pH meter (Denver instrument, Germany). The density, refractive index and viscosity of dextran solutions were measured by a density meter (DMA 35N, Anton Paar, Austria), a refractometer (ATAGO, Japan) and an automated micro viscometer (AMVn, Anton Paar, Austria), respectively.

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