# INFLUENCE OF PALLADIUM α-LIPOIC ACID COMPLEX ON THE MECHANICAL PROPERTIES OF BLOOD EXPOSED TO GAMMA RADIATION

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*Abstract*. This work studies the effect of palladium α-lipoic acid complex on the mechanical properties of blood and its effect as radioprotector *in vitro*. Whole blood was exposed to 100 Gy. The results showed that exposure to gamma radiation increased membrane rigidity and decreased membrane permeability. The interaction of palladium α-lipoic acid complex with red blood cells membrane produced significant changes in their biophysical properties and increased membrane permeability. Its addition before irradiation was shown to provide significant protection to the red blood cells.

*Key words*: palladium α-lipoic acid complex, rheological properties, red blood cells, gamma radiation, radioprotector.

## INTRODUCTION

Red blood cells (RBCs) are more than an oxygen transporter, they can be considered as an oxygen sensor and may augment blood flow by liberation of ATP and $O_2$ delivery wherever and whenever the need might arise [46]. Mechanical properties are part of their main properties, since they are regularly subjected to a complex of mechanical forces in a form of varying stresses, strains, pressures, and flows. These mechanical properties play a key role in the ability of RBCs to withstand mechanical loading while performing their physiological functions [58]. The mechanical behavior of the RBC cannot be characterized simply in terms of fixed "properties", as its structure is a dynamic system that adapts to its local mechano-chemical environment [59]. At constant hematocrit and temperature, low shear blood viscosity is primarily determined by RBC aggregation, while high shear viscosity is dependent on their deformability [39]. The aggregation is

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dependent on the magnitude of shearing forces acting on the cells, and it is determined by both the properties of plasma, fibrinogen concentration, hematocrit and RBCs characteristics such as surface electric charges and shape [8]. Deformability depends on fluid-electrolyte balance and hence on its volume and cytoplasmic viscosity, the mechanical and electrical properties of their membranes [29]. It was shown to be one of the important parameters related to their viability [49], as it is also the most important factor affecting the flow of blood [8]. Maintenance of normal RBCs deformability depends on the availability of metabolic energy in the form of ATP ($Na^+$-$K^+$ATPase and $Ca^{2+}$ATPase) that serve to regulate intracellular cation and water content thereby maintaining cell volume and thus the cell's surface to volume ratio [44].

Cell membranes are particularly sensitive to the effects of radiation-induced oxidative damage and lipid peroxidation which can alter membrane structure and function [54]. In addition, numerous experiments have suggested that radiation may modify the transport mechanisms and affect membrane-bound proteins [57]. Importantly, several membrane biophysical parameters may also be altered by radiation induced oxidative stress (e.g., shape, permeability, and osmotic fragility) [28]. Hence, it is important to test the influence of radioprotectors on the mechanical properties of RBCs. This work is a part of study conducted for testing the effect of palladium α-lipoic acid complex (PLAC) on the RBCs *in vitro* and to evaluate its radioprotective effects. PLAC was formulated to act as a non-toxic chemotherapeutic agent. The active ingredient in this complex is the palladium-lipoic acid polymer, which allows palladium-lipoic acid to be water and lipid soluble and exists as liquid crystal. It acts as a liquid electrical transistor that transfers electrical current from the cell membrane to the mitochondria which redistributes the electrical current throughout the cell via the existing electrical pathways. Hence, it inhibits anaerobic cells without damaging healthy ones. It can influence the electrochemical process occurring in the body, consequently "electrocuting" cancer cells (being anaerobic cells) while leaving healthy cells alive [21]. However, RBCs, the main organelles that supply all the body's tissues and cells with oxygen, do not have mitochondria and depend on anaerobic respiration, instead of aerobic respiration. Only glycolysis supplies energy to this cell. In the previous study, the effects of PLAC on the electrical properties of RBCs were concerned, and it was shown that the PLAC increased the RBCs' AC conductivity and dielectric permittivity, and increased cellular energy production in the form of ATP. It also showed that it provided significant protection against the damaging effects of gamma radiation [56]. In this work the effects of PLAC on the mechanical properties of RBCs were studied, as well as its effect as radioprotector.

## MATERIALS AND METHODS

### PREPARATION OF RED BLOOD CELLS SAMPLES

Blood samples were obtained from adult male Swiss Albino rats, weighing 120–150 g, after dissection using heparnized needles to prevent coagulation. To retain cell viability 5% glucose isotonic solution (commercially available, pyrogen free, prepared for intravenous infusion) was added to blood samples to reach concentration of 20 mM. Each sample was divided into four groups to provide self control comparison: control, control with PLAC (positive control), irradiated and irradiated with PLAC. Each group consisted of 6 samples of volume 8 mL. They were incubated in water bath at 37 $^{o}$C for 30 min before irradiation. Whole blood samples were irradiated in glass bottles tightly closed at room temperature. The short duration of irradiation (about 2 minutes) avoids heating or oxidation of the samples during irradiation. After irradiation, the samples were centrifuged at 3 000 rpm for 10 minutes to remove plasma and buffy coats. RBCs were washed by phosphate-buffer saline (PBS, pH 7.4) and centrifuged at 3 000 rpm for another 10 minutes at 4°C, and then the supernatant was removed to obtain the packed cells ready for the experimental measurements.

### RADIOPROTECTOR

Palladium lipoic acid complex (PLAC), a liquid supplement of concentration 11.65 mg/mL, was obtained as a gift from Garnett Mckeen laboratory, Inc., USA. It was added to the whole blood in concentration of 2%v/v without further purification as described by Menon and Nair [42].

### GAMMA IRRADIATION

The irradiation process was carried out in the National Center for Radiation Research and Technology (NCCRT), Atomic Energy Authority, Cairo, Egypt. The irradiation dose was 100 Gy from Cobalt-60 source **(**dose rate was 3.089 kGy/h) at the beginning of the experimental work. The calibrations of the sources and doses calculation were performed by the Egyptian high-dose reference laboratory. The chosen dose in this study (100 Gy) was based on its significant effects on the measured factors as reported in previous studies [17, 55].

## RHEOLOGICAL PROPERTIES

The rheological properties were measured by means of Brookfield DV-III Programmable Rheometer. It is a cone-plate viscometer that measures fluid parameters of shear stress and viscosity at given shear rates. The applied shear rate values were 7.5 to 375 $s^{-1}$. The body temperature varies from 29°C at the surface to 37°C in the blood stream. RBCs properties are well known to be temperature-dependent, so that statistically significant differences were present in a wider range of temperatures between 25–37°C [7]. In this study, the temperature was set at 35°C. The data was collected from the rheometer by means of software program "Rheocalc for Windows".

## THERMAL ANALYSIS

Variation of viscosity with shear rates is a thermally activated process. To move, the molecules have to overcome activation energy $E_a(T)$ barrier created by the resistance of the surrounding building units [4]. In this study, the viscosity of blood was measured over an extended temperature range from 25 to 45°C at shear rate 37.5 $s^{-1}$.

## OSMOTIC FRAGILITY MEASUREMENTS

Whole blood samples were added to hypotonic saline buffered to pH 7.4, with different concentrations, in the proportion of 1 to 100. The samples were incubated for 30 minutes at 37°C, and centrifuged at 3000 rpm for 5 min, to precipitate the non hemolyzed red blood cells. The absorbance of the solubilized hemoglobin, resulting from cell rupture, was read spectrophotometrically at 540 nm to characterize the degree of hemolysis [14].

## LIGHT SCATTERING

Light scattering gives information about size and shape of the cell suspension. When monochromatic light passes through cell suspension, a part of the incident light is scattered. It can be defined in terms of the turbidity, which is the absorption coefficient due to scattering after subtracting the scattering due to solvent alone. Changes of the cell suspension turbidity (indicating swelling or shrinking) were registered by measuring the transmittance at 610 nm [53] using a UV-visible spectrophotometer CECIL-3041. The samples were prepared as 3% RBCs suspension.

STATISTICAL ANALYSIS

Results were expressed as mean ± standard deviation. Student t-test was applied to evaluate the significant difference between treated and control groups. Values of $p < 0.05$ were considered as significant value.

## RESULTS

Blood is a non-Newtonian shear thinning fluid, i.e. the apparent viscosity decreases as the applied shear rate increases. The change in viscosity with shear rate gives the flow curve (Fig. 1), which is characterized by two regions: in the low shear rate up to 100 $s^{-1}$, and high shear region from 100 $s^{-1}$ up to the shear rate at which no change in viscosity is obtained.

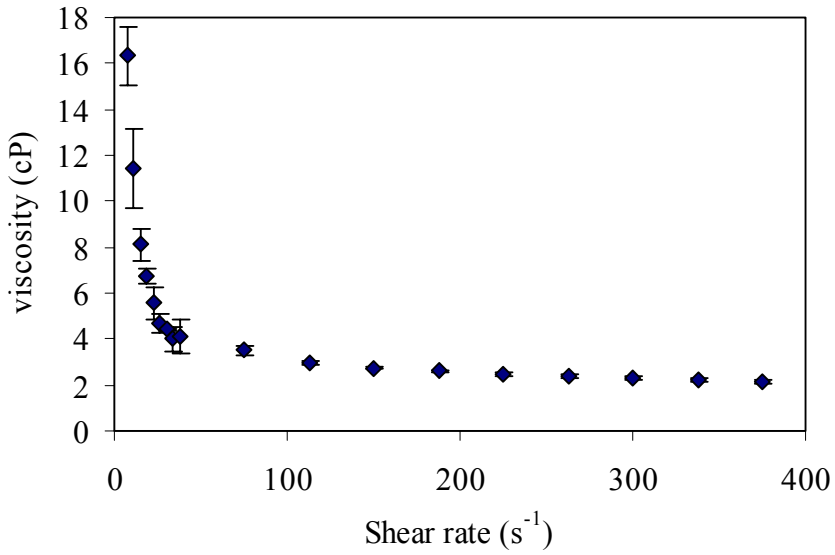


Fig. 1. The flow curve for control blood.

For analysis of the flow curve, the Bingham plastic model was applied to calculate the yield stress ($F_0$ ) and viscosity (η) as follows:

$$F = F_0 + \eta D \tag{1}$$

where $F$ is the shear stress (dyne/cm$^2$) and $D$ is the shear rate ($s^{-1}$) (Fig. 2).

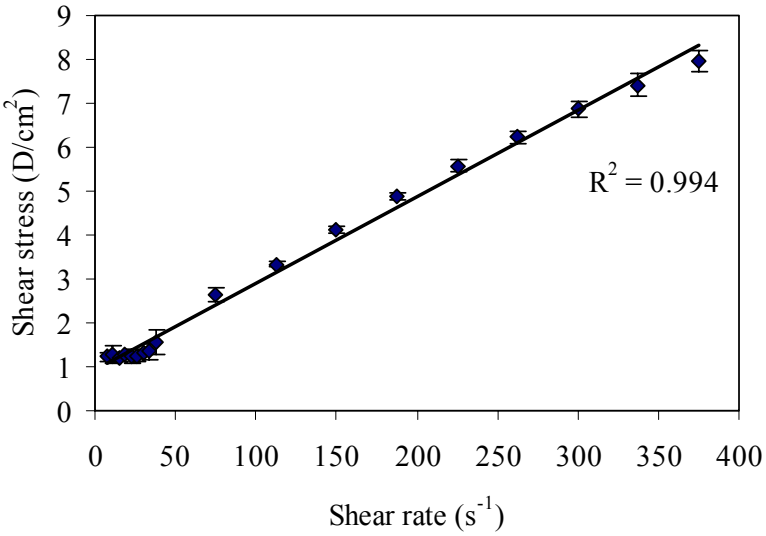


Fig. 2. Bingham fit for control blood.

The yield stress, the minimum stress the applied force has to overcome to start the flow, is the rheological parameter relevant to cell aggregation, as it represents a resistive force that the fluid can maintain under static conditions [9]. Addition of PLAC to blood did not show significant changes in the viscosity and yield stress compared to control. Exposure to 100 Gy gamma radiation resulted in significant increase in blood viscosity and yield stress. Incubation of blood with PLAC prior to gamma irradiation resulted in decrease in viscosity and yield stress from the irradiated group; however, it remained significantly higher than control (Table 1).

*Table 1*

Statistical analysis of the Bingham viscosity, yield stress and flow activation energy for control, treated, irradiated and treated irradiated groups

| **Groups** | **Statistics** | **Control** | **PLAC** | **100 Gy** | **100 Gy + PLAC** |
|---|---|---|---|---|---|
| Bingham viscosity (cP) | mean | 2.047 | 2.311 | 3.035* | 2.858* |
| | S. D. | 0.111 | 0.322 | 0.317 | 0.420 |
| yield stress ($D/cm^2$) | mean | 0.913 | 1.077 | 1.709* | 1.404* |
| | S. D. | 0.941 | 1.257 | 1.732 | 1.438 |
| $E_a$ (kJ/mol K) | mean | 16.55 | 22.58* | 19.64* | 15.99 |
| | S. D. | 1.08 | 2.80 | 2.00 | 1.42 |

S.D.: standard deviation;
*: statistically significant.

The viscosity decreases with increasing temperature; the relationship can be expressed in the form of an Arrhenius-type equation:

$$\eta = A\ e^{(Ea/RT)} \tag{2}$$

where η is the viscosity (cP), $A$ is the pre-exponential factor (cP), R is the gas constant (J/mol/K) and $T$ is the absolute temperature (K) [22]. The plot of experimental data in Arrhenius coordinates; log η against $1/T$ is shown in Fig. 3, the activation energies calculated for all groups are shown in Table 1. Low shear rate exists at the venous side of the circulation and in the center of the flow in arteries [16]. The important aspect of whole blood viscosity at low shear is that it reflects inflammatory activity [63, 64] and it is strongly correlated with the aggregation tendency of red blood cells [1, 62]. The activation energy of positive control and irradiated groups showed significant increase from control value, while the addition of PLAC before irradiation did not change the activation energy compared to control.

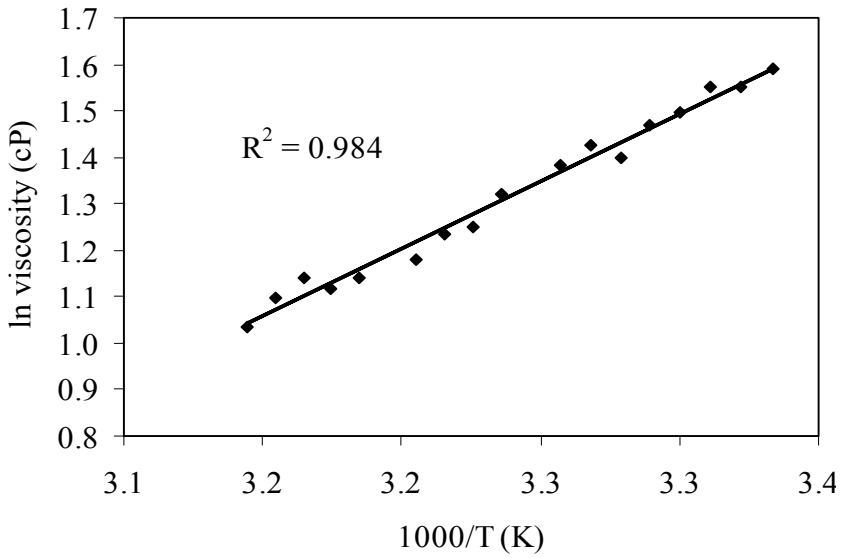


Fig. 3. Arrhenius plot for the viscosity of normal blood.
It represents the variation of ln viscosity *versus* $1/T$.

Changes in the ion transport and in the osmotic fragility are considered as an indicator for alterations in the RBCs membrane [5, 32]. The fragility curve can be evaluated by the average osmotic fragility ($H_{50}$): the NaCl concentration ($C$) producing 50% hemolysis. The experimental curves were normalized to 100% hemolysis to facilitate the comparison between different samples without the

interference of the hematocrit changes (Fig. 4). The differentiation of the fragility curve, which represents a Gaussian curve (the rate of hemolysis d*H*/d*C* as a function of *C*), is shown in Fig. 5. The width at half maximum of these curves reflects the dispersion of hemolysis process (low dispersion than normal indicates sudden rupture of the RBCs, while higher values of dispersion reflect the abnormal increase in the membrane elasticity).

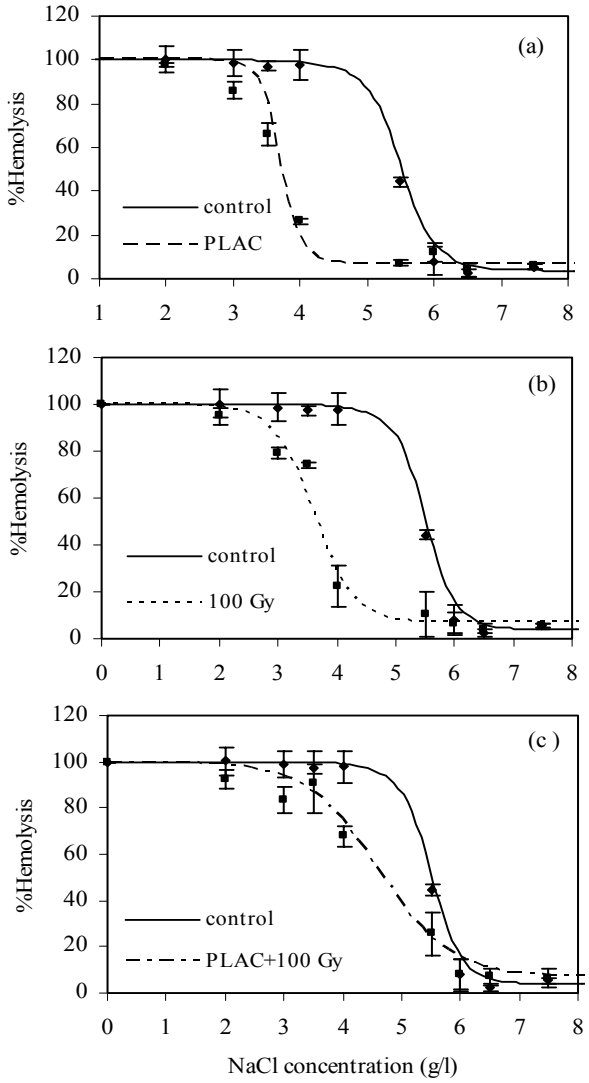


Fig. 4. Osmotic fragility curves for control (♦ and solid line) and treated with PLAC (a), irradiated with 100 Gy (b) and treated with PLAC before exposure to 100 Gy (c) groups (■ and dashed line). The dot with error bar is the experimental data and the solid and dotted lines are the fitted data.

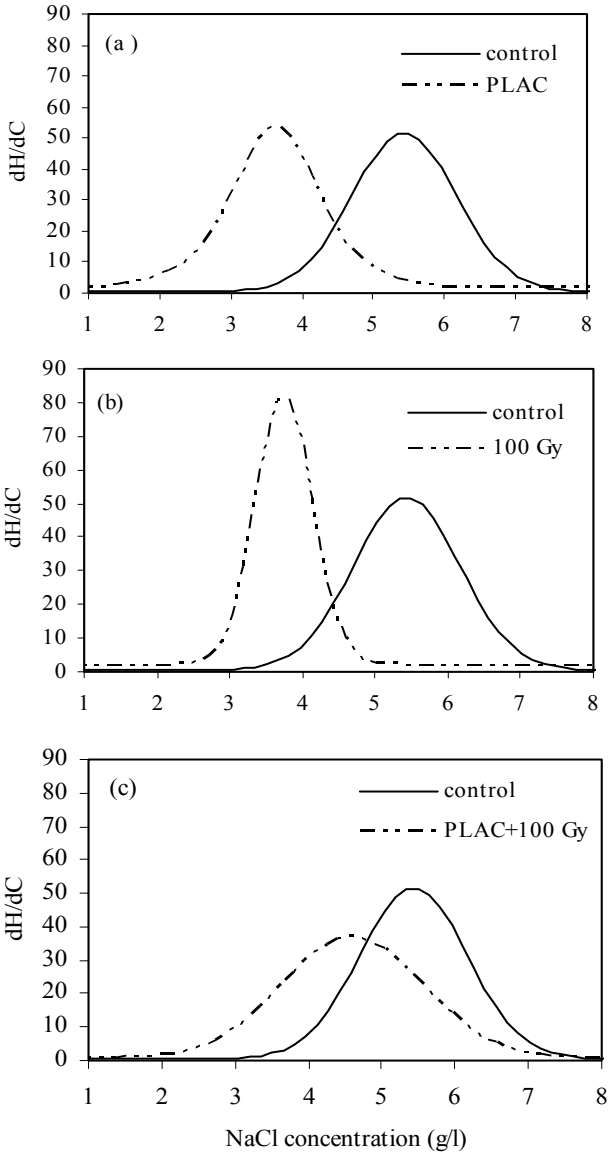


Fig. 5. Differential curves of hemolysis for control (solid line) and treated with PLAC (a), irradiated with 100 Gy (b) and treated with PLAC before exposure to 100 Gy (c) groups (dashed line).

The addition of PLAC to blood decreased the average osmotic fragility and dispersion hemolysis. The exposure to 100 Gy gamma radiation resulted in significant decrease in the average osmotic fragility but increased significantly the dispersion of hemolysis. The addition of PLAC prior to irradiation

increased the average osmotic fragility compared to irradiated group but remains significantly below the control value. The dispersion of hemolysis showed remarkable increase compared to control (Table 2).

An indirect measure of cell diameter can be performed by measuring the turbidity of cell suspensions using spectrophotometer at a wavelength that is not absorbed by hemoglobin but, rather, by the intact cells whose membranes reflect light. This particular procedure is an example of a light-scattering technique. The turbidity ($T$) is given by:

$$I = I_o \, e^{-Tl} \tag{3}$$

where $I_0$ and $I$ are the incident and transmitted light, respectively, and $l$ is the length of the light path through the scattering solution [15]. As the cells shrink, they reflect greater amounts of light; therefore, the per cent transmittance decreases as the turbidity of the solution increases. On the other hand, as the cells swell to diameters greater than normal the turbidity of the cell suspension decreases. The $\%T$ increases because the cell membranes reflect less light [40]. The exposure to 100 Gy increased the turbidity of RBCs suspension significantly, while the addition of PLAC acid did not induce significant change in the turbidity of both positive control and irradiated groups (Table 2).

*Table 2*

Statistical analysis of the average membrane hemolysis, dispersion of hemolysis and %turbidity for control, treated, irradiated and treated irradiated groups

| Groups | Statistics | Control | PLAC | 100 Gy | 100 Gy +PLAC |
|---|---|---|---|---|---|
| Average membrane hemolysis (g/L) | mean | 5.433 | 3.513* | 3.688* | 4.600* |
| | S.D. | ± 0.066 | ± 0.177 | ± 0.0448 | ± 0.005 |
| Dispersion of hemolysis (g/L) | mean | 0.783 | 0.508* | 0.954* | 1.868* |
| | S.D. | ± 0.044 | ± 0.099 | ± 0.124 | ± 0.115 |
| % Turbidity | mean | 4.001 | 4.049 | 4.312 | 4.063 |
| | S.D. | ± 0.394 | ± 0.291 | ± 0.193 | ± 0.215 |

S.D.: standard deviation;
*: statistically significant.

## DISCUSSION

Palladium lipoic acid complex presents a three-dimensional structure, with palladium in the centre of the complex, covalently coordinated with both oxygen of the lipoic acid carbonyl and the two sulfurs of the thiolane ring and

the carboxyl of the pentanoic chain in a 1:1 ratio (Fig. 6) [13, 18, 20]. Different preparations give different morphologies of this compound ranging from 60 to 100 nm in size [30]. The interaction of polymer nanoparticles with biological membranes is a complex process due to the heterogeneity of both the nanoparticles and the cell membranes [33].

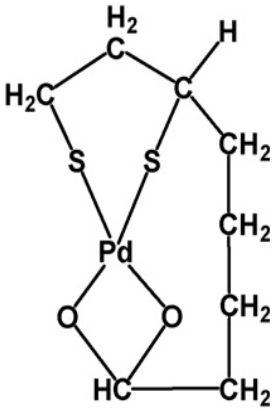


Fig. 6. Proposed structure of palladium covalently coordinated with both oxygen of the lipoic acid carbonyl and the two sulfurs of the thiolane ring and the carboxyl of the pentanoic chain [13].

Recent studies indicated that nanoparticles can strongly interact with cell membranes, either by adsorbing onto the membrane or compromising its integrity [10, 26, 35–38]. Li *et al*. [34] reported that a hydrophobic nanoparticle can result in the inclusion into the bilayer, whereas a semi-hydrophilic nanoparticle is only found to adsorb into the membrane. Also, the deformation of the lipid bilayer induced by the addition of nanoparticles is short-range, and the rearrangement of lipid molecules plays a significant role for morphological variations of nanopaticles-containing lipid membrane. Being soluble in water and lipid, PLAC can be found in both cell membrane and cytosol. Adhesive interactions due to electrostatic forces, van der Waals, or steric interactions are suspected to be involved in these processes [24, 50, 52]. The entering mechanism of nanoparticles into RBCs is different from phagocytosis and endocytosis, since they have neither phagocytic receptors on their surface nor the structures necessary for phagocytosis [51]. It may occur by unspecific means, including diffusion, trans-membrane channels, and electrostatic, van der Waals, hydration forces, or adhesive interactions [51].

It was found that engineered nanoparticles are capable of inducing cellular perturbations by interacting directly with biological membranes [33]. It may cause wide range of structural changes that could lead to enhanced permeability ranging

from the formation of an actual hole in the membrane to more subtle changes in content of the membrane leading to enhanced diffusion [3]. The interaction of PLAC with RBCs membrane produced significant changes in their biophysical properties. It was shown to increase the number of dipoles in the cell membrane and increase membrane permeability [56]. It was suggested that addition of PLAC may result in ATPase activation, and increase ATP production in RBCs.

The average membrane hemolysis showed a significant decrease by 35% from control value, the dispersion of hemolysis also decreased by the same ratio. It has been postulated that when the zwitterionic head group, of the membrane lipid bilayer, is approached by charged nanoparticles, the head group can reorient itself such that either the positively charged choline group or the negatively charged phosphatidic acid group terminates the surface, depending on whether the approaching particle is negatively or positively charged, respectively [60]. This reorientation is accompanied by a change in the effective "size" of the head group and thus produces an overall change in lipid packing. Positive nanoparticles bend the head group over, increase the area occupied per head group, and expand the lipid bilayer [23]. The increase in the surface area to volume ratio also gives the cell decreased osmotic fragility [11], as it allows it to take up more water for a given amount of osmotic stress. This rearrangement in the membrane bilayer, and the increase of total membrane mass reported in the previous study as a result of interaction of PLAC with cell membrane, may explain the significant increase in the calculated flow activation energy. However, this interaction did not affect the viscosity and yield stress which could be attributed to the increase in the membrane permittivity reported in the previous study [56], since the increase in the membrane surface charge increases the repulsive force between neighbored cells, facilitates their flow. The non-significant change in the turbidity of the RBCs suspension suggests that the change in the internal ordering of the cell membrane did not result in significant morphological change.

Cell membranes are particularly sensitive to the effects of radiation-induced oxidative damage and that of lipid peroxidation which can alter membrane structure and function [54]. In addition, numerous experiments have suggested that radiation may modify the transport mechanisms, react with membrane-bound proteins [57]. Importantly, several membrane biophysical parameters may also be altered by radiation induced oxidative stress (e.g., shape, permeability, and osmotic fragility) [28]. In this study, exposure of blood to 100 Gy resulted in increase of its viscosity and yield stress. Alterations of blood flow behavior may take place as a result of free radicals generated during exposure to ionizing radiation. Oxygen free radicals and their derivatives are known to damage RBCs resulting in important functional alterations, and both membrane and cytoplasmic structures are affected

by such oxidant attack. It has also been reported that generation of oxygen free radicals may result in cross-linking within membrane proteins and/or between membrane proteins and hemoglobin. Protein degradation and increased membrane rigidity were found to increase in RBCs exposed to oxygen free radicals [6]. Blood viscosity is increased with a decrease in red blood cell deformability [12]. The increase in viscosity and yield stress may be due to radiation induced rigidity in the RBCs membrane. Ionizing radiation is well known to generate oxygen radicals within biological systems which become higher in well oxygenated tissues than in tissues deficient in oxygen. From oxidative damages is Heinz body formation which can attach to the inner surface of the cell membrane and increase its rigidity [48]. The induced rigidity in the cell membrane as a result of exposure to radiation is supported by the observed increase in its activation energy. The decrease in the membrane surface charge of RBCs leads to the decrease in the deformation and orientation indices as well as the increase in blood viscosity [61] and may produce alterations in blood flow properties, deformability, aggregation and adhesion of circulating cell at vessel walls. In the previous study [56], it was reported that exposure of blood to 100 Gy gamma radiation decreased the relative permittivity and dielectric loss, which could be one of the causes of the increase in viscosity and yield stress. Significant decrease was also observed in the AC conductivity at 5 MHz, reflecting decrease in membrane permeability [56]. This decrease in permeability increases the membrane tolerance to osmotic pressure, as appeared in the decrease in the average osmotic fragility observed in this study. It also decreased membrane deformability which resulted in increasing the viscosity and yield stress. The decrease in the dispersion of hemolysis ($S$) was explained by the presence of unusually flattened red cells in which the surface area to volume ratio is increased [31]. Also, the increase in dispersion could be attributed to protein cross linking and shrinkage of cell membrane, which is confirmed by the significant increase in the turbidity of irradiated group.

While radiation protection was not the original therapeutic design for PLAC, it exhibited high efficiency as a radioprotector. It was shown to reduce the γ-radiation-induced mortality in mice and aided recovery from the radiation-induced loss of body weight after 8 Gy exposure. It also reduced the radiation-induced DNA damage when it was administered to animals exposed to a gamma radiation [47]. In another *in vivo* study, the administration of PLAC for seven days prior to whole body gamma radiation significantly reduced the damage to cellular DNA in bone marrow and blood leukocytes, as well as preventing the radiation-induced lowering of tissue antioxidant levels [41]. *In vitro*, addition of PLAC to the blood before exposure to 100 Gy gamma radiation provided a significant radioprotection of the dielectric properties and permeability of the cell membrane These effects can

be attributed to its ability to neutralize the free radicals formed by the radiation and the enhancement of the cellular energy production in the form of ATP [56]. PLAC possesses a powerful antioxidant effect, which can be partly attributed to its lipoic acid fraction. Since this naturally occurring acid is soluble in both lipid and water, PLAC are able to pass across cell membranes and work intracellularly [43]. The activation energy and percentage turbidity of the treated group before irradiation showed normal values. In the previous study, the relative permittivity and the total dipole moments of the membrane were normal [56]. These results may support the idea of radioprotector action of PLAC, and the increase in Bingham viscosity, yield stress, average membrane hemolysis and the dispersion of hemolysis could be attributed to the induced change in the cell membrane due to interaction of PLAC as previously discussed. This point needs more investigations with different concentrations of PLAC and other doses of gamma radiation.

Investigations of the effect of a certain radioprotector on living organisms, especially humans, have certain limitations. The *in vitro* studies can provide a basis for the evaluation of radioprotective activity. The simplest tests could be evaluation of lipid peroxidation, assays of free radicals and antioxidants status of the pharmacological product. If the product is found to inhibit lipid peroxidation and scavenge free radicals, it may act as radioprotector [27]. The oxygen radical absorbance capacity (ORAC) analysis of PLAC demonstrates that it is approximately a five times more potent antioxidant than α-lipoic acid [2]. The next step is to evaluate its radioprotective potential *in vitro* on cell line or suspension such as blood. If it shows a significant effect in reducing the radiation-induced damage, it could be considered as radioprotector. The *in vitro* tests on red blood cells carried out in this study and the previous one [56] showed that PLAC provide significant degree of protection against the radiation damage. In addition to its unique arrangement as a liquid crystal structure which allows it to store a great deal of energy and thus serve as a semiconductor [19], it can present a new category of radioprotectors.

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