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# The distribution of polyphosphoinositides in lipid films

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**Abstract**

Fluorescence microscopy of Langmuir films is used to determine the effect of polyphosphoinositides (PPIs) on the structure of phosphatidylcholine-containing monolayers. Dramatic alterations in the texture of these films occur as the fraction of PPI in the film is altered. These changes depend on the ionic strength of the underlying subphase and can be accounted for by considering the electrostatic interactions among PPIs. Surface adsorption of a fluorescent peptide derivative based on the PPI binding site of the protein gelsolin co-localizes with PPI-rich domains. Localization of polyphosphoinositides in domains within the inner leaflet of the plasma membrane is proposed to be a key element in some aspects of intracellular signaling, and these data have implications for explaining the cause of restructuring of such domains. 

*Keywords:* Polyphosphoinositides; Langmuir monolayers; Phase transitions; Phosphatidylinositol

## 1. Introduction

Phosphorylated derivatives of phosphatidylinositol, also called polyphosphoinositides (PPIs), are essential components of many cellular signal transduction pathways [1–3]. In addition to the function of $PIP_2$ (phosphatidylinositol-4,5-bisphosphate) as a substrate for phospholipase C (PLC) to produce diacylglycerol (DAG) and inositol trisphosphate ($IP_3$) [4], phosphorylated inositol lipids themselves function to activate, inactivate, or localize a large number of proteins involved in cytoskeletal organization, cell adhesion, ion transport, vesicle trafficking, and cell division. In a few cases, binding of a cytosolic protein to $PIP_2$ involves sole interaction with the headgroup of a single lipid molecule [5], but in most cases the PPI must be contained within a multimolecular complex of lipids to bind its ligand, even if the

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resulting stoichiometry of binding is almost 1:1 [6].

In biological membranes, the sum of PPIs (including all isomers of PIP, $PIP_2$ and $PIP_3$) constitute no more than a few percent of total lipid; but in vitro, several proteins do not bind $PIP_2$ unless it is present in bilayers at approximately 10 mol% [6,7]. This result and the finding that specialized regions of the plasma membrane such as caveoli or lipid rafts may be highly enriched ($> 20\%$) in PPI [8] suggest that clustering of inositol lipids in specialized regions of a lipid bilayer may be an important aspect of their ability to activate specific ligands. Moreover, transitions in the packing of PPI induced by one protein may alter the lipid's reactivity with other proteins.

While a great number of studies have characterized binding of various PPIs to many proteins or implicated these interactions in specific cellular functions, there is very little known about how PPIs are distributed in a lipid bilayer, and the assumption that these lipids randomly diffuse through the membrane is at odds with its apparent ability to stimulate activities that require its concentration or clustering. Neither $PIP_2$ (phosphatidylinositol-4,5-bisphosphate or phosphatidylinositol-3,4-bisphosphate) nor $PIP_3$ (phosphatidylinositol-3,4,5-trisphosphate) are stable in a bilayer vesicle and instead, form spherical or rod-like micelles in water [9,10], and ribbon-like lamellar aggregates in the presence of divalent cations [11]. The large size of the inositol rings relative to other lipid headgroups, together with the large net negative charge of PPIs suggest that these lipids may strongly perturb the distribution of other lipids that are stable in flat bilayers and so, might influence or initiate changes in lipid texture or curvature that could affect cellular functions.

In this paper, it is shown that the effect of PPIs on the texture of reconstituted Langmuir films is dramatic and can be explained using electrostatic arguments. The effect of the polyphosphoinositides on the structure of lipid films was explored using Langmuir monolayers as a model system with the goal of isolating the effect of charged lipids on membrane domains. A large change in the shape of domains in the gas/liquid-expanded region was observed as the ratio of phosphatidylinositol monophosphate (PIP) to phosphatidylcholine (PC) was increased. This effect is reversed by screening electrostatic interactions using a monovalent salt, confirming that the effect is due to electrostatics and not steric interactions. This result can be explained using a framework that is, at a fundamental level, similar to the theory of the effect of cholesterol on lipid films. This earlier theory has been elegantly tested in a series of experiments conducted by McConnell et al. [12–15].

## 2. Materials and techniques

L-α-Phosphatidylcholine and DTPC (Ditridecanoylphosphatidylcholine) were purchased from Avanti (Alabaster, AL, USA), L-α-phosphatidylinositol 4-monophosphate, and sodium salt was purchased from Sigma (St. Louis, MO, USA). These two lipids are natural products containing a variety of acyl chains with a significant fraction of unsaturated chains preventing the formation of gel phases. *N*-(Texas Red sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphatidylethanolamine (referred to here as Texas Red-PE) was purchased from Molecular Probes (Eugene, OR, USA). The lipids were dissolved in a 4:1 chloroform–methanol solution. The solvents were purchased from Sigma and were of spectrophotometric grade (99% + pure). Fluorescein-QRLFQVKGRR, a fluorescent derivative of a peptide based on the $PIP_2$-binding site of gelsolin [18] was kindly provided by Dr Rolands Vegners, Latvian Institute of Organic Synthesis.

A custom built teflon trough, milled from a single piece of Teflon and graciously provided by C. Schmidt of the University of Michigan, was used to manipulate the films in this study. The trough was rectangular, constructed from a single piece of Teflon, with a barrier at one end, having an area of 26.17 $cm^2$ and a depth of 0.5 cm. Before measurements, the trough was rigorously cleaned using NoChromix (GODAX Laboratories, New York, NY, USA) and sulfuric acid (Trace Metal Grade, Fisher Scientific, Pittsburgh, PA, USA) and subsequently, rinsed with Milli-Q (Mil-

lipore Corp., Marlborough, MA, USA) water for a minimum of 20 min. Lipids were applied in a chloroform–methanol solution to the surface of an aqueous compartment consisting of 10 mM HEPES, 0.1 mM EDTA, pH 7.4 with additional ionic content, as listed for individual experiments. The amount of lipid applied was adjusted to produce a film in the gas/liquid-expanded region.

Fluorescence microscopy was performed using a Zeiss II RS upright microscope fitted with Texas Red and fluorescein filters from Omega Optical (Brattleboro, VT, USA) and a Zeiss Plan 16/0.35 objective.

## 3. Theory

Taking into account both dipole–dipole and charge–charge interactions, the free energy of a mixed lipid monolayer system is

$$F = \lambda \oint \mathrm{d}r + \frac{\mu^2}{2} \int\int \frac{\mathrm{d}A\mathrm{d}A}{|r-r'|^3} + \frac{\rho^2}{2} \int\int \frac{\mathrm{d}A\mathrm{d}A}{|r-r'|}$$

This can be re-written in an alternative form using the following steps:

Green's theorem

$$\int\int \left( \frac{\partial^2 Q}{\partial x' \partial x} + \frac{\partial^2 P}{\partial y' \partial y} \right) \mathrm{d}A\mathrm{d}A' = \oiint (P\mathrm{d}x\cdot\mathrm{d}x' + Q\mathrm{d}y\cdot\mathrm{d}y')$$

results in the identity

$$\int\int \xi^{-(m+2)}\mathrm{d}A\mathrm{d}A' = -m^2 \oiint \xi^{-m}\mathrm{d}r\cdot\mathrm{d}r' + \frac{\Delta(m+2)}{m} \int\int \xi^{-(m+4)}\mathrm{d}A\mathrm{d}A'$$

where $\xi = (|r-r'|^2 + \Delta^2)^{1/2}$ and $\Delta$ is small.

Thus,

$$F = A\varepsilon + (\lambda - \mu^2) \oint \mathrm{d}r - \frac{\mu^2}{2} \oiint \frac{\mathrm{d}r\cdot\mathrm{d}r'}{|r-r'|} - \frac{\rho^2}{2} \oiint |r-r'|\,\mathrm{d}r\cdot\mathrm{d}r'$$

Where $\lambda$ is the line tension, $\mu$ is the dipole density, $\rho$ is the charge density, and $\varepsilon$ is the electrostatic energy per unit area for a domain of infinite size. The first three dipolar terms were originally calculated by McConnell and de Koker [16].

Absent from this argument is explicit consideration of the counter-ion layer that exists beneath an electrostatically-charged membrane [17]. Because the effects discussed in this report depend on forces between molecules within the membrane, not between two membranes as is usually considered in the Gouy–Chapman theory, the presence of counterions may quantitatively affect the ratios of charged to uncharged lipid at which lateral demixing occurs, but are unlikely to give rise to qualitatively different effects.

Now, to take a specific example, we evaluate the energy term on an ellipse:

The term $\frac{\rho^2}{2} \int\int \frac{\mathrm{d}A\mathrm{d}A}{|r-r'|}$ can be evaluated [13], resulting in $\frac{16}{3}\rho^2 \frac{(ab)^2}{b} K(k)$ where $K(k)$ is the complete elliptical integral of the first kind and $k = \sqrt{\left(\frac{b}{a}\right)^2 - 1}$. This term in energy can be factorized into $(4\rho^2/3\pi)(\pi ab)[4aK(k)]$. Note that the area of an ellipse $= \pi ab$ and the circumference is $4aE(k)$ where $E(k)$ is the complete elliptical integral of the second kind.

The final task is to minimize the energy with respect to the ratio of $b/a$ (the distortion of the ellipse). The energy is:

$$F = 4\pi(\lambda - \mu^2)E(k) - \frac{\mu^2}{2} \oiint \frac{\mathrm{d}r\cdot\mathrm{d}r'}{|r-r'|} + \left(\frac{4\rho^2}{3\pi}\right)(\pi ab)(4aK(k)).$$

Using the general arguments and results from

Keller et al. [13], it is possible to express this result analytically:

Expanding the energy in powers of $\Delta = \ln(b/a)$:

$$4\pi(\lambda - \mu^2)E(k) = 2\pi(\lambda - \mu^2)(ab)^{1/2} \times\left(1 + \frac{3}{16}\Delta^2 + \frac{1}{2^{10}}\Delta^4 + ...\right)$$

$$\left(\frac{4\rho^2}{3\pi}\right)(\pi ab)(4aE(k)) = \frac{8\pi}{3}\rho^2(ab)^{3/2} \times\left(1 - \frac{1}{16}\Delta^2 + \frac{11}{3\cdot 2^{10}}\Delta^4 + ...\right)$$

$$-\frac{\mu^2}{2}\oiint\frac{dr\cdot dr'}{|r-r'|} = -2\pi\mu^2(ab)^{1/2} \times\left(\ln\left(\frac{4(ab)^{1/2}}{e^2 d}\right) + \frac{3}{16}\ln\left(\frac{4(ab)^{1/2}}{e^{10/3}d}\right)\Delta^2 + \frac{1}{2^{10}}\ln\left(\frac{4(ab)^{1/2}}{e^{46/3}d}\right)\Delta^4 + ...\right).$$

The energy can then be written in the form $F = e_0 + e_2\Delta^2 + e_4\Delta^4 + ...$

It can be argued, on physical grounds, that it is reasonable to take $e_4 > 0$. If $e_4 < 0$, we have a minimum of energy at large $\Delta$ and the system is unstable. In the case that $e_4 > 0$, elementary calculus provides the result that if $e_2 > 0$, the minimum is at $\Delta = 0$; while for $e_2 < 0$, the minima are at $\Delta = \pm e_2/2e_4$.The transition occurs when

$$e_2 = 0 = \frac{2\pi(ab)^{1/2}}{16}\left(3(\lambda - \mu^2) - \frac{\rho^2(ab)}{2}\right) - 3\mu^2\ln\left(\frac{4(ab)^{1/2}}{e^{10/3}d}\right).$$

With an increase in the charge density of the system, it goes through a second order phase transition, from a phase where round domains minimize the energy to a phase where distorted domains minimize the energy. Consequently, it is the balance of the product of the area ($\pi ab$) and charge density squared vs. the line tension that determines the texture of the film. The dipole density, which was kept constant in this study, also influences texture.

## 4. Results

### 4.1. The effect of PIP on PC monolayer texture

Lipid films were produced on a HEPES-buffered (pH 7.0) solution containing 10 mM HEPES, 0.1 mM EDTA underlying PC and PIP with PC:PIP ratios of 100:0, 90:10, 50:50 and 0:100, respectively. Under these conditions the headgroup of PIP carries a nominal charge of approximately −2.5. In most experiments, 1% Texas Red-labeled PE was added to the mixtures to allow observation of the films using florescence microscopy. As the concentration of PIP was increased, the texture of the film changed, from one in which the shape of the observed domains was governed by line tension (Fig. 1a) to one in which the domains possess jagged edges (Fig. 1e). A similar transition also occurred in films of DTPC:PIP films (data not shown).

The transitions seen in Fig. 1 were primarily driven by electrostatic effects. By adding NaCl to the subphase of a monolayer containing only PIP, the irregular appearance of domains with jagged edges (Fig. 2a) was reversed to a state in which the shape of the domains was governed by line tension (Fig. 2b) similar in appearance to a monolayer low in PIP.

### 4.2. The localization of PIP

In order to determine the localization of PPIs directly, monolayers were prepared with NBD-labeled $PIP_2$ (a generous gift of Glenn Prestwich) and Texas Red-labeled PE. In these measurements, the Texas Red-labeled PE was found to concentrate in the same domains as the NBD−$PIP_2$ (Fig. 3a,b).

In addition, a fluorescein-labeled peptide, based

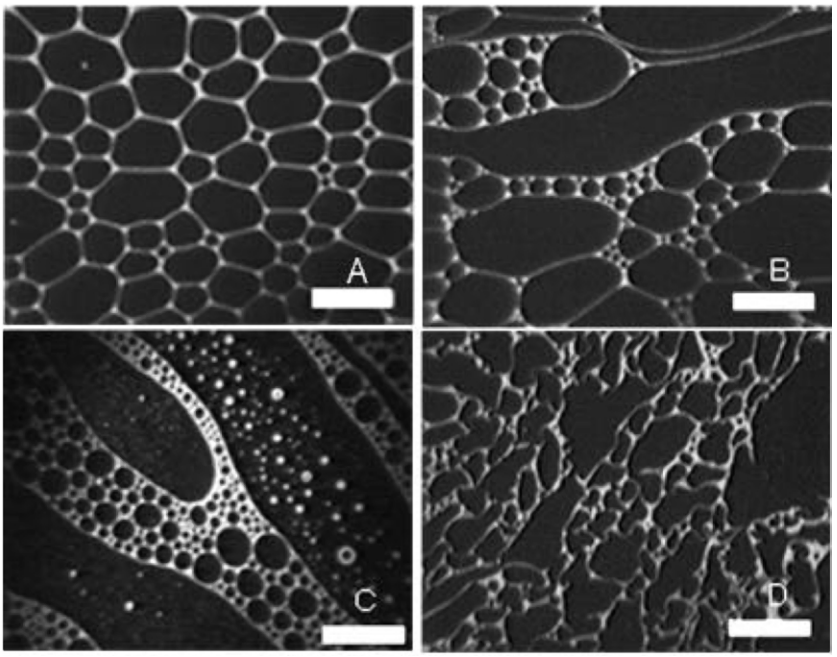


Fig. 1. Brain-PC Langmuir films containing 1 mol% Texas Red-PE and (a) 0, (b) 10, (c) 50, and (d) 100% PIP, respectively; scale bars represent 100 μm. Light regions depict areas enriched in the fluorescent lipid Texas Red-PE, and dark regions are areas relatively devoid of the fluorescent lipid.

upon the PIP-binding domain of gelsolin [18], was introduced into the subphase of a monolayer containing 10% PIP. Fig. 4a shows that the Texas Red-labeled PE is selectively depleted from the small circular domains suggesting that PIP is localized to the surrounding area of the monolayer similar to the result shown in Fig. 1b. The PIP-binding peptide fluorescein-QRLFQVKGRR preferentially segregates to the same domains as the Texas Red-labeled PE (Fig. 4b), as would be expected if the PIP segregated.

## 5. Discussion

The phosphorylated inositol phospholipids are key components of signal transduction pathways as well as being important in the regulation of numerous enzymes. For example, the actin binding protein gelsolin is regulated by its interaction with phosphorylated PI. Also, specific inositol lipid-binding structures, such as the pH and FYVE domains, are thought to have a role in localizing proteins that contain these domains to membranes. Overexpression of either the kinases that produce cellular $PIP_2$ [20] or the phosphatase that degrades it [21], profoundly affects cell function, including cytoskeletal organization. Finally, steric arguments have been used to suggest that clustering of PIP should produce membrane curvature [19] in cell membranes.

This study shows that the presence of PIP in Langmuir monolayers dramatically alters the texture of the film and that this alteration can be accounted for physically via electrostatic arguments. These results suggest a physical mechanism whereby the interaction between PIP-binding proteins and clusters of charged PIP lipids can take place.

Several of the proteins that bind $PIP_2$ in vitro, do so in a manner that strongly depends on the manner in which these lipids are packed into bilayers. In some cases, lateral demixing or a critical mole fraction of PPIs appears to facilitate

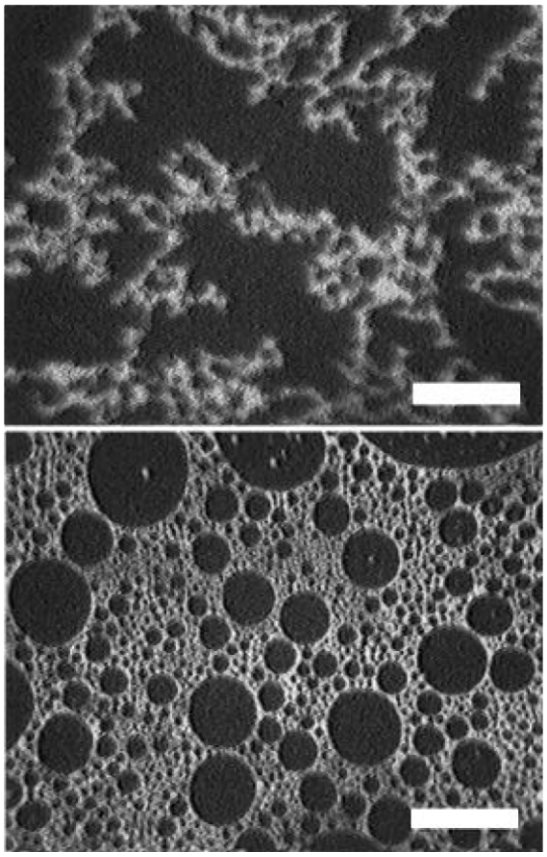

Fig. 2. A PIP film before (a) and after (b) adding NaCl to the subphase (conc. = 0.65 M). Scale bars represent 100 μm.

their interaction with proteins such as the actin-binding protein gelsolin [6] or the protein kinase β-ARK [7]. Moreover, the covalent modification of PPIs by enzymes like PLC-γ or PI 3-kinase depends on such structural features as surface pressure and membrane curvature, and can be either enhanced or suppressed by small concentrations of PPI-binding peptides and proteins [19]. Such in vitro results suggest that PPIs are not randomly distributed in mixed bilayer systems and that proteins preferentially target boundaries or regions of high PPI content. The recent finding that most of the cellular PI(4)P and PI(4,5)$P_2$ reside in less than 5% of the total plasma membrane [8], further supports the hypothesis that segregation of PPIs in membranes is an important aspect of their biological function.

One consequence of concentrating acidic lipids within small domains is to form regions of locally increased membrane potential that could stabilize binding of specific peripheral proteins. As was pointed out by MacLaughlin [17], the reduction of dimensionality at the membrane interface favors association and the low-dielectric interface approximately doubles the potential, allowing association of cationic protein domains caused by electrostatics to be stronger at the surface of a membrane enriched in anionic lipids. Such binding affinity is complicated by contributions from both the electrostatic energy and the entropy

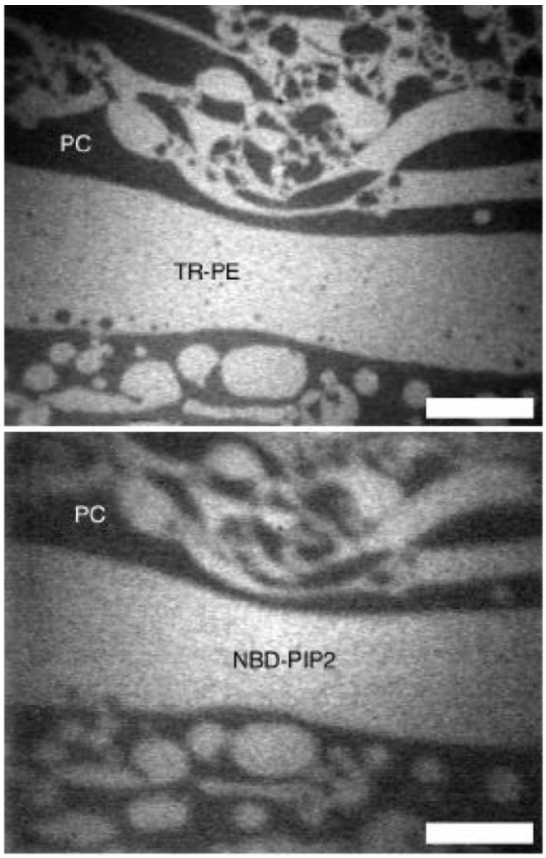


Fig. 3. A NBD–$PIP_2$/brain-PC film (1:1) containing 1 mol% TR-PE when viewed in the (a) Texas Red and (b) NBD channel; scale bars represent 100 μm. Areas enriched in particular lipids (PC, TR-PE, NBD–$PIP_2$) are illustrated, assuming that the fluorescence intensity is proportional to the relative concentration of the labeled lipid.

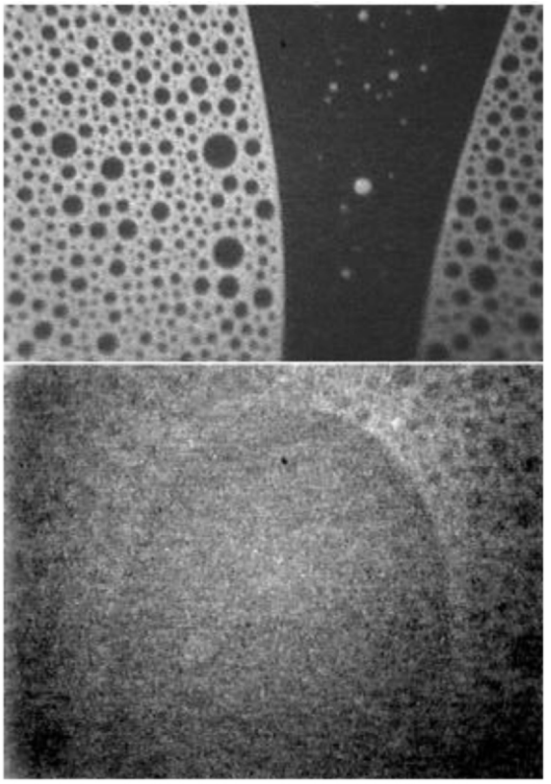

Fig. 4. A brain-PC film, containing 10 mol% PIP and 1 mol% TR–PE, with 10 μM fluorescein-labeled, gelsolin-derived peptide in the aqueous subphase when viewed in the (a) Texas red channel to reveal areas enriched in TR–PE and PIP, and the (b) fluorescein channel to reveal localization of the gelsolin-derived $PIP_2$-binding peptide at the liquid/monolayer interface.

gained as regions of a protein bearing multiple positive charges displace multiple lower valence ions from the membrane double layer. It is hoped that the equations described in this investigation can be further modified to account for these additional factors.

Our results demonstrate that electrostatic arguments can account for the segregation of highly charged lipids into domains in which they are concentrated and those in which they are depleted. Thus, segregation of highly charged lipids such as PIP, can be accounted for physically. Once these clusters of PIP have formed (via the electrostatics described here), steric interactions can be used to argue that this clustering of PIP would lead to a protrusion of the membrane with a small radius of curvature. The formation of these protrusions may be important for further segregation of membrane-bound proteins and is consistent with a central role for PI-3-kinase and PI(4)P 5-kinase in cellular vesicle traffic. PPI-mediated effects on membrane curvature may also contribute to the activity of PI3-kinase [19], since this activity is greater on vesicles with a smaller radius of curvature.

The most general implication of these results is that the large electrostatic charge on PPIs, rather than keeping them randomly dispersed within a membrane, can lead to segregation into domains and form boundaries where local curvature or enhanced accessibility of the lipid headgroups is likely. Such structures can be altered by binding of specific PPI ligands, and may be sites at which PPI turnover or regulation of PPI-dependent enzymes is particularly active.

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