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ORIGINAL PAPER

Influence of Freezing and Low Molecular Weight Cryoprotectants on Microsomal Membrane Structure: A Study by Multiparametric Fluorescent Probe

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Abstract The influence of low molecular weight cryoprotectants (CPs) such as glycerol (GL), 1,2-propanediol (PD) and dimethylsulfoxide (DMSO) on the structure of rat liver microsomal membranes on the stages of equilibration and upon freezing up to $-196^{\circ}C$ was studied using a multiparametric fluorescent probe of flavonol nature. It was estimated that the studied CPs have individual concentration ranges defining low amplitude of their action on biomembranes. An exceeding of these ranges strongly increases the violation of membrane native structure already at the stage of incubation with CPs, strengthening it during the freezing procedure. According to the perturbation effect on microsomal membranes the studied CPs can be arranged in a sequence: DMSO > PD > GL.

Keywords Microsomes · Cryoprotectants · Glycerol · 1,2-propanediol · Dimethylsulfoxide · Freezing · Fluorescent probe · 3-hydroxyflavone · Flavonol · Fluorescence

Introduction

The long-term storage of the human and animal cells at low temperatures now finds wide applications in biology, medicine, biotechnology and other spheres of human activity. Thus, successful choice and optimal requirements to artificial cryoprotecting materials introduced into cell suspensions in many respects define the outcome of cryoconservation process [1]. The important problem on this field is the preservation of structural and functional integrity of one of the most cryolabile cell components which are the membranes [2].

The data accumulated on the modification of biomembranes functional properties at the presence of low molecular weight CPs, which are barrier function [2], passive and active carrier [3], binding and receptor activity, proteins and nucleic acids metabolism etc. [4] can be an indirect illustration in favour of their interaction with biomembranes. Depending on their chemical structure, low molecular weight CPs can act as inhibitors of membrane enzymes of competitive (glycerol) or non-competitive (DMSO) types [5].

At the same time the collected data allows us to consider as an established fact the immediate interaction of CPs molecules with biomembranes [6–8]. CPs are capable to modify lipid-lipid and lipid-protein interactions, reducing, smoothing or eliminating the phase transition processes in membranes [9]. They can be partially adsorbed on the membranes or can interact with their charged groups changing the surface potential [10]. The essential influence on the results of the CPs interaction with lipid bilayer has lipid phase physicochemical properties and the presence of cholesterol in membranes [11].

However, despite considerable amount of works devoted to the study of cryoprotectants' action on membranes, at present time there is no complete comprehension of molecular mechanisms their action on cryoconservation stages.

Fluorescent methods are widely applied in the analysis of bioobject structural modifications, including biomembranes, due to their high sensitivity and informativity. In addition, the use of artificially created fluorescent labels or probes with

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known location in membrane allows to receive detailed, often unique information about bilayer structural state [12, 13]. In particular, a series of new fluorescent probes from 3-hydroxyflavones (flavonols) recently have shown to be highly sensitive to the parameters of environment [14–16].

The fluorescent probes of 3-hydroxyflavone class are capable to isomerization in the excited state, forming normal (N*) and tautomer (T*) species [17]. This reaction is known as an excited state intramolecular proton transfer (ESIPT). Each of the forms has fluorescent properties, owing to which the observation of two bands of green-blue (N* form) and yellow-orange emission (T* form) becames possible in the fluorescence spectrum. The position and intensity of each form depends not only on the probe chemical structure [18–20], but also on the parameters of its molecule microenvironment, such as polarity, viscosity and the existence of intermolecular hydrogen bond donors [21–24].

3-Hydroxyflavones favourably differ from other classes of fluorescent probes as they are multiparametric molecular devices (see [23, 25]), allowing simultaneous determination of several physical parameters such as medium local dielectric permeability and refractive index, and also–to distinguish environment polarity changes caused by hydrogen bonding from effects of non-specific intermolecular interactions [26]. The most successfully fluorescent probes of this class are applied for the study of processes in lipid membranes on the molecular level [27–31]. The above mentioned data have prompted us to use one of the probe–3-hydroxy-4′-(*N*,*N*-dimethylamino)flavone (FME) in the present work.

Microsomal membranes are convenient model for the study of CPs action mechanisms on biomembranes. They are natural lipid-protein formations and, despite the presence of haemoproteins, which are included in the microsomal electron transport chain, they do not absorb the light in the visible range so intensively, as haemoglobin in erythrocytes. Thus, using the spectrofluorimetry method with FME probe application in the present work we have studied an influence of low molecular weight cryoprotectants (CPs) such as glycerol (GL), 1,2-propanediol (CH_3–CHOH–CH_2OH, PD) and dimethylsulfoxide (Me_2SO, DMSO) on rat liver microsome membranes both in native state and after freeze-thawing procedure.

Materials and methods

Materials

FME probe was synthesized as described in [32]. According to the TLC analysis (carried out on "Silufol" plates 150 mm × 50 mm, eluent–chloroform-methanol mixture with the component ratio from 98:2 to 9:1), the probe didn't have any impurities. Before using the FME probe solution was diluted with ethanol to 1 mM concentration and 10 μl of the last solution was added to the 2 ml of membrane suspension. The FME probe final concentration in samples was 5×10^{-6} M. The fluorescence spectra were recorded immediately after probe addition, since it binds with membranes very fast.

1,2-Propanediol, dimethyl sulfoxide, glycerol, tris (hydroxymethyl)aminometane (tris), EDTA and KCl (res. grade) were from "Sigma-Aldrich Russia". All solutions were prepared in twice distilled water, and CPs were additionally purified before using. Glycerol was purified by two-fold vacuum distillation [33]. DMSO and PD were previously kept over aliminium oxide or over absorbite of the brand "A" [34] and then purified in the same manner. All CPs solutions were prepared by weighing. Their concentrations are expressed in mass percentage (mas. %).

Preparation of microsomes

Microsomes of rat livers were obtained by differential centrifugation method during 90 min at 105,000*g* in a solution containing 1.15% KCl and 0.5 mM EDTA, pH 7.4 [35]. After this microsomes were resuspended in 50 mM tris-buffer, pH 7.4. The samples were kept on ice and used immediately after isolation. The experiments were carried out on five separately obtained samples with protein content of 0.1–0.75 mg/ml. The content of protein in microsomal suspension was determined by the Lowry method [36].

Microsomal membranes suspension was frozen in 3.0 ml polystyrole ampules by immersion in liquid nitrogen (–196°C) and were cooled at a middle rate of about 200°C/min. After finishing of crystallisation the samples were thawed in a water bath (40°C).

Fluorescence measurements

Fluorescence spectra were registered on the Hitachi F-4010 spectrofluorimeter (Japan) with authomatical spectrum correction. The monochromator slits width was 5 and 3 nm respectively. FME fluorescense was excited at 405 nm and registered at 425–700 nm. All measurements were done in thermostated sample holder at 20 ± 1°C.

The analysis of obtained spectra was carried out using Microcal Origin 6.1 software. The positions of N*-band maxima were calculated according to its second derivatives.

Results and discussion

Comparative characteristics of FME microenvironment in aqueous solutions and microsomal membranes

The fluorescence spectra of FME probe in the presence of microsomal membranes have the main intensive

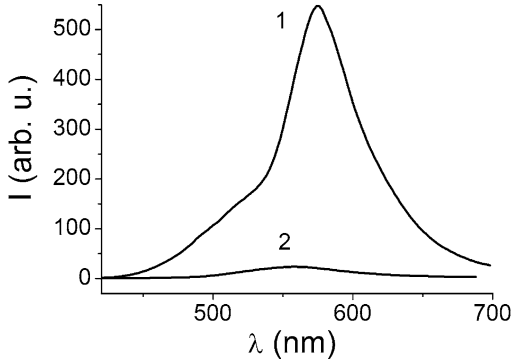

Fig. 1 Fluorescence spectra of FME probe in microsomal membranes (1) and in 50 mM tris buffer, pH 7.4 (2)

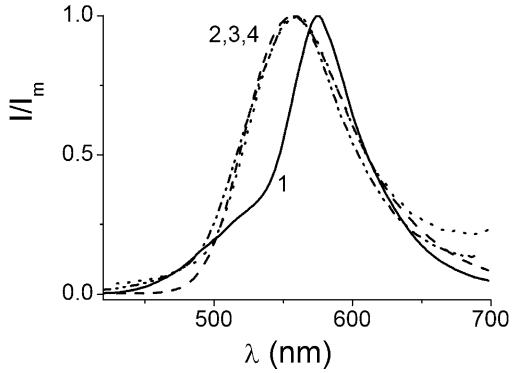

Fig. 2 Normalized fluorescence spectra of FME probe in microsomal membranes (1) and in 20% solutions of CPs (2, 3, 4)

longwavelength band located at 575 ± 1 nm (T*-band) and a shoulder near 510–520 nm (N*-band). In the last case the 2nd derivative of the spectrum points on the position of maximum at 515 ± 1 nm. Fluorescence intensity of the probe in tris-buffer is low and consists less than 5% of the emission intensity in microsomal membranes (Fig. 1). This is connected mainly with high viscosity of probe environment in the membrane [37]. The ratio of fluorescence band intensities I_{N^*}/I_{T^*}, (defined as I_{515}/I_{575}) in microsomal membranes depends on the content of proteins and lipids and can change from 0.27 ± 0.02 (at high protein level) to 0.56 ± 0.02 (at low protein level), while in tris-buffer there is no any expressed T*-band, or the shoulder in the spectra.

The spectrum of FME probe in microsomal membranes is presented by two bands where the longwavewlength T*-band has in several times higher intensity (see Fig. 1). This fact reflects a relatively low polarity of probe microenvironment. At the same time the position of N*-band, considerably shifted to the red and thus expressed as a shoulder in the spectrum can point on highly polar and hydrated surroundings of a part of probe molecules. All together the observations testify about quite heterogeneous distribution of probe in the membrane [38]. This conclusion is in a good agreement with the known data about high heterogeneity of microsomal membrane lipids and protein compositions [39, 40].

In the presence of CPs (GL, PD and DMSO) the fluorescence spectra of FME probe are presented by one-component curves (Fig. 2). The maximum position in each spectrum is located on several nanometers at shorter wavelengths than in tris-buffer ($\lambda_{max} = 555$ nm) and correlate well with the hydrophobicity parameter of the used CPs (Table 1). The hydrophobicity of CPs is reflected by their distribution coefficients in chlorophorm-water system [41]. Meantime, the probe quantum yield depending on the viscosity of solution [42], is the highest for GL and the smallest for DMSO solutions.

Taking into attantion the short wavelength positions and higher quantum yields of FME probe in aqueous solutions of CPs comparing to that in tris-buffer, one can conclude that in a aqueous solutions all CPs form a solvation shell around the FME molecules and, depending on the nature of CPs, these shell differs on the composition, viscosity and polarity. The most polar environment makes up the tris-(hydroxymethyl)-aminometane zwitterions, if to take into account the most longwavelength position of emission maximum, whereas the most rigid environment for FME molecules is created by the glycerol surroundings. This suggestion follows from the highest value of quantum yield of the probe in glycerol solution (see Table 1).

Additionally in all the spectra from aqueous solutions no dual fluorescence is observed for the FME probe, as in the 50 mM tris-buffer as in the solutions of CPs. In all of these cases the FME fluorescence is presented by a wide single band that can be an evidence of homogeneous microenvironment of the probe molecules. Taking into account the fluorescent properties of FME probe in so high polar environment it may be concluded, that in all cases only the fluorescence of the N*-form of FME is observed, the position of which is shifted considerably toward to longwavelengths. The two-band fluorescence can be observed in much less

Table I Fluorescence parameters of FME probe in aqueous solutions of cps and tris-buffer

Solution composition	Relative QY (%)	Maximum position (nm)	CPs distribution coefficients in chloroform–water system	Viscosity of 10% CP solution at 0°C, sP
10% GL	260	554 ± 0.2	0.00001	3.02
10% PD	240	552 ± 0.5	0.002	2.83
10% DMSO	190	546 ± 0.2	0.05	2.50
50 mM tris-buffer, pH 7.2	100	555 ± 2	–	–

polar surroundings, for example–in biomembranes [27–31]. In these cases the probe incorporation into membranes may be detected not only by the appearance of the second band, but also by the multi-fold increase of total fluorescence intensity (see Figs. 1 and 2).

Influence of CPs and freeze-thawing procedure on the FME probe fluorescence in microsomal membranes

The most appropriate parameter that alows to monitor the microsomal membrane structural changes is the fluorescence intensity ratio I_{515}/I_{575}. This parameter directly reflects the polarity of FME probe surroundings [43]. With the increase of concentration of CPs in bulk solution the changes of the I_{515}/I_{575} ratio shows the changes of polarity of FME probe surroundings both towards some its decrease (reflected as a fall of the I_{515}/I_{575} ratio) or towards its increase (reflected as the growth of the ratio, Fig. 3). In each case the changes of the polarity corresponds to the changes of the concentration of water in the sites of probe location [27, 30, 38]. The direction of the changes of the I_{515}/I_{575} ratio depends on the chemical structure of the CP as well as on its concentration in solution. Some delay of the changes of I_{515}/I_{575} ratio that is obserwed at high CP concentrations can be ascribed to aggregation of the vesicles.

All the investigated CPs in their concentrations up to 10% do not cause any essential influence on the I_{515}/I_{575} intensity ratio of the FME emission from microsomal membranes. The I_{515}/I_{575} parameter changes lightly depending on the chemical structure of the added CPs. For the GL in its concentration range from 5 to 10% a trend to decrease of the I_{515}/I_{575} ratio is observed that, probably, is a result of the decrease of the polarity of membrane surface due to a sorption of CP molecules and partial substitution of water molecules by the CP ones [44, 45]. For all the investigated CPs at their concentrations higher than 10% the more expressed changes of the I_{515}/I_{575} ratio is observed (see Fig. 3). These changes are different on amplitude and reflect different level of the

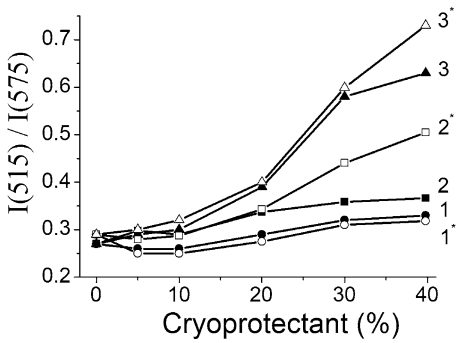

Fig. 3 Influence of CPs and freeze-thawing procedure on the intensity ratio I_{515}/I_{575} of FME probe fluorescence in the membranes of rat liver microsomes: 1—GL; 2—PD; 3—DMSO. The index (*) corresponds to the curves that were obtained after freeze-thawing

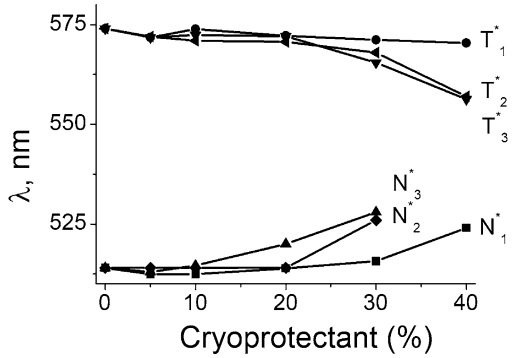

Fig. 4 Influence of CPs on the position of N* and T* band fluorescence maxima of FME probe in the membranes of rat liver microsomes. The indexes 1, 2 and 3 correspond to GL, PD and DMSO respectively

influence of CP on the microsomal membranes. For example, the GL and PD produce smaller perturbations of the membrane state in the whole range of their concentrations, as it can be seen from the changes of I_{515}/I_{575} ratio. The influence of GL is the smallest. At the same time an increased concentrations of DMSO cause a sharp raise of the I_{515}/I_{575} ratio.

To evaluate more precisely the position of N* band we used the second derivatives of fluorescence spectra. The obtained data show that the increase of concentration of CP in the incubation medium of microsome results in the red shift of N*-band and in the opposite shift of T*-band in the spectrum (Fig. 4). The most pronounced are the changes of position of N*-band. They take place for the solutions of GL starting from its content of 25–30%, for PD—starting from 20%, and for DMSO—starting from 10% concentrations. After the analysis of spectral behavior of FME probe in more simple systems [46] such spectral shift can be attributed to the influence of increased quantities of hydrogen bond donor molecules in probe surroundings, that under the experimental conditions corresponds to the raise of water molecule concentration (probably, bound with CPs molecules) in lipid-protein membranes.

The investigations carried out in a wide kit of solvents for the analog of FME probe have shown, that the position of N*-band has linear correlation with the medium polarity index E_T^N and with function of local dielectric constant $f(\varepsilon)$. This feature allows to utillize two mentioned parameters for the evaluation of polarity of probe microenvironment [47]. Since the changes of N*-band maximum position in the presence of CPs reflects the classical positive solvatochromism of the probe, it is possible to conclude that the polarity of probe microenvironment in the membrane sites increases with the raise of CP concentration. Taking into account the changes of T* band position it can be suggested that the increase of polarity is not caused by the CPs molecules themselves, but rather water molecules involved into hydrogen bonded complexes with them.

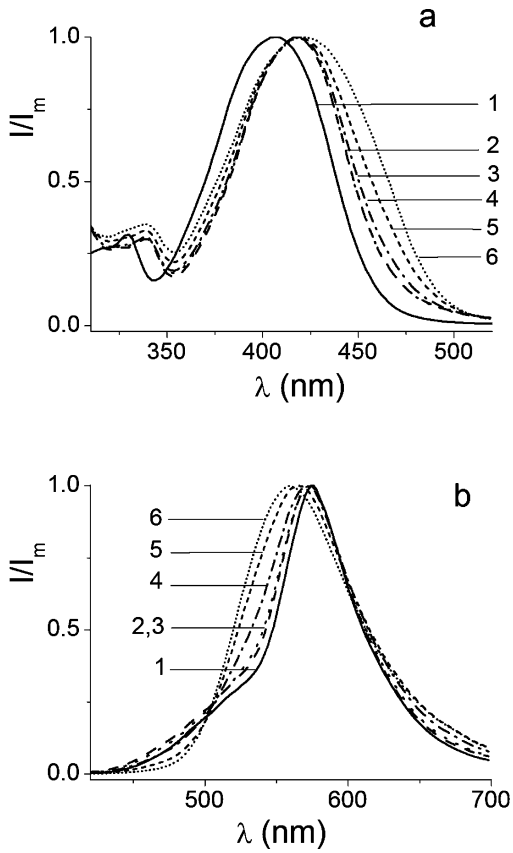

Fig. 5 Normalized excitation (a) and emission (b) spectra of FME probe in microsomal membranes in the presence of different concentrations of PD: 1–0%; 2–5%; 3–10%; 4–20%; 5–30%; 6–40%

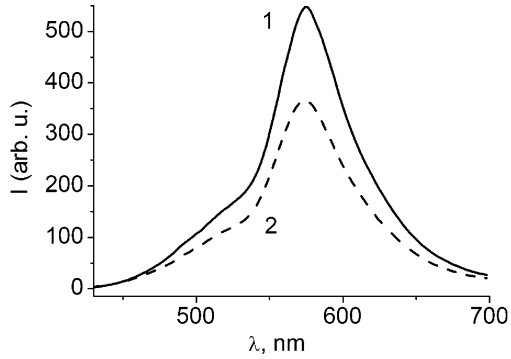

Fig. 6 Effect of freeze-thawing procedure on the FME fluorescence spectra in microsomes: 1–control; 2–after freeze-thawing

The smaller distance between N* and T* bands of FME observed in PD and DMSO at their concentrations of 30–40% results in disappearance of two-band structure of the spectrum (Fig. 5). Probably, more hydrophobic molecules of PD and DMSO in their concentration ranges displace the FME probe molecules out of the hydrophobic sites of the membrane toward to the aqueous medium, where the probe display single-band fluorescence. The essential decrease of probe fluorescence intensity at high CP concentrations in the incubation medium supports this assumption. At these concentrations some perturbations of native packaging of lipid bilayer can occur in the region of location of fatty acid chains, where the FME probe is partially localized [43]. Our results on the changes of light scattering by the microsomal membrane suspension, obtained during the spectrophotometric study are in line with this assumption [48].

After freeze-thawing procedure of microsomes in tris-buffer the decrease of fluorescence intensity together with some rise of two band fluorescence ratio is observed (Fig. 6). This testifies the decrease of rigidity and some increase of polarity in the sites of probe location, which can be caused by the raise of surface area of microsomal membranes and their accessibility for water molecules initiated by temperature-dependent conformational changes of proteins and lipids.

Freezing and subsequent thawing of microsomal suspension in the presence of glycerol practically do not cause any additional spectral effects in the whole range of concentrations, whereas PD in the concentrations more than 20% and DMSO at the concentrations more than 30% cause further increase of the I_{N^*}/I_{T^*} ratio, if to compare the data with and without freezing (see Fig. 3). The merging of N* and T* bands of FME fluorescence is also observed and is the most expressed after freezing at high concentrations of CPs (not shown).

Comparison of the dependences of I_{515}/I_{575} ratio versus cryoprotectant concentration (see Fig. 3) shows that all of them are smooth and the course of each is defined mainly by the nature of the organic additive themselves. The additional modification of biomembranes by freesing has small influence expressed only in a course of the curve. Well-expressed influence of freeze-thawing procedure take place only at high concentrations of CPs and connected probably with intense perturbations of biomembrane packaging that can results in essential alterations of their structural and functional properties.

The investigated CPs can be refered to the substances which are capable to create intermolecular hydrogen bonds with water molecules as hydrogen bond acceptors. Additionally polyols GL and PD can serve as hydrogen bond donors with water molecules or with themselves. It is considered that the mechanism of action of these materials has mainly colligative basis stipulated by their hydrophylic nature [49]. Meantime the DMSO molecules contain two hydrophobic CH_3-groups and have more hydrophobic properties. This allows them to penetrate deeply in lipid membrane as in pure state as in the complex with water molecules [50].

Minor changes of the fluorescence parameters of FME probe at the moderate concentrations of CPs can be connected mainly with the effects stipulated by the ability of these substances to be localized near the membrane surface, partially substituting the water molecules [51, 52], and thus

changing the viscosity and dielectric properties of nearest microenvironment, including the polar head groups of phospholipids [53], changing their mobility and influencing the membrane surface potential [10]. The deaper penetration of DMSO molecules can increase the withdrawing of charged phosphate groups and result in modification of the bilayer packing density [54]. The ability of DMSO to influence the stability of lipid matrix of cell membranes and to change its permeability is described elsewhere [55–57].

Thus, the obtained results point that the explored CPs disturb the microsomal membrane structure already at the equilibration stage, and these perturbations take place on both polar and hydrophobic regions of the membrane, influencing on functional properties of membrane integral proteins. The amplitude of disturbing influence of CPs is defined by their hydrophobic properties, having the most expressed effect in the case of the most hydrophobic substances like PD and DMSO.

Also it is possible to assume on the collected data that specific interactions such as hydrogen bonds both between the molecules of water and CPs and lipid-protein components of membranes (OH-groups of thyrosine residues, or NH-groups of tryptophan, or polar head groups of lipids etc.) play an important role in the mechanisms of influence of the CPs on the microsomal membranes state and functions.

Conclusions

Using the multiparametric probe of flavonol nature it was established, that low molecular weight cryoprotectants (CPs) such as GL, PD and DMSO in the concentrations less than 20% do not influence essentially on the state of membranes of rat liver microsomes. They cause perturbations of the membranes at concentrations of 30% and more. The violation of the membrane native structure is caused by penetration of considerable amounts of the hydrated molecules of CPs in the region of fatty acid ester groups. In this process the hydrophobic properties of CPs play the key role. More hydrophobic molecules like PD or DMSO penetrate more easily into non-polar region of the membrane, in a greater scale disturbing its structure. The less hydrophobic molecules of GL influence dominantly on the lipid membrane surface.

For all explored CPs the character of structural changes of microsomal membranes is defined mainly by the chemical nature of CPs and their concentration. The CPs have an influence on the membrane structure already on the equilibration stage. The additional perturbation of biomembranes by freesing procedure changes their state in a little degree only. At the same time high concentrations of CPs, equal to 30–40% coupled with the action of freeze-thawing brings to the irreversible transformations of biomembranes structure reflected in the loss of their functional properties.

Thus, the fluorescent probe FME that belongs to the class of flavonols has served as an indicator of biomembrane structural integrity perturbations, initiated by the action of organic cryprotectants and low temperatures. In this direction it can be successfully applied in cryobiology.

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