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Real-time monitoring of mechanical changes during dynamic adhesion of erythrocytes to endothelial cells by QCM-D†

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A quartz crystal microbalance with dissipation monitoring is used to measure changes in mechanical properties of diabetic red blood cells (RBCs) and normal RBCs. Moreover, the adhesion interaction between these two kinds of RBCs and endothelial cells (ECs) is further investigated using a proposed QCM-D biosensor for the first time.

Erythrocytes (RBCs), accounting for about 45% of total blood volume, realize their basic function by transporting oxygen and carbon dioxide in the blood. Erythrocyte membranes surround cells and separate cellular contents from the external environment, and they also control all communication between the inside and outside.[1] The erythrocyte membrane consists of an overlaying asymmetric phospholipid bilayer membrane, supported by an underlying spectrin–actin cytoskeletal complex.[2] It is generally believed that the erythrocyte membrane together with its cytoskeletal support is responsible for the maintenance of the shape and stability of the cell and also for allowing extensive deformations when needed. Changes in the shape, mechanical characteristics or the integrity of the erythrocyte have severe implications for the functionality of the cell, as can be seen in several dysfunctional states of the erythrocyte, whether environmentally induced, or due to hereditary defects or disease.[3] Since diabetes was defined by Wautier as a major risk factor for vascular thrombosis,[4] there has been a growing interest in study of the properties of RBCs in diabetic patients. Buys *et al.* studied RBC membrane structure in type 2 diabetes using scanning electron microscopy and atomic force microscopy.[5] Foresto *et al.* evaluated red blood cell aggregation in diabetes by computerized image analysis.[6] Yang *et al.* demonstrated that sialic acids from RBC surfaces can induce erythrocyte adhesion to endothelial cells and that such adhesion is significantly enhanced in the presence of high-molecular weight dextran.[7] Unfortunately, many of these methods failed to measure in real time intrinsic properties such as viscoelasticity and rigidity of RBCs in diabetic and healthy individuals, which are very interesting indicators of the health state of cells. Thus, developments of a sensitive and label-free method for detecting real-time cell properties are still needed.

Most importantly, the interactions between red blood cells and vessels are essential in inflammatory situations, infectious and thrombotic diseases.[8] Abnormal red blood cell adhesion to human umbilical vein endothelial cells (HUVECs) has been correlated with vascular complications in diseases such as diabetes, sickle cell anemia and malaria.[4] A variety of physiological and physical changes of cells occur in these cell–cell interactions, and could be indicative in evaluating the effect of drugs, mutations or diseases on cell structures. Up to now, many techniques have been used to measure changes in cell adhesion, such as immunofluorescence assay,[9] optical microscope imaging,[10,11] cell counting kit-8 assay,[12] fluorescence resonance energy transfer (FRET)[13,14] and photonic crystal biosensors.[15] These assay methods are, however, rather time consuming and labour intensive. Moreover, many of these methods are based on end-point detection which fails in real-time detection, or are based on the introduction of foreign objects which can affect the response of cells. Therefore, a novel assay to investigate cell–cell adhesion is worth developing and still at the forefront of the field.

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a non-invasive, real-time and label-free technology that has been applied in much cell research, such as cell attachment, adhesion and proliferation.[16–18] Combined dissipation and frequency shift (ΔD–Δf) measurements using the QCM-D provide information about the structure and dynamic viscoelastic properties, as well as mass, of surface-adsorbed cell layers.[19] However, to the best of our knowledge, there have been almost no reports of the application of QCM-D for the sensitive, label-free and real-time monitoring of red blood cell adhesion to endothelial cells.

In the present work, we evaluated the viscoelasticity and rigidity of RBCs from type 2 diabetes, which is correlated with the severity of vascular injury.[20] For the sake of comparison, an equivalent study was conducted with RBCs from healthy individuals. Knowing that the existence of adhesion between RBCs and endothelial cells is correlated with the severity of vascular injury,

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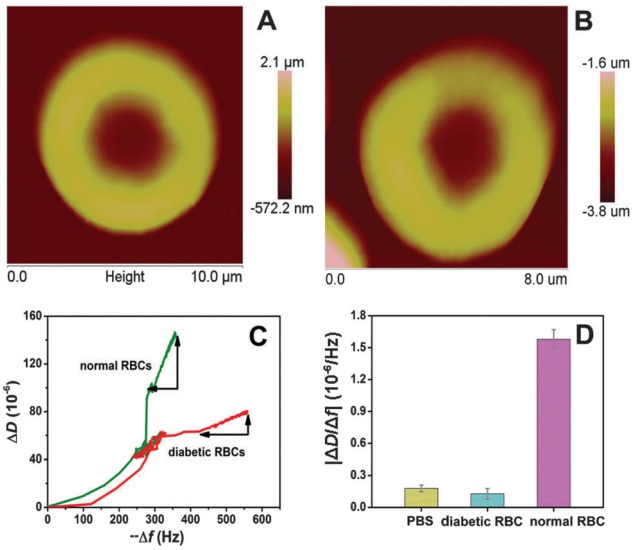

Fig. 1 AFM images of (A) diabetic erythrocytes and (B) normal human erythrocytes; (C) plot of ΔD as a function of Δf; (D) the histogram of $|\Delta D/\Delta f|$.

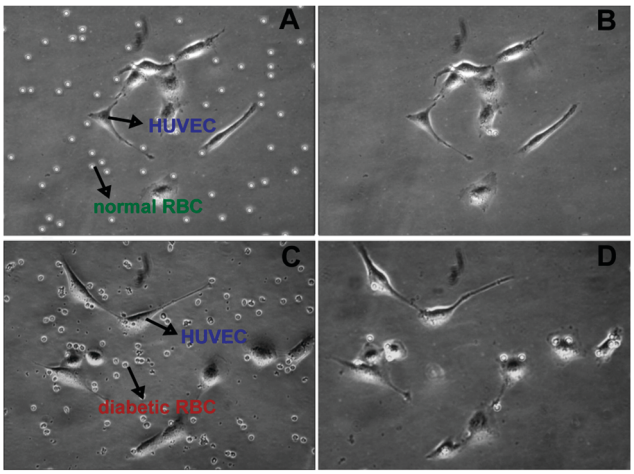

Fig. 2 The adhesion experiment visualized by inverted fluorescence microscope. (A) Normal RBCs and (C) diabetic RBCs were dispersed on the adhered HUVECs; after rinsing with PBS, (B) no normal RBCs but (D) many diabetic RBCs adhered to HUVECs.

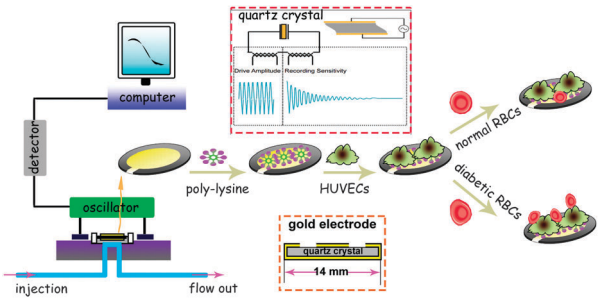

Fig. 3 Schematic illustration of the QCM-D experimental set-up and the label-free QCM-D biosensor for the detection of cell adhesion.

we performed cell adhesion experiments using QCM-D monitoring. Electrochemical techniques further confirmed the results obtained.

Erythrocyte deformability is a critical determinant of morphology, and the morphology is fundamental to hemorheology. Thus, the size and shape of human RBCs were observed by atomic force microscopy (AFM). Compared with the typical concave shape in healthy RBCs (Fig. 1A), diabetic RBCs are tumid and distorted (Fig. 1B). Further, a plot of ΔD–Δf for erythrocyte motion on a poly-lysine modified gold electrode was constructed for a better understanding of the cell membrane properties and the capacity for remodelling of the cytoskeleton. As shown in Fig. 1C, after injection of buffer solution for blank measurement, an increase in dissipation with decreasing frequency was observed both for the diabetic and normal RBCs. However, the slope ($|\Delta D/\Delta f|$) for diabetic RBCs was lower than that for normal RBCs, as shown in Fig. 1D, indicating that the diabetic RBCs were stiffer, and showed decreased deformation. This is due to glucose oxidation and protein glycosylation inducing a number of modifications in the mechanical and morphological properties of erythrocytes.[21,22]

Next, we observed if the diabetic RBCs indeed tend to adhere to endothelial cells. Fig. 2 shows the adhesion experiment visualized by inverted fluorescence microscope. RBCs from healthy and diabetic individuals were dispersed on the adhered normal HUVECs as shown in Fig. 2A and C, respectively. The cells were co-cultured for 30 min than rinsed with PBS. Fig. 2B shows no normal RBCs adhered to HUVECs. However, obvious adhesion to HUVECs occurred for diabetic RBCs (Fig. 2D). The results show that the diabetic RBCs tended to adhere to endothelial cells, which is the major cause of diabetes-derived complications.

Further, for the first time, an easily-constructed QCM-D biosensor was proposed for non-invasive and real-time study of the cell adhesion (Fig. 3). Here, the resonator oscillates with some constant amount of energy, and as the amount of energy in the system remains constant, the oscillation frequency changes with addition of any mass onto the resonator surface. Changes in the amount of mass adsorbed are then measured through change in frequency (Δf), and changes in dissipation (ΔD), which give a qualitative measure of how rigidly a cell adheres to the underlying substrate. An increase in dissipation indicates more energy being lost to the surrounding environment, suggesting a relatively softer cell layer. Herein, we noninvasively measured the mechanical changes during cell adhesion in real time and in a label-free manner through Δf and ΔD. As shown in Fig. 4A and B, after obtaining stable baselines in stage I, flow was resumed by changing to HUVECs. Stages II and III show Δf and ΔD responses reflecting time-dependent changes in mechanical properties of HUVECs adhering to substrate and RBCs adhering to HUVECs. In stage II, HUVECs underwent sedimentation and reached the electrode surface under the influence of gravity; immediately after, these cells could react with poly-lysine on the electrode surface. This complicated procedure resulted in a notable decrease of frequency ($\sim$350 Hz) and increase of dissipation ($\sim 6.0 \times 10^{-5}$). Subsequently, the trends of Δf and ΔD were reversed; both the frequency and dissipation shifts were $\sim$30% of what was observed before, suggesting the cells changed or rearranged their cytoskeletons. Moreover, the frequency and dissipation shifts were coherent with each other, which can only be correlated to changes in the cytoskeleton and cell contacts to the surface, without involving any shrinkage of the cells,

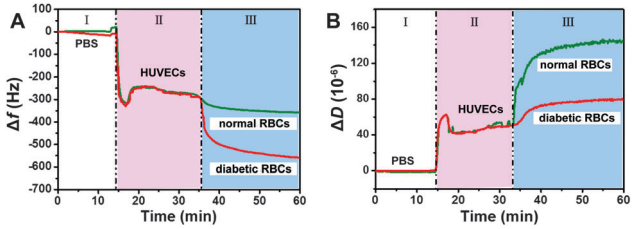

Fig. 4 Real-time (A) Δf and (B) ΔD responses to the successive addition of 3×10^5 cells per mL HUVECs and subsequent adherence to 2×10^5 cells per mL normal RBCs and diabetic RBCs.

eventually reaching a dynamic equilibrium.[23] The addition of RBCs led to obviously different QCM-D shifts (stage III). The Δf was negative while ΔD was positive, gradually reaching plateaus. For normal RBCs, the values of the frequency and dissipation responses were -380 Hz and 1.50×10^{-4}. Such an obvious large shift of the dissipation in comparison to the frequency change suggests that the cell layer was softer after RBC adhesion. Interestingly, for diabetic RBCs, the frequency decreased to -600 Hz and the dissipation increased only to 7.5×10^{-5}, showing the frequency shift is larger than the dissipation change. Table S1 (ESI†) shows that diabetic RBCs were recognized by the QCM-D biosensor with good selectivity. Table S2 (ESI†) shows the Δf and ΔD values obtained from all the healthy and diabetic RBCs when adhering to HUVECs in stage III. The above results indicate that some changes in the fluidic cell interior may also occur which can be associated with the expression of the adhesion molecule or specific proteins and lead to the specific adhesion between diabetic RBCs and HUVECs.[8,24,25] In addition, the specific adhesion further induced endothelial cell activation, shrinkage and then release of water from the cortex due to changes in osmotic pressure,[23] the consequence of which is increased contact area of diabetic RBCs with HUVECs and thus increased mass on the biosensor and a more rigid cell layer. These results suggest that in diabetes there is an intrinsic erythrocyte abnormality that is related to vascular disease.

Finally, the Young's modulus of the healthy and diabetic RBCs was detected by using AFM (Table S2, ESI†). The results showed Young's moduli of diabetic RBCs were larger than those of healthy cells, indicating that the diabetic RBCs had lost their original elasticity leading to increased stiffness of the cell. In addition, we designed an electrochemical biosensor to confirm our above findings that diabetic RBCs easily adhere to HUVECs (Fig. S1 and S2, ESI†).

In summary, the mechanical properties of diabetic RBCs and normal RBCs were studied on a QCM-D gold electrode. Moreover, the adhesion interaction between these two types of RBCs and HUVECs was further investigated by a proposed QCM-D biosensor. The results showed obvious Δf and ΔD shifts that can be related to mechanical energy changes of these two kinds of erythrocytes. Diabetic RBCs appeared stiffer than normal RBCs, which could be due to the glycosylation of proteins on cell membranes. In addition, the diabetic RBCs adhered more easily to HUVECs accompanied by the possibility of activated HUVECs, leading to a decline of viscoelasticity of the adhesion layer, which was also revealed by AFM, CV and DPV measurements. Furthermore, the mechanism of erythrocyte adhesion to endothelial cells is of great importance for the prognosis and therapy of diseases such as thrombosis and vascular complications, which can be a focus of further study. This study validates that QCM-D techniques can sensitively and quickly measure changes in cell activity, particularly cell–cell interactions, and provides real-time data in comparison to traditional techniques. Additionally, this label-free biosensor can find wide application in the real-time and non-invasive investigation of intercellular actions *in vitro* which can help clinicians better understand treatment response.

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