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Opinion

Phase separation enhances probability of receptor signalling and drug targeting

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The probability of a given receptor tyrosine kinase (RTK) triggering a defined cellular outcome is low because of the promiscuous nature of signalling, the randomness of molecular diffusion through the cell, and the ongoing nonfunctional submembrane signalling activity or noise. Signal transduction is therefore a 'numbers game', where enough cell surface receptors and effector proteins must initially be engaged to guarantee formation of a functional signalling complex against a background of redundant events. The presence of intracellular liquid–liquid phase separation (LLPS) at the plasma membrane provides a mechanism through which the probabilistic nature of signalling can be weighted in favour of the required, discrete cellular outcome and mutual exclusivity in signal initiation.

High-fidelity signalling is a low probability occurrence

The traditional view of a tightly regulated and highly selective signalling cascade from cell surface receptors to cellular response (e.g., gene expression) is being supplanted by more complex models as our knowledge expands. The textbook description of the initiation of a reproducible signal from a RTK begins with extracellular ligand binding to a receptor followed by the upregulation of kinase activity leading to phosphorylation of tyrosine residues (pYs) to provide binding sites for downstream effector proteins. The signal is transduced by a relay of largely bimolecular binding events which, via a linear pathway, result in a defined and prolonged cellular outcome. In other words the signal transduction process is portrayed as being 'cranked up' from zero to full activity.

This simplistic picture is not easy to reconcile in the light of a number of features that emerge from deeper mechanistic studies of receptor signalling. For example, the expression of receptors on the plasma membrane is often very high (frequently of the order of up to 10^5 to 10^6 copies). This suggests that signal initiation requires, or has to process, multiple potential inputs and/or there is a high degree of failure of stimuli to produce a mutually exclusive cellular response. Furthermore, RTKs are often found in a partially phosphorylated state prior to extracellular ligand binding suggesting that the RTK is not in a 'ground zero' state and that signalling is possible even under nonstimulated conditions [1,2]. Thus, in the absence of a clear signalling on/off switch, as represented by ligand stimulation, signalling is less well regulated. Moreover, the binding of downstream effector proteins to pY sites is generally of moderate affinity. Interactions of these sites have the potential to recognise and bind to >100 SH2 (or PTB) different domain-containing proteins expressed in human cells within a limited range of equilibrium dissociation constants (0.1–10 μM) [3]. Hence, there is a potential for promiscuity in the binding, raising the possibility of aberrant interactions or initiation of redundant pathways. In addition, to facilitate the bimolecular relay of binding events the required downstream effector proteins have to move through the concentrated 'soup' of biomolecules which constitute the cytoplasm. This process, in the absence of long-range attractive forces (i.e., electrostatic forces are short, measured in nanometres), is dominated by Brownian diffusion leading to erratic temporal control of signal transduction. All of these

Highlights

Liquid–liquid phase separation (LLPS) of biomolecules into condensed phases is associated with multiple functions in cells. The importance of this in receptor tyrosine kinase (RTK) signalling is explored.

The condensation of selected molecules results in elevated local concentrations limiting the potential for aberrant interactions; hence, LLPS enhances the probability of functional signal transduction.

LLPS involving RTKs are likely to be dominated by an electrostatically charged environment which is exacerbated by the lipid surface of the plasma membrane. As a result LLPS at the plasma membrane has unique features not found in condensed phases elsewhere in the cell.

RTK-mediated LLPS has an evolutionary advantage imposing an additional level of regulation on signal transduction through excluding proteins that could initiate aberrant, pathological signals.

RTK-mediated LLPS has become the focus of intense study in the area of pharmaceutical development. Targeting small molecules to these condensed phases is a novel but challenging paradigm in drug development.

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features in this noisy picture of signalling invoke additional uncertainty and suggest that functional signal transduction is a process driven by probability [4–6]. Therefore, since we know that stimulation of a RTK by specific ligands invariably leads to a defined and measurable downstream response and cellular outcome, the unpredictable, random nature of signalling must be reduced; that is, the probability must be stacked in favour of the predetermined outcome.

There are a number of ways in which molecular mechanisms can affect the probability of a signal being transduced. The probability of a discrete signalling event prevailing under the unpredictable conditions highlighted previously is dramatically enhanced by elevation of the respective concentrations of the required signalling molecules in their active states. Contributions to this include downstream amplifier feedback loops that elevate the concentrations of, for example, phosphorylated signalling proteins required to activate a specific pathway; regulation of functional signalling protein concentration through endocytosis and degradation mechanisms; and a requirement for specific multiprotein complex formation to act as stop–go decision points [7]. The current view of RTK-mediated signal transduction ignores the importance of the outcomes being probabilistic. In this Opinion, we focus on how despite potential for random behaviour, initiation of signalling does deliver largely predictable outcomes. In our opinion, LLPS into protein condensates contributes to the probability of mutually exclusive RTK signalling and has an impact on potential therapeutic intervention.

LLPS and membrane receptors

LLPS describes the condensation of macromolecules into confined membraneless nano- to microscale structures that separate into a distinct phase [8–11]. These LLPS states have been identified in intracellular functions including regulation of signalling associated with, for example, nephrin [12], the T-cell receptor [13], mammalian target of rapamycin [14], Sos-Ras [15], cell migration [16], and autophagy [17]. These structures are formed by the accumulation of biomolecules (either individually or as functional complexes) through weak/moderate affinity, multivalent interactions (typical K_ds 0.1–100 μM: including moderate affinity interactions of phosphotyrosine residues on RTKs with SH2 domains of downstream proteins [3]). The transient nature of these interactions means that they are incapable of forming static interactions driven by intra- or intermolecular stable structure formation or ordering. The favourable enthalpy change associated with the multitude of weak interactions dominates the unfavourable change in entropy from concentrating proteins within the cytoplasm. The entropic cost of LLPS can also be alleviated by dehydration that can ultimately promote properties that are more gel-like in nature [18]. LLPS is sensitive to temperature, pressure and environmental stress. Along with the component proteins and their respective concentrations, these extraneous influences can modulate functional output from the condensed phase.

RTK-mediated LLPS localises to the plasma membrane (Figure 1) [19]. Within a typical kinase-mediated condensate there are multiple pY sites on the receptors and downstream proteins. This highly charged milieu of the RTK-associated droplets can be amplified by the phospholipids of the cytoplasmic leaflet of the plasma membrane which become more negatively charged on receptor oligomerisation [20,21]. Therefore, the initiation of receptor-mediated LLPS at the plasma membrane is distinct from other intracellular droplet formation in that the phosphorylation of multiple residues is a prerequisite, or a significant amplifier, for the rapid formation and prolonged condensation of selected downstream effector proteins [13,19,22–24]. This is exemplified for *in vitro* condensation of a number of RTKs with downstream effector proteins [e.g., fibroblast growth factor receptor (FGFR)1, FGFR2, epidermal growth factor (EGFR), HER2, HER4, vascular endothelial growth factor receptor (VEGFR)1, and VEGFR2 with SHP2 and SHC [19]]. Phosphorylation of EGFR immobilised in a supported membrane was also shown to be key in the condensation-

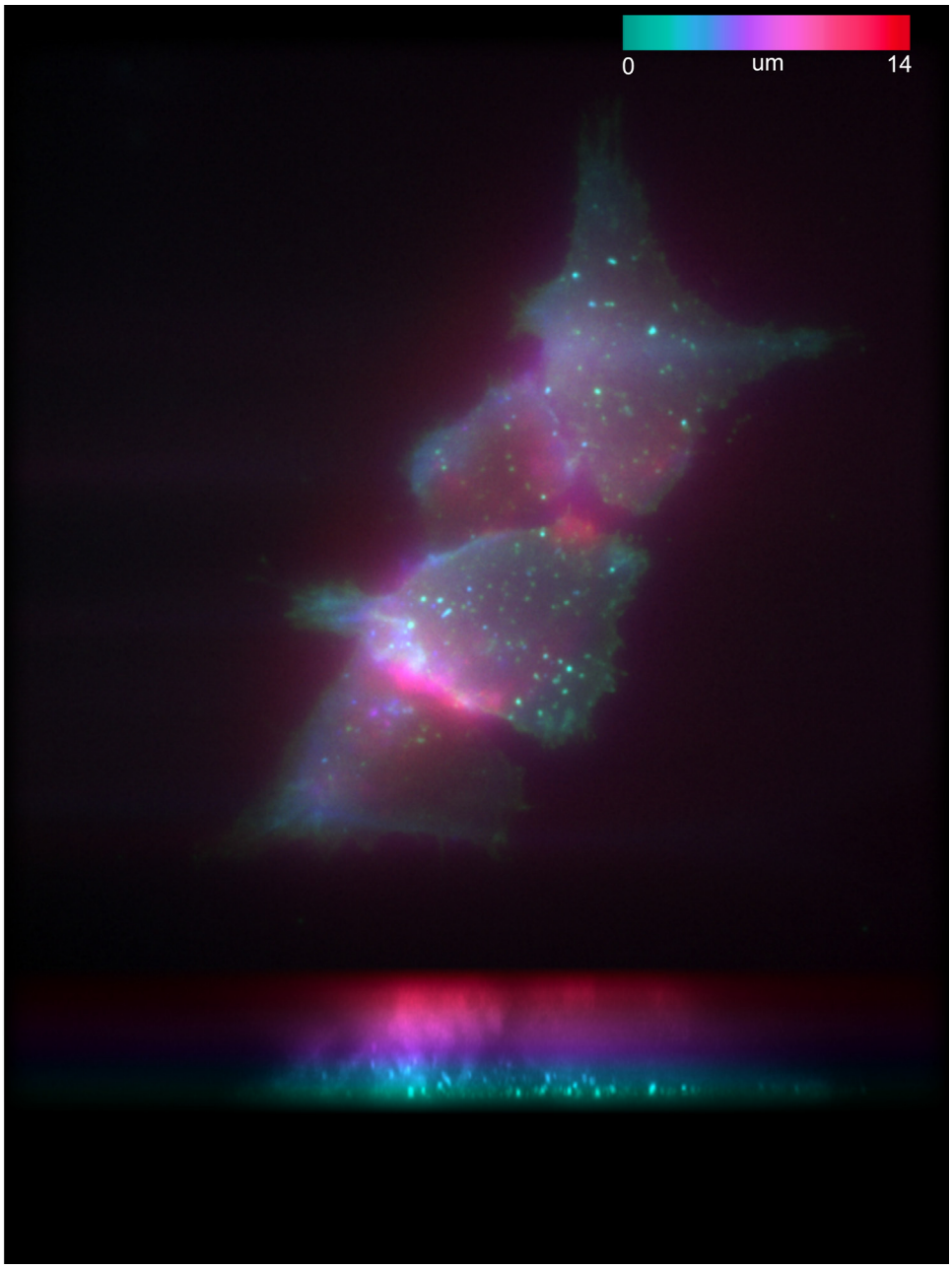

Figure 1. The presence of receptor tyrosine kinase (RTK)-mediated liquid–liquid phase separation (LLPS) at the plasma membrane is confirmed through microscopy images. This localisation is unique to RTK association and fundamental to downstream signalling. The figure shows membrane-localised droplets in HEK293T cells. Highly inclined laminated optical sheet image showing that GFP-tagged phosphorylated fibroblast growth factor receptor 2 droplets (coexpressed with untagged SHP2 and phosphorylated phospholipase Cγ1) are localised proximal to the cell membrane. Data are presented as depth-coded images (Top): XY view and (Bottom): XZ view, colour bar: 0–14 μm. In the XZ view (Bottom), it is clear that many of the droplets are visible along the flat line of the coverslip, and hence localised on the plasma membrane [13].

phase transition with the dimerised adaptor protein GRB2 [25]. Additionally, the prerequisite of phosphorylation in LLPS has also been demonstrated for non-RTK membrane receptors. For example, mutation of the pY-containing motifs on the C-terminal tail of Nephrin impairs phase separation with NCK [22,23,26]. Similarly, the presence of pY is also necessary in condensates of plasma membrane-bound LAT with GRB2 and SOS. Formation of LLPS with membrane-immobilised phosphorylated LAT is rapidly dissipated on addition of a phosphatase [13,15].

Protein condensation is enhanced by the presence of intrinsically disordered regions (IDRs) commonly found at the C termini of RTKs. These are dynamic, unstructured extended amino acid sequences that can provide multiple sites for binding partners. IDRs often include patches of polar, similarly charged or aromatic residues that are capable of weak, nonspecific intermolecular binding. Although electrostatic interactions are fundamental, additional multivalency of receptors can be enhanced by other forms of weak interactions. For example, hydrophobic proline-rich sequence motifs (PRMs), which are present on >40% of RTKs and are recognised by proteins containing, for example, SH3 and WW domains.

Chimeric fusion proteins involving RTKs, for example EML–ALK and CCDC6–RET, also undergo high-order assembly into actively signalling protein condensates [27–29]. However, the loss of the transmembrane regions in these fusions results in the condensed state appearing distal from the membrane.

Membrane receptor clusters as platforms for LLPS

Diverse families of membrane receptors such as RTKs, cell adhesion receptors, and immune cell receptors are able to transition from freely diffusing monomers or discrete dimers into higher-order oligomers of indefinite stoichiometry [12]. Such organisation of receptors into clusters, which can extend to 100s of nanometres, plays an important role in downstream signalling [23,30–32].

Although the involvement of RTKs in LLPS has only recently been characterised, the phenomenon of clustering of plasma membrane receptor proteins has been widely reported [33–35]. Constraining RTKs in the 2D membrane increases the probability of self-association which is elevated and stabilised through binding of multivalent extracellular and/or intracellular ligands. The constituent lipids of the membrane also constrain transmembrane protein localisation though the formation of lipid microdomains (sometimes referred to as rafts). Indeed, phase separation of plasma membrane lipids can initiate clustering of constituent proteins because these regions display properties required for receptor sequestration, such as charge and topology, that are different from the bulk membrane [20]. Formation of such clusters could then drive LLPS with cytoplasmic proteins through increasing the surface area of available binding sites and decreasing the dimensionality of motion of receptors. This increases the probability of an interaction with randomly diffusing downstream effector proteins [36,37].

Preclustering of nonstimulated signalling receptors represents a primed state; a condition from which they can rapidly assemble into the fully active constellation. In this state background phosphorylation can occur, although signal transduction is inhibited [1,2]. This has a significant impact on signalling reaction times and intensity, such that, rather than having to go from zero activity to above the noise level, signalling networks only have to transition from just below to just above the noise threshold [4]. Ligand-based activation within a cluster tips the balance toward a higher percentage of fully active receptors, which is sustained by the reduced dynamics and an increase in rebinding events [12] (e.g., rebinding of GRB2 to EGFR [38]). However, this small increase in the percentage of ligand-bound, and hence activated receptors, makes all the difference in

terms of signalling outcome; in particular, promoting the recruitment of proteins to form a condensed droplet.

The degree to which cytoplasmic proteins assemble in the third dimension when recruited to membrane-associated clusters is unknown, however it has been observed that increasing the dimensionality of *in vitro* systems from two to three increases the threshold concentration required for phase separation by as much as 30-fold [22,23]. Thus, if a cytoplasmic multivalent effector protein is at a concentration below the threshold for 3D droplet formation, but above the threshold for membrane-associated phase separation, the latter could be specifically triggered while constitutive phase separation in the cytoplasm is avoided. This provides a mechanism for concentration-dependent regulation of downstream signalling [12].

Impact on downstream signal transduction

The weak/moderate binding between component molecules involved in LLPS sustains an environment within a defined volume where interactions are probabilistically influenced by the relative concentrations. Amongst the random collisions within the phase, functional complexes form and initiate, or promote signalling outcomes which occur with higher probability than outside the condensed phase (Box 1).

The elevated concentrations of a subset of signalling proteins proximal to the membrane receptors in LLPS allow a rapid, high fidelity response to receptor upregulation. The molecular components of the signalling-competent LLPS include proteins that are able to interact to facilitate signal transduction. In addition, the molecular milieu within the phase-separated droplet excludes molecules that could interfere with signal transduction, such as molecules that might compete for binding sites on receptors or enzymes that function to inappropriately switch off signals [29], such as specific phosphatases [19].

The basis for selection of molecules that are able to prevail within the phase-separated droplet is not entirely clear, however the availability of complementary nonspecific, multivalent binding sites,

Box 1. Probability and encounter rate

Within the phase-separated droplet the elevated apparent concentrations of the component molecules in a defined volume profoundly affect the probability of the complex product being present. This is based on the equilibrium binding constant for an interaction as well as kinetic constants which can define a state where enzyme binding sites are continuously occupied by the substrate and the reaction kinetics are always at maximum velocity. Thus, even though the overall copy number of a biomolecule might represent a relatively low concentration within the context of the entire cell, if these are localised via LLPS, the concentration can be effectively elevated by several orders of magnitude.

Mathematical approaches can be applied to describe probabilistic concepts in LLPS. For example, asking whether size/number of clusters of receptors on the plasma membrane affect the probability of an effector protein binding (encounter rate). In the simplest mathematical abstraction, effector proteins diffuse in the cytoplasm until they encounter a cluster of receptors. If molecules have diffusivity, D, the cell is assumed spherical with radius R, and the cluster is a small circular patch with radius, a, then the mean time to encounter is given by $\pi R^3/3aD$. We may say that the rate of such encounters is proportional to a. When there are multiple well-separated clusters, the encounter rate is the sum the rates for each cluster. For example, if we replace a single cluster of radius, a, by two clusters of radius $a/\sqrt{2}$ (so that the total area is conserved and hence cellular concentration of receptors is constant) then the encounter rate is reduced by a factor of $1/\sqrt{2}$ but the total rate is increased by a factor of $\sqrt{2}$. Similarly for n clusters the reduction is by a factor of $1/\sqrt{n}$. Thus, the number of distributed clusters on the membrane has a modest impact on encounter rate.

A more dramatic increase in encounter rates is found if, instead, we consider the confinement radius of the molecules through LLPS into droplets. As described previously the mean encounter rate is proportional to the third power of R. Suppose that the molecules do not diffuse throughout the cell but only in smaller volume, adjoining the receptor cluster, with radius equal to one percent of the cell's radius and other parameters are unchanged. Then the encounter rate is increased by a factor of 1 million.

charge distribution and stability are likely to be important features. It is interesting to speculate that the properties of proteins found within the condensates are able to isolate specific signalling molecules while forming a barrier to entry of other cytoplasmic molecules. This would ensure that the probability of a defined signal is hugely increased over a background signal. In this way LLPS at receptors could ensure the observed mutual exclusivity in signal initiation. A good example of selection of proteins by droplets that has pathological consequences is demonstrated by electrostatic mutations in the phosphatase, SHP2, promoting its ability to engage in LLPS [39].

An extracellular-stimulated RTK can be responsible for several signalling outputs; however, the cell response is usually limited to one of these. LLPS would enable the initiation of one signal to dominate through providing the availability of only the downstream effectors required. For example, EGFR signalling has a large number of outputs resulting in diverse endpoints (e.g., cell proliferation, invasion, and survival), and perhaps LLPS helps to regulate these individually under predefined cellular conditions. Extending this idea of LLPS being a controller of a cellular response suggests that properties of LLPS have evolved to reduce the random nature of receptor function; that is, there is an evolutionary pressure for LLPS to enhance the deterministic outcome of signalling events.

Recruitment of important enzymes to the membrane, often via post-translational modification by lipid transferases (e.g., Src family kinase, and RAS), or binding to RTK-bound scaffold proteins has been demonstrated within phase-separated droplets. For example, the impact of LLPS is observed in the increased phospholipase activity of phospholipase C (PLC)γ within the condensate formed with FGFR2 and SHP2 (the phosphatase, SHP2 plays the role as a scaffold protein [19]). Condensation of EGFR, GRB2, and SOS enhances RAS activation at the membrane [25]. An important outcome of the inclusion of different proteins that are sequential in signalling pathways to the phase separated condensate is that, for each additional protein that is required in the linear pathway, the probability of interaction is dramatically increased compared to the recruitment of each protein in turn through random diffusion in the cytoplasm in the absence of LLPS. This is well exemplified in the recruitment of downstream effector proteins associated with insulin receptor signalling and activation of signalling outputs from LLPS [40,41]. IRS, which is a substrate of insulin receptor, mediates the formation of multiprotein LLPS formation that functions as a hub for processing of insulin signalling.

Impact on inhibitor development

Because of their role in multiple pathologies, the targeting of membrane receptors, particularly RTKs, has been the focus of exhaustive pharmaceutical development. Despite optimistic preclinical and initial patient responses, receptor-directed treatment outcomes often end in failure. In many cases the reasons for failure are unclear; however, our knowledge gap is expanded by the additional complexities of signal transduction from phase-separated condensates. For example, do the elevated local concentrations of signalling proteins within droplets have an impact on the targeting of inhibitors to receptors, or their effector proteins? Could targeting drugs to droplets increase the probability of binding, hence elevating efficacy and reducing off-target toxicity? There are a number ramifications associated with LLPS that could impinge on efficacy as well as provide opportunities to develop novel approaches to drug design through targeting droplets. Currently, it is unknown whether it is possible to reliably and efficaciously target small molecules to specific droplets within an animal model. However, there are some encouraging examples of separation of some therapeutically relevant molecules into droplets within specific cellular environments [42].

There are several ways in which LLPS could be targeted; however, two themes emerge (Figure 2). These are currently largely unproven even at the preclinical stage. The first approach is to

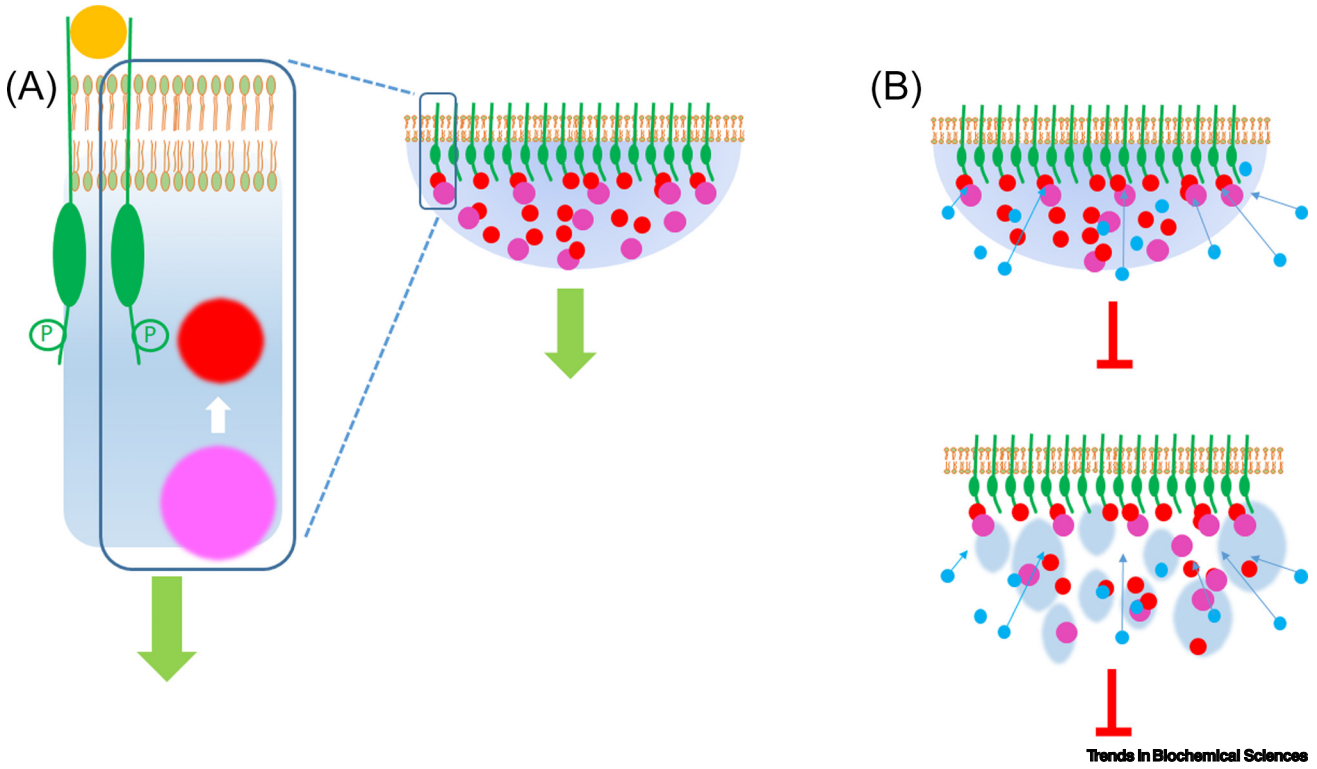

Figure 2. Targeting of liquid–liquid phase separation in drug development. (A) Schematic of initiation of signal from receptor tyrosine kinase (RTK) (green). On binding of extracellular growth factor (orange), the intracellular kinase activity is upregulated, resulting in phosphorylation of tyrosine residues on the receptor (circled green 'P'). These post-translationally modified residues contribute to recruitment of downstream effector proteins (red) and a signal transduction pathway through subsequent interactions with other proteins (purple). Within a droplet (Right, inset) multiple RTKs cluster at the membrane and are exposed to elevated concentrations of downstream effector proteins to drive mutually exclusive signal transduction initiation (green arrow). (B) Cluster of membrane receptors (green) interact with downstream effector proteins condensed in droplet (red and magenta). (Top) Targeting of small molecule inhibitor (blue) to membrane receptor in droplet. Phase separation of inhibitor into droplet increases targeting of interaction between RTK and downstream effector molecules. Efficacy is raised through elevation of local apparent concentration of drug. (Bottom) Targeting of small molecule inhibitor to droplet abrogates multivalent interactions between droplet protein constituents resulting in dissolution of droplet, hence reducing probability of signal initiation.

enhance compound binding to the protein target through synthesizing inhibitor molecules such that they phase separate with the target molecule. This approach requires greater understanding of the thermodynamic and electrostatic conditions within the specific droplet in which the target is localised. The interior of an RTK-mediated droplet, because of, for example, the high levels of phosphorylation and membrane lipid inclusion, is likely to be a highly charged environment. Is it possible, therefore, to develop compounds or pro-drugs that preferentially separate into these environments? If so this could have a big impact on our approaches to dosing and cell permeation.

The second approach is to target compounds that dissolve phase separated droplets and hence disperse the component signalling molecules into the cytoplasm: hence reducing the probability of pathological signal initiation. Although, this approach is perhaps more difficult to achieve, it does seem to have been validated through *in vitro* evidence of downregulation of signal transduction though dispersal of LLPS with small molecules such as hexanediol [19,37,43].

There are a number of challenges associated with the preliminary and preclinical validation of these approaches. For example, defining the environment within the target droplet is complicated. The

formation and sustainability of a condensate is highly dependent on the relative concentrations of the component proteins [44]. However, the droplet size and the concentration of functional complexes of the component proteins are not directly correlated (Figure 3). In other words, droplets of different sizes can contain varying amounts of target proteins, hence droplet dosing of directed inhibitors will be difficult. Thus, targeting large, droplets in cells does not necessarily lead to efficacious outcomes. The demonstration of inhibitor translocation to droplets does not validate therapeutic outcome. Another challenge is the development of methods to assess phase partition of compounds into droplets in cells [45–47]. Current approaches have been focused on observation of fluorescent compounds; however, the development of alternative methods will be needed for high-throughput screening protocols to investigate interactions of large chemical libraries. Recent years have seen substantial funding directed at targeting LLPS, but further biological understanding and development of novel tools is required to mitigate the apparent high risk [48,49].

Concluding remarks

Protein condensates mediated by plasma membrane receptors, particularly RTKs, constitutes a unique niche in the broader study of the biology of LLPS. The recruitment of a subset of specific signalling proteins to phase-separated droplets elevates their apparent concentration within the limited volume, increasing the rate of collision compared to the bulk cytoplasm. LLPS therefore increases the probability of interactions occurring between component molecules while reducing the probability of aberrant interaction through exclusion of redundant molecules. Although the full extent of LLPS involvement in receptor signalling is still unknown, it is possible that we will have to revisit many of the tenets that support our understanding of RTK function. This will have a huge impact on our holistic picture of signal transduction within cells and define novel approaches for pharmaceutical intervention. Future discoveries will decide whether the textbooks need to be rewritten (see Outstanding questions).

Outstanding questions

Identification of LLPS in cells is becoming more functionally diverse. Can we clarify the differences in phase properties of these different functions and subcategorise these?

Can we obtain a greater understanding of the conditions within the LLPS droplets and how these control selectivity? For example, what are the key requirements of a protein to enable it to enter the condensate droplet, or be excluded?

In addressing all of the above questions, will we obtain a greater understanding of the opportunities for targeting of LLPS condensates in the pharmaceutical intervention?

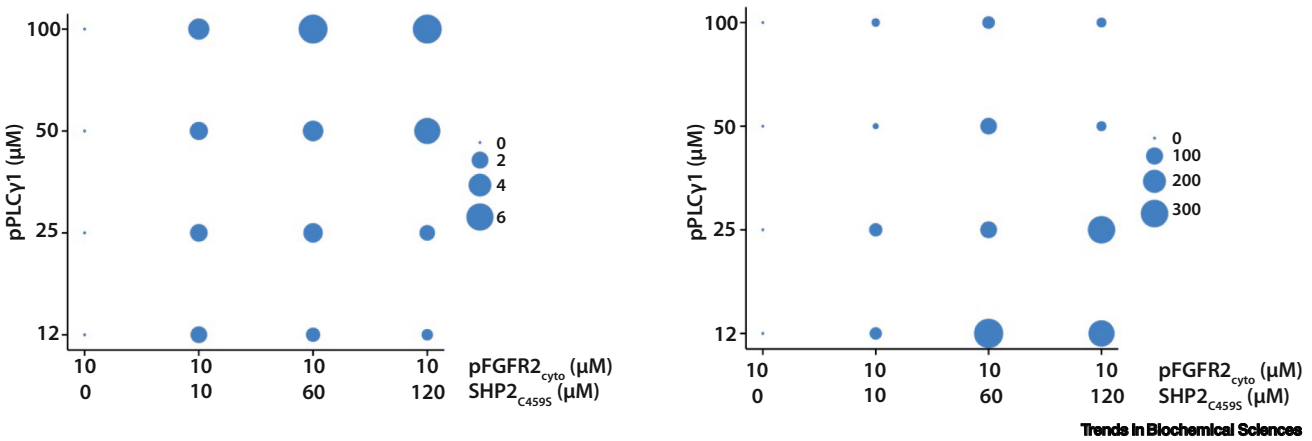

Figure 3. The concentration of functional complex does not correlate with the size of the phase separated droplet. This provides a challenge for drug screening, dosing and efficacy. (Left) Data based on the formation of the ternary complex between phosphorylated fibroblast growth factor receptor 2 (pFGFR2), SHP2 and phosphorylated phospholipase Cγ1 (pPLCγ1) which undergoes liquid–liquid phase separation (details of mathematical modelling of complex concentration [19]). The plot shows the concentration of the ternary complex (circle size coded according to scale from 0–6.0 μM) with varying concentration of individual components shown on graph axes. (Right) Phase diagrams of pFGFR2 and SHP2 with concentrations shown (x axis), and pPLCγ1 (y axis). The sizes of the circles represent the average sizes of droplets (μm^2) [19]. From comparison of the two graphs it can be seen that the concentration of ternary complex under a given concentration regime does not correlate with the average size of the phase separated droplet. This underscores the need to determine relative concentrations of protein components in droplets to optimise targeting of inhibitors. Importantly, large droplet sizes do not correlate with high concentrations of functional complex [e.g., compare values at pPLCγ1 (25 μM), FGFR2 (10 μM), and SHP2 (120 μM)].

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Declaration of interests

None are declared by the authors.

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