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## THE INFLUENCE OF NATURAL AND MODEL FORMS OF HUMIC ACIDS ON THE DYNAMIC PARAMETERS OF MODEL MEMBRANES

### WPŁYW NATURALNYCH I MODELOWYCH KWAŚÓW HUMINOWYCH NA DYNAMICZNE PARAMETRY BŁON MODELOWYCH

**Abstract:** In this paper the influence of humic acid concentrations extracted from Histosols (HA-A) and their model forms (HA-B) separated from humic substances commercially produced by Carl Roth GmbH + Co.KG on the dynamic properties of liposome membranes was determined. Differences in the quality of the humic acids (HA-A and HA-B) were determined by the <sup>1</sup>HNMR and FTIR methods. Liposomes from the sonication of egg yolk lecithin (EYL) in an aqueous solution and synthetic Dipalmitoylphosphatidylcholine (DPPC) were used. Fluidity of liposome membranes was determined by the EPR technique with spin probes (TEMPO, 16DOIXYL). The electrical parameters of membranes were found using a Keithley 6517 electrometer. Our study showed significant differences in the influence of HA-A and HA-B on the membranes. In the bilayer membranes of the liposomes of HA-A admixture there was slightly more stiffening of the interior of the membrane in comparison to HA-B. A similar effect was observed in the surface layer of the liposome membranes. This difference is particularly evident for DPPC liposomes, however, the EYL liposomes admixture with HA-B slightly increased the fluidity of the surface layer. Electrical study confirmed this effect. The study shows that natural and model forms of humic acids differ in their effects on the activity of tested membrane models. The strong differences in the interaction of HA-A and HA-B on parameter F in DPPC liposomes can be result from the transport of humic acids connected to the metal ions inside the membranes (xenobiotics present in the environment).

**Keywords:** lecithin liposomes, ESR, <sup>1</sup>HNMR, humic acid, spin label

## Introduction

Frequently humic substances (HS) are referred to as the most comprehensive and most 'elusive' compounds in nature [1-3]. According to the commonly used definition proposed by IHSS, 'humic substances are complex and heterogeneous mixtures of polydispersed materials formed in soils, sediments and natural waters by biochemical and chemical

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reactions during the decay and transformation of plant and microbial remains' [4]. Their activity and mobility in the environment depend on the amount and quality of functional groups and their degree of aromaticity and aliphaticity. The fractional groups, in decreasing order of typical content are: carboxyl, phenolic and alcoholic hydroxyl, quinone and ketonic carbonyl, nitrogen- and sulphurcontaining groups [5, 6]. The prevalence of carboxyl and phenolic hydroxyl groups is responsible for their net negative charge in natural waters and their behavior as polyelectrolytic compounds.

Thus, as research has shown, their structures can be modified by the quality of the environment where they are formed [1, 2, 7, 8]. Commonly used methods of extracting these compounds from the natural environment can also modify their internal structure (changes in pH, ionic strength, oxidation, hydrolysis, etc.). As a result it is difficult to study their natural structure and to compare their effects on living organisms through biological membranes.

Due to the great structural, phasal and dynamic complexity of the biological membranes, research on their biophysical properties is very often performed on model systems. Experimental models of biological membranes are usually layers (or multilayered), double formed from one or more types of lipids, sometimes containing a controlled amount of other components. Liposomes serve as a good model for biological membranes. Studies carried out with liposomes have made it possible to predict, for example, the strong biological activities of tin - organic compounds [8-12]. It seems that research using membrane models can play an important role in understanding the mechanisms which determine the condition of living organisms, since biological membranes are involved in almost all aspects of cell activity.

They regulate the transport of external substances from the environment to the cell interior and going the opposite direction, they determine the formation of tonicity and functionality. They are involved in energy metabolism, simultaneously enabling the competitive overlap of chemical reactions, which is reflected in their dynamic properties [13].

Researchers have shown [3, 8, 14-17] that HS are not just components of the soil but are also found in various biological substances and are indication factors of biochemical processes that significantly modify the fluidity of membrane models. Our previous studies [18] found that structural differences of HS have varying effects on the fluidity of biological membrane models - liposomes.

The aim of this work was to demonstrate the structural diversity of humic acids extracted from peat and model forms of HS as well as their varying effect on the dynamic properties of the liposome membranes formed from lecithin EYL (natural) and synthetic lecithin DPPC.

## **Materials and methods**

### **Humic substances (HS)**

Peat, an organic soil, was taken from surface horizons of Histosols. Model forms of HS, containing 30-40% of humic acids (HA-A) was made by Carl Roth GmbH + Co. KG. Humic acid extraction and purification were carried out by the IHSS method [14] as follows: humic substances were isolated by extraction of 10 g of peat with an aqueous solution of NaOH 0.5 mole followed by centrifuging at room temperature and was precipitated at pH < 1.5. Humic acids were separated from fulvic acids by centrifugation,

washed with distilled water in order to get a neutral pH, shaken with a mixture of HF and HCl at room temperature for 24h, washed again with distilled water to a neutral pH. Humic acids of model humic substances (HA-B) were isolated in the same way, using 1 g of humic substances made by Carl Roth GmbH + Co. KG. For FTIR analysis both humic acids were dried at 60°C under vacuum and weighed. FTIR spectra of humic acids at 4000-400 cm<sup>-1</sup> absorption region were taken using fourier transformation spectrometer Nicolet NEXUS FT-IR spectrometer (Thermo Nicolet, USA 2002).

<sup>1</sup>H NMR analyses were done using a Bruker NMR spectrometer (Germany 2005) 400 MHz UltraShield at 22°C. For the measurements, a 0.5 cm<sup>3</sup> sample containing 20% D<sub>2</sub>O (v/v) was used.

<sup>1</sup>H NMR spectra were taken with a spectral width  $sw = 9615$  Hz, acquisition time  $aq = 3.4$  s and relaxation delay  $d1 = 3$  s. During the measuring time, 14 minutes, 128 scans were collected, which included 65,536 points. The strong water signal was suppressed by water suppression by presaturation. The same methodology was used in our previous work [18].

The content of organic carbon in the solutions of used humic acids was determined by Analyzer Multi N/C 3100 (Analytikjena, Germany 2013). The analyzed humic acid contents were respectively: HA-A: 2951 mg/dm<sup>3</sup>, HA-B 1722 mg/dm<sup>3</sup>.

## Liposomes

The liposomes used in this experiment were prepared by sonication of Egg Yolk Lecithin (EYL, isolated from fresh egg yolks) and synthetic lecithin (DPPC, produced by Sigma - Aldrich Company) in distilled water using ultrasonic disintegrator UD-20 (TECHPAN, Warsaw, Poland). The procedure used for isolation and purification of lecithin was a modification of the method described by Singleton et al [19]. The total time of sonication of single samples (2 cm<sup>3</sup>) was 10 min and took place in 10 alternating cycles of 30 s of sonication, 30 s of cooling. The concentration of lecithin in the samples was 0.04 M. After sonication, spin probes at a concentration of 1000 ppm in relation to the lecithin were added to the sample.

## EPR technique

To study changes in liposome membrane fluidity with admixtures of humic acid the ESR technique was used with spin probes: 2,2,6,6-tetramethylpiperidine-1-oxylane (TEMPO) and 2-ethyl-2-(15-methoxy-15-oxopentadecyl)-4,4-dimethyl-3-oxazolidinyloxy (16-DOXYL stearic acid methyl ester) (Fig. 1).

The probes were purchased from Sigma-Aldrich Company, Poland. Spin probe concentrations in the samples ranged from 0.01 to 0.005% of mol in relation to the lecithin. Figure 2 presents examples of spin probe spectra placed in an aqueous dispersion of liposomes.

Based on the fission of the high-field amplitude line of probe TEMPO (a) partition coefficient  $F$  was determined. This parameter indicates the allocation of the probe between the liquid phase (line P), and the lipid phase (line H). Changes of parameter  $F$  indicate the migration of the probe from one environment to another, which may indicate changes in fluidity [11]. On the basis of the spectra of probe 16-DOXYL (b) rotation correlation time  $\tau$  was determined ( $\tau = 5.95\Delta H((I_0/I_{+1})^{1/2} + (I_0/I_{-1})^{1/2} - 2)10^{-10}$ ). An parameter  $\tau$  increase was followed by increased stiffness of the liposome membranes [20]. Due to the complex structure of humic acid, concentrations by weight were used, which in relation to lecithin

varied from 0 to 12%. In order to thoroughly mix the ingredients, each of the samples was shaken 600 s (using a microshaker with polypropylene tubes) before measuring and then placed in a measuring chamber spectrometer (in glass capillaries of 1 mm diameter and capacity approx.  $0.04 \text{ cm}^3$ ). The ESR measurements were performed at a constant temperature of  $20^\circ\text{C}$ , and the total time of the measurement series was no more than 3 h for each sample. ESR spectrometer (MX-201R ESR spectrometer TU Wroclaw, Poland) operating parameters were: microwave power  $P = 50 \text{ mW}$ , sweep range  $\Delta H = 7 \text{ mT}$ , amplitude modulation  $dH = 0.08 \text{ mT}$ , time constant  $dt = 0.3 \text{ s}$ , sweep time  $t = 128 \text{ s}$ .

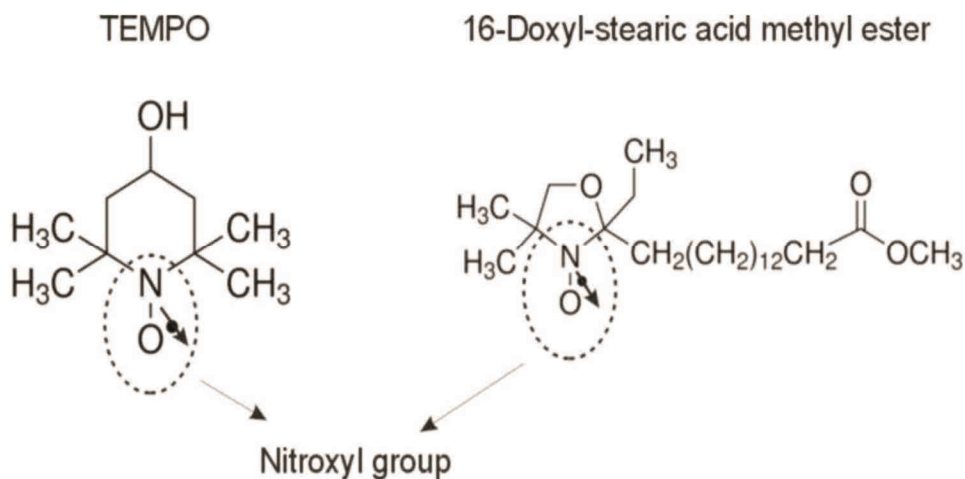


Fig. 1. Structural formulas of used spin probes

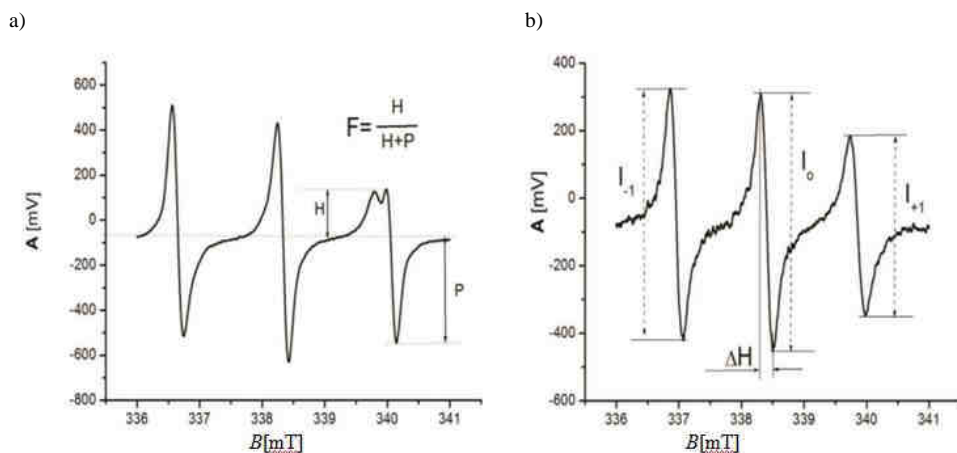


Fig. 2. ESR spectra of spin probes placed in the liposome membrane: a) TEMPO probe and spectroscopic partition parameter ( $F$ ); b) 16-DOXYL - stearic acid methyl ester probe and parameter ( $\tau$ )

## Electric method

Parallel to the ESR studies, the electrical properties of membranes modified by selected humic acid were investigated. The membranes were made of nitrocellulose filters, produced by Synpor. Before the measurement they were then impregnated with lauric acid butyl ester ( $\text{C}_4\text{H}_9\text{COOC}_{12}\text{H}_{23}$ ). Filter diameter was 1.5 and 50  $\mu\text{m}$  thick. Membrane M (Fig. 3) separates the reference chamber (2) from the measuring chamber (1).

Initially the chambers were filled with 0.01 M KCl solution. The trans-membrane voltage, due to the presence of the two humic acids in the measuring chamber, was determined using Keithley 6517 electrometer and Ag/AgCl electrode system (Fig. 3). The concentration of compounds in the test chamber was changed 0-0.024% relative to the water.

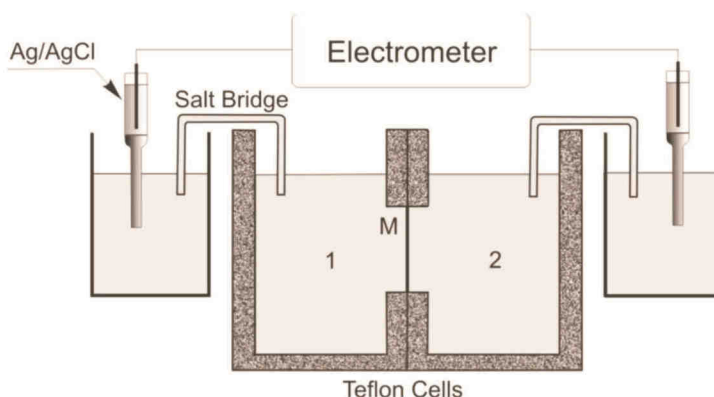


Fig. 3. A scheme of voltage measuring system, where M indicates a membrane separating the measuring (1) and reference (2) chambers

## Results and discussion

### IR spectroscopy

As illustrated in Figures 4 and 5, and Table 1, spectra of humic acids extracted from peat and model forms of HS indicate the presence of similar absorption zones with differing intensities.

In both substances the strongest expression in the absorption zone was in the range of  $3600\text{--}3000\text{ cm}^{-1}$ , corresponding to the  $-\text{OH}$  group of phenols and alcohols. Generally, next to this zone, there is a marked absorption at  $3000\text{--}2800\text{ cm}^{-1}$ , characteristic of the  $-\text{CH}$  bonds extending methyl groups  $-\text{CH}_3$ . In the analyzed spectra this signal was not observed. The absorption zones corresponding to vibrations extending in the carboxyl groups occurred in the range of  $1705$  and  $1706\text{ cm}^{-1}$ . The area ranging from  $1500$  to  $1107\text{ cm}^{-1}$  has a low intensity in the HA-B, as well as the presence of vibrations of the scissor groups  $-\text{CH}$  ( $1455\text{ cm}^{-1}$ ), and deformation of the  $-\text{OH}$  groups of phenols (less than  $1401\text{ cm}^{-1}$  in the HA-B, and less than  $1405\text{ cm}^{-1}$  in the HA-A). The absorption bands in the area of  $1158\text{ cm}^{-1}$  in the 'model forms' and in both areas of  $1207$  as well as  $1078\text{ cm}^{-1}$  in the peat are responsible for the presence of ester bond stretching vibrations of  $\text{C-O}$ . Spectral range from  $900$  to  $675\text{ cm}^{-1}$  together with the presence of vibrations in the range of  $1600\text{ cm}^{-1}$

may indicate the presence of aromatics. This range has low absorbance, reflecting the strongly expressed aliphatic nature of the structures of both forms of humic acids. Moreover, the absence of an absorption band extending slightly above  $3000\text{ cm}^{-1}$  also confirms that both fractions of humic acids have a highly expanded aliphatic part of their structures [14, 15, 18, 21-23].

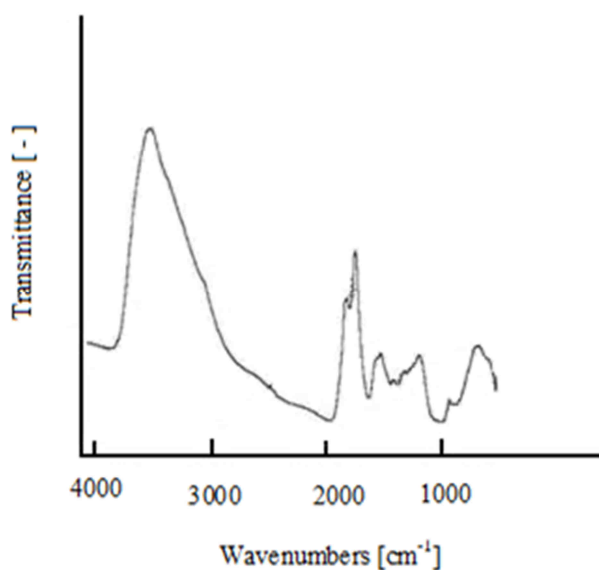


Fig. 4. FTIR spectra of humic acids extracted from peat (HA-A)

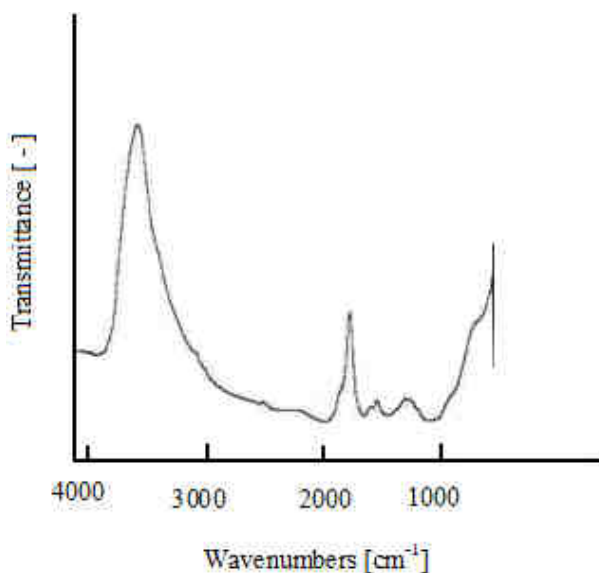


Fig. 5. FTIR spectra of humic acids extracted from humic substances (HA-B)

Table 1

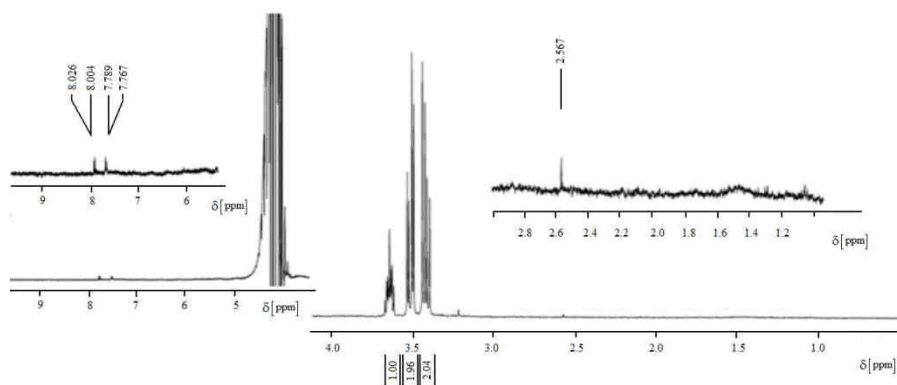
Absorption zones of humic acids [15, 21, 22]

Functionality	FTIR [cm <sup>-1</sup> ]	Type	HA-A	HA-B
CH <sub>3</sub>	3000-2850	extending	no	no
CH <sub>2</sub>	2950-2850 2875-2800	extending extending	no	no
CH	1300-1100	deformation	no	no
HC=CH	3100-3000 1420-1290	extending deformation	no	no
≡C-OH	3500-3100 2260-2100 1370-1220	extending extending deformation	no	no
OH alcohols and phenols	3600-3000 1210-1120 1125-1090 1075-1050	extending extending extending extending	3405	3426
-COH	2900-2700 (H) 1750-1650 (C=O)	extending extending	no	no
COOH	3300-2900 (OH) 1775-1675 (C=O) 1725-1700 (C=O)	extending deformation deformation	1706 1730	1705
≡CNH	3500-3200	deformation	no	no
Ar	~3030 ~1600 ~1500 ~1450 ~750 ~700	extending framework extending framework extending framework extending deformation deformation	1629	1455 1630
fingerprint region	1450-1250 (OH) 1430-1050 (C-O) 600-800 (C-Cl)	deformation extending extending	1078 1207 1305 1405	1158 1401

no - not observed

### <sup>1</sup>H NMR spectroscopy

Analysis of <sup>1</sup>H NMR spectra of humic acids extracted from peat and model forms of HS (Figs. 6 and 7) confirms the aliphatic nature of their structural composition.

Fig. 6. <sup>1</sup>H NMR solution spectra of HA - humic acids extracted from used peat

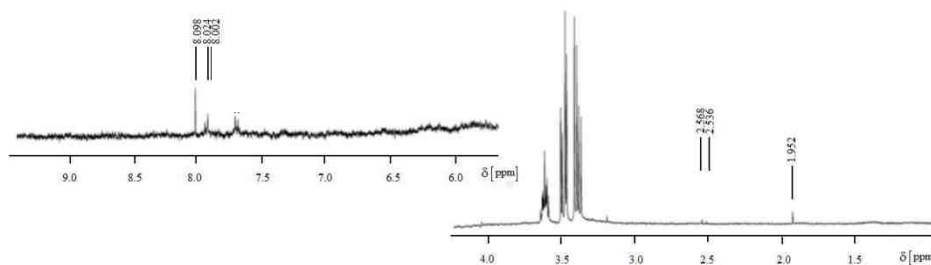


Fig. 7.  $^1\text{H}$  NMR solution spectra of HA - humic acids extracted from used model forms of HS

Table 2 presents important diagnostic absorption of  $^1\text{H}$  NMR spectra in general and analyzed humic acids.

Table 2

Chemical shifts of  $^1\text{H}$ NMR of humic acids [15, 18, 21-23]

Functionality	$\delta$ $^1\text{H}$ [ppm]	HA-A	HA-B
$\text{CH}_3$	0-1	no	no
$\text{CH}_2$	1-2	no	+
$\text{CH}$	1.5	no	no
$\text{HC}=\text{CH}$	4.6-5.9	+	+
$\equiv\text{C}-\text{OH}$	2-3	+	+
OH alcohols and phenols	2-6	+	+
$\text{COOH}$	6-8.5	+	+
$\equiv\text{CNH}$	1-5	no	no
Ar	6-9.5	+	+
$\text{HC}-\text{Ar}$	2.3-2.9	no	no
$\text{HC}=\text{O}$	9-10	no	no
$\text{HO}-\text{Ar}$	4-12	no	no

no - not observed, + - presence

Taking into consideration chemical shifts, humic substances were used which are characterized by the presence of broad signals in the range of 3.4 to 3.7 ppm, associated with protons located on the carbon atom in alcohols. Simultaneously, the spectrum is attributed to the presence of glycerol, which may be the result of lipid hydrolysis during the extraction and purification of the humic acids, emphasized in our previous work [18].

$^1\text{H}$  NMR spectra of humic acids, extracted from peats and model compounds, give signals connected with protons of aliphatic compounds  $\delta \leq 2.5$  ppm (the presence of these signals was previously reported by Man et al [18, 23]). Comparative analysis of spectra of humic acids extracted from peat (HA-A) and the model forms (HA-B) indicates a high degree of similarity in their structure. There was a signal which was related to alkyl in the aliphatic chain ( $2.5 < \delta \leq 8.6$  ppm) as well as the aromatic protons - signal in the range of  $6.6 \leq \delta \leq 8.6$  ppm. The area of the spectrum giving signals between 2.5-2.7 ppm includes a system consisting of several functional groups: amino, amide or methylene groups.

The signals, in the range of 3.75-3.92 ppm and associated with the presence of functional groups of protons, are typical for HS. They indicate the presence of repeating units of methylopropyl groups, which are structural units of lignin, and appropriate humic



compounds [24]. This signal was not seen in any of the analyzed spectrum, confirming their aliphatic character.

### EPR spectroscopy

Figure 8 shows the effect of the concentration of humic acids HA-A and HA-B on the spectroscopic properties of probe TEMPO (F), placed in an aqueous dispersion of liposomes prepared from DPPC (Fig. 8a) and EYL (Fig. 8b).

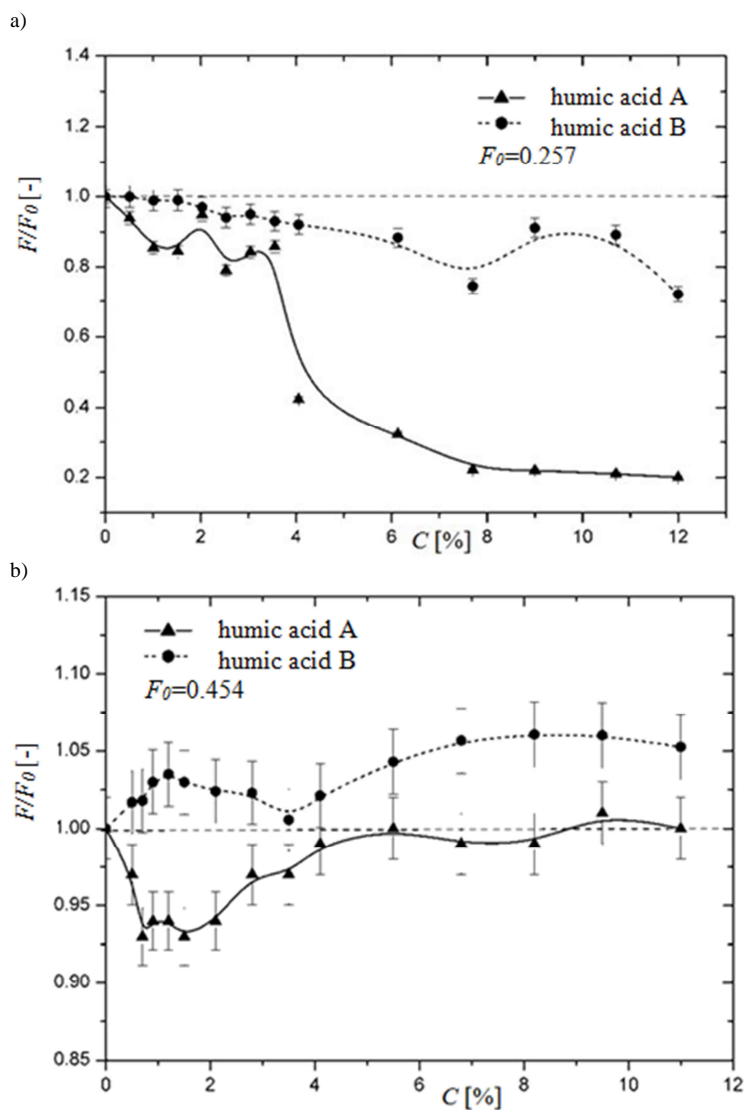


Fig. 8. The dependence of relative value of spin probe TEMPO placed in: a) the membrane of DPPC liposome, b) EYL liposome

To highlight the changes caused by introducing the humic acids into the membranes, the charts show the portiton coefficient of the probe parameter ( $F/F_0$ ). The broken line shows the values of these parameters for liposomes without admixtures HA-A and HA-B. Points below this line mean that the membranes under the influence of humic acids are stiffening: the smaller the  $F/F_0$ , the greater the stiffness of the membrane.

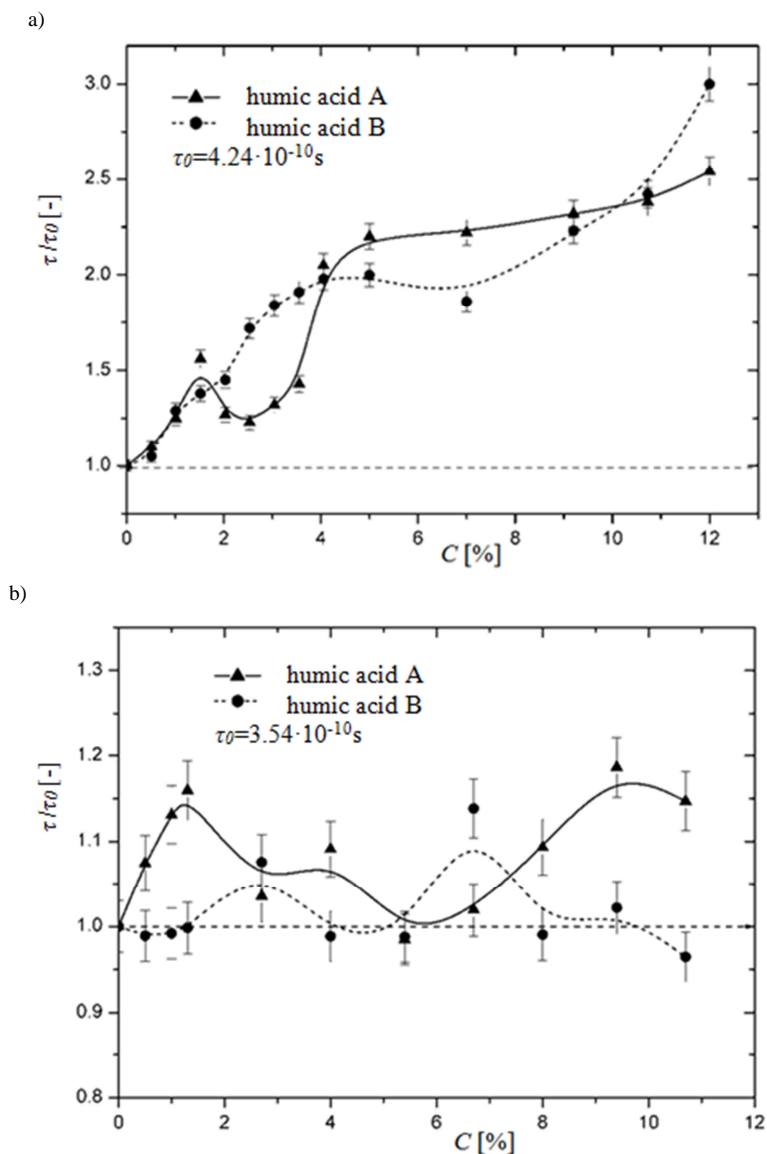


Fig. 9. The dependence of relative value of spin probe 16-DOXYL placed in: a) the membrane of DPPC liposome, b) EYL liposome

From the data shown in Figure 8, we can observe that liposomes formed from DPPC were stiffened for both the HA fractions. There was much stronger stiffening for HA-A than HA-B. This is particularly evident for concentrations above 6%, where parameter  $F/F_0$  has a value of about 0.2, whereas HA-B oscillates around 0.8. Liposomes formed from EYL are less susceptible to changes in their fluidity under the tested HA. In the case of HA-A admixtures a small minimum value of parameter  $F/F_0$  (0.93) was found in a concentration range of 0.5-1.5%.

Concentrations above 4% of HA-A admixture do not significantly alter membrane fluidity. Surprisingly, the EYL liposome membrane fluidity was affected by the admixture of HA-B. In contrast to previous cases, it slightly increased membrane fluidity - data arranged above the dotted line (Fig. 8). Changes in parameter  $F/F_0$  stabilized at a level of 1.05 for concentrations above 6%.

Figure 9 shows the effect of humic acid concentrations (HA-A and HA-B) on the rotation correlation time ( $\tau$ ) of probe 16-DOXYL which was placed in the center of the bilayer of the liposome from DPPC (Fig. 9a) and EYL (Fig. 9b). To highlight the changes caused by the introduction of HA into the membranes, the charts present the rotation parameter correlation time ( $\tau/\tau_0$ ). The dotted line shows the value of these parameters for liposomes without admixture HA. Points located above the line indicate that the membrane under the influence of the admixtures was stiffening. The higher the value of the  $\tau/\tau_0$  parameters, the greater the stiffening of the membrane.

Both fractions of HA greatly stiffen liposome membranes formed from DPPC (Fig. 9). The increase is approximately proportional to an increase in the concentration of HA, except for concentrations of HA-A between 0.5 to 1.5%, in which parameter  $\tau/\tau_0$  reaches a local maximum (1.5). For a concentration of 12% admixtures HA-A and HA-B, parameters  $\tau/\tau_0$  reach their maximum values of 2.5 and 3. A decidedly weaker effect on membrane fluidity was observed in liposomes formed from EYL. With increasing HAS concentrations, values oscillated between 0.95-1.05 for HA-B and 1-1.2 for HA-A. There was no clear relationship between an increased HAS concentration and changes of parameter  $\tau/\tau_0$ .

### Voltage experiment

Figure 10a and b present the influence of humic acid concentrations (HA-A and HA-B) on the transmembrane voltage generated between the measurement chamber and the control.

The dividing membrane M (Fig. 3) resulted from the impregnation of the filter with lauric acid butyl ester. HA-A and HA-B concentrations are expressed as a per cent of weight to volume ratio:

$$c_x = \frac{m_x}{V_c} \cdot 100\% \quad (1)$$

where:  $m_x$  - humic acid weight,  $V_c$  - measurement chamber volume.

The graphs indicate that together with increased admixture concentration the transmembrane voltage also increases. The influence of HA-A on the voltage was significantly greater than for the HA-B admixture. According to the Nernst equation:

$$U = \frac{RT}{zF} \ln \frac{f_1 c_1}{f_0 c_0} \quad (2)$$

where:  $R$  - gas constant,  $T$  - absolute temperature,  $F$  - Faraday constant,  $z$  - valence of ions,  $c_1$  and  $c_0$  - concentrations of redox fraction in the measurement and reference chambers - respectively:  $f_1$  and  $f_0$  - ions activity coefficients.

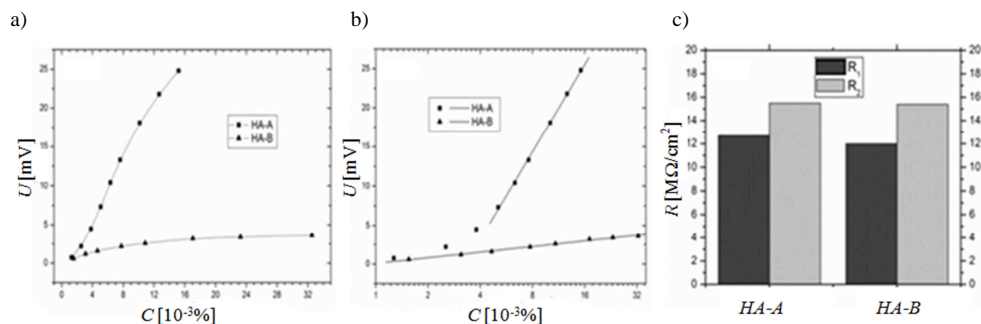


Fig. 10. Effect of humic acid concentrations on trans-membrane voltage in  $C$  [%]: a) normal scale, b) logarithmic scale, c) changes in the resistance of  $R$  membranes [MΩ/cm<sup>2</sup>]:  $R_1$  - initial,  $R_2$  - final

The diagram of dependence  $U = f(\ln c)$  should be a straight line. This occurred for the H-AB admixture for all concentrations, however, for the H-AA admixture it was not found for the first three measurements (low concentrations). This may indicate that for the reactions occurring in the measurement chamber after an application of a small amount of H-AB the Nernst equation is not applicable - they are more complex. The linear nature of dependence  $U = f(\ln c)$ , in agreement with the Nernst equation, which was observed by Podolak et al [25] and others, dealt with the influence of KCl on changes in the transmembrane voltage using similar measuring equipment as in the presented experiment. This suggests that the influence of the humic acids on model membranes is similar to the influence of KCl on the membranes. Figure 10c presents changes in the resistance of  $R$  membranes (resistance on 1 cm<sup>2</sup> of surface), before and after adding humic acid. The initial  $R_1$  membrane resistance is shown in black while gray shows the final  $R_2$  resistance after reaching the maximum concentration for a given humic acid in the measurement chamber. The presence of both HA-A and HA-B caused a definite increase in membrane resistance. Probably the humic acid admixtures first combine with cations in the measurement chamber. Then they are attracted to the surface of the membrane. Such an interaction can generate both transmembrane voltage and increased membrane resistance in the presence of humic acids.

## Conclusions

The study shows that natural and model forms of humic acids differ in their effects on the activity of tested membrane models. This effect is particularly evident in changes of the  $F$  parameter for DPPC liposomes. In EYL liposomes, the changes were minor. This effect was probably related to the state of the phase in which the membranes was found. The study was conducted at room temperature (22°C), in which membranes of DPPC liposomes were in the gel phase, while EYL membranes were in the liquid crystal phase. Presumably, a membrane - based liquid crystal can not 'see' HAs molecules as clearly as when they are

in the gel phase. Computer simulations have shown [26-28] that in the liquid crystal phase, the membrane may have numerous gaps in the hydrophilic part. This is related to the mobility of molecules, and therefore the ability to dissolve the admixtures in a given phase. Our previous work indicates [26] that macromolecules may differ substantially in their effect on the membranes of liposomes DPPC and EYL. We would argue, that strong differences in the interaction of HA-A and HA-B on parameter F in DPPC liposomes can result from the transport of humic acids connected to the metal ions inside the membranes (xenobiotics present in the environment and humic acids forming stable organo-metallic complexes).

Alloway [29] showed that the bioaccumulation ability of organometallic complexes may cause changes in the electrical charge of the membrane. An additional factor which describes the interactions between the components of the membrane and the environment is the pH of the system and the mobility of humic substances in different environments and the shifting balance of acid-base groups present in the lipid membrane [13]. Simultaneously, the content of organic carbon in the applied solutions of humic acids showed considerable variation in quantity, which determined their mobility and ability to form complexes with metals.

The influence of organo-metallic complexes on the surface layer of membranes and theoretical considerations have been described [27, 28]. Experiments with trans-membrane voltage confirmed significant differences between the effects of HA-A and HA-B on the membranes. When the membrane separating the measuring chamber was under the influence of HA-A admixtures there was increased electrical resistance. This can attest to the stiffening structure and gradual blocking of the transportation of ions located on both sides of the membrane.

A clear understanding of physical, chemical and electrical properties of cell membranes can be useful in the study of mechanisms involved in the transport of substances and information across biological membranes. This has also been suggested by other authors [13-16].

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