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Applications of metal ions and liquid crystals for multiplex detection of DNA

Yanyang Liu, Kun-Lin Yang *

Department of Chemical and Biomolecular Engineering, National University of Singapore, 4 Engineering Drive 4, Singapore 117576, Singapore

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ABSTRACT

Many cations such as sodium ions have strong influence on anchoring behaviors of liquid crystals (LC). Since DNA is negatively charged and forms complex with metal ions, it is possible to form DNA/metal ions complex on surfaces to disrupt orientations of LC. This phenomenon is used to establish a principle for detecting surface immobilized DNA by using LC. In contrast, peptide nucleic acid (PNA) is electroneutral. It does not complex with metal ions or affect the orientations of LC. Therefore, PNA can be used as a probe to hybridize with specific DNA with a unique sequence, and the principle mentioned above can be used to detect the DNA target by using metal ions and LC. Based on this method, a 600 bp DNA target in buffer can be detected with a limit of detection at 10 fM. Unlike other fluorescence-based DNA assays, this LC-based detection method does not require labeling of DNA, and the test result can be viewed with the naked eye under a polarized microscope.

1. Introduction

Liquid crystals (LCs) are materials in which molecules can self-align to have a long-range directional order [1]. In the past, many chemical and biomolecular sensors based on the unique properties of LCs have been developed. For example, Abbott's group reported LC sensors for detecting peptides, lipids and other chemical compounds [2–7]. Other groups also developed a series of LC sensors for the detection of DNA, proteins and small molecules including thiol, amines and metal ions [8–11]. An interesting observation arising from these studies is that when a thin layer of LC is in contact with a liquid or a solid interface, the orientation of LC becomes very sensitive to ionic species (proton, electrolytes, and metal ions) present at the interface [12–14]. Some possible mechanisms have been proposed to explain how ionic species affect the orientation of LC. Firstly, when LC 4-cyano-4′-pentylbiphenyl (5CB) is in contact with an aqueous solution, simple electrolytes such as sodium chloride are able to partition into the interfacial region of LC and create an electrical double layer which aligns LC homeotropically [15]. Meanwhile, some chaotropic anions such as perchlorate can also interact with the nitrile group of 5CB and triggers an ordering transition [16]. Secondly, when 5CB is in contact with a solid surface decorated with copper ions, nitrile group of 5CB is able to complex with copper ions [17,18] and causes a homeotropic orientation. Molecular interactions between nitrile groups of 5CB and copper ions have also been characterized in detail by using PM-IRRAS [19]. Finally, metal salts deposited on the surface may dissociate and dissolve in LC, forming an electrical double layer inside LC [17]. Electric field associated with the electrical double layer also causes the orientations of LC to become homeotropic.

Detection of DNA is of great importance in molecular biology and bioanalytical chemistry. Currently, most detection methods are fluorescence-based due to their high sensitivity [20]. However, labeling DNA with fluorophores means additional working steps, and such labeling may affect the activity of DNA. Recently, several label-free methods for the detection of DNA by using LCs have been reported. Price et al. reported that hybridization of DNA at a LC/aqueous interface laden with DNA and cationic surfactants resulted in the reorientation of LC. Park et al. showed that DNA hybridization on a polymeric surface with sinusoidal structures can lead to disruption of LC orientation [21,22]. Recently, Chen et al. showed that the concentration of DNA can be estimated by using the number of bright LC spots on the surface, and Lai et al. used LCs to examine the quality of a DNA microarray chip [23,24]. However, effect of DNA on the orientation of LC varies in different systems. For instance, Price found that single-stranded DNA (ssDNA) causes planar/tilted orientation in LC in contact with aqueous solutions, whereas double-stranded DNA (dsDNA) leads to a homeotropic orientation. In contrast, Kim reported that ssDNA keeps LC in a homeotropic orientation on a solid surface whereas dsDNA causes a planar orientation in the LC [25]. Since LC is very

* Corresponding author.
E-mail address: cheyk@nus.edu.sg (K.-L. Yang).

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sensitive to surface properties, a detailed study is required to elucidate factors that lead to different orientations of LC in contact with DNA.

On the basis of literature review and our observation, we hypothesize that the presence of metal ions is an important factor for determining the orientation of LC on a surface decorated with DNA. Depending on the types of metal ions and their concentrations, the LC may adopt planar, tilted or homeotropic orientations on a surface. To test this hypothesize, we incubated solid surfaces into buffers containing different metal ions and compared the orientations of LC supported on these surfaces. Since DNA is negatively charged and can complex with metal ions easily, we also immobilized PNA, which is electroneutral and does not complex with metal ions, for comparison. This study can help us better understand how metal ions affect the orientations of LC. This phenomenon can also be applied to the detection of unlabeled DNA and DNA hybridized to PNA probes.

2. Material and methods

2.1. Materials and reagents

Glass slides were purchased from Fisher (U.S.A). *N*, *N*-dimethyl-*N*-octadecyl(3-aminopropyl)trimethoxysilyl chloride silane (DMOAP), sodium cyanoborohydride, sodium chloride, sodium carbonate, potassium chloride, magnesium chloride, calcium chloride, ammonium chloride, ammonium sulfate, ammonium carbonate were purchased from Sigma–Aldrich (Singapore). DNA was purchased from Sigma–Aldrich. PNA was purchase from Panagene (Korea). LC 4-cyano-4′-pentylbiphenyl (5CB) was purchased from Merck (Singapore). All solvents used in this study were analytical or HPLC grades.

2.2. Preparation of DMOAP modified surface

To clean the surface, glass slides were immersed in a 5% Decon-90 solution (a commercially available detergent) for 12 h. Then, they were rinsed with copious amount of deionized water and cleaned in an ultrasonic bath twice, each time for 15 min. After this, the slides were dried under a stream of nitrogen. The cleaned glass slides were immersed in an aqueous solution containing 0.1% (v/v) of DMOAP. After 5 min, they were rinsed with copious amounts of deionized water and dried under a stream of nitrogen. To cross-link the immobilized DMOAP, the slides were placed in a vacuum oven at 100 °C for 15 min. Finally, the slides were placed 3 cm below an UV lamp (254 nm, Sigma–Aldrich, model 11SC-1) for 50 s to activate the surface.

2.3. Surface characterization by X-ray photoelectron spectroscopy (XPS)

XPS was performed by using an AXIS-His spectrometer (Kratos Analytical, Japan) to determine compositions of surface. More details of this analysis can be found in our previous study [26].

Since glass slides always contain sodium, aluminum, magnesium etc, we use silicon wafers instead of glass slides as supporting substrate for XPS test. The C1s hydrocarbon peak at 285.0 eV was used as the reference peak for all binding energy measurements.

2.4. Fabrication of LC cells

A hybrid LC cell was made by sandwiching two glass slides with two strips of spacer (~6 μm) and two binder clips. The top surface was modified with DMOAP. The bottom surface was the analytic surface, which was functionalized by droplets of DNA solutions on an UV modified DMOAP glass slide. After the LC cell was made, LC was drawn into the cavity formed between the two glass slides by using capillary force. Optical appearance of the sample was observed by using a polarizing optical microscope (Nikon ECLIPSE LV100POL, Japan) in the transmission mode. Each image was captured by using a digital camera mounted on the microscope with an exposure time of 40 ms.

2.5. Immobilization of DNA and PNA

DNA or PNA was first dissolved in 0.5 M of ammonia carbonate buffer containing 5 mM of sodium cyanoborohydride as a reducing agent. Then, the DNA or PNA solution was spotted on a glass slide in an array format by using a spotting robot (AD1500 from Biodot, U.S.A). Each spot contains approximately 100nL of DNA solution. Humidity was maintained at 90% throughout the printing process. Then, the glass slide was kept in a humidified chamber for 18 h, allowing the covalent immobilization of DNA or PNA on the surface. After the immobilization, the glass slide was washed by using a 4-step cleaning process as follows: (1) in 0.1% SDS solution for 5 min, (2) in 0.1 M H_2SO_4 solution for 20 min, (3) in deionized water for 5 min, and (4) in hot water (90–100 °C) for 10 min. Finally, the slide was dried under a stream of nitrogen. In some cases, the slide with immobilized DNA was immersed in 0.5 M of sodium chloride solution for 30 min, allowing the sodium ion to complex with DNA. Then, the slide was dried by blowing nitrogen on the surface.

2.6. PCR amplification of DNA

Template genome DNA for PCR was extracted from *Escherichia coli* by using Gentra Puregene Yeast/Bact. Kit (Qiagen, Singapore). This genome DNA was amplified by using a forward primer (5′-GGCATAACCACCACGCTCA-3′) and a reverse primer (5′-CGTCCCCTCAAACTGGCAGA-3′) which flank a 600 bp fragment. To obtain a single-stranded DNA for hybridization, asymmetric PCR technique was used. Every 50 μl of PCR reaction mixture contains: 1 × PCR buffer, 2 μM of $MgCl_2$, 250 μM dNTPs, 500 pM of Cy5 labeled reverse primer and 5 pM of forward primer, 1 unit of Taq DNA polymerase (Promega, Singapore) and 10 ng of extracted genome DNA. The PCR program began with a 10 min denaturing step at 95 °C followed by 40 cycles of denaturing at 95 °C for 45 s, annealing at 52 °C for 45 s and extension at 72 °C for 1 min, with an additional 10 min of extension at 72 °C. PCR products were confirmed by using gel electrophoresis and then purified by using MinElute Purification Kit (Qiagen, Singapore). Purified single-stranded DNA product was quantified by using a NanoDrop 2000 spectrometer (Thermo Scientific, U.S.A).

2.7. DNA hybridization

Before hybridization, DNA target was incubated in a hot water bath (100 °C) for 10 min to denature double strands, and then transferred to a cold water bath (0 °C) for 5 min. Next, DNA target was added to a hybridization solution containing 2 × SSC. Then, 50 μl of hybridization solution was added to a hybridization chamber which is made of a slide functionalized with PNA and a cover slide. After hybridized for 2 h, the chamber was disassembled, and the slide was washed with 2 × SSC containing 0.1% SDS (5 min) and 2 × SSC (5 min), respectively. Finally, the slide was dried under a stream of nitrogen.

2.8. Fluorescence detection

Fluorescence signals on the slides were detected by using a microarray scanner (MS200) manufactured by Roche (Singapore). Slides were scanned with the highest speed while resolution is

40 μm. Laser (532 nm) intensity is 100% and gain value is 1%. The same parameters were used for all images and analysis.

3. Result and discussion

3.1. Effect of sodium chloride on orientations of LCs

It has been reported that metal ions affect orientations of LC under several possible mechanisms [9,27–29]. To better understand this phenomenon, droplets of sodium chloride solution (0.5 M) were dispensed onto three types of surfaces. After incubation for 18 h, surfaces were washed with 0.1% SDS solution and deionized water (5 min each) and boiled in water for 10 min, then blown dried with nitrogen. Subsequently, LC cells were made from these surfaces and filled with LC 4-cyano-4′-pentylbiphenyl (5CB) to study how sodium chloride affects the anchoring behavior of LC. The first two types of surfaces were DMAOP with UV treatment (UV-DMOAP) and without UV treatment. The last one is TEA/DMOAP surface which had been described in our previous study [24]. Interestingly, Fig. 1A shows that sodium ions only affect the orientation of LC on the UV-DMOAP surface and cause two bright spots. On the other hand, sodium ions do not affect the orientations of LC on the other two types of surfaces (Fig. 1B and C). Apparently, UV treatment contributed to this result. In the literature, it has been reported that UV treatment on a DMOAP surface can lead to generation of some functional groups such as alcohol, aldehyde and carboxylic acid on the surface [26]. One possible reason for the bright spots in Fig. 1A is that carboxylic acid groups complex with sodium ions, and then sodium ions affect the orientation of the LC (they change the easy axis of the LC from homeotropic to planar/titled on the UV-DMOAP surface, see Supporting Information). The other two types of surfaces lack functional groups to complex with sodium ions. Therefore, sodium ions do not adsorb on the surface. To test the hypothesis that surface adsorbed sodium ions are responsible for the bright spots in Fig. 1A, we characterized an UV-DMOAP surface with XPS. Fig. 1D shows that even after the washing procedure, the sodium ions are still present on the UV-DMOAP surface. This result is consistent with the bright spots shown in Fig. 1A.

3.2. Effect of metal ions on LCs' orientation

Because sodium chloride has strong influence on the anchoring behavior of LC on the UV-DMOAP surface, we also deposited solutions of other inorganic salts including sodium sulfate, sodium carbonate, potassium chloride, magnesium chloride and calcium chloride ammonium carbonate, ammonium chloride and ammonium sulfate on the UV-DMOAP surface. Optical images of LC in contact with these surfaces were shown in Fig. 2. By using optical appearance of LC (bright or dark), we can conclude that the presence of sodium ion and other cations such as potassium, magnesium and calcium ions, regardless of the counter anions, always adsorb on the UV-DMOAP surface and disrupt the orientation of LC (Fig. 2A–E). On the other hand, ammonium ion, regardless of the anions, does not adsorb on the surface nor affect the orientation of LC (Fig. 2F–H). Ammonium ion is unique may because it can decompose into ammonia vapor upon heating such that it does not adsorb on the surface. Another important conclusion that can be drawn from Fig. 2A–H is that LC is sensitive to surface metal ions only but not anions. However, all anions tested in our experiment are kosmotropic in nature. Chaotropic anions such as perchlorate might influence the orientation of LC as shown in a past study [16].

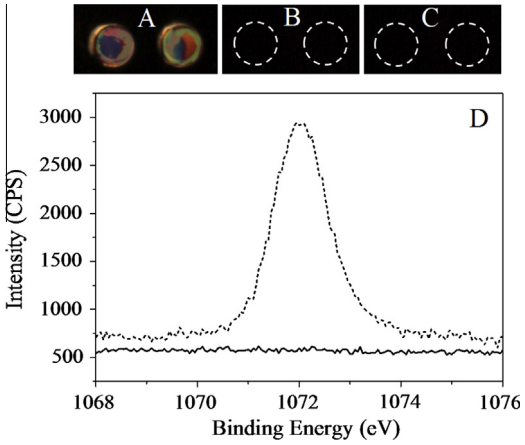

Fig. 1. Optical images of LC supported on (A) UV-DMOAP, (B) DMOAP and (C) TEA/DMOAP surfaces decorated with droplets of sodium chloride solutions. LC only shows bright on the UV-DMOAP surface. (D) XPS results showing the presence of sodium on the UV-DMOAP surface, after it was immersed in a sodium chloride solution deposition and then rinsed with DI water (dotted line). The control is a UV-DMOAP surface without immersion into sodium chloride solution (solid line).

3.3. Detection of DNA using LC

Since LC is very sensitive to sodium ion, this phenomenon can be used to detect DNA which is negatively charged and can complex with sodium ions. We designed a set of experiments to test the feasibility of this idea. First, 1 μM of DNA (5′-NH_2-AAATG TGAGC GAGTA ACAAC CCGTC-3′) was immobilized on an UV-DMOAP surface, and then the surface was subjected to a 4-step washing process as described in experimental section. Subsequently, the surface was immersed into 2 × SSC buffer which contains sodium ions. After 2 h, the surface was blown dried and an LC cell was made to study the orientation of LC. Fig. 3A shows that DNA spots become bright. This result suggests that sodium ions complexed to the surface disrupt the LC, and that leads to a planar/titled orientation of LC. In contrast, if the surface with immobilized was immersed into DI water (free of sodium ions), then DNA spots remain dark (Fig. 3B). This result shows that immobilized DNA cannot disrupt LC by itself. Only DNA complexed with sodium ions is able to disrupt the LC. This result also shows that the orientation of LC responds to immobilized DNA on the surface, but the surface must be incubated in buffer containing sodium ions first. The principle of detection is shown in Fig. 3A and B.

Even though the method mentioned above allows us to detect immobilized DNA on a solid surface, it is not practical to use LC for detecting a specific DNA target hybridizing to the surface. This is because LC cannot distinguish DNA target from immobilized DNA probe if they both complex with sodium ions. Thus, to prevent this problem, we first immobilized electroneutral PNA on the surface as a probe, and used it to hybridize with DNA targets. Since PNA is electroneutral and does not complex with sodium ions, we anticipate that the immobilized PNA probe alone does not attract sodium ions nor disrupt LC. Thus, PNA probe can an ideal

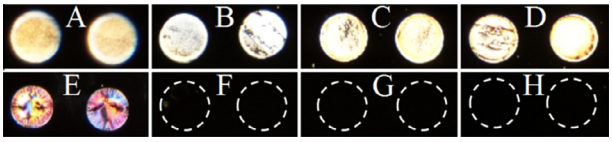

Fig. 2. Effects of inorganic salts and surface-adsorbed metal ions on the orientation of LC. Droplets of aqueous solution containing 0.5 M of (A) sodium sulfate, (B) sodium carbonate, (C) potassium chloride, (D) magnesium chloride, (E) calcium chloride, (F) ammonium carbonate, (G) ammonium chloride and (H) ammonium sulfate were applied to UV-DMAOP surface and examined by using LC.

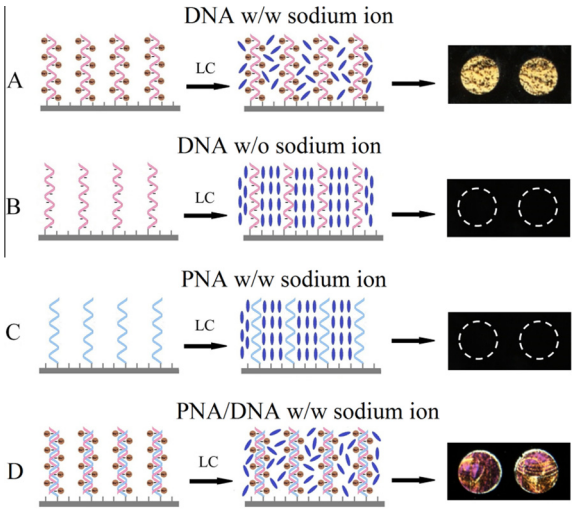

Fig. 3. Principles for detection of DNA by using LC. Red probes represent DNA whereas blue probes represent PNA. (A) DNA contact with sodium ion (B) DNA contact without sodium ion (C) PNA contact with sodium ion (D) PNA/DNA complex contact with sodium ion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

candidate to detect DNA if the LC-based detection method is used. The detection principle is shown in Fig. 3C and D. To test this hypothesis, we first immobilized PNA (5′-AAATGTGAGCGAGTAA-CAACCCGTC-3′) on a UV-DMOAP surface. After immobilization of PNA, the surface was immersed in 2 × SSC for 2 h and then XPS was employed to characterize the surface (Fig. 4). From the carbon, nitrogen, and phosphor peaks, we can confirm that PNA was successfully immobilized on the surface. However, sodium peak was not observed. These results support our hypothesis that electroneutral PNA does not attract sodium ions.

Next, to ensure that DNA target can hybridize to immobilized PNA probe, we designed a fluorescently-labeled DNA target T1 (5′- GACGGGTTGTTACTCGCTCACATTT-Cy3-3′), which is complementary to the sequence of PNA probe. Then, DNA hybridization was performed in 2 × SSC buffer. Fluorescence image in Fig. 5A shows that T1 successfully hybridized to PNA. Subsequently, we used LC to compare the surface before and after DNA hybridization. Fig. 5B shows that before DNA hybridization, there is no bright LC spot, suggesting that PNA probes alone do not disrupt LC. However, after hybridization of DNA to the surface, two bright spots can be seen (Fig. 5B). This is a strong proof showing that DNA hybridized to PNA can complex with sodium ions and disrupt the orientation

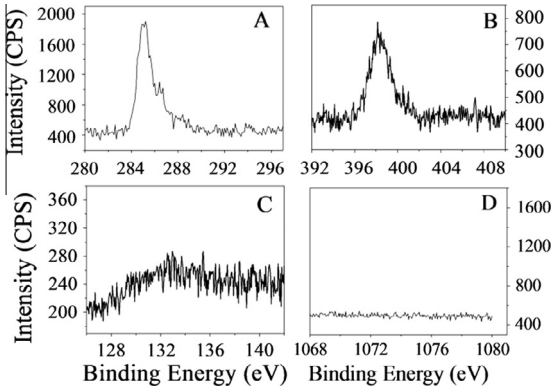

Fig. 4. XPS results for UV-DMOAP surfaces with immobilized PNA. Elements detected include (A) carbon (B) nitrogen (C) phosphorus and (D) sodium.

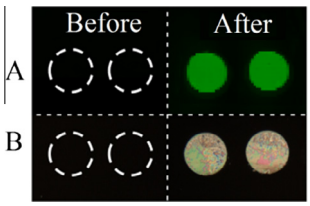

Fig. 5. Before and after hybridization of Cy3-DNA target to immobilized PNA probe. (A) fluorescence image (B) LC image.

of LC. This result provides a new principle for the detection of DNA target with LC.

3.4. Detection limit of DNA by using sodium ion and LC

To test detection limit of this method, 5 μM of PNA was immobilized on a UV-DMOAP surface. Since the orientation of LC is dependent on the amounts of sodium ions complexed with DNA, we synthesized DNA targets of different lengths including 15 bp (5′-TACTCGCTCACATTT-3′), 25 bp (5′- GACGGGTTGTTACTCGCT-CACATTT-3′) and 40 bp (5′-TCCCA CGGAG AATCC GACGG GTTGT TACTC GCTCA CATTT-3′). These three targets share the same sequences in their last 15 bp which is complementary to the PNA sequence. After hybridization of these DNA targets with immobilized PNA probes, LC was used to probe the surface. Fig. 6A, B and C shows that the detect limit decreases with the increasing target length. When the DNA target length is 15 bp, the detection limit is 0.2 μM. When the DNA target is 40 bp, the detection limit is 0.01 μM. This result indicates that the amounts of sodium ions complexed to the surface increase with increasing target lengths. Based on this principle, the detection limit is lower if the DNA target is longer.

In most DNA microarray applications such as gene expression or phenotype classification, DNA targets are amplified from genomic DNA by using PCR, and the DNA target length is at least few hundreds base pairs. Because of this reason, we amplified a 600 bp DNA target from genomic DNA of *E. Coli*. The PCR-amplified DNA target contains a 25 bp fragment which is complementary to the sequence of the PNA probe. Thus, the same PNA probe can be used to detect the 600 bp PCR-amplified product. After DNA hybridization, LC imaging protocol was conducted and LC image was taken. Fig. 6D shows the LC is sensitive enough to detect the DNA target at a concentration of 10 fM. This result is more sensitive than most fluorescence-based detection method. Because the length of the target DNA is 600 bp, it can be detected at a much lower concentration than shorter DNA targets shown in Fig. 6A, B and C.

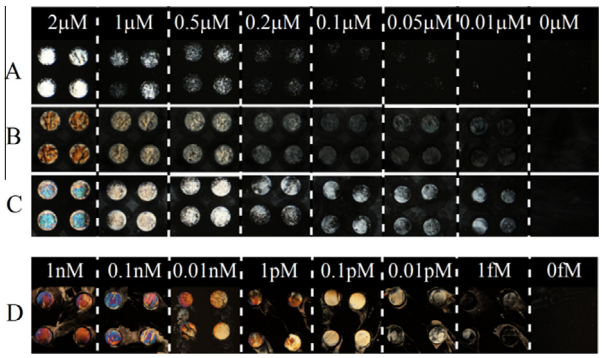

Fig. 6. Effect of DNA target length on the detection limit. DNA target length is (A) 15 bp, (B) 25 bp, (C) 40 bp and (D) 600 bp.

4. Conclusions

When a thin layer of LC is supported on a UV-DMOAP surface, the orientation of LC is very sensitive to metal ions adsorbed on the surface. In particular, a small amount of sodium ion adsorbed on the surface can disrupt the orientation of LC and cause a bright spot. Because DNA is negatively charged and attracts sodium ions, this phenomenon is exploited to detect surface-immobilized DNA by using LC. Moreover, PNA is not charged and does not attract metal ions. Thus, immobilized PNA does not disrupt the orientation of LC, making it an ideal probe for detecting complementary DNA targets. By using PNA as a probe and LC as an optical readout, 10 fM of DNA (~600 bp, amplified from genomic DNA with PCR) can be detected by using this method.

Acknowledgment

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcis.2014.10.038.

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