The mechanical properties of stored red blood cells measured by a convenient microfluidic approach combining with mathematic model

Ying Wang,¹ Guoxing You,¹ Peipei Chen,² Jianjun Li,² Gan Chen,¹
 Bo Wang,¹ Penglong Li,¹ Dong Han,^{2,a)} Hong Zhou,^{1,a)} and Lian Zhao^{1,a)}

¹*Institute of Transfusion Medicine, Academy of Military Medical Sciences,
 No. 27 Taiping Road, HaiDian, Beijing 100850, China*

²*National Center for Nanoscience and Technology, No. 11, Beiyitiao, Zhongguancun,
 Beijing 100190, China*

(Received 2 December 2015; accepted 29 February 2016; published online 11 March 2016)

The mechanical properties of red blood cells (RBCs) are critical to the rheological and hemodynamic behavior of blood. Although measurements of the mechanical properties of RBCs have been studied for many years, the existing methods, such as ektacytometry, micropipette aspiration, and microfluidic approaches, still have limitations. Mechanical changes to RBCs during storage play an important role in transfusions, and so need to be evaluated pre-transfusion, which demands a convenient and rapid detection method. We present a microfluidic approach that focuses on the mechanical properties of single cell under physiological shear flow and does not require any high-end equipment, like a high-speed camera. Using this method, the images of stretched RBCs under physical shear can be obtained. The subsequent analysis, combined with mathematic models, gives the deformability distribution, the morphology distribution, the normalized curvature, and the Young's modulus (E) of the stored RBCs. The deformability index and the morphology distribution show that the deformability of RBCs decreases significantly with storage time. The normalized curvature, which is defined as the curvature of the cell tail during stretching in flow, suggests that the surface charge of the stored RBCs decreases significantly. According to the mathematic model, which derives from the relation between shear stress and the adherent cells' extension ratio, the Young's moduli of the stored RBCs are also calculated and show significant increase with storage. Therefore, the present method is capable of representing the mechanical properties and can distinguish the mechanical changes of the RBCs during storage. The advantages of this method are the small sample needed, high-throughput, and easy-use, which make it promising for the quality monitoring of RBCs. © 2016 AIP Publishing LLC.

[<http://dx.doi.org/10.1063/1.4943861>]

I. INTRODUCTION

Red blood cells (RBCs), which make up about 45% of the bulk blood volume, contribute significantly to microcirculation perfusion. This is because the mechanical properties of RBCs can determine the rheological and hemodynamic behavior of blood.¹ For example, deformability, the critical mechanical parameter, plays an important role in transfusion effects because the RBCs are approximately 8 μm in diameter and must deform to traverse capillaries of similar or even smaller diameters to deliver oxygen. Even in larger blood vessels, RBCs deformability is also important in decreasing blood viscosity at high shear rates.² Thus, reduced deformability can result in impaired perfusion and reduced oxygen delivery in peripheral tissues.^{3–5} However, the mechanical properties of RBCs can be altered by numerous pathologic states, like sickle

^{a)}Authors to whom correspondence should be addressed. Electronic addresses: dhan@nanoctr.cn; zhoutht1966@163.com; and zhaolian@bmi.ac.cn

cell anemia and malaria,⁶ and are critical for the evaluation of therapy effect. For example, the plasmodium infection of RBCs results in impaired deformability,⁷ which is recovered after an effective treatment with artesunate.⁸ Therefore, these mechanical properties are also of interest outside of transfusions. Stored RBCs (SRBCs) also undergo significant mechanical changes,⁹ which can limit the effectiveness of transfusions.¹ Despite the need, the existing detection methods of the mechanical properties of RBCs are not suitable for online testing and need improvement. Therefore, we aimed to find a convenient detection method for the mechanical properties of RBCs and apply it in the detection of stored RBCs.

Several methods have been developed to detect the mechanical properties of RBC, such as ektacytometry,¹⁰ micropipette aspiration,¹¹ optical tweezers,¹² and microfluidic techniques.^{13,14} Among them, ektacytometry is a high-throughput method and reveals the mechanical properties of the RBC population via laser-diffraction patterns, while ignoring the heterogeneity or size differences within samples.¹⁵ More recently, micropipette aspiration and optical tweezer techniques have been developed to give a single-cell test. Unfortunately, the measurements depend on the conditions of the experiments.¹⁵ Furthermore, they are highly time-consuming, have excessive technical requirements, and can only represent the mechanical properties of the RBCs in static conditions. To obtain the mechanical properties of RBCs under flow, microfluidic devices have been developed, which can test a large number cells and focus on single-cell variations. For example, Shin *et al.* developed the RheoScan-D to measure RBC deformability, which combined microfluidic and ektacytometer.¹⁶ However, it is an ektacytometry method substantially and needs laser and special buffer. Huang and colleagues⁸ have developed a simply microfluidic device with arrays of triangular pillars in parallel capillary channels to evaluate RBC deformability via the time required to pass through the gaps (from 3 to 4 μm). Cluitmans *et al.*¹³ and Zheng *et al.*¹⁴ designed microchannels to detect the RBCs deformability under flow. However, these microfluidic devices are based on high-speed cameras and complex microfluidic designs, which make them difficult to operate and restrict their application.^{13,14}

Recently, Zheng *et al.*¹⁷ and Alapan *et al.*¹⁸ developed simple microchannels to test the mechanical properties via the stretching of adherent RBCs under flow. However, the shear environment cannot simulate the shear stress in microcirculation factually, and the analyzed cell number is small. Inspired by them, in this study, we improved the convenient analytical method on the basis of adhesion model and a simple microfluidic device mimicking physiological microcirculation. The advantages of this method are the small sample needed (10 μl RBC suspension with hematocrit (Hct) at 2%), high-throughput (hundreds of cells in each test), and ease of use. Combined with mathematic models, changes in several mechanical parameters, such as deformability distribution, morphology distribution, normalized curvature, and membrane elasticity under physiological shear flow (1384 s^{-1}), can be obtained. And we first present that the normalized curvature could represent the surface charge of RBCs better. The results demonstrate that the present approach is able to give the meaningful results in terms of membrane changes of RBCs under physiological shear and distinguish RBCs that have had different storage times.

II. EXPERIMENTAL METHODS

A. Animals

All the studies were performed with permission of the Laboratory Animal Center of the Academy of Military Medicine Sciences (No. IACUC of AMMS-2014-028). The male C57 mice (25–30 g) were purchased from Vital River Laboratories (Beijing, China) and were given access to food and water *ad libitum* until the experiments.

B. RBC sample preparation

The C57 mice were anaesthetized through intraperitoneal injection using sodium pentobarbital (Peking Chemical Agent Co., Beijing, China) (57.5 mg/kg) and put on a heating pad (TMS-202, Softron, Beijing, China) in a supine position to be kept at 37 °C. The blood collection and storage was performed as described by Yu *et al.*¹⁹ Briefly, the blood was withdrawn

aseptically by cardiac puncture and mixed with CPDA-1 (Sigma-Aldrich, St Louis, MO) to give a final concentration of CPDA-1 to 14%. The blood was leukoreduced by passing through a leukoreduction filter (BengBu Medical College, China), centrifuged for 15 min at 400 g to remove the supernatant, and stored at 4 °C.

C. Biochemical and hematologic changes in stored RBCs

The concentrations of Na^+ and Cl^- of the fresh RBCs (FRBCs, 0d) and SRBCs (for 7d and 14d) were tested using a blood gas analyzer (Radiometer ABL90COOX, Copenhagen, Denmark). Via the same test, we can get Hct and total hemoglobin concentration (tHb) to calculate the hemolysis. On 0d and 14d of storage, the free hemoglobin concentrations (FHb) were measured using a kit (Nanjing Jiancheng Bioengineering Institute, China), which was used to calculate the hemolysis using the formula

$$\text{Hemolysis (\%)} = \text{FHb} \times (1 - \% \text{Hct}) / \text{tHb}.$$

The mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the red blood cell volume distribution width (RDW) of the FRBC and SRBC were obtained via a Multispecies Hematology Systems (HEMAVET 950, Drew Scientific Ltd., Cumbria, UK). The zeta potential of stored RBCs was tested via Zeta sizer (Malvern, UK) in 0.09% NaCl/0.5% Glucose after storing for 0d, 3d, 7d, 10d, and 14d.

D. Microchannel fabrication and experimental setup

The microchannel was made using soft lithographic techniques. An SU-8 photoresist structure was fabricated on a silicon wafer. The PDMS (polydimethylsiloxane) (Dow Corning, Midland, MI, USA), mixed with an initiator, was molded against the silicon wafer and then adhered to a microscope slide (Thermo Fisher Scientific, Somerset, NJ, USA) via $-\text{OH}$ to assemble the microfluidics device with straight microchannels (1 cm long, 500 μm wide, and 17 μm high), whose area of section is approximately equivalent to a microvessel with a 100 μm diameter (Fig. 1(a)). Every microfluidic device had four sealed microchannels. Before testing, the microchannel was coated with Poly-L-Lysine (PLL) (0.01% w/v, molecular weight 150 000–300 000, Sigma-Aldrich, St Louis, MO) at 37 °C for 30 min. Phosphate buffered saline (PBS) was injected to wash the microchannel, followed by air to dry it. The experimental procedure is shown in (Fig. 1(b)). The stored RBCs (0d, 7d, and 14d) with 2% Hct were perfused and adhered to the substrate of the microchannels. A computer-controlled syringe pump (Harvard Apparatus, Holliston, MA, USA) was used to infuse PBS into the microchannel at a rate of 120 $\mu\text{l/h}$ for 30 min to wash the suspended RBCs away and simulate the physiological flow. The corresponding shearing rate (r') is 1384 s^{-1} according to the equation

$$r' = 6Q/WH^2, \quad (1)$$

where Q is the total flow rate, W is the width of the microchannel, and H is the height. All experiments were carried out at 25 °C. The images were obtained via an inverted biological microscope (XDS-1B, COIC, China) under a 40 $\times$ long working distance objective lens (LPL40/0.60 160/1.5). After shearing for 30 min, the photos of stretched RBCs were taken. The images of stretched RBCs were further evaluated via software to extract the geometrical shape. Meanwhile, the fresh RBCs with 1% Hct were infused into the microchannel to obtain the original morphology of fresh RBCs at static (Fig. 1(c)).

E. Data analysis

1. Deformability index (DI), morphology distribution, and normalized curvature

Before analysis, the images need to be preprocessed. During shooting, we adjusted the microscope to ensure the gray value is appropriate to separate the RBCs from the background.

Then, the images were turned into binary mask representation via Image J. In order to insure the accuracy, the incomplete and aggregated RBCs were wiped off. Finally, the preprocessed images were imported into the software Celltool to extract the outlines of the RBCs and analyze, as described by Pincus and Theriot.²⁰ Different images in the same experiment were analyzed, respectively, and the results were compared mutually to prevent the possible error. The length (L)/width (W) ratio, representing the DI, of each cell was calculated and represented in a histogram. The cell morphologies were analyzed via principal component analysis (PCA) in the software package. In brief, Celltool aligned the extracted contours automatically and distinguished the characteristic of each RBC. The two principal characteristics of all RBCs (about 95% of total variance) were obtained, and using which as the horizontal and vertical axis, the RBCs were plot on a two-dimensional coordinate system according to their morphologies. Then, the cell contours were divided into 10 equal parts by point ordering (Fig. 1(d)), and the normalized curvature from points 41 to 51 was computed by Celltool automatically. To further evaluate the correlation between zeta potential and curvature, the RBCs stored for 0d, 3d, 7d, 10d, and 14d were evaluated again.

2. Calculation of membrane elasticity

The Young's modulus (E), which represents membrane elasticity, was calculated according to the mathematical model established by Hochmuth *et al.*²¹ The RBC stretches as a rectangular

FIG. 1. Schematic of the microfluidic device, the experiment program, and the graphical analysis. (a) The structure of the microfluidic device. (b) The experiment program. The stored RBCs were infused and stick to the substrate via PLL. After subjecting to the shear flow, the RBCs were stretched. The images were recorded and analyzed. (c) The fresh RBCs were infused into the microchannel to show the original morphology of RBCs at static. (d) The schematic of dividing the contours by point ordering, using color.

strip. For simplicity, the stress $\tau(x)$ is assumed to be linearly related to the strain $\epsilon(x)$ at any point x on the membrane. Then, the modulus of elasticity E is written as

$$E = \tau(x)/\epsilon(x). \quad (2)$$

It is assumed that the shear stress acting over the surface of the membrane is the same as the shear stress at the wall (τ), and that the net force acting on the RBC is equal to τ times the area of the cell projected onto the surface. So

$$\tau(x) = \tau A(x)/tW(x), \quad (3)$$

where the area $A(x)$ is the integral of the width function $W(x)$ between 0 (the tail edge) and x , and t is the thickness of the membrane (approximately $0.01 \mu\text{m}$ (Ref. 21)).

The local strain in the membrane can be expressed in terms of the displacement function $u(x)$ as follows:

$$\epsilon(x) = du(x)/dx. \quad (4)$$

Integrating Eq. (4) over the entire length of the membrane yields

$$\int_0^L \epsilon(x) dx = u(L) = L - L_0. \quad (5)$$

When Eq. (3) is substituted into Eq. (2) and integrated (see Eq. (5)), we get

$$E = \frac{\tau A(x)}{tW(x)(L - L_0)} = \frac{\tau}{(L - L_0)t} \int_0^L \frac{A(x)}{W(x)} dx. \quad (6)$$

For a rectangular strip, $W(x) = W_0$ and $A(x) = W_0 \times x$. Thus, Eq. (6) becomes

$$E = \frac{\tau L^2}{2t(L - L_0)}. \quad (7)$$

λ , the extension ratio, is defined as the length (L) of the cell at a given τ divided by the length (L_0) of the cell when $\tau = 0$. When written in terms of λ and rearranged slightly, Eq. (7) becomes

$$E = \tau \lambda^2 \left/ \frac{2t}{L_0} (\lambda - 1) \right. \quad (8)$$

The data from the images were substituted into Eq. (8) to calculate E .

F. Statistical analysis

Each experiment was repeated three times, and two visual fields were recorded (about 200 RBCs included in each image) and analyzed in each experiment. Data were analyzed using Statistical Analysis System software (SAS Version 9.3, SAS Institute, USA). A t -test was used to compare the hemolysis between analyses at 0d and 14d. One-way analysis of variance (ANOVA) was used to calculate statistical significance of other parameters among the three groups (0d, 7d, and 14d). When normality and homogeneity of variance assumptions were not satisfied, non-parametric Kruskal-Wallis test was applied. $p < 0.05$ was considered to be statistically significant. The relation between the normalized curvature and the zeta potential of RBCs was evaluated by means of correlative analysis.

TABLE I. The variation in the hemolysis of SRBC (14d).

	FRBC (0d) (n = 3)	SRBC (14d) (n = 3)
Hemolysis	0.10% $\pm$ 0.05%	0.76% $\pm$ 0.11% ^a

^a $p < 0.01$ vs. FRBC.

III. RESULTS

A. Biochemical and hematologic variation of RBCs during storage

The biochemical and hematologic parameters of RBCs were detected to confirm the reliability of the RBC storage model. The hemolysis of SRBCs stored for 14d was under 1%,

FIG. 2. The variation of (a) Na^+ , (b) Cl^- , (c) MCV, (d) MCH, and (e) RDW of RBCs during storage. **, $p < 0.01$ vs. 0d.

which agrees with the results of Yu *et al.*,¹⁹ despite it being significantly higher than that of FRBC ($p < 0.01$, Table I). The concentration of Na^+ decreased with the storage time, while the Cl^- increased. As shown in Figs. 2(a) and 2(b), both the Na^+ and Cl^- concentrations in SRBC (7d and 14d) were significantly different from those in FRBC ($p < 0.01$), while there is no significant difference between the two SRBC groups. There were no apparent changes in MCV and MCH with the storage time (Figs. 2(c) and 2(d)). However, the RDW (Fig. 2(e)) increased significantly after storage ($p < 0.01$ 7d, 14d vs. 0d). There was also no significant difference between the two SRBC groups.

B. Images of stored RBCs before and after shearing

As shown in Fig. 3(a), the RBCs were normal concave disks before shearing. Then, they were stretched by shearing for 30 min. However, the level of stretching was different for each of the three groups. Almost all RBCs were deformed in the 0d group (Fig. 3(b)). Hypodeformable RBCs only appeared after being stored for 7d (Fig. 3(c)), and the number of hypodeformable RBCs increased in the 14d group (Fig. 3(d)).

C. DI distribution and deformability changes with prolonged storage time

The DI value was evaluated, and the histograms are shown in Fig. 4. Fig. 4(a) shows that the DI of FRBC displays an approximately normal distribution, with a median of 1.78. As the storage time increases, the DI decreased significantly. The DI distribution shifted to the left gradually, and the distribution became non-normal (Figs. 4(b) and 4(c)). The median of RBCs DI in the 7d group is 1.49, and this further decreased to 1.28 in the 14d group. The DI cumulative curve (Fig. 4(d)) showed that the RBC storage induced decreased deformability and gave a DI distribution that is significantly different at longer storage times ($p < 0.01$).

FIG. 3. The RBCs morphology in microchannel. (a) The original morphology of fresh RBCs. (b)–(d) The images of stored RBCs subjected to physiological shear flow.

FIG. 4. The distribution of DI with the storage time. (a)–(c) The distribution of DI at 0d, 7d, and 14d, presented by the percentage of RBC at a discrete value. (d) The DI cumulative curve.

D. Morphology distribution of stored RBC after shear

Base on PCA, Celltool makes a morphology model with enough “principal morphology modes” to account about for 95% of the total variance (Fig. 5(a)). Among them, mode 1 and mode 2 represent the two most important dimensions of the RBC that give differential morphologies between the three groups. The first, a combined size and shape mode that appears to reproduce the cell cycle, accounts for 66.2%, and the second, which describes how bent any individual cell is, accounts for 28.1%. The software then exports the image (Fig. 5(b)), which shows the distribution of RBCs after different storage times. Mode 1 could better distinguish the RBC morphology among three groups, while mode 2 could not. In detail, the RBCs in the 0d group mainly present on the left two quadrants and mean the cells’ shapes close to the black line of mode 1 in Fig. 5(a). However, the storage groups (7d and 14d) display variant morphologies. After storing for 7d, most of the RBCs appear in the right two quadrants, meaning they have a shorter long axis. The RBCs in the 14d group mainly appear on the right lower quadrant and have further variations. The cells’ shapes in 7d and 14d group more close to the yellow line of mode 1 in Fig. 5(a). Most RBCs appear circular and smaller, which mean they are hypo-deformable RBCs under shear. Mode 2 cannot distinguish the RBC morphology among three groups because the cells plot at the same position of vertical axis.

E. The normalized curvature variation of stored RBC

The normalized curvature distributions of the RBCs were evaluated, and the histograms are presented in Fig. 6. The normalized curvature decreased significantly with storage time. The curvatures of RBC stored for 7d and 14d were lower than for 0d ($p < 0.01$), and there is also a significant difference between 7d and 14d ($p < 0.01$). The curvature cumulative curve (Fig. 6(d)) showed that the RBC storage induced decreased normalized curvature and gave a normalized curvature distribution that is significantly different at longer storage times ($p < 0.01$). The curves were used to represent the surface charge of the RBCs and imply the negative surface charge decreases significantly with storage.

FIG. 5. PCA analysis of RBCs. (a) The three principal modes and contributions. (b) The distribution of the morphology of RBCs along with the first two principal models. The horizontal axis represents mode 1, and the vertical axis represents mode 2. The morphology of each cell in the three groups is plotted at its appropriate location. 0d, blue; 7d, orange; and 14d, green.

F. The relation between normalized curvature and zeta potential of RBCs

As shown in Fig. 7, the zeta potential increased significantly with storage time. The zeta potential of RBC stored for 7d was higher than for 0d and 3d ($p < 0.05$), and there is also a significant difference between 7d and 10d, 14d ($p < 0.05$). The curvature and the zeta potential showed a good linearity relationship ($r = -0.9730$).

G. Changes in the calculated Young's modulus with storage time

At a given volume flow rate Q ($120 \mu\text{l/h}$), the shear stress τ is given by

$$\tau = \eta r', \quad (9)$$

where η is the fluid viscosity (approximately 0.001 Pa s). Hence, when Eq. (1) is substituted into Eq. (9), τ is 1.384 Pa . L_0 is analyzed in the present study as $6.5 \mu\text{m}$. In our experiments, λ is calculated as 1.30 ± 0.04 in the 0d group, 1.14 ± 0.09 in the 7d group, and 1.13 ± 0.05 in the 14d group. The substitution of these data into Eq. (8) gives

$$E_{0d} = 2.54 \pm 0.17 \text{ kPa}; \quad E_{7d} = 4.83 \pm 1.27 \text{ kPa}; \quad E_{14d} = 5.54 \pm 2.87 \text{ kPa}.$$

And the Young's moduli in 7d and 14d group are significantly higher than it in 0d group ($p < 0.01$).

IV. DISCUSSION

In the present study, we use the RBCs of C57 mouse to study the mechanical properties of stored RBCs. The RBCs have a lifespan of ~ 120 days in humans and about ~ 45 days in

FIG. 6. The normalized variation of the curvatures of the RBCs. (a) 0d, (b) 7d, (c) 14d, and (d) the curvatures cumulative curve.

mice.^{22,23} Gilson *et al.*²⁴ as well as Hod *et al.*²⁵ reported that after 14 days of storage in CPDA-1, murine RBCs had an *in vivo* 24-h RBC recovery of 64% to 65%, which is similar to the data from human RBCs stored for 42 days.²⁶ Yu *et al.* reproduced the mature model, characterized the biochemical and functional effects, and studied the mechanisms responsible for the adverse effects of transfusing stored RBCs.¹⁹ In the present study, we reproduced the model to detect the mechanical properties via the geometry of stretched RBCs. Although the diameter of mice RBCs is smaller than that of human RBC,²⁷ no data could verify the difference of mechanical properties between mice and human.

FIG. 7. The variation of the zeta potential of stored RBCs and its' relation with curvature. (a) Changes of zeta potential of stored RBCs and (b) the correction between the zeta potential and the curvature. *, $p < 0.05$ vs 0d; &, $p < 0.05$ vs 3d; and #, $p < 0.05$ vs 7d.

A. Advantages of the present method

We have developed a microfluidic approach that is combined with a mathematic model to evaluate the mechanical properties of stored RBCs. This method requires a small sample volume, has high-throughput, and is easy-to-use. First, only 10 μl of RBC suspension with a Hct of 2% is required in the present method, while the ektacytometry needs 30 μl RBC suspension with a Hct of 40%. Second, it is capable of observing hundreds of cells and recording the change in each RBC in a single visual field, which is also superior to ektacytometry. Third, being easy-to-use is an often overlooked advantage of the present method. The RBCs could be measured within 30 min. A special buffer called polyvinyl pyrrolidone (PVP) with high viscosity must also be used in ektacytometry. The pipette aspiration requires the RBC to be detected one at a time. Hence, the present method based on a microfluidic device measures the mechanical properties of RBCs under shear flow and is suitable for the online detection during RBC storage.

In comparison to other microfluidic methods in the literature, some aspects of our designs ensure the method is convenient and effective. First, the present method does not rely on a high-speed camera and does not have a complex microfluidic design. Researchers only need an ordinary biological microscopy to record the RBCs stretched by physiological shear flow. The simple, straight microchannel of the device makes it easy-to-use. Second, the design of the present method is significant to represent the mechanical properties of the RBCs under shearing. (1) Korin *et al.*²⁸ have also found that the DI varied with the RBC layer depth in a microchannel. In our system, the RBCs were adhered to the substrate, which removes the depth effect. (2) The data analysis showed that when flowing in the microchannel, most of the RBC gathered in a small region of approximately 15 μm from the center line, independent of the flow rate.²⁹ The height of the present microchannel is 17 μm , which ensures a steady velocity. (3) The microfluidic devices could evaluate cells under flows that accurately mimic those in microcirculation. Cluitmans and colleagues¹³ suggested that the storage did not alter the RBC deformability capacity during passage through the capillary system using a stenosis microfluidic. Zheng and colleagues¹⁴ also found no significant changes of deformability, but a decrease in relaxation capacity after storage via applying 2.5 kPa to drive RBC flow in microchannels. We guess that the nature of the applied shear stress can affect the results.² Hence, the shear rate and the flow velocity in the microchannel must be taken into account. The flow-induced shear rate changes greatly among vessels. Tangelder *et al.* have reported that least estimates of wall shear rate ranged from 472 to 4712 s^{-1} with a median value of 1700 s^{-1} in mesenteric arterioles of rabbit.³⁰ RBC center-line velocities varied between 1.3 and 14.4 mm/s (median 3.4).³⁰ The present study using PLL to coat the substrate and ensure RBC can endure a physiological shear rate (1384 s^{-1}) for enough time and not detach, produce the approximate physiological condition in arteriole. Under these conditions, the computational flow velocity is 3.9 mm/s, which is close to that in the arteriole (~ 3.4 mm/s). Hence, the present approach evaluates the mechanical properties of the RBCs conveniently and effectively.

B. Explanation of the results and comparison to the literature

Using the present approach, we have evaluated the mechanical properties of stored RBCs, including DI distribution, morphology distribution, surface charge, and Young's modulus (E). The results are in accordance with the literature and can distinguish the RBCs depending on their storage time.

Deformability, which was first recognized by van Leeuwenhoek in 1675,⁵ is a critical mechanical parameter of RBCs and studied widely. Decreases in RBC deformability can disturb blood flow and oxygen delivery.^{4,5} Hence, the deformability is a significant measure in evaluating the quality of RBCs. The decreased DI value of stored RBCs presented in our results corresponds to the results from previous studies.^{11,31} Moreover, we also observed the DI distribution changed with the storage time. The DI distribution of FRBCs (0d) is nearly symmetrical and close to a normal distribution, while in SRBCs (7d and 14d) it is significantly non-normal and

shows an increase in hypodeformable cells. Therefore, the DI distributions may better represent the changes in the mechanical properties of RBCs than mean deformability.⁶ Using the present approach, we have obtained the DI values and distributions of RBCs, and the results effectively distinguished the different storage times of the RBCs.

Further, the RBCs morphology distribution under physiological shear was analyzed after different storage times. The morphology distribution appears to be helpful in illustrating variations in the mechanical properties. The results show that the fresh RBCs (0d) display a uniform stretched morphology, which mean the fresh RBCs possess a healthy membrane and cytoskeleton, and deformed normally when subjected to physiological shear rates. In the PCA analysis, the RBCs' location shifts to the right in the 7d group, which means the deformability has decreased. With even longer storage times, the RBCs undergo further, irreversible damage to the membrane and cytoskeleton, and vesiculation occurs, which reduce the area-to-volume ratio and alter the cell shape to spherical and spinous.³² Therefore, in 14d group, the RBCs were smaller and more hypodeformable. The morphology distributions of the stored RBC agree with the DI and could visually distinguish the RBCs with different storage times.

The numerical curvatures of RBCs were also calculated and showed significant decreases with storage times. Chien *et al.*³³ studied the relationship between geometric parameters and membrane properties via the adhered RBCs in a flow channel. They observed that each RBC showed a single small area of attachment when elongated. Thus, it is possible that the curvature of the RBCs' tail could represent the adhesive force between the cells and the substrate. The adhesive force in the present experiment depends on the attraction between the negative charge of the RBCs' surface and the positive charge of the substrate from the PLL. Therefore, we hypothesize that the downstream curvature of the RBCs under flow could represent the surface charge. We calculated the curvature, tested the zeta potential of stored RBCs, and evaluated the relationship between them. Our data show the curvature of the RBCs decreased significantly during storage, which is in negative correlation with the increased surface charge.³⁴ These results support our hypothesis and make the numerical curvatures of adherent RBCs under flow become a novel mechanical parameter to represent the surface charge.

The membrane properties are the critical factors in determining the deformability and morphology of the RBCs.^{5,35} During storage, oxidative stress profoundly damages the membrane of the RBCs.³⁶ This can induce membrane lipid peroxidation and protein cross-linking.³⁷ The further oxidation of hemoglobin results in the formation of hemichromes, which bind to the membrane and cause further changes in the membrane.³⁸ Finally, the vesiculation occurs, the RBCs lose part of their membrane and become smaller and more spherocytic.³² These variations also arise in our RBCs model. The changes of ion concentration suggest that the membrane proteins in control of ion-exchange are damaged specifically. For example, the increased Cl^- in extracellular solution suggests that band 3, the major membrane proteins mediating the exchange of Cl^- and HCO_3^- ,³⁹ is damaged. Significantly decreased RDW suggests that the morphology of the RBCs changes during storage. All of the variations indicate membrane damage and increased rigidity. Therefore, evaluating the membrane elasticity is important and could represent a series of lesions during the storage of RBCs. In a recent study, an atomic force microscope (AFM) was used to evaluate the Young's modulus of the cell membrane, despite difficulties in detecting the suspended RBCs. Moreover, it was reported that the Young's modulus of 4.0 kPa at low levels of deformation could be as high as 300 kPa at a higher levels of deformation,⁴⁰ which means the Young's modulus of RBCs has a nonlinear correlation with the extension ratio. Therefore, both the detection and the analysis of RBCs using AFM detection need further study. In the present study, we have developed a microfluidic approach combined with a classical adherence model²¹ to calculate the Young's modulus of stored RBCs. Under the applied shear stress, the extension ratio (λ) detected in the present experiment is 1.13–1.30, which fits the whole cell stretching model.²¹ The calculated Young's modulus of FRBC is 2.54 kPa, which agrees with the result from Ref. 21. Through this calculation, we found that under physiological shear, the Young's modulus of SRBC increased with storage, which means a more rigid membrane and decreased deformability. Moreover, the calculated Young's modulus of stored RBCs suggests progressive damage to the membrane and cytoskeleton proteins

during storage. The mechanical properties of RBCs depend on their cytoskeletal protein networks.⁴¹ The RBC cytoskeleton network is predominantly composed of spectrin tetramers, actin, and protein 4.1R, along with adaptor proteins,³⁶ which be attached to membrane by Band 3 via ankyrin.⁴² The cytoskeleton network plays a crucial role in maintaining the normal morphology of the RBC, which allows RBCs to deform and pass through narrow capillaries.⁴³ Disrupting the network via protein 4.1R phosphorylation to alter its spectrin-actin binding affinity results in a significant increase in the Young's modulus.⁴¹ During the first days of storage, the oxidation of cytoskeletal proteins 4.2 and 4.1, spectrin, and band 3 is observed. These are followed by breakdown of actin, GAPDH, band 4.9, and ankyrin, as well as the cross-linking of spectrin.⁴⁴ Therefore, the membrane becomes unsteady and rigid and shows an increased Young's modulus, as in our results.

C. Possible applications of the method

These results confirm that our microfluidic approach combined with mathematic model revealed the distribution of the mechanical properties of RBCs under physiological shear flow, easily and effectively distinguishing RBCs with different storage times. Based on the advantages of this method, it could be applied to online tests of the mechanical properties of RBCs during blood collection, processing, storage, and pre-transfusion, which would help evaluate the effect of preservative solutions and operational methods. Moreover, this method also could help to evaluate the hemocompatibility of plasma expanders, diagnose some RBC-related diseases, such as malaria, sickle cell anemia, and hereditary disorders, and estimate therapy effects.

V. CONCLUSION

In this paper, we have developed a simple and capillary-mimetic microfluidic device which provides a physiological shear flow via the immobilization of RBCs. The device can yield effective information on the mechanical properties via single cell detection, without the need for any advanced device, such as high-speed cameras. The present method is simple, convenient, and high-throughput, and could distinguish RBCs with different storage times via the distribution of several mechanical properties, including deformability distribution, morphology distribution, surface charge, and Young's modulus. These advantages make it a promising prospect in quality online monitoring of RBCs.

VI. PERSPECTIVE

The prospective experiments are required to promote the application of this method. (1) According to conventional studies, methodology validations should be performed to demonstrate the sensitivity and accuracy of the present method. (2) The method should be optimized focusing on convenience. Automation technology should be used to further shorten the testing time. (3) Applying the present method to perform the large scale testing to determine the reference ranges of mechanical properties in normal and pathological RBCs. Via further experiments, we expect that this simple microfluidic detection and analytical method will be used in online RBCs detection.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 31271001) and a grant from the National High Technology Research and Development Program of China (No. 2012AA021902).

¹O. Yalcin, D. Ortiz, A. G. Tsai, P. C. Johnson, and P. Cabrales, *Transfusion* **54**, 1015 (2014).

²N. Nemeth, F. Kiss, and K. Miszti-Blasius, *Scand. J. Clin. Lab. Invest.* **75**, 213 (2015).

³G. K. Driessen, C. W. Haest, H. Heidtmann, D. Kamp, and H. Schmid-Schonbein, *Pflügers Arch.* **388**, 75 (1980).

⁴K. Parthasarathi and H. H. Lipowsky, *Am. J. Physiol.* **277**, H2145 (1999).

⁵N. Mohandas and J. A. Chasis, *Semin. Hematol.* **30**, 171 (1993).

- ⁶J. G. G. Dobbe, M. R. Hardeman, G. J. Streekstra, J. Strackee, C. Ince, and C. A. Grimbergen, *Blood Cells, Mol., Dis.* **28**, 373 (2002).
- ⁷J. P. Mills, M. Diez-Silva, D. J. Quinn, M. Dao, M. J. Lang, K. S. W. Tan, C. T. Lim, G. Milon, P. H. David, O. Mercereau-Puijalon, S. Bonnefoy, and S. Suresh, *PNAS* **104**, 9213 (2007).
- ⁸S. Huang, A. Undisz, M. Diez-Silva, H. Bow, M. Dao, and J. Han, *Integr. Biol. (Camb.)* **5**, 414 (2013).
- ⁹D. J. Kor, C. M. Van Buskirk, and O. Gajic, *Bosnian J. Basic Med. Sci.* **9**, S21 (2009).
- ¹⁰M. Bessis, N. Mohandas, and C. Feo, *Blood Cells* **6**, 315 (1980).
- ¹¹P. L. La Celle, *Transfusion* **9**, 238 (1969).
- ¹²S. Hénon, G. Lenormand, A. Richert, and F. Gallet, *Biophys. J.* **76**, 1145 (1999).
- ¹³J. C. Cluitmans, V. Chokkalingam, A. M. Janssen, R. Brock, W. T. Huck, and G. J. Bosman, *BioMed Res. Int.* **2014**, 764268.
- ¹⁴Y. Zheng, J. Chen, T. Cui, N. Shehata, C. Wang, and Y. Sun, *Lab Chip* **14**, 577 (2014).
- ¹⁵T. Giovanna, *Biomicrofluidics* **8**, 051501 (2014).
- ¹⁶S. Shin, J. X. Hou, J. S. Suh, and M. Singh, *Clin. Hemorheol. Microcirc.* **37**, 319 (2007).
- ¹⁷Y. Zheng, M. A. Cachia, J. Ge, Z. Xu, C. Wang, and Y. Sun, *Lab Chip* **15**, 3138 (2015).
- ¹⁸Y. Alapan, J. A. Little, and U. A. Gurkan, *Sci. Rep.-UK* **4**, 7173 (2014).
- ¹⁹B. Yu, C. Lei, D. M. Baron, A. U. Steinbicker, K. D. Bloch, and W. M. Zapol, *Transfusion* **52**, 1410 (2012).
- ²⁰Z. Pincus and J. A. Theriot, *J. Microsc.* **227**, 140 (2007).
- ²¹R. M. Hochmuth, N. Mohandas, and P. L. Blackshear, Jr., *Biophys. J.* **13**, 747 (1973).
- ²²Y. Gottlieb, O. Topaz, L. A. Cohen, L. D. Yakov, T. Haber, A. Morgenstern, A. Weiss, K. C. Berman, E. Fibach, and E. G. Meyron-Holtz, *Haematologica* **97**, 994 (2012).
- ²³S. Wang, G. L. Dale, P. Song, B. Viollet, and M. H. Zou, *J. Biol. Chem.* **285**, 19976 (2010).
- ²⁴C. R. Gilson, T. Kraus, E. A. Hod, J. Hendrickson, S. L. Spitalnik, C. D. Hillyer, B. Shaz, and J. C. Zimring, *Transfusion* **49**, 1546 (2009).
- ²⁵E. A. Hod, N. Zhang, S. A. Sokol, B. S. Wojczyk, R. O. Francis, D. Ansaldi, K. P. Francis, P. Della-Latta, S. Whittier, S. Sheth, J. E. Hendrickson, J. C. Zimring, G. M. Brittenham, and S. L. Spitalnik, *Blood* **115**, 4284 (2010).
- ²⁶L. J. Dumont and J. P. AuBuchon, *Transfusion* **48**, 1053 (2008).
- ²⁷K. Namdee, M. Carrasco-Teja, M. B. Fish, P. Charoenphol, and O. Eniola-Adefeso, *Sci. Rep.-UK* **5**, 11631 (2015).
- ²⁸N. Korin, A. Bransky, and U. Dinnar, *J. Biomech.* **40**, 2088 (2007).
- ²⁹A. Bransky, N. Korin, Y. Nemirovski, and U. Dinnar, *Biosens. Bioelectron.* **22**, 165 (2006).
- ³⁰G. J. Tangelder, D. W. Slaaf, T. Arts, and R. S. Reneman, *Am. J. Physiol.: Heart Circ. Physiol.* **254**, H1059–H1064 (1988).
- ³¹E. Bennett-Guerrero, T. H. Veldman, A. Doctor, M. J. Telen, T. L. Ortel, T. S. Reid, M. A. Mulherin, H. Zhu, R. D. Buck, R. M. Califf, and T. J. McMahon, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 17063 (2007).
- ³²B. Blasi, A. D'Alessandro, N. Ramundo, and L. Zolla, *Transfus. Med.* **22**, 90 (2012).
- ³³S. Chien, L. A. Sung, M. M. Lee, and R. Skalak, *Biorheology* **29**, 467 (1992).
- ³⁴D. C. Silva, C. N. Jovino, C. A. Silva, H. P. Fernandes, M. M. Filho, S. C. Lucena, A. M. Costa, C. L. Cesar, M. L. Barjas-Castro, B. S. Santos, and A. Fontes, *PLoS One* **7**, e31778 (2012).
- ³⁵O. K. Baskurt and H. J. Meiselman, *Semin. Thromb. Hemostasis* **29**, 435 (2003).
- ³⁶A. G. Kriebardis, M. H. Antonelou, K. E. Stamoulis, E. Economou-Petersen, L. H. Margaritis, and I. S. Papassideri, *J. Cell. Mol. Med.* **11**, 148 (2007).
- ³⁷T. Yoshida and S. S. Shevkoplyas, *Blood Transfus.* **8**, 220 (2010).
- ³⁸R. Kannan, R. Labotka, and P. S. Low, *J. Biol. Chem.* **263**, 13766 (1988).
- ³⁹H. Passow, *Rev. Physiol., Biochem. Pharmacol.* **103**, 61 (1986).
- ⁴⁰K. E. Bremmell, A. Evans, and C. A. Prestidge, *Colloids Surf., B* **50**, 43 (2006).
- ⁴¹F. Tang, X. Lei, Y. Xiong, R. Wang, J. Mao, and X. Wang, *J. Biomech.* **47**, 3400 (2014).
- ⁴²V. Bennett and P. J. Stenbuck, *Nature* **280**, 468 (1979).
- ⁴³D. Kabaso, R. Shlomovitz, T. Auth, V. L. Lew, and N. S. Gov, *Biophys. J.* **99**, 808 (2010).
- ⁴⁴G. M. D'Amici, S. Rinalducci, and L. Zolla, *J. Proteome Res.* **6**, 3242 (2007).