Review

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Targeting spectrin redox switches to regulate the mechanoproperties of red blood cells

<https://doi.org/10.1515/hsz-2020-0293>

Received August 25, 2020; accepted November 17, 2020;

published online December 2, 2020

Abstract: The mechanical properties of red blood cells (RBCs) are fundamental for their physiological role as gas transporters. RBC flexibility and elasticity allow them to survive the hemodynamic changes in the different regions of the vascular tree, to dynamically contribute to the flow thereby decreasing vascular resistance, and to deform during the passage through narrower vessels. RBC mechanoproperties are conferred mainly by the structural characteristics of their cytoskeleton, which consists predominantly of a spectrin scaffold connected to the membrane via nodes of actin, ankyrin and adducin. Changes in

redox state and treatment with thiol-targeting molecules decrease the deformability of RBCs and affect the structure and stability of the spectrin cytoskeleton, indicating that the spectrin cytoskeleton may contain redox switches. In this perspective review, we revise current knowledge about the structural and functional characterization of spectrin cysteine redox switches and discuss the current lines of research aiming to understand the role of redox regulation on RBC mechanical properties. These studies may provide novel functional targets to modulate RBC function, blood viscosity and flow, and tissue perfusion in disease conditions.

Keywords: hemoglobin; molecular simulations; nitric oxide; reactive species interactome; spectrin structure.

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Introduction

Red blood cells (RBCs) are anucleate, biconcave-shaped, disc-like cells, which are specialized in compartmentalization and transport of respiratory gases from the lungs to the peripheral tissues and back (Hall 2015). The biochemical, biological, and biophysical characteristics of RBC are highly specialized for this purpose (Kuhn et al. 2017). A loss of RBC integrity (also termed hemolysis) and the release of hemoglobin (Hb) into the circulation may induce toxic effects, including systemic nitric oxide (NO) scavenging and oxidation reactions (Kuhn et al. 2017).

The mechanical and biophysical properties of RBCs are particularly important for their physiology (Kuhn et al. 2017). Their biconcave shape increases the surface for a more efficient gas exchange. Moreover, RBCs are also extremely elastic and flexible, two properties that allow them to survive the dramatic changes in flow conditions along the vascular tree, as well as to deform in the narrower vessels of the microcirculation (Hall 2015).

These mechanical properties are mainly conferred by the RBC cytoskeleton, which has a unique protein composition and structural characteristics. The main protein component of the RBC cytoskeleton are spectrin fibrils,

which are connected to each other by nodes of actin and anchored to the membrane by ankyrin and adducin. This forms a very elastic and flexible scaffold stabilizing the

membrane and providing the RBCs with their typical biconcave shape (Figure 1).

Figure 1: Cytoskeleton of RBCs.

(A) Schematic view of the biconcave shape of RBCs. (B) The structure of the cytoskeleton is enlarged, showing the lipid double membrane with spectrin and actin building hexameric units. Adapted from Goodman et al. (2019). (C) The hexameric arrangement is enlarged. α -spectrin and β -spectrin are stretched between a complex of actin, adducin, and band 3. Ankyrin binds at the center of the spectrin tetramer and connects it to the membrane via band 3. Adducin caps the barbed end of the actin polymer. (D) Detailed representation of the complex mentioned in panel (C). Prx2 peroxidorexin 2; CH calponin homology; GEC glycolytic enzyme complex; Glut1 glucose transporter 1; GPA glycoporphin A; GPB glycoporphin B; GPC/D glycoporphins C and D; RhAG Rh-associated glycoprotein. Adapted from Lux (2016). (E) Surface representation of our structural model of the spectrin tetramer. The hollow double-start helix is clearly visible. α -spectrin chains are colored in red and orange; β -spectrin chains are colored in blue and purple. (F) Our tetramer model with cartoon representation of the spectrin repeat (SR) domains. Cysteine sulphur atoms are shown as yellow spheres (van-der-Waals radii are increased for visibility). (G) Part of the multiple sequence alignment of human α -spectrin and other vertebrate sequences of erythroid spectrin which was used to assess the evolutionary conservation of cysteines. This example corresponds to residues 1390–1468 of human α Spl and shows two conserved cysteines, 1394 and 1404 (yellow, marked with numbers). Not all sequences are shown for clarity. Colored residues indicate high identity according to the 'identity' coloring option of Mview; colors vary according to physicochemical properties as specified in the MView documentation (Madeira et al. 2019). Full alignments of erythrocytic α - or β -spectrin sequences with their UniProt accession numbers are available at <https://doi.org/10.25838/d5p-13>.

Interestingly, changes in cellular redox state and/or oxidative modifications of cytoskeletal proteins induced by pathological conditions like diabetes or genetic defects of antioxidant enzymes are known to affect cell shape, RBC deformability, membrane stability, and resistance to osmotic stress, as well as half-life of RBCs in the circulation and/or induce hemolytic anemia (Kuhn et al. 2017). Such effects are also induced by treating RBC *in vitro* with reagents targeting thiol groups (Becker et al. 1986; Fischer et al. 1978; Smith and Palek 1983; Sullivan and Stern 1984), including *N*-ethylmaleimide (NEM) and diamide (Becker et al. 1986; Sinha et al. 2015; Smith and Palek 1983; Zaccarin et al. 2014). These observations indicate that the stability of the RBC membrane and RBC mechanoproperties may be regulated by specific cysteine redox switches, which may be present in the RBC cytoskeleton.

In this perspective review, we summarize current knowledge about the structural and functional characterization of spectrin cysteine redox switches and discuss the current lines of research aiming to understand the role of redox regulation on RBC mechanical properties. In particular, we will describe (1) structural and functional characteristics of spectrin, including general biochemical characteristics, structural models at different resolutions, and its main binding partners, (2) evidence for potential redox switches in spectrin, (3) how cysteine redox switches in spectrin may contribute to controlling RBC deformability and influencing blood rheology, and (4) the (patho)physiological implications. The study of redox switches in RBCs may reveal potential molecular targets for pharmacological intervention in diseases such as hemoglobinopathies or malaria.

Structural and functional characteristics of spectrin

The RBC integrity, biconcave shape (Figure 1A), and mechanical properties are mainly defined by the peculiar structural dynamics of their cytoskeleton (Mohandas and Gallagher 2008). A major component of the cytoskeleton is spectrin (Marchesi and Steers 1968). There are two isoforms of spectrin, the erythroid isoform (SpI), expressed only in erythroid cells, and the non-erythroid isoform (SpII), which is found in other cell types, including platelets and neuronal cells (Goodman et al. 2019).

In the native RBC cytoskeletal organization, spectrin fibrils are arranged to form the sides and diagonals of a hexagonal unit (Figure 1B and C) and are connected to each other via nodes of short actin polymers, stabilized by the cytoskeleton protein adducin and the cytoskeleton-associated protein 4.1 (also termed band 4.1) (Figure 1C

and D). The spectrin hexagonal units form a flexible network under the lipid bilayer (Liu et al. 1987) and are connected to the lipid bilayer via the scaffolding protein ankyrin (Bennett 1992), which interacts with the transmembrane protein band 3 (Goodman et al. 2013; Mohandas and Gallagher 2008). In this section, we describe the general structure of the RBC cytoskeleton, with a specific focus on the structural characteristics of spectrin including spectrin repeat domains and domain organization, and the available structural models of spectrin (Figure 1).

Structure of the spectrin fibrils

The spectrin fibril consists of α I (2419 amino acids, 280 kDa) and β I (2,137 amino acids, 246 kDa) spectrin (for the context of RBC hereafter referred to as α and β), encoded by the SPTA1 and SPTB gene, respectively. One α and β subunit each form a heterodimer (Shotton et al. 1979; Speicher et al. 1992), where the C-terminus of α -spectrin and the N-terminus of β -spectrin interact with each other. Two $\alpha\beta$ -dimers associate in a head-to-head manner with their β -spectrin C-terminus and α -spectrin N-terminus, creating a tetramerization region on each $\alpha\beta$ -dimer (DeSilva et al. 1992; Ipsaro et al. 2010). The mass of the physiological tetramer structure is ~ 1000 kDa (Goodman et al. 2019). A main focus has been the investigation of the spectrin mesh in membrane patches of RBC or RBC ghosts, RBC depleted of hemoglobin and other cytoplasmic proteins, via different electron microscopic imaging methods, atomic force microscopy, or super-resolution microscopy, leading to coarse-grained structural representations of spectrin. From early electron micrographs of partially extended RBC ghosts, the helicity of the spectrin fibril was evident and subsequently modeled as a two-start helix (McGough and Josephs 1990). In these stretched conformations, spectrin fibrils are visible as straight lines connecting the actin nodes and having a maximum end-to-end distance of ~ 200 nm (Liu et al. 1987; McGough and Josephs 1990). In the resting state, however, spectrin was found to be much shorter with estimations of approximately 45–65 nm length (Brown et al. 2015; Nans et al. 2011; Swihart et al. 2001). Nonetheless, the question of the accurate preservation of the physiological resting state in electron and atomic force microscopic (AFM) experiments remains a challenge. More recent experiments using super-resolution microscopy represent so far the most native-like state of the RBC membrane and suggest a physiological resting length of ~ 80 nm (with minimal and maximal values of 50 and 150 nm) (Pan et al. 2018).

McGough and Josephs calculated the diameter of the spectrin fibril via Fourier-analyzed electron micrographs

(McGough and Josephs 1990). In their model, they observed a negative correlation between the diameter and the pitch of the two-start helix, with a diameter of 3.6 nm and a pitch of 16.6 nm for the stretched state and a diameter of 5.2 nm and a pitch of 10.4 nm for the resting state. A similar diameter of 4.0–4.5 nm was reported from electron tomography studies (Nans et al. 2011).

Spectrin repeat domains and domain organization

The spectrin repeat (SR) domain is the main structural subunit of spectrin. The SR is an antiparallel, three-helix (a–c) coiled-coil domain of about 106 amino acids length. 20 and 16 SR make up α and β spectrin, respectively. Obtaining molecular structures of (multiple connected) SR together with detailed information about the coiled-coil helix arrangement and other atomistic properties is a current research area in the study of spectrin and its mechanical properties. Although sequences of the SR can vary substantially (from ~9 to ~36% sequence identity using the SR of α - and β spectrin), the overall SR structure is highly conserved (see for example Brown et al. 2015, for a structural alignment of SR crystals structures).

The linker region between two SR is α -helical as well and continues helix c to the helix a of the following SR (Grum et al. 1999). Nonetheless, the linker region is able to bend in different angles (Brown et al. 2015) and, thus, influences the pitch of the helical spectrin structure. For some of the SR, it was shown that co-translational folding occurs (Nilsson et al. 2017) and that neighboring repeat domains exert an effect on the co-translational folding that is specific for different SR (Kemp et al. 2020). Individual measurements of the melting temperature of each individual SR in erythroid spectrin support the idea that, despite their similarity in secondary and tertiary structure, SR actually differ significantly in some of their physicochemical properties (An et al. 2006).

High-resolution structures of different SR are available (Grum et al. 1999; Yan et al. 1993), including structures of up to three consecutive SR (SR β 13–15, 15% coverage of β -spectrin (Ipsaro and Mondragón 2010) and SR β 14–16, 14% coverage of β -spectrin (Davis et al. 2009)) and of the tetramerization domain (covering SR α 0–1 and SR β 16–17); the latter is the largest substructure of spectrin that has been crystalized (Ipsaro et al. 2010). Units of up to four consecutive SR have been resolved so far for α -actinin (Ylänne et al. 2001) and the Plakin domain of Desmoplakin, containing an additional SH3 domain (Choi and Weis 2011). Constructs larger than that become rapidly more flexible

with each additional SR, making it more and more difficult to determine a crystal structure.

The dimerization of the α and β chain is initiated at SR β 1–2 and α 20–21 via long-range interactions (Li et al. 2008). After this specific step, the chains bind together via weaker interactions of the following SR (Li et al. 2007), coiling around each other.

Domain organization and interaction with other cytoskeletal proteins

Spectrin is indirectly connected to the RBC membrane via the erythrocyte-specific isoform of ankyrin (Bennett 1992), which binds with its ZU5 domain at SR 14–15 of β -spectrin (Ipsaro and Mondragón 2010), such that there are two potential binding sites for ankyrin roughly in the middle of the spectrin tetramer. In erythrocytes, ankyrin binds to the membrane protein band 3 (Pinder et al. 1995). Having an integral membrane domain as well as a cytoplasmic domain (Lux et al. 1989), band 3 thus connects the RBC plasma membrane to the spectrin mesh (Figure 1).

The N-terminal CH domains of β -spectrin and the C-terminal EF hand domain of α -spectrin together form both equivalent ends of tetrameric spectrin. The CH domains bind to the short actin filaments (An et al. 2005; Karinch et al. 1990), supported by the EF hand domain (Korsgren and Lux 2010). Additionally, protein 4.1 is binding at the calponin homology (CH) domain (An et al. 2005) and facilitates the binding of spectrin and actin (Ohanian et al. 1984). Six spectrin tetramers can bind one actin filament (Liu et al. 1987), which gives rise to the hexagonal pattern of the cytoskeletal mesh. Adducin caps the barbed ends of the actin filaments, preventing further growth of the actin polymer structure (Kuhlman et al. 1996) and, at the same time, can interact with band 3 as well to give another anchoring point to the plasma membrane (Anong et al. 2009). The adducin binding site on band 3 as identified in mutational studies suggests, however, that adducin and ankyrin likely are not able to bind to band 3 at the same time due to steric hindrance (Franco et al. 2016).

The SH3 domain of spectrin (also referred to as SR α 10) is generally capable of forming various protein-protein interactions (Mayer 2001). Yet, its specific functions in erythroid spectrin is not yet fully understood. The spectrin-actin cytoskeleton furthermore interacts with a range of additional proteins, including tropomyosin, tropomodulin, and dematin, which are discussed elsewhere in more detail (Goodman et al. 2019; Lux 2016).

Structural models of spectrin

The exact mechanism of how spectrin is able to change between its resting and extended lengths, and how it thus provides flexibility to the RBC cytoskeleton, remains a topic of debate. Structural models of spectrin, ideally at the atomistic level, bridge the gap between coarse-grained representations of full-length spectrin and the atom-level information obtained from SR structures available for parts of the spectrin sequence only. That way, the structural models can contribute to scrutinizing the mechanisms how the spectrin fibril in its physiological state performs its function as the main component for RBC deformability.

The molecular origins of spectrin flexibility and its elastic properties were investigated by atomistic and coarse-grained molecular simulations (Mirijanian et al. 2007), based on crystal structures of up to four SRs (Autore et al. 2013) and the tetramerization domain complex (Ipsaro et al. 2010). Note that such simulations may miss the influence of further structural parts that had not been structurally resolved at that time as well as information about the physiological conformation of SR in the spectrin tetramer.

There are three main models of how spectrin stretches from a compressed to an extended state. The first one assumes the unfolding of SR domains to be responsible for the extension, based on unfolding experiments of multi-SR units with AFM (Law et al. 2003) and related steered MD simulations (Paramore and Voth 2006). While this unfolding might occur physiologically under extreme conditions, it is unlikely to play a role in the earlier stages of extension and short, compressed states, which can be better explained by one of the other two models.

The second model proposes a soft-polymer, worm-like behavior of the spectrin tetramer under compression; a regular, linear double helix is only obtained in stretched states (Shotton et al. 1979). This type of model is also supported by the most detailed simulations of the spectrin tetramer so far (Mirijanian and Voth 2008).

Mirijanian et al. conducted atomistic molecular dynamics simulations of double SR units (Mirijanian et al. 2007), which allow for the helical linker between two SR to bend and twist without unfolding. The observed SR dynamics was transferred to a coarse-grained (Mirijanian and Voth 2008) representation of the spectrin tetramer model for further simulations. However, the lengths of the molecular dynamics simulations of only 45 ns used to parameterize the SR dynamics appear too short by current standards to have reached convergence. Furthermore, the parametrization of the coarse-grained model did not take into account the interaction between the individual

spectrin chains, which could be crucial for a stable linear double helix arrangement.

The third type of models assumes a spring-like extension mechanism in which the linear double helix conformation stays intact across a wide range of lengths, as suggested from the early electron micrographs of stretched RBC ghosts (McGough and Josephs 1990). The *Chinese finger trap* model (Brown et al. 2015) belongs to this category and is likely the most advanced published quaternary structure model of the full-length spectrin heterotetramer so far. It uses atomistic homology models of all SR with the crystal structure of SR $\alpha 16$ as a template.

The SR were arranged to fulfill different length and helix pitch constraints. The linker region of SR was not explicitly modeled in the complete structure but would have to adopt a 90° angle in the most compressed state, with which it loses its α -helical structure. This model is consistent with the linear shape of the tetramer found at different lengths (Nans et al. 2011). Nonetheless, it was not yet subjected to a test in simulations, and the challenge remains to acquire high resolution structures of the whole spectrin tetramer in its near physiological state to verify details about the models.

In own, unpublished work, we constructed a structural model of the $\alpha/\beta/\alpha$ -complex of human SpI at the atomistic level to determine the accurate interacting surfaces between each complementary pair of SRs. We did so to investigate the localization of cysteine switches and their role on spectrin properties. We exploited information on the structural dynamics of SRs as done before, but based on ~10-fold longer molecular dynamics simulations, together with distance information derived from cross-linking experiments between the $\alpha 3$ and $\alpha 4$ units of SpI, the length of the spectrin $\alpha/\beta/\alpha$ -complex derived from the protein composition of the erythrocytes (Brown et al. 2015; Li et al. 2008), as well as cross-linking experiments on the full $\alpha/\beta/\alpha$ -complex of SpI. The helical structure of one spectrin α/β chain was generated by repeatedly superimpositioning the copies of an SR according to this information and using the 3 SR unit crystal structure (Davis et al. 2009) as a structural alignment template, yielding a model of 36 full SR of SpI similar to previous approaches (Brown et al. 2015). The 36-SR model was used then as a template for comparative modeling with the in-house tool TopModel (Mulnaes and Gohlke 2018; Mulnaes et al. 2020). Finally, two α/β -chains were manually arranged such that they fulfill cross-links information (Li et al. 2008), resulting in the tetrameric $\alpha/\beta/\alpha$ -complex built of ~126,000 atoms (Figure 1E and F). The model obtained has an overall length, SR number per turn, handedness, hollowness, and intra-subunit and inter-subunit distances in agreement with experimental data (Brown et al. 2015).

Our model thus belongs to the third type of models mentioned above, which is expected to remain mainly linear and with a regular helicality of the antiparallel double helix. Still, as is the case for the coarse-grained model of the soft-polymer category (Mirijanian and Voth 2008), missing protein-protein interactions found under native conditions, such as with actin oligomers, may lead to non-linear conformations in simulations.

Another promising method to evaluate specific structural models of spectrin is (single-particle) cryo-EM, which was used on several cytoskeletal and filamentous proteins such as F-actin (Chou and Pollard 2019; Merino et al. 2018), tropomyosin (Ecken et al. 2016) or microtubules (Debs et al. 2020) in recent years. For proteins of the spectrin family, however, only the actin binding domain of a neuron specific isoform of spectrin was structurally resolved by cryo-EM so far (Avery et al. 2017). In this regard, further pursuing to resolve the structure and different elongation states of the spectrin tetrameric fibril with cryo-EM will be very valuable.

Mesoscopic and macroscopic models

The biconcave shape of the RBC is due to the constant tension of the spectrin fibrils (Park et al. 2010). In order to model red cell dynamics on a cellular level (i.e., deformability, shear properties, and membrane/cytoplasm viscosity), mechanical representations of the spectrin mesh as networks of springs are frequently employed (Fedosov et al. 2010a). The macroscopic models, however, generally do not use atomistic information but rather rely on experimental techniques such as optical tweezers to deform the RBC or dynamic scattering microscopy to track the microrheology of RBC to obtain parameters for subsequent simulations (Amin et al. 2007).

Mesoscopic RBC models (Fedosov et al. 2010b; Noguchi and Gompper 2005) together with particle dynamics techniques to simulate fluid flow (Español and Revenga 2003; Müller et al. 2015) are able to scrutinize a variety of RBC mechanical properties (Chien et al. 2019). These approaches present an opportunity to link ultimately the atomistic properties in the spectrin molecule to the behavior of RBC and blood flow.

Potential cysteine redox switches in spectrin – evidence and functional significance

There is evidence that chemical modification of thiols in spectrin affects the structure and stability of the RBC

cytoskeleton, membrane symmetry, and RBC shape (Arduini et al. 1989; Becker et al. 1986; Fischer et al. 1978; Smith and Palek 1983). The localization of cysteine redox switches on spectrin is still unknown, also because of the lack of a structural spectrin model with sufficient resolution, as discussed in the previous section. Nature, reversibility and the physiological/pathological conditions leading to spectrin modifications have remained elusive either. In this section, we summarize the existing evidence of the presence of cysteine redox switches in spectrin and discuss their potential functional significance.

Putative cysteine redox switches in spectrin

There is preliminary evidence that spectrin carries cysteine redox switches affecting the deformability and the membrane stability of RBCs, but their physiological significance is unknown. In the 1970/80, a number of studies demonstrated that treating isolated spectrin with monovalent or divalent thiol group-targeting molecules such as NEM or diamide leads to significant changes in electrophoretic behavior of spectrin, cell structure, and resistance to various stresses (like osmotic pressure, temperature and shear) (Becker et al. 1986; Fischer et al. 1978; Go et al. 2015).

Interestingly the nature of the chemical modifiers of the thiols of spectrin determines the outcome on the mechanical and physiological properties of the cells. In a pioneering paper, Fischer and colleagues showed that monofunctional reagents such as NEM are less potent as these compounds require that more than 20% of the membrane SH-groups are modified in order to diminish elongation of RBCs by 50% (Fischer et al. 1978). In contrast, divalent compounds such as diamide, which is able to also induce cross-links, induce the same effects on RBC elongation by blocking less than 5% of the membrane SH-groups.

The marked differences in effectivity between cross-linking and monofunctional agents could be interpreted by assuming that monofunctional reagents irreversibly block thiols and, thus, prevent formation of disulfide bonds; in turn diamide promotes formation of disulfide bonds, which appear to be essential for regulation of deformability and membrane stability.

The interaction of spectrin with membrane proteins such as band 3 and band 4.2 were proposed to be the node of regulation of shape, cytoskeletal flexibility, and deformation (Becker et al. 1986; Fischer et al. 1978; Go et al. 2015). Interestingly, labeling experiments by Fisher et al. (1978) identified band 3 and band 4.1 as targets of cross-linking induced by diamide. It is important to point out that, although these studies are compelling, the exact

position of targeted thiols as well as the physiological relevance of the thiol modifications need further extensive investigation.

Regulatory cysteines in proteins are often highly conserved among vertebrates (Go et al. 2015). Goodman found conserved cysteines in the C-terminal region of α chains of all erythrocytic and non-erythrocytic isoforms of spectrin (SpI and SpII), including Cys 2072 and 2101, which are targeted by ubiquitination after cysteine oxidation (Kakhniashvili et al. 2001). Preliminary analysis carried out by us comparing α and β -SpI sequences of different vertebrates revealed a few highly conserved cysteines in both chains, including Cys 167, 965, 1394, 1404, 2072, 2299, 2388 in the α -chain and 603, 618, 860, 2012 in the β -chain (for an example see Figure 1G; full alignments of erythrocytic α - or β -spectrin sequences are available at <https://doi.org/10.25838/d5p-13>). Shear stress induces exposure of Cys 1877 and 595, respectively, of mouse α and β -SpI, which are conserved in the human sequences (Krieger et al. 2011).

Potential influences of redox modifications on the spectrin structure and mechanical properties were not extensively studied so far at the atomistic level, and many open questions remain. For another large, elastic structural protein, titin, AFM experiments showed a softening effect of cysteine S-glutathionylation upon extension (Herrero-Galán et al. 2019). The specific details of the effect, however, may be different in spectrin: Although cysteine modifications likely prevented the refolding of the reversibly unfolded domains of titin upon relaxation, unfolding of SR does not seem to be the main driver of spectrin's flexibility.

Targeting spectrin cysteine redox switches with NO – S-nitrosation

There is also some evidence that hemoglobin, cytoskeletal, and membrane proteins of RBCs may be targeted by S-nitrosation reactions (Diederich et al. 2018; Grau et al. 2013). The presence and physiological significance of a cysteine redox switch in hemoglobin (Cys63 of the β -chain) was studied by Stemler and colleagues and is still very lively discussed in the literature (Hess et al. 2005; Premont et al. 2020; Sun et al. 2019).

To answer the question whether human spectrin thiols can be targeted by nitrosation reactions in RBCs, purified spectrin or RBC ghosts were treated with high, non-physiological concentrations of the nitrosothiol S-nitroso cysteine (CysNO). Nitrosation was analyzed by (I) reduction of S-nitrosospectrin by using acetic triiodide solution and detection of released NO by chemiluminescence, applying a

method established for albumin (Marley et al. 2000), and (II) biotin switch assay, followed by Western blotting. As a positive control, human serum albumin was analyzed in parallel. Treatment with CysNO resulted in nitrosation of both purified spectrin and spectrin within the intact cytoskeleton of RBC ghosts (Diederich et al. 2018).

Clearly these experiments indicate that nitrosation may occur on spectrin. Note, however, that physiological formation of nitrosospecies in RBCs, such as nitrosothiols, and their physiological significance are still highly debated (Hess et al. 2005; Premont et al. 2020; Rassaf et al. 2003; Sun et al. 2019). Localization and effects of nitrosation of spectrin and other cytoskeletal proteins on RBC structural and functional properties under physiological conditions need to be further studied.

Targeting spectrin cysteine redox switches with glutathione – S-glutathionylation

A highly interesting thiol modification that was proposed to occur on spectrin and other cytoskeletal proteins is glutathionylation (Giustarini et al. 2019; Rossi et al. 2006). Glutathionylation is a reversible posttranslational redox modification occurring at a low- pK_a cysteine by spontaneous or enzymatically catalyzed addition of a glutathione (GSH) molecule from glutathione disulfide (GSSG) (Checconi et al. 2019; Ghezzi 2013; Zhang et al. 2018). Depending on the protein target, glutathionylation can evoke a variety of effects by controlling activity, function, and structure of proteins (Cooper et al. 2011; Groitl and Jakob 2014; Townsend 2007).

GSH is the most abundant intraerythrocytic thiol and is present at millimolar concentrations, and strongly contributes to maintain cellular redox homeostasis by participating in enzymatic reactions as a cofactor (Figure 2). Enzymes using GSH as reducing equivalent are glutaredoxin (GRx) and glutathione peroxidase (GPx). The enzyme responsible for GSH recycling is glutathione reductase, which reduces GSSG to GSH via consumption of NADPH (Figure 2). The enzyme catalyzing glutathionylation is glutathione S-transferase, which is highly abundant in RBCs (Krohne-Ehrich et al. 1977). GRx are small cytoplasmic enzymes also catalyzing deglutathionylation, reduction of protein disulfides, or Fe-S linkage formation (Hanschmann et al. 2013; Mieyal et al. 1991).

Glutathionylation of cytoskeletal proteins and, in particular, of spectrin in RBCs was shown to occur as a result of treatment with diamide (Rossi et al. 2006) or by treating RBCs with oxidants such as *t*-BuOOH (Giustarini et al. 2019). As discussed above, these treatments lead to an

Figure 2: Superoxide production and breakdown in erythrocytes.

Hemoglobin undergoes an auto-oxidation reaction leading to the formation of methemoglobin (Hb-Fe^{3+}). If methemoglobin is not converted back into Hb-Fe^{2+} , it is degraded leading to the formation of superoxide anion radical ($\text{O}_2^{\bullet-}$). $\text{O}_2^{\bullet-}$ is dismutated into hydrogen peroxide by superoxide dismutase (SOD). Formation of $\text{O}_2^{\bullet-}$ and H_2O_2 and derived products may cause oxidative damage. Hydrogen peroxide can be reduced to water by catalase (Cat), peroxiredoxin 2 (Prx 2) thioredoxin (Trx(SH)_2) or glutathione peroxidase (GPx). To carry out the reaction, Trx(SH)_2 is oxidized into TrxS_2 , while GPx will oxidize reduced glutathione (GSH) into glutathione disulfide (GSSG). To reduce GSSG and TrxS_2 the enzymes glutathione reductase (GSR) and thioredoxin reductase (TrxR) need NADPH as a hydrogen donor, which is delivered by the pentose phosphate pathway.

increase in GSSG and a decrease in GSH/GSSG ratio, which promote oxidative modification of protein thiols and also glutathionylation. Glutathionylation of cytoskeletal proteins were related to changes in osmotic fragility and in cell morphology (Giustarini et al. 2019; Rossi et al. 2006). However, the impact of glutathionylation on structure/function relationships is unknown.

Physiological sources of reactive species and their role in control of RBC deformability and blood rheology

The chemistry and biochemistry of reactive species in RBCs is very complex (Figure 2) (Kuhn et al. 2017). There is accumulating evidence that a complex network of reactive species are enzymatically produced and metabolized in RBCs, which include superoxide anion radicals ($\text{O}_2^{\bullet-}$) and H_2O_2 , NO and derived nitrosating species (like N_2O_3), and sulfide, persulfide and thiosulfate (Kuhn et al. 2017). These reactive species may contribute to intracellular signaling and regulation of RBC physiology also via modification of cysteine redox switches.

In this section we summarize the physiological sources of reactive species and discuss the evidence of their control of RBC deformability and rheology.

Physiological sources of reactive species in RBCs

Mature RBCs lack mitochondria. The major source of reactive species in RBCs is degradation of Fe^{3+} hemoglobin (Alayash et al. 2001). During heme degradation reactive oxygen species (ROS), including superoxide anion radicals ($\text{O}_2^{\bullet-}$), $\text{OH}^{\bullet}$, H_2O_2 and other reactive species derived by reaction of these species with other molecules, are rapidly formed in the RBCs. Other – less abundant – enzymatic sources of ROS in RBCs are also xanthine oxidoreductase and NADPH oxidase (George et al. 2013; Webb et al. 2008). Therefore, RBCs are very well equipped with a battery of antioxidant systems comprising SOD, catalase, GPx, and the GSH recycling system, as well as thioredoxin and glutaredoxin and ascorbic acid (Figure 2). The main source of redox equivalents for recycling of NADPH needed to feed the antioxidant system are provided by the pentose phosphate pathway from glucose. The latter is the only source of both redox equivalents and energy (in the form of ATP) in RBCs, which are also needed to maintain the transmembrane ion equilibria and membrane integrity.

Under normoxic conditions RBCs are known to be powerful scavengers of endothelial NO via reaction with oxyhemoglobin, but also to transport stable NO metabolites (including nitrite, nitrate, nitroso species, and nitrosyl-hemoglobin). Under hypoxic conditions RBCs were shown to produce NO and/or reactive nitrogen

species (RNS), including N_2O_3 and other nitrosating species, from nitrite and to release NO bioactivity leading to hypoxic vasodilation (Cosby et al. 2003; Pawloski et al. 2001). In addition, we demonstrated that RBCs carry a catalytically active endothelial NO synthase (eNOS) and produce NO / nitrosating species under normoxic conditions (Cortese-Krott et al. 2012a,b). Chimeric mice lacking eNOS in the blood have decreased circulating nitrite and nitrate levels and increased blood pressure, indicating that blood cell eNOS activity may contribute to the regulation of circulating levels of NO metabolites *in vivo* (Wood et al. 2013).

Interestingly, RBC were shown to metabolize and produce sulfide and reactive sulfur species (Bianco et al. 2016; Vitvitsky et al. 2015). Recently, we also found that the chemical interaction between NO or nitrosothiols with sulfide leads to formation of S/N hybrid species, including nitrosopersulfide (SSNO^-) and SULFI/NO ($[\text{ONN}(\text{OH})\text{SO}_3]^-$). These species were shown to carry bioactivity of both sulfide and NO by their ability to modulate blood pressure and heart contractility (Cortese-Krott et al. 2015). In a following study, SSNO^- was shown to participate in redox regulation by activation of Nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated gene expression, likely via post-translational modifications of cysteine redox switches in Kelch-like ECH-associated protein 1 (Keap-1) (Cortese-Krott et al. 2016).

All these reactive species and their thiol targets, which were defined recently as *reactive species interactome* (Cortese-Krott et al. 2017), may modulate the cellular redox state and control cysteine redox switches in RBCs and, thereby, contribute to modulate structural and functional properties of proteins (Cortese-Krott et al. 2017). A schematic representation of the RSI and the

possible cysteine redox switches modifications is depicted in Figure 3.

Modulation of RBC deformability and blood viscosity by NO and oxidants

It is well established that oxidants (such as peroxides), cell permeable thiol reactive molecules (such as NEM or diamide) or reducing agents (such as phenylhydrazin) that induce degradation of hemoglobin strongly affect RBC deformability and integrity by affecting the RBC cytoskeletal proteins and inducing formation of hemoglobin aggregates, defined as Heinz bodies (Arduini et al. 1989; Becker et al. 1986; Fischer et al. 1978; Smith and Palek 1983). Several studies also indicate that intracellularly produced NO and derived reactive species in RBCs may affect RBC deformability (Belanger et al. 2015; Bor-Kucukatay et al. 2003; Grau et al. 2013) and RBC velocity (Horn et al. 2011). It was found that a specific range of concentrations of NO donors increase RBC deformability and that treatment with a nitric oxide synthase inhibitor decreases RBC deformability (Bor-Kucukatay et al. 2003). One work proposed that nitrosation of spectrin may play a role in the effects of NO on promoting RBC deformability (Grau et al. 2013). However, the effects of extracellular NO on RBC deformability are not without controversy, as they were not confirmed by other laboratories including ours (Barodka et al. 2014; Belanger et al. 2015; Cortese-Krott et al. 2018).

Recently, Diederich et al. studied the effects of oxidative modifications of the spectrin cytoskeleton on RBC rheology which were assessed by using ectacytometry in a laser rotational cell and a low-shear rotational viscometer (Diederich et al. 2018). Oxidation of spectrin with *tert*-

Figure 3: Possible chemical modifications of cysteine redox switches and the reactive species interactome.

Reactive oxygen species (ROS), reactive sulfur species (RSS), and reactive nitrogen species (RNS) can react with free thiol groups leading to a number of post-translational modifications. The interaction of reactive species with their thiol target was defined as 'reactive species interactome' (RSI). Post translational modifications include nitration ($-\text{SNO}_2$), S-nitrosation ($-\text{SNO}$), formation of sulfinic ($-\text{SO}_2\text{H}$) and sulfonic acids ($-\text{SO}_3\text{H}$) or disulfide ($-\text{SS}-$), or formation of mixed disulfide ($-\text{SSR}'$) and persulfide ($-\text{SS}^-$).

buthylhydroperoxide (*t*-BuOOH) was shown to affect structure and electrophoretic properties of spectrin (Sullivan and Stern 1984). We found that *t*-BuOOH induces a concentration-dependent decreased RBC deformability and increased blood viscosity (Diederich et al. 2018). Although erythrocyte deformability and blood viscosity were not affected by treatment with increasing concentrations of CysNO, pretreatment with CysNO protected RBCs from *t*-BuOOH-induced decrease of deformability, indicating that CysNO may protect cytoskeletal proteins from oxidation. Since treatments were not added at the same time, this indicates that the antioxidant effects of CysNO were not due to direct reactions with *t*-BuOOH, but rather may be dependent on protection of defined cysteine redox switches (Diederich et al. 2018). The targeted proteins and cysteine redox switches, which may be responsible for these effects, were not identified yet.

Pathophysiological and clinical implication of spectrin modifications

Hereditary hemolytic anemia and hereditary elliptocytosis are conditions characterized by increased membrane fragility, increased susceptibility to mechanical or oxidative stress, loss of RBC integrity, and intravascular hemolysis. These are generally linked to hemoglobinopathies (which increase the rate of oxidation/degradation of hemoglobin), congenic defects of antioxidant enzymes or enzymes responsible for production/recycling of redox equivalents (such as GPDH), as well as linked to cytoskeletal defects. In particular, hereditary elliptocytosis is characterized by defects in the spectrin-actin-4.1 complex, which may be due to mutations in the genes coding for α or β spectrin or protein 4.1.

The mutations in spectrin are typically located in ‘mutational hotspots’ such as the tetramerization sites of α spectrin subunits, in the first six SR of α spectrin, and the C-terminally truncated β subunit (CpG islands) (Eber and Lux 2004; Gallagher 2004). In diabetes mellitus, glycation and oxidation of spectrin and other cytoskeletal proteins were observed and correlated to defects in RBC deformability, microcirculatory function and tissue perfusion (Schwartz et al. 1991). Other oxidative modifications of spectrin were also observed in infectious diseases, such as visceral Leishmaniasis (Samanta et al. 2011).

Malaria is an infectious disease caused by the apicomplexan parasite *Plasmodium*. During malaria infection, the immune system of the human host counteracts the

parasite’s invasion into the RBC with high levels of oxidative stress. The parasite, on the other hand, has developed an excellent oxidative defense system, which is crucial to deal with oxidative disbalance throughout its life cycle. Glutathione metabolism, as well as targets for glutathionylation, in *Plasmodium falciparum* were already thoroughly investigated (Becker et al. 2003, 2004; Kehr et al. 2011). Many currently used antimalarials disrupt the parasite’s redox regulation during the intraerythrocytic stage by an increase of oxidative stress.

This knowledge is also the basis of many current research projects wishing to identify novel potential drug targets (Becker et al. 2004; Rahbari et al. 2017). An infection with *Plasmodium* inevitably leads to damage of the erythrocyte plasma membrane, decreased deformability, and hemolysis mainly due to lipid peroxides in both infected and uninfected RBCs (Becker et al. 2004; Mourão et al. 2018; Müller 2015). Hemoglobinopathies causing decreased erythrocyte deformability are known to have advantageous, but not yet completely understood, effects on the outcome of malarial infections (Huisjes et al. 2018; Taylor et al. 2012; Williams 2006).

Taking all these facts into account, one may hypothesize that thiol switches such as glutathionylation of erythrocyte membrane skeletal proteins caused by redox imbalances may be ‘naturally occurring protective mechanism[s]’ against malaria, putatively contributing to decreased deformability of RBCs and eryptosis (Cyrklaff et al. 2016; Gupta et al. 1982; Pantaleo et al. 2017).

Summary and perspective

The mechanical properties of RBCs are fundamental for their cellular physiology and their function as oxygen transporters. Flexibility and deformability of RBCs allow those cells to survive rapid hemodynamic changes within the vascular tree, to dynamically contribute to the flow thereby decreasing vascular resistance, and to properly deform during the passage through narrower vessels.

The RBC mechanoproperties are conferred mainly by the structural characteristics of their cytoskeleton, which mainly consists of a spectrin scaffold connected to nodes of actin and being linked to the plasma membrane via ankyrin and adducin.

There is compelling evidence that changes in redox state and treatment with thiol-targeting molecules decrease the deformability of RBC and affect the structure and stability of the spectrin cytoskeleton, indicating that there might be potential cysteine redox switches. Surprisingly, there is only a very limited number of studies

focusing on the molecular localization of redox switches in the RBC cytoskeleton, or on the structural/functional relationship of redox switches in spectrin and RBC mechanoproperties, blood viscosity, and tissue perfusion. Our working hypothesis is that cysteine redox switches on the spectrin cytoskeleton may be targeted by redox signaling in RBC and thereby modulate RBC mechanical properties; thus, redox regulation of spectrin/cytoskeletal redox switches may contribute to the modulation of blood viscosity, blood flow, and tissue perfusion in health and disease. These studies may provide novel functional targets to modulate RBC function, blood flow and viscosity, and tissue perfusion with potentially important clinical implications.

Acknowledgements: The authors are grateful to Dr. Beate Hutzler and Dr. Lenka Tomasova for their help with experiments related to this subject. We thank Dr. Markus Dick for generation of the atomistic-level spectrin model and Birte Schmitz for help with analyses. This work was supported by the German Research Foundation (DFG) within SPP1710 ‘Dynamics of Thiol-based Redox Switches in Cellular Physiology’ (grant numbers CO 1305/3-1 and GO 1367/3-1). H.G. is grateful to the Jülich Supercomputing Centre at the Forschungszentrum Jülich for computing time on the supercomputer JURECA and JUWELS (NIC project IDs: CHDD19, HKF7) and to the ‘Zentrum für Informations- und Medientechnologie’ (ZIM) at the Heinrich Heine University Düsseldorf for providing computational support.

Author contribution: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: This research was supported by Deutsche Forschungsgemeinschaft (CO 1305/3-1, GO 1367/3-1, SPP 1710).

Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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