An AFM Approach of RBC Micro and Nanoscale Topographic Features during Storage

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Abstract—Blood gamma irradiation is the only available method to prevent transfusion associated graft versus host disease (TA-GVHD). However, when blood is irradiated, determine blood shelf time is crucial. Non irradiated blood have a self-time from 21 to 35 days when is preserved with anticoagulated solution and stored at 4°C. During their storage, red blood cells (RBC) undergo a series of biochemical, biomechanical and molecular changes involving what is known as storage lesion (SL). SL include loss of structural integrity of RBC, decrease of 2,3-diphosphatidylglyceric acid levels, and increase of both ion potassium concentration and hemoglobin (Hb). On the other hand, Atomic force Microscopy (AFM) represents a versatile tool for a nano-scale high resolution topographic analysis in biological systems. In order to evaluate SL in irradiated and non-irradiated blood, RBC topography and morphometric parameters were obtained from an AFM XE-BIO system. Cell viability was followed using flow cytometry. Our results showed that early markers as nanoscale roughness, allow us to evaluate blood quality since other perspective.

Keywords—AFM, Blood γ-irradiation, roughness, Storage lesion.

I. Introduction

BLOOD transfusion is one of the most common therapeutic procedures for patients in oncology, hematology as well as in emergency room [1]. Red blood cells (RBC) concentrates can be preserved in blood banks around 42 days at 4°C before donation. However, during this time RBC normal morphological, biochemical and physiological features are affected. Stored RBC exhibit loss of their biconcave shape and membrane integrity [2], lactic acid and adenosine triphosphate (ATP) accumulation, as well as 2,3-diphosphoglycerate (2,3-DPG) concentrations depletions [3]. Some of these alterations results in irreversible and prejudicial effects referrers to as storage lesion (SL) [4].

The main function of RBC is oxygenation of cells and tissues. This is possible because it contains a molecule capable of catching and carrying oxygen, called Hemoglobin (Hb). Hb can be present in two allosteric states differing both the number and energy of interactions with their subunits. In this way, conformational changes are activated by the presence or absence of oxygen in the Hb ligands. Binding of oxygen molecules (O_2) into Hb active sites induces a displacement of Fe phorphyrin ring due to spin change in their "d" electrons [5]. As consequence of this change, oxygens are attached very tightly, therefore it is said that has O_2 high affinity. This high O_2 affinity Hb is called oxygenated Hb (Oxy-Hb) and its spatial configuration is a relaxed (R) state. On the other hand, deoxygenated Hb (Deoxy-Hb) lacks O_2 affinity and behaves as the tense (T) form, thus oxygen can be released in tissues [6]. Nonetheless, T-state is very unstable, and only get stable in presence of 2,3-DPG by forming salts bridges with the Hb β-subunits [7]. This mechanism occurs on homeostasis in healthy RBC. Moreover, when they are stored, R state of Hb is more favored.

It is a well-known fact that during SL 2,3-DPG concentrations decrease, leading out Hb increases their oxygen affinity [8]. However, clinical effects after blood transfusion are controversial. On one hand, clinical evidence suggests that this oxygen availability reduction in aging RBC induce immunommodulatory and proinflamatory effects [9]. On the other hand, it has been considered has a reversible condition and normalized in circulation in a few days after transfusion [10], [11]. Even in a work made by Tsai AG and colleagues, has been considered has a positive factor during the treatment of hemorrhagic shock resuscitation [12]. Nonetheless, a fact that cannot be ignored is that alterations in biochemical characteristics of RBC, results in oxidative damage, leading to hemolysis of RBC [13].

There are several morphological alterations has been reported during RBC-SL. Geometrical changes include shape and a size abnormality apparent at micrometric scale [14]. However, cellular geometry changes are the result of metabolic processes modifications. Recent evidence revealed that these alterations impact their hemodynamics [15]. Also, a number of biophysics parameters related to RBC fragility has been reported, as increased agreeability and viscosity [16]. When the viscosity increases, the osmotic pressure existing in the cell membrane is higher. As a result, RBC loses their ability to regulate intra and extracellular water flow. Due to this, the increased concentration of intracellular water leads to membrane breakage, and this is known as hemolysis. Hemolysis can be associated with morphological deformities at microscale, which have been well studied [17]. In this work, nanoscale surface topography of RBC irradiated and normal during storage are investigated using atomic force microscopy. The RBC morphometric features reported in this paper are intended to exhibit the first changes due to RBC-SL which cannot be seen with micrometer scale. This is an analysis of

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the nano-biology of the cell membrane as a first signal of storage RBC quality.

II. PROCEDURE

A. Blood Storage

A blood unit equivalent to 450 ml of whole blood from a healthy volunteer was taken into bag containing 63 ml of Citrate Phospahte Destrose Adenine (CPDA) anticoagulant. A portion of 3 ml was taken for the control measurements and the rest was stored at 4°C during 21 days. Thus, 3 ml of the stored blood were collected and alicuoated for hematic biometry, flow cytometry and AFM measurements every day. In order to maintain normal storage conditions the unit was refrigerated with a regular mixed system. Irradiation was performed using a 220E-Gammacell model, equipped with ^{60}Co source. The dose of irradiation was of 25 Gy.

B. Hematic Biometry (HB)

1 ml of whole blood was place in a BD tube and 12 different blood constituents were determined with the Beckman Coulter equipment ACT-5-DIF. The measured parameters include hemoglobin (Hb), hematocrit, red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHM), platelets, white blood cells (WBC), monocytes, lymphocytes and granulocytes concentrations. After this, the blood was centrifuged at 1500 rpm for 10 min at room temperature and serum was separated, then hemolysis was analyzed in a UV-Spectrophotometer.

C. Atomic Force Microscopy

About 20 μl of sample was slipped into a glass slide and air-dried for 15 minutes and topographic images were performed. An XE-BIO AFM (Park System) was used in true contact mode to obtain topographic images. Aluminum coated nitride tips (TESPA, Bruker, USA) with a spring constant of 20–80 N/m, a resonant frequency between 382 405 kHz and a nominal tip radius of 8 nm was employed in all AFM measurements. 25 cells of each sample were scanned at the following fields: 0.01x0.01, 10x10 and 100x100 μm. The XE image processing program was used to generate both qualitative and quantitative information from individual RBC during their storage. Morphometric characteristics including measurements of cell counts, surface area, volume and size (length, width and height) as well as texture features as roughness and skewness were carried out. Standard deviation (SD) was applied in order to obtain the mean weights of the samples.

III. RESULTS AND DISCUSSION

To assess RBC stored quality after γ-irradiation, classical cell blood counts were studied. The parameters of interest were hemoglobin (Hb) as a fundamental factor in their oxygen transport role. Additionally, hematocrit (Hct) which represent the volume of whole blood that is comprise of RBC. Since previous studies recommended use irradiated blood within 48 hours after their treatment, in this study irradiated blood

samples will be stored and analyzed during three days.

Hct relative counts were found under their normal values for blood γ-irradiated and unirradiated control samples before 21 days. However, decreases in Hb concentration were observed for irradiated blood samples as can be depicted in Fig. 1 (a). These results are consistent with the literature reporting that blood for transfusions can be stored during a period from 21 and 35 for therapeutic use [18]. Nevertheless this information has been controversial, since clinical complications associated with old blood transfusions have been reported [19]. Furthermore, the percentage of RBC hemolyzed increased slowly by 1.2 after 16 days of storage. By 3 days of storage in γ-irradiated samples, RBC hemolysis number increased significantly and faster than unirradiated control (Fig. 2 (b)).
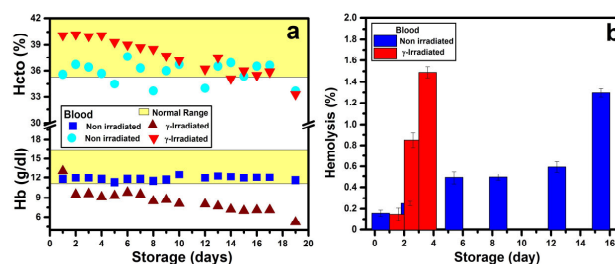

Fig. 1 Hb and hematocrit levels in non-irradiated samples (a) Percentage of RBC hemolyzed during blood storage increases faster in irradiated than non-irradiated samples (b)

Since Hb is a critical molecule for maintaining the shape of RBC, morphological characteristics were studied. Micro-scale topography was obtained using the AFM true-contact mode in scan sizes of 10x10 and 100x100 μm. After scanning, images were processed using the XEI software and 3D reconstructed.

After storage, RBC undergo morphologically deformed as achantocyes in two modalities: type 1 with small central pale, and type 2 with flat surface (Fig. 2 (a)). Alterations in the central depth of these cells, seems to be associated with the presence/absence of oxygens on their surface. Normally, oxygen molecules bind to the available heme group of hemoglobin, taking the form of oxygenated Hb (Oxy-Hb) as described above. The presence of oxygen on the surface gives to RBC the pale central appearance. Abnormally shaped RBC are observed in some pathologies [20], which trigger erythrocyte impairment and poor functioning. When irreversible changes occur in RBC morphology, they lose their ability to deform. Cell deformation capability can be evaluated through their roughness.
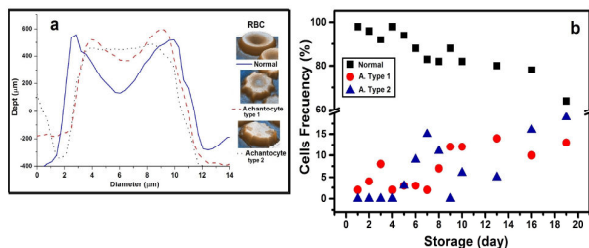
Fig. 2 Geometrical profile of RBC after storage (a) and frequency (b) Acanthocytes exhibit irregular spicules projected on the cell surface

There is a number of works about cell deformation response after external factors [21], and as a mean of cellular sorting [22] using the atomic force microscopy (AFM). These studies consider roughness as a pivotal factor in biomechanical processes as motility, deformation, adhesion, etc., thus as a mean of cellular quality. In this work, we have determined surface roughness of RBC using AFM nanoscale analysis.

RBC nano-topography of stored blood is compared in Fig. 3. In general, membrane roughness depletion was observed in both irradiated and non-irradiated samples after storage. In the case of unirradiated blood, roughness starts to decrease significantly after six days of storage (from 2.21±0.06 nm to 1.38±0.04 nm), whereas that irradiated samples show a drastic decay since the first 24 hours of storage (from 2.42±0.05 nm to 1.40±0.02 nm). RBC deformability is associated with the roughness of the membrane. When cell loses roughness becomes more rigid and therefore more susceptible to breakage and waste functions. So, we can summarize that irradiation enhanced aging of RBC. Also, small changes in the membrane topography of non-γ-treated RBC can be observed, but this can only be perceived through an analysis of the mechanical properties of the membrane since a nanometric approach.
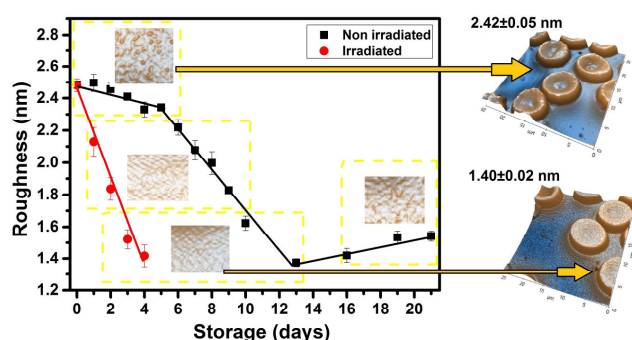

Fig. 3 Roughness as function of storage of RBC

Despite variations in the HB parameters, such as hematocrit, were observed, these were not founded outside the normal range, even in the irradiated samples. In the case of Hb, significant decreases were observed only for the irradiated samples related to storage. However, analyzing RBC surface at nanometric scale (through roughness) allow us to observe significant variations between fresh and old samples, showing

enhanced effect in irradiated samples. This suggests that stored RBCs undergo morphological changes associated with cell damage processes, which may be early-detectable if the nano-topography of the membrane is analyzed. However, more studies are required.

IV. CONCLUSION

Stored RBC exhibits morphometric alterations suggesting quality reduction. This effect seems to be enhanced in irradiated blood. However, these changes can only be observed in early stage by analyzing their structure at nanoscale. This is possible with the use of the Atomic Force Microscopy.

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