Biochemical and Biophysical Research Communications 512 (2019) 303–309

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Microfluidic assemblies designed for assessment of drug effects on deformability of human erythrocytes

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ARTICLE INFO

Article history:
Received 22 February 2019
Received in revised form
8 March 2019
Accepted 11 March 2019
Available online 16 March 2019

Keywords:
Microfluidics
Human erythrocytes
Deformability
Hydrogen peroxide
Ethanol
Pentoxifylline

ABSTRACT

Extreme deformability of human erythrocytes is a prerequisite for their ability to squeeze through narrow capillaries of the blood microcirculation system. Various drugs can modify this deformability and consequently provoke circulation problems. We demonstrate that microfluidic assemblies are very convenient platforms for *in vitro* study of the associated processes. Two types of microfluidic channels were designed to quantitatively investigate modifications of erythrocyte deformability induced by hydrogen peroxide, ethanol and pentoxifylline based on transit velocity measurements. With a high sensitivity our microfluidic assemblies show that hydrogen peroxide decreases erythrocyte deformability in a dose-dependent manner. Then, results on ethanol resolve a biphasic nature of this reactant on the deformability of single erythrocyte cells. Results on pentoxifylline provide evidence that, similar to ethanol, also this medical drug has a double-sided effect on the erythrocyte deformability, i.e. increasing the deformability at low concentrations, while decreasing it at higher ones. Taken together, our microfluidic designs propose a potent measurement method for the erythrocyte deformability, as well as providing a perspective to evaluate effects of drugs on it.

1. Introduction

Human erythrocytes (red blood cells) possess a characteristic shape of biconcave disks with a diameter of ~8 μm, which is governed by a special architecture of their membrane-skeleton system. Despite their relatively small size, erythrocytes have to strongly modify their shape to be able to travel through the tiny blood capillaries with diameters as small as 3 μm in purpose to transport oxygen and carbon dioxide between blood and tissues. This extreme deformability is a crucial property by which erythrocytes maintain their physiological functions [1,2]. It has been well known that the pathophysiology of several common diseases, such as diabetes [3], anemia [4] and malaria infection [5], is associated with decreased erythrocyte deformability. Consequently, the development of fast and reliable assessment methods focused on this distinctive property of erythrocytes is considered to be of great clinical significance in diagnosis and treatment.

Different techniques have been used to evaluate the erythrocyte deformability *in vitro*. For instance, ektacytometry, which can be used either with the whole blood or with erythrocyte suspensions, measures elongation of erythrocytes imposed by a shear stress [6]. The blood filtration techniques infer deformability from the flow rate of erythrocyte suspensions [7]. These methods are easy-to-use, cost-effective and generally provide a high-throughput. However, they are not suitable for detection at the level of single cells. In contrast, different micromanipulation methods, such as micropipette aspiration [8] or optical tweezers [9], provide highly sensitive assessment of the deformability of single erythrocytes, but they are relatively inefficient and laborious. As an alternative, microfluidics has recently emerged as a new promising method for testing erythrocyte deformability at a single cell level [10–13]. Nonetheless, further advancement of microfluidic designs that imitate human capillaries is needed to accurately and convincingly evaluate the effects of different deformability-associated drugs on the

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https://doi.org/10.1016/j.bbrc.2019.03.066
0006-291X/

erythrocyte capability to travel through narrow capillary channels.

In the present study, we investigate mobility of erythrocytes in two specially designed microfluidic channels: a meandering microchannel and a microchannel with repeated cylindrical pillars. They are used to quantitatively assess modifications of erythrocyte deformability induced by hydrogen peroxide, ethanol and pentoxifylline. Our results demonstrate that the investigated microfluidic configurations provide an efficient and sensitive platform for the determination of erythrocyte deformability at a single cell level. This makes them very promising for assessment of medical drugs associated with erythrocyte deformability modifications.

2. Methods and materials

2.1. Human erythrocytes isolation and sample preparation

Aliquots of 20 μL of fresh finger blood from healthy individuals were diluted in 1 mL of phosphate-buffered saline (PBS, NaCl 137 mM, Na_2HPO_4 8 mM, KCl 2.7 mM, KH_2PO_4 1.47 mM) containing 10 mM glucose and washed once with PBS by centrifugation at 1000 rpm for 5 min. The final suspension was adjusted to concentration of ~10^7 cells/mL in PBS. For drug treatment analysis, erythrocytes were preincubated with hydrogen peroxide (H_2O_2) (Acros Organics, New Jersey, USA), ethanol (EtOH) (Precision Chemical Reagent Factory, Tianjin, China) and pentoxifylline (PTX) (Tokyo Chemical Industry, Tokyo, Japan) for 2 h at room temperature. All the drugs were washed out with PBS before experiments.

2.2. Fabrication of microfluidic channels

The silicon template was designed with AutoCAD and fabricated using a deep silicon etching process by Wenhao chip technology corporation, Suzhou, China. The polydimethylsiloxane (PDMS) precursor and curing agent (Sylgard184, Dow Corning, Michigan, USA) were mixed (10:1) and poured onto the silicon template. After curing of PDMS for 40 min at 80 °C, the PDMS was peeled off the template. Then PDMS replica was placed onto a clean glass coverslip followed by oxygen plasma treatment. The microfluidic device was punched at both ends of the microchannel as the 'inlet' and the 'outlet' reservoirs for introducing fluids.

2.3. Deformability measurement

The microchannel device was fixed to an inverted fluorescence microscope (IX71, Olympus, Japan). Observations of fluid flow were performed with a high-speed video camera (Retiga R1, QImaging, Canada) that captured the images through a 60× oil immersion objective. The maximum acquisition rate was 100 frames per second. Approximately 200 μL of the selected erythrocyte suspension was loaded to the inlet reservoir of the microchannel assembly that was connected to the vertically oriented 20-mL injector containing PBS (Fig. S1). The pressure gradient in the microchannel was adjusted by changing the height between the liquid level in the injector and the inlet of the microchannel. Videos of erythrocytes travelling through various microchannel configurations were recorded. Their transit velocity and other properties were obtained by analysis of the video data with Image J (National institutes of health, USA), Origin (version 8.5, OriginLab, USA) and SPSS programs (version 22, IBM, USA).

2.4. Statistical analysis

To obtain suitable statistics on the transit velocity, more than 80 cells from at least three donors were analyzed in each experiment. The obtained data are presented as a mean value ± standard deviation (SD). Statistical comparison between different groups was carried out by using the Student's *t*-test (SPSS 22, IBM, USA). The probability values $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Hydrogen peroxide decreases erythrocyte deformability in a dose-dependent manner

The meandering microchannel designs used in our investigations are shown in Fig. 1A. The height of the microchannel was 4 μm. It began with a 1000-μm-diameter circular reservoir at its inlet. The entrance of the microchannel was narrowed to the width of 30 μm, while the meandering segment itself had a width of 3 μm (Fig. S2A). At exit, the microchannel opened into another 1000-μm-diameter reservoir. The meandering segment consisted of 16 semicircles with the diameter of 13.7 μm and a total length of 343.8 μm. A continuous extrusion force was acting on the erythrocytes during their travel through the channel. The erythrocyte deformability is elucidated from the average transit velocity measured for their passing through the microchannels, i.e. higher transit velocities are associated with larger deformability.

Pressure-gradients of 0.05, 0.1, 0.2 and 0.4 Pa/μm were used to investigate the transit process of erythrocytes through the meandering microchannel. In the control experiments with untreated erythrocytes it was found that inside the channel they deformed from the natural disk shape to a strip-like elongated shape (Fig. 1B, Movie. S1). Then, after coming out of the channel, they rapidly recovered to their original shape (Fig. 1C, Movie. S2). As shown in Fig. 1D, the measured transit velocity increases with increasing pressure gradient. The obtained average velocities are 304.4 ± 14.2, 570.0 ± 48.4, 1163.7 ± 68.7 and 2549.2 ± 189.8 μm/s at the pressure gradients of 0.05, 0.1, 0.2 and 0.4 Pa/μm, respectively.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2019.03.066.

In the next series of experiments the erythrocytes were treated with H_2O_2, which is a well-known oxidant that affects erythrocyte deformability in oxidative stress processes [14]. First, there is no evident influence of H_2O_2 with different concentrations on the profile of erythrocytes (Fig. S3A). Furthermore, as can be resolved from Fig. 2A and Movie. S3, higher concentrations of H_2O_2 lead to lower velocities of erythrocytes, demonstrating a dose-dependent effect on deformability. After leaving the meandering microchannel, the erythrocytes pretreated by 0.1 mM and 1 mM H_2O_2 rapidly recovered their native disk shape, while those pretreated by 10 mM H_2O_2 recovered the original shape more slowly (Fig. 2B and Movie. S4). The obtained dependences of the erythrocyte velocity on the pressure gradient for different concentrations of H_2O_2 are given in Table 1 and are shown in Fig. 2C. Specially, one can notice that at the pressure gradient of 0.4 Pa/μm there exists an evidently distinguishable difference in the transport velocity between the control group and the group pretreated by 0.1 mM H_2O_2. A statistically significant decrease of deformability for the 1 mM and 10 mM H_2O_2 concentrations was detected also at a pressure gradient as low as 0.05 Pa/μm.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2019.03.066.

3.2. Ethanol induces a biphasic impact on erythrocyte deformability

The effect of alcohol consumption on blood microcirculation is a problem of wide concern. Thus, we investigated alteration of the deformability of EtOH-treated erythrocytes by analyzing their transit motion through the meandering microchannel. It showed

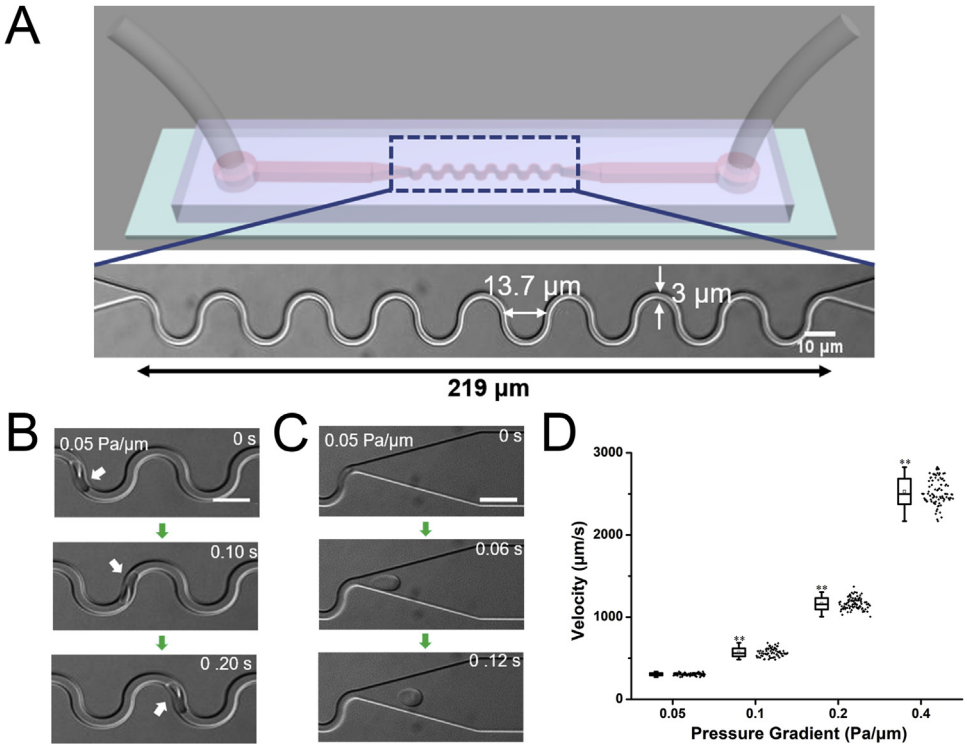

Fig. 1. Normal erythrocytes travelling through a meandering microchannel. (A) Schematic of the meandering microchannel designs. (B) Image sequences obtained at a pressure gradient of 0.05 Pa/μm. The white arrow indicates the deformed erythrocyte. (C) Image sequences of the erythrocytes coming out from the microchannel at a pressure gradient of 0.05 Pa/μm. Scale bars are 10 μm. (D) Statistical data on the transit velocity of erythrocytes measured at different pressure gradients. $^{**}P < 0.001$, compared to 0.05 Pa/μm group.

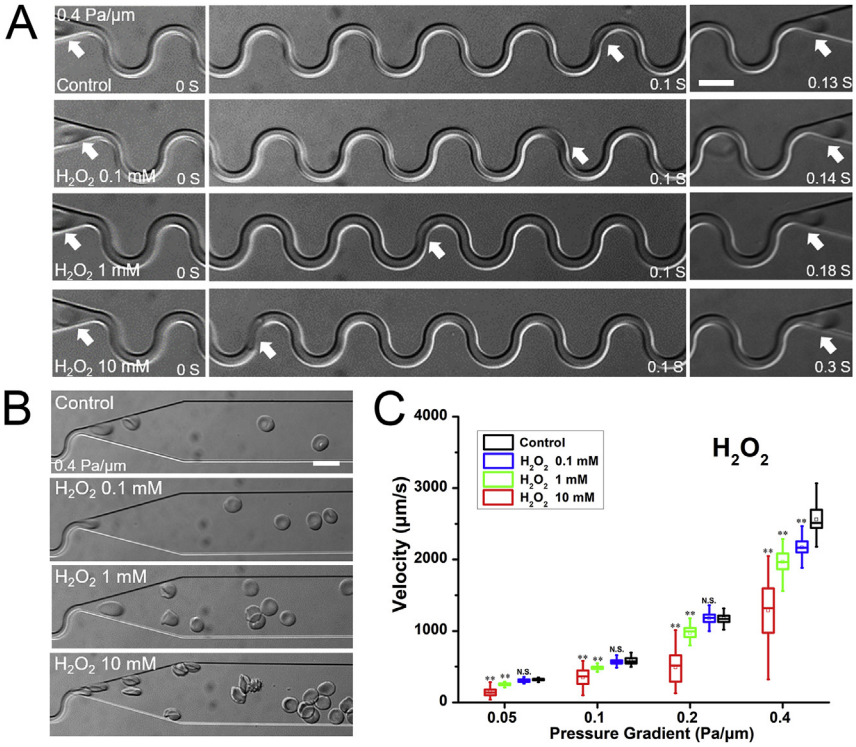

Fig. 2. H_2O_2 reduces erythrocyte deformability depending on concentration. (A) The transit velocity of erythrocytes significantly decreases with the increasing concentration of H_2O_2 from 0.1 mM to 10 mM. (B) Erythrocytes treated with H_2O_2 travelled out of the microchannel at 0.4 Pa/μm. 10 mM H_2O_2-treated erythrocytes cannot rapidly recover to the biconcave shape. Scale bars are 10 μm. (C) Statistical data on transit velocity of H_2O_2-pretreated erythrocytes. $^{**}P < 0.001$, compared to the control group at the same pressure gradient. N.S., no significant difference.

Table 1
Velocities of H_2O_2-treated erythrocytes (μm/s).

H_2O_2 Concentration				
Pressure Gradient	0 mM	0.1 mM	1 mM	10 mM
0.05 Pa/μm	304.4 ± 14.2	303.8 ± 11.1	253.5 ± 17.5	132.5 ± 26.0
0.1 Pa/μm	570.0 ± 48.4	560.5 ± 43.8	480.9 ± 42.7	336.9 ± 47.9
0.2 Pa/μm	1163.7 ± 68.7	1166.7 ± 71.2	971.7 ± 106.2	478.2 ± 93.3
0.4 Pa/μm	2549.2 ± 189.8	2156.1 ± 121.4	1968.5 ± 166.4	1272.8 ± 200.8

that low concentrations of EtOH (0.003%, 0.03%, and 0.3%, v/v) had no evident effect on the shape of erythrocytes, however, high concentration (3%, v/v) turned approximately 10% of the cells into acanthocytes (Fig. S3B). For a pressure gradient of 0.4 Pa/μm, it was observed that erythrocytes treated by low concentrations of EtOH (0.003%, 0.03%, and 0.3%, v/v) travelled faster than the control group, however, for the highest investigated concentration (3%, v/v) the transit velocity decreased (Fig. 3A, Movie. S5). For all investigated EtOH concentrations, the erythrocytes rapidly recovered their shape after leaving the meandering channel (Movie. S6). The average velocity of erythrocytes as a function of pressure gradient for various EtOH concentrations is given in Table 2 and shown in Fig. 3B. From the obtained results it follows that EtOH has a biphasic impact on the erythrocyte deformability, i.e. lower EtOH concentrations increase the deformability, while higher ones decrease it.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2019.03.066.

3.3. Pentoxifylline has a double-sided effect on erythrocyte deformability

PTX, which is widely clinically used for treatment of peripheral vascular diseases, has been reported to improve microcirculation [15]. Therefore, we decided to investigate the deformability of PTX-treated erythrocytes using the meandering microchannel at single cell level. The obtained results show that erythrocytes pretreated with 0.3 mg/mL PTX travel significantly faster than the control

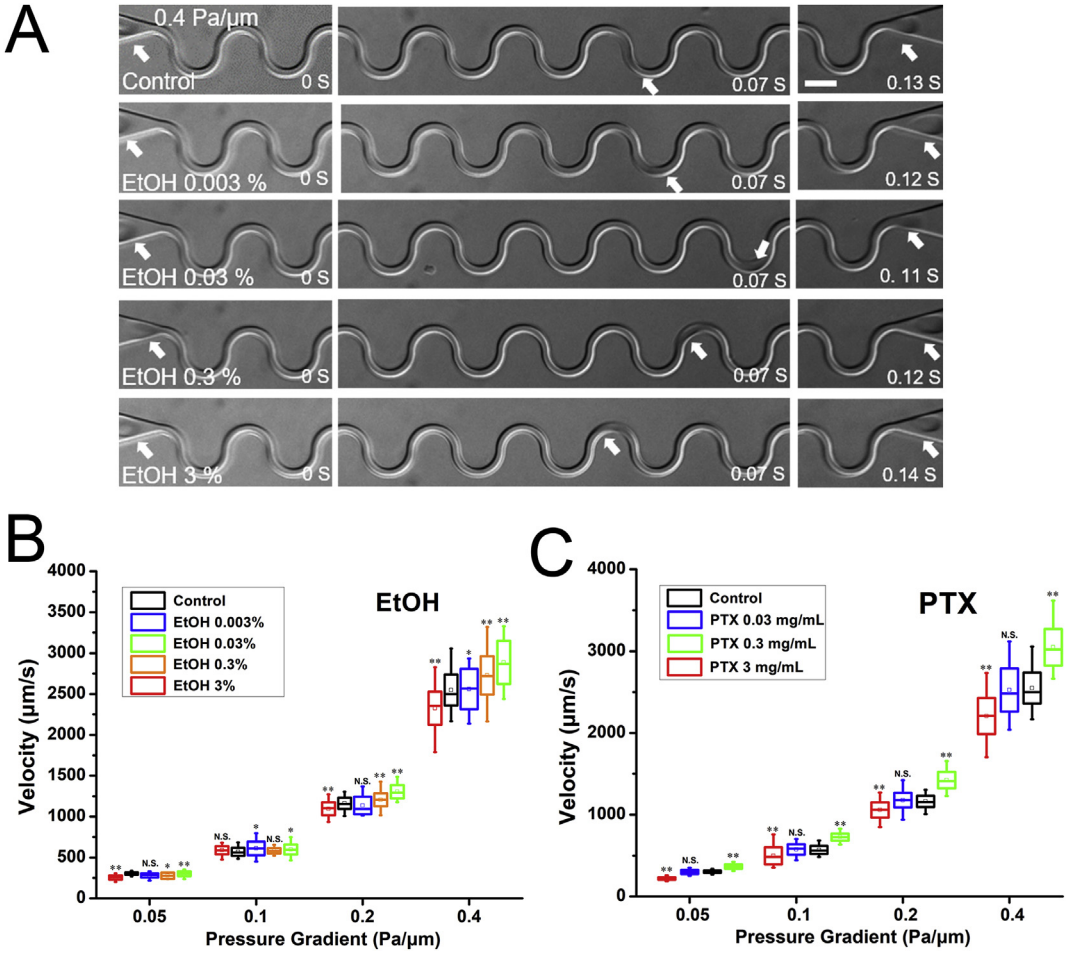

Fig. 3. Pretreatment with EtOH and PTX has a biphasic effect on the erythrocyte deformability. (A) Low concentrations of EtOH (0.003%, 0.03% and 0.3%, v/v) increase the transit velocity, while high concentration (3%) decreases it. The data are given for pressure gradient of 0.4 Pa/μm. White arrows indicate deformed erythrocytes. (B, C) Statistical data on the measured transit velocity of erythrocytes pretreated with EtOH (B) and PTX (C). $^{**}P < 0.001$, compared to the control group at the same pressure gradient. N.S., no significant difference.

Table 2
Velocities of EtOH-treated erythrocytes (μm/s).

Ethanol Concentration (v/v)					
Pressure Gradient	0%	0.003%	0.03%	0.3%	3%
0.05 Pa/μm	304.4 ± 14.2	280.0 ± 26.2	336.6 ± 29.5	277.9 ± 38.3	253.7 ± 26.4
0.1 Pa/μm	570.0 ± 48.4	610.5 ± 83.0	632.1 ± 60.6	582.2 ± 33.1	588.7 ± 50.2
0.2 Pa/μm	1163.7 ± 68.7	1135.6 ± 106.5	1206.4 ± 78.9	1338.7 ± 80.3	1096.3 ± 79.3
0.4 Pa/μm	2549.2 ± 189.8	2561.1 ± 246.7	2921.4 ± 262.9	2727.2 ± 233.6	2326.1 ± 201.9

erythrocytes, whereas the transit velocity of 3 mg/mL-pretreated erythrocytes is significantly lower than that of control erythrocytes (Movie. S7). The erythrocytes treated with 0.03 mg/mL of PTX showed a similar velocity as the untreated ones. After exiting the meandering microchannel, they rapidly recovered to their natural shape for all the investigated PTX concentrations (Movie. S8). The average velocity as a function of pressure gradient for different concentrations is given in Table 3 and shown in Fig. 3C. In addition, there is no obvious influence of PTX with different concentrations on the shape of erythrocytes (Fig. S3C). The conclusion following from our observations is that low concentrations of PTX increase the erythrocyte deformability, whereas high concentrations have an opposite effect.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2019.03.066.

3.4. Microchannels with array of cylindrical pillars can also detect deformability modifications

A microchannel design with repeated array of cylindrical pillars also began with a 1000-μm-diameter circular reservoir. Its entrance was narrowed to a width of 39 μm (Fig. S2B). The height of the microchannel was 4 μm. At the center of the microchannel device there were 4 rows of 14 micropillars with diameter of 6 μm (Fig. 4A). The distances between the rows and the columns in the micropillar array were 3 μm and 10 μm, respectively. The erythrocytes were extruded between the rows and then recovered between the columns.

In experiments we observed that erythrocytes squeezed themselves between the pillars. This process was followed by a rapid recovery of their natural shape (Fig. 4B, Movie. S9). The squash and recovery events significantly reduced the transit velocity of erythrocytes through the microchannel. Therefore, control measurements were performed at a relatively high pressure gradient of 2 Pa/μm. The obtained distribution of the transit velocities is shown in Fig. S4 and has the value of 611.2 ± 97.4 μm/s.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2019.03.066.

After this, the erythrocytes preincubated with the three investigated drugs, namely H_2O_2, EtOH and PTX, were introduced into the microchannel. The obtained results on transit velocity for H_2O_2 pre-treatment are shown in Fig. 4C (Movie. S9), for EtOH pre-treatment in Fig. 4D (Movie. S10) and for PTX pre-treatment in Fig. 4E (Movie. S11), respectively. The average velocities for the three reactants are given in Table 4. It is evident that H_2O_2 monotonically decreases the erythrocyte deformability in a dose-dependent manner. EtOH and PTX, in contrast, exhibit a biphasic impact on the deformability modifications.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.bbrc.2019.03.066.

4. Discussion

An important issue in development of microfluidic assays for determination of erythrocyte deformability is the optimization of the microchannel geometry. For instance, the cell behavior in common straight microchannel was investigated to resolve the viscoelasticity of single erythrocytes according to their parachute-like shape [16]. A network of parallel straight branches was fabricated to simulate human capillaries and measure erythrocyte deformability [17]. Such various straight microchannels have been applied to assess erythrocyte deformability in several conditions including malaria infection [18,19] and blood storage lesion [20,21]. For our design, the meandering geometry imitates microcirculation in real blood capillaries better than the common straight microchannels. In addition, longer travelling length within them increases the available observation time within the restricted microscopic field of view. The individual differences of deformability among healthy erythrocytes could be illustrated through the dispersion of the transit velocities when cells travelled through the microchannel (Fig. 1D).

As the most sensitive method for deformability measurement, optical tweezers were able to detect single erythrocyte deformability modification induced by 0.1 mM H_2O_2 [22]. In our work, the results showed that the meandering microfluidic assembly was also competent to detect the erythrocyte deformability decrease provoked by as low as 0.1 mM H_2O_2 in a higher throughput (Fig. 2C), thus demonstrating the sensitivity and efficiency for our microfluidic design. Furthermore, our investigation clearly showed a nonmonotonic effect of EtOH treatment on erythrocyte deformability at the single cell level too, i.e. lower EtOH concentrations increase the deformability, while higher ones decrease it (Fig. 3B). A possible mechanism of this interesting biphasic impact is that at low concentrations, EtOH locates on the lipid membrane surface of erythrocytes, giving rise to an increase in overall fluidity of the membrane, leading to an increase in erythrocyte deformability [23]. At high concentrations, in contrast, EtOH molecules enter into the hydrophobic core of the inner layers of the membrane, resulting

Table 3
Velocities of PTX-treated erythrocytes (μm/s).

PTX Concentration				
Pressure Gradient	0 mg/mL	0.03 mg/mL	0.3 mg/mL	3 mg/mL
0.05 Pa/μm	304.4 ± 14.2	299.3 ± 24.8	365.0 ± 26.0	219.3 ± 15.4
0.1 Pa/μm	570.0 ± 48.4	574.8 ± 63.7,	726.2 ± 42.5	497.5 ± 104.6
0.2 Pa/μm	1163.7 ± 68.7	1423.6 ± 98.9	2207.8 ± 120.9	1178.1 ± 88.9
0.4 Pa/μm	2549.2 ± 189.8	2524.7 ± 263.6	3046.9 ± 223.6	2207.8 ± 220.9

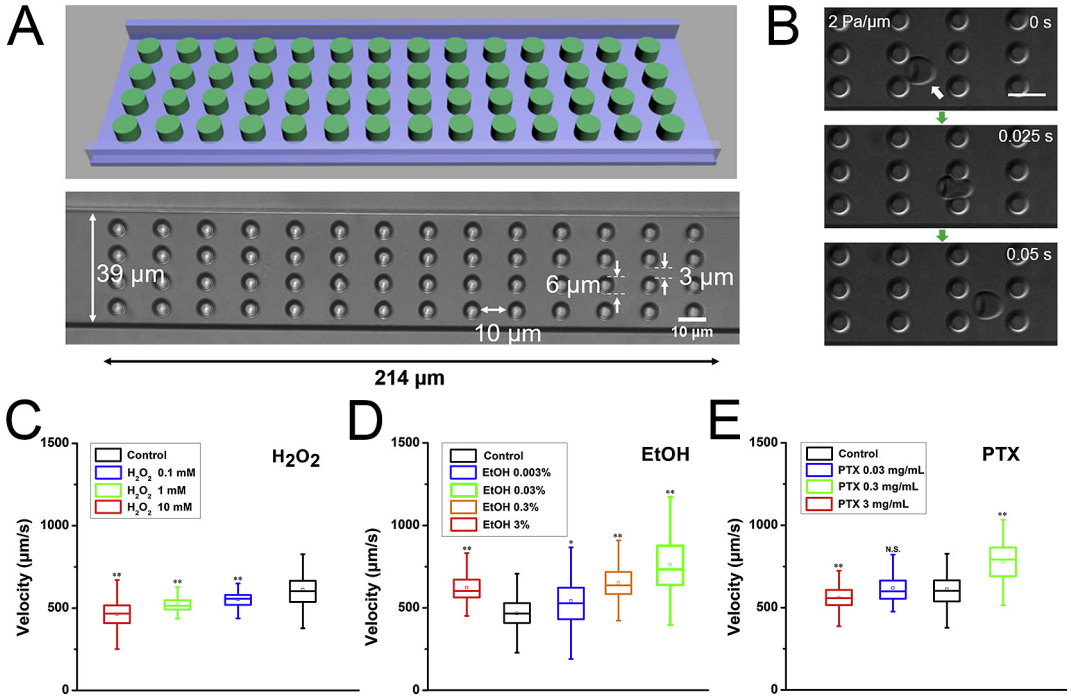

Fig. 4. A microchannel assembly with repeated cylindrical pillars can be used to detect deformability modifications of drug-pretreated erythrocytes. (A) Schematic of the microchannel with repeated funnel-shaped structures. (B) Image sequences of erythrocytes travelling through the microchannel at pressure gradient of 2 Pa/μm. The erythrocytes are repeatedly squashed and recovered. A white arrow indicates the deformed erythrocyte. Scale bare is 10 μm. (C–E) Statistical data for the transit velocity of erythrocytes pretreated with (C) H_2O_2, (D) EtOH and (E) PTX travelling through the microchannel with repeated cylindrical pillars. $^*P < 0.05$, $^{**}P < 0.001$, compared to the control group at the same pressure gradient. N.S., no significant difference.

Table 4
Velocities of erythrocytes in the microchannel with repeated cylinders at 2 Pa/μm (μm/s).

H_2O_2	0 mM	0.1 mM	1 mM	10 mM	
	611.2 ± 97.4	552.5 ± 55.4	522.3 ± 45.5	461.8 ± 98.4	
EtOH	0 v/v %	0.003 v/v %	0.03 v/v%	0.3 v/v %	3 v/v %
	611.2 ± 97.4	622.1 ± 89.6	758.3 ± 111.6	683.5 ± 84.2	560.9 ± 79.4
PTX	0 mg/mL	0.03 mg/mL	0.3 mg/mL	3 mg/mL	
	611.2 ± 97.4	619.0 ± 92.9	777.3 ± 117.2	559.8 ± 78.1	

in an expansion of the cytoskeleton and a decrease in deformability [24]. According to our results, low dose of alcohol consumption appears to be of benefit to the blood microcirculation system. Nevertheless, one needs to take into account that a recent study based on meta-analysis revealed that the level of alcohol consumption that minimizes health loss is zero [25]. Previous studies based on filtration techniques reported that PTX were capable of increasing the erythrocyte deformability [26]. In our experiments, a nonmonotonic behavior similar with EtOH was also found for PTX (Fig. 3C), which, to the best of our knowledge, for the first time reveals a double-sided action of PTX on deformability of single erythrocytes *in vitro*. This result provides a guideline for optimal dosage of PTX in clinical usages, as well as reveals a potential harm of overusing PTX because a high dose of this drug may lead to microcirculation damage. However, more studies are still in need to uncover the detailed mechanism of its double-sided action on deformability.

Besides this, microchannels structured with intra-channel repeated arrays of some obstacles, such as cylindrical [27,28] and tapered pillars [29,30], were used in purpose to measure the deformability of erythrocytes. For details, a biomimetic microchannel with cylindrical arrays was fabricated to study the mechanical retention of erythrocytes in the spleen [28]. Experiments in the microchannel with repeated tapered pillars revealed that ethanol was able to reverse the deformability of iron dextran-impaired erythrocytes [30]. In our second design, the repetitive extrusion-recovery process provided by an array of cylindrical pillars amplified the evidence on deformability differences among the erythrocytes (Fig. 4). Moreover, the transit velocity of erythrocytes was significantly decreased by the repeated pillars, which also reduced the requirements for the CCD's speed performance. Results using this microchannel design are in very good agreement with the results obtained in the experiments with the meandering microchannel assembly, proving the rationality and validity of these two microchannel designs.

In summary, this work presented two potent microfluidic designs to quantitatively assess the deformability change between control and drug-treated (H_2O_2, EtOH and PTX) erythrocytes based on transit velocity detection. We believe that the presented designs of microfluidic assemblies are very promising for application in assessment and dosage optimization of different drugs that affect the erythrocyte deformability.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (no. 11874231, 11574165 and 31801134), Tianjin Natural Science Foundation (no. 18JCQNJC02000), the PCSIRT (no. IRT_13R29), the 111 Project (no. B07013), and the project of bilateral cooperation between Slovenia and China (no. BI-CN/17-18-018).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.03.066.

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