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Erythrocyte death *in vitro* induced by starvation in the absence of Ca^{2+}

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ABSTRACT

Human erythrocytes (RBCs), stored at 4 °C under nominal absence of external energy sources and calcium ions, show a gradual decrease in membrane roughness (R_{rms}) at the end of which the appearance of morphological phenomena (spicules, vesicles and spherocytes) is observed on the cell membrane, phenomena that can mainly be ascribed to the ATP-dependent disconnection of the cortical cytoskeleton from the lipid bilayer. After depletion of the intracellular energy sources obtained under the extreme conditions chosen, treatment with a minimal rejuvenation solution makes the following remarks possible: (i) RBCs are able to regenerate adenosine triphosphate (ATP) and 2,3-bisphosphoglycerate only up to 4 days of storage at 4 °C, whereas from the eighth day energy stocks cannot be replenished because of a disorder in the transmembrane mechanisms of transport; (ii) the RBCs' roughness may be restored to the initial value (*i.e.* that observed in fresh RBCs) only in samples stored up to 4–5 days, whereas after the eighth day of storage the rejuvenation procedure appears to be inefficient; (iii) membrane physical properties – as measured by R_{rms} – are actually controlled by the metabolic production of ATP, necessary to perform the RBCs' basic functions; (iv) once energy stores cannot be replenished, a regulated sequence of the morphological events (represented by local buckles that lead to formation of spicules and vesicles of the lipid bilayer with generation of spherocytes) is reminiscent of the RBCs' apoptotic final stages; (v) the morphological phenomenology of the final apoptotic stages is passive (*i.e.* determined by simple mechanical forces) and encoded in the mechanical properties of the membrane-skeleton; and (vi) necrotic aspects (*e.g.* disruption of cell membrane integrity, so that intracellular protein content is easily released) ensue when RBCs are almost totally (≥90%) depleted in an irreversible way of the energetic stores.

1. Introduction

During banked shelf-life of human red blood cells (RBCs), the molecular consequences of the *in vitro* ageing process appear to be partially different from those observed in the similar sequence of events occurring *in vivo* [1–3].

The dissimilarities lie in lesions as well as in a lowered mean survival time of RBCs after transfusion [4,5] and may be postulated to partially result from the accumulation of toxic metabolites [6]. Indeed, in contrast with the effective elimination machinery operating *in vivo*, senescent cells (erythrocytes as well as residual leukocytes and platelets) are not removed from the storage bag and therefore are expected (i) to release bio-active substances that may accelerate the *in vitro* ageing process, (ii) to favour the rapid disappearance from circulation shortly after transfusion, and (iii) to contribute to many adverse clinical outcome of transfusion (such as organ failure and mortality) [7–9]. In any case, the constellation of biochemical (*e.g.* depletion of 2,3-bisphosphoglycerate (BPG), which reduces O_2 delivery) and biomechanical (*e.g.* decreased deformability, that can impede microvascular flow) changes to the RBCs that occur during bank storage (known as 'storage lesion') (i) progresses with duration of shelf-life, (ii) eventually results in irreversible damages, (iii) limits the banking period and (iv) lessens RBC viability after transfusion. Therefore, a simple and non-destructive approach, capable of systematically measuring the consequences of bio-active substances as a whole on the structural stability of RBCs, is highly auspicable.

A general consensus has emerged that RBC ageing is a form of apoptosis [10–14], that is concentrated – at least in its final stages – at the plasma membrane [10] level (such as membrane blebbing and vesicle formation). Therefore, remodelling of RBC membrane over storage time can be proposed to manifest some relevant structural signal that determines the fate of RBCs after transfusion [14,15]. Thus, the objective of the present investigation has been designed to directly measure reversible and irreversible features at a nanometer scale seen on the surface of RBCs stored under extreme conditions (*i.e.*

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in the total absence of external energy sources, except of inosine-carried ribose supplied in the occasion of rejuvenation treatments) as a function of shelf-life time. Moreover, since a correlation between ATP level and RBC morphology has been observed [16,17], the determination of ATP (and of BPG, the oxygen affinity modulator of haemoglobin) content could give additional information useful in assessing the viability of stored RBCs. In order to get through this morphological problem, the use of an intrinsically three-dimensional, high resolution, non-destructive surface characterizing technique such as atomic force microscopy (AFM) is very appropriate. In fact, AFM has emerged as a powerful tool to provide a quantitative description of human RBCs' morphology (with a lateral resolution approaching few nanometers and a vertical sensitivity of the order of 1 Å), under para-physiological and non-physiological conditions [18–20]. Recently, a direct correlation between the plasma membrane roughness and the connectivity of membrane-skeleton units has been demonstrated [20], thus offering a powerful tool to investigate changes in the cortical cytoskeleton network actively controlled by metabolic pathways of the RBCs.

Finally, extreme conditions of storage (explicitly, the total absence of energy sources in the extracellular additive solution) were chosen with the assumption that under such unfavourable circumstances many systems become highly simplified because of the elimination of any possible compensatory energy-driven mechanisms, and accordingly only a few storage damages could be hidden due to reduced repairing capacity. Indeed, adult RBCs lack intracellular organelles (*e.g.* nucleus, mitochondria) and thus are incapable of *de novo* synthesis of proteins and lipids; hence, total absence of external energy sources has been considered a propitious condition for investigating the life to death transition of RBCs. Moreover, it is well known that moderate to severe drop in ATP can stimulate the initiation of RBC apoptosis or programmed (suicidal) death or eryptosis [11]; such an effect, however, may be either direct or indirect, the latter mediated through an impact on another cellular process like ion homeostasis (*e.g.* that of Ca^{2+} [12]). Therefore, in order to reduce to the only ATP-dependent chain of events the intricate biochemical factory that governs the final stages of RBC apoptosis *in vitro*, even calcium ions have been successfully excluded from the investigated system. In fact, the reported results are in line with the view [21] that the elastic properties of the network of RBC cortical cytoskeleton and its state of steady remodelling sustained by a continuous expenditure of hydrolysable sources of free energy (*i.e.* ATP) constitute the structural basis of the final phase of RBC self-destruction, even in the nominal absence of Ca^{2+}. Such a result (*i.e.* RBC programmed death observed at $[Ca^{2+}]$ nominally equal to 0 M) is a relevant novelty in the investigations on eryptosis. In fact, it is well known (see *e.g.* Ref. [11]) that RBC suicidal death is triggered and/or enhanced by a wide variety of diseases (such as sepsis, renal insufficiency, malaria, Wilson disease) and a large number (more than 20, to date) of xenobiotics (*e.g.* valinomycin, chlorpromazine, lead, mercury); and just as the energy depletion [22] applied in the present work, all the other stressors – *i.e.* pathological conditions and chemical agents influencing eryptosis – are known to stimulate a complex molecular machinery that elicits an increase in cytosolic Ca^{2+} activity [11] (even though exception(s) may exist [23] or at least cannot for certain be ruled out): it is the entry of Ca^{2+}, both *in vivo* and *in vitro*, that is followed by the typical sequences of molecular events involved in RBC senescence and leading to its death (such as: (i) stimulation of cysteine endopeptidase calpain, which degrades the membrane-skeleton; and (ii) activation of Ca^{2+}-sensitive potassium channels, KCl exit, osmotic loss of cellular water, cell shrinkage and phosphatidylserine exposure at the RBC surface). In other words, the reported results provide an unexpected evidence, namely that the final stages of the RBC apoptotic process can also operate through a mechanism different from that activated by the increase of cytosolic Ca^{2+} (the only one known to date).

2. Materials and methods

2.1. Blood collection and RBC isolation

Blood samples (10 mL) were obtained from healthy human volunteers using venipuncture into Vacutainers (Becton-Dickinson, Franklin Lakes, NJ, USA) containing ethylenediaminetetraacetic acid (EDTA) and were centrifuged at 1500 rpm for 10 min at 4 °C. The yellowish supernatant (*i.e.* platelet rich-plasma) and the white coat on the pellet (*i.e.* most of leukocytes) were discarded, and the erythrocytes were re-suspended and washed 4 times (volume ratio: 1 to 10) under sterile conditions in glucose-free PBS (potassium phosphate, 4 mM; NaCl, 140 mM; EDTA, 1 mM) adjusted with NaOH to pH 7.35. In order to avoid proteolytic degradation, a protease inhibitor (phenyl-methyl-sulfonyl-fluoride, 1 mM) was also added to the RBC suspension; moreover, with the aim of reducing oxidative damage during shelf-life, RBCs were stored under carbon monoxide (CO) atmosphere (760 Torr) at 4 °C. This treatment was performed immediately after the blood collection, in order to preserve as much as possible RBCs in their native state.

2.2. Immobilization of RBCs on a glass surface

At the chosen times, an aliquot (6 μL) of the RBC suspension (from the batch or the rejuvenated specimens) was taken from the containers for preparing the samples for biochemical and AFM measurements as a function of *in vitro* ageing. In order to get samples with uniform RBC distribution upon each slide (*i.e.* not piled up), smears were made by manual spreading of 6 μL of suspension of RBCs diluted with plasma (ratio, 1:5) on poly-L-lysine microscope glasses (Menzel Glazer, Germany). Poly-L-lysine assures the attachment of RBCs on the slide surface due to the electrostatic interactions between the negative charged RBCs and the positively charged poly-L-lysine. Unattached cells were removed by gentle rinsing of the slides a few times with PBS solution, just before air-drying. To follow the structural alterations taking place during the shelf-life, RBC smears were prepared at different storage time.

2.3. Depletion of intracellular energy source

RBC suspensions (packed RBCs/PBS:1 to 5, in volume) were incubated in a thermostatic shaker at 37 °C gently agitated for 24 h under CO atmosphere (760 Torr). In order to avoid bacterial contamination during the incubation period, a mixture of three antibiotics at suitable concentration (gentamicin, 5 mL/L; penicillin, 100 U/mL; streptomycin, 100 μg/mL) was added to the RBC suspension.

2.4. Treatment of stored RBC with rejuvenation solution

Incubations for rejuvenation were carried out in a thermostatic shaker at 37 °C gently agitated for 4 h, after mixing of 5 volumes of additive solution (see below) to 1 mL of RBCs drawn aseptically from the storing container after various days of storage. The additive rejuvenation solution (IPP medium) was made up as follows: 10 mM inosine (Sigma grade), 10 mM pyruvate (sodium salt, Sigma) and 75 mM phosphate [24,25]. Controls were performed treating RBCs with saline solution at the same temperature and for the same time. After 4 h incubation, RBCs were washed and centrifuged 4 times with PBS buffered solution and then re-suspended in PBS (ratio in volume, 1:10); the concentrations of ATP and BPG were measured by enzymatic assays.

2.5. Evaluation of intracellular organic phosphates (ATP and BPG)

Batch analysis for the polyphosphates ATP and BPG was conducted using spectrophotometric assays based on the oxidation of NADH to NAD^+ (Roche Diagnostic, Mannheim, Germany). Briefly, sample aliquots

were extracted at the indicated time points, and the RBC specimens were deproteinized according to the manufacturer's instructions. The lower limits of detection for ATP and BPG were 3 μmol/dL and 0.2 μmol/mL, respectively, values that are well below the measured range.

2.6. Atomic force microscopy (AFM)

The AFM measurements were performed using a home-built microscope (described in detail elsewhere [26]) operating in contact mode under strictly controlled environmental conditions (room temperature and constant 30% relative humidity). The instrument, that has already been applied in the study of erythrocytes [18–20], operates in the weak repulsive regime of constant force with a probe force below 1 nN from zero cantilever deflection using Veeco probes (Camarillo, CA, USA — model MSCT): these are silicon nitride cantilevers with asymmetric pyramidal shape and nominal tip radius of 10 nm. The high resolution images were collected at a scanning speed of about 3–4 s/row. The reproducibility of data was carefully tested. Concerning the reported images, no noise filter was applied to the raw data which were treated only by a software background subtraction and, when required to have a flat non-tilted surface, by a plane alignment and/or an *X* axis linearization performed using the Gwyddion software (www.gwyddion.net). For additional information (*e.g.* observation time), see [18–20].

2.6.1. RBC's roughness calculation

After acquiring an AFM image, the comparison of the distinctive features at the nanometer scale seen on the surface of other samples can be best described in terms of statistical analysis rather than by comparison of tiny structures strictly related to the local sample arrangement. In this framework, roughness which is related to the distribution of the heights on the RBC surface and therefore to the vertical displacement (*i.e.* the *Z* component) of each data point, is a useful tool. In particular, the roughness value of the surface can be described in terms of the root-mean-square value of the height distribution (symbolised by R_{rms}). The standard deviation of this distribution is, of course, the associated experimental error. Most studies make use of the R_{rms} since such a quantitative definition (i) has the advantage of being a simple mathematical approach and (ii) conserves the intuitive concept of a rugged surface. When describing the surface roughness from an AFM image, several parameters of the acquisition method have an influence on the measured value. These parameters include the dependence on probe size (constant in our case), on the scanned area and, due to the surface statistics (*i.e.* the fractal or scale-dependent behaviour [20]), on the number of data points. As a consequence, when using the value of roughness for comparison between two or more samples, the analysed area must be specified and kept at a constant value as well as the sampling density. The constraints placed on the number of sampling points per image (*i.e.* the product: image size × sampling density) is, however, less critical provided that they are large enough to ensure a solid statistical set (10,000–60,000) with minor difference from image to image. In particular, a reference sampling density able to ensure about 20,000 points in a frame of $1 \times 1\ \mu m^2$ images was used. The data treatment was performed using the software package PWWAVE which allows the analysis of AFM images and the calculation of roughness value using integrated routines.

3. Results and discussion

3.1. Maximal acceleration of RBC ageing in vitro as evaluated by roughness of plasma membrane

During the banked shelf-life of RBCs, a most central biochemical variation seems to be a decrease in ATP concentration, the physiological level of which is important for the maintenance of membrane structure and cytoskeleton integrity [21]. In order to check such a correlation, a reference RBC sample – completely lacking of energy sources in the external solution – was prepared by washing it 4 times under sterile conditions in PBS. Therefore, the only energy at disposal is that stored up as intracellular ATP content: after 24 h incubation in PBS at 37 °C, the ATP concentration decreased by 82,4% (from 0.91 ± 0.03 mM of freshly drawn RBCs to 0.16 ± 0.03 mM) and a lessening of BPG level by 98.2% (from 3.37 ± 0.016 mM to 0.06 ± 0.016 mM) were observed; accordingly, a parallel and rapid disjointing of the membrane-skeleton architecture was expected.

A representative membrane surface of RBCs, as observed by AFM after 1 day of storage at 37 °C under the extreme conditions chosen, is reported in Fig. 1.A: this is a typical image (similar to those reported previously [18,20,27]) constituted by small grain-like structures, homogeneously distributed all over the surface and representing the main fabric of the plasma membrane as influenced by the underlying cytoskeleton architecture. Even from visual observation, it is evident that Fig. 1.A is significantly different from Fig. 1.B, which shows the relatively smooth RBC membrane topography (its roughness being ~50% of the initial value reported in Fig. 2.A) after 6 days of storage at 4 °C in the total absence of external energy sources. Indeed, the two cross sections shown in panels C and D (related to panels A and B of Fig. 1, respectively) are significantly different: the profile in C spans over ~8 nm, twice that in panel D, traced on a 6 days aged cell. The intuitive idea that the plasma membrane surface becomes less uneven as RBCs grow older *in vitro* finds a precise mathematical expression in the study of the roughness parameter.

The changes in the surface roughness of the very same RBC specimen (washed 4 times with PBS) as a function of shelf-life time at 4 °C are shown in Fig. 2. It is plain that, in the total absence of external energy sources, the large majority of the *in vitro* ageing effects on the cytoskeleton network occurs within 6–7 days, revealing themselves with a drastic decrease of the average sample unevenness that drops to ~50% of its initial value. Furthermore, after 7 days of storage, the measured roughness reaches a minimum value, which remains constant within the experimental error. As extensively shown elsewhere [20], the roughness of the RBC plasma membrane is sensitive to the integrity of the cytoskeleton: in particular, low R_{rms} values can be correlated to a partial disruption of the protein network architecture resulting in the alteration of nano-mechanical properties of the cell. In the present experiments, large oxidative damages [28] resulting from reactions with denatured haemoglobin and O_2 as well as relevant proteolytic degradation events can be excluded, respectively, because of the CO atmosphere and the presence of a protease inhibitor (PMSF) as well as the absence of calcium ions. Thus, the observed decrease in the average sample roughness can mainly be ascribed to a gradual disconnection of membrane-skeleton network [21] due to the intrinsic ageing dynamics (related to the ATP level). In particular, the effectiveness of the anti-oxidant treatment (i.e. CO atmosphere) finds also support (i) in the absence of large holes (~150 nm in diameter) that usually are observed on RBC membrane [29] due to serious peroxidation, and (ii) in the fact that only minor leakage of haemoglobin and other molecules contained in RBCs was observed (as a rule, in the sample containers haemolysis was $\ll 0.05\%$ per week).

The rectangular box in Fig. 2 (marked by the letter 'a') reports the average roughness value (the box height being defined by the experimental error) measured on a RBC sample stored for 45 days under the conditions indicated in the standard protocol for banked blood. In terms of changes in the membrane roughness (*i.e.* in terms of alterations in the cytoskeleton structure and nanomechanics properties), 5–6 days under starvation conditions are equivalent to 45 days of ageing in banked RBC storage.

3.2. Representative morphological patterns at nanometer level

Morphological parameters of whole RBCs – such as shape, height, diameter and other similar characteristics obtainable also by AFM

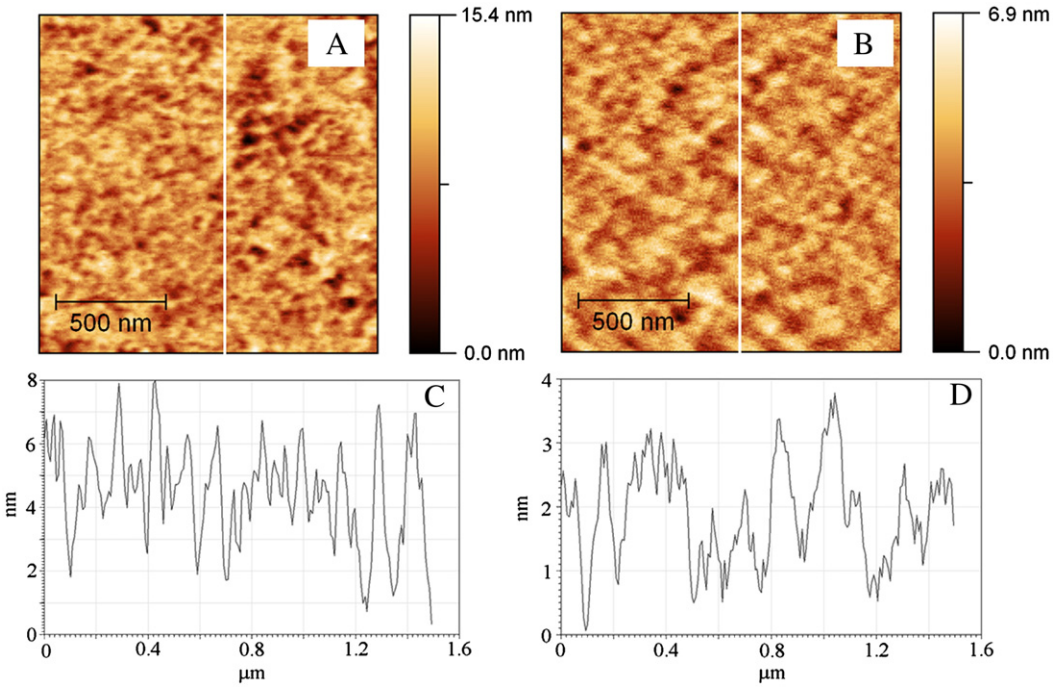

Fig. 1. AFM topographies of RBC surfaces taken, respectively, after 1 day at 37 °C (panel A) and after 6 days at 4 °C (panel B) of ageing. A homogeneous surface (panel C) indicates that the cell is still in a preliminary stage of ageing and the membrane-skeleton is still intact, or nearly so. Large invaginations are clearly visible (panel D) and a low value of roughness is measured on the surface; as a consequence, the deep and structured invaginations can be associated to a local disruption of the membrane-skeleton.

measurements [18,19] – have not been investigated in the present study, since these are features influenced by too many, simultaneously acting ageing effectors [18]. Instead, attention has focused on changes in the structure of the membrane-skeleton network at nanometer level, *i.e.* on a feature that is independent of the overall cell geometry (*e.g.* discocyte or spherocyte) [19,30].

The strong falling apart of cytoskeleton connectivity (Fig. 1.B) appears to be associated with additional morphological features frequently detected in the samples (see Fig. 3.B–C): polip-like protrusions, that may be interpreted as proto-spicules and proto-vesicles. Such a view is in line [21,31] with the evidence that the cytoskeleton shrinks to a 3–5-fold smaller area when the whole membrane is removed under physiological conditions [31], indicating that the cortical skeleton network is stretched by its attachment to the cell membrane and accordingly exerts a pulling and/or a compression force on the membrane. As a consequence, after a partial detachment of the cortical cytoskeleton from lipid membrane (a processes related to ATP concentration; see under Changes in roughness of RBC membrane during accelerated ageing and after rejuvenation), the lipid bilayer is pulled over a smaller area with respect to the previous one; it is the need to accommodate the bilayer area over a smaller projected region that results in the appearance of the spicules [33].

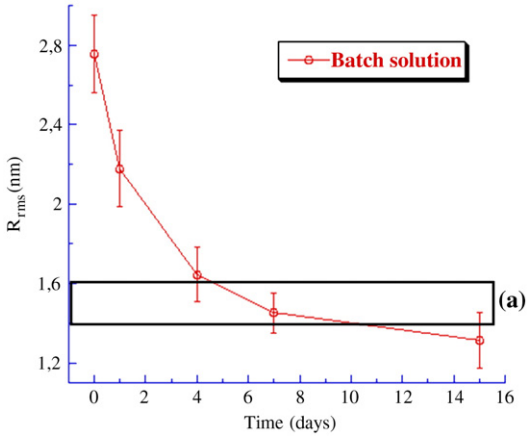

Fig. 2. Behaviour of the RBC roughness values as a function of *in vitro* ageing (measured in days) in the total absence of external energy sources. After the 6–7 days at 4 °C, the exponential decrease of R_{rms} reaches a minimum value, which remains constant within the experimental errors.

The sequence of events, displayed in Fig. 3, develops towards the production of vesicles observed in large amount after 10–15 days of shelf-life (Fig. 3.D). Therefore, at the end of the chain of morphological events (Fig. 3.A–D) — (proto)spicules, proto-vesicles, spherocyte with released vesicles, the RBCs have suffered from a loss of patches of plasma membrane that leads to a cell volume decrease. In this landscape, the extracellular vesicles depicted in Fig. 3.D are portions of plasma membrane detached from the cell [33] and rearranged, according to the their amphiphilic nature, in a spherical shape.

During blood bank storage, RBCs undergo complex structural (see, *e.g.* Figs. 2 and 3) and biochemical (see under Regeneration of ATP and BPG in RBCs preserved under shortage of energy sources) changes, which may be seen as an accelerated and/or aberrant form of the physiological RBC ageing process (resulting in the storage lesion [4,6,7]); indeed, all currently available data suggest that the storage lesion of RBCs under blood bank conditions may be similar, but is far from identical to ageing *in vivo* [14,17]. Therefore, from this viewpoint, data reported in Fig. 3.B and C (in particular, the presence of clusters of protrusions in restricted areas) appear to constitute morphological alterations specifically induced by the extreme conditions chosen. However, no matter how things stand, to the best authors' knowledge the reported images show for the first time at a very high resolution the initial development of spicules/vesicles from RBC membrane.

RBCs' senescence (and their self-destruction) is associated with cell shrinkage, plasma membrane micro-vesiculation, a progressive shape change from a discocyte to a spherocyte, and loss of plasma membrane phospholipid asymmetry [10]; in particular, during the lifespan of a mature erythrocyte the surface area decreases by approximately 30 μm^2 by blebbing of (micro)vesicles with an average diameter of 0.5 µm from the tips of echinocytic spicules. If this is how

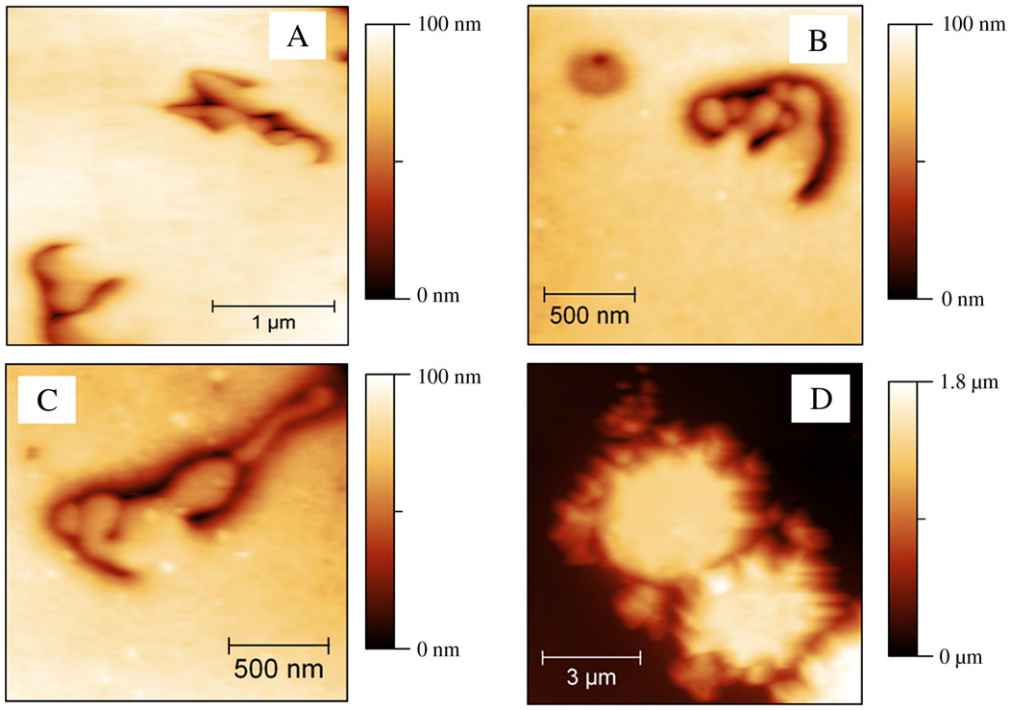

Fig. 3. Structural patterns appearing on the cells' surface after 15 days of ageing at 4 °C in the absence of external energy sources. The morphological hallmarks of RBC apoptosis, representing the execution phase of this physio-pathological process, are mostly constituted by membrane blebbing (with formation of (proto)spicules, panel A, and (proto)vesicles, panels B and C), and by shedding of vesicles with consequent cell volume shrinkage associated to the production of smaller spherical cells or spherocytes (a spherocyte surrounded by released vesicles is represented in panel D). A more or less scheme of the apoptotic process, that leads to the removal of senescent and/or ill RBCs without release of intracellular proteins (which would otherwise cause inflammation *in vivo*), can be illustrated in its bare essentials by the sequence of panels (A, B, C and D).

things stand, the images reported in Figs. 2 and 3 (taken together) describe phenomena considered as important aspects of RBC apoptosis [10,11,14] and therefore may be regarded the expression of a valuable model for studying the execution phase of a sort of RBC apoptosis, observed under the peculiar experimental conditions chosen (*i.e.* when the final stages of apoptosis are only caused by energy depletion): in fact, a huge portion of RBCs is removed by the vesicle formation system in such a way that no release of cellular constituents in the environment can occur [14].

3.3. Regeneration of ATP and BPG in RBCs preserved under shortage of energy sources

The biochemical rejuvenation studies have been carried out to assess whether and how long the operating capacity of the RBC main metabolic pathway, *i.e.* the glycolysis, is preserved during storage in the total absence of external energy sources. After 1 (at 37 °C), 4 and 8 days of shelf-life at 4 °C, (i) a suitable amount of RBCs was isolated by centrifugation from each sample, (ii) the levels of ATP and BPG in those specimens were measured and (iii) the remaining RBCs of each sample incubated in IPP medium for 4 h at 37 °C. Subsequently, the rejuvenated RBCs were washed 4 times with PBS and re-suspended in the washing buffer, checked the biochemical indicators (*i.e.* ATP and BPG) to evaluate the rejuvenation efficiency and a new cycle of shelf-life ageing at 4 °C was started and monitored.

Fig. 4 depicts the time-related changes of two metabolites, BPG and ATP, (i) in the fresh sample under accelerated depleting conditions at 37 °C for 24 h (after which, *i.e.* from the second day on, kept at 4 °C), and (ii) in specimens of RBCs treated with the rejuvenation solution after 1 (at 37 °C), 4 and 8 days of shelf-life at 4 °C. An almost complete depletion of both BPG (panel A) and ATP (panel B) is achieved in fresh RBCs within 24 h incubation in buffered PBS at 37 °C.

Rejuvenation treatments indicate (Fig. 4.A) the capacity of BPG-depleted RBCs of synthesizing BPG up to supra-normal levels (2.6 to 1.7 fold of the initial value) after 1 (at 37 °C), 4 and 8 days of storage at 4 °C in the total absence of external energy sources. In all three samples, BPG concentration remains roughly steady for three days of shelf-life, after which a gradual (for the 1 and 4 days old samples) or quickened (for the specimen stored for 8 days) decline is observed. Human RBCs, ageing under blood bank conditions [34], follow a different time-related trend in concentration decrease: under these latter conditions, in fact, a progressive and rapid deterioration towards a minimum is always monitored in contrast with the lack of large variations observed in the present study, at least during the first 3–4 storage days. Only RBCs, preserved for 8 days in shortage of external energy sources and then rejuvenated, are in some way reminiscent of the banked ones: after a week storage, in both samples (*i.e.* those stored under banked conditions and those in shortage of external energy sources) BPG level has failed by 60%.

The results for ATP concentration decline in fresh (or batch) and rejuvenated RBCs are reported in Fig. 4.B, where is shown that the regenerated ATP never reaches the baseline concentration (0.91 ± 0.03 mM). In particular, RBCs – treated with rejuvenation solution on day 1 and 4 of storage in the absence of external energy sources – exhibit half of synthesizing ATP capacity (~0.45 mM), whereas those incubated in IPP medium after 8 days of shelf-life produces only $1/6^{th}$ (~0.15 mM) of the initial value. Once the maximal ATP level has got by rejuvenation treatment, a progressive declension of ATP concentration starts immediately at 4 °C: this kinetic behaviour is markedly diverse relative to that of the banked human RBCs [34], in which ATP levels remain almost steady for the first 15 days and then a gradual declension is observed. In conclusion, the results of the ATP and BPG measurements as depicted in Fig. 4 show trends of concentration depletions just reversed in the curve shape with respect to those observed in banked human RBCs.

Both ATP and BPG are products of one metabolic pathway, the glycolysis, and therefore it is apparently surprising that RBCs – stored up to 4 days in the total absence of external energy sources – point out

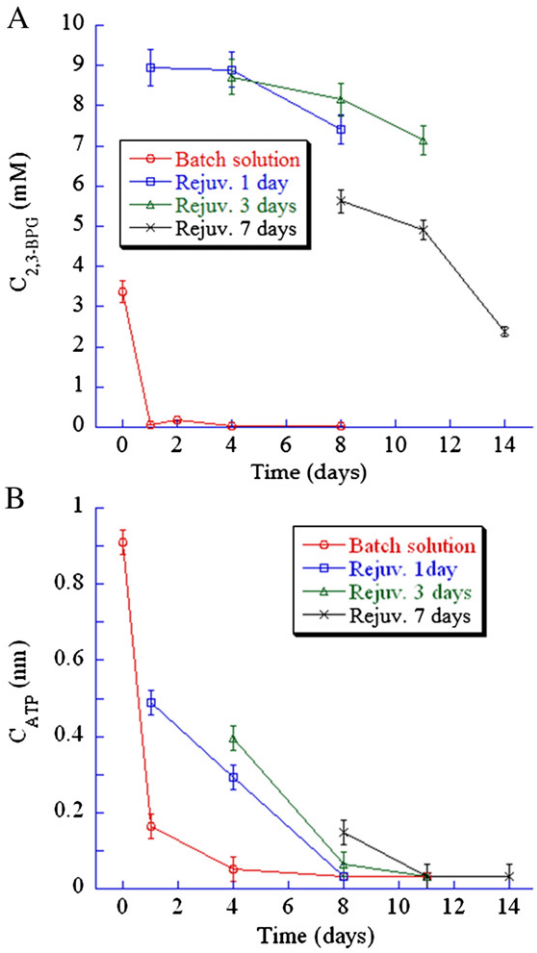

Fig. 4. 2,3-BPG (panel A) and ATP (panel B) concentration trends after the rejuvenation procedure performed at 37 °C and different days of ageing. Fresh RBCs (○) show a quick decrease in both 2,3-BPG and ATP during the incubation at 37 °C in PBS. Changes in 2,3-BPG and ATP concentration in RBCs rejuvenated after 1 (□), 3 (△) and 7 (X) days, respectively, of *in vitro* ageing show a different time course.

a full capacity to produce BPG and half of the normal potentiality to fabricate ATP; in fact, under the rejuvenating conditions, the *de novo* synthesis of both metabolites (BPG and ATP) can only make use of the energy source coming from inosine-carried ribose, a sugar metabolized through the pentose phosphate pathway that produces fructose-6-phosphate and glyceraldehyde-3-phosphate (both intermediates of glycolysis). It may be proposed that the main cause of data reported in Fig. 4 is the nominal absence of Mg^{2+} in the washing and rejuvenating solutions. BPG is in fact generated by the Lapoport–Leuebering shunt, which is a glycolytic stage that lies just before the chemical processes able to harness free energy to synthesize ATP. In particular, the flux of metabolites through the glycolytic pathway is controlled as follows: phosphoglycerate kinase catalyzes the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate that results in the formation of the first ATP molecule, and competes with phosphoglycerate mutase (the enzyme designed to generate BPG) for 1,3-diphosphoglycerate. Data in Fig. 4 suggest a relatively higher activity of phosphoglycerate mutase, which may preferentially direct 1,3-bisphosphoglycerate to BPG, thereby possibly explain the diminished effect of rejuvenation solution on ATP production. Since the phosphoglycerate kinase activity is Mg^{2+}-dependent, the shortage of magnesium ions (due to the nominal absence of Mg^{2+} in both washing and storage solutions) can partially account for the accumulation of BPG as well as the reduced capacity of ATP synthesis delineated in Fig. 4. Moreover, it has been suggested [12] that cation channels in erythrocytes sense cell age and accordingly an increased passive permeability of both Mg^{2+} and Ca^{2+} is expected with the consequent, continuous leakage of intracellular cations as a function of shelf-life duration leading to the release of Mg^{2+} and Ca^{2+} in the extracellular environment (with a parallel reduction of intracellular cation concentration). Lastly, an additional factor, contributing to the explanation of the low regeneration of ATP by incubation in IPP medium, is the catabolism of adenine nucleotides that proceeds from AMP to hypoxanthine (thus limiting the regeneration of ADP and hence of ATP), very slowly under physiological conditions but much more expeditiously by suppression of glucose [35].

Aside from furnishing the substrates (with IPP medium) required to produce ATP and BPG, it is also necessary for the energizing compound (*i.e.* inosine) to pass through the RBC membrane in the cytoplasm in order to indirectly supply the glycolytic pathway. Data replaced in Fig. 4.A shows that, after treatment with the rejuvenation solution, RBCs – stored for 8 days in an energy-lacking medium at 4 °C – manifest a reduction by 1.6 fold of the maximal regeneration potentiality for BPG (Fig. 4.A) compared to the younger ones and appear marginally capable of still producing ATP (Fig. 4.B). Such a failure of 8 days stored RBCs to rejuvenate seems to suggest that, compared to the younger ones (*i.e.* 1 and 4 days stored RBCs), less substrates necessary for the synthesis of both organic phosphates enter into these cells. Indeed, a functional decline of the pentose phosphate pathway is unlikely, since young RBCs

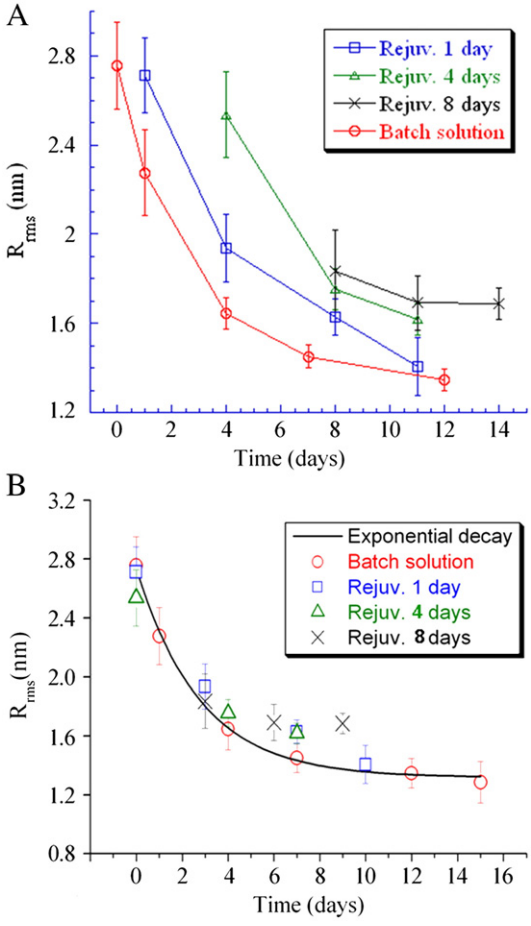

Fig. 5. Decreasing trends of the roughness values after the rejuvenation treatment performed at different days of ageing. Decline of R_{rms} (panel A) in fresh (○) RBCs and rejuvenated after 1 (□; at 37 °C), 4 (△) and 8 (X) days (at 4 °C), respectively, shows a similar trend, underlined in panel B (where all data are normalized with respect to the negative exponential of the batch sample). After 8 days of storage at 4 °C, the rejuvenation procedure appears to be inefficient, since the initial value of R_{rms} cannot be restored: this means that irreversible phenomena have occurred and the membrane-skeleton has permanently been damaged.

in vivo are able to metabolize 2.5 times more glucose than old ones through glycolysis; on the other hand, the amount of glucose utilized via the pentose phosphate pathway is not known to show any age dependence [36].

3.4. Changes in roughness of RBC membrane during accelerated ageing and after rejuvenation

The membrane of RBCs [37] is a fluid sheet of lipids and proteins, which is connected through node complexes to a two-dimensional cytoskeleton primarily composed of flexible spectrin filaments linked together and to integral proteins (the main ones being band-3 proteins and sialoglycoproteins, embedded in the lipid membrane) mainly by short stiff actin filaments, band-4.1 protein and ankyrin.

Since (i) ATP is important for facilitating cytoskeleton flexibility and rearrangement [37,38] (in that it modulates the degree of dissociations in the cytoskeleton network), and (ii) since rejuvenation solutions rapidly restore ATP concentration in RBCs kept for a long time under blood bank conditions [12], it seems plausible that the changes in RBC surface roughness observed as a function of the shelf-life duration be partially or totally reverted after treatment with IPP medium. The molecular mechanism generating the changes in membrane roughness is possibly dependent on AMP-activated protein kinase (AMPK). In fact, previous studies [22] showed that glucose depletion in RBCs leads to a stimulation of AMPK activity, which in turn phosphorylates [39] cytoskeletal proteins (such as protein-4.1) and induces transient dissociation of the network of membrane-attached proteins; interestingly, as ATP levels drop phosphorylation of these proteins (corresponding to a parallel increase in the network dissociation) increases [40], since the AMPK activity is stimulated by the binding with AMP [39]. It is highly probable that even under these extreme conditions the cytoskeleton protein phosphorylation is considerably modulated by AMPK.

Fig. 5.A shows the decline of roughness with time in the batch sample and in rejuvenated RBCs, all kept in energy source-free PBS at 4 °C and under CO atmosphere. The decrease of R_{rms} values of the specimens, rejuvenated at the first and the fourth day of the *in vitro* ageing at 4 °C, follows a kinetic behaviour practically superimposable (Fig. 5.B) to that of the batch sample. In particular, rejuvenation treatment after 1 and 4 days of storage (i) restores the roughness (Fig. 5.A) to the initial value observed in the fresh sample (within the statistical errors) showing that ATP is necessary for the recovery of RBC mechanical properties after tether extrusion, and (ii) does not modify the typical cycle of morphological patterns that progressively appears on the surface and around the rejuvenated RBCs (data not shown) – *i.e.* (proto)spicules, (proto)vesicles and vesicles – observed after 4–6 days of re-ageing, just as noticed in the batch sample (see Fig. 3). In other words, the order of appearance of these structural events seems to be encoded in the mechanical properties of the membrane-skeleton [40]: once the correct integrity of the cytoskeletal network is re-established by rejuvenation treatment (as measured by the roughness value) due to the reattachment of cortical cytoskeleton to the integral proteins, the consumption of ATP results in a decrease of the cell mechanical stability [38,41].

A different situation is evidenced in the structural alterations observed as a function of time in RBCs rejuvenated after 8 days of shelf-life with respect to those of the batch sample (as well as the other rejuvenated specimens): in the first case, indeed, (i) only a very small restoration of roughness is measured (the value raising by only 30%), and (ii) the lowest mean value of R_{rms} is visibly higher than the corresponding one of the other curves (see Fig. 5.B). This latter peculiar behaviour may partially be explained through a direct analysis of the roughness morphologies of the RBC surfaces. In Fig. 6.A an AFM image, typical of RBCs rejuvenated after 8 days of storage at 4 °C and collected after further 3 days of *in vitro* ageing, is shown: a massive presence of local damages of the plasma membrane integrity can be observed, consisting of ruptures, deep furrows and inhomogeneities on the surface. The evaluation of the R_{rms} of similar cells can be tricky: in fact, the basic hypotheses for a correct measure of roughness (*i.e.* homogeneous surface and Gaussian-like distribution of heights) cannot be satisfied any longer [20]. In particular, the heights distribution results over-spread around its mean value and

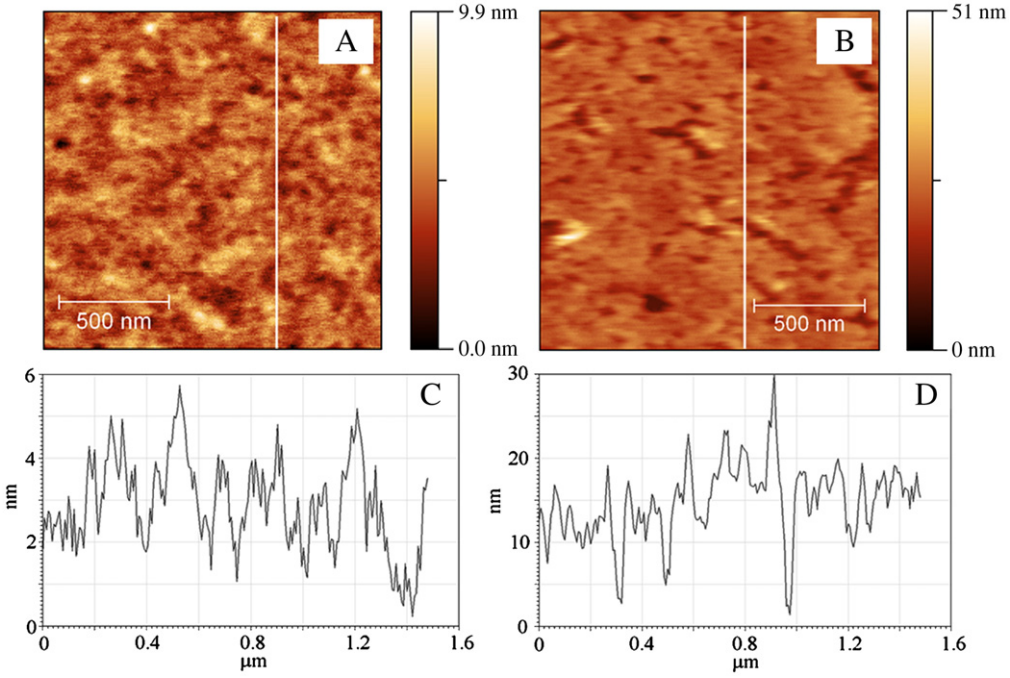

Fig. 6. AFM image ($2 \times 2\ \mu m^2$; 400 points per row) of a cell surface after 7 days of accelerated ageing in the absence of external energy sources, taken before (panel A) and after the rejuvenation procedure (panel B), respectively. The two surfaces appear to be topographically very different: (a) homogeneous in the absence of rejuvenation (panel C); and (b) rich in furrows and ruptures, distinctly evident also in the section profile (panel D), after treatment with IPP at 37 °C (these latter features affect the roughness estimations and suggest a possible damage even at level of the lipid bilayer).

the measured R_{rms} value is partially overestimated. It is relevant that when the quantitative roughness evaluation is affected by such age-related morphological disorders of the cell membrane fabric, the overall structural and biochemical damages of the RBCs have become irreversible.

Next, a comparison of data reported in Figs. 5 and 6 suggests that most of the serious storage lesions (*i.e.* the irreversible ones), localized on the membrane surface, appear when ATP synthesizing capacity declines to apparently sub-viable levels.

Lastly, it is known [14] that RBC storage is associated with numerous cytoskeletal changes, such as abnormal spectrin-protein-4.1-actin complex formation and loss in the band-3-ankyrin anchorage of the cytoskeleton to the lipid bilayer. Therefore, at very low ATP content a highly dissociation of membrane-skeleton proteins can be anticipated, and consequently a strong aberrant re-association among these proteins – due to their intrinsic high association constants [21] – is expected. Thus, such an aggregation of the dissociated structural units is inevitably random, and accordingly the network connectivity experiences extensive deformations leading to the inhomogeneous surface roughness measured after 8–15 days (Fig. 6.B). However, ATP depletion by itself does not account for all storage lesions: in fact, comparison of images collected from the very same RBC population (i) kept for 11 days at 4 °C (Fig. 6.B) and (ii) rejuvenated at 37 °C after 8–11 days at 4 °C (Fig. 6.A), both specimens containing ~0.5 mM ATP, suggests that some peculiar aspects (*e.g.* membrane fractures) are possibly induced by a temperature and other environmental effects. In other words, incubation in IPP medium at 37 °C for 4 h appears to favour the production of tears and lacerations in the lipid bilayer, possibly as a result of stresses and strains due to the vibratory motions (classically attributed to thermal mechanisms) [41,42] of the still partially anchored membrane to the stiff cortical cytoskeleton or, alternatively, to phenomena of water transport [43,44]. In fact, while thermal dissociations in the cortical cytoskeleton network are highly unlikely [21], increases in temperature are expected to have a marked stimulatory effect on (i) the kinetic energy of particles undergoing Brownian motion [45] and, as a consequence, the fluctuation of the lipid bilayer, and (ii) the permeability to ions [44]. As a consequence, the presence of cracks and holes (all >10 nm width) on the RBC surface – through which cell protein content may easily pass with consequent inflammatory response *in vivo* – adds necrotic elements to the entire picture of RBC death under the extreme conditions chosen. These observations might be similarly effective *in vivo*.

As a whole, it is apparent that when the maximal ATP concentration of rejuvenated RBCs (see Fig. 5) is lower than 25% of the initial value (*i.e.* that of fresh drawn RBCs), no storage lesion is outwardly reversible. In other words, the transition between reversible and irreversible biophysical (*i.e.* R_{rms}) and biochemical (ATP and BPG levels in RBCs) modifications appears revealed (not always controlled) by a minimum of ATP concentration: in particular, the ATP effect seems mediated through a blocking impact on cellular pumps. When storage at 4 °C in the total absence of energy sources reaches the eighth day, the final stages of RBC death seem to occur in a passive way as a result of increasing stiffening of untidily aggregated cytoskeleton proteins.

4. Conclusions

(i) Many of the storage modifications are considered as stages of an intrinsic program of cell death [10,12,14] (held apparently in check by normal concentration of ATP [46]) that may be elicited by several stressors, including osmotic shock, oxidative stress and energy depletion, all activating Ca^{2+}-permeable channels with subsequent entry of calcium ions into RBCs. Indeed, it is well known that a major mechanism stimulating RBC apoptosis in RBCs is just an increase in cytosolic Ca^{2+} activity [11]: which leads to membrane vesiculation and to stimulation of cysteine endopeptidase calpain, whose catalytic action degrades the cytoskeleton and thus facilitates membrane blebbing. However, since mature RBCs lack intracellular calcium stores, elevation in its intracellular concentration must stem from Ca^{2+} influx: such an event however must be ruled out in this work, for extracellular Ca^{2+} has been removed by repeated washing with isotonic solutions without nominal calcium ions. Therefore, even though the molecular trigger(s) of the RBC apoptotic process studied in the present investigation remains elusive, a Ca^{2+}-stimulation can definitely be excluded. At the best authors' knowledge, this is the first evidence of an ordered chain of morphological events reminiscent of human RBC apoptosis *in vivo*, spontaneously occurring in the nominal absence of Ca^{2+} or without the stimulation of any xenobiotic (such as thymoquinone [23]).

(ii) Since, under the extreme experimental conditions chosen in this work (*i.e.* total absence of external energy sources during RBC storage), the overall proteolytic activity (by addition of PMSF and extracellular removal of Ca^{2+}), the oxidative stress (by CO atmosphere) and the transglutaminase activity leading to cross-linking of proteins (by nominal absence of Ca^{2+}) have been reduced to a minimum, the RBC membrane alterations – quantified by changes in the roughness values – may largely be attributed to the pure dynamics of cytoskeleton network, modulated by the ATP regulation. These results argue that even for RBCs kept in a minimal additive solution membrane physical properties – as measured by R_{rms} – are actually controlled by the cell metabolism, until necessary ATP to perform some basic functions can be regenerated. Necrotic aspects (*e.g.* disruption of cell membrane integrity, so that intracellular protein content is easily released) ensue when RBCs are almost totally (≥90%) depleted in an irreversible way of the energetic stores [47].

(iii) It is simple mechanical forces that give rise to the morphologic phenomenology represented by a local buckling that leads to vesiculation of the lipid bilayer [21,40], when RBCs are severely or totally depleted of the energetic stores, possibly because of the disruption of transmembrane mechanisms of transport. Therefore, RBCs represent a sort of living matter as long as they retain the capacity of taking energy sources from the environment, whereas they die by passive collapse when the membrane transport pathways do not work any longer and the cytoskeleton is stiffer [21,32,40,48] (see also under Representative morphological patterns at nanometer level) than normal (and the surface roughness has become extremely patchy).

(iv) Lastly, the analysis of RBC specimens using AFM can be applied to confirm and to advance the understanding of erythrocyte death gained from biochemical studies [10,11,14]. In particular, the reported results demonstrate how AFM can be employed to interrogate the cytoskeleton behaviour by taking advantage of nano-scale phenomena, and the simplified method here proposed is expected to be able to dissect and to probe the machinery leading to RBC death by checking the effects on cytoskeleton of a wide variety of drugs, environmental pollutants, xenobiotics and endogenous substances that trigger the death of RBCs.

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