BENZOATE GROUP ATTACHMENT TO TEMPO PROVIDES ENHANCED DISCRIMINATION OF LIPOSOMES FABRICATED USING HUMAN LUNG NORMAL AND CARCINOMA CELLS

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Abstract. It is widely accepted that the lipid compositions of the plasma membranes of healthy and cancer cells significantly differ from each other. During the cancer progression, cancer cells change the lipid constituent of the membranes resulting in the loss of lipid asymmetry between the membrane leaflets. Consequently, physicochemical properties of the cell membranes are also changed in response to altered lipid organization. Partitioning of the spin probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) into the membranes of the cells has broadly been applied to characterize membrane properties of various cells in health and disease conditions. In this work, we used liposomes fabricated using lipids extracted from normal and carcinoma cells. This system permits the determination of the properties of the healthy and cancer cell membranes provided exclusively by its lipid components. Application of TEMPO-benzoate, in which the benzoate group is attached to the TEMPO, indicates significantly enhanced discrimination of liposomes between cancer and normal cells. Partitioning experiments with TEMPO-benzoate revealed relatively enhanced incorporation efficiency for liposomes of cancer cells. On the contrary, TEMPO incorporation efficiency in the same liposomes of cancer cells was not much different compared to healthy cells. Data indicate that TEMPO-benzoate as a probe is more suitable than TEMPO to discriminate cancer cells from healthy cells. Free energy gain observed for TEMPO-benzoate resulted mainly from the hydrophobic effect of the benzoate group.
***Key words**: Electron paramagnetic resonance, TEMPO, TEMPO-benzoate, partitioning, lung carcinoma, cell membrane lipid composition.*

INTRODUCTION

During the disease progression cancer cells obtain new capabilities. Because cancer cell development and transformation is a multi-step process, a solid tumor mass shows cell heterogeneity at both phenotypic and functional levels [1,2]. Functional reprogramming of the cancer cells provides new features, such as evading apoptosis, sustained growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, uncontrolled replicative potential and sustained angiogenesis. These features are common for all types of human tumors [3].

The mortality of cancer patients is mainly related to metastatic progression. Cancer cell mesenchymal transition is a critical step for the development of metastasis. Metastatic progression of the cells manifested by loss of cell polarity, cell-to-cell adhesion, etc. [3,4]. The significant changes observed in the cancer cell membranes play a direct role in conferring new capabilities. The various lipids, mainly composed of assorted phospholipids, sphingolipids, glycolipids, cholesterol, etc. arranged asymmetrically in the healthy cells. As a result, the outer leaflets of healthy cells are more negatively charged compared to that of inner leaflets [5-7]. Phospholipid transportation proteins, such as flippases, floppases and scramblases, play a pivotal role in maintaining the asymmetry the healthy cells. However, cancer cells show significant loss of asymmetry that manifested in decreased negatively charged phospholipid head group in the outer leaflet. Therefore, the zeta potential of cancer cells is decreased compared to that of healthy cells [8].

The membrane proteins play a major role in signaling pathways that regulate the metabolism of both cancer and healthy cells [4,9-12]. The membranes, such as those of cancer and healthy cells, composed of dissimilar lipids are adopted to modulate functions of particular cell types [5,13,14]. The cell membrane permeability is an important parameter to characterize healthy and cancer cells.

As indicated above, the lipid compositions of the membranes of healthy and cancer cells significantly differ from each other [15-17]. The lipids in the membrane are arranged non-uniformly and described with domain structures that have distinct properties in both cell types [18-21]. The membrane proteins, as well as lipid compositions, may play a critical role in assembling these domains [16,17,22].

In this study, taking a reductionist approach, we have evaluated if differences in lipid compositions of the membranes of healthy and cancer cells are enough to discriminate between both cell types. For this reason, we have studied the permeability properties of the liposomes produced from the lipids extracted from the healthy and cancer cells of human lung tissue.

The spin probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) has widely been applied to investigate membrane permeability properties of various cell types in health and diseased conditions. Particular properties of Electron Paramagnetic Resonance (EPR) spectra of TEMPO in aqueous (hydrophilic) and membrane (hydrophobic) environments

allow easy detection of the spin probes located in these environments. TEMPO-benzoate, along with TEMPO, was also tested to evaluate its partition properties in healthy and cancer cells. The experiments were performed at various temperatures to examine the temperature sensitivity of the lipid extracted from both cell types. Data indicate relatively more enhanced incorporation of TEMPO-benzoate into the liposomes of cancer cells compared to that of TEMPO. Unlike TEMPO-benzoate, TEMPO partitioning constants in the liposomes were similar to healthy and cancer cells.

MATERIAL AND METHODS

2.1. Human lung tissue collection

Since experiments were performed with liposomes, formalin-fixed lung tissues were adequate for providing reliable data for analysis. Human lung tissues were collected and used in accordance with the tenets of the Declaration of Helsinki and approved by the review board of the Azerbaijan National Center of Oncology. The cancer diagnosis was provided by a histopathology report. Here we report, as an example, a case of a 30-year-old female who did not receive chemo- or radiation therapy before the surgery. The pathology findings on the surgical lung tissue were consistent with a XBT-0-3:8070/3, (squamous cell carcinoma) lung cancer. Cancer grade was classified as G2 (intermediate grade/moderately differentiated). The separation of lung tissue into cancer and normal (also referred to as healthy) parts was provided by the pathologist.

2.2. Preparation of epithelial cell suspension from lung tissue

The lung tissue was washed thoroughly with PBS buffer to remove blood. Afterward, the tissue was cut into small pieces. To remove formalin fixation, the sample was incubated in PBS buffer for 2 hours. During this procedure, the buffer was refreshed every 15 minutes. Then, the samples were homogenized in PBS buffer using a glass homogenizer. The homogenized lung tissues were washed three times with PBS solution and then centrifuged (Eppendorf 5418) to remove the cell debris.

2.3. ***Extraction of lipids and preparation of liposomes from healthy and cancer cells***

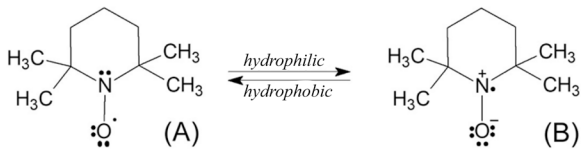
The epithelial cell suspensions obtained from healthy and cancer tissues of the lung were used to extract the lipids. Total lipid extraction was performed using a widely accepted three-component system (water, chloroform and methanol) [23]. The liposomes from the lipids extracted from healthy and cancer cell suspensions were produced using the probe-tip sonication method [24].

2.4. EPR spectroscopy

EPR measurements were performed using a Bruker ELEXSYS E580 spectrometer at X-band frequency with a variable temperature accessory. The liposomes fabricated from the lipids extracted from lung carcinoma and normal cells were incubated with TEMPO or TEMPO-benzoate for 30 min and then were placed into Pyrex capillary tubes with an I.D. of 0.6 mm. The following instrumental parameters were chosen for recording the EPR spectra. Scan width: 100 Gauss, sweep time: 40 s, modulation amplitude: 1 Gauss, modulation frequency: 100 kHz, microwave power: 0.47 mW, time constant: 0.1 s. The samples were kept for 5 minutes at each temperature to make sure that the temperatures of the samples matched the set temperatures.

2.5. Partitioning of TEMPO and TEMPO-benzoate in the liposomes constructed using the lipids extracted from human lung normal and carcinoma cells

TEMPO and TEMPO-benzoate contain the same nitroxide group that shows a well-known EPR spectrum with three-component resulting from nitrogen hyperfine interactions. The hyperfine splitting constants in EPR spectra of TEMPO and its derivatives depend on the polarity of the solvent. As it will be shown below the hyperfine splitting constant of nitroxide is larger in a hydrophilic environment compared to that in a hydrophobic one. TEMPO derivatives dissolved in the system that contains two phases with distinct polarities, such as liposomes and aqueous environments, will be distributed in these phases according to accessibility and polarity. Obtained EPR spectra, which are composed of the partitioning of TEMPO derivatives, show resolved components at the high magnetic field. The following consideration explains the features of EPR spectra observed in the partitioning experiments. The values for isotropic hyperfine coupling constants (A_{iso}) and *g* factors of the nitroxide spin probe are slightly different in hydrophobic (liposome) and aqueous environments which can be explained by differences in spin densities of two canonical structures of TEMPO [25].



The aqueous solution trend to stabilize the structure (B) in which unpaired electron density is localized on N-atom. In contrast, the structure (A), in which unpaired electron density is localized on the oxygen atom, is preferentially stabilized in a hydrophobic environment. Therefore, the nitrogen hyperfine coupling constant is lower in a hydrophobic environment compared to that in a hydrophilic environment [25].

The EPR spectra of TEMPO and TEMPO-benzoate incubated in the solutions of liposomes fabricated from the lipids extracted from lung carcinoma and healthy cells were analyzed using the LabVIEW program developed by Christian Altenbach https://sites.google.com/site/altenbach/) employing the spectral simulation code written in FORTRAN [26]. Two-component analysis was adequate to describe the EPR spectra of both TEMPO and TEMPO-

benzoate incubated in various liposome systems. The resolved components of EPR spectra were used to calculate a partition coefficient (K) according to the following formula:

$$K = I_{lip}/(I_{lip} + I_{aq}). \quad (1)$$

I_{lip} and I_{aq} are double integrals of EPR components of TEMPO or TEMPO-benzoate localized in liposome and aqueous environments, respectively. The same liposome (by mass of lipids) and spin probes (TEMPO or TEMPO-benzoate) concentration were used for direct comparison of the partition coefficients [27]. In partition experiments, the concentrations of TEMPO or TEMPO-benzoate were 150 □M.

For quantitative characterization of thermodynamic parameters of liposomes constructed using the lipids extracted from lung carcinoma and healthy cells, the free energy required to transfer a TEMPO or TEMPO-benzoate from an aqueous to a liposome environment was calculated using the values of the equilibrium constants, i.e. partition coefficients, determined at various temperatures

$$LogK = -\Delta G/RT. \quad (2)$$

Similarly, the rotational correlation times of TEMPO or TEMPO-benzoate determined at various temperatures were used to determine the activation energies for rotational motions in the lipid fraction of the liposomes fabricated from healthy and cancer cells.

RESULTS AND DISCUSSION

Detection of cancer at the earliest stage is most important for successful cancer treatment. Today various screening methods exist to reduce mortality or prevent various types of cancer, such as colon cancer, breast cancer, etc. However, such of screening method is not yet available for wide-range application for lung cancer, one of the deadliest diseases. Recent developments utilizing artificial intelligence applied to the FTIR spectra of human blood plasma samples for the classification of lung cancer patients are encouraging [28,29]. Evaluating of distinct properties of the healthy and cancer cell may provide the information for selected drug delivery, i.e., incorporation into the membranes.

Lipid constituents play a significant role in conferring specific properties to cell membranes, such as permeability, membrane dynamics, etc. It is well documented that the lipid composition of healthy and cancer cells significantly varies from each other [15–17]. Liposome model systems used in this study allow us to determine the properties of the healthy and cancer cell membranes provided exclusively by its lipid components. The distinct features of the liposomes composed of the lipids from the healthy and cancer cells may provide very important information for drug delivery applications. Comparison of the results obtained from the applications of TEMPO and TEMPO-benzoate permits the evaluation of the contribution of a benzoate group in partitioning the lipid phases composed of different components. Below, we provide TEMPO and TEMPO-benzoate partitioning experiments into the liposomes constructed from healthy and cancer cells at the temperature of 295K – 317K interval.

EPR spectra of TEMPO resulted from partitioning in liposomes using the lipids extracted from human lung normal (healthy) cells at pH 7.3 are shown in Figure 1.

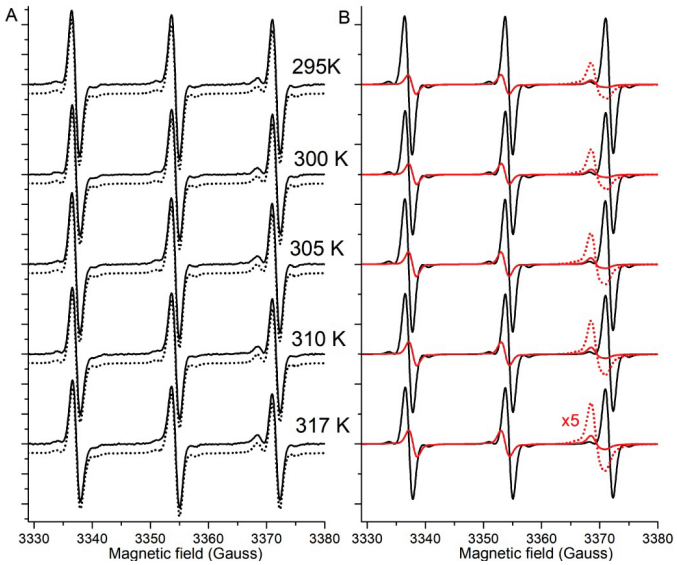


Figure 1. EPR spectra of TEMPO resulted from partitioning in the liposomes fabricated using the lipids extracted from human lung normal (healthy) cells at pH 7.3. (A) EPR spectra of TEMPO were recorded at various temperature values as indicated on the graph. Dotted lines are simulated spectra obtained from the fitting to a two-component model. For better visualization, simulated spectra were shifted vertically. (B) EPR spectra of resolved components. Black and red lines indicate EPR spectra of TEMPO localized in hydrophilic (aqueous) and lipophilic (liposome) environments, respectively. The amplitudes of high field components of EPR spectra of TEMPO in a liposome environment were increased 5-fold (dotted red lines) for better judgments

As can be seen from Figure 1A (vertically shifted dotted lines), two components are enough for adequate characterization of the spectra. The small fractions of TEMPO molecules are localized in the lipid fraction of the liposome system (Fig. 1B, red lines). TEMPO partitioning into the lipid fraction of the liposome is increased at higher temperatures. TEMPO partitioning experiment in liposomes constructed from the lipids extracted from cancer cells of human lung tissue shows similar results (Fig. 2).

Partitioning of TEMPO into the liposomes constructed from the cancer cell lipids is almost identical to that of healthy cells (Fig. 1, Fig. 2). The parameters recovered from the fitting procedures are shown in Tables 1 and 2.

The A_{iso} values for TEMPO in the lipid and aqueous environments in the liposomes fabricated from the lipids of cancer and healthy cells show identical values, 34.5± 0.1 and 31.4± 0.1, respectively. The identical A_{iso} values for TEMPO in lipid fractions of liposomes from cancer and healthy cells indicate that TEMPO molecules in both cases are localized in regions with similar hydrophobicities. The Arrhenius plots reveal that free energies required to transfer a TEMPO from an aqueous to the liposomes of cancer and healthy cells are very close, 7.7± 0.6 kcal/mol and 6.6± 0.6 kcal/mol, respectively (Fig. 3). Thus, TEMPO does not discriminate between the liposomes fabricated from cancer and healthy cells.

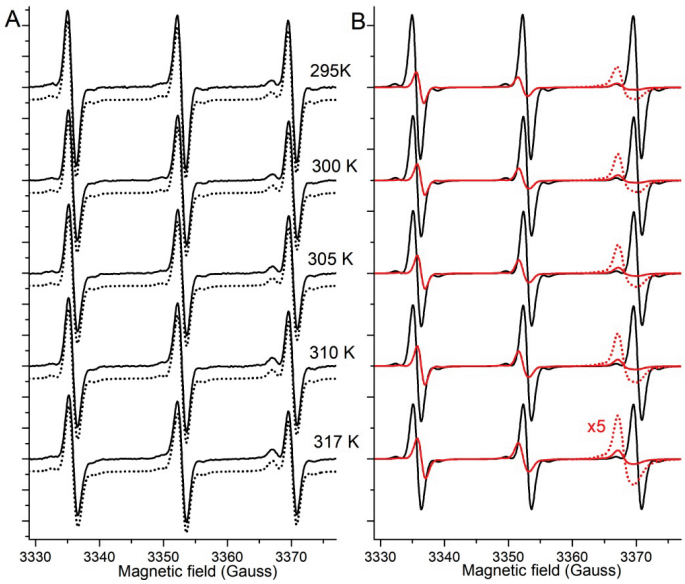


Figure 2. EPR spectra of TEMPO resulted from partitioning in liposomes using the lipids extracted from human lung carcinoma cells at pH 7.3. (A) and (B) are the same as in Figure 1, but obtained from the lipid extracted from lung carcinoma cells

Table 1. Parameters obtained from the EPR studies on the liposomes, lipids of which were extracted from healthy and cancer cells of the human lung

Sample		$2A_{iso}$ (Gauss) (aqueous)	$2A_{iso}$ (Gauss) (liposome)	ΔG_K (kcal/mol)
Liposome (Healthy), pH 7.3	TEMPO	34.5± 0.1	31.4± 0.1	6.6± 0.6
Liposome (Cancer), pH 7.3	TEMPO	34.5± 0.1	31.4± 0.1	7.7± 0.6
Liposome (Healthy), pH 7.3	TEMPO-benzoate	33.7± 0.2	31.4± 0.1	2.9± 0.7
Liposome (Cancer), pH 7.3	TEMPO-benzoate	33.7± 0.1	30.8± 0.1	4.5± 0.7

Table 2. Rotational correlation times for TEMPO-benzoate located in lipid fractions of the liposomes. The liposomes were fabricated using the lipids extracted from healthy and cancer cells of the human lung

Temperature (K)	τ (ps) Cancer, Component B	τ (ps) Healthy, Component B
295	466.8	278.8
300	312.5	223.0
305	291.5	202.0
310	244.0	108.3
317	181.8	45.8

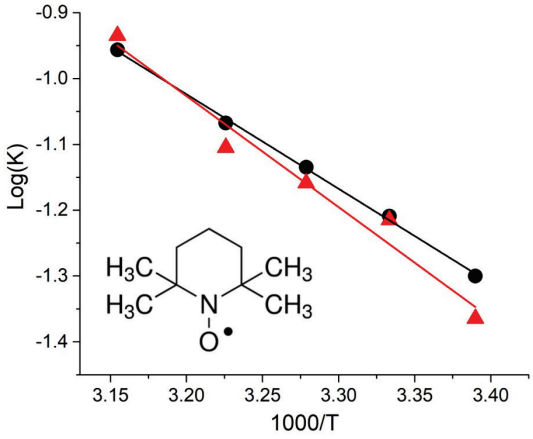


Figure 3. Arrhenius plots for the TEMPO partitioning coefficients obtained for the liposomes constructed using the lipids extracted from the healthy (black circles) and carcinoma (red circles) cells of the human lung tissue. Experiments were conducted at pH 7.3

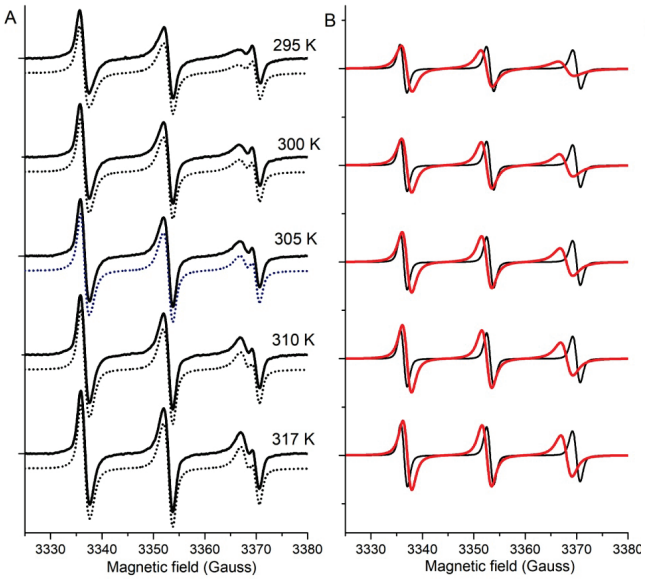


Figure 4. EPR spectra of TEMPO-benzoate resulted from partitioning into the liposomes fabricated using the lipids extracted from human lung normal (healthy) cells at pH 7.3. (A) EPR spectra of TEMPO-benzoate were recorded at various temperature values as indicated on the graph. Dotted lines are simulated spectra obtained from a two-component model. For better visualization, simulated spectra are shifted vertically. (B) EPR spectra of resolved components. Black and red lines indicate EPR spectra of TEMPO-benzoate localized in hydrophilic (aqueous) and lipophilic (liposome) environments, respectively

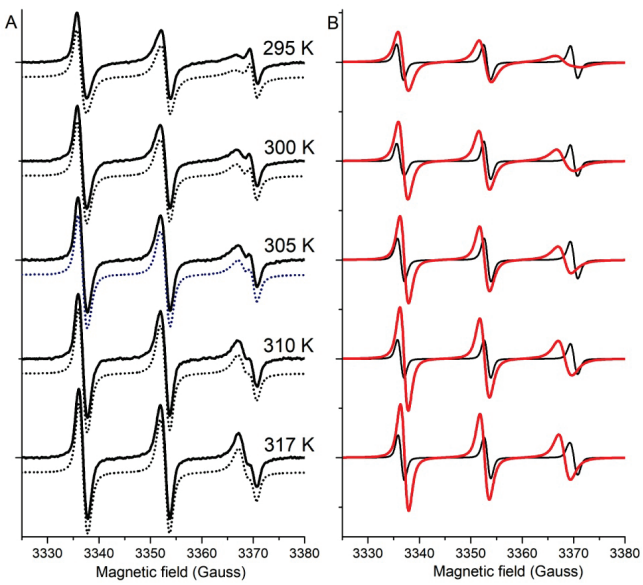


Figure 5. EPR spectra of TEMPO-benzoate resulted from partitioning in liposomes using the lipids extracted from human lung carcinoma cells at pH 7.3. (A) and (B) are the same as in Figure 4, but obtained from the lipid extracted from lung carcinoma cells

The same liposomes were also tested with TEMPO-benzoate, in which the benzoate group is attached to TEMPO. The EPR spectra of TEMPO-benzoate incubated in human lung healthy and cancer cell suspensions at pH 7.3 are shown in Figures 4 and 5, respectively. As in the case of TEMPO, a model with two components sufficiently characterize the EPR spectra of TEMPO-benzoate at various temperatures (Fig. 4A, dotted lines and 4B).

Significantly enhanced partition for TEMPO-benzoate compared to that of TEMPO is evident (Fig. 1 and 4). Partitioning of TEMPO-benzoate into liposomes from healthy cells is increased at higher temperatures. In contrast to TEMPO, TEMPO-benzoate demonstrates much higher partitioning into liposomes of cancer cells compared to that of healthy cells (Fig. 4 and 5). The parameters recovered from the fitting procedures indicated that A_{iso} value for TEMPO-benzoate in liposomes from cancer cells decreased from 31.4± 0.1to the value of 30.8± 0.1. The decreased value of A_{iso} for the liposomes of cancer cells can be attributed to lipid rafts that are more hydrophobic compared to the phospholipid parts. At higher temperatures, relatively higher partitioning of TEMPO-benzoate to the liposomes of cancer cells is evident (Fig. 6).

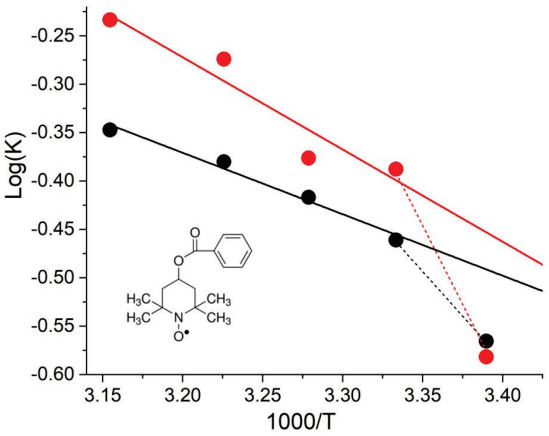


Figure 6. Arrhenius plots the TEMPO-benzoate partitioning coefficients obtained for the liposomes constructed using the lipids extracted from the healthy (black circles) and carcinoma (red circles) cells of the human lung tissue

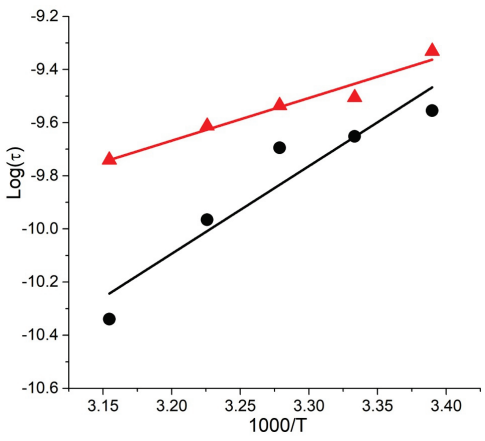


Figure 7. Arrhenius plots for rotational correlation times of the TEMPO-benzoate in the liposomes constructed using the lipids extracted from the healthy (black circles) and carcinoma (red circles) cells of the human lung tissue

The Arrhenius plots for partitioning values of TEMPO-benzoate indicate significant differences in energies required to transfer TEMPO-benzoate from aqueous to liposome environments for cancer and healthy cells. The energy transfer values of TEMPO-benzoate are 2.9 ± 0.7 kcal/mol and 4.5 ± 0.7 kcal/mol for liposomes of healthy and cancer cells, respectively. The rotational correlation times of TEMPO-benzoate and their temperature dependence are appreciably different for cancer and healthy cells (Fig. 7, Table 2). Activation energies for the rotational motion in liposomes of cancer and healthy cells are 7.4± 0.9 kcal/mol and 15.1± 2.8 kcal/mol, respectively. Free energy difference values for TEMPO and TEMPO-benzoate transfer to the same system are revealing. □□ G (TEMPOTEMPO-benzoate) for the liposomes from healthy and cancer cells are 3.7 kcal/mol and 3.2 kcal/mol, respectively. Free energy gain from the transfer of the –CH_2 group from an aqueous to a hydrophobic environment is about 0.6 kcal/mol [30]. The free energy gain from the benzoate group can be approximated as 6 of –CH_2 groups, i.e. 3.6 kcal/mol. This value is very close to the □□ G (TEMPO TEMPO-benzoate) values obtained by the experiments. Thus, free energy gain obtained for TEMPO-benzoate partitioning for both liposomes from healthy and cancer cells can be explained primarily by the hydrophobic effect.

CONCLUSIONS

A comparison of TEMPO and TEMPO-benzoate partitioning into the liposomes fabricated from the lipids using the healthy and cancer cells of the human lung reveals important differences in their properties. In contrast to TEMPO, the use of TEMPO-benzoate allows the discrimination of liposomes between fabricated from cancer and healthy cells. The temperature-dependent partitioning coefficients for TEMPO-benzoate and energies required to transfer TEMPO-benzoate from aqueous to lipid phase are adequate parameters for cell discrimination. Data indicate that not TEMPO but TEMPO-benzoate is a good probe to characterize and/or discriminate between cancer and healthy cell systems. Free energy gain observed for TEMPO-benzoate mainly resulted from the hydrophobic effect of the benzoate group.

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