

Antioxidant properties of ganglioside micelles

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Abstract

Antioxidant activity of gangliosides GM1 and GT1b in the Fenton type of reaction was investigated by EPR spectroscopy using DMPO as a spin trap. Hydroxyl radical spin adduct signal intensity was significantly reduced in the presence of gangliosides at their micellar concentrations. Mean micellar hydrodynamic diameter was not changed, whereas significant changes in negative Zeta potential values were observed as evidenced by Zetasizer Nano ZS. This study showed that the primary mode of ganglioside action was not due to direct scavenging of OH·, but rather to the inhibition of hydroxyl radical formation. This phenomenon is related to the ability of ganglioside micelles to bind oppositely charged ferrous ions, thus reducing their concentration and consequently inhibiting OH· formation.

Keywords: Gangliosides, micelles, free radicals, antioxidants, electron paramagnetic resonance, Zeta potential

Introduction

Gangliosides, multifunctional molecules of a glycosphingolipid class, are associated with the plasma membrane of all mammalian cells and have been found to be involved in the regulation of a wide range of biological processes [1]. They provide cells with antigenic and adhesive properties and modulate signal transduction [2]. In addition to these naturally occurring, endogenous manifestations, exogenously administered gangliosides have been observed to also affect many cellular functions. Several reports have suggested that gangliosides might exhibit antioxidant activity, particularly on lipid peroxidation [3–7], enzymatic antioxidant defences [8,9] and viability of rat brain neurons [10]. Beside findings of the protective effects of gangliosides on neuronal injuries, some studies have also reported on the protective

action of gangliosides against reactive oxygen species formation in isolated rat heart [11], epithelial lens and retinal cells [12], human LDL [13] and hepatocytes [14]. Evidence of the antioxidant activity of gangliosides has been obtained with either an individual ganglioside type or a mixture of brain gangliosides. The mode of action of gangliosides includes different mechanisms, such as changes in membrane fluidity and membrane stabilization. Scavenging of hydroxyl radicals by gangliosides has also been reported based on an indirect radical detection method [7]. In our previous studies we have established the protective role of gangliosides on sperm membrane fluidity changes induced by external oxidative stimulus [15,16] and demonstrated the anti-oxidative activity of some types of gangliosides against the production of reactive oxygen species [17]. We have also reported on the inhibitory effect

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of trisialoganglioside GT1b, but not monosialoganglioside GM1, on propagation of experimentally induced lipid peroxidation chain reaction in plasma membrane [17]. Studies on the molecular structure of gangliosides have shown that the number of sugar units, position and linkage type of sialic acids distinguish one ganglioside from another [18] and affect their physicochemical properties [19]. Yamaguchi et al. [20] have reported that the effect of gangliosides may be attributed not only to the size of carbohydrate chain, but also to the positional distribution of the anionic charge, such as the terminal sialic acid. The chemical structure of GM1 and GT1b molecules is shown in Scheme 1 [21]. Both gangliosides have the same hydrophobic and differently structured hydrophilic parts, the latter being made up of several sugar rings, some of which are sialic acid residues. GM1 has five sugar rings and only one carboxyl group linked to the inner galactose, whereas GT1b has seven sugar units with a second carboxyl group attached at A and a third carboxyl group attached in 4. Gangliosides form micelles at very low concentrations (of 10^{-9} – 10^{-8} M order) with a relatively large aggregation number having a bulky headgroup layer, the size of which is comparable to that of the hydrophobic core [1,22]. Different properties of ganglioside micelles (the aggregation number and size) can be attributed to the hydrophilic headgroups, i.e. to the number of sugar rings and carboxylic groups [23]. The decrease in the number of sugar rings and carboxylic groups is accompanied by the decrease of the interfacial area required by the molecule, an increase in packing parameter and a consequent increase in micellar size and transition to vesicles [1,22,24–26].

In this study, the effect of GM1 and GT1b on free radical generation was investigated in two typical *in vitro* oxidation models. Hydroxyl radical yield was studied in the Fenton type of reaction, while superoxide radicals were generated using a xanthine/xanthine oxidase system. Both of these free radicals, although of extremely short lifetime, were detected directly by electron paramagnetic resonance spectroscopy (EPR) using spin trapping techniques [27]. Mean hydrodynamic diameter and Zeta potential (comparable to the potential of micellar surface) of

GM1 and GT1b micelles were measured to elucidate their different antioxidant activity.

Materials and methods

Chemicals

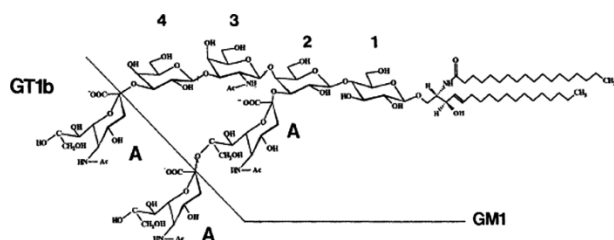
The spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO), xanthine, xanthine oxidase (grade III), superoxide dismutase, deferoxamine mesylate, chelex 100 sodium (50–100 dry mesh), dimethyl sulphoxide (DMSO) and gangliosides GM1, GD1a, GD1b and GT1b of a guaranteed reagent grade were purchased from Sigma Chemical Co. (St. Louis–Aldrich Corp., MO, USA). Ammonium iron (II) sulphate hexahydrate, hydrogen peroxide (analytical grade) and phosphate salts were from Kemika (Zagreb, Croatia). DMPO was purified with activated charcoal and stored under nitrogen at -70°C before use.

EPR experiments

Hydroxyl and superoxide radicals were detected by EPR spectroscopy using a DMPO spin trap. The experiments were performed in glass capillaries (inner diameter of 1 mm) on an X-band Varian E-109 spectrometer. Data were collected using the supplied software [28]. The spectra were recorded at room temperature and analysed by EasySpin software package [29].

Spin trapping of the hydroxyl radicals. Hydroxyl radicals ($\text{OH}\cdot$) were generated by decomposition of H_2O_2 by ferrous ions (Fenton reaction), which in the presence of the spin trap DMPO form the DMPO-OH spin adduct, exhibiting a characteristic EPR spectrum [30]. Dimethyl sulphoxide was used as an $\text{OH}\cdot$ scavenger to verify that the reaction system was actually producing $\text{OH}\cdot$ radicals. DMPO was dissolved in chelex-pre-treated phosphate buffer (PB) (0.1 M, pH 7.4), while ammonium iron (II) sulphate hexahydrate was dissolved in distilled water and deoxygenated with N_2 . Fresh Fe^{2+} and H_2O_2 stock solutions were prepared prior to each experiment. The reaction mixture contained PB, DMPO (0.1 M), H_2O_2 (0.5 mM) and ammonium iron (II) sulphate hexahydrate (0.075 mM), which were added to the reaction mixture following spin trap in the experiments including gangliosides. Exactly 2 min after ammonium iron (II) sulphate hexahydrate had been added, EPR spectra of DMPO-OH were detected with the following EPR spectrometer settings: 10 mW microwave power, 0.1 mT modulation amplitude and 100 kHz modulation frequency. The EPR spectral intensity was estimated from the low field peak amplitude.

Spin trapping of superoxide radicals. Superoxide radicals were generated by xanthine/xanthine oxidase



Scheme 1. Structure of gangliosides GM1 and GT1b [21]; 1, 2, 3 and 4 denote the sugar units and A denotes sialic acid residues.

system reaction. Xanthine oxidase catalyses the oxidation of xanthine in the presence of molecular oxygen, yielding uric acid and O_2^- as reaction products [31]. In the presence of the DMPO spin trap, O_2^- leads to the production of DMPO-OOH adduct, which can be analysed by EPR spectroscopy [27]. Superoxide trapping in the xanthine/xanthine oxidase reaction was verified by adding 65 U/ml superoxide dismutase which inhibits O_2^- generation. The reaction mixture contained PB, deferoxamine mesylate (2 mM), which prevents any reaction of superoxide radicals with traces of iron [32], DMPO (0.1 M), xanthine (0.32 $\mu\text{M}/\text{ml}$) and xanthine oxidase (0.33 U/ml). In the experiments which included gangliosides, these were added to the reaction mixture following the spin trap. Exactly 58 s after the addition of xanthine oxidase, EPR spectra of DMPO-OOH were detected with the following EPR spectrometer settings: 20 mW microwave power, 0.1 mT modulation amplitude and 100 kHz modulation frequency.

The mean hydrodynamic diameter of GM1 and GT1b micelles

The mean hydrodynamic diameter (using dynamic light scattering (DLS)) of ganglioside micelles was measured by a Zetasizer Nano ZS (Malvern, UK) equipped with a 532 nm 'green' laser. Detection occurred at 173° angle in glass cuvettes. A latex standard of uniform particle size of 20 nm was used to evaluate the accuracy of the measurements. The mean hydrodynamic diameter (d) was estimated using Debye-Einstein-Stokes equation:

$$d = k_B T / 3\pi \eta D$$

where k_B is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the dispersing medium and D is the apparent diffusion coefficient.

The Zeta potential measurements of GM1 and GT1b micelles

The Zeta potential (using Laser Doppler Electrophoresis) of ganglioside micelles was measured by Zetasizer Nano ZS (Malvern, UK). The Zeta potential (ζ/mV) was estimated from electrophoretic measurements using Henry equation:

$$U_e = 2\varepsilon\zeta f(Ka) / 3\eta$$

where ζ is the zeta potential, ε is the dielectric constant, U_e is the electrophoretic mobility and η is the viscosity. $f(Ka)$ is in this case 1.5 and is referred to as the Smoluchowski approximation. Deviations ranged within ± 1 mV.

Statistics

Statistical analysis was performed using a statistical package (Complete StatSoft CSS, Tulsa, OK, USA).

Data are expressed as mean \pm SEM of at least five experiments. A Wilcoxon matched pairs test was used to reveal significant differences between samples with and without gangliosides. P -values < 0.05 were considered statistically significant.

Results

Effect of GM1 and GT1b on hydroxyl radical generation in the Fenton reaction

The effect of different gangliosides on hydroxyl radical generation in the Fenton reaction was tested by the addition of 200 μM of each of the substances to the reaction mixture and analysed by EPR spectroscopy (Figure 1A). The four-line spectrum, characteristic of hydroxyl radical spin adduct, with the hyperfine coupling constants from nitrogen, $a^N = 1.498$ mT and β -hydrogen, $a^H = 1.475$ mT, was in accordance with the published data [33]. In the presence of GD1a, GD1b, GT1b and GM1, the amount of radicals was decreased by 30, 42, 50 and 52%, respectively, in comparison to control samples (bar graph in Figure 1B). As gangliosides GM1 and GT1b exhibited a stronger inhibitory effect, they were used in further experiments. The effect of different concentrations of these two gangliosides (5–200 μM) on the DMPO-OH spin adduct formation was also investigated (Figure 2). The comparison of the effects of GM1 and GT1b revealed a significant decrease of EPR signal intensity already at a concentration of 75 μM of GT1b, while the effect of GM1 was observed at a concentration of 150 μM and above (both $p < 0.05$; $n = 5$).

In an attempt to distinguish between the inhibitory effect of gangliosides on hydroxyl radical generation and ganglioside scavenging of hydroxyl radicals produced by Fenton reaction, the concentration of DMPO spin trap was varied [34–36], as presented in Figure 3. This approach was based on the underlying idea that the substance acting as a free radical scavenger would compete with spin trap for free radicals in such a way that the reaction rate would depend on spin trap concentration. However, the change in the concentration of DMPO by one order of magnitude did not influence the concentration of gangliosides required to induce a drop in the hydroxyl radical spin adduct formation to 50% of its value as compared to the samples without gangliosides. It could therefore be concluded that gangliosides inhibit radical formation in the Fenton type of reaction rather than act as their scavengers.

Effect of GM1 and GT1b on superoxide radical formation in the xanthine/xanthine oxidase system

The EPR study of spin adduct formation in xanthine/xanthine oxidase system is presented in Figure 4. The superoxide radicals were identified by spectral

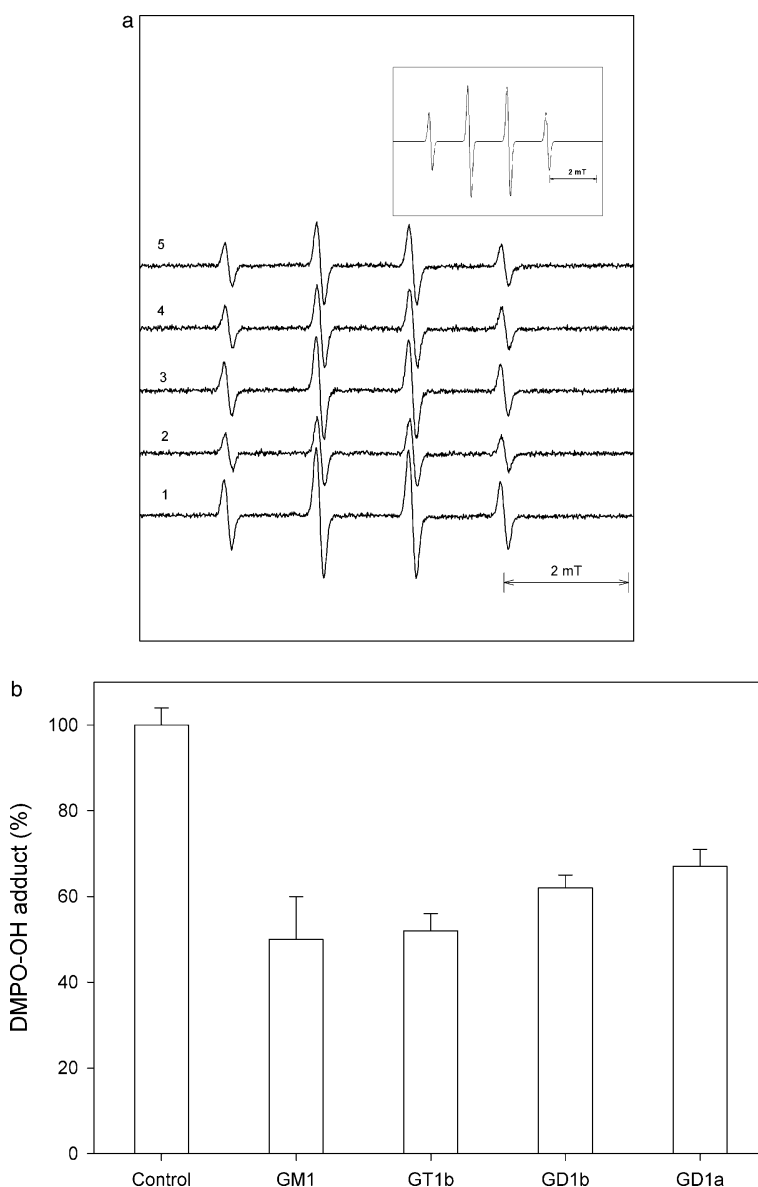


Figure 1. The EPR study of the influence of different gangliosides (200 μM) on the formation of DMPO-OH in the Fenton type of reaction. The following EPR spectra (a) are presented: sample without gangliosides (1) and samples containing GM1 (2), GD1a (3), GD1b (4) and GT1b (5). In the insert to the figure a typical simulation of the experimental spectrum is presented. The analysis of experimental data (b) is presented as the percentage of DMPO-OH spin adduct signal decrease in the presence of GM1, GT1b, GD1b and GD1a. Data are normalized to the EPR intensity of control samples without gangliosides.

simulation, revealing the apparent hyperfine splitting constants for DMPO-OOH spin adduct ($a^{\text{N}} = 1.43$ mT, $a^{\text{H}\beta} = 1.14$ mT and $a^{\text{H}\gamma} = 0.115$ mT), the result being in conformity with the published literature [37]. As the lifetime of superoxide is short with respect to the EPR time scale, hydroxyl radicals as superoxide 'end-products' contributed to the acquired spectra. In the presence of 200 μM GT1b no clear change in DMPO-OOH spin adduct signal could be observed as compared to the sample without gangliosides. The same was obtained with GM1 (data not shown).

The measurements of mean hydrodynamic diameter of GM1 and GT1b micelles

In this study GM1 and GT1b were applied at concentrations higher than their critical micellar concentrations so as to relate their respective micellar properties to their potential anti-oxidative effects. The mean hydrodynamic diameter of ganglioside micelles in phosphate buffer (0.1 M) was 10.9 ± 0.3 and 8.9 ± 0.2 nm for GM1 and GT1b, respectively. These results are in agreement with those from the literature showing that GM1 micelles are slightly larger than GT1b micelles [22]. The addition of the

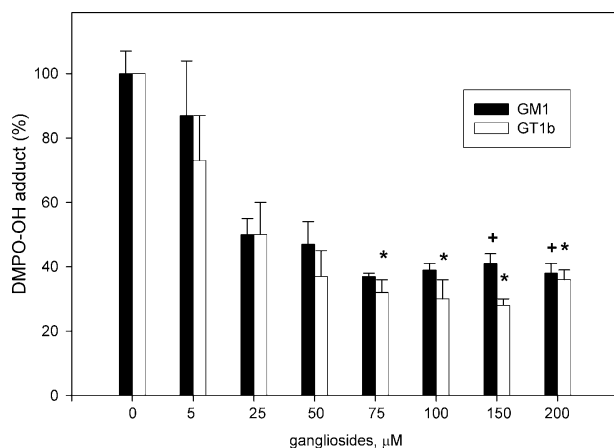


Figure 2. The EPR study of DMPO-OH spin adduct formation in the Fenton type of reaction in the presence and absence of different concentrations of GM1 (■) and GT1b (□). Data are normalized with respect to the EPR intensity of control samples without gangliosides. Significant differences are $^+p < 0.05$ for GM1 and $*p < 0.05$ for GT1b as compared to control values.

Fenton's reagent to ganglioside micellar solutions did not significantly affect mean hydrodynamic diameters of both ganglioside micelles (11.1 ± 0.01 and 9.4 ± 0.4 nm, respectively).

The measurements of the Zeta potential of GM1 and GT1b micelles

Results of Zeta potential measurements for two different GM1 and GT1b concentrations before and after the addition of ferrous ions (at a concentration corresponding to that of Fenton's reagent) are shown in Table I. Ganglioside micelles are negatively charged due to the presence of sialic acid. In comparison to GM1, GT1b micelles exhibit a slightly higher negative charge due to their sialic acid content. The addition of ferrous ions changed the absolute values of Zeta potential. Table I shows that these

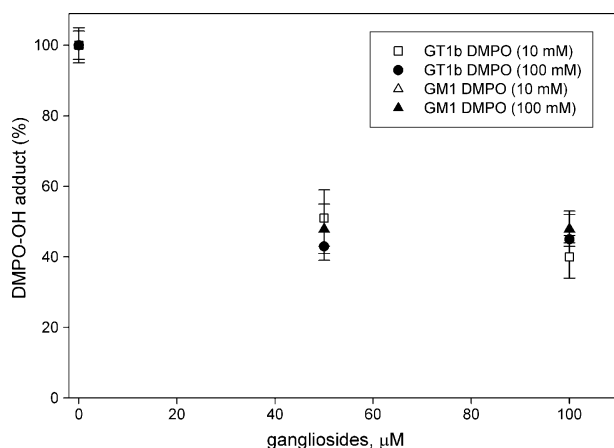


Figure 3. The EPR study of DMPO-OH formation as a function of spin trap concentration in the Fenton type of reaction in the presence and absence of gangliosides. Data are normalized with respect to the EPR intensity of the samples not containing gangliosides.

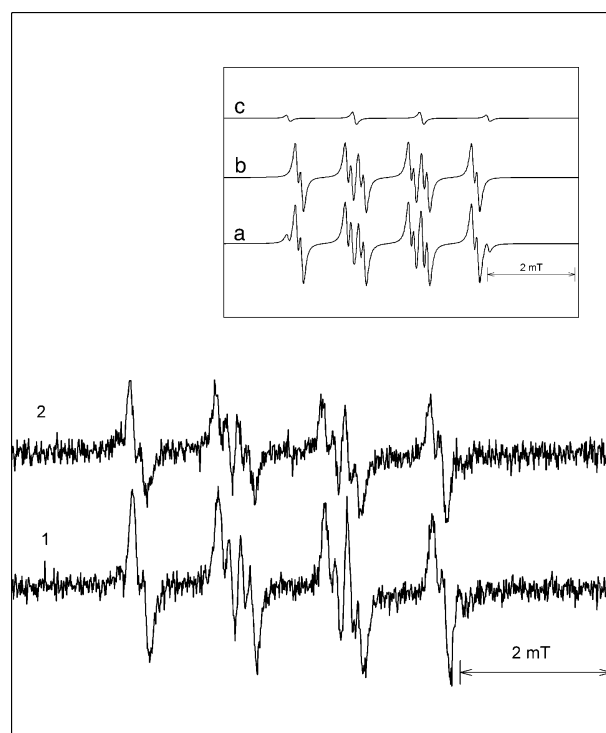


Figure 4. The EPR study of DMPO-OOH spin adduct formation in xanthine/xanthine oxidase system. The presented spectra refer to the samples: without gangliosides (1) and those in the presence of $200 \mu\text{M}$ GT1b (2). The results of the simulation of the experimental spectrum without gangliosides (1) by EasySpin software package reveal that in the acquired spectrum (a) the superoxide spin adduct contributes with 85% (b) and hydroxyl radical spin adduct with 15% (c) (insert to the figure).

changes were more pronounced in the presence of GM1 micelles than in the presence of GT1b micelles.

Discussion

In this investigation we focused on the mechanism of action of gangliosides involved in oxidative processes in a cell-free system. The research was prompted by our previous observation that certain types of gangliosides in *in vitro* lipid peroxidation model could inhibit changes in cell membrane molecular ordering [17]. Therefore, we studied the involvement of gangliosides in free radical oxidation processes using two different model systems generating either hydroxyl radicals or superoxide anions.

The analysis of EPR signal intensity indicated that the content of hydroxyl radical spin adducts was lower in the presence of gangliosides at concentrations above their respective critical micellar concentrations in comparison with the samples without gangliosides. This effect was studied in detail for GM1 and GT1b, as their specific molecular structure differs both in the number of sialic acid residues and in their linkage type. The experimental evidence showed that the influence of GT1b on the detected EPR signal intensity was significant at a lower

Table I. The Zeta potential (ζ /mV) of ganglioside (Gn) micelles in systems: Gn+PB (phosphate buffer) and Gn+PB+Fe²⁺ (75 μ M).

System	Zeta potential (ζ /mV)	
	Gn+PB	Gn+PB+Fe ²⁺
GM1 (20 μ M)	-2.4	-8.3
GM1 (200 μ M)	-4.8	-11.9
GT1b (20 μ M)	-7.1	-22.0
GT1b (200 μ M)	-10.2	-23.2

concentration (75 μ M) as compared to that of GM1 (150 μ M). This result is in accordance with the published data [14] which has shown that 60 μ M GM1 was unable to scavenge hydroxyl radicals in a cell-free system and inhibit iron-induced lipid peroxidation of rat hepatocytes. Moreover, an insignificant effect of 100 μ M GM1 on sperm membrane lipid peroxidation has been detected [17].

In order to clarify whether changes in EPR signal intensity of DMPO-OH spin adduct in the presence of gangliosides were due to the scavenging of hydroxyl radicals or to direct inhibition of hydroxyl radical formation in Fenton reaction, further experiments were performed. The change in DMPO concentration by one order of magnitude showed that the spin trap did not compete with gangliosides in the elimination of free radicals from the system, indicating that the primary mode of ganglioside action in the Fenton reaction was *via* the inhibition of hydroxyl radical formation. This might suggest that an interaction between gangliosides and ferrous ions in this system renders metal ions inaccessible to the reaction.

Binding of ferrous ions to negatively charged ganglioside micelles might have caused the decrease in their concentration in bulk solution, thus decreasing OH \cdot production. There might be at least two processes responsible for ferrous ion binding to ganglioside micelles: (i) complexation with polysaccharides and (ii) electrostatic binding of Fe²⁺ ions as counterions to ganglioside micelles. The antioxidant activity of various polysaccharides has been found to arise from their ability to complex Fe²⁺ ions, rendering them inactive or poorly active in a Fenton reaction [38]. On the other hand, the addition of an inorganic electrolyte to the ionic micelles causes electrostatic binding of counterions at the micelle/solution interface. As in a surfactant solution containing counterions of uni- and multivalent type ionic micelles predominantly bind multivalent ions [39], ferrous ions could be considered to strongly bind to negative ganglioside micelles. It should be stressed that for both types of gangliosides micellar size remained almost the same upon ferrous ions binding, whereas the Zeta potential exhibited more negative instead of expected more positive values.

The measured increase in absolute Zeta potential value indicated that binding of Fe²⁺ ions to ganglio-

side micelles represented a more complex phenomenon than just a 'simple' binding. This can be attributed to the specificity of bulky ganglioside headgroup and the extreme variability of the Fe⁺³/Fe⁺² redox potential. Ganglioside micelles differ significantly from the majority of conventional ionic surfactants due to their bulky headgroup. Charged oligosaccharide heads with a large conformational flexibility can adopt two main spatial arrangements corresponding to tighter (α arrangement) or looser (β arrangement) packing [40]. The conformational transition between two main arrangements is very sensitive to variations occurring in the bulk phase, such as ganglioside concentration and/or concentration and type of added electrolyte. GT1b, carrying a bulkier head than GM1, shows a conformational transition between two main packing states at a lower concentration than GM1. A conformational transition between two packing states following binding of ferrous ions may cause redistribution of charges, thus contributing to changes of electrophoretic mobility, i.e. Zeta potential values. Pasquale et al. [41] have pointed to the effect of the position of charges in the micelle/solution interface on the electrophoretic mobility, showing that charges at the hydrophobic micellar core exert a smaller effect on the mobility than charges located somewhere in the hydrophilic layer of micelle.

It is well known that a series of chemical and photochemical reactions involving iron complexes and hydrogen peroxide followed by the formation of many reactive species occurs in the bulk phase. The Fe³⁺/Fe²⁺ redox potential at physiological pH depends on the concentration and type of complexing agent. The formation of hydroxyl radicals can be catalysed only if two conditions are simultaneously met: (i) the ferric complex is reduced into its ferrous state and (ii) the ferrous complex has a redox potential allowing the Fenton reaction [42]. In the presence of ganglioside micelles the redox potential of ferrous complex allowing Fenton reaction in bulk solution, i.e. the transfer of one electron to H₂O₂ and OH \cdot production, is not completely realized. On the other hand, changes in Zeta potential values indicated that ferrous ion binding initiated some processes inside the hydrophilic layer of micelles, which requires further investigation. Molecular damage of ganglioside amino sugar by a localized Fenton reaction seemed unlikely, as the Fe²⁺/H₂O₂ system has not been found to destroy N-acetyl aminosugars, as opposed to the Cu²⁺/H₂O₂ system [43].

The study of the effect of gangliosides on superoxide anion production in a cell-free system revealed no significant decrease in the EPR signal of DMPO-OOH adduct could be observed in the presence of gangliosides even at a concentration of up to 200 μ M. This indicated that gangliosides did not act primarily as superoxide anion scavengers.

In conclusion, data obtained in our study using an EPR technique clearly demonstrated that the ganglioside micelles inhibited iron-catalysed hydroxyl radical formation due to their iron chelation potential, rather than to their hydroxyl radical scavenging capacity. The increase in the absolute Zeta potential values could be ascribed to the binding of ferrous ions to the oppositely charged ganglioside micelles and processes occurring inside the bulky headgroup layer. Greater antioxidant activity of GT1b micelles than that of GM1 micelles can be attributed to their differently structured hydrophilic parts, i.e. to the higher content of sugar units and sialic acid residue as well as to conformational transition at a lower concentration of GT1b.

Acknowledgements

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