X-Ray Reflectivity and Diffraction Studies of Doxorubicin Binding to Model Lipid Membranes

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Abstract

The effect of anticancer antibiotic doxorubicin on structural organization of anionic lipid monolayers has been studied. X-ray reflectivity and grazing incidence diffraction techniques were applied to monitor the changes in 2D structure and electron density distribution of Langmuir monolayer composed of negatively charged dipalmitoylphosphatidylglycerol (DPPG) and dioleoylphosphatidylserine (DOPS). For comparison, monolayer of zwitterionic dipalmitoylphosphatidylethanolamine (DPPE) also was investigated. The presented experimental results suggest that doxorubicin interaction with anionic lipid monolayers (DPPG and DOPS) proceeds preferentially via electrostatic attraction—positively charged amino groups of doxorubicin bind to negatively charged head groups of phospholipid molecules. Based on the obtained data, the penetration of doxorubicin into the hydrophobic part of anionic lipid monolayers does not occur. X-ray measurements on DPPE monolayer indicated that doxorubicin did not cause any significant alterations of molecular packing in condensed monolayer of zwitterionic DPPE molecules.

Keywords Doxorubicin · Langmuir monolayer · X-ray reflectivity · GID

1 Introduction

The anthracycline antibiotic doxorubicin (also known as Adriamycin) has proven to be very effective in anticancer therapy as a cytostatic and cytotoxic agent [1]. At the same time, medical applications of doxorubicin often are limited by its serious drawbacks, particularly cumulative cardiotoxicity and frequent cases of tumor resistance [2]. The main mechanism of doxorubicin cytotoxicity is related to its inhibitory effect on DNA and DNA-associated enzymes. Another critical factor that influences significantly the pharmacological action of this drug is interaction of doxorubicin with cell membranes. Oxidative membrane damage is a well-known side effect of doxorubicin [3]. Of crucial importance is doxorubicin-induced alterations of structural characteristics in cell membranes, such as lipid packing and membrane fluidity [4].

At the same time, doxorubicin membrane binding plays a central role in drug transport process, its distribution and accumulation in the cell, that is, highly relevant to doxorubicin delivery efficacy [4]. Considerable efforts have been directed to elucidate the differences in doxorubicin delivery to resistant/sensitive cancer cells. Since the early 1980s, these studies have received great attention in the context of multidrug resistance problem [4–7]. As demonstrated in [4, 5], cell's susceptibility to doxorubicin depends strongly on biophysical properties of membrane (lipid packing, membrane fluidity, electric charge) and, ultimately, on doxorubicin-lipid interaction.

Doxorubicin-lipid interaction has been extensively studied in the model experiments on membrane-mimicking systems, such as supported lipid bilayers, and liposomes [7–9]. Much

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attention in the literature is paid to interaction of doxorubicin with Langmuir monolayers of synthetic phospholipids [10, 11, 12] and lipids isolated from the cancer cells [5]. Brewster angle microscopy, compression isotherm measurements, and fluorescence microscopy are the most widely used experimental techniques that allow for detailed characterization of monolayer morphology, thermodynamic properties, phase behavior, and so on. However, doxorubicin-induced alterations in monolayer structure have not been addressed systematically yet.

In the present studies, X-ray reflectivity and grazing incidence diffraction techniques were applied to follow the changes in molecular organization of lipid monolayer, caused by doxorubicin. The main focus was on electrostatic interaction of doxorubicin with anionic phospholipids. We studied Langmuir monolayer composed of dipalmitoylphosphatidylglycerol which has been chosen as one of the essential negatively charged phospholipids in the cell membranes. To elucidate the role of acyl chain unsaturation in doxorubicin-lipid interaction, we also examined monolayer of dioleoylphosphatidylserine with two unsaturated acyl chains. As a reference, X-ray experiments have been performed also on monolayer of zwitterionic dipalmitoylphosphatidylethanolamine. The experimental measurements were carried out at the ID10 beamline (ESRF Grenoble, France).

2 Materials and Methods

Doxorubicin hydrochloride was purchased from Boryung Pharmaceutical (South Korea). 1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol) sodium salt (DPPG), 1,2-Di-(cis-9-octadecenoyl)-sn-glycero-3-phospho-L-serine sodium salt (DOPS), and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were purchased from Sigma and used without further purification. All solutions were prepared using ultrapure water (Milli-Q Advantage A10 Water Purification System, Millipore, France). All measurements were performed at 21 °C.

2.1 Formation of Phospholipid Monolayer on Liquid Surface

Solutions of DPPG and DPPE were prepared using 9:1 v/v chloroform-methanol at a concentration of 0.45 mg/ml. DOPS was dissolved in chloroform at a concentration of 0.42 mg/ml.

To form a phospholipid monolayer, a solution of phospholipid was spread on the surface of pure water in a Langmuir trough. The solvent was allowed to evaporate for 15 min. The layer was compressed to a surface pressure of $\pi = 25$ mN/m which is close to physiologically relevant pressure [13]. After the monolayer was compressed, 300 μl of doxorubicin

solution (concentration of 1.47×10^{-3} M) was injected underneath the monolayer by microsyringe. The monolayer was left for 40 min to allow for interaction of doxorubicin with the lipid molecules. Note that a distinct increase in surface area of anionic lipid monolayers (DPPG and DOPS) was detected, while we were injecting doxorubicin solution at constant surface pressure.

2.2 X-ray Measurements

X-ray reflectivity (XRR) and grazing incidence diffraction (GID) measurements were carried out at the beamline ID10 (ESRF) equipped with a custom designed Langmuir trough. We used a 0.564 Å wavelength. GID measurements were performed at fixed incidence angle $\theta = 0.8 \times \theta_C$ (θ_C is the critical angle of total external reflection for water). The diffracted intensity in the q_z direction was recorded using a linear position-sensitive detector (Mythen) with angular scans in q_{xy} (in-plane direction). Experimental measurements were carried out during (for at least) 3 h in each series. At every XRR and GID scan, the Langmuir trough was shifted by 200 μm horizontally across the X-ray beam that allowed to reduce radiation damage of the phospholipid monolayer. The surface pressure was kept constant during X-ray measurements.

2.3 Data Analysis

The conventional approach to XRR data analysis is based on varying the parameters of the a priori known model for the investigated films. In the present studies, we have used the free-form approach (FFA) for fitting experimental XRR scans. The main advantage of FFA is the possibility to reconstruct the electron density profile without any additional assumptions about the parameters of the lipid monolayer. In the frame of this approach, the electron density profile is defined as a continuous function passing through the set of base points. The variation of the base points allows obtaining susceptibility profiles in the physical meaning area. The strategy for choosing the number of base points, physical and mathematical constraints, as well as solution of the inverse problem is discussed in [14].

3 Results and Discussion

3.1 Negatively Charged Phospholipids

DPPG Monolayer The experimental results, obtained in GID measurements on DPPG monolayer, clearly demonstrated the disastrous changes that doxorubicin produced in the monolayer structure (Fig. 1). Very weak diffraction peak could be hardly seen on the first GID scan, recorded 40 min after
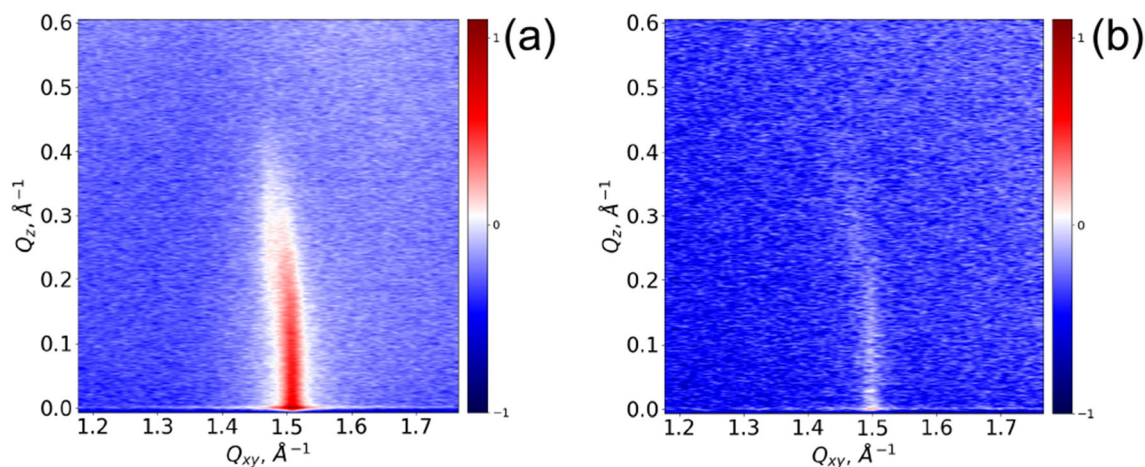
Fig. 1 Two-dimensional diffraction scattering patterns from the DPPG monolayer before (**a**) and after (**b**) doxorubicin injection (the GID intensity color scale is logarithmic)

doxorubicin solution was injected underneath the DPPG monolayer (Fig. 1b). Just 30 min later (the second GID scan), no diffraction signal has been detected, that is an obvious indication of complete disordering of crystalline phase in monolayer.

The XRR scans recorded on DPPG monolayer before and after doxorubicin injection are presented in Fig. 2a (curves 1 and 2, respectively). The significant differences between these experimental data evidence the pronounced alteration of electron density distribution in DPPG monolayer in the presence of doxorubicin. The most remarkable feature, observed in XRR scans after doxorubicin injection, is the considerable increasing of the Kiessig fringes period as well as more complicated shape of the interference oscillations. In addition, curve 2 exhibits more rapid intensity decline with angle. Note that these characteristic changes were seen already on the first XRR scan, recorded 1 h after doxorubicin injection. Over the course of further measurements (2 h), only slight

transformations in the shape of XRR scans were observed: the first minimum became deeper.

Figure 2b shows the electron density distribution profiles for DPPG monolayer, reconstructed using FFA approach. Referring to this figure, the air/lipid monolayer interface smeared drastically upon doxorubicin injection, while the overall thickness of tail region reduced. These findings can be attributed to the increasing of tilt angle dispersion of acyl chains in DPPG monolayer. Furthermore, the increasing of electron density in the head region is clearly visible in Fig. 2b that implies the incorporation of doxorubicin into the head region of DPPG monolayer.

DOPS Monolayer Monolayers of unsaturated lipids are known to possess no long-range lateral order. Accordingly, diffraction measurements do not provide any details about lateral molecular organization in monolayer of DOPS, which has two unsaturated acyl chains in cis configuration.

Fig. 2 XRR results for the DPPG monolayer. **a** X-ray reflectivity data (o) and best fit(−) before (curve 1) and after (curve 2) doxorubicin injection. **b** The electron density distribution before (curve 1) and after (curve 2) doxorubicin injection. The electron density profiles are normalized to the water density. For clarity, the curves have been offset vertically. For comparison, electron density profile before doxorubicin injection is shown as gray line
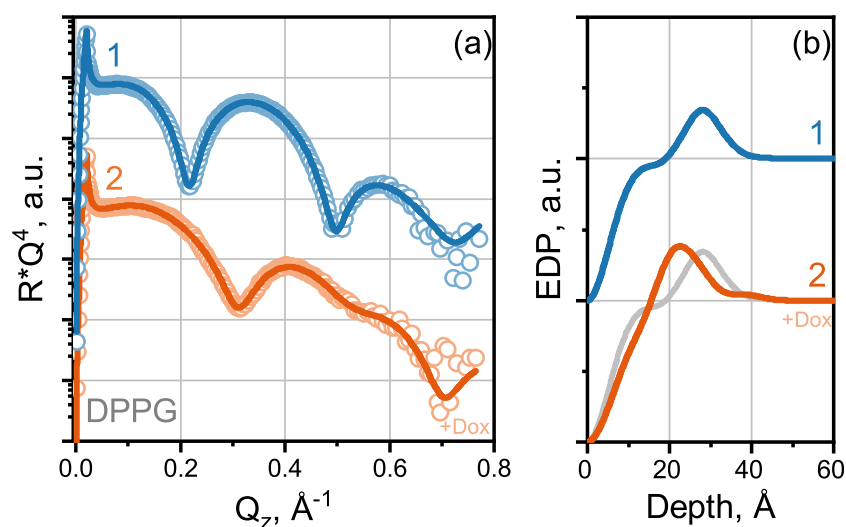
Fig. 3 XRR results for the DOPS monolayer. **a** X-ray reflectivity data (o) and best fit (−) before (curve 1) and after (curve 2) doxorubicin injection. **b** The electron density distribution before (curve 1) and after (curve 2) doxorubicin injection. The electron density profiles are normalized to the water density. For clarity, the curves have been offset vertically. For comparison, electron density profile before doxorubicin injection is shown as gray line
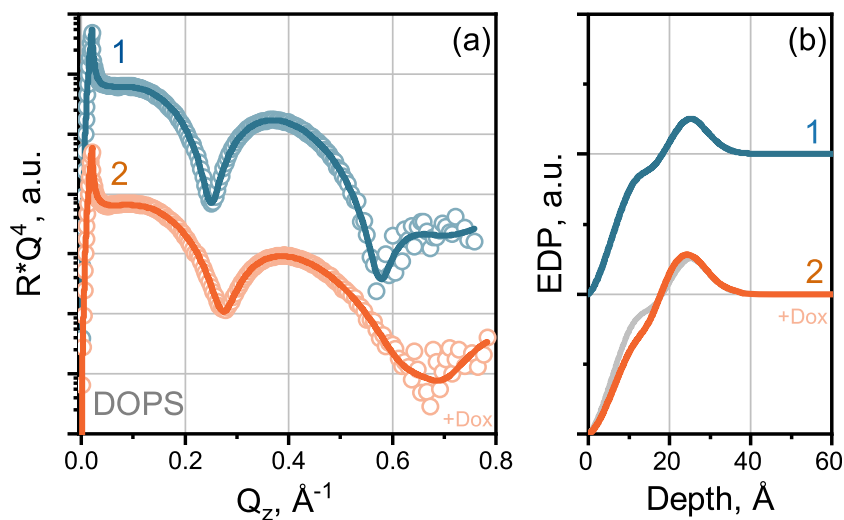
Fig. 4 Two-dimensional diffraction scattering patterns from the DPPE monolayer before (**a**) and after (**b**) doxorubicin injection (the GID intensity color scale is logarithmic). The integrated diffraction intensity as a function of q_{xy} and q_z before (**c** and **e**) and after (**d** and **f**) doxorubicin injection
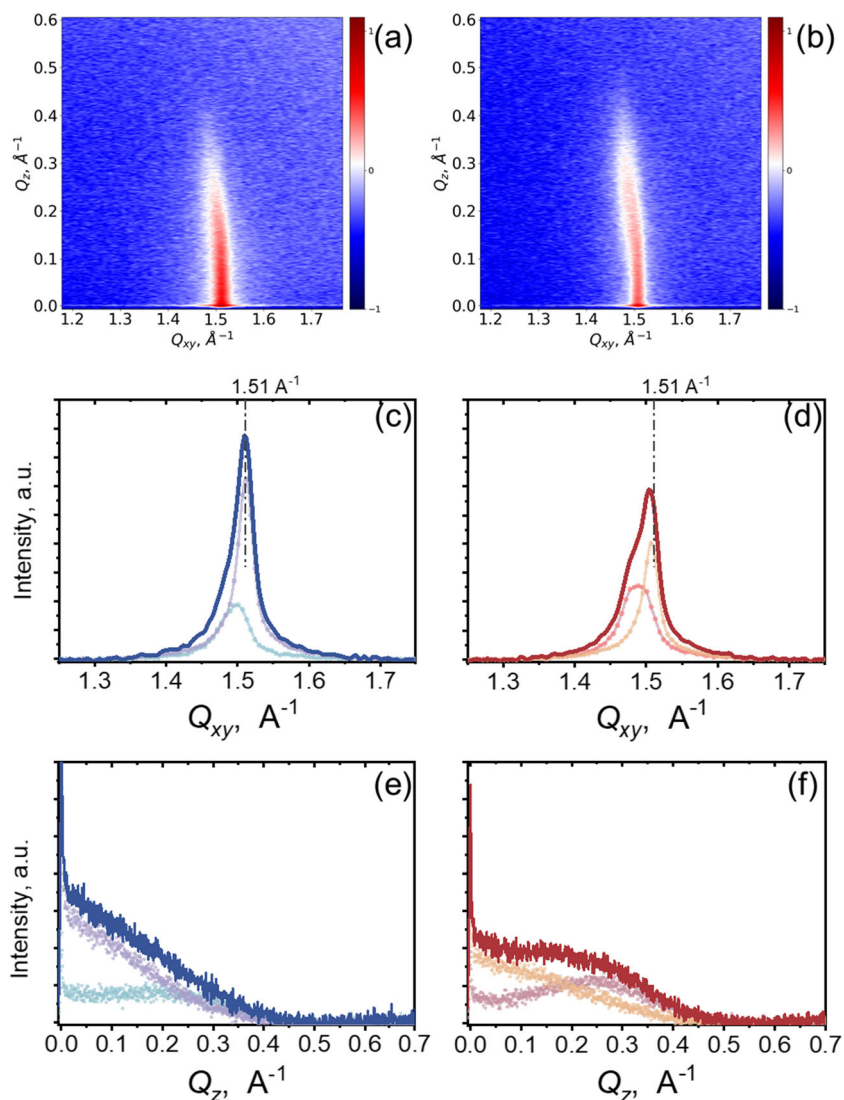
Table 1 2D lattice parameters for DPPE monolayer before and after doxorubicin injection

	a, b (Å) γ (deg)	Tilt angle (deg)	Area/chain (Å^2)	Unit cell	Tilt direction
Before injection	$a = 4.82$ $b = 8.39$ $\gamma = 90.6$	9.2	20.22	Centered oblique	NN (along diagonal)
After injection	$a = 4.84$ $b = 8.45$ $\gamma = 90.7$	11.1	20.45	Centered oblique	NN (along diagonal)

Important information on doxorubicin-induced alterations in DOPS monolayer was obtained in XRR measurements. Figure 3a shows XRR scans recorded for DOPS monolayer before and after doxorubicin injection, and the corresponding electron density profiles are presented in Fig. 3b. As can be seen, doxorubicin changes the molecular packing in DOPS monolayer considerably. Quite remarkable is the fact that these changes are very similar for both saturated DPPG and unsaturated DOPS: the smearing of the air/monolayer interface, the decreasing of tail region thickness, and the increasing of the electron density in the headgroup region. These observations imply that interaction of doxorubicin with negatively charged monolayers proceeds via the similar mechanism.

Taken together, the X-ray data, obtained for DPPG and DOPS monolayers, can be explained by the incorporation of doxorubicin molecules into lipid monolayer. Doxorubicin molecules have been shown to be positively charged in water at neutral pH values [15]. Thus, the interaction of doxorubicin with anionic phospholipids would be expected to occur preferentially due to electrostatic forces—positively charged amino groups of doxorubicin can bind to negatively charged phosphate groups of DPPG and carboxyl groups of DOPS that exert a pronounced effect on molecular packing in lipid monolayer by increasing the distance between the lipid

molecules and consequent increasing of tilt angle dispersion of acyl chains. Note that both DPPG and DOPS monolayers show the enlargement of surface area after doxorubicin injection that supports the conclusion about the insertion of doxorubicin between the negatively charged lipid molecules.

It is important to emphasize that in the case of negatively charged monolayer, the insertion ability of doxorubicin does not depend on the acyl chain saturation. Indeed, the obtained results demonstrated that doxorubicin molecules incorporate into the condensed monolayer of saturated DPPG just as efficiently as into the fluid monolayer of DOPS with two unsaturated acyl chains.

It also should be taken into account that doxorubicin is an amphiphilic compound that contains both hydrophobic and hydrophilic parts; thereby, coordination with hydrocarbon tails of phospholipid molecules can play an important role in interaction of doxorubicin with lipid monolayer [4, 5]. The presented XRR data do not provide a direct information on the localization of the dihydroxyanthraquinone moiety of doxorubicin. Nevertheless, the obtained results, first of all the observed decrease in thickness and density of tail region, imply that the penetration of doxorubicin into the hydrophobic part of the DPPG or DOPS monolayers does not occur. Most probably doxorubicin molecules, incorporated into the polar

Fig. 5 XRR results for the DPPE monolayer. **a** X-ray reflectivity data (o) and best fit (−) before (curve 1) and after (curve 2) doxorubicin injection. **b** The electron density distribution before (curve 1) and after (curve 2) doxorubicin injection. The electron density profiles are normalized to the water density. For clarity, the curves have been offset vertically. For comparison, electron density profile before doxorubicin injection is shown as gray line
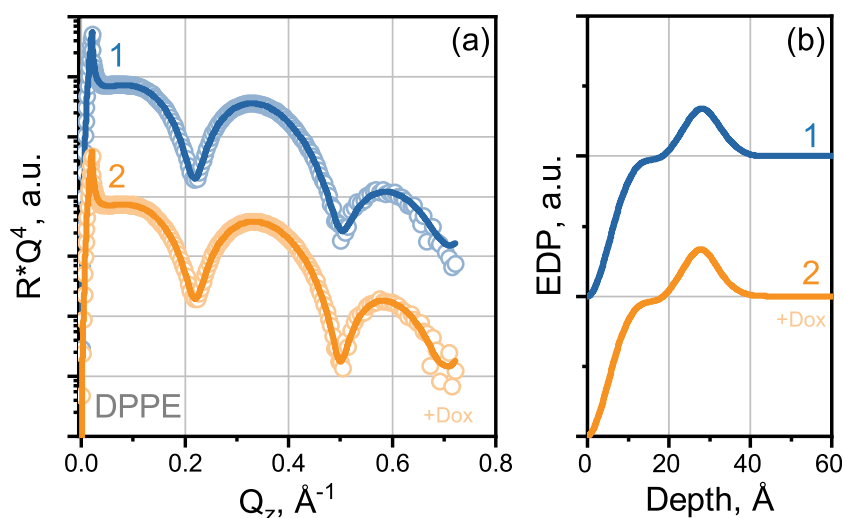
regions, remain trapped between the head groups of lipid molecules due to electrostatic attractive interaction.

3.2 Zwitterionic Phospholipids

DPPE Monolayer Of special interest in diffraction experiments for DPPE monolayer is the presence of strong Bragg peak on GID scans, recorded upon doxorubicin injection. This result is very different from that observed for DPPG monolayer, when the Bragg peak completely disappeared over a period of ~ 1 h.

GID scans for DPPE monolayer and the integrated diffraction intensity as a function of q_{xy} and q_z are presented in Fig. 4; the corresponding structural parameters are listened in Table 1. X-ray data were recorded during 2 h after doxorubicin injection, and only minor changes in GID scans were observed over this period: The intensity of Bragg peak decreased slowly with time. According to the data shown in Table 1 after doxorubicin injection, the 2D lattice parameters in DPPE monolayer increased slightly compared with those in DPPE monolayer on pure water, and small inclination of the acyl chains from the vertical was observed as well. This subtle structure changes indicate the extension of the lipid packing in ordered phase of DPPE monolayer that might be attributed to conformational modifications of the polar group of DPPE molecules in the presence of doxorubicin.

XRR measurements, carried out on DPPE monolayer during 2 h after doxorubicin injection, show no any changes in the reflectivity profiles. Furthermore, the shape of these curves appeared to be essentially identical to that of DPPE monolayer on pure water (Fig. 5). Thus, X-ray experimental data obtained for DPPE clearly indicated that electron density distribution in DPPE monolayer remained unchanged after doxorubicin injection, and also crystalline part of the monolayer has roughly the same 2D structure as in the DPPE monolayer on pure water. Therefore, we conclude that doxorubicin did not cause any significant alterations of molecular packing in condensed monolayer of zwitterionic DPPE molecules.

To summarize, the presented GID and XRR studies revealed the distinct effect of doxorubicin on negatively charged phospholipid monolayers. The observed changes have the same tendency for both saturated DPPG and unsaturated DOPS; thus, we conclude that interaction of doxorubicin with anionic lipids is mainly controlled by electrostatic attraction between positively charged amino groups of doxorubicin and negatively charged polar head of phospholipids. Based on the obtained data, doxorubicin penetration into the negatively charged monolayer occurs mostly in the head group regions. In contrast, the molecular packing of zwitterionic DPPE monolayer appeared to be largely unaffected by doxorubicin.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Availability of Data and Material None.

Code Availability None.

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