

Lipid rafts make for slippery platforms

What's in a raft? Although cell membranes are certainly not homogeneous mixtures of lipids and proteins, almost all aspects of lipid rafts—how to define them, their size, composition, lifetime, and biological relevance—remain controversial. The answers will shape our views of signaling and of membrane dynamics.

In the influential “fluid mosaic” model of Singer and Nicolson, a “mosaic” of integral transmembrane proteins floats about in a “fluid” sea of lipids (Singer and Nicolson, 1972). More recently, researchers have shifted to a view in which membrane lipids are not randomly distributed, but instead show local heterogeneity. One might imagine this as a two-dimensional projection of a lava lamp, with different types of greasy globules in constant motion, endlessly separating and rejoining into distinct but transient domains. These domains are now referred to under the general heading of lipid rafts and domains, a subset of which are the morphologically identifiable “caveolae.”

The study of lipid domains has exploded since the debut of the “raft hypothesis” only about fifteen years ago. This torrent of research notwithstanding, there remains heated discussion concerning matters as fundamental as what lipid domains look like—a discussion that peaked but reached little in the way of resolution at a recent conference (Euroconference on Microdomains, Lipid Rafts, and Caveolae; Tomar, Portugal, May 17–22, 2003). Regardless of their actual form, evidence is mounting that lipid rafts are essential participants in signal transduction, membrane and protein sorting, and the pathogenesis of several human diseases.

Operational definitions

Membrane lipid heterogeneity was explored in the late 1970s, but a cellular

role for membrane domains was first proposed in the late 1980s to explain lipid sorting. The three general classes of membrane lipids are glycerophospholipids, sphingolipids, and sterols (including cholesterol). In polarized epithelial cells, sphingolipids are strongly enriched on the apical side of the cell. van Meer and Simons suggested that sphingolipids might aggregate into a distinct domain in the Golgi (van Meer et al., 1987), which could then sort apically as a unit–membrane patch. Glycophosphoinositol (GPI)-anchored proteins were subsequently found to sort through the same pathway (Lisanti et al., 1988), suggesting an association of GPI-anchored proteins with the sphingolipid-rich domains.

The first operational definition of lipid rafts came with the demonstration that sphingolipids and GPI-anchored

Rafts have proven harder to tie down *in vivo* than *in vitro*.

proteins are insoluble in a cold detergent extraction (Brown and Rose, 1992) and literally “float” like a raft to the top of a density gradient as a separable, cholesterol-dependent fraction. This was the basis for a large body of work in which the composition and properties of cold, detergent-insoluble and cholesterol-dependent preparations were characterized.

Caveolae are the only morphologically identifiable type of lipid domain, and have been recognized as stable flask-like invaginations of the plasma membrane for over fifty years (Palade, 1953; Yamada, 1955). These sphingolipid- and cholesterol-rich structures are distinguished from bulk lipid rafts by the presence of caveolin. This protein is required for and can induce the characteristic shape of caveolae, which are

often seen as ~50–100-nm pits, although they may form an extensive reticulum in muscle cells (Parton, 2003).

Unidentified floating objects

Despite all of the work published on lipid rafts, it is not clear whether everybody is studying the same thing or even studying what they intend to study. Lipid domains cannot be isolated in a native state and, with the exception of caveolae, are presumed to be too small and transient to be directly observed in unperturbed living cells. But the relationship between their operational definition—detergent-insoluble and cholesterol-dependent *in vitro* entities—and any real world counterpart is not clear.

The cold detergent procedure for preparing membranes is very convenient, and continues to be used. These analyses have perhaps reached their apex with a recent proteomic definition of raft complements (Foster et al., 2003). But how informative are these studies? Serious caveats do apply. *In vitro*, the method can yield large, >1- μm^2 sheets. These are generally taken to be an artifact, but may reflect aggregation of smaller, bona fide rafts. Other concerns include contamination or loss of components during extraction, and nonstandardized extraction procedures (Edidin, 2003).

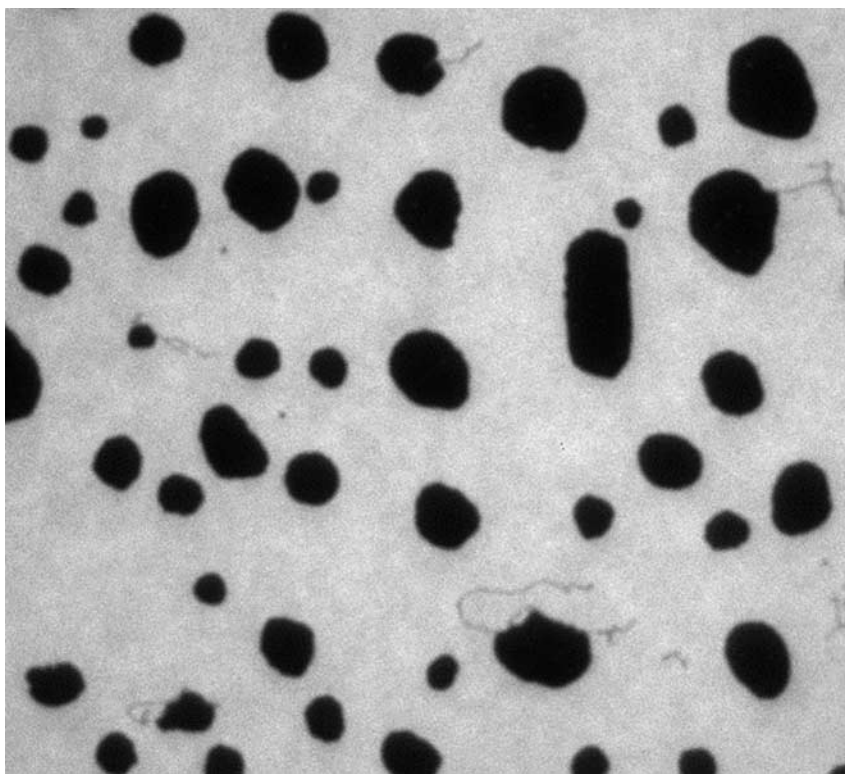
Still, some researchers argue for the practical value of the method. Gerrit van Meer (Utrecht University, Utrecht, Netherlands) concedes that “this is a relatively blunt tool, and not everything you see is real,” but says, “one has only to see that many proteins defined by this method are functionally involved in raft signaling...so it has been a useful tool.” Debbie Brown (State University of New York, Stony Brook, NY) believes that “detergent insolubility does reflect the affinity of certain proteins for an ordered lipid domain.” Such associa-

tions are dynamic (e.g., during signal transduction) and thus probably not a nonspecific membrane property.

Dick Anderson (University of Texas Southwestern Medical Center, Dallas TX), however, specifically blames much of the confusion regarding lipid domains on the wide usage of the extraction procedure and suggests that the procedure itself “creates the phenomenon” of rafts. “This type of cell fractionation can be very misleading, and is not necessary,” he says. He encourages others “to just give up on the detergent extraction method” in favor of other methods such as detergent-free fractionation after sonication or, in the case of caveolae, α -caveolin immunopurification.

Cholesterol dependence also defines rafts. But cholesterol depletion can alter cell morphology, exocytosis, and trafficking and, according to new work from Michael Edidin (Johns Hopkins University, Baltimore, MD), disrupt the actin cytoskeleton, which could have any number of pleiotropic effects. Sandy Schmid (Scripps Research Institute, La Jolla, CA) says that “cholesterol depletion makes cells sick.”

Others are more confident in the directness of cholesterol depletions, if they are performed judiciously. Unlike cholesterol, sphingolipids and GPI-anchored proteins are restricted to the outer leaflet of the membrane. Chris Fielding (University of California, San Francisco, CA) believes that “cholesterol is flipped slowly enough between the leaflets that careful experiments should allow relatively specific depletion of cholesterol from the exofacial leaflet,” thus specifically disrupting rafts on the outer leaflet. Arnd Pralle (University of California, Berkeley, CA) agrees, stating that “acute effects of cholesterol extraction on rafts can be observed within ten minutes, with perhaps only 50% loss of cholesterol”—a time period and decrease that should not strongly affect other cellular processes. Nevertheless, the speed with which cholesterol flips is contested and cholesterol is likely to be important for raft phenomena on the inner leaflet, so appropriate treatments are not agreed upon by researchers.



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Figure 1. **In vitro mixtures of lipids form large rafts, but do these have an in vivo correlate?**

Playing the shell game

Raft formation could be driven by lipids or proteins. Evidence for the lipid side comes from in vitro studies in which cholesterol segregates away from bulk glycerophospholipids, which contain shorter, kinked, unsaturated acyl chains, and has affinity for the longer, largely saturated, acyl chains of sphingolipids. In defined lipid mixtures that approximate cell membranes, these properties drive the formation of visible aggregates with increased viscosity, which are taken to be in vitro correlates of lipid rafts (Silvius, 2003) (Fig. 1). When GPI-anchored proteins are added to these lipid mixtures, they can associate with the rafts, suggesting that preexisting rafts can recruit proteins.

But the biological significance of the model membrane studies is unclear. Cellular lipids differ from in vitro lipids: they have ~ 100 times greater diversity, and are strongly influenced by their protein content. Perhaps most significantly, cells are not systems at stable equilibrium. In fact, Edidin believes that “in the face of all of the turbulent ATP-driven processes going on in the plasma membrane, the extremely weak lipid–lipid interactions aren’t

likely to contribute meaningfully to hypothesized 50–100-nm-scale entities.”

An alternative view is that lipid organization is driven by protein–lipid and protein–protein interactions. This idea grew out of the “boundary lipid” model from the 1970s, which suggested that transmembrane proteins might organize a limited domain of a few surrounding lipid molecules. Although this model seems to have long fallen from favor, it is worth reexamining the evidence. Certain lipids appeared “frozen” to proteins by electron spin resonance (EPR), but then nuclear magnetic resonance (NMR) studies showed no evidence of boundary lipid preference. Aki Kusumi (Nagoya University, Nagoya, Japan) reminds us that “EPR gives nanosecond time resolution, while NMR gives only microsecond resolution.” Thus, “the time scales are completely different. People wanted to compare these results directly but one cannot do that.” He believes that neighboring lipids may exchange rapidly, but that proteins can still strongly affect the dynamics of neighboring lipids and exhibit a lipid preference.

In the more recent “lipid shell” hypothesis, it is both transmembrane and peripheral membrane proteins that are proposed to associate with a larger preferred cohort of perhaps up to 50 lipid molecules (Anderson and Jacobson, 2002). Indeed, many proteins are now known to exhibit strong and specific affinities for different lipids. Protein clustering might then induce a lipid heterogeneity, which could also attract additional shelled proteins. Thus, the “shell” might be the quantal unit of the “raft,” with the distinction in this perspective that a raft could not exist without protein.

The opposing views of what holds a lipid domain together are not mutually exclusive, but controversy over the relative contributions rages. Edidin rules out the idea of protein-free rafts; van Meer insists that lipids can do it by themselves; and Brown straddles the middle ground, suggesting that “lipids can do it all, but [it] is almost inconceivable that proteins aren’t also involved *in vivo*.”

How to see a ghost

For most people, seeing is believing, and the fact remains that no one has ever “seen” a noncaveolar lipid domain in an unperturbed cell. For example, even though certain GPI-anchored proteins can show nearly exclusive partitioning into a detergent-resistant “raft” phase and can be clustered in the presence of antibodies, their distributions in resting cells as assessed by immunofluorescence or immunoelectron microscopy are uniform and show no evidence of clustering. Thus, the idea of large rafts, at least in resting cells, does not seem to hold.

However, more sophisticated techniques involving depolarization FRET, single fluorophore tracking (SFT) and single particle tracking (SPT) that give resolution on the nanometer–nanosecond, millisecond, and microsecond scales, respectively, are now allowing researchers to infer the existence of membrane domains in living cells. FRET detects energy transfers between two molecules only a few nm apart. If FRET efficiency is linear with density, then one presumes a random

distribution of targets, whereas density-independent FRET is taken to reflect clustering, presumably due to raft association. SFT and SPT techniques allow the direction and speed of single or clustered molecules to be followed. Observation of target slowing may be inferred to reflect its movement into a more viscous raft environment.

The original data were highly variable, but measurements appeared to converge on a domain diameter of 70–100 nm (Anderson and Jacobson, 2002). However, more recent SPT studies from Kusumi and FRET measurements by Satyajit Mayor’s group (National Center for Biological Sciences, Bangalore, India) are now indicating that lipid domains are much smaller—up to 25 nm in diameter on the high end and perhaps involving only ~ 5 molecules on the low end (Subczynski and Kusumi, 2003). This view may be close to the lipid shell idea, except that raft lipid molecules may be rapidly exchanging with outside lipid molecules, so that rafts are not considered as fixed domains or shells but are highly dynamic.

Kusumi’s high-resolution SPT analysis indicates that operationally defined raft proteins, such as GPI-anchored CD59, are temporarily confined within apparent compartments, but frequently “hop” to new compartments. However, CD59 kinetics are identical to those of single labeled, nonraft phospholipids (Subczynski and Kusumi, 2003). The nonrandomness of the movement appears to derive not from raft constraints but from “picket fences” of transmembrane proteins that, because of their attachment to the actin cytoskeleton, form temporary barriers and compartments. Kusumi therefore believes that resting state rafts must be extremely small and/or unstable, lasting for less than a millisecond.

However, other data suggest that distinct membrane domains do exist. Using a variety of monomeric proteins with different inner leaflet lipid anchors, Roger Tsien’s group (University of California at San Diego, La Jolla, CA) observed FRET between acylated proteins and between acylated proteins and caveolin, but not between either of

these and prenylated proteins (Zacharias et al., 2002). Notably, prenylated proteins clustered separately in a cholesterol-independent and detergent-soluble manner, thus providing evidence of a distinct lipid domain not included in the traditional operational definition of a lipid raft.

FRET only detects a state of molecular interaction on the order of 10 ns, so the domains detected by this method are still potentially short lived, in accord with Kusumi’s data. This is countered by diffusion measurements of single molecules confined by a laser trap. This method indicated that nonraft associated molecules exhibit low viscous drag, whereas raft-associated molecules diffuse as part of discrete lipid domains with high viscous drag (Pralle et al., 2000). Consistent with the properties of rafts, acute cholesterol depletion affected only the viscous drag on raft molecules and reduced it to that of nonraft molecules. However, the high viscosity domains, which were tracked at microsecond resolution, appeared stable on the order of minutes. So researchers are divided as to the stability of rafts in the resting state, although Kusumi believes that the large beads used by Pralle are almost certainly cross-linking their target and thus stabilizing rafts.

Biological and regulatory significance of rafts

Even if researchers cannot yet agree on the shape, size, and lifetime of rafts *in vivo*, all agree that biological membranes are not homogenous mixtures of lipids. Given this, a second axis of debate concerns the biological significance of this phenomenon. Could the existence of membrane domains possibly be incidental, a by-product of preferential interactions amongst the hundreds of lipid types? Clinical observations make the case for biological relevance: a variety of human diseases affect sphingolipid storage, transport, or trafficking (Marks and Pagano, 2002), and thus perhaps raft function, and caveolin mutations underlie other human disorders (Parton, 2003). However, in most cases, the connection between phenomenon and pathology is not well

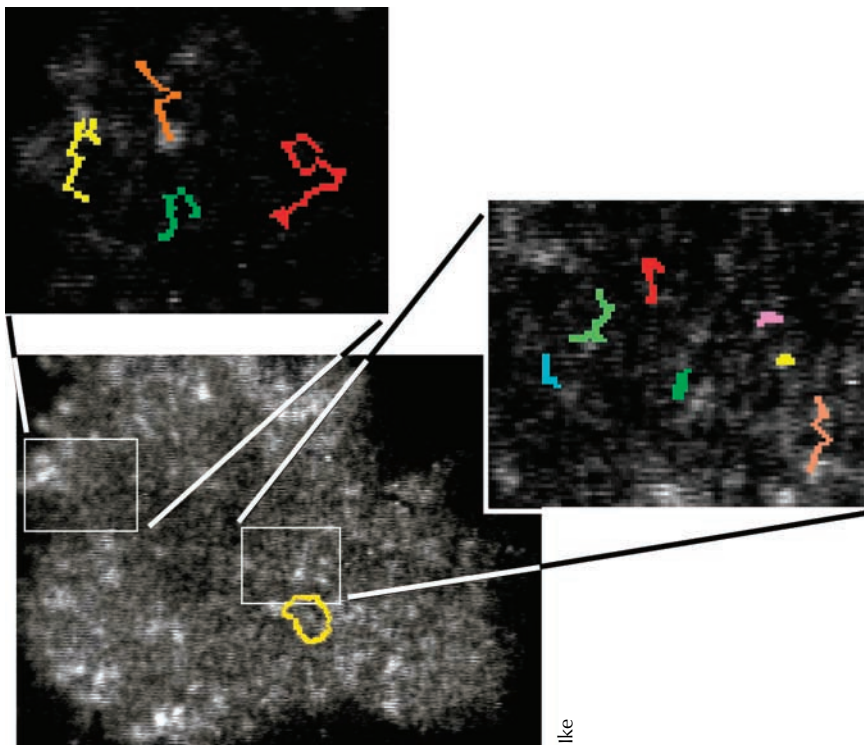


Figure 2. Lck near a site of activation (yellow) is more constrained than Lck farther away.

understood. Insight may come from studying the two areas of cell biology in which lipid domain function has been implicated: signal transduction and membrane dynamics.

Rafts as signaling platforms

A great deal of excitement was generated by the finding that many raft-associated proteins mediate signal transduction (Stefanova et al., 1991; Anderson et al., 1992; Lisanti et al., 1994). Rafts may concentrate signaling proteins, thus potentially increasing the output from receptors that require cross-activating interactions and increasing local concentrations of other, downstream signaling components. These lipid domains may also create a microenvironment that is locally protected from other transmembrane negative regulators of signaling, such as phosphatases. Finally, coordination of the inner and outer leaflets by rafts has been proposed as a theoretical link between extracellular GPI-anchored signaling proteins and intracellular signaling proteins, in cases where no transmembrane protein is implicated.

Some of the strongest cases for involvement of lipid domains in signal-

ing come from studies in hematopoietic cells. Cross-linking of many GPI-anchored proteins or transmembrane receptors in T cells and B cells by various means induces clustering of components of signal transduction, and often correlates with an increase in the raft association of relevant signaling components, at least as defined by detergent insolubility and/or cholesterol dependence (Viola et al., 1999; Wilson et al., 2000). Although subject to the operational caveats listed above, “the correlation of signal activation with the movement of signaling proteins into the detergent-insoluble fraction makes a compelling case that rafts are involved in signaling,” says Brown.

The new imaging techniques are now providing more direct visualization of raft involvement in these signaling events. During T cell activation, the T cell receptor (TCR) moves into rafts upon interaction with its cognate antigen and is phosphorylated by the Lck kinase. Kusumi and colleagues tracked single molecules of Lck-GFP by SFT and observed that it slows down following TCR activation, consistent with its association with an ordered raft domain (Ike et al., 2003)

(Fig. 2). Kusumi sees similar dynamics by SPT for CD59, a complement-activated GPI-anchored protein that activates signaling to protect a cell against autoimmune attack. Following its clustering with antibody-coated particles to activate signal transduction, he says that “CD59 slows down eightfold following activation, and the clusters concentrate cholesterol,” which together strongly suggests raft formation (Subczynski and Kusumi, 2003).

These types of data suggest a dynamically unstable view of rafts, where subtle changes can rapidly translate into bigger local effects by coalescence of rafts, which may be just as rapidly terminated by raft dissolution. Says Brown, “cells may be poised on the brink, with the least bit of coalescence inducing a phase separation.” She reasons that this is important for making signaling responsive but transient.

Moving membranes and sorting proteins

With the interest in signal transduction, it is sometimes forgotten that rafts were originally proposed to underlie membrane sorting. Recent work from Kai Simons’ group implicates lipid rafts in yeast protein sorting and mating polarity (Bagnat and Simons, 2002). And van Meer has proposed that lipid domain properties could either sort or curve membranes (Sprong et al., 2001). The different heights of phospholipid-rich and sphingolipid-rich domains could in principle lead to a variety of membrane-bilayer thicknesses, which could sort integral membrane proteins based on the length of their transmembrane domain. Plus, different lipids have distinct shapes, either largely cylindrical or conical, depending on the relative sizes of the head and tail groups. Thus, distinct lipid domains might inherently direct membrane convexity or concavity, leading to either budding or invagination. However, van Meer concedes that experimental evidence for these ideas is wanting and that unidentified proteins could underlie internalization of “smooth” vesicles, devoid of caveolin and clathrin.

Multifunctional caveolae

Caveolae, as with rafts, have been implicated in membrane dynamics and signal transduction. And the visual analogy to clathrin-coated pits brings to mind an endocytic role. Although caveolae are relatively stable and immobile, they can associate with the GTPase dynamin, which is involved in membrane fission of clathrin-coated pits, and caveolar internalization can be stimulated by certain treatments. It is now clear that a variety of toxins and pathogens use caveolae as a specific route of cellular entry, although less is known about endogenous cargo traffic. Dick Anderson believes that they internalize at least some GPI-anchored proteins, but it is also clear that GPI-anchored proteins are internalized by caveolin- and caveolar-independent mechanisms.

Many signaling proteins are present in caveolar cell fractions, and many signaling proteins associate with caveolin itself (Couet et al., 1997). Functional support for a signaling function has come from tissue culture studies, although in vivo tests are now possible with caveolin-1 knock-out mice. These mice, which lack almost all caveolae, have constitutive NO production and impaired vasoconstriction, presumably because caveolin binds to and inhibits endothelial nitric oxide synthase (eNOS) (Drab et al., 2001; Razani et al., 2001). However, since these mutant mice are mostly healthy and fertile, it seems unlikely that caveolin and caveolae can have an essential role in regulating the great number of signaling processes suggested by the in vitro work. Nevertheless, Schmid suggests that “caveolae don’t have to be essential to still have a regulatory role,” and says that many phenotypic measurements are not well-suited to assess quantitative differences. Indeed, more detailed studies of the caveolin knock-outs continue to reveal additional defects (Parton, 2003). Anderson also cautions that the “caveolin knock-outs are equivocal since they do not remove all rafts.” He thinks that compensatory mechanisms mediated by rafts may keep things going without caveolin, and that at least

some caveolae can exist without caveolin-1.

Finally, caveolae appear to be sites of cholesterol transport from internal stores, and caveolin itself binds cholesterol and fatty acids. This could connect them to maintenance or even formation of lipid rafts.

Moving forward

The concept of lipid domains has generated tremendous interest across a broad range of disciplines, including cell biology, membrane biophysics, and signal transduction. Richard Pagano (Mayo Clinic, Rochester, MN) agrees that “it is a good thing that the raft concept has stimulated so much research and has gotten people excited about lipids.” However, Pagano’s excitement is tempered by the concern that the “definitions of rafts are not clear even amongst the field...and that has gotten in the way of progress.” For example, the relationships, if any, amongst caveolae, rafts, and smooth invaginations free of caveolin and clathrin are debated in the absence of consensus views on what constitutes a raft, whether there are different subclasses of rafts, whether a caveola becomes a raft in the absence of caveolin, and so forth. Anderson emphasizes that good raft markers will be essential to resolve these issues.

Fielding wants more information on the three-dimensional structure of lipid rafts, including lipid–protein and especially lipid–lipid interactions. van Meer agrees, and eagerly awaits the development of improved optics and new fluorescent cholesterol analogs that better mimic the behavior of native cholesterol in living cells. The true movement and distribution of cholesterol, both laterally and between membrane leaflets, is not known; these data would influence models for raft formation and stability. The development of practical methods to track multiple fluorophores or particles simultaneously could also validate rafts by demonstrating correlated movements.

Many researchers insist that a move toward animal studies will be crucial for in vivo testing of functions ascribed to rafts. “Given that there may be lipid

heterogeneities,” says Pagano, “what is the evidence that the cell uses it in some way?” He would like to see genetic studies of raft phenomena using knock-outs and endogenous modulation of lipids, given that exogenous lipids can disrupt rafts in cultured cells (Simons et al., 1999). Simple knock-outs of genes involved in lipid synthesis may be lethal, but controlled strategies that would allow manipulation of lipid ratios in vivo at will would open up a world of studies. This may potentially be approached in model systems with sophisticated genetic tools such as nematodes and flies.

Finally, Kusumi stresses that much heat has been generated by apparent conflicts between experiments that are not even comparable, and that careful thinking about what constitutes relevant data sets is necessary. In particular, he says, “we need to always keep in mind the time scale and space scale,” and be aware that many biological processes are quantitative and not all-or-nothing. With these issues in mind, and some new tricks in the bag, he hopes that rafts and the field will keep floating forward.

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