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Effect of Curcumin on Lateral Diffusion of Phosphatidylcholines in Saturated and Unsaturated Bilayers

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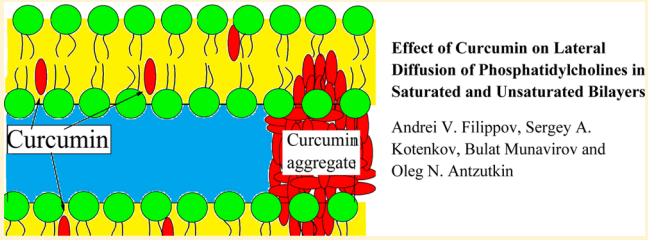
Supporting Information

ABSTRACT: Curcumin, a dietary polyphenol, is a natural spice with preventive and therapeutic potential for neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Curcumin possesses a spectrum of antioxidant, anti-inflammatory, anticarcinogenic, and antimutagenic properties. Because of this broad spectrum of pharmacological activity, it has been suggested that, like cholesterol, curcumin exerts its effect on a rather basic biological level, such as on lipid bilayers of biomembranes. The effect of curcumin on translational mobility of lipids in biomembranes has not yet been studied. In this work, we used ^{1}H NMR diffusometry to explore lateral diffusion in planar-oriented bilayers of dimyristoylphosphatidylcholine (DMPC) and dioleoylphosphatidylcholine (DOPC) at curcumin concentrations of up to 40 mol % and in the temperature range of 298−333 K. The presence of curcumin at much lower concentrations (∼7 mol %) leads to a decrease in the lateral diffusion coefficient of DOPC by a factor of 1.3 at lower temperatures and by a factor of 1.14 at higher temperatures. For DMPC, the diffusion coefficient decreases by a factor of 1.5 at lower temperatures and by a factor of 1.2 at higher temperatures. Further increasing the curcumin concentration has no effect. Comparison with cholesterol showed that curcumin and cholesterol influence lateral diffusion of lipids differently. The effect of curcumin is determined by its solubility in lipid bilayers, which is as low as 10 mol % that is much less than that of cholesterols 66 mol %.



1. INTRODUCTION

Curcumin (CUR), a yellow pigment, is the active ingredient of the natural spice turmeric, which is also used in traditional medicines.[1,2] The broad antioxidant, anticarcinogenic, antimutagenic, and anti-inflammatory properties of CUR and its derivatives explain its traditional medicinal use and have made CUR of particular interest for pharmaceutical drug development.

CUR has shown prophylactic and therapeutic capacity for Alzheimer's and Parkinson's diseases, which has been attributed to its interaction with fibrils of Alzheimer's amyloid β (Aβ) peptides,[3−5] as well as oligomers and protofibrils of Aβ.[3] Because of its broad spectrum of pharmacological activity for such a small molecule, it has been proposed that CUR, like cholesterol, acts on a rather basic biological level, such as on biomembranes. Indeed, CUR has a protective effect on erythrocyte membranes against primaquine-induced oxidative damage[6] that has been explained by complex formation of CUR with membrane proteins.[7] Furthermore, it has been proposed that CUR can regulate the action of membrane proteins indirectly by changing the physical properties of the membrane rather than by directly binding to the proteins.[8]

Liposomes from phospholipids can be used not only as a model to study lipid-CUR interaction, but also as nanocarriers allowing for stabilization and increasing solubility of CUR in aqueous environment.[9] CUR exerted a number of effects in experiments performed on lipid liposomes. Work by several groups[10−13] has shown that CUR can bind to the membrane in two modes: a surface-associated mode at low CUR concentrations and a trans-membrane mode at higher concentrations. A combination of solid-state NMR and DSC showed that CUR has a strong effect on membrane structure at low concentrations. CUR inserts deep into the membrane in a trans-bilayer orientation, anchored by hydrogen bonding to the phosphate group of lipids in a manner analogous to that of cholesterol,[14] increasing segmental ordering in the membrane.[9,14] CUR also can promote formation of the highly curved inverted hexagonal phase.[14] On the other hand, some spectroscopic experiments have demonstrated that encapsulation of CUR at higher concentrations disrupts the packing of the bilayer, leading to a looser and more flexible structure.[15] This phenomenon has been confirmed by a decrease in the gel-to-liquid phase transition temperature.[15]

The effect of CUR on the translational mobility of lipids, an important property of lipids in biomembranes, has not yet been studied, however. In this work, we selected phosphatidylcho-

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lines with saturated and unsaturated chains. ^{1}H NMR diffusometry was employed as a method to investigate the effect of CUR on lateral diffusion of these lipids in a wide range of temperatures and CUR concentrations in membranes.

2. MATERIALS AND METHODS

2.1. Sample Preparation. Phospholipids DOPC (dioleoylphosphatidylcholine), DMPC (dimyristoylphosphatidylcholine), and DMPC with deuterated hydrocarbon chains (DMPC-d_{54}) were purchased from Avanti Polar Lipids (Alabaster, AL). DMPC is the typical saturated-chain lipid, while DOPC contains one double bond in each of its hydrocarbon chains. Curcumin (>94% curcuminoid content, >80% CUR) and deuterated water (^2H_2O, 99.7%) were also purchased from Sigma. Structures of DOPC, DMPC, and CUR molecules are shown in Figure 1.

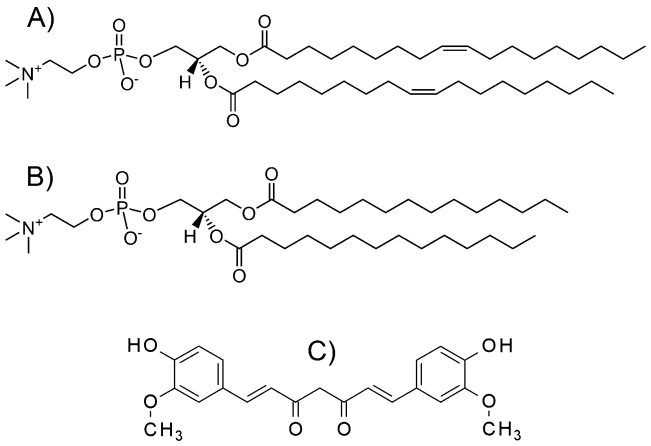

Figure 1. Structures of DOPC (A); DMPC (B); and curcumin (keto form) (C) molecules.

To prepare a vesicular sample the lipid and curcumin were dissolved in a sufficient amount of ethanol. The ethanol was then evaporated under a constant flow of dry nitrogen, then the sample was vacuum-pumped overnight to remove solvent traces. The resulting lipid film was hydrated using deionized water in 5:1 ratio. Five freeze−thaw cycles were applied using liquid nitrogen and warm water, which resulted in the formation of a homogeneous vesicular sample.

Macroscopically glass-plate-oriented lipid multibilayers with CUR were prepared following a previously described procedure.[16,17] The amount of DMPC or DOPC in each sample was 20 mg, while the CUR concentration varied from 0 to 40 mol %. The usage of the broad range of CUR concentration was motivated from one side by high loading capacity of egg yolk phosphatidylcholine vesicles, around 64 mol %.[9] Moreover, the maximum solubility of cholesterol, which supposed to have similar effect on lipid membrane as CUR, is around 66 mol %.[18] To prepare the sample, a solution of DOPC (or DMPC) with certain amount of CUR, containing 10 mg of lipid and 700 μL of ethanol was deposited by 25 μL on approximately 40 glass plates (5 × 14 × 0.08 mm^3). The solvent was evaporated first in air and then under vacuum overnight. The plates were stacked, placed in square cross-section tube, and hydrated in a humid atmosphere (D_2O) at 35 °C for 3−5 days. The degree of hydration, which reached around 23 wt %, was controlled by weighing the samples. The process of multibilayer formation was confirmed by ^{1}H NMR spectrum of the sample oriented at the "magic angle", i.e., 54.7° (similar to Figure S1 in Supporting Information (SI)).[19] The final adjustment of the amount of water (45 wt %) was made through a piece of filter paper placed on the top of the glass stack. At these conditions samples were fully hydrated, this was confirmed by comparison (lateral diffusion measurements) with overhydrated samples. These samples contained more than 60 wt % of water, partly in droplets inside the NMR tubes. Afterward, the sample was sealed. Views of oriented samples are shown in Figure S2 in SI. The square sample tube containing macroscopically oriented lipid multibilayers was placed in a specifically designed goniometer probe (Cryomagnet system, Indianopolis, IN).[19] By rotating the stack of glass plates, the bilayer normal can be oriented at the "magic angle" with respect to the magnetic field. This causes the dipolar interactions to vanish with a significant reduction of the line widths.[19]

2.2. ^{2}H and ^{31}P NMR Spectroscopy. The ^{2}H NMR spectra of DMPC-d_{54} vesicles were recorded in Bruker AVANCE III operating at a proton frequency of 400 MHz applying solid-echo pulse sequence[20] at 308 K. ^{2}H NMR frequency was 61.402 MHz, relaxation delay was 0.25 s, RF pulse duration was 25 μs, sweep width was 39 kHz, and acquisition time was 0.21 s; a total of 16k scans were collected. The ^{31}P NMR spectra were recorded at 145.70 MHz without proton decoupling, relaxation delay was 2 s, RF pulse duration was 33 μs, sweep width was 40 kHz, and acquisition time was 0.25 s. Totally 25k scans were collected.

2.3. ^{1}H NMR Diffusometry. A Chemagnetic InfinityPlus CMX-360 (Agilent, Fort Collins, CO) NMR spectrometer operating at proton frequency of 360 MHz was used. Details of the NMR diffusion measurements in oriented lipid membranes can be found elsewhere.[19] An NMR goniometer probe that enables macroscopically aligned bilayers to be oriented with the lipid bilayer normal at the magic angle (54.7°) with respect to the main magnetic field was used. Thermal rotation of the lipid molecules around their axis leads to the dipolar protons interactions to vanish; thus, the ^{1}H NMR spectrum of lipid molecules can be obtained without any additional averaging.[17] For all measurements, the stimulated echo pulse sequence was used (Figure S3 in SI).[21] The main stationary magnetic field and the pulsed field gradient were aligned in the same direction. Diffusion decays $A(k)$ were obtained, where $A(k)$ is the spectrum integral, $k = \gamma^2\delta^2g^2t_d$, γ is the ^{1}H gyromagnetic ratio, δ is the duration, and g is the amplitude of the gradient pulse, $t_d = (\Delta - \delta/3)$ is the diffusion time, and Δ is the duration between identical gradient pulses. $A(k)$ in the case of a one-component bulk liquid (e.g., pure water) depends on the experimental parameters as

$$A(k) = A(0)\exp(-k\cdot D) \quad (1)$$

where D is the diffusion coefficient. The amplitude of the pulsed field gradient was 1.15 T/m. In our experiments T_1 time delay (τ_1) was set to either 100 or 500 ms. At τ_1 = 100 ms we varied gradient pulse duration δ in the range of 1.1−8.7 ms stepwise (by 10 to 20 steps), while T_2 time delay (τ) in the pulse sequence was set to 11 ms. In the second case(τ_1 = 500 ms) to get the same dynamic range of the diffusion decay we varied δ in the range of 0.5−4.1 ms and τ was set to 6.5 ms. No effects of τ and τ_1 on measured diffusion decays were observed; therefore, most of the experiments were performed at τ_1 = 100 ms. The diffusion time at smallest delta (1.1 ms) is ∼110.6 ms, while at the largest delta (8.7 ms) it is ∼108.1 ms.

For a lipid membrane the observed diffusion is a combination of two processes, along the membrane (D_L) and perpendicular to the bilayer ($D_\perp$). Therefore, the observed diffusion coefficient D in the pulsed field gradient NMR experiment is[19]

$$D = D_L\cdot\sin^2\theta_{LD} + D_\perp\cdot\cos^2\theta_{LD} \quad (2)$$

where θ_{LD} is the angle between the bilayer normal and the main magnetic field (B_0) and $D_\perp$ is of orders of magnitude smaller than D_L. In our experiment B_0 was collinear with the field gradient direction, while θ_{LD} is the magic angle 54.7°; thus, $\sin^2\theta_{LD}$ = 2/3 and finally lateral diffusion coefficient of lipids can be obtained from D as D_L = 1.5·D.

3. RESULTS AND DISCUSSION

3.1. Lipid Phases and Ordering. Observation of resolved ^{1}H NMR spectra of oriented bilayers, which are "sensitive" to the orientation at magic angle in the entire range of CUR concentrations (from 0 to 40 mol %) (Figure S1 in SI), confirmed the formation of a lipid lamellar phase in all samples.

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As the magic angle of sample orientation θ_{LD} deviatesd by more than 3° from 54.7°, the spin−echo and stimulated echo signals diminished to zero. Therefore, no symptoms of nonlamellar phases, such as hexagonal or cubic phases, were observed. While the stimulated echo signal, which we use in the NMR diffusion experiment, originated entirely from the lamellar phase of lipids. Additionally, experiments performed on vesicles of DOPC (^{31}P NMR) and DMPC-d_{54} (^{31}P NMR and ^{2}H NMR) in the presence of CUR (Figures S4, S5 in SI) demonstrated that phosphorus and deuterium spectra have shapes typical for the lamellar phase of lipids.

Figure 2 shows a central part of the ^{2}H NMR spectra of vesicular DMPC-d_{54} with CUR and cholesterol, which is related

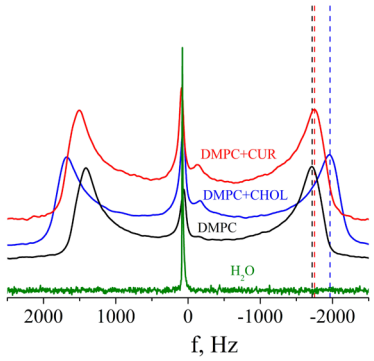

Figure 2. ^{2}H NMR spectra of pure DMPC-d_{54}, 5 mol % of cholesterol, 5 mol % of curcumin, and water (with natural ^{2}H content). T = 308 K.

with methylene's CD_3 groups of the lipid alkyl chains. The presence of CUR in the whole range of concentrations studied only slightly changed the quadrupolar splitting of the spectra (from 1.73 to 1.76 kHz), while the presence of 5 mol % of cholesterol (Figure 2) increased the quadrupolar splitting considerably more (to 1.96 kHz). A similar effect was observed in all the lines in ^{2}H NMR spectra of DMPC, corresponding to CD_2 groups of the lipid alkyl chains (Figure S6 in SI).

Integral values of ^{1}H NMR signals for samples oriented at the "magic angle" were dependent on the used lipid and on the concentration of CUR (Figure S1 in SI). NMR signal decreased with increasing CUR concentration. This might be conditioned by different interactions of lipid molecules with CUR, which is particularly apparent in the ordering of the lipid molecules and correlation times of the molecules' rotational mobility. We may note the gradual reduction in the NMR signals in both DOPC/CUR and DMPC/CUR series, corresponding to the progressive increase in the amount of CUR molecules interacting with lipid bilayers. The gradual reduction in ^{1}H and ^{31}P NMR signals (Figures S1 and S4, respectively) may also demonstrate that some fraction of lipid molecules have very short NMR relaxation. This is probably because of their participation in the formation of hard aggregates being associated with curcumin. But in this case they will not give any contribution in the signal of diffusing molecules.

3.2. Lipid Lateral Diffusion. Lamellar structure of both DOPC/CUR and DMPC/CUR systems allowed us to do lateral diffusion measurements. Diffusion decays of the ^{1}H spin−echo NMR for all lipid bilayers were exponential and slopes were not dependent on diffusion time, demonstrating homogeneity of the translational mobility in the bilayers in the time scale of these diffusion experiments (t_d >111 ms). Lateral diffusion coefficients of lipids (D_L) in DOPC/CUR and DMPC/CUR bilayers are shown in Figure 3A and B, respectively, by solid lines and symbols. It can be seen from

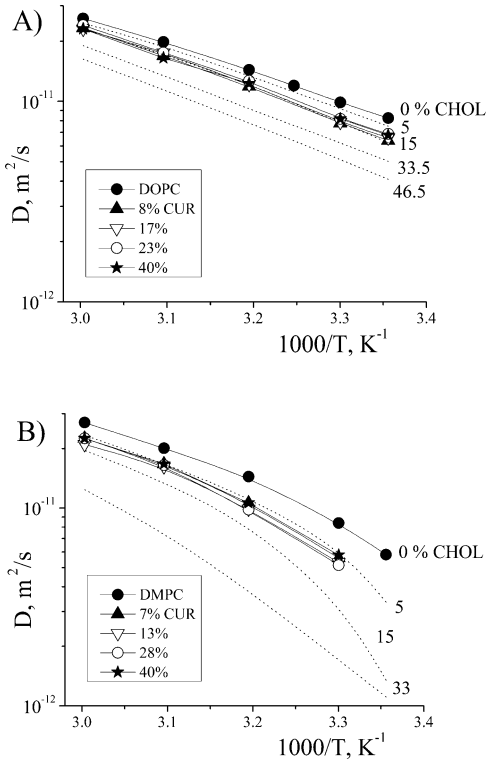

Figure 3. Arrhenius plots of temperature dependences of the lateral diffusion coefficient for DOPC/CUR (A) and DMPC/CUR (B) multibilayers at CUR concentrations from 0 to 40 mol % (solid symbols and solid lines). Errors of measurements do not exceed symbol sizes used in the figure. Dotted lines and legends on the left correspond to DOPC/CHOL and DMPC/CHOL bilayers,[16] shown for comparison.

these figures that the presence of CUR even in the smaller concentrations used in our study leads to a decrease in D_L. The second intriguing observation is that CUR offers the same effect on lateral diffusion independent of its concentration in the range from 7 to 40 mol % of CUR. An analysis showed that the presence of CUR (>8 mol %) decreases D_L of DOPC by a factor of 1.3 at lower temperatures and by a factor of 1.1 at higher temperatures. For DMPC bilayers, the presence of CUR (>7 mol %) decreases D_L by a factor of 1.5 at lower temperatures and by a factor of 1.2 at higher temperatures (see Figure 3).

From data presented in Figure 3, one can see that temperature dependences of D_L do not follow the Arrhenius law in the whole temperature range studied here. However, it is possible to estimate apparent activation energies of the diffusion process based on our experimental data (for example, at higher temperatures) and using the following Arrhenius equation:[22]

$$D_L = D_0 \exp\left(-\frac{E_D}{RT}\right) \quad (3)$$

where D_0 is the pre-exponential factor independent of temperature, E_D is the apparent activation energy for diffusion, RT is the thermal energy, R is the universal gas constant, and T is the temperature in Kelvin. Our calculations give $E_D \sim 29$ kJ/mol for DOPC/CUR bilayers and $E_D \sim 32$ kJ/mol for DMPC/

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CUR bilayers. By comparing with the corresponding values of "pure" bilayers (27 kJ/mol for DOPC and 31 kJ/mol for DMPC[16]), we may conclude that the presence of CUR slightly increases (around 3−8%) activation energies of lateral diffusion of lipids.

3.3. Comparison with Cholesterol. In a number of previous studies, the effect of CUR on the physical properties of lipid membranes has been compared with that of cholesterol (CHOL).[9,14] Indeed, both of these molecules, as suggested, may influence organism functions through changing properties of lipid membranes. Let us compare lateral diffusion in phospholipid membranes in the presence of CUR with that of CHOL. In Figure 3A,B, the temperature dependences of D_L at different CHOL concentrations (approximately in the same range as CUR concentrations in our study) are shown by dotted lines.[16,22] Increasing the CHOL concentration leads to a gradual decrease in the diffusion coefficients of both systems, DOPC/CHOL and DMPC/CHOL. At low concentrations of CHOL (<5 mol %), the corresponding dependences for bilayers with CHOL and CUR are close to one another. We can see from these figures that CUR reaches its maximum effects, which corresponds to ca. 15 mol % CHOL for DOPC and ca. 5 mol % CHOL for DMPC.

We also note that at the maximal concentrations of CHOL, D_L for DOPC decreases by a factor of 1.6−2, while for DMPC it decreases by a factor of 2.3−5.1. Therefore, the influence of CHOL at concentrations around 40 mol % on saturated bilayers of DMPC is more than twice as high in comparison with that on unsaturated bilayers of DOPC. This phenomenon has been related previously to the ordering of lipid chains and characterized as "saturated chains are more susceptible to ordering by CHOL than the unsaturated ones".[23] The influence of lipid chain unsaturation on D_L in the presence of CUR is smaller (1.1−1.3 for DOPC and 1.2−1.5 for DMPC).

Activation energies of diffusion in the presence of CHOL also gradually increase from 27 to 32 kJ/mol for DOPC and from 31 to 55 kJ/mol for DMPC,[16,22] which is much higher than those for CUR. Thus, the effect of CUR on lateral diffusion in bilayers of saturated and unsaturated lipids is comparable at low concentrations (<7−8 mol %) to that of CHOL, but much less comparable at higher concentrations. Therefore, CUR and cholesterol have a different effect on diffusion in studied lipid bilayers. The effect of CHOL is usually explained by the ordering of hydrocarbon "tails" of lipids near flat and rigid CHOL molecules without any specific interaction between lipids and CHOL.[24] This leads to a decrease in the lipid molecule-free area, which causes a decrease in the diffusion coefficient.[25] CUR is a more flexible molecule that possibly cannot order neighboring lipids to the same extent as CHOL even if CUR fully or partly penetrates into the lipid bilayer. Most probably, the CUR molecule simply binds to lipids through hydrogen bonding of −OH groups of CUR phenolic rings with the headgroup of phosphatidylcholine,[14,15] thereby increasing the size of the diffusing entity. This may explain effects that were observed in this study: no visible change in the ^{2}H NMR spectra and similar factors leading to the decrease of D_L in saturated and unsaturated bilayers.

3.4. Low Limit of CUR Solubility in Lipid Bilayers. What causes the observed difference of effects of CUR and cholesterol on the lateral diffusion of lipids in bilayers? It is known that the increase of concentration of cholesterol in lipid bilayers is limited by ∼66 mol %, its solubility limit.[18] Concerning the CUR solubility limit in lipid bilayers, it is known that the loading capacity of CUR liposomes prepared from the egg yolk phosphatidylcholine is 0.84 w/w %, i.e., 64 mol %. However, Figure S2 in SI shows that at concentrations of CUR higher than 7−8 mol % samples contain dark inclusions, which are aggregates of CUR. Further we suggested that solubility of CUR is quite low, as far as ∼10 mol % in both saturated and unsaturated lipid bilayers; therefore, the increase in concentration of CUR above this limit really does not change CUR molecule concentration solved inside the bilayer.

There is an apparent contradiction between our work and the previously published work of Karewicz et al.[9] results concerning solubility of CUR in lipid bilayers, as mentioned above. In our opinion, the high loading capacity of egg yolk PC liposomes can be conditioned by one of the three or all of these factors working together: (i) rather broad distribution of the hydrophobic chains lengths of egg yolk PC,[26] which is not typical for synthetic DOPC and DMPC; (ii) inclusion of insoluble CUR aggregates inside the liposomes; (iii) crude phospholipid extract from egg yolk may also contain some small amounts of different lipids, for example, phosphatidylethanolamine,[15] which can also modify CUR solubility. Any aspects of solubility of CUR in lipid systems have not been studied before; therefore, we cannot make a conclusion at this moment. Further work to investigate it is in progress and the results will be published elsewhere.

4. CONCLUSION

This study has shown that the lateral diffusion coefficient of lipids in phospholipid bilayers is reduced by addition of CUR. However, the effect of CUR is different from that exerted by CHOL. The presence of CUR even in low concentrations (up to 7−8 mol %) leads to a moderate decrease in the lateral diffusion coefficients, while further increase in the concentration has no effect. The factors leading to the diminishing effect of curcumin on lateral diffusion of lipids are similar in saturated and unsaturated lipid bilayers; it is the limited solubility of curcumin in lipid bilayers by around 10 mol %.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR spectra of oriented samples, photos of these samples, as far as ^{31}P and ^{2}H NMR spectra of multilamellar vesicles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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