Surveying the Response of Transport Channels of Intact RBC Membranes upon AgNO_3 Administration: an Atomic Force Microscopy Study

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Key Words

RBC membrane • Transport channels • Inhibition processes • Reversibility of inhibition • Atomic force microscopy

Abstract

Background/Aims: Cell membranes facilitate the transport of water, ions, and necessary nutrients by hosting a great variety of transport channels that have either a 'simple' pore-like structure or more complex architecture that is based on the utilization of specific receptors. The present study reveals the impact of AgNO_3 , a well-known inhibitor of water channel activity, on transport channels that emerge at the membrane of intact red blood cells (iRBCs). **Methods:** Atomic force microscopy is employed to survey the morphological modification of all transport channels by directly comparing the respective images obtained on the *exact same* iRBCs prior to and after spraying the AgNO_3 solution. **Results:** Small pores of mean size 50 nm that were assigned to water channels, and extended orifices of mean size 300 nm that exhibit a neck-like extracellular segment were observed at the iRBC membrane. **Conclusion:** Our results reveal that AgNO_3 exerts noticeable influence on all transport channels so that its selective water channel inhibi-

tory action should be reconsidered. For low AgNO_3 concentrations extended recovery of the small pore network was observed upon waiting, giving strong evidence that iRBCs have a recovery potential upon simply removing the inhibition cause without the need for specific reducing agents.

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Introduction

Cells possess enormous biochemical potential thus it is not surprising that they are the building units of every living organism. They store and supply vital nutrients to other cells, they mediate specific signals that ultimately trigger subsequent cascade processes, they are responsible for either the repair or the elimination of other cells, etc. Owing to the diversity of their intense biological activities, cells are equipped with comprehensive biochemical machineries capable of responding to exceptionally robust demands [1-3]. Most of these machineries are hosted at the cell membrane which is a crowded biochemical interface separating the intracellular space from the extracellular environment. The cell membrane hosts a great variety of transport channels of either 'simple'

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pore-like structure or more complex architecture ultimately based on the utilization of specific receptors -see the schematic image of Fig. 1A, below. These transport channels facilitate the bi-directional flow of water, ions and all necessary nutrients [1-3].

The membrane of the most abundant type of blood cells, namely red blood cells (RBCs), consists of a lipid-lipid bilayer that incorporates a protein interlayer and has a total thickness of 5 nm [4-7]. By hosting haemoglobin, RBCs are mainly responsible for delivering oxygen to, and removing carbon dioxide from tissues. Since the biochemical activity of RBCs is by no means limited to these two primary functions their membrane is extremely crowded; an enormous number of different proteins that relate to the membrane facilitate vital biochemical activities -see the atomic force microscopy (AFM) image of Fig. 1B, below. Very recently both the membrane and cytoplasmic RBC proteome were surveyed in great detail [8]. M. Mann and colleagues identified and categorised 340 membrane-related and 195 cytoplasmic proteins by means of mass spectrometry methods [8]. From the membrane-related proteins, 105 were annotated as membrane spanning, 54 as membrane bound, 40 as cytoskeletal, and 20 of extracellular origin that are somehow bound to the membrane. Referring to the membrane-spanning proteins, well-documented studies have revealed their importance for the selective transport of water. In the late '60s it was shown that the RBC membrane exhibits both rapid and selective water permeability characterised by low Arrhenius activation energy [9]. Later on it was revealed that the permeability of the RBC membrane to water molecules can be temporarily inhibited upon treatment with HgCl_2 , while further addition of a reducing agent restores water permeability [10]. Agre and colleagues were the first who identified this water channel when they isolated a 28 kD protein integral to the RBC membrane that was called CHIP28 owing to its annotation as Channel-forming Integral Protein of 28 kD molecular weight [11]. By employing the newly identified CHIP28 in *Xenopus* oocytes, Agre and colleagues unambiguously documented that this protein was related to the water transport function of RBCs since water permeability was drastically increased in the CHIP28 injected *Xenopus* oocytes compared to the control ones [12]. Since it was clearly shown that CHIP28 behaves as a selective 'water pore' the term 'aquaporin' (AQP) was widely adopted. Nowadays, 10 families of AQPs have been identified in mammals, AQP0-AQP9. The one that relates to the RBC membrane is termed AQP1 and during the last decade has been studied in great detail [13-

Fig. 1. Biochemical landscape of the iRBC membrane: Schematic illustration (A) and AFM image (B) of an iRBC membrane.

16]. A nice review on these topics is given in [17].

In this work we study the response of all possible transport channels, while specifically focusing on water channels, hosted at the membrane of *intact RBCs* (iRBCs) upon administration of AgNO_3 , a well-known water-channel inhibitor. Non-intact RBCs (niRBCs) have been also examined. Our study is based on a powerful topographic technique, namely AFM, capable of revealing details of the cell membrane down to the *nanometer* (10^{-9} m) level. This unique technique was employed very recently for studying the biocompatibility of magnetic nanoparticle-bovine serum albumin conjugates with blood cells [18] and the activation of IP_3 receptors that span

the nuclear cell membrane under calcium depleted conditions [19, 20]. Fig. 1B shows a representative AFM image of an iRBC membrane. By focusing on an area of $3000 \times 3000 \text{ nm}^2$, this image clearly reveals the existence of different transport channels having diverse architecture; tetramer-like structures (upper corner), strand-hosting orifices (right corner) and more simple pore-like structures are all observed on each iRBC membrane. We emphasize that our study was performed on iRBCs, that is RBCs which were not washed - and subsequently redispersed in an aqueous medium - but were left in the blood plasma environment, in order to (a) preserve most of their overall biological activity, and (b) to capture their response to AgNO_3 administration while being in an intact state unperturbed by any extra laboratory treatment that could motivate slight modifications in the transport channels under investigation even in the case where cell-compatible media are used.

Our efforts were focused on the exact same iRBCs that were monitored prior to and after the delivery of an AgNO_3 solution by means of a spraying device. This 'scan-spray a test solution-rescan' process not only provides direct and reliable visual information for the exact same cells under investigation but also allows a quite accurate estimation of the timescales during which cell biochemical responses are triggered by an externally applied cause. Standard experiments based on a conventional procedure during which iRBCs were matured under the presence of AgNO_3 have also been conducted for the direct comparison with the 'scan-spray a test solution-rescan' process. Standard optical microscopy (OM) was employed as a supplementary technique for the pre-evaluation of the overall shape morphology and possible shape variations of reference iRBCs prior to and after the detailed examination by AFM. niRBCs have been also examined. In this work we present results focused exclusively on the AFM technique owing to its superiority upon OM, and on iRBCs since the studied features are obscured in the niRBCs owing to the slight unphysiological conditions that they experience during their treatment.

Materials and Methods

Materials

All chemicals were reagent grade. AgNO_3 , KCl, and NaCl were purchased from Aldrich [St. Louis, MO, USA]. Water was purified by means of an analytical-grade water purification system [Millipore Corporation, Billerica, MA, USA]. Sodium chloride saline for injection 0.9% w/v [Fresenius] and syringes were

purchased from a local pharmacy, while phosphate-buffered saline (PBS) was purchased from Pierce [Rockford IL, USA]. Anticoagulant-containing, ethylenediaminoethanetetraacetic acid (EDTA), Vacutainer test tubes [BD, NJ, USA] at a concentration of 1 mg/mL were used for temporary blood storage. Standard microscope slides [Corning, NY, USA] were used for the preparation of blood films.

Blood collection and preparation of blood films

Whole blood was drawn by venipuncture from 7 healthy volunteers of different blood groups after informed consent and deposited in standard anticoagulant-containing, EDTA test tubes. According to our experimental protocol blood was always used fresh or temporarily stored at $T=4^\circ\text{C}$ and used within 48 hours. By means of an electronically controlled rotating platform [Headway Research INC MOTOR CONTROL-101] we were able to spread the blood samples onto the ethanol-cleaned surface of standard microscope slides in the form of a thin film. More specifically, by adjusting both the rotation frequency (3000-3500 rounds per min) and duration (10-15 sec) of the platform we managed to uniformly distribute the blood sample onto the microscope slide so that only a single layer of closely packed blood cells was formed. This detail is very crucial; for high throughputs by means of AFM and OM techniques the reliable examination of both the overall shape morphology and specific membrane characteristics in as many cells as possible of each sample is a necessity. The blood cells studied in this work were 'dried' in the sense that they were spread onto the slides without being preserved in a buffer liquid medium. However, the blood cells retained biochemical activity during our experiments (maximum 12 hours) as this was clearly evidenced by their intense and distinct response upon administration of various test solutions, for instance KCl, NaCl, PBS, and AgNO_3 , that we have examined. All solutions were prepared in purified water and used fresh daily. In this work we focus on AgNO_3 . However, representative results for other test solutions are also discussed. The nominal concentration of the AgNO_3 solution sprayed onto blood cells was 32 mg/mL. However, the effective concentration ultimately obtained at the area of a single iRBC can only be roughly estimated since we know that during our experiments an amount of 5-20 μL is sprayed onto a total area of 20-40 cm^2 . Not knowing the exact concentration of the AgNO_3 solution delivered to each studied cell is a disadvantage of our experimental method.

OM and AFM techniques

OM images were obtained by means of a light microscope [LEICA DMRXP] in the transmission mode. Detailed AFM images were obtained by means of a scanning probe microscope NT-MDT Solver PRO [NT-MDT Co, Moscow, Russia] since this technique enables the evaluation of specific membrane characteristics of blood cells down to the nanometer level. Images of iRBC membranes were acquired in the non-contact scanning mode with cantilevers that end to silicon nitride tips [Nano and More GmbH, Wetzlar, Germany] having nominal force constant of 0.07 Nm^{-1} and resonance frequency $f=330 \text{ kHz}$, at line frequencies 2-4 Hz and 256 to 512 lines per image. We emphasize that during this study the same

Fig. 2. Comparative AFM and OM images of RBCs, WBCs, and Plts. A two-dimensional AFM (A),(D), three-dimensional AFM (B),(E), and OM (C),(F) images of the exact same area of freshly prepared (A)-(C) and degraded (D)-(F) blood films. In the OM images (C) and (F) the magnification of the objective lens and of the camera was 100 $\times$ and 3 $\times$, respectively.

gain was selected and the exact same tip was used in all investigated iRBCs in an effort to minimize the parameters that could falsify our results.

Spray device

The homemade spray device employed for the delivery of small amounts of test solutions onto blood films or other cell cultures is based on a propellant gas-microcapillary

reservoir-microneedle apparatus. Initially the microcapillary reservoir is loaded with a very small amount of the test solution, as low as 5 μ L, by means of a micropipette. Its content is released by the microneedle under the assistance of the propellant gas (usually neutral nitrogen). Regarding the employed experimental procedure we emphasize that this 'scan-spray a test solution-rescan' process allows a rather accurate estimation of the timescales where cell biochemical response

is triggered since the exact same cells can be rescanned within seconds immediately after the spray of the test solution.

Isolation of niRBCs

In order to investigate differences that could possibly exist between iRBCs and niRBCs that were treated with cell-compatible media such as saline and PBS we isolated niRBCs by means of sequential wash/centrifugation cycles. The wash was performed either with saline or PBS, while centrifugation referred to the mild conditions of 3000 rpm for 3 min so that mechanical distress of the niRBCs is prevented.

Standard maturation procedure

A standard procedure during which whole blood is matured upon mild incubation (20 rpm) at room temperature under the presence of a test solution has also been employed for a direct comparison with the results obtained by means of the 'scan-spray a test solution-rescan' process. During these maturation experiments typical values for AgNO_3 concentration and incubation duration were 3 mg/mL and 2 min, respectively.

Results

OM is traditionally employed by haematopathologists, along with staining methods, as an efficient tool for the diagnosis of various haematological diseases such as blood malignancies. The great advantages of this conventional technique are easy realisation, high throughputs and reliable evaluation owing to the direct visual observation of the stained blood cells. OM was employed in our study as a supplementary technique, prior to the detailed examination by AFM, for the pre-evaluation of the overall shape morphology and possible shape variations of iRBCs that could be induced by AgNO_3 administration.

Figures 2A, B and C show a two-dimensional AFM image, its three-dimensional reconstruction, and the relevant OM image, respectively, for a freshly prepared blood film, while Figs. 2D, E and F show the respective images for a blood film of damaged cells. All three types of blood cells, namely RBCs, white blood cells (WBCs) and platelets (Plts) are observed by both OM and AFM. However, we clearly see that the information provided by the OM technique can be entirely erroneous owing to its intrinsic restriction to two-dimensional imaging. As a result the ghost residual of a completely damaged WBC and significantly degraded RBCs appear to have all the characteristics of normal cells if we are exclusively based on OM (Figs. 2C and F). In contrast, access to three-dimensional reconstructed images, provided by the AFM technique, restores this inefficiency by ultimately unveiling

Fig. 3. AFM image of a blood film prior to and after the AgNO_3 spraying. A specific blood film area of $100 \times 100 \mu\text{m}^2$ (A) prior to, and (B) after the spraying of the AgNO_3 solution and 'head' remounting. The relative shift of the two frames is below $5 \mu\text{m}$ in both x and y directions. Dotted rectangle focuses on one of the iRBCs investigated in this particular blood film.

the true nature of the studied cells (Figs. 2B and E).

Furthermore, OM enables access to information restricted to the micrometer (10^{-6} m) level; only the evaluation of the overall shape morphology of RBCs, WBCs and Plts can be achieved, while access to specific characteristics is not possible. In contrast, as clearly proved below, AFM enables us to obtain information down to the nanometer (10^{-9} m) level; the detailed evaluation of specific characteristics of RBCs, WBCs and Plts can be readily performed by AFM. Accordingly in this study we focus on results that were obtained by means of AFM.

Our investigations were focused on the exact same iRBCs prior to and after the spraying of AgNO_3 solution since this is the most direct and subsequently convincing way to reveal the impact of this water channel inhibitor on iRBC. To that effect, after surveying a specific area of a blood film we removed the ‘head’ of the AFM instrument where the cantilever is mounted and we mildly sprayed the AgNO_3 solution onto the blood film by means of a homemade spray device able to deliver solution amounts as low as 5 μL . The ‘head’ was then immediately remounted and the same blood film area was scanned once again. Remarkably, only a small shift was observed in the scanned blood film area that was less than 5%. Thus, not only the same blood film area, but also the exact same iRBCs can be reliably monitored prior to and after the spraying of any test solution.

This is clearly evidenced by the AFM images presented in Figs. 3A and B which survey the specific blood film area of $100 \times 100 \mu\text{m}^2$ where the investigations were performed. Panel A shows the scanned area prior to the removal of the ‘head’, while panel B shows the respective image after the ‘head’ was remounted. We emphasize that the relative shift of the two frames in both x and y directions is well below 5 μm (taking into account that the mean size of an iRBC is approximately 8 μm). Dotted rectangle focuses on one of the five iRBCs that were investigated in great detail for this particular blood film.

Figures 4A and B show a specific iRBC prior to and after the spraying of the AgNO_3 solution. We see that there are no obvious changes in the overall shape of that specific iRBC after the spraying of the AgNO_3 solution. This fact makes clear that the total amount of AgNO_3 delivered to each iRBC is sufficiently low so that intense structural/morphological changes are not motivated. This was the aim of the present study: to directly reveal the modification of iRBC membrane transport channels upon administration of small amounts of a well-established water channel inhibitor, namely AgNO_3 . The features observed on the iRBC membrane by means of the AFM technique cannot be revealed by any conventional technique which is effective only at the micrometer level such as OM and phase contrast microscopy. These techniques can only detect extended changes that emerge when high concentrations of a test compound are used. The combination of AFM with the ‘scan-spray a test solution-rescan’ approach enables us to reveal features that exclusively emerge at the nanometer level on the membrane of iRBCs only when extremely low AgNO_3 amounts are delivered to each iRBC. We emphasize that these features are

Fig. 4. Focusing on a representative iRBC prior to and after the AgNO_3 spraying. A representative iRBC (A) prior to, and (B) after the spraying of the AgNO_3 solution.

observed (i) in niRBCs, that is ones treated with laboratory media that are considered as optimally cell-compatible, such as saline and PBS, and (ii) when relatively high amounts of AgNO_3 that are most commonly examined in conventional experimental methods, are used. These issues are discussed in detail below (see Fig. 6 and the respective discussion).

Figures 5A and B present images obtained on the membrane of the iRBC shown in Figs. 4A and B. The scanned area is $3000 \times 3000 \text{ nm}^2$. We emphasize that in all ‘large scale’ images that range from 2000×2000 to $4000 \times 4000 \text{ nm}^2$ a nearly parabolic contribution originating from the biconcave shape of iRBCs is subtracted so that specific details that possibly emerge on the membrane are revealed more clearly. However, when relatively small areas of the iRBC membrane are scanned,

Fig. 5. Response of the small pores and extended orifices of the cell membrane upon AgNO_3 spraying. Images obtained on the membrane of the particular iRBC shown in Fig. 4, (A) prior to, and (B) after the spraying of the AgNO_3 solution. The scanned area is $3000 \times 3000 \text{ nm}^2$. A nearly parabolic contribution originating from its biconcave shape is subtracted so that any specific structure that possibly emerges at the membrane is revealed more clearly. (C) Image of a smaller area so that the small pores are revealed more clearly.

typically below $1000 \times 1000 \text{ nm}^2$, we do not perform any mathematical treatment since at these length scales the iRBC membrane is already sufficiently flat. Panel A reveals the iRBC membrane prior to the spraying of the AgNO_3 solution, while panel B shows exactly the same membrane area after the treatment is accomplished. Clearly two different features are resolved on the membrane of this particular iRBC: an approximately 500 nm -sized ring-shaped orifice that lacks any additional structural symmetry dominates the landscape that is crowded by significantly smaller pores of size around 50 nm that seem to span the iRBC membrane (see below). Panel C presents an image of a smaller region of the iRBC membrane, prior to the AgNO_3 spraying, so that the size of small pores is revealed more clearly. The size of small pores ranges within $20\text{--}80 \text{ nm}$ (white arrows focus on the smaller pores, while black arrows on larger ones). For the reliable estimation of the pore size distribution we employed the software of the instrument. All the reported results have been cross-checked by examining many iRBCs so that reliable statistical data are eventually obtained.

Except for their quantitative difference the two features also have qualitative ones; the ring-shaped orifice possesses a structure along the normal direction since it exhibits a neck-like segment that extends to the extracellular environment for about $15\text{--}20 \text{ nm}$. Though the exact nature of these features cannot be straightforwardly identified, we can proceed by making assumptions that are fairly justified on the basis of standard knowledge that comes from cell biology. For instance, the extended network of small pores can be ascribed to water channels that, according to well-established biophysical analyses, are the most abundant structure spanning the iRBC membrane [17]. The image presented in panel B advocates this assignment; clearly, the network of small pores is strongly affected by AgNO_3 since it completely disappears after spraying of the solution. Furthermore, the

overall size of the water channel tetramer as determined by both theoretical modelling [14, 15] and electron/atomic force microscopy experiments [16] performed on reconstituted two-dimensional crystals of AQP1 was estimated to be approximately 10 nm . Our observations give a mean value of the pore size around 50 nm that is significantly higher than the value of 10 nm reported in [14–16]. To rectify this discrepancy we emphasize that our study is performed on transport channels captured in their pristine form on the iRBC membrane, while in [14–16] the ideal case of purely reconstituted two-dimensional water channels that form well-ordered AQP1 crystals was studied.

Fig. 6. Modification of iRBC membrane characteristics upon use of cell-compatible media. Comparative images obtained for (A-C) an iRBC, and (D-F) a niRBC that was treated with saline. (A) and (D) present the whole cell, (B) and (E) show a typical scanned area of its membrane, and (C) and (F) show the reconstructed side view section of the images presented in (B) and (E), respectively.

Slight variations in the experimental conditions employed among different studies can probably explain reasonable quantitative discrepancies. To further investigate this possibility we examined the robustness of the features observed on the iRBC membrane under slight variation of the experimental conditions. Thus, we performed additional experiments on niRBCs treated with media that

are most commonly used in laboratory practice to maintain cell-compatible pH conditions, such as saline and PBS. Representative results are shown in Figs. 6A-F. Panels A, B, and C show results for iRBCs, while D, E, and F refer to niRBCs, all coming from a single blood sample obtained from one of the donors. Panels A and D show the whole cell, B and E show a typical scanned area of

its membrane, and C and F show the reconstructed side view section of the images presented in B and E, respectively. The niRBC presented here was isolated by means of sequential saline-wash/centrifugation cycles and was redispersed in saline. The same results were observed when PBS was employed during the wash/centrifugation cycles. First of all we see that on the membrane of the niRBC presented in panel D small granules have emerged, possibly motivated by osmotic processes due to the slightly non-physiological conditions experienced by the cell. In contrast the iRBC presented in panel A maintains the same membrane appearance as in all other cases studied here. Focusing on the membrane of the niRBC, panel E, we clearly see the structure of the granules, while the respective membrane of the iRBC, panel B, exhibits no such features. Probably the most important information is shown by the reconstructed side view sections presented in panels C and F for the iRBC and niRBC, respectively. We see that the 5 nm thick [4-7] membrane of the iRBC is totally penetrated by the small pores, while the membrane of the niRBC appears smooth from the side of the intracellular space. These experimental results reveal that noticeable morphological changes emerge on the membrane of niRBCs, giving evidence that the characteristics of the transport channels are very sensitive to the exact experimental conditions. This fact can explain reasonable discrepancies between the results reported here and in articles that are published in the recent literature. Also, we once again want to emphasize the great potential of the AFM technique. The nanometer size granules that emerge on the membrane of niRBCs cannot be detected by any other conventional technique such as OM and phase contrast microscopy that are effective in the micrometer level.

Returning to the AFM data presented in Figs. 5A and B we note that surprisingly the 500 nm-sized orifice is strongly affected by AgNO_3 ; its size decreases, while its extracellular segment is completely suppressed. Extra information can be revealed by the images shown in Figs. 7A and B that present a reconstructed side view section of the images presented in Figs. 5A and B, respectively. We see that the extracellular segment of the 500 nm-sized orifice is completely suppressed, while its membrane integral part is clearly narrowed. Owing to the absence of the extracellular segment of the 500 nm-sized orifice, now the tip of the cantilever is able to penetrate deeper into its interior as much as 20 nm. This value is significantly higher than the thickness of the iRBC membrane [4-7], thus the 500 nm-sized orifice completely spans the membrane of this iRBC. We see that at a dis-

Fig. 7. Response of the small pores and extended orifices across the cell membrane upon AgNO_3 spraying. Side view sections of the images presented in Fig. 3, (A) prior to, and (B) after the spraying of AgNO_3 solution. Intracellular and extracellular space is represented by *is* and *es*, respectively.

tance of 10 nm from the outer surface of the membrane, the 500 nm-sized orifice gets as narrow as 50 nm. The same holds for the network of small pores since the ones observed in Fig. 7A penetrating at a depth of a few nm (see white arrowheads) is almost totally suppressed in Fig. 7B. This fact adds an additional clue towards identifying these small pores as water channels of iRBC. Finally, regarding the identity of the 500 nm-sized orifice we stress that it could be related to the physiological aging process of iRBCs. Since iRBCs lack of both nuclei and organelles they do not synthesize RNA and as a consequence they neither divide nor repair themselves, thus having a life span of almost 120 days. According to knowledge existing in the literature the aging process of iRBCs

relates to membrane microvesiculation, shape changes, and cytoskeleton degradation [21, 22]. Thus, we could invoke that extended orifices relate to the physiological aging and ultimate death process of iRBCs. However, this assignment is not fairly justified; extended orifices may also relate to specific receptors that facilitate intracellular transport of high molecular weight nutrients or to membrane-bound proteins of extracellular origin [8]. This issue needs farther investigation. No matter what the 500 nm-sized orifice identity is, the surprising fact is that it clearly responds to the spraying of the AgNO_3 solution. The same behavior was observed for all iRBCs studied in this work.

Probably the most important piece of information raised by our AFM investigations on water channel response comes from the examination of the exact same iRBCs within a time window of a few hours after the spraying of the AgNO_3 solution. An important question related to the water channel activity is the possible reversible/irreversible character upon removal of the triggering cause. Figures 8A and B show comparative images obtained right after AgNO_3 administration and 4 hours later for the representative iRBCs discussed in this work. Clearly, after sufficient time a recovery effect is detected; the pore network, that is the water channels, nicely evolves once again so that these pores reopen. This observation gives strong evidence that upon removing the triggering cause iRBCs have the ability to recover, so that water channel inhibition of iRBCs by AgNO_3 is a reversible biochemical process, at least partially. In contrast to what is observed for the recovery/reopening of small pores, for the extended orifices we do not observe a noticeable difference since their extracellular segment is not recovered/restructured. Finally, additional recovery upon further waiting (8 hour time window) was not observed for both the small pores and extended orifices so that we conclude that the reversibility of these inhibition processes is partial since the initial state of iRBC cannot be fully recovered. The same behavior was observed for all iRBCs studied in this work.

Owing to the unconventional character of our 'scan-spray a test solution-rescan' approach, AFM data on iRBCs that were matured by means of a standard procedure have been also obtained for comparison reasons. Figures 9A and B show results that refer to the membrane of an iRBC matured by means of the conventional procedure for 2 min under AgNO_3 concentration of 3 mg/mL. Panel A presents the AFM image obtained right after the blood film preparation, while panel B presents the respective image 4 hours later. We do not

Fig. 8. Time evolution of iRBC membrane characteristics upon removal of the inhibition cause. Comparative images obtained (A) immediately, and (B) 4 hours after the AgNO_3 spraying for a representative iRBC.

see noticeable differences between these two images. Either these conventionally matured iRBCs reach biochemical equilibrium during the maturation interval, or the important time window during which morphological changes emerge on the membrane is lost since noticeable time intervenes during the blood film preparation (approximately 10 min). In contrast, the results obtained with the 'scan-spray a test solution-rescan' process during which the iRBC membrane is scanned at short times right after the spray of the AgNO_3 solution (approximately 1 min) exhibit noticeable morphological changes of the iRBC membrane as discussed above. Thus, the combination of the 'scan-spray a test solution-rescan' approach with the AFM technique as proposed in this work could be employed as a standard procedure for investigating the time evolution of morphological changes emerging on cell membranes at very short time intervals after the application of a biochemical substance.

Fig. 9. Time evolution of conventionally matured iRBC membrane characteristics upon removal of the inhibition cause. The membrane of an iRBC matured by means of the conventional approach for 2 min under AgNO_3 concentration of 3 mg/mL (A) right after the blood film preparation, and (B) 4 hours later. The scanned area is $3000 \times 3000 \text{ nm}^2$. A nearly parabolic contribution originating from its biconcave shape is subtracted so that any specific structure that possibly emerges at the membrane is revealed more clearly.

Discussion

Recently Niemietz and Tyerman reported a detailed study referring to the inhibitory potency of various compounds, including AgNO_3 , for three different AQP classes, including AQP1 of human RBC [23]. The authors clearly revealed the increased potency of silver compounds, AgNO_3 and silver sulfadiazine, when compared to other transition metal based candidates. Furthermore, in Ref. [23] it was reported that inhibition caused by silver compounds was (a) specific to water channels and (b) not reversible upon treatment with mercaptoethanol. Our AFM data clearly reveal that these

two observations are not met in iRBC membranes. We directly documented that in iRBC membranes (a) the AgNO_3 compound triggers noticeable biochemical response to all candidate transport channels, and (b) is significantly reversible upon simple removal of the triggering cause. Explanations for these seemingly conflicting observations can rely on the different experimental conditions where the two studies were conducted. First, in [23] the reversibility of AgNO_3 inhibitory action was examined for relatively high AgNO_3 concentrations and only for a single concentration of mercaptoethanol, while in this work we examined a quite low AgNO_3 effective concentration that does not impose noticeable structural/morphological changes such as shrinking. Probably at high AgNO_3 effective concentrations even in our case the inhibition process is no longer reversible as was observed in [23]. The results presented in Figs. 9A and B concur toward this direction. Second, in [23] washed RBCs were diluted in HEPES-KOH medium, while in this work iRBCs were investigated in an absolutely physiological blood plasma environment. The results presented in Figs. 6A-F give strong evidence that slight variations in the experimental conditions and media that are most commonly used to maintain cell-compatible pH conditions can obscure the respective morphological modifications that emerge on cell membranes, ultimately resulting in both quantitative and qualitative discrepancies between techniques that operate in the nanometer and micrometer level.

The lack of selectivity of the biochemical action of AgNO_3 and the significant reversibility of the process observed in this study raise new questions on the underlying mechanism that governs water channel function. The AQP1 tetramer consists of four structurally identical and biochemically equivalent monomers that all mediate water transport [13-17]. To that effect each monomer has a narrowing along its membrane integral segment, called the selectivity filter that has an effective diameter of 3 Å slightly higher than that of water molecule, 2.8 Å. Accordingly, inhibition of water channels by HgCl_2 is ascribed to the mechanical blocking of the selectivity filter by Hg^{2+} that has an effective diameter of 2.2 Å possibly owing to its interaction with a cysteine residue. This makes the inhibitory action of HgCl_2 reversible under treatment with a reducing agent [17]. However, in the case of AgNO_3 we observed significant reversibility without any treatment except for exposition to atmosphere. Owing to its high oxygen content air could possibly act as an oxidative environment for the extremely low amount of Ag^+ ions (2.5 Å effective diameter)

employed in our experiments, thus reopening the selectivity filter of water channels. Surely, these issues need farther examinations in the future.

Conclusions

Summarising, in this work we presented a thorough AFM study on the modifications observed in transport channels residing on the membrane of iRBCs upon administration of AgNO₃. Our topographic technique was focused on the exact same iRBCs prior to and after the spraying of a AgNO₃ solution. Small pores of mean size around 50 nm that were observed in the iRBC membrane were assigned to water channels since they completely seal upon AgNO₃ administration. Extended ori-

fices with size ranging within 200-500 nm, exhibiting a neck-like extracellular segment were also observed in the iRBC membrane. These extended orifices also respond to AgNO₃ administration. Our results reveal that AgNO₃ exerts an inhibitory action on all possible transport channels observed on the iRBC membrane so that any claims referring to selective water channel inhibition by AgNO₃ should be reconsidered. Furthermore, extended recovery of the small pores network, but not of extended orifices, was observed upon waiting, giving strong evidence that iRBC has a recovery potential upon removal of the inhibition cause without the need for the administration of a reducing agent. The lack of selectivity in the inhibitory action of AgNO₃ and the significant reversibility of the inhibition process raise new questions on the underlying inhibition mechanism.

References

- Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P: Molecular Biology of the Cell. ed 5. Garland Science, New York, 2007.
- Becker WM, Kleinsmith LJ, Hardin J: The World of the Cell. ed 6. Benjamin Cummings, New York, 2006.
- Lodish H, Berk A, Zipursky LS, Matsudaira P, Baltimore D, Darnell JEL: Molecular Cell Biology. ed 4. W H Freeman & Co, New York, 1999.
- Sackmann E: Membrane bending energy concept of vesicle-and cell-shapes and shape-transitions. FEBS Lett 1994;346:3-16.
- Tsukita S, Ishikawa H: Cytoskeletal network underlying the human erythrocyte membrane. Thin-section electron microscopy. J Cell Biol 1980;85:567-576.
- Gov N, Zilman AG, Safran S: Cytoskeleton confinement and tension of red blood cell membranes. Phys Rev Lett 2003;90(22):228101.
- Popescu G, Ikeda T, Goda K, Best-Popescu CA, Laposata M, Manley S, Dasari RR, Badizadegan K, Feld MS: Optical measurement of cell membrane tension. Phys Rev Lett 2006;97(21):218101.
- Pasini EM, Kirkegaard M, Mortensen P, Lutz HU, Thomas AW, Mann M: In-depth analysis of the membrane and cytosolic proteome of red blood cells. Blood 2006;108:791-801.
- Rich GT, Sha'afi I, Romualdez A, Solomon AK: Effect of osmolality on the hydraulic permeability coefficient of red cells. J Gen Physiol 1968;52:941-954.
- Macey RI: Transport of water and urea in red blood cells. Am J Physiol 1984;246:C195-203.
- Agre P, Saboori AM, Asimos A, Smith BL: Purification and partial characterization of the M(r) 30,000 integral membrane protein associated with the erythrocyte Rh(D) antigen. J Biol Chem 1987;262:17497-17503.
- Preston GM, Carroll TP, Guggino WB, Agre P: Appearance of water channels in Xenopus heterodimers. J Biol Chem 1992;269:10417-10422.
- Yool AJ, Weinstein AM: New roles for old holes: ion channel function in aquaporin-1. News Physiol Sci 2002;17:68-72.
- Sui H, Han BG, Lef JK, Walian P, Jap BK: Structural basis of water-specific transport through the AQP1 water channel. Nature 2001;414:872-878.
- Walz T, Hirai T, Murata K, Heymann JB, Mitsuoka K, Fujiyoshi Y, Smith BL, Agre P, Angel A: The three-dimensional structure of aquaporin-1. Nature 1997;387:624-627.
- Walz T, Tittmann P, Fuchs KH, Muller DJ, Smith BL, Agre P, Gross H, Engel A: Surface topographies at subnanometer-resolution reveal asymmetry and sidedness of aquaporin-1. J Mol Biol 1996;264:907-918.
- Gonen T, Walz T: The structure of aquaporins. Q Revs Biophys 2006;39:361-396.
- Stamopoulos D, Manios E, Gogola V, Benaki D, Bouziotis P, Pissas M, Niarchos D: Bare and protein-conjugated Fe₃O₄ ferromagnetic nanoparticles for utilization in magnetically assisted haemodialysis: biocompatibility with human blood cells. Nanotechnology 2008;19:505101.
- Moore-Nichols D, Arnott A, Dunn RC: Regulation of nuclear pore complex conformation by IP(3) receptor activation. Biophys J 2002;83:1421-1428.
- Mooren OL, Erickson ES, Moore-Nichols D, Dunn RC: Nuclear side conformational changes in the nuclear pore complex following calcium release from the nuclear membrane. Phys Biol 2004;1:125-134.
- Bratosin D, Estaquier J, Petit F, Arnould D, Quatannens B, Tissier JP, Slomianny C, Sartiaux C, Alonso C, Huart JJ, Montreuil J, Ameisen JC: Programmed cell death in mature erythrocytes: a model for investigating death effector pathways operating in the absence of mitochondria. Cell Death Differ 2001;8:1143-1156.
- Berg CP, Engels IH, Rothbart A, Lauber K, Renz A, Schlosser SF, Schulze-Osthoff K, Wesselborg S: Human mature red blood cells express caspase-3 and caspase-8, but are devoid of mitochondrial regulators of apoptosis. Cell Death Differ 2001;8:1197-1206.
- Niemietz CM, Tyerman SD: New potent inhibitors of aquaporins: silver and gold compounds inhibit aquaporins of plant and human origin. FEBS Lett 2002;531:443-447.