The Contribution of Storage Medium and Membranes in the Microwave Dielectric Response of Packed Red Blood Cells Suspension

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Abstract: During cold storage, packed red blood cells (PRBCs) undergo slow detrimental changes that are collectively termed storage lesion. The aging of the cells causes alterations in the composition of the storage-medium in the PRBC unit. In this paper, we present the comparison of the dielectric response of water in the primary (fresh) storage medium (citrate phosphate dextrose adenine solution, CPDA-1) versus the storage medium from three expired units of PRBCs. Dielectric response of the water molecules has been characterized by dielectric spectroscopy technique in the microwave frequency band (0.5–40 GHz). The dominant phenomenon is the significant increase of the dielectric strength and decrease the relaxation time τ for the samples of the stored medium in comparison with the fresh medium CPDA-1. Furthermore, we demonstrated that removing the ghosts from PRBC hemolysate did not cause the alteration of the dielectric spectrum of water. Thus, the contribution associated with water located near the cell membrane can be neglected in microwave dielectric measurements.

Keywords: microwave dielectric spectroscopy (MDS); CPDA-1; red blood cells (RBC); packed red blood cells (PRBC); ghosts; malondialdehyde (MDA) water main relaxation peak; cole-cole (CC) function; broadening parameter α; relaxation time τ; dielectric strength $\Delta\varepsilon$

1. Introduction

Red blood cell (RBC) transfusion is a life-saving procedure whose primary objective is to sustain tissue and organ oxygenation in patients with massive bleeding or acute anemia. Packed red blood cell (PRBC) donations for transfusion are routinely stored for up to 35 to 42 days, depending on the preservation solution [1].

During cold storage, PRBCs undergo slow detrimental changes that are collectively termed storage lesion [1-3]. Aging-related processes (that are at least partially due to the sensitivity of cells to oxidative stress [4]) lead to significant metabolic and structural changes in red blood cells [5-8], and involve global biochemical and biophysical alteration, remodeling of the cell membrane, and cytoplasm composition [3,9]. The most studied changes include: adenosine triphosphate (ATP) and 2,3-bisphosphoglycerate (2.3-DPG) depletion [9], loss of cellular antioxidant capability [10,11], changes in K+ and Na+ concentration [12,13], loss of membrane and skeleton proteins [14,15], loss of membrane lipids, and changes in their in/out distribution, vesicle generation [16,17], oxidation and remodeling of skeleton proteins [18],

clustering of band 3 proteins [19,20], alteration of nitric oxide signaling [21,22], decrease in antioxidant activity [23-25], etc.

A number of these changes are interrelated and initiate a cascade of biochemical and structural changes, which in turn lead to impairment in RBC functionality, specifically—alteration in the biophysical/mechanical properties of cells [5,7].

Each of the reported storage-induced alterations in PRBC properties occurs on its specific time-scale [26-34]. Some of these changes take place at an early stage of storage (during the first seven days), while others occur later.

Changes in the composition and property of the cell membrane begin at the very early stages of PRBCs storage [35-38]. Freitas Leal et al. summarized this cascade of changes as follows [36]: oxidative damage-induced, high-affinity binding of hemoglobin to the cytoplasmic domain of band 3, activation of Ca^{2+}-permeable channels, phosphorylation-controlled alterations in morphology and metabolism affecting ATP production and redox status, degradation of band 3 and/or aggregation of band-3 fragments, binding of immunoglobulin-G, and micro vesicles generation. Obviously, such changes in the composition of the cell membrane, its shape, and properties must inevitably lead to a change in its hydrophobicity [39].

Furthermore, bio-active substances accumulate in storage medium [40]. The accumulation of immunomodulatory factors in PRBC concentrates [41] has been implicated as a potential cause of transfusion reactions associated with the use of PRBCs [42,43]. These include lipids (that prime recipient neutrophils and have been implicated in transfusion-related acute lung injury [44]), cytokines [26,41], arachidonic acid [45], and malondialdehyde (MDA, a marker of lipid peroxidation) [2]. In addition, PRBC lesion causes the alteration of sodium/potassium balance [46], accumulation of lactate [41,46], lactate dehydrogenase [2], iron [26,46,47], and free hemoglobin [2,41,46] in the supernatant in parallel with decreasing of glucose concentration [46] and reducing of medium pH [26,40].

Our recent Microwave Dielectric Spectroscopy (MDS) studies have shown that it is possible to evaluate the dielectric response of cytoplasmic water by measuring the suspension of stored red blood cells (RBC) [48]. From the analysis of the dielectric relaxation as a function of storage time (ex vivo aging), it was assumed that the behavior was rooted in the delicate interplay between bound and bulk water in the cellular interior. Based on these results, we hypothesized that the state of intracellular water might be used as a marker of the functionality of stored cells. Furthermore, the RBC aging is associated with a specific pattern of changes in intracellular water. It was shown the broadening parameter α of the main dielectric peak of cytosolic water shows a linear correlation with the storage time and allows to predict the RBC deformability [48]. This observation was recently supported by Petrovic et al. [49], who monitored changes of intracellular water dynamics (during packed RBC (PRBC) cold storage) by H^1 NMR.

To calculate the dielectric spectrum of the cell interior, we used a Kraszewski mixed formula [48], where the spectrum of buffer was subtracted from the spectrum of RBC suspension. We assumed [48] that (1) storage medium does not change within the period of storage, and (2) the contribution associated with the water molecules located near the surface of the cell membrane can be neglected. However, the change in the composition (alteration of ions and dipoles concentration) of the medium described above should lead to a transformation in the water molecules state and will lead to variation in the number of bounded water molecules; these changes should come out in the alteration of dielectric water response. The present study was undertaken to examine this hypothesis.

Note that in our previous work [48], we did not take into account water in the vicinity of the membrane, which may affect the dielectric signal of the cell interior. Therefore, in this study, we also tested the role of water molecules located near the surface of the membrane on the dielectric response of water molecules in the cytoplasm of RBCs. All-in-all, in the presented study, we assessed the microwave response of water molecules, which are in the storage buffer and those located near to the cell membrane. Thus, for the first time, we evaluated the changes in the composition of the storage-medium caused by PRBCs aging using MDS. The results open up the avenues of non-invasive monitoring of RBC lesion during their storage in a blood bank.

2. Materials and Methods

2.1. Study Design

In the present study, we compared the dielectric response of the water in the primary (fresh) storage medium versus the storage medium from PRBC units and compared microwave dielectric response of water in hemolysate that contains cell membranes with dielectric behavior of water in membranes-free hemolysate. The dielectric response of the water molecules has been characterized by the MDS in the frequency range (0.5–40 GHz) at 25 °C.

2.2. Samples Preparation

The institutional review board approved the study for human experiments (Helsinki Committee Regulations Permit 98290, Hadassah Hospital, Jerusalem, Israel). To examine the alteration of citrate phosphate dextrose adenine solution (CPDA-1, Macopharma, Tourcoing, France) PRBC samples (7.0 mL) were taken from five outdated un-filtrated units (in CPDA-1) stored 35–37 days under the standard condition in the blood bank. Cells were mixed with an equal volume of fresh CPDA with the following separation by centrifugation under 500 g for 7 min. After centrifugation, the supernatant was separated and re-centrifuged to remove the cells completely. For examination, the dielectric response of water molecules in the vicinity of the membrane surface 20 mL of hemolysate was prepared by sequential freezing (at −78 °C) and thawing of the cells. After thawing 10 mL of hemolysate was centrifuged (14,000× *g*, 7 min.) to remove ghosts.

2.3. Microwave Dielectric Spectroscopy

Dielectric measurements were carried out in the frequency range from 500 MHz to 40 GHz using a microwave vector network analyzer N5234B PNA-L (Keysight, Santa Rosa, CA, USA), together with a flexible cable and slim-form probe (Keysight N1501A Dielectric Probe Kit). The calibration of the system was performed with the aid of three references: air, a Keysight standard short circuit, and pure water at 25 °C. Calibration was maintained by Ecal mode. A special stand for the slim-form probe was designed and combined with a sample cell holder for liquids (total volume 7.8 mL). The holder was enveloped by a thermal jacket and attached to a Julabo CF 41 (Julabo, Seelbach, Germany) oil-based heat circulatory system. The cell was held at 25 °C by the circulator-thermostat, with temperature fluctuations less than 0.1 °C. The whole measuring system was placed in an air-conditioned room maintained at 25 ± 1 °C. Each sample was measured at least six times. The real and imaginary parts $\varepsilon'(\omega)$ and $\varepsilon''(\omega)$ were evaluated using the Keysight N1500A Materials Measurement Software with an accuracy of $\Delta\varepsilon'/\varepsilon' = 0.05$, $\Delta\varepsilon''/\varepsilon'' = 0.05$.

2.4. Statistical Analysis

The results are presented as mean ± SEM and tested for statistical significance using the nonparametric Mann–Whitney test. Statistical differences, examined with the SPSS 21 software package, were considered significant at $p < 0.05$.

3. Results and Discussion

The measurements of storage solutions were carried out at 25 °C. The dielectric spectrum of the storage-medium in comparison with fresh CPDA in the frequency band 0.5–40 GHz is presented in Figure 1a.
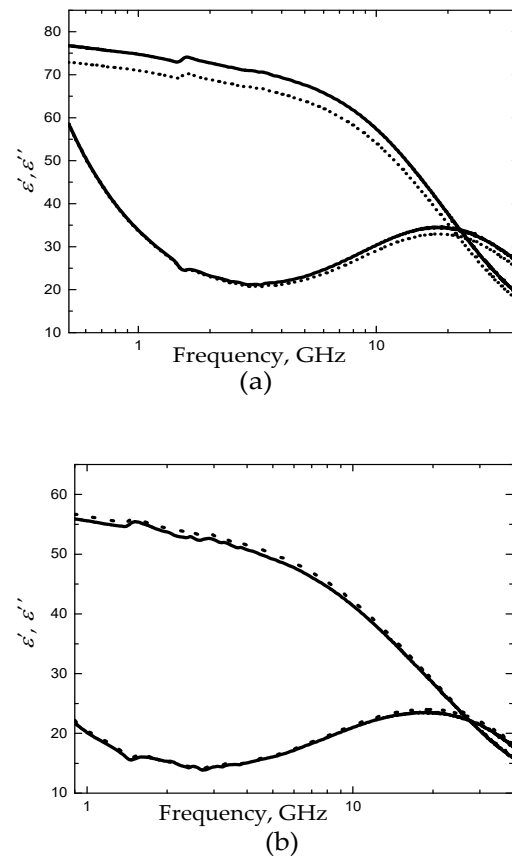
(a)

(b)

Figure 1. (**a**) Dielectric spectra of fresh citrate phosphate dextrose adenine solution (CPDA-1) (dot line) and the storage-medium for sample C (black line). (**b**) Dielectric spectra of membrane-containing (H) (dot line) and membrane-free (MF-H) (black line) hemolysates for sample C.

Figure 1a shows the typical dielectric spectra of the storage medium (dielectric spectrum of sample C is plotted) versus fresh CPDA. The relaxation time τ, the broadening parameter α, and the relaxation amplitude $\Delta\varepsilon$ of water for fresh/pure and stored medium are demonstrated in Table 1 and Figure 2.

In Figure 2, dielectric features of water molecules for all tested fluids are presented. It is shown that dielectric parameters of fresh/pure CPDA-1 and storage-medium that separated from PRBCs units are different. In Table 1, we summarized the obtained averaged dielectric parameters. For all samples of the stored-medium, the dominant feature is the significant increase of the dielectric strength $\Delta\varepsilon$ and decrease the relaxation time τ in comparison with the fresh medium CPDA-1.
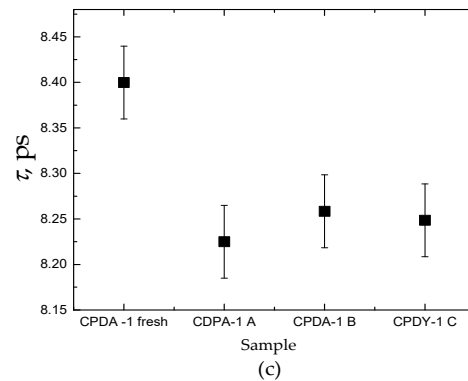

(a)

(b)

(c)
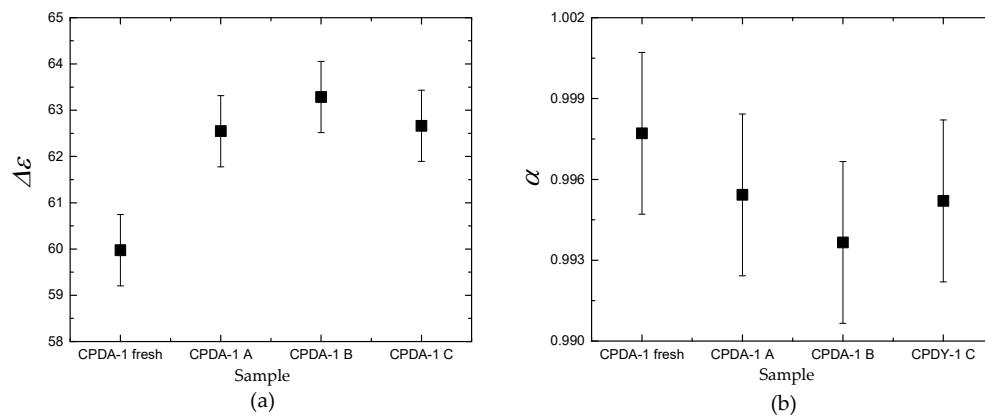
Figure 2. Comparison of dielectric parameters $\Delta\varepsilon$ (**a**), α (**b**), τ (**c**) for fresh CPDA-1 with storage medium for three samples A, B, and C. Each datum is mean ± SEM from six repeated measurements.

Table 1. The mean values of relaxation times τ, the broadening parameter α, and the relaxation amplitude $\Delta\varepsilon$ of water for fresh CPDA-1 and stored-medium. Each datum is the mean ± SEM from six repeated measurements.

Medium	Relaxation Time, τ, picoseconds (mean ± SEM)	Broadening Parameter, α (mean ± SEM)	Relaxation Amplitude, $\Delta\varepsilon$ (mean ± SEM)
CPDA-1 (from stored units)	8.24 ± 0.04	0.975 ± 0.003	62.83 ± 0.8
CPDA-1 (fresh)	8.40 ± 0.04	0.998 ± 0.003	59.97 ± 0.77

Changes in dielectric response of water, observed in this study, can be assigned to alteration of ions and dipoles concentration that occur during the storage in the medium in the PRBC unit. We can assume that alteration of Na+/K+ balance, decreasing of pH, and formation of lactate ion in the medium (during the storage) can cause disarrays of the H-bond network of water. In this case, the water's main relaxation peak shifts to lower relaxation times [50]. In parallel, the accumulation of cytokines, arachidonic acid, and free hemoglobin can induce ordering the H-bond network of water. It shifts the water main relaxation peak to the lower frequencies or higher relaxation times [50]. In this regard, special attention must be paid to the possible effect of the accumulation of MDA in the storage-medium. This is since the molecule of MDA molecule is small, polar, and highly water-soluble [50], and its accumulation in solution [2] should decrease water relaxation time as it was obtained in the experiment.

In this table, we show the average values of the primary dielectric parameters (that obtained for three samples of PRBC) for both types of hemolysates. Each value is presented as a median from six repeated measurements with standard error values. We have not observed a significant difference (pair test) between dielectric features that characterized water behavior in membrane-containing and membrane-free hemolysates.

As can be seen from Table 2, the presence of membranes does not affect the dielectric response of water in hemolysates. Thus, we can conclude that changes in the intracellular ionic composition can be attributed to the highest factors determining the dielectric response of water molecules in the cytoplasm of RBC.

Table 2. Comparison of the relaxation time τ, the broadening parameter α, and the relaxation amplitude $\Delta\varepsilon$ of water for membrane-containing (H) and membrane-free (MF-H) hemolysates. Each datum is the mean ± SEM from six repeated measurements.

Samples	Membrane-Containing Hemolysate (H)	Membrane-Free Hemolysate (MF-H)
	Relaxation Time, τ, ps (Mean ± SEM)	
A	8.12 ± 0.04	8.09 ± 0.04
B	8.18 ± 0.04	8.23± 0.04

C	8.12 ± 0.04	8.11± 0.04
Broadening Parameter, α (Mean ± SEM)		
A	0.952 ± 0.003	0.954 ± 0.003
B	0.941 ± 0.003	0.939 ± 0.003
C	0.951 ± 0.003	0.949 ± 0.003
Relaxation Amplitude, $\Delta\varepsilon$ (Mean ± SEM)		
A	43.15 ± 0.8	42.43 ± 0.8
B	44.14 ± 0.8	45.08 ± 0.8
C	46.94 ± 0.8	47.60 ± 0.8

The phenomenon we discovered can be explained in the following way. Water present in biological tissues has been defined as bound or free water depending upon its proximity to neighboring macromolecules, membranes, or other interfaces. In a classical paper [51] entitled, "What retains water in living cells?" Ling & Walton concluded that cell proteins located on the surface of a biological membrane polarize most of the cell water in the form of multilayer layers. Water forming the hydration shell of the macromolecule is considered as bounded. The remaining water in the system is defined as bulk. As we can expect that the number of water layers in which the mobility of the molecules is limited (differs from) does not exceed 4–5. This should mean that the volume of such water, in comparison with the intracellular water volume, is insignificant. Therefore, the presence of a membrane in the hemolysate should not lead to a change in its dielectric spectrum.

Limitation of the study: It is well known that the PRBCs lesion is affected by the protocol of PRBCs preparation and storage [1,5,26]. The results presented here, were obtained for units of PRBCs that are non-leukoreduced and cells are stored in CPDA-1. In the future, we plan to examine PRBCs units that are produced with leukoreduction and gamma-irradiation, in which cells are storage in SAGM (Saline, Adenine, Glucose, Mannitol).

Thus, from the results demonstrated in this study, and based on our previously published data [48], it follows that:

1. The main factors determining the microwave dielectric response of the PRBC suspension is the condition of water molecules in the cytosol and storage medium;
2. The contribution associated with the water molecules located near the surface of the RBC membrane can be neglected in microwave dielectric measurements.

Thus, having summed up our results, we conclude that using MDS, it is possible to control the functionality of PRBCs by the response of water molecules located both inside and outside the cell.

4. Conclusion

We showed that MDS is sensitive to the alterations in the composition of the storage medium of the suspension of PRBC. Since CPDA-1 undergoes significant changes during the storage period, senescent buffer (buffer of the same age of PRBC suspension) has to be subtracted from the spectrum of PRBC suspension in Kraszewski's formula [48]. Thereby it seems interesting to repeat the experiment carried out by Levy et al. [48] using the current knowledge of the storage medium. The results allow us to conclude that the contribution associated with the water molecules located near the surface of the cell membrane can be neglected in microwave dielectric measurements.

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