Integrative Biology

Cite this: *Integr. Biol.*, 2011, **3**, 972–981

www.rsc.org/ibiology

REVIEW ARTICLE

Red blood cell dynamics: from cell deformation to ATP release

Jiandi Wan, Alison M. Forsyth and Howard A. Stone*

Received 13th May 2011, Accepted 17th August 2011
DOI: 10.1039/c1ib00044f

The mechanisms of red blood cell (RBC) deformation under both static and dynamic, *i.e.*, flow, conditions have been studied extensively since the mid 1960s. Deformation-induced biochemical reactions and possible signaling in RBCs, however, were proposed only fifteen years ago. Therefore, the fundamental relationship between RBC deformation and cellular signaling dynamics *i.e.*, mechanotransduction, remains incompletely understood. Quantitative understanding of the mechanotransductive pathways in RBCs requires integrative studies of physical models of RBC deformation and cellular biochemical reactions. In this article we review the physical models of RBC deformation, spanning from continuum membrane mechanics to cellular skeleton dynamics under both static and flow conditions, and elaborate the mechanistic links involved in deformation-induced ATP release.

Introduction

The mechanical features of a red blood cell (RBC) are of physiological and pathological significance. For example, highly deformable RBCs can transverse capillaries that have smaller diameters than that of the cell in order to delivery oxygen to tissues. The ability of red cells to deform also allows the cells to participate in the flow and is known to reduce the effective viscosity of the blood.[1] Meanwhile, pathological factors that decrease the deformability of red cells can cause serious vascular complications. Indeed, red cells stiffened by the infection by *Plasmodium falciparum* (malaria) can result in microvascular occlusion and subsequent organ damage;[2] also, impaired deformability of red cells due to diabetes mellitus induces insulin-dependent platelet aggregation.[3] Moreover, transfusion of stored red cells with decreased deformability can impede microvascular flow and cause complications and mortality.[4] In addition, studies have shown that, upon deformation, red cells can also release chemicals, such as ATP, to participate in vascular signaling and control the systemic circulation.[5] Therefore, quantitative understanding of the viscoelasticity of the cell, the cell–fluid interaction in blood, and the correlation of these properties to chemical release, vascular diseases, and blood storage will be of great interest not only to fundamental research, but also for clinical applications.

Although the deformability of red blood cells (RBCs) was observed more than three hundred years ago,[6] the earliest quantitative description of the deformation of RBCs was reported in 1964, when the mechanical properties of the cell membrane were studied experimentally using micropipette aspiration technology (Fig. 1A).[7] In the intervening decades, continuum mechanical models have been developed along side experimental studies, and it is standard practice to extract mechanical parameters of the cell, such as the bending modulus, shear modulus and membrane viscosity.[8–10] Indeed, in the 1990s, the quantitative understanding of the mechanical properties of red cells at the whole cell level led to the invention of a new type of dynamic force spectroscopy, where

Department of Mechanical and Aerospace Engineering, Princeton University, Princeton, NJ, USA. E-mail: hastone@princeton.edu

Insight, innovation, integration

A unique feature of the human red blood cell is its ability to deform significantly during the passage through the microvasculature. The deformability of red cells, therefore, plays a key role in regulation of cell function and survival, and is crucial for exchange of gases in the human circulatory system. On the other hand, research has also shown that red cells can release signaling molecules, *e.g.*, ATP, upon cell deformation and participate in the regulation of vascular tone. This article presents a thorough review of red cell deformation and deformation-induced ATP release. Additionally, by integrating physical models of red cell deformation and cellular biochemical reactions, mechanisms of deformation-induced ATP release are described. The combined features of red cell deformation and deformation-induced ATP release provide insight to red cell dynamics and strategies for the integrative study of blood and vascular diseases.

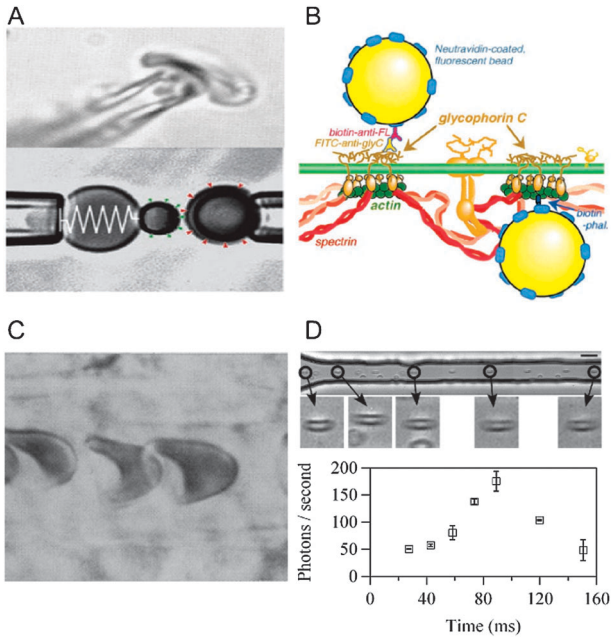

Fig. 1 Overview of RBC dynamics. (A) (top) Micropipette aspiration setup for determination of the stiffness of the RBC membrane[7] and (below) a RBC is used as a force transducer to measure weak biological interactions.[11] (B) Schematic of the use of fluorescence nanoparticles to measure the local elasticity of the red cell's spectrin-actin network.[12] (C) *In vivo* images of red blood cells flowing in a capillary,[13] where most of the cells exhibit a parachute shape. (D) (top) Deformation of red cells flowing through a constriction channel and (below) the corresponding ATP release reported by a bioluminescent reaction.[14]

RBCs are used in a micropipette arrangement as an effective spring to measure weak biological forces (Fig. 1A).[11,15,16]

Over the past five decades, since the first quantitative description of the deformation of RBCs, research on cell deformation has continued systemically with studies moving from macroscopic, at the scale of a cell, to molecular, and from purely mechanical to mechano-chemical. Combinations of fluorescent labeling technology, micropipette aspiration, and optical tweezers, for example, have been utilized to investigate the deformation of red cells at the molecular level (Fig. 1B). The investigations, focusing on membrane skeletal filaments and transmembrane proteins, provide a precise description of RBC deformation in terms of the changes of connectivity and orientations of the actin-spectrin network.[15] Based on these results, detailed microstructural models of the deformation of the cell membrane have been developed.[17–20] In addition, these studies provide a basis for understanding the relationship of bioactivity of red cells to mechanical forces, *e.g.*, shear stress induced cell deformation and ATP release.[21]

Since RBCs flow in the circulatory system, how red cells respond to hydrodynamic stresses is crucial for understanding the basic physiology of blood. RBCs under flow have distinguishing features that are not observed under static conditions,

Jiandi Wan

Jiandi Wan is currently a Research Associate in the Department of Mechanical and Aerospace Engineering at Princeton University and will start as an assistant professor at Rochester Institute of Technology in January 2012. His degrees are in chemistry from Wuhan University (BS, 1998, MS, 2001) and Boston University (PhD, 2006). Dr Wan worked as a post-doctoral researcher in the School of Engineering and Applied Sciences at Harvard University from 2006 to 2009 and moved to Princeton University in 2009. Dr Wan's research includes microfluidic approaches for studying red blood cell dynamics and multiphase emulsions.

Alison M. Forsyth

Alison M. Forsyth is a PhD candidate at Harvard University in the School of Engineering and Applied Sciences and is currently a visiting scholar at Princeton University in the Department of Mechanical and Aerospace Engineering. She completed her BS in bioengineering at Syracuse University in 2006. Her research involves red blood cell deformation and dynamics with implications for physiological responses in the cardiovascular system.

Howard A. Stone

Howard A. Stone is the Donald R. Dixon '69 and Elizabeth W. Dixon Professor in Mechanical and Aerospace Engineering at Princeton University. He received his SB degree in chemical engineering from the University of California, Davis, and a PhD in chemical engineering from Caltech. From 1989 to 2009 he was on the faculty in the School of Engineering and Applied Sciences at Harvard University. His research interests are in fluid dynamics, especially as they arise in research and applications at the interface of engineering, chemistry, and physics. He was the first recipient of the G. K. Batchelor Prize in Fluid Dynamics, which was awarded in August 2008. In 2009 and 2011 he was elected to the National Academy of Engineering and the American Academy of Arts and Sciences, respectively.

e.g., linear aggregation into rouleaux,[22] lateral migration,[23] tank-treading where the cell maintains a constant orientation to the flow direction while the membrane rotates around the cell body,[1,24] and tumbling of the cell.[22] During flows in capillaries, RBCs can deform as a symmetric parachute-like shape or a nonsymmetric slipper-like shape depending on the capillary radius, apparent fluid viscosity and other parameters (Fig. 1C).[13,25] These dynamic responses have inspired corresponding mathematical studies that couple the hydrodynamics to the mechanics of the cell membrane.[26–28,44]

In the mid-1990s, it was proposed that RBCs are able to respond to shear stress, and release chemicals to increase blood flow.[29] The idea was introduced after detecting released ATP from RBCs that were forced to flow through filter paper with pore sizes comparable to the diameter of a red cell. The results suggested that high shear stress induced increased ATP release from RBCs. Further experimental work conducted under controlled microenvironments by using microbore capillaries[30,31] and microfluidic channels[32,33] supported the original filter paper experiments. In addition, several key elements in the mechanotransductive pathways of ATP release from red cells, including G-protein coupled receptors[34] and hemichannels,[35] have been proposed. We recently demonstrated a microfluidic approach to study the time-dependent dynamics of deformation-induced ATP release from RBCs (Fig. 1D).[14]

In this review, we survey mechanical studies of the deformation of RBCs under static and flow conditions. In addition, we discuss recent work on ATP as a signaling molecule following its stress-induced release from red cells. We present the main experimental approaches and theoretical studies of the deformation of RBCs, and provide an integrative understanding of deformation-induced ATP release from RBCs. These steps take advantage of interdisciplinary approaches from biology, chemistry, and engineering and so we integrate the existing physical models, biological evidence, and engineering approaches to offer an improved understanding of the deformation of RBCs and its physiological roles.

Red blood cell deformation: theoretical models and experimental observations

RBCs have a non-nucleated, hemoglobin-rich cytoplasm encapsulated by a molecularly thin membrane composed of a lipid bilayer, membrane skeleton network, and transmembrane proteins. The most well-recognized mechanical responses of RBCs are ascribed to the mechanical properties of the cell membrane. In this section, we briefly discuss the cell deformation using continuum mechanical and microskeletal approaches. We then extend the discussion to cell deformation under flow conditions where hydrodynamics and cell membrane mechanics are coupled.

RBC deformation under static conditions

From the stand point of mechanics, the cell membrane can be treated as a two-dimensional incompressible elastic sheet. Classical ideas from three-dimensional continuum mechanics can be applied to a two-dimensional system, in which case the tensions, a force per length, are defined on the surface of the membrane, and from which mechanical properties of the membrane can be calculated.[8,9] For example, the three independent modes of deformation (Fig. 2A), dilation, shear elongation, and bending, correspond to the three elastic moduli of the membrane, respectively, the area expansion, shear and bending moduli. Each of the elastic moduli measures the resistance to a different mode of deformation. The resistance to the rate of deformation, on the other hand, represents the membrane viscosity. The constitutive relations between the membrane tensions, the independent modes of deformation, and the rate of deformation, quantitatively characterize the viscoelastic response of the cell membrane.[9]

Determining the intrinsic mechanical properties of the RBC membrane requires the measurement of deformation at the cellular and subcellular scales and a control system through which external stresses can be applied. Micropipette aspiration is the most common experimental system, in which the external stress can be adjusted by changing the suction pressure and the corresponding deformations, *e.g.*, the length of membrane aspirated into the pipette, can be measured (Fig. 2B). Since the shear modulus is proportional to the slope of a curve of applied pressure *versus* the aspirated length, which can be converted to membrane tension *versus* the fractional change in surface area, the shear and area expansion moduli can be obtained.[8,9] Moreover, the bending modulus can also be derived from the measured pressure where the cell buckles or folds. Therefore, all three of the elastic moduli of the cell membrane can be extracted from a single micropipette aspiration experiment.

For example, for a normal RBC at room temperature, the area expansion, shear and bending moduli obtained from micropipette aspiration experiments are 0.3–0.6 N m^{-1}, 5–7 $\times$ 10^{-6} N m^{-1} and 10^{-19} N m, respectively,[8,9] where bending stiffness is expressed as an energy. The larger these

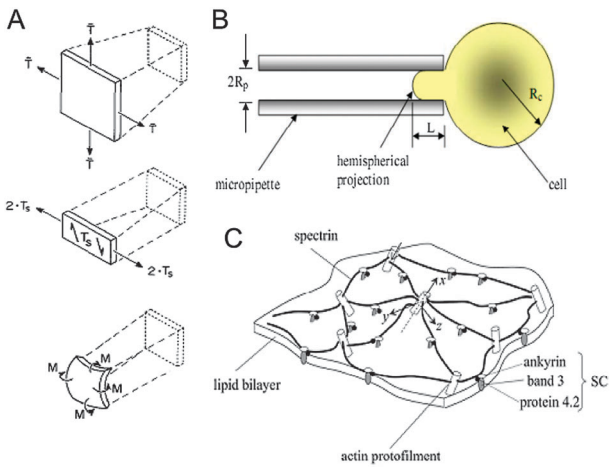

Fig. 2 RBC deformation under static conditions. (A) Schematic illustration of the area expansion, elongation, and bending modes of an element of a membrane.[8] (B) Micropipette aspiration was used to determine the mechanical properties of the RBC membrane.[10] L = aspiration length. (C) A three-dimensional model of the RBC membrane and spectrin network.[20] A junction complex, composed of a central short actin protolament and six long spectrin dimmers, is coupled to the lipid bilayer. SC: suspension complex, which consists of ankyrin, band 3, and protein 4.2.

values, the more resistance is offered to the static mode of deformation. Based on these results, it is concluded that RBCs are easy to bend and elongate, but can hardly be expanded. Therefore, the cell membrane is approximated as incompressible. In addition, micropipette studies of the viscous dissipation process of RBCs, *e.g.*, the timescale required for a cell to recover its stress-free shape upon the release of applied stress, indicate that it is the cell membrane that dominates the dissipation process; the corresponding surface viscosity is on the order of 10^{-6} N s/m.[9]

One application of membrane mechanics is to use RBCs as an ultrasensitive force transducer: the so-called "biomembrane force probe (BFP)" is used to study the weak forces occurring in biological interactions, *e.g.*, receptor–ligand interactions (Fig. 1A).[11,16] The BFP works in the platform of a micropipette aspiration setup, and the tension of the red cell membrane is controlled by the suction pressure. The stiffness of the transducer, which equals the applied axial force divided by a small displacement of the membrane, is defined by the membrane tension. Therefore, the force sensitivity of the BFP can be tuned *via* the pipette aspiration pressure over several orders of magnitude, *i.e.*, from 10^{-2} to 10^{3} pN, which covers a wide range of biological forces, such as myosin/actin and kinesin tractions and actin filament stretching.[15,36] Combining these advantages of the BFP with recently developed techniques, such as optical tweezers,[37] horizontal force microscopy[36,38] and reflection interference contrast microscopy,[39] BFP-based dynamic force spectroscopy has been applied for a variety of biophysical force studies and is a novel application of the mechanical properties of RBCs.

Although the two-dimensional continuum model provides an accurate description of RBC deformation at the whole cell level, it does not provide a detailed picture of the changes of local sub-cellular structures and specific molecules during cell deformation. Microskeleton models, on the other hand, focus on the spectrin-actin network and study the collective responses and connectivity of membrane skeletal proteins during cell deformation.[12] Such molecular models offer a more complete picture of cell deformation.

Fluorescence labeling technologies, such as fluorescence imaging microdeformation,[40] fluorescence recovery after photobleaching,[41] and fluorescence nanoparticle tracking[12] are used to study the spatial and temporal changes of membrane skeleton deformation at the molecular level. These approaches provide new experimental results for the development of theoretical models.[17,19,42] For example, using fluorescence polarization microscopy and micropipette aspiration, it has been shown that actin protofilaments maintain a membrane-tangent orientation whether or not the cell is deformed,[43] which indicates a strong interaction between the protofilament and its associated transmembrane protein.

Along with the experimental approaches, theoretical models have been developed to understand the dynamics of the microskeleton network in the cell membrane. For example, one study establishes a model of the RBCs membrane by coupling the actin-spectrin network with the lipid bilayer.[20] The authors investigated the static and dynamic responses of a junction complex, which was composed of a central short actin protolament and six long αβ spectrin dimers, when coupled with a lipid bilayer under external loads (Fig. 2C). The model demonstrated that the actin and spectrin interactions at the junction complex affected their structural response to cell deformation and predicted the nanomechanics of the protein network and lipid bilayer. In addition, a three-dimensional spectrin-level microskeleton model has also been developed based on a study of cell deformation using optical tweezers.[19]

These quantitative approaches, spanning from membrane skeletal structures to whole cell dynamics, have also motivated studies of the coupling of cell membrane mechanics with cellular biochemical reactions.[21] For example, one of the hypothesis for deformation-induced ATP release proposes that the defects of the spectrin network induced by cell deformation will expose actin molecules to the transmembrane proteins, which will be activated upon binding to actin and subsequently trigger ATP release.[21] In order to verify or modify this proposed mechanism, understanding of cell deformation at the molecular scale is critical, as discussed further below.

RBC deformation under flow

RBCs experience a wide range of shear stresses *in vivo* and deform constantly in the circulatory system, particularly in microvessels and capillaries. During flow, RBC deformation is dependent on hydrodynamic parameters, such as shear rate, the effective fluid viscosity outside the cell, and the boundary conditions. The challenge, therefore, is to couple the external hydrodynamics with the cellular mechanics and then to predict the cell's response. Since steady, uniform shear is the simplest flow, we start our discussion there and then extend it to more specific flow conditions, such as those in microvessels/capillaries.

It has been experimentally observed that in steady shear flow RBCs exhibit complex dynamics, such as tumbling, tank-treading,[24] and swinging, which refers to an oscillation of the cell's inclination angle superimposed to tank-treading.[44] Among these dynamic behaviors, tank-treading is believed to play a role in the shear shinning property of blood.[1] The detailed picture of tank-treading can be described as follows (Fig. 3A): with an increase of the shear rate, red blood cells deform and reach an equilibrium ellipsoidal shape with a fixed angle to the flow direction while the membrane undergoes a tank-treading motion around the cell body. By tank-treading, the external shear stress is transmitted across the cell membrane to the cytoplasm and induces internal circulation. As a consequence, the whole cell participates in the flow and the apparent blood viscosity decreases.[1]

One class of mathematical models describing how RBCs deform and adapt to the shear flow is based on an analogy to liquid drops: RBCs are treated as a deformable capsule with an elastic membrane.[28,44,45] By coupling the internal and external hydrodynamics with membrane mechanics, theoretical models are able to predict the conditions for RBCs to exhibit different dynamics[46] and where the transitions between these motions occur.[27,44] For example, for a simple shear flow, by numerically calculating the velocity and stress distributions over the surface of the cell, conditions for deformation and periodic flipping motion, similar to swinging where the cell oscillates

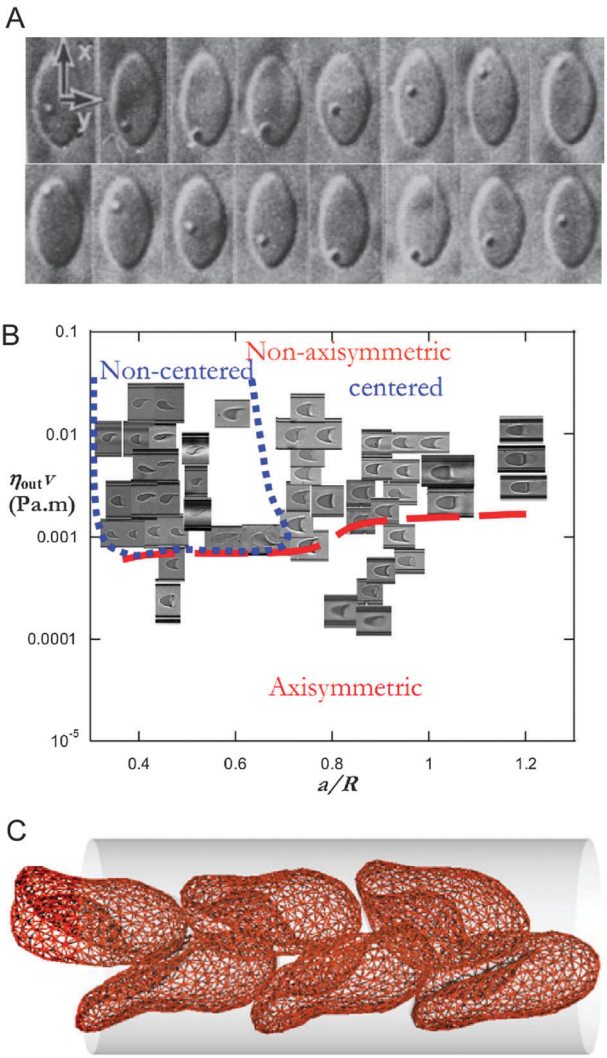

Fig. 3 RBCs deformation under flow. (A) Time-lapse images of a tank-treading RBC under shear flow conditions.[24] x and y indicate the flow direction and radial direction in the cone-and-plate chamber, respectively. The tank-treading motion is visualized by the motion of a membrane-bound latex particle. (B) RBCs flowing in circular glass capillaries show a symmetric parachute-like shape and a non-axisymmetric slipper-like shape as a function of the flow speed (v) and external viscosity (η), and nondimensional particle size a/R, where R is the radius of the capillary tube.[50] (C) Numerical simulations of flow-induced clustering of RBCs in a circular capillary.[51]

along the flow direction, can be established.[46] Moreover, in contrast to cell deformation under static conditions where the effect of the cytoplasmic viscosity is negligible, the transitions, tumbling and tank-treading, are dependent on the viscosity contrast between the internal and external fluids.[27,44] In a few cases, bending resistance of the membrane (*e.g.*, studied in the case of liquid capsules) is taken into account for the shape transitions of RBCs.[47]

Although mechanical properties of the RBC membrane have been characterized experimentally (summarized in the previous section), the measured mechanical parameters are based mainly on micropipette aspiration experiments, in which the conditions are far from what the cells are subjected to in the circulation. It is therefore important to examine the applicability of the obtained membrane parameters under flow conditions. Membrane viscosity, for example, has been studied experimentally under shear flow conditions using a rheoscope, and a surface average-value of membrane viscosity can be extracted based on hydrodynamic models. In this scenario, the membrane viscosity is provided by an energy balance involving the tank-treading, which drives the motion of the outer fluid, and the energy dissipated by the viscous effects in the membrane and cytoplasm.[48,49] The obtained membrane viscosity ($0.5–1.2 \times 10^{-7}$ N s m^{-1}) under flow conditions is little lower than that derived by micropipette experiments ($\sim 10^{-6}$ N s m^{-1}).

RBCs also exhibit distinguished hydrodynamic characteristics in microvessels and capillaries due to the confined microenvironment. It has been shown that RBCs are not uniformly distributed across the microvessel; typically the concentration of red cells is lower in microvessels relative to that in large vessels (Fahraeus effect) and the apparent blood viscosity decreases (Fahraeus-Lindqvist effect).[23] RBCs also deform differently when passing through the capillaries, *e.g.*, the existence of a non-axisymmetric slipper-like shape and a symmetric parachute-like shape.[25,52–55] Since these hydrodynamic characteristics of RBCs in microvessels and capillaries are related to the change of blood viscosity and flow resistance, it is desirable to provide a well-controlled microenvironment where deformation and RBC motion under flow conditions can be studied quantitatively at the single cell level.

Our group has experimentally studied the cellular-scale hydrodynamics of RBCs in confined spaces, *e.g.*, microfluidic channels, and characterized the deformation and motions of RBCs.[50] We established a phase diagram illustrating a shear-stress dependent shape transition of RBC inside microfluidic channels (Fig. 3B). We also quantified the drift speed of the lateral migration of RBCs in a constriction channel and developed microdevices for affecting separation of RBCs from whole blood.[58] In addition, we studied the mechanical responses, *e.g.*, the time-dependent pressure changes, of individual cells flowing inside the microfluidic channels and exploited these features for fast mechanical cell lysis.[59] Also, we recently reported the dynamic behavior of chemically "stiffened" red blood cells in pressure-driven microchannel flows and demonstrated that viscous effects in the cytoplasm and/or lipid membrane are a dominant factor in dictating dynamic responses of RBCs.[60]

The experimentally observed evolution of the changes of the cell shapes and the lateral migration of RBCs in microvessels or capillaries have inspired a large number of theoretical studies.[22,61] Mathematical models suggest that the cell deformation in microvessels is the consequence of a force balance between the bending and stretching forces of the cell membrane and the lubrication forces between the cell membrane and the capillary walls.[25] Numerical simulations of capillary flows show that the velocity and the position of RBCs are the two most important parameters that determine RBC shape transition, *e.g.*, from a discocyte shape to a slipper-like or parachute-like shape.[54,62] Cells that are off the center-line of the capillaries develop asymmetric shapes after a finite time and such asymmetries in shape lead to the lateral migration of RBC.[52,63] Accounting for the effects of cell-cell interactions, a recent

study has shown the formation of flow-induced clustering, with specific arrangements of RBCs in the capillary depending on the hematocrit concentration (Fig. 3C).[51]

ATP release from RBCs

The behavior of RBCs mentioned above, either under static or flow conditions, is a passive response to external stimuli. The idea that RBCs can respond to external stimuli, such as oxygen stress, by releasing signaling chemicals, *e.g.* ATP, was proposed and tested experimentally in 1995.[64] Later, mechanical stress-induced ATP release from RBCs was observed experimentally.[14,30–33] Since it is well known that ATP can induce vasodilation, the observation of ATP release from RBCs implies that the red blood cell can act as a possible blood flow regulator. Here we will discuss how this idea developed and then focus on mechanisms of ATP release from RBCs.

ATP release from RBCs as a precursor for vasodilation

Extracellular ATP, through purinergic receptors, is able to regulate a wide range of biological activities, such as neurotransmission,[65] vasodilation,[66] pain sensation,[67] inflammation and immune responses.[68] Purinergic receptors are membrane ion channels that are distributed throughout tissues in the body and, upon activation, allow flow of ions across the cell membrane and changes of transmembrane potentials. Purinergic receptors can be divided into two classes: P1 and P2, according to the substrates that they can recognize.[65,69] P1 is the class of receptors for nucleoside adenosine and P2 is the class of receptors primarily for ATP and ADP. For example, in the case of ATP-mediated vasodilation, extracellular ATP binds and activates the P2Y receptors (a subclass of P2 receptors) on the vascular endothelial cells (Fig. 4A),[5] and induces the synthesis and release of nitric oxide (NO).[70] Nitric oxide is a well known vasodilator, which causes relaxation of the surrounding smooth muscle cells. Physiologically, vasodilation increases the diameter of a blood vessel, which allows an increase in blood flow with no change in pressure drop.

One source of extracellular ATP in blood is the circulating erythrocytes. RBCs have millimolar concentrations of intracellular ATP and are able to release ATP under a variety of external stimuli, *e.g.*, hypoxia and hypercapnia,[71] mechanical stress,[32] pH and osmotic pressure.[72] The hypothesis of RBC-released ATP as a link to NO and local control of pulmonary vasodilation has been tested under hypoxic conditions,[64] which we discuss next, and provides a new understanding of local blood flow regulation and oxygen delivery.

Sprague, Ellsworth, and their colleagues proposed that RBCs can act as an oxygen sensor to regulate blood flow by releasing ATP.[5] This hypothesis was based on the experimental observations that oxygen content, *i.e.*, the hemoglobin oxygen affinity, rather than oxygen tension, *i.e.*, the driving force for the diffusive transfer of oxygen from red blood cells to tissue, was more important for maintaining the supply of oxygen in conditions of severe hypoxia.[73] Since the oxygen content in RBCs is directly linked to the oxygen utilization in tissues, it is possible that the RBCs themselves can be part of the regulation system for blood flow. Also as RBCs are able to release ATP under hypoxic conditions,[71] it was suggested that RBC may play a direct role in regulation of vascular tone by releasing ATP.[64] Inspired by this idea, experimental and theoretical investigations were under taken and the results are in a good

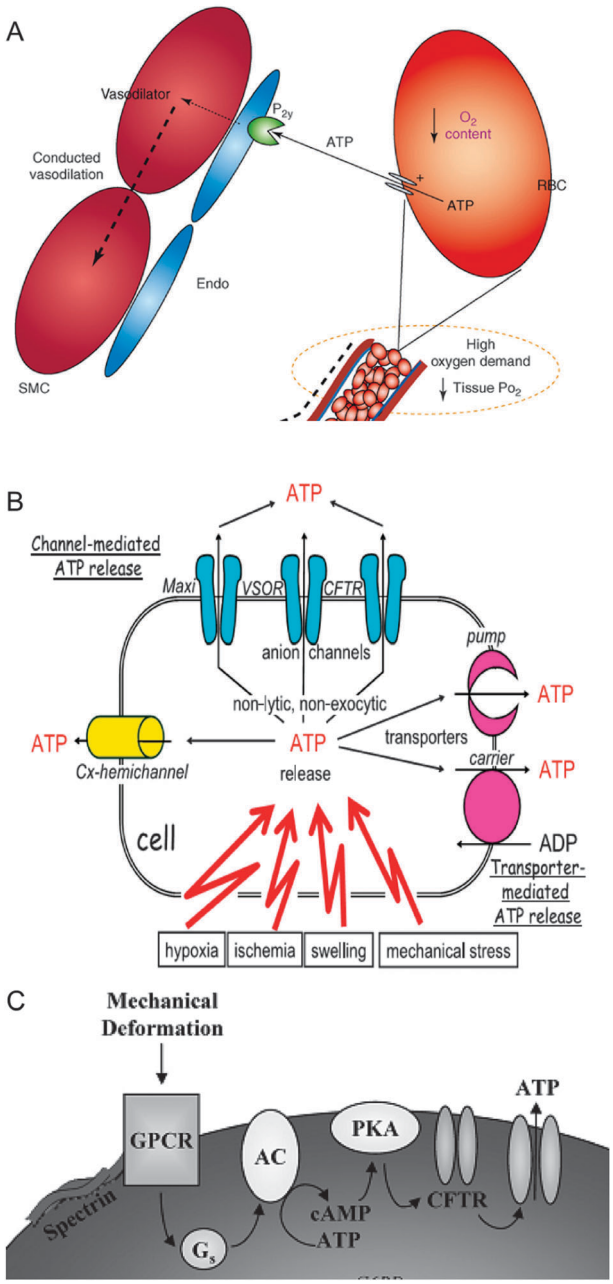

Fig. 4 Molecular-scale mechanisms of ATP release from RBCs. (A) Proposed hypoxia-induced ATP release from RBCs as a precursor for vasodilation.[5] Released ATP from RBCs, due to the decrease of oxygen content, binds the purinergic receptors (P2Y) on the vascular endothelial cells and induces the synthesis and release of vasodilators for conducted vasodilation. SMC: smooth muscle cells. (B) Proposed non-lytic ATP release pathways: transporter mediated, channel and hemichannel-mediated.[56] (C) Proposed G-protein mediated ATP release from RBCs.[57] Mechanical-deformation of the cell membrane activates G-protein coupled receptor (GPCR) and G_s, which subsequently activates adenylyl cyclase (AC) to convert ATP to cAMP. cAMP activates protein kinase A which phospholates CFTR and stimulates ATP release.

agreement with the hypothesis.[74–80] For example, when perfusing a cerebral arteriole under low oxygen conditions, the vessel diameter and the concentration of ATP in the effluent were increased only in the presence of RBCs, which indicates that the ATP released from RBCs actively participates in the process of microvascular regulation.[77] However, it is also possible that more than one factor contributes to the control of blood flow in the microcirculation. For instance, it has been reported that there is metabolic regulation of blood flow in the absence of red blood cells.[81] Therefore, more studies are needed to clarify the mechanisms of blood flow regulation.

Since RBCs regularly encounter a wide range of hydrodynamic shear stress *in vivo* and deform significantly on entering arterioles and capillaries, deformation of the cells was proposed as the trigger for ATP release and the topic has been investigated experimentally and theoretically.[14,21,30–33] Indeed, when changes in oxygen tension and pH are not significant, deformation-induced release is considered the major contributor to extracellular ATP.[29] Early studies of deformation-induced ATP were conducted by sending RBCs through porous filter paper.[29] The results showed that more ATP was released when the pore size of the filter paper was decreased, which suggested that high shear stress or deformation enhances ATP release. Further investigations using devices with controlled shear stress such as microbore tubing[30,31] and microfluidic channels,[14,32,33] confirmed that the amount of released ATP was dependent on the magnitude of shear stress and the duration of the shear stress, *e.g.*, length of the tubing or channel constriction. The addition of chemicals that stiffen RBC membranes decreased the amount of ATP released, which suggested that deformation of the cell membrane was a necessary trigger.

Deformation-induced ATP release from RBCs

With respect to the mechanism of ATP release from different cell types, such as endothelial cells and neuronal cells, there are at least three suggested pathways for non-lytic ATP release (Fig. 4B):[56] (1) transporter-mediated release through ATP-binding cassette (ABC) transporters, which includes the cystic fibrosis transmembrane conductance regulator (CFTR), multidrug resistance transporter (MDR), and other ABC transporters; (2) channel-mediated release including connexin and pannexin hemichannels, maxi-anion channels and volume-regulated channels, and (3) exocytosis-mediated release. Since RBCs do not contain organelles such as the golgi complex, which is involved in vesicle formation, and hence can not form vesicles under normal physiological conditions, the study of ATP release from RBCs focuses on the ABC transporter-mediated and channel-mediated pathways, respectively the CFTR and pannexin-1 hemichannels.

The exact role of CFTR in the process of ATP release, however, has been debated for a long time. CFTR is an ATP-dependent ion channel that allows Cl^- ions to diffuse through the membrane.[82,83] RBCs express CFTR on their membrane and there is evidence showing that CFTR activity is involved in the process of deformation-induced ATP release.[84] Thus, a signal transduction pathway has been proposed:[57] mechanical deformation of the cell membrane activates the G-protein (G_s) coupled receptor GPCR, and the activated GPCR triggers adenylyl cyclase (AC) to convert ATP to cAMP,[85] which directly leads to the phosphorylation of CFTR by the protein kinase A (PKA) and stimulates ATP release through a yet unknown separate ATP releasing channel (Fig. 4C).[86–89] In this case, CFTR acts as a regulator for ATP release instead of being the actual conductance channel, as suggested by its name. Other experimental evidence, however, suggests that CFTR is the conductance channel through which ATP can be released.[56,90] It has been proposed, therefore, that CFTR may act as both an ATP conductance regulator and an ATP transport channel,[91] where the rates of ATP transported across the channel determine the functionality of CFTR.

Pannexins, on the other hand, are channel-forming proteins in vertebrates[92] and they have been shown to provide a conduit for ions to flow across the cell membrane. Pannexin-1 assembles into hexameric hemichannels in a cell's membrane to allow efflux of large organic molecules, such as ATP[93,94] and ions such as calcium.[95] In addition, when pannexin-1 was expressed in *Xenopus* oocytes, it was found that mechanical stimuli in the form of suction into a pipette of cell-attached membrane patches with pannexin-1 hemichannels could cause ATP release.[96] This result leads to the hypothesis that pannexin-1 acts as a mechanosensing ATP release channel. Further research on human erythrocytes showed that RBCs expressed pannexin-1 on their membrane and ATP release due to osmotic stress was attenuated by carbenoxolone, a highly effective pannexin channel blocker.[35] Moreover, the observed channel currents from patch-clamp experiments on RBCs exhibited similar properties with the patch-clamp experiments on pannexin-1 expressed *Xenopus* oocytes (Fig. 5A). Collectively, the experimental results suggest that pannexin-1 might be one of conductance channels responsible for deformation-induced ATP release.

We recently studied ATP release from RBCs,[14] tested the hypothesis that cell deformation induces ATP release, and provided experimental evidence for a model based on a CFTR-actin pathway for ATP release (Fig. 5B).[21] In our work, a microfluidic channel with a constriction was used to tune the magnitude and duration of the shear stress in the flow. We used a high-speed camera to track the deformation of individual cells. Also, to determine the average ATP release, we used a bioluminescent reaction and performed identical experiments in a setup with a photon counting photomultiplier tube. In this way, we were able to measure the dynamics of ATP release with millisecond resolution and correlate the time dependence of the ATP release with the typical deformation of the cells.

We observed two distinct timescales associated with the mechanotransductive release of ATP from RBCs. First, under a constant applied shear stress, significant ATP release occurs only when the duration of applied shear stress is above a certain time scale. Second, there is a delay period between the onset of elevated shear stress and ATP release. The first time scale, which we estimate to be 3–6 ms based on the minimum constriction length necessary to induce significant ATP release, is the activation time. The second time scale, after activation, is associated with the time required for the release of ATP in response to the onset of increased shear stress. This latter time scale varies from 25–75 ms depending on the magnitude of the shear stress.

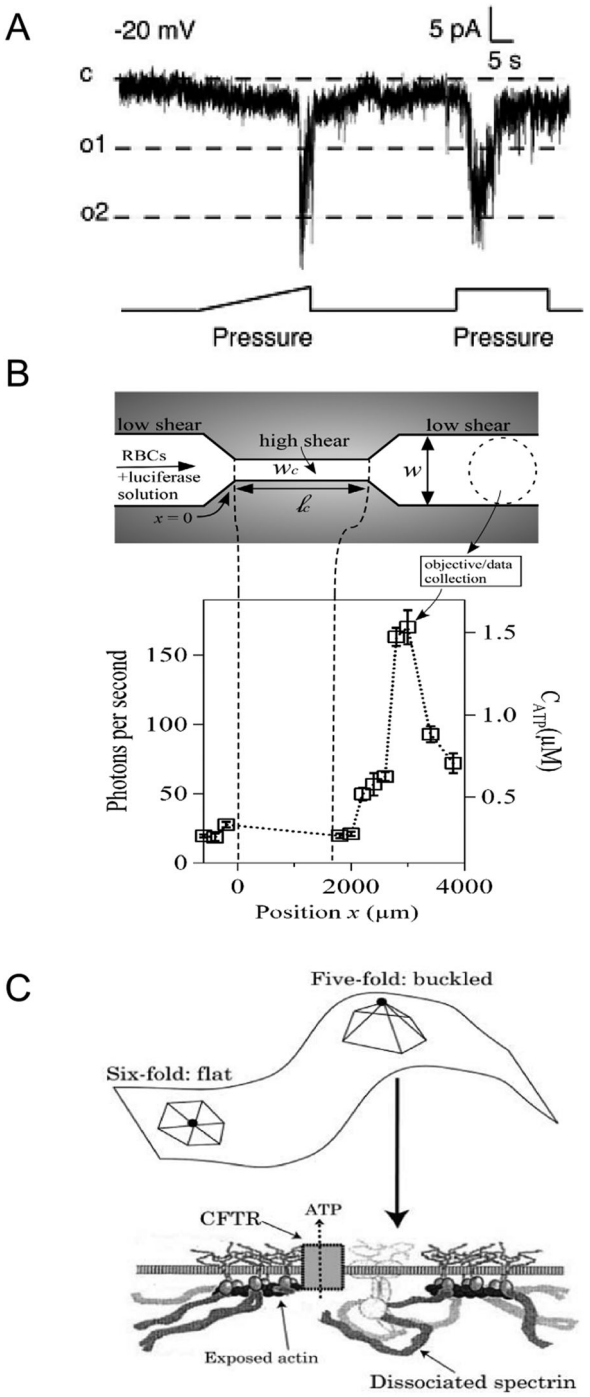

Fig. 5 Quantifying ATP release from RBCs. (A) Large conductance channels on the RBC membrane show similar mechanosensitive properties with those of pannexin-1 channels expressed in *Xenopus* oocytes.[35] (B) Illustration of the experimental setup for a microfluidic study of the dynamic process of deformation-induced ATP release from RBCs.[14] A mixture of RBCs and luciferase/luciferin solution are pumped through a microuidic constriction and the photon emission rate, which results from the reaction between luciferase/luciferin and released ATP is measured *versus* position along the channel using a photomultiplier setup. (C) Schematic of the CFTR-actin based ATP release model, where a membrane skeleton defect due to membrane deformation induces the binding of exposed actin filament to CFTR, which leads to ATP release.[21]

The two time scales observed in our experiments match the two distinct physical processes described in the CFTR-actin model.[21] In this model, the deformation of the cell causes defects in the spectrin network and exposes actin filaments, which can attach to the freely diffusing CFTR protein in the cell membrane; this attachment activates the CFTR[97] and induces ATP release (Fig. 5C). As shown in our experimental results, the 3–6 ms activation time can be interpreted as the reorganization (*i.e.*, relaxation) of the spectrin-actin network in response to cell deformation. The second time scale in our data can be interpreted as the time required for CFTR to diffuse along the cell membrane and bind to the exposed actin. Given the diffusion constant of CFTR ($D \approx 10^{-13}$ m^2 s^{-1})[98] and the actin-actin junction distance ($l \approx$ 60–80 nm),[99] the estimated time scale ($l^2/D \approx$ 30–70 ms) for CFTR to find available actin is comparable to our measured delay time.

While progress has been made in understanding ATP release by mechanotransduction processes, the precise mechanism for release into the extracellular space remains unclear. Indeed, many critical questions still need to be addressed. For example, if CFTR and pannexin-1 channels are both responsible for the deformation-induced ATP release from RBCs, do CFTR and pannexin-1 work cooperatively or sequentially? In either case, what is the effect of shear rate? Are these channels sensitive to the rate of change in mechanical stress? Also, are there any feedback loops, which control the release?

Outlook and future work

We have provided an integrative perspective of the studies of RBC dynamics, RBC deformation, and ATP release. The experimentally documented deformation-induced ATP release from RBCs demonstrates that cells can respond actively to mechanical stress. The released ATP, however, has to communicate with surrounding endothelial cells so as to regulate vascular tone. It is, therefore, important to explore the spatiotemporal response of surrounding endothelial cells to the released ATP and obtain quantitative understanding of the process of red cell-mediated vascular tone. Moreover, in addition to cell deformation, there are a wide range of cell motions in flow, *e.g.*, tumbling, swinging, and tank-treading, which contribute significantly to the blood flow dynamics and the changes of blood viscosity. It will be of great interest to know whether these behaviors of red cells play roles in vascular signaling, *e.g.*, do they affect ATP release? Furthermore, given that ATP is one of the metabolites from glycolysis inside human RBCs, it might be possible that other metabolites with small molecular structures can also be released from RBCs upon deformation. Last, since mechanical stress has been shown to affect cellular metabolism in other cell lines, we wonder whether mechanical stress will influence the red cell's metabolism.

RBCs constantly experience shear stress *in vivo* and most of the cardiovascular diseases are associated with RBC metabolism, signaling, and deformation.[100–102] We believe that the combination of studies of physical models and biochemical processes in RBCs will deepen our understanding of the physiological roles of RBC, provide a new perspective to examine vascular homeostasis, and offer creative therapeutic approaches to treat vascular diseases.

Acknowledgements

We thank the Princeton School of Engineering and Applied Science for support of this research. We also thank G. Guidotti, W. Ristenpart, J. Grotberg, and M. Abkarian for helpful discussions.

Notes and references

1 H. Schmid-Schoenbein and R. Wells, *Science*, 1969, **165**, 288–291.
2 H. C. van der Heyde, J. Nolan, V. Combes, I. Gramaglia and G. E. Grau, *Trends Parasitol.*, 2006, **22**, 503–508.
3 P. Vague and I. Juhan, *Diabetes*, 1983, **32**(Suppl 2), 88–91.
4 J. Ho, W. J. Sibbald and I. H. Chin-Yee, *Crit. Care Med.*, 2003, **31**, S687–S697.
5 R. Sprague, A. H. Stephenson and M. L. Ellsworth, *Trends Endocrinol. Metab.*, 2007, **18**, 350–355.
6 N. Mohandas and P. G. Gallagher, *Blood*, 2008, **112**, 3939–3948.
7 R. P. Rand and A. C. Burton, *Biophys. J.*, 1964, **4**, 115–135.
8 E. A. Evans, *Methods Enzymol.*, 1989, **173**, 3–35.
9 R. M. Hochmuth and R. E. Waugh, *Annu. Rev. Physiol.*, 1987, **49**, 209–219.
10 C. T. Lim, E. H. Zhou and S. T. Quek, *J. Biomech.*, 2006, **39**, 195–216.
11 E. Evans, A. Leung, V. Heinrich and C. Zhu, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 11281–11286.
12 J. C. M. Lee and D. E. Discher, *Biophys. J.*, 2001, **81**, 3178–3192.
13 R. Skalak and P. I. Branemark, *Science*, 1969, **164**, 717–719.
14 J. Wan, W. D. Ristenpart and H. A. Stone, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 16432–16437.
15 E. Evans, K. Ritchie and R. Merkel, *Biophys. J.*, 1995, **68**, 2580–2587.
16 R. Merkel, P. Nassoy, A. Leung, K. Ritchie and E. Evans, *Nature*, 1999, **397**, 50–53.
17 M. Dao, J. Li and S. Suresh, *Mater. Sci. Eng., C*, 2006, **26**, 1232–1244.
18 D. E. Discher, D. H. Boal and S. K. Boey, *Biophys. J.*, 1998, **75**, 1584–1597.
19 J. Li, M. Dao, C. T. Lim and S. Suresh, *Biophys. J.*, 2005, **88**, 3707–3719.
20 Q. Zhu, C. Vera, R. J. Asaro, P. Sche and L. A. Sung, *Biophys. J.*, 2007, **93**, 386–400.
21 N. S. Gov and S. A. Safran, *Biophys. J.*, 2005, **88**, 1859–1874.
22 H. L. Goldsmith, *Science*, 1966, **153**, 1406–1407.
23 H. L. Goldsmith, G. R. Cokelet and P. Gaehtgens, *Am. J. Physiol.*, 1989, **257**, H1005–1015.
24 T. M. Fischer, M. Stohr-Lissen and H. Schmid-Schonbein, *Science*, 1978, **202**, 894–896.
25 C. Pozrikidis, *Phys. Fluids*, 2005, **17**, 031503/031501–031503/031514.
26 S. R. Keller and R. Skalak, *J. Fluid Mech.*, 1982, **120**, 27–47.
27 J. M. Skotheim and T. W. Secomb, *Phys. Rev. Lett.*, 2007, **98**, 078301/078301–078301/078304.
28 Y. Sui, Y. T. Chew, P. Roy, Y. P. Cheng and H. T. Low, *Phys. Fluids*, 2008, **20**, 112106/112101–112106/112110.
29 R. S. Sprague, M. L. Ellsworth, A. H. Stephenson and A. J. Lonigro, *Am. J. Physiol.*, 1996, **271**, H2717–H2722.
30 D. J. Fischer, N. J. Torrence, R. J. Sprung and D. M. Spence, *Analyst*, 2003, **128**, 1163–1168.
31 R. Sprung, R. Sprague and D. Spence, *Anal. Chem.*, 2002, **74**, 2274–2278.
32 A. K. Price, D. J. Fischer, R. S. Martin and D. M. Spence, *Anal. Chem.*, 2004, **76**, 4849–4855.
33 A. K. Price, R. S. Martin and D. M. Spence, *J. Chromatogr., A*, 2006, **1111**, 220–227.
34 R. S. Sprague, A. H. Stephenson, E. A. Bowles, M. S. Stumpf and A. J. Lonigro, *Diabetes*, 2006, **55**, 3588–3593.
35 S. Locovei, L. Bao and G. Dahl, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 7655–7659.
36 V. Heinrich and C. Ounkomol, *Appl. Phys. Lett.*, 2008, **92**, 153902.
37 B. Heymann and H. Grubmuller, *Phys. Rev. Lett.*, 2000, **84**, 6126–6129.
38 C. Ounkomol, H. Xie, P. A. Dayton and V. Heinrich, *Biophys. J.*, 2009, **96**, 1218–1231.
39 V. Heinrich, W. P. Wong, K. Halvorsen and E. Evans, *Langmuir*, 2008, **24**, 1194–1203.
40 D. E. Discher, N. Mohandas and E. A. EVans, *Science*, 1994, **266**, 1032–1035.
41 J. C. M. Lee, D. T. Wong and D. E. Discher, *Biophys. J.*, 1999, **77**, 853–864.
42 S. K. Boey, D. H. Boal and D. E. Discher, *Biophys. J.*, 1998, **75**, 1573–1583.
43 C. Picart, P. Dalhaimer and D. E. Discher, *Biophys. J.*, 2000, **79**, 2987–3000.
44 M. Abkarian, M. Faivre and A. Viallat, *Phys. Rev. Lett.*, 2007, **98**, 188302.
45 C. D. Eggleton and A. S. Popel, *Phys. Fluids*, 1998, **10**, 1834–1845.
46 C. Pozrikidis, *Ann. Biomed. Eng.*, 2003, **31**, 1194–1205.
47 C. Pozrikidis, *J. Fluid Mech.*, 2001, 269–291.
48 T. M. Fischer, *Biophys. J.*, 2007, **93**, 2553–2561.
49 R. Tran-Son-Tray, S. P. Sutera and R. P. Rao, *Biophys. J.*, 1984, **46**, 65–72.
50 M. Abkarian, M. Faivre, R. Horton, K. Smistrup, C. A. Best-Popescu and H. A. Stone, *Biomed. Mater.*, 2008, **3**, 13–35.
51 J. L. McWhirter, H. Noguchi and G. Gompper, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 6039–6043.
52 T. W. Secomb, B. Styp-Rekowska and A. R. Pries, *Ann. Biomed. Eng.*, 2007, **35**, 755–765.
53 T. W. Secomb and R. Skalak, *Microvasc. Res.*, 1982, **24**, 194–203.
54 H. Noguchi and G. Gompper, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 14159–14164.
55 P. Gaehtgens, C. Duhrssen and K. H. Albrecht, *Blood cells*, 1980, **6**, 799–812.
56 R. Z. Sabirov and Y. Okada, *Purinergic Signalling*, 2005, **1**, 311–328.
57 N. V. Tolan, L. I. Genes, W. Subasinghe, M. Raththagala and D. Spence, *Anal. Chem.*, 2009, **81**, 3102–3108.
58 M. Faivre, M. Abkarian, K. bickraj and H. A. Stone, *Biorheology*, 2006, **43**, 147–159.
59 M. Abkarian, M. Faivre and H. A. Stone, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **103**, 538–542.
60 A. M. Forsyth, J. Wan, W. D. Ristenpart and H. A. Stone, *Microvasc. Res.*, 2010, **80**, 37–43.
61 D. Lominadze and G. Mchedlishvili, *Microvasc. Res.*, 1999, **58**, 187–189.
62 C. Pozrikidis, *Ann. Biomed. Eng.*, 2004, **33**, 165–178.
63 P. Olla, *Phys. Rev. Lett.*, 1999, **82**, 453–456.
64 M. L. Ellsworth, T. Forrester, C. G. Ellis and H. H. Dietrich, *Am. J. Physiol.*, 1995, **269**, H2155–H2161.
65 G. Burnstock, *Trends Pharmacol. Sci.*, 2006, **27**, 166–176.
66 W. T. McCullough, D. M. Collins and M. L. Ellsworth, *Am. J. Physiol.*, 1997, **272**, H1886–H1891.
67 K. Inoue, M. Tsuda and H. Tozaki-Saitoh, *Purinergic Signalling*, 2007, **3**, 311–316.
68 M. J. L. Bours, E. L. R. Swennen, F. Di Virgilio, B. N. Cronstein and P. C. Dagnelie, *Pharmacol. Ther.*, 2006, **112**, 358–404.
69 B. S. Khakh and A. North, *Nature*, 2006, **442**, 527–532.
70 D. Janigro, T. S. Nguyen, E. L. Gordon and H. R. Winn, *Am. J. Physiol.*, 1996, **270**, H1423–H1434.
71 G. R. Bergfeld and T. Forrester, *Cardiovasc. Res.*, 1992, **26**, 40–47.
72 D. B. Light, T. L. Capes, R. T. Gronau and M. R. Adler, *Am. J. Physiol.*, 1999, **277**, C480–C491.
73 J. C. Stein and M. L. Ellsworth, *J. Appl. Physiol.*, 1993, **75**, 1601–1607.
74 J. E. Jagger, R. M. Bateman, M. L. Ellsworth and C. G. Ellis, *Am. J. Physiol.*, 2001, **280**, H2833–2839.
75 A. Faris and D. Spence, *Analyst*, 2008, **133**, 678–682.
76 M. L. Ellsworth, *Med. Sci. Sports Exercise*, 2004, **36**, 35–41.
77 H. H. Dietrich, M. L. Ellsworth, R. Sprague and R. G. Dacey Jr, *Am. J. Physiol.*, 2000, **278**, H1294–H1298.
78 J. C. Arciero, B. E. Carlson and T. W. Secomb, *Am. J. Physiol.*, 2008, **295**, H1562–H1571.
79 Z. Cao, J. B. Bell, J. G. Mohanty, E. Nagababu and J. M. Rifkind, *Am. J. Physiol.*, 2009, **297**, H1494–H1503.
80 M. Farias, III, M. W. Gorman, M. V. Savage and E. O. Feigl, *Am. J. Physiol.*, 2005, **288**, H1586–H1590.
81 A. T. Ngo, L. J. Jensen, M. Riemann, N.-H. Holstein-Rathlou and C. Torp-Pedersen, *Pfluegers Arch.*, 2010, **460**, 41–53.

82 M. Sugita and J. K. Foskett, *Membrane Structure in Disease and Drug Therapy*, 2000, 439–459.
83 G. Decherf, G. Bouyer, S. Egee and S. L. Y. Thomas, *Blood Cells, Mol., Dis.*, 2007, **39**, 24–34.
84 R. Sprague, M. L. Ellsworth, A. H. Stephenson, M. E. Kleinhenz and A. J. Lonigro, *Am. J. Physiol.*, 1998, **275**, H1726–H1732.
85 R. Sprague, M. L. Ellsworth, A. H. Stephenson and A. J. Lonigro, *Am. J. Physiol.*, 2001, **281**, C1158–C1164.
86 J. J. Olearczyk, A. H. Stephenson, A. J. Lonigro and R. Sprague, *Am. J. Physiol.: Cell Physiol.*, 2004, **286**, H940–H945.
87 J. J. Olearczyk, A. H. Stephenson, A. J. Lonigro and R. Sprague, *Med. Sci. Monit.*, 2001, **7**, 669–674.
88 C. Li, M. Ramjeesingh and C. E. Bear, *J. Biol. Chem.*, 1996, **271**, 11623–11626.
89 G. M. Braunstein, R. M. Roman, J. P. Clancy, B. A. Kudlow, A. L. Taylor, V. G. Shylonsky, B. Jovov, K. Peter, T. Jilling, I. I. Ismailov, D. J. Benos, L. M. Schweibert, J. G. Fitz and E. M. Schweibert, *J. Biol. Chem.*, 2001, **276**, 6621–6630.
90 P. Linsdell and J. W. Hanrahan, *J. Gen. Physiol.*, 1998, **111**, 601–614.
91 E. M. Schwiebert, *Am. J. Physiol. Cell Physiol.*, 1999, **276**, C1–C8.
92 Y. Panchin, I. Kelmanson, M. Matz, K. Lukyanov, N. Usman and S. Lukyanov, *Curr. Biol.*, 2000, **10**, R473–474.
93 R. Iglesias, G. Dahl, F. Qiu, D. C. Spray and E. Scemes, *J. Neurosci.*, 2009, **29**, 7092–7097.
94 F. B. Chekeni, M. R. Elliott, J. K. Sandilos, S. F. Walk, J. M. Kinchen, E. R. Lazarowski, A. J. Armstrong, S. Penuela, D. W. Laird, G. S. Salvesen, B. E. Isakson, D. A. Bayliss and K. S. Ravichandran, *Nature*, 2010, **467**, 863–867.
95 V. I. Shestopalov and Y. Panchin, *Cell. Mol. Life Sci.*, 2008, **65**, 376–394.
96 L. Bao, S. Locovei and G. Dahl, *FEBS Lett.*, 2004, **572**, 65–68.
97 B. Chasan, N. A. Geisse, K. Pedatella, D. G. Wooster, M. Teintze, M. D. Carattino, W. H. Goldmann and H. F. Cantiello, *Eur. Biophys. J.*, 2002, **30**, 617–624.
98 P. M. Haggie, B. A. Stanton and A. S. Verkman, *J. Biol. Chem.*, 2002, **277**, 16419–16425.
99 T. J. Byers and D. Branton, *Proc. Natl. Acad. Sci. U. S. A.*, 1985, **82**, 6153–6157.
100 E. Ragone, P. Strazzullo, A. Siani, R. Lacone, L. Russo, A. Sacchi, P. Cipriano, M. Mancini, G. Zhao, X.-Y. Yan, D.-Y. Li and L. Gong, *Am. J. Hypertens.*, 1998, **11**, 935–941.
101 T. Rassaf, P. Kleinbongard and M. Kelm, *Kidney Blood Pressure Res.*, 2005, **28**, 341–348.
102 E. Ch. Mokken, M. Kedaria, Ch. P. Henny, M. R. Hardeman and A. W. Gelb, *Ann. Hematol.*, 1992, **64**, 113–122.