NUKLEONIKA 2013;58(3):439–442

ORIGINAL PAPER

The impact of humic substances on the liposome structures: ESR method

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Abstract. In this paper the changes of membrane fluidity of liposome with additions of humic substances (humic and fulvic acids) were examined. Liposome were done by the sonication of lecithin EYL. Concentrations of humic substances in attitude to EYL varied between 0–10% of weight. The technique of electron spin resonance (ESR) were used for the examination followed by three spin probes with a variety placement of the membrane located. TEMPO probe melted in the hydrophobic membrane and in the aquatic solution which allowed to determine the spectroscopic partition parameter (F), indicating the changes that occur in water-lipid interphase. Probe 5-DOXYL placed directly under the heads of polar lipids and the order parameter measuring by the T_{II} showed the changing of membrane fluidity at surface area. 16-DOXYL probe penetrated the middle of the lipid bilayer membrane and allowed to determine the rotational correlation time τ parameter, which gives us information about changing of the liquidity lipid bilayer. Studies showed that the tested humic substances significantly changed the membrane fluidity of liposome. The dynamics of this process depends on both: the fraction of humic substances and its quality and quantity as well as the placement area of the membrane.

Key words: EYL liposome • ESR method • humic substances

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Received: 4 December 2012
Accepted: 20 January 2013

Introduction

Humic substances are important components of soils, aquatic ecosystems, shallow-sea sediments, surface sediments of lakes, which besides biogenic elements, can control the matter and energy cycle in ecosystems. Humic substances are amorphous, yellow, brown or black, hydrophilic, acidic, polydisperse substances with molecular weights ranging from several hundreds to tens of thousands [2]. Based on their solubility in alkali and acid humic substances usually are divided into two fractions: humic acids (soluble in alkali) and fulvic acids (soluble in alkali and acids). Soil humic substances may stimulate or inhibit the activity of microorganisms and plant growth through the activation of several mechanisms at microorganisms cell or root-cell membrane level and affect their metabolism [2, 13, 14]. Usually different interactions are observed as the impact of humic and fulvic acids on a variety of biological elements in the environment. Many review articles show that organic substances, including humic substances, intake to lipid membrane can change their physical properties [4–7]. Described investigations focused on the changing after induce variety concentration of organic substances introduced to membranes [1, 8, 11, 12] and different time of impact it on the lipid bilayer [9, 10].

The aim of our work was to determine physical processes of liposome under the admixture of humic substances: humic acids and fulvic acids. The liposome

of lecithin EYL were used. Concentration of humic substances attitude to lecithin were changed from 0 to 10% of weight. The electron spin resonance (ESR) using spin probes penetrating three different regions of the lipid bilayer was used to determine the changes in membrane fluidity. This allowed to obtain information about the impact of humic acid and fulvic acid on membrane fluidity in its different regions: the water--lipid interphase, the outer lipid layer just beneath the polar part of the membrane and in the middle, strongly hydrophobic parts.

Materials and methods

Liposome was originated by the sonication process of natural lecithin in distilled water originated from hen egg yolk (EYL). Lecithin was processed at the Institute of Chemistry, Opole University. The sample volume of 1 cm^3 was sonicated by a ultrasonic disintegrator (TECH-PAN UD-20) in six alternating cycles in order to prevent its overheating – 30 s of sonication, 30 s of cooling. The EYL concentration in the sample was 40 μM. Spin probes were added to the liposome at a concentration of 0.001 M to attitude of lecithin (one molecule of probe in a thousand molecules of lecithin). Three spin probe were used in our work (TEMPO, 5-DOXYL and 16-DOXYL) which penetrated the different areas of the membrane. Humic substances were extracted from peat using 0.5 M NaOH solution. Humic and fulvic acid extraction and purification was carried out by the Schnitzer method [16]. The optical properties of 0.001% of humic substances in the alkaline extract were determined (light absorption coefficient at wavelength: 280, 472, 664 nm) by the Sapek and Sapek method [15]. Also, ratios of $A_{2/4}$, $A_{2/6}$, $A_{4/6}$ were calculated. Humic substances were inducted into liquid dispersion of liposomes in water solutions. Since the structure of humic substances is complicated, the concentration of humic and fulvic acids attitude to lecithin was varied from 0 to 10%.

Measurements were performed at a constant temperature of 22°C. Spectra were recorded using the following instrumental settings: time constant – 0.3 s, modulation amplitude – 0.8×10^{-1} mT, scan time – 256 s (spectrometer EPR SE/X-28 Wrocław University of Technology). The technique of electron spin resonance (ESR) was used to study the changes in membrane fluidity of liposome under the impact of humic substances. Used spin probes were chosen in order to give information from a different areas of membrane. The probe TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), located in both the hydrophobic part of the membrane and in the water solution, showed changes occurring in lipid-water interphase. According to the spectrum of this probe (Fig. 1a) the spectroscopic parameters – spectroscopic partition parameter (F) was determined, which is associated with membrane fluidity [19]. A measure of parameter (F) is the ratio of the line of high – field amplitude EPR dissolved in an aqueous medium (P) (observed in the spectrum) to the amplitude of the low – field line in lipid treatment (H). The increase of parameter F indicates an increase in membrane fluidity. The spectrum of the probe 16-DOXYL (2-ethyl--2-(15-methoxy-15-oxopentadecyl)-4,4-dimethyl-3-

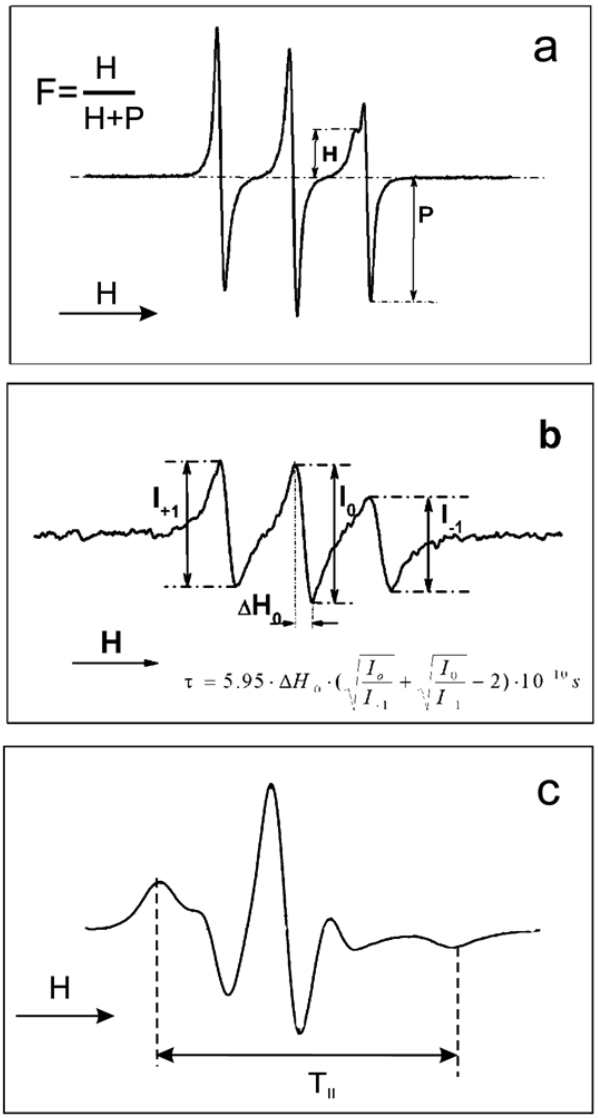

Fig. 1. ESR spectra of spin probes placed in a water dispersion of liposome EYL (a) TEMPO, (b) 16-DOXYL, (c) 5-DOXYL.

-oxazolidinyloxy) gave information about the interior of the lipid bilayer through the so-called parameter τ (rotation correlation time) (Fig. 1b). This parameter, inter alia, depends on the degree of membrane fluidity [3]. The decrease in τ indicates an increase in liquidity of the interior of the lipid bilayer. The probe 5-DOXYL (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy stearic acid) was located in the surface of the lipid bilayer, indicating changes of the area between the polar part of the membrane and its hydrophobic interior (Fig. 2c). Based on the spectrum of this probe, the T_{II} spectroscopic parameter (coefficient ordering), associated with this area of membrane fluidity [17] was determined.

Analysis of the spectroscopic parameters (F, T_{II}, and τ) provides information about the dynamic properties of the tested membrane under the impact of varying concentrations of humic substances. All measurements were performed in three replicates. The results presented in the paper are the arithmetic means. The relative measurement errors were, respectively: 2% for the parameter F and T_{II}, and 3% for the parameter τ.

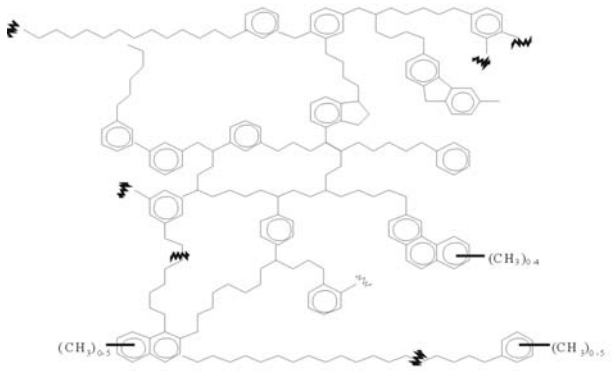

Fig. 2. Proposed chemical structure for humic acids by Schulten [18].

Results and discussion

Data available suggest that structurally humic and fulvic acids are similar to each other although they differ in molecular weight, ultimate analysis, have a variety of functional groups and their content. Besides, the chemical structure of humic and fulvic acids have been a subject for much research, still their definition constitute a scientific problem [2]. To date, a hypothesis for humus formation and the possible molecular structure of humic substances, is presented in Fig. 2.

Analysis of the fractional composition of organic matter in peat bases using the Sapek and Sapek method [15] indicates diversity in the quality of used humic substances. In this method, a single peat extraction with 0.5 M sodium hydroxide is used. The solution prepared with sodium hydroxide and humic substances, showed a light absorption coefficient in ultraviolet (A_{280}) and in visible light (A_{472} and A_{664}), which allowed for the assessment of the degree of humification of organic matter. The value of A_{280} depends on the content of the alkali extract of the lignin-type compounds. A higher value of this parameter indicates the presence of compounds which are not easily humificated. Alkali extract absorbance value at a wavelength of 472 nm (A_{472}), indicates the presence of humic substances in the early stages of humification. The size of the alkali extract absorbance value index is positively correlated with the number of early forms of humic acids formed in the beginning stages of the transformation of organic matter. This fraction of humic substances is characterized by a high mobility. A_{664} value of the coefficient, indicates the participation of compounds of a darker colour and, therefore, the highest degree of humification. Used fulvic acids were characterized by lower stages of humification and a higher mobility than in humic acids. It was also confirmed by $A_{2/4}$, $A_{2/6}$, $A_{4/6}$ ratios, which determines the degree of humification of humic substances (Table 1).

In Fig. 3 the changes of spectral parameters of spin probes located in the membranes of liposome (obtained by a sonic process EYL) in the water treatment under the impact of additions of humic and fulvic acids were presented.

In Fig. 3a the changes of spectroscopic parameters F, distribution coefficient for spin probe TEMPO as a function of the concentration of humic acids was showed. The increase of the value of this parameter indicates an increase in the surface layer of membrane fluidity. Reduction of its value indicates a lower liquidity of this part of the membrane. Analyses of the figure show that both fractions: humic and fulvic acids stiffened surface area of the lipid bilayer, as demonstrated by decreasing the value for F also with increasing of concentration of humic substances. There was a slightly stronger impact of humic acids on the liquidity of the surface layer – a faster value decline of the parameter F, especially for fulvic acid concentrations from 0–1.5% and 9–10%. The range of humic and fulvic acid concentrations from 2.5 to 9% of the effect of was similar.

The changes of spectroscopic parameters – the rotation correlation time τ as a function of humic substances concentration was showed in Fig. 3b. The increase of value of the parameter τ indicates stiffening of the central area of lipid bilayer membranes (longer probe rotation time), while reducing the value of the parameter indicates an increase of their liquidity (faster rotation time). The figure shows that the humic acids significantly stiffening central region of lipid bilayer, as demonstrated by the increase τ parameter values with higher concentration of humic acids. Fulvic acids showed differences in activity and significantly increased the liquidity of the lipid bilayer center. A particularly

Table 1. Optical properties of used humic substances

Humic substances	A_{280}	A_{472}	A_{664}	A_2/A_4	A_2/A_6	A_4/A_6
Fulvic acids	6.49	0.68	0.09	9.54	68.32	7.16
Humic acids	17.64	2.18	0.35	8.10	51.13	6.32

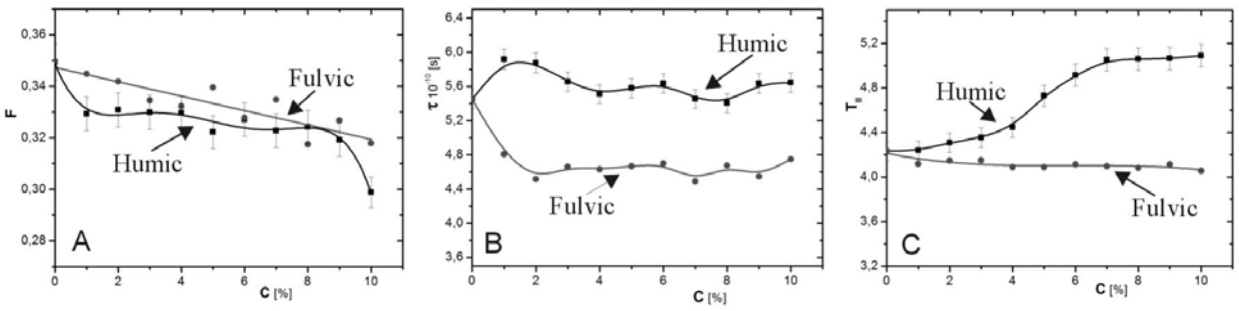

Fig. 3. Changing of the spectroscopic parameters as a function of humic substances concentration. (a) F – for probe TEMPO, (b) τ – for probe 16-DOXYL, (c) T_{II} – for probe 5-DOXYL.

strong influence of the parameter τ on the value of fulvic acids concentration range from 0 to 2% was observed. Above this level of concentration parameter τ changed slightly (changes in the margin of error). The changes in spectroscopic parameters T_{II} order parameter as a function of humic and fulvic acids concentration showed in Fig. 3c. Increase the value of parameter T_{II} indicates the lipid bilayer stiffening located between the polar heads of membrane and its central part. The figure also showed that the humic acids significantly stiffening surface area of the lipid bilayer, as demonstrated by increase in the value of the parameter T_{II}. This effect was strongly state with increasing concentration of humic acids. Fulvic acids did not show impact on changes in membrane fluidity of the analyzed area – the curve almost parallel to the axis of concentrations throughout the range.

Conclusions

UV-vis spectroscopy, especially at 280, 472 and 664 nm, has been successfully applied to the study of maturity of humic substances. The composition of humic substances of used humic and fulvic acids were characterized by the optical properties that showed the changes of the content of lignin, carboxyl and alkyl carbon and level of aromatic structures. Qualitative optical differences were reflected in the values at ratios of $A_{2/4}$, $A_{2/6}$, $A_{4/6}$.

The results of ESR analysis showed differently affect fulvic and humic acids on the fluidity of the membrane of liposome. Fulvic acids made the liquid of the central area of the membrane, also the surface area was slightly changed. This phenomenon may indicate that humic substances take placement in the middle part of the lipid bilayer. This process could show the loosening of bonds between hydrocarbon chains of molecules what build the membrane phospholipids. Humic acids made stiff the membrane surface area, and slightly affected the central, hydrophobic part of lipid bilayer. This may provide that humic compounds took the placement on the surface of the membrane, stringing it with additional bonds, which could lead to stiffening of the structure. A similar phenomenon was observed in the literature for membranes doped with cholesterol, in which takes place a stabilizing membrane fluidity.

Acknowledgment. We thank Dalia Gosik, native speaker, for proofreading.

References

1. Boniewska-Bernacka E, Man D, Słota R, Broda M (2011) Effect of tin and lead chlorotriphenyl – analogues on selected living cells. J Biochem Mol Toxicol 25;4:231–237
2. Choudhry GG (1984) Humic substances: structural, photophysical, photochemical and free radical aspects and interactions with environmental chemicals. Gordon and Breach Science Publishers, New York
3. Hemminga MA (1983) Interpretation of ESR and saturation transfer ESR spectra of spin labeled lipids and membranes. Chem Phys Lipids 32:323–383
4. Immordino ML, Brusa P, Arpicco S, Stella B, Dosio F, Cattel L (2003) Preparation, characterization, cytotoxicity and pharmacokinetics of liposomes containing docetaxel. J Control Release 91:417–429
5. Kleszczyńska H, Sarapuk J, Przestalski S (1999) Destabilization of model membranes by organotin compounds. Folia Histochem Cytobiol 37:1–31
6. Man D (2008) Fluidity of liposome membranes doped with organic tin compounds: ESR study. J Liposome Res 18:225–234
7. Man D, Olchawa R, Kubica K (2009) Membrane fluidity and the surface properties of the lipid bilayer: ESR experiment and computer simulation. J Liposome Res 20:211–218
8. Man D, Podolak M, Engel G (2006) The influence of tin compounds on the dynamic properties of liposome membranes: A study using the ESR method. Cell Mol Biol Lett 11:56–61
9. Man D, Podolak M, Engel E, Boniewska E (2009) Effect of chlorotriphenyl derivatives of Sn and Pb upon biophysical properties of membranes. J Biomed Biotechnol, doi:10.1155/2009/969480 (7 pp)
10. Man D, Słota R, Broda MA, Mele G, Li J (2011) Metalloporphyrin intercalation in liposome membranes: ESR study. J Biol Inorg Chem 16;1:173–181
11. Man D, Słota R, Mele G, Li J (2008) Fluidity of liposome membranes doped with metalloporphyrins: ESR study. Z Naturforsch C 65:440–444
12. Mitrus S, Man D (2012) Effect of tin and lead chlorotriphenyl analogues on fruit fly Drosophila hydei and liposomes membrane. J Biochem Mol Toxicol 26;4:162–167
13. Moliszewska EB, Pisarek I (2004) Activity of bacteria strains originated from sewage sludge against some soil fungi. Soil Sci Plant Nutr 50;6:807–814
14. Pisarek I (2007) The influence of sewage sludge on the direction of the humic substances transformation and some soil fertility of the Cambisol developer from silt. Studia i Monografie Uniwersytetu Opolskiego no. 385 (in Polish)
15. Sapek B, Sapek A (1986) The use of 0.5 M sodium hydroxide extract for characterizing humic substances from organic formations. Roczniki Gleboznawcze 37;2/3:139–147 (in Polish)
16. Schnitzer M, Khan SU (1978) Soil organic matter. Elsevier, Amsterdam
17. Schreier S, Polnaszek CF, Smith ICP (1978) Spin labels in membranes problems in practice. Biochim Biophys Acta 515:375–436
18. Schulten HR, Plage B, Schnitzer M (1991) A chemical structure for humic substances. Naturwissenschaften 78:311–312
19. Shimshick EJ, McConnell HM (1973) Lateral phase separation in phospholipid membranes. Biochemistry 12:2351–2360