Enhanced Separation of Aged RBCs by Designing Channel Cross Section

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Prolonged storage will alter the biophysical properties of red blood cells (RBCs), and it decreases the quality of stored blood for blood transfusion. It has been known that less deformable aged RBCs can be separated by margination, but the recognition of the storage time from the separation efficiency of the stiff RBCs is still a challenge. In this study, we realized enhanced separation of aged RBCs from normal RBCs by controlling the channel cross section, and demonstrated that the storage time can be deduced from the percentage of the separated RBCs in the stored RBCs. This separation technology help to reveal the regulation of time on RBC aging mechanism, as well as offer a new method to separate stiffened cells with high efficiency.

I.INTRODUCTION

Red blood cells (RBCs) undertake the function of delivering oxygen to the body tissues, and the deformability of RBCs enables them to get through small capillaries.[1] The deformability of RBCs can be altered naturally by various pathophysiological conditions, including malaria,[2] sickle cell disease,[3] diabetes,[4] sepsis,[5] as well as artificially by chemicals.[6-8] RBCs will lose deformability with growing age when they are circulating in venules,[9] until hemolysis or cleaned by spleen. Recently, the deformability of stored RBC raises much concern for safe blood transfusion.[10-11] Transfusion with high-quality is essential to modern health care,[12] however, known as the "storage lesion",[13-15] complex biochemical and physiological changes caused in the storage process increase the transfusion risks. Significant loss of deformability will happen after the RBCs are stored above 14 days, and the loss is not reversible.[11] The aged RBCs after storage will be harmful to the blood transfusion, with the increasing risk of RBC retention, thrombosis and haemolysis.[16] Moreover, the extracted blood contains RBCs with different ages, the old RBCs will lose most of their deformability and have extreme high stiffness in the same storage time. These aged RBCs would be especially harmful to the blood transfusion. Recent microfluidic work on RBCs also emphasized the effect of RBCs deformability in health and disease[17-19]. Therefore, it is necessary to know the dependence of deformability on the storage time and separate the aged RBCs from the blood for safer blood transfusion.

Margination is originally used to describe the migration of leukocyte and platelets to the vessel wall in blood vessel, and the margination mechanism has been revealed in many ways.[20-23] Size, shape and deformability affect the margination performance of objects in shear flow, and the margination theory has been applied on the study of cell sorting technology and drug delivery i*n vivo* and *in vitro*. As a viscoelastic fluid, blood in vessel offers the first normal stress for rigid objects to perform

margination. Under the effect of the first normal stress, RBCs will approach the wall in a shear flow, the interaction between a soft membrane and the wall generates a high lift force that will pushes the cell away from the wall.[20] While, a rigid RBC lacks such a high lift force to push it away from the wall, resulting the separation from soft RBCs and the realization of margination. As a rigid particle, drug delivery processes in blood are also largely dependent on margination theory.[23] As a new technology, microfluidic devices offered a fast and simple method for cell sorting with high efficiency and low cost. Recently, the separation of stiffened cells has been realized in microfluidic devices based on the margination theory. It has been used to separate diseased RBCs for better diagnosis and therapy, for instance, the purification of malaria-infected RBCs and sepsis-infected RBCs from normal RBCs.[24-25] The stiffened RBCs would perform lateral motions toward the channel wall under the first normal stress generated in the viscoelastic RBCs suspension or polymer solution.[24] Also, the deformability-based cell sorting method was applied to enrich less deformable RBCs from stored blood.[26] Yet, the sorting efficiency is not high enough for the next commercial application, and it is still unclear that whether the storage time affect the separation efficiency. It inspires us to study the enhanced separation of aged RBCs and the recognition of the storage time from the separation efficiency of aged RBCs.

In this paper, particles performances in different viscoelastic fluids were firstly investigated to find the best flow condition for the separation of aged RBCs. Microchannels with different cross section shape were then designed to obtain the enhanced separation efficiency. It should be noted here that traditional rectangular microchannel is compared to a new designed triangular microchannel on the separation efficiency, and the triangular channel is demonstrated to be a better one to separate and detect aged RBCs. Furthermore, RBCs performances in triangular microchannel was compared to investigate the relationship between storage time and separation efficiency, and the mechanism was clarified by analyzing the RBCs' modulus changing pattern over storage time. This research may not only help to understand the metabolism of RBCs during their span life, but also bring us a new insight of the cell separation technology to reduce the risk in blood transfusion.

II. METHODS

A. Microfluidic device.

Devices with triangular cross section were fabricated using a metal mode, as shown in Figure 1(a). The cross section shape was controlled through mechanical processing to an isosceles triangle with bottom angel of 30°, and the base length is 100μm. The liquid PDMS was poured on the mode, and after air exhausting, the mode with PDMS was put on the hot plate with temperature of 150°C for baking.5mins later, PDMS channel was solidified and removed from the metal mode. This PDMS mode was then bonded with a PDMS slide with height less than 1mm using a plasma gun (BD-20ACV, Electro-Technic). After 30mins baking at the temperature of 100°C, the microchannel with triangular cross section was fabricated. Devices with rectangular cross section were fabricated using the standard polydimethylsiloxane (PDMS) lithographic method.[27] The structure of the microfluidic device was shown in Figure 1(b). The height of the microchannel used in Figure 2 is 10μm and in Figure 3 is 25μm. The microchannel has 3 sections, the first section is the inlet with a converge shape. The second section is a straight microchannel with the widths of 45μm for Figure 2 and 100μm for Figure 3. The third section has a diverge shape and it is the outlet of the constriction channel. The top views at these three

sections show the margination process of particles in rectangular channel, and the cross section views (inserted picture i and ii) show the effect of margination on particles' distribution in different sections of the channel. Moreover, there are three reservoirs at the outlet of the device, distributed in the side and center of the channel to collect the separated RBCs suspensions. The separated particles were observed at this section, as the inserted picture (iii) show in Figure 1(b). Before use, all the channels were pumped into PBS solution with 2.5g/l BSA to prevent cell adhesion. And the mean flow rate was controlled as 1mm/s in all flowing experiments.

B. Particle sample.

Fluorescent particles (polystyrene-based, 7%v/v, Excitation max=441nm, Emission max=486nm, Polysciences, Inc.) with the diameter of 6μm were mixed with PBS buffer, PEO solution, as well as the RBCs solution, at the concentration of 0.03%v/v, resulting a ratio of RBCs (30%Hct) to particles in the sample is 1000 (30%/0.03%), which matches the ratio of RBCs to leukocytes in the blood. The shear modulus of the particles is 700-800MPa. and the size of particle is chosen to match the size of RBCs to obtain cell sorting condition for cell margination without size difference effect.[28]

C. PEO solutions.

PEO is a commonly used polymer to enhance the viscoelasticity of fluid in the study of particle margination, and its viscoelasticity has been well analyzed in previous research.[28-29] Polyethylene oxide (PEO, Mw=4000000, Sigma Aldrich) was suspended in phosphate-buffered saline (PBS, 1X without Ca^{2+} and Mg^{2+}, Hyclone) buffer with the concentration of 0.2%wt to achieve the PEO solution.

D. RBCs solutions.

The blood used in this experiment were taken from healthy adults and washed by PBS buffer on a centrifuge (Eppendorf AG) at 3000r/min for 75s each time, and the centrifuge was repeated 3 times to get the packed RBCs. The packed RBCs were re-suspended in PBS buffer to achieve the RBCs solution with the corresponding hematocrit (Hct) for the next experiment, and 30%Hct were used in Figure 2.

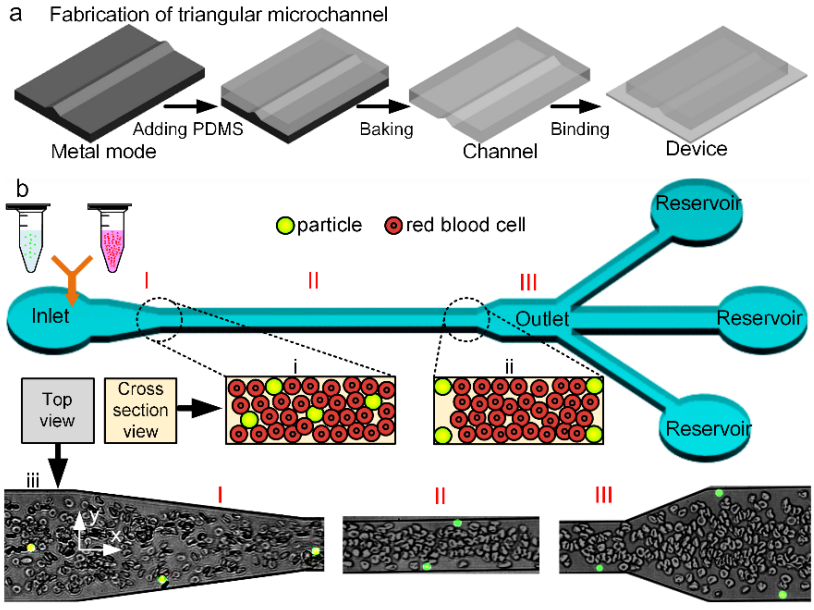

FIG.1. The schematics of the microfluidic device for the margination experiment. (a) The fabrication process

of triangular microchannel. (b)The structure of the rectangular channel, and the inserted figures of (i) and (ii) illustrate the distribution of the particles in different sections of the channel, and (iii) shows the observed margination of the particles at three sections of microchannel with red blood cells.

E. Aged RBCs sample.

After washing, the leukocytes and platelets are removed from the fresh taken blood, and then the main part of the packed RBCs are re-suspended in PBS buffer to compose the RBCs solution in point 'D', the other part of the packed RBCs are suspended in PBS with 1mg/ml bull serum albumin (BSA, Sigma), and then stored in a refrigerator at 3~4°C to prepare the aged RBCs sample. The fresh taken RBCs are the 0day RBCs, and it is also called normal RBCs in this paper, and 1day RBCs means the storage time is 24 hours. Before use, the stored RBCs suspension will be washed again using PBS buffer to get the target aged RBCs, and the washing protocol is the same to point 'D'. In order to distinguish the target RBCs from normal RBCs during their flowing in microchannel, before mixing with normal RBCs solution, the target RBCs were stained by 1mg/ml fluorescein isothiocyanate isomer I (Sigma-Aldrich) suspending in PBS buffer. After 3hours incubation in water bash at 37℃, the target RBCs were re-washed using PBS buffer and then they were added into the RBCs solution at the concentration of 0.03%v/v to achieve the aged RBCs sample for sorting experiment.

F. Modulus test process.

The Young's modulus of RBCs was measured to investigate the revolution of RBCs stiffness on storage time. As described in previous study, a standard biological Nanoindenter (Piuma, Optics11, Probe stiffness: 0.46N/m, Tip radius:52.5μm)[30] was applied on this study. Applying the Hertzian model to describe the indentation of elastic RBCs,[31] the Nanoindenter uses loading section of the load-displacement curve to determine the Young's modulus of RBCs during testing. Allowing for the depth of RBCs, a fit of all data points from the contact point to 5% of the maximum load point is chosen to obtain the Young's modulus, and this ratio was set in the testing software before testing, and after test, you will get an automatic fitting modulus value. For experiments, the Piuma Nano-indenter is placed on the top of a regular lab bench, and the testing is automatically performed through the specific software control. As for the testing target, RBCs with different storage time (from 0 day to 29 days) were mixed with PBS buffer at the concentration of 0.2%v/v, and the concentration was chosen to ensure the effect of cell adhesion on the glass slide in the next step. Then the solutions were placed on the glass slide coated by Poly-L-Lysine (LIUSHENG). After 10mins, the glass slides were washed by the PBS buffer, while the cells were kept on the glass slide. PBS buffer was added again on the glass slide to support RBCs, and then, the prepared glass slide with the adhered RBCs was placed on the bottom of indenter, after the indenter merged into the droplet of PBS, more PBS buffer was added to confirm that the measurement could be finished with indenter and RBCs fully merged in PBS buffer.

G. Experiment procedures and image analysis.

The motions of the flowing particles/RBCs in the channel were captured by the fast camera (M710, Phantom Co.) under the fluorescent field provided by an inverted microscope (LEICA DME6000 B), while the background was captured in the bright field at the same position. The frame rate was set as 24fps to get the biggest exposure time of 41000μs of the camera, and under this exposure time, the flowing of fluorescent target RBCs could be clearly captured. First of all, the fast camera was connected

to the microscopy to replace the microscopy's original CCD. Switching the microscopy lens to X20 and regulating the focal length and the observation platform to find the channel, and then the flow of particles and RBCs could be observed from the camera's software. Regulate the parameters of camera to achieve clear flowing of particles and RBCs in microchannel. The motions of particles in PBS buffer and PEO solution were captured under bright field, the motions of particles and target RBCs in RBCs solution were captured under both bright field and fluorescent field. After the shooting, the obtained videos were analyzed through Image J. Specifically, the trajectory of particle in RBCs solution was extracted from a video of particle flowing in RBCs solution. The images of particle in RBCs solution at different frame were output from Image J firstly, and then the images were montaged by using Photoshop software to exhibit the trajectory of particle flowing in RBCs solution. In order to show the relative position of particle to RBCs, the background captured in bright field at the same position was added at the back of the fluorescent image, and the transparency of the fluorescent image was regulated to clearly show the bright image. The final impression drawing is shown in Figure 2(c). As for the sorting efficiency and particles distribution, we marked a position on the channel when playing the video in the Image J, therefore, we could count the target particles and RBCs at the same position. The distance of target particles and RBCs to the channel wall was measured through Image J, and the measuring results were saved into Excel file. Then the data were input into the Origin to obtain the particles distribution. At last, the figures are plotted in Origin.

H. Fluorescent dots observation from reservoir.

The reservoir was fabricated by a hole puncher with diameter of 4mm before the channel was bonding on a PDMS slide. 30μl PBS buffer was pre-added in the reservoirs before experiment. After the experiment, the separated RBCs suspension collected at the reservoir was taken out by the pipette and put on a glass slide to conduct the fluorescent dots observations.

III.RESULTS

A. Margination of particles in RBCs suspension.

To find the best separation condition, the performance of solid particles in PBS, PEO and RBCs solutions were firstly investigated. The fluorescent particles were mixed with three solutions, respectively, and they were pumped into the microchannel by a syringe pump. The particles suspended in the PBS solution and PEO solution were used as the control groups. It is found that particles in PBS solution are randomly distributed across the channel, and the margination does not happen, as shown in Figure 2(a). The margination of particles happens in the PEO solution, and the particles have three equilibrium positions in the channel, the wall on the both sides and the center of the channel, as shown in Figure 2(b). This result is consistent with the previous studies that stiffen particle will perform margination in viscoelastic fluid.[24-25] The rectangular channel width (W) and height (H) is 45μm and 10μm, respectively. Allowing for the definition of hydraulic diameter $D_w=2WH/(W+H)$, the blockage ratio$\kappa=a/D_w=0.37>0.25$, under this flow condition, normal stresses push the particles toward the wall.[28,32] However, the center equilibrium position does not appear for the particles flowing in the RBCs solution, as shown in Figure 1(c). It is considered that the particles staying in the center would be pushed aside by the interactions with RBCs and perform the margination to the channel wall.

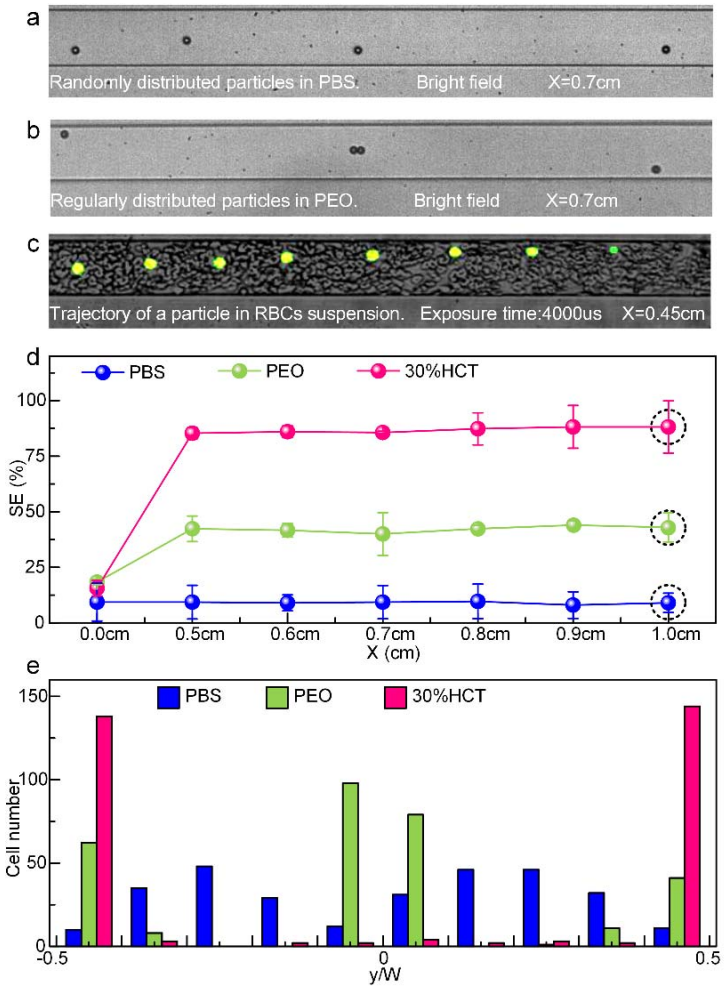

FIG. 2. Margination of particles in RBCs, PEO and PBS solution. (a) Random distribution of particles flowing in PBS solution. (b) Particles motion in PEO solution with three equilibrium positions. (c) Migration trajectory of a particle in RBCs solution, and the picture is the superposition of the fluorescent particles under fluorescent field with the bright field, and the trajectory of the particles is from the different positions of one particle at different moment, as described in Method "G". (d) Separation efficiency of particles flowing in different solutions along the channel length. The dashed circles represent the observation conditions in (e). (e) Lateral distribution of particle across the channel at the length of 1cm in the 3 kinds of solutions.

The locations of particles and cells along the channel width at the different channel length were counted and represented by the separation efficiency (SE), which is defined as $SE=N_{wall}/N_{total}$, where N_{wall} is the number of the particles flowing close the channel wall with the distance less than 4.5μm (1/10W, W is the channel width and represented by "y", this area is also defined as the margination zone), and N_{total} is 100 observed particles at the same observation. The SE provides a quantitatively result on the margination of particles in the three kinds of solutions. It is obviously higher in RBCs solution than that in PEO solution, while there is no margination effect in PBS solution, as shown in Figure 1(d). The particle's lateral distribution across the channel at the length of 1cm (the dashed circle in Figure 1(d)) was measured and plotted in Figure 1(e). The particles have 3 equilibrium positions in PEO solution, 2 equilibrium positions in RBCs solution, while they present homogeneous distribution in PBS solution.

This study has demonstrated that the viscoelasticity of solution could trigger the margination of particle in rectangular microchannel, and the collisions between the RBCs and the particles during flowing would push the particles away from the center equilibrium position, resulting in the disappear of the center equilibrium positions of particles in RBCs solution. Therefore, more stiff particles would migrate to the side wall of the channel when they are flowing in the RBCs suspension. Accordingly, RBCs suspension has the priority to realize cell sorting with higher separation efficiency than viscoelastic PEO solution.

B. Cell separation in rectangular and triangular microchannels.

According to the margination experiment for particles shown in Figure 2, the channel length of 1cm is enough for the RBCs to migrate to the wall in a 100μm×25μm rectangular channel. Allowing for the consumption of blood during the detection, RBCs suspension with 20%Hct is used in this experiment. RBCs with storage time of 0 day, 6 days, 18 days, 24 days and 29 days were prepared and fluorescently dyed as the target cells.

Since the first normal stress could be expressed as: $F_N \sim \mu\lambda \nabla\dot{\gamma}^2$.[33] The direction of the normal stress is the same as the gradient of the shear rate, $\nabla\dot{\gamma}$, and the gradient of the shear rate on the cross section of microchannel could be obtained through Comsol Multiphysics. Figure 3(a) shows the first normal stress distribution on the rectangular cross section, and the low stress zone (blue color) predicts the equilibrium positions of stiffened RBCs in microchannel. The RBCs performance in microchannel under bright and fluorescent field is shown in Figure 3(b). SE was calculated to clarify the separation of aged RBCs. Following the counting rules in Figure 2, 100cells was counted in every point in Figure 3(c), and each point was repeated three times in the experiment. It is obvious that the SE is increasing along the channel length, and the 29day RBCs has the highest SE. However, the SE is so low that could not be used as a separation method to sort RBCs. Moreover, the difference between the SE of each sample is dedicate, and not enough to be used as a bio-mark to detect aged RBCs. Actually, our previous study has demonstrated that the cross section shape of microchannel could affect the margination of RBCs in viscoelastic solution, and an acute angle could result in a high separation efficiency.[33] Therefore, triangular microchannel was applied in this experiment. As shown in Figure 3(d), the first normal stress distribution over the triangular cross section, and the flow rate was controlled as the same with that in rectangular microchannel. This distribution shows a bigger margination zone (blue color) near the channel wall in triangular channel than that in rectangular channel. Under the same flow condition, the RBCs performance in triangular microchannel under bright and fluorescent fields are shown in Figure 3(e), and the SE variations of target RBCs samples along channel length are shown in Figure 3(f). Obviously, the SE in triangular channel is enhanced comparing with that in rectangular channel. In addition, the aged RBCs performed visible distinction over storage time in triangular microchannel between 6day, 18day, 24day and 29day. While, the SE of 0day and 6day RBCs are similar, it is considered that 6 days is not enough for RBCs to perform a significant change on deformability and behave like stiff cells. Furthermore, at the same position along the length of microchannel, the RBCs with longer storage time have higher sorting efficiency. That is, at this location, RBCs with different storage time distribute at distinct position along channel width, from channel wall to channel center, the storage time of the distributed RBCs is from long to short, and the age of the distributed RBCs along the channel width is in a descending order. Allowing for the high SE and the visible distinction between

aged RBCs, the SE of aged RBCs from normal RBCs could be enhanced by controlling the channel cross section shape, and the triangular channel has the priority to be used as the cell separation channel for the elimination of aged RBCs.

To clarify the relationship between SE and storage time for cell detection, the marginated cells at the position of the 1cm of triangular channel were counted in every 100 observed cells, and the results from 300 cells are shown in Figure 3(g). The marginated cell is defined as the cell flowing in the margination zone. It should be noted that some RBCs is also separated from 0day blood, it can be understood because blood may contain some old RBCs with high modulus, and some target cells might already stay in the margination zone when they entranced the channel. The number of marginated cells is increasing from 25±3 to 74±4 per 100 as the storage time is elongated from 0 day to 29 days. That is, RBCs with different storage time perform distinct level of margination, as the storage time increased, more and more RBCs entranced into the margination zone when they were flowing in the triangular microchannel.

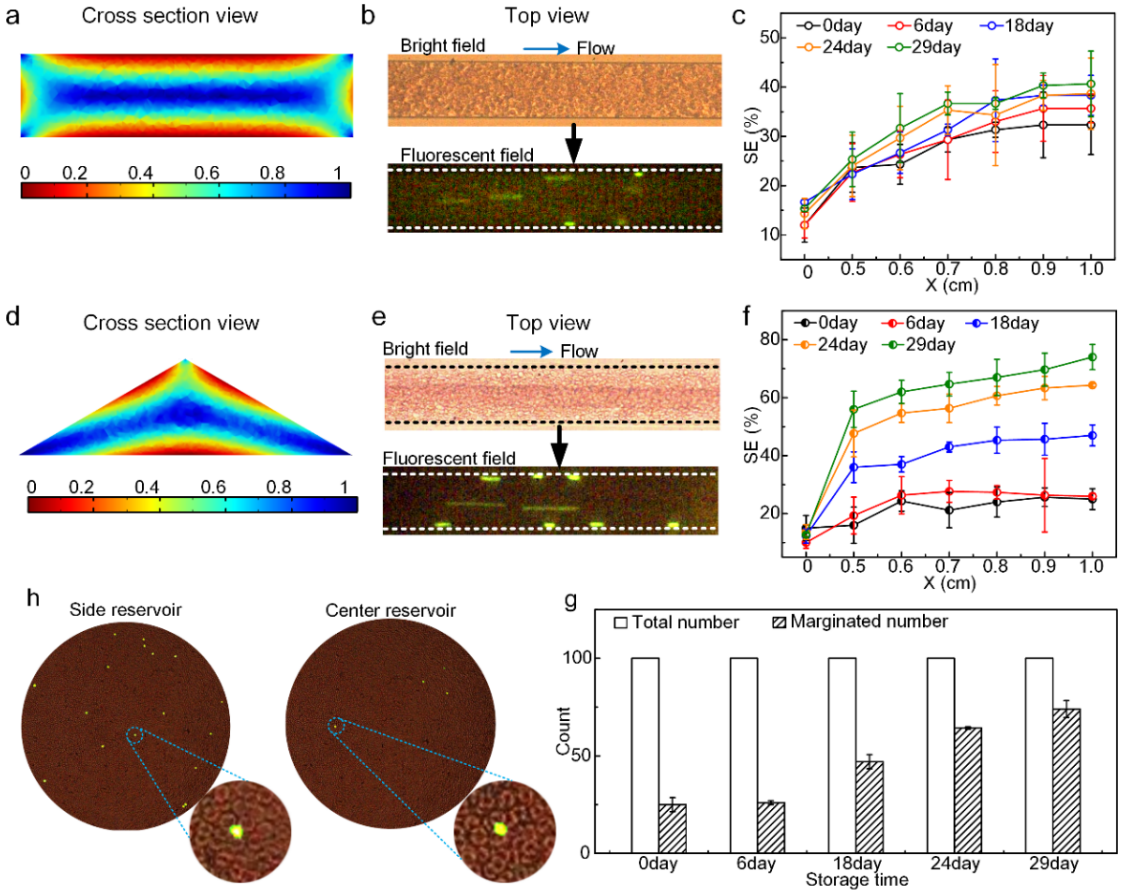

FIG. 3. Margination of aged RBCs' in rectangular and triangular channel. (a)The first normal stress distribution on rectangular channel cross section, and the color represent the relative magnitude of the first normal stress. (b)The performance of aged RBCs in rectangular channel under bright and fluorescent field. The dashed lines represent the channel edges, the bright dots represent the fluorescent 29day RBCs, and the figures were captured at 1cm of the channel. (c)The variation of SE of aged RBCs with different storage time in rectangular channel. (d)The first normal stress distribution on triangular channel cross section. (e)The performance of aged RBCs in triangular channel under bright and fluorescent field. (f)The variation of SE of aged RBCs with different storage time in triangular channel. (g) Cell number alternation of marginated RBCs

along storage time at the position of 1cm of triangular channel. (h)The fluorescent dots observation of 29day RBCs from side and center reservoirs, respectively. The dashed circles represent the partial enlarged views.

On the other hand, SE could describe the probability of aged RBCs' margination happening in microchannel, and allowing for the visible distinction between aged RBCs with different storage time, the SE in triangular channel could be used as a bio-mark to detect the storage time of blood. However, SE is a dynamic counting value, and it is hard to be used allowing for the detecting application. Therefore, the fluorescent dots observation at the outlet of separation device was performed to prove the utility of this detecting method. 29day RBCs were prepared and mixed with normal RBCs, under the same flow condition with Figure 3(e), the separated RBCs were collected at the outlet reservoirs of the device with triangular microchannel. The fluorescent cells separated in the side reservoir were compared to those unseparated in the center reservoir. As shown in Figure 3(h), the bright dots are the stained 29day RBCs, and the picture was obtained under fluorescent field, and the background was obtained under bright field at the same position. It is obvious that the separation of 29day RBCs from normal RBCs were realized. Furthermore, 251 target cells from 10 obtained pictures were counted, and the ratio of target cells from side is 80.88%, which is a little higher than the results in Figure 3(f). It can be understood that we counted the target cells that flowing in the margination zone in Figure 3(f), which is strict. While, during the observation in Figure 3(h), some targets cells that were close to the margination zone might entrance into the side collecting reservoir, which increased the ratio in Figure 3(h). This result verified our margination theory on detecting aged RBCs, and showed its potential application in recognition of the aged RBCs with high efficiency.

C. Dependence of SE on RBCs modulus

It has been knowing that storage process would cause the deformability loss of RBCs, and the aged RBCs could be separated from normal RBCs based on margination.[16,26] While, in the above study, we have demonstrated that RBCs with different storage time performed different level of margination, resulting in distinct SE. That is, the recognition of storage time from separation efficiency of aged RBCs is realized, while, the mechanism between them is still unclear. If we define the process of RBCs' deformability loss as the stiffened process by storage time, it is hypothesized that the stiffened rate of storage time on RBCs regulate the margination of aged RBCs in microchannel. As described in Method "F", the Young's modulus of RBCs was measured to investigate the dependence of SE on RBCs stiffness. RBCs with storage time of 6 days, 18 days, 24 days, and 29 days were prepared, and the RBCs without storage (0 day) were used as the control group. For each kind of aged RBCs, their modulus was measured 3 times, and 40 cells were measured in each time.

The RBCs in different storage time express different Young's modulus distributions, as shown in Figure 4(a). As the storage time increased, more RBCs became stiffer. We defined the area with modulus higher than or equal to the maximum of 0day modulus (E_{0max}) is the stiffened zone, as the colored zone in Figure 4(a) showed, and the RBCs entranced the stiffened zone is the stiffened cells (SCs). It is obvious that the number of SCs is growing along the storage time. However, not every RBC showed obvious loss of deformability during storage, some RBCs still maintained their low stiffness and not entranced the stiffened zone. Especially for the 6day RBCs, only a few RBCs become stiffened, which verified the margination behaviors of 6day RBCs in Figure 3(f). Because of the similar modulus

distribution, the SE of 0day and 6day RBCs are close, and it could not distinguish 0day RBCs and 6day RBCs by their SE.

The normal distribution curve of the cell modulus is presented in Figure 4(b). It is found that, as the storage time was elongated from 0 day to 29 days, the average modulus increases, and the modulus distributions move to the higher region and become more disperse. This result indicates that the RBCs with different storage time have distinct modulus distribution, and the older RBCs have much higher stiffness. Allowing for the margination performance of aged RBCs in microchannel in Figure 3(f), the normalized separation efficiency (NSE) of RBCs ($NSE_1=SE_1-SE_0$, "1" means the position of 1cm of the channel and "0" means 0cm) was then calculated and plotted with SCs% in Figure 4(c). SCs% means the percentage of SCs in each RBCs sample with different storage time. As shown in the black symbols, eliminating the impact of entrance on the SE, NSE is also increased over the storage time. As shown in the red symbols, the percentage of stiffed cells is increased by the storage time. Specifically, the variation of NSE and SCs% along storage time are consistent, that is, the increasing trends of separation efficiency and cell modulus along the storage time are consistent, especially the results of 18day, 24day and 29day, the percent reached the same level. On the other hand, as the storage time increased, more RBCs become stiff, and once they reached the stiffened zone, they could be separated from the normal RBCs by the margination technology. That is, the stiffening rate of RBCs by storage time could regulate the margination of aged RBCs in microchannel.

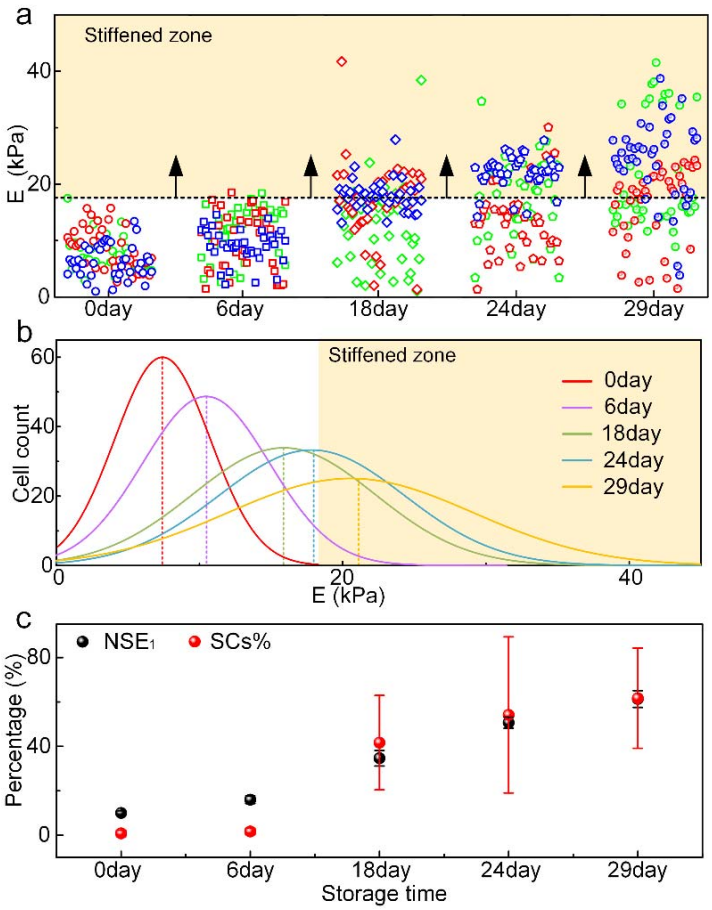

FIG. 4. Stiffness shifting of RBCs over storage time. (a) Young's modulus of RBCs sample, and the dots color represents experiment group. The colored zone represents the stiffened zone where the modulus of RBCs is higher or equals to the maximum of 0day RBCs' modulus. (b) Normal distribution of RBCs Young's modulus, and the color represents RBCs type with different storage time. (c) The relationship between NSE_1 and SCs% along storage time. Especially, $NSE_1=SE_1-SE_0$, and "SCs%" means the percentage of stiffened cells.

IV. CONCLUSIONs AND DISCUSSIONs

In this study, we observed the approaching process of particle to the channel wall during its margination, and the disappear of center equilibrium position of particles flowing with RBCs suspension. Therefore, as a viscoelastic fluid, the RBCs solution has its priority to realize the separation of aged RBCs from blood based on the deformability dependent margination, and the separation efficiency can be further substantially enhanced by the triangle cross-sectioned microchannel. Allowing for the visible distinction of separation efficiency between RBCs with different storage time obtained in triangular microchannel, the recognition of storage time from the separation efficiency of aged RBCs could be realized. Then the modulus of RBCs was investigated to clarify the mechanism between separation efficiency and storage time. It is found that significant increase of stiffness of RBCs happened after 18 days' storage time, while 6 days is not enough for RBCs to become stiff. Because the disperse distribution on Young's modulus, aged RBCs could not be all separated, and only those with modulus higher than the normal RBCs can be separated. More importantly, we demonstrated that the probability of aged RBCs' margination happening in triangular channel, characterized by the SE of aged RBCs from normal RBCs, would increase along the storage time. The increasing variation is consistent with the stiffening rate of RBCs by different storage time, which is characterized by the increase on modulus. The regulating role of storage time on the margination of aged RBCs is confirmed.

As a deformability-based cell separation method, margination has been used to study cell sorting technology for decades. Tomaiuolo[19] reported biomechanical changes of RBCs in health and disease based on microfluidics, which offered some instructions of RBCs deformability in margination-based RBCs separation. Gordon et al[35] reported that the electrical property changes of cell enable effective discrimination through dielectrophoresis, which offered a sensitive method to detect bovine red blood cell starvation age. Gagnon et al[36] reported a buffer electric relaxation time tuning technique to discriminated bovine red blood cell starvation age based on dielectrophoresis, which offered a new sensitive method to detect the changes of cell membrane and cytoplasm conductivity changes. Zhou[37] et al reported the sensitivity of capillary loading of blood suspensions on the RBCs deformability, which suggested some designs for the diagnosis of sepsis by microfluidics. Our work in this paper verified the overall deformability revolution of RBCs along storage time as reported before,[35-36] and quantitatively investigated the relationship between aged RBCs' sorting and storage time. The modulus test indicated the sensitivity of each RBCs to storage time, and revealed the aging mechanism of RBCs during stored. On the other hand, the quantitative relationship between sorting efficiency and RBCs storage time could be used to recognize the storage time of blood. The advantage of our work is to put up with a rapid simple and low cost means for separating aged RBCs with high efficiency, and further detecting the quality of RBC products from a small fraction of RBCs sample, as well as could be used to discriminate between specific aged blood abnormalities. More importantly, the separation, analysis and detection

could be finished on one microfluidic device without impairment on RBCs. The mechanism could also be applied on the separation of aged RBCs to decrease the blood transfusion risks, as well as on the future commercialization of deformability-based cell sorting technology with high efficiency. However, the storage time used in this paper is not consecutive day by day, and it is still unclear that the minimum interval of storage time that could be distinguished by the sorting efficiency. Therefore, there are still a lot of work to confirm more accurate relationship between sorting efficiency and storage time for the detection of stored blood. The RBCs solution not only provide viscoelasticity for aged RBCs to perform margination, but also enhanced the sorting efficiency by the collisions between RBCs. While, not every aged RBCs could be separated from normal RBCs, and the sorting efficiency still cannot reach 100%, because there are always some RBCs that maintain their deformability even the storage time is elongated to 29 days. On the other hand, as the supporter of cell sorting, the application of RBCs solution on blood detection is not convenient and safe, while the utilization of viscoelastic polymer will lower the sorting efficiency. Therefore, it is necessary to find the perfect substitute of RBCs for cell sorting in blood detection.

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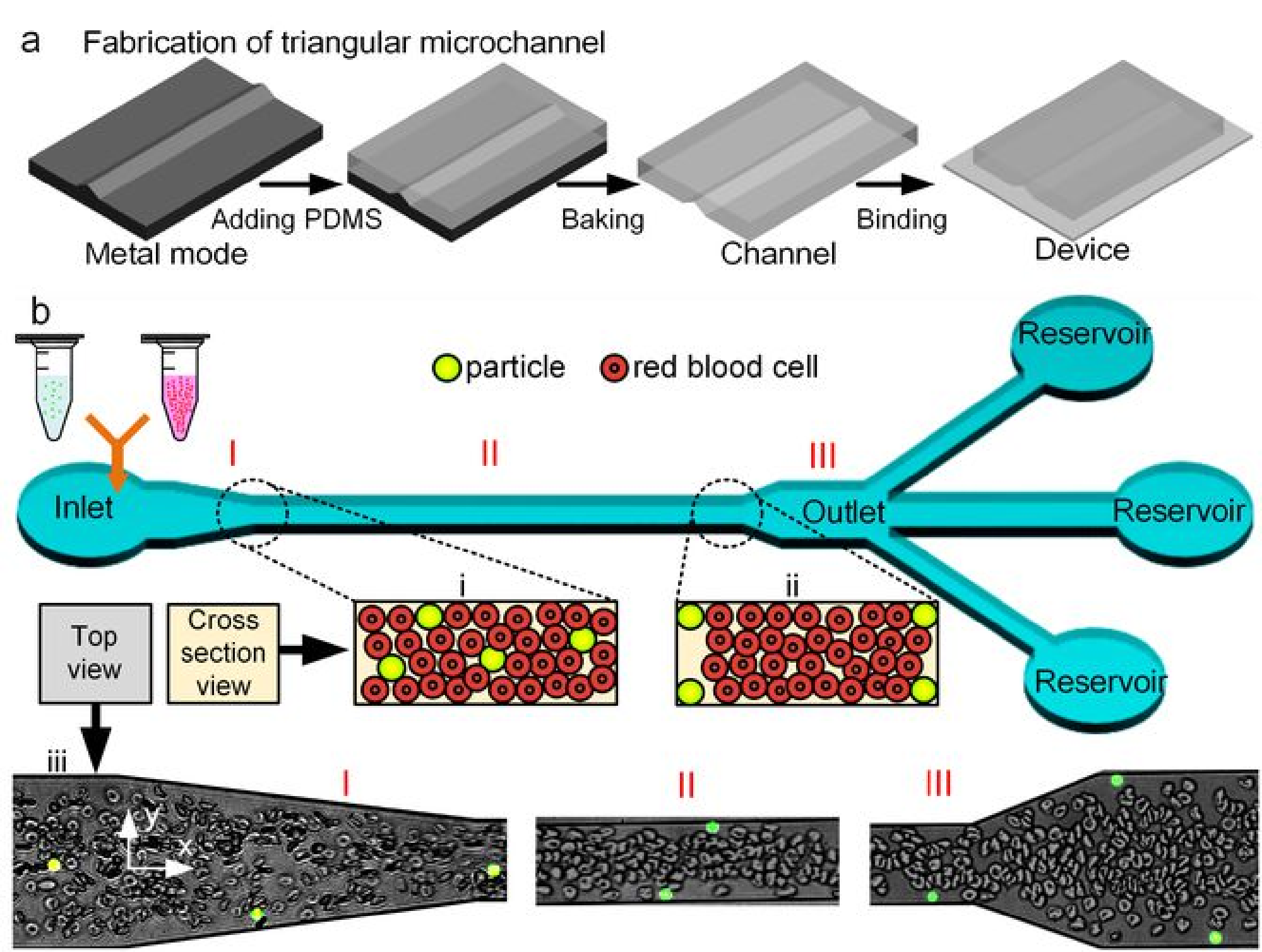
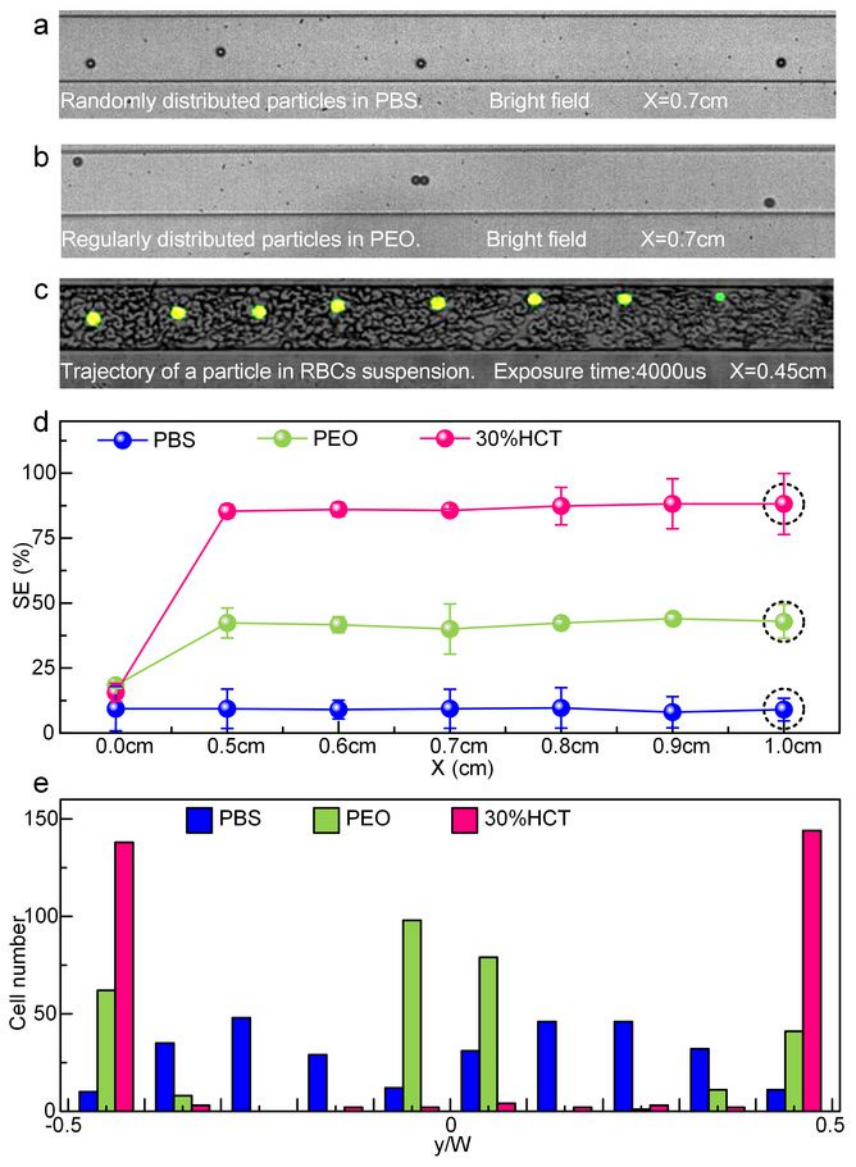
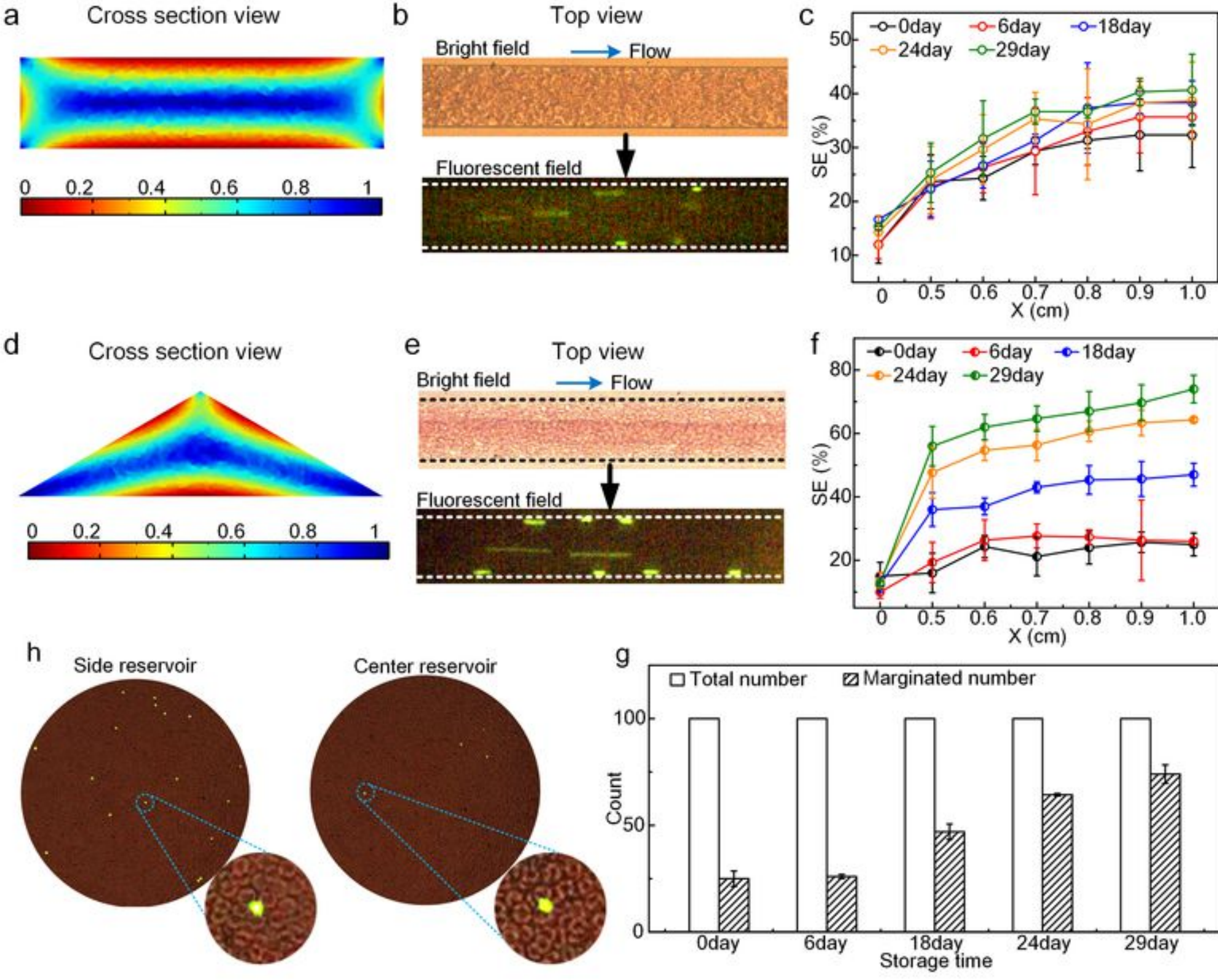
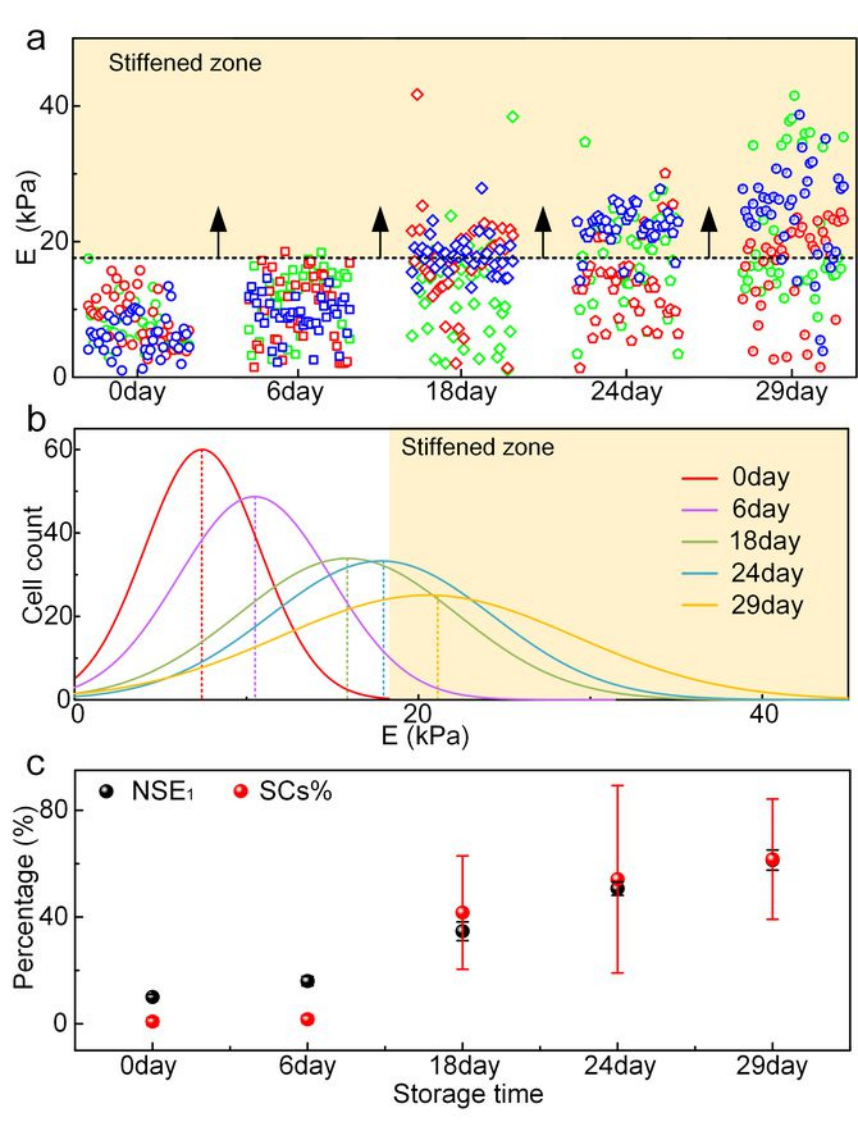

a
Fabrication of triangular microchannel
Metal mode
Adding PDMS
Baking
Channel
Binding
Device
b
particle
red blood cell
Reservoir
Inlet
I
II
III
Outlet
Reservoir
Reservoir
i
ii
Top view
Cross section view
iii
I
II
III
y
x


a
Randomly distributed particles in PBS. Bright field X=0.7cm
b
Regularly distributed particles in PEO. Bright field X=0.7cm
c
Trajectory of a particle in RBCs suspension. Exposure time:4000us X=0.45cm
d
PBS
PEO
30%HCT
SE (%)
100
75
50
25
0
0.0cm
0.5cm
0.6cm
0.7cm
0.8cm
0.9cm
1.0cm
X (cm)
e
PBS
PEO
30%HCT
Cell number
150
100
50
0
-0.5
0
0.5
y/W


a
Cross section view
0
0.2
0.4
0.6
0.8
1
b
Top view
Bright field
Flow
Fluorescent field
c
0day
6day
18day
24day
29day
SE (%)
X (cm)
d
Cross section view
e
Top view
f
h
Side reservoir
Center reservoir
g
Total number
Marginated number
Count
Storage time


a
Stiffened zone
E (kPa)
0
20
40
0day
6day
18day
24day
29day
b
Stiffened zone
Cell count
0
20
40
60
0day
6day
18day
24day
29day
E (kPa)
c
NSE1
SCs%
Percentage (%)
0
40
80
0day
6day
18day
24day
29day
Storage time