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Chemical tools for study and modulation of biomolecular phase transitions

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Biomolecular phase transitions play an important role in organizing cellular processes in space and time. Methods and tools for studying these transitions, and the intrinsically disordered proteins (IDPs) that often drive them, are typically less developed than tools for studying their folded protein counterparts. In this perspective, we assess the current landscape of chemical tools for studying IDPs, with a specific focus on protein liquid–liquid phase separation (LLPS). We highlight methodologies that enable imaging and spectroscopic studies of these systems, including site-specific labeling with small molecules and the diverse range of capabilities offered by inteins and protein semisynthesis. We discuss strategies for introducing post-translational modifications that are central to IDP and LLPS function and regulation. We also investigate the nascent field of noncovalent small-molecule modulators of LLPS. We hope that this review of the state-of-the-art in chemical tools for interrogating IDPs and LLPS, along with an associated perspective on areas of unmet need, can serve as a valuable and timely resource for these rapidly expanding fields of study.

Introduction

Intrinsically disordered proteins and intrinsically disordered protein regions (henceforth collectively referred to as IDPs) are common in the proteome.[1,2] All IDPs share a relatively flat folding energy landscape, and often completely lack a detectable folded low-energy state.[3] Unlike proteins constructed from random amino acid sequences, which are also often intrinsically disordered, proteomic IDPs typically exhibit low sequence complexity with primary sequences enriched in a small number of amino acids.[4–6] Despite their unfolded or conditionally folded nature, IDPs can act as important regulators of cellular functions, often through low-affinity interactions that are driven by the bulk chemical properties of residues in the IDP.[2] Many IDPs can also undergo a process called liquid–liquid phase separation (LLPS) that allows them to concentrate into dynamic and spatially resolved condensates.[7] This capability allows IDPs to

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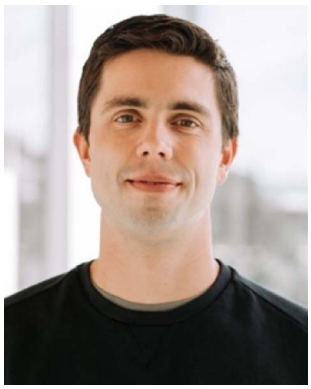

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rapidly and selectively recruit other biomolecules and entrenches their role as regulators of intracellular organization, with many common IDPs such as p53, FUS, and α-synuclein exhibiting pleiotropic regulatory behaviors that are essential for cell viability.[8,9]

IDPs present unique challenges to structural biologists – their conformational plasticity and the range of dynamics that they can exhibit are difficult to characterize with tools that have been optimized for folded proteins. While early structural studies of IDPs often focused on proteins that fold upon binding, this behavior is not universal. For example, some IDPs engage in high affinity interactions while retaining their disorder, while others engage in nonspecific low affinity interactions that drive biomolecular LLPS and are dependent on their intrinsically disordered nature.[10–12] In addition to the conceptual challenges brought on by the unstructured nature of these proteins, the sensitivity of IDPs to environmental conditions requires special consideration when working with them *in vitro*. Characterizing the structure–function relationship of IDPs, therefore, necessitates a shift in the way that we think about protein structure and a coincident shift in the methods and tools that we use to interrogate it, especially in the context of LLPS.[13,14]

In this perspective, we discuss how chemical biology can aid structural studies of IDPs, with an emphasis on chemical tools that are compatible with LLPS. This will include strategies for the recombinant preparation of IDPs, tools that enable the efficient and site-selective introduction of chemical probes and isotopic labels, and chemical modulators of IDPs and LLPS. We take an application-centric approach to highlight real use cases that are enabled by the current state-of-the-art in IDP chemical biology. We hope to provide insight into best practices for handling and studying these systems, along with a call for the development of minimally perturbative small-molecule chemical tools to aid their analysis and functional manipulation.

IDPs present distinct challenges for biophysical investigations *in vitro*

When working with IDPs *in vitro*, their intrinsic disorder brings considerations that are not present for folded proteins. IDPs are often less soluble than their globular counterparts near their isoelectric points due to the lack of a distinct fold, and they are often prone to LLPS or aggregation under these conditions due to nonspecific interactions between hydrophobic residues.[15] On the other hand, the lack of a need to maintain a fold can facilitate IDP survival at extreme pH levels and temperatures that would lead to denaturation and aggregation of folded proteins, which can enable unique purification and manipulation strategies. The conditions under which IDPs are handled must therefore be chosen with these considerations in mind. In this section, we will discuss challenges associated with working with IDPs *in vitro*, along with approaches for purification that are distinct from those of typically folded proteins. We will highlight examples of IDP preparations that strategically employ pH, temperature, and other abiotic factors in ways that would not be applicable for folded proteins. This section will also provide some context for the challenges that need to be overcome when developing chemical tools to aid the structural and functional analysis of IDPs. For a more comprehensive overview of strategies and recommendations for purifying IDPs, the interested reader is referred to two excellent resources by Graether and Alberti *et al.*[16,17]

Whether expressed in *E. coli* or other recombinant expression systems, one of the challenges in handling IDPs is avoiding undesirable aggregation or LLPS during the purification process. Fusing solubility tags such as MBP or GST to an IDP of interest can be a useful strategy for maintaining its solubility.[18,19] As these solubility tags are large and may interfere with downstream functional and biophysical assays, they are typically removed at the end of the purification process through a suitably engineered cleavable site. This requires an additional purification step to remove the cleaved tag. Cleavage of a solubility tag after purification can be a facile approach for performing LLPS studies.[20] In a compelling recent example of this approach, Morin *et al.* use an MBP-Klf4 fusion protein to construct a model describing the role of prewetting in the sequence-specific surface condensation of the transcription factor Klf4, which forms small LLPS condensates on DNA. In this work, the MBP-Klf4 fusion is capable of adsorbing onto DNA, but does not undergo LLPS. After adsorption, the MBP tag is removed and Klf4 condenses into droplets around sequences known to promote Klf4 binding.[21] This strategy allows for the disambiguation of Klf4 adsorption and LLPS. In this case, a solubility tag is used to enable an LLPS study in an environment that would be sensitive to other means of LLPS initiation, such as a pH jump or changing the IDP or salt concentration.

Since IDPs are often enriched in hydrophilic and charged residues, tuning the pH can be a straightforward way to improve the performance of a purification protocol. Although there are reports that leverage pH in order to purposefully precipitate the IDP of interest,[22] in most cases the selection of a buffer that optimizes solubility is preferred. Buffers that maintain unusually high or low pH conditions are often advantageous, and the disordered nature of the IDP means that pH-induced denaturation is not an issue. As an example, some purification approaches for the low-complexity domain of Fused in Sarcoma (FUS LC), which undergoes LLPS and/or aggregation at neutral pH, involve the extensive use of *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS), a buffer that maintains a pH of 10–11. At such high pH, the tyrosine residues on FUS are deprotonated and the protein is highly charged, which promotes solubility and enables the subsequent purification of the LC domain by size-exclusion chromatography without the need for a denaturant.[18,23–25] On the other hand, low-pH conditions are often used to purify α-synuclein.[26] This first step in the purification process precipitates many undesired cellular proteins, thereby leveraging α-synuclein's resistance to pH-dependent denaturation. A potential disadvantage of pH-based purification protocols is that they may not be compatible with chemical biology approaches for protein labeling or modification, as the reactions are typically sensitive to pH.

Temperature is another practical consideration that may require a different approach than that taken for a typical globular protein. Since there is no fold to maintain, some IDPs are resistant to high temperatures, a unique property that can be exploited with high-temperature protein purification approaches. Tau and α-synuclein, for example, are often purified by boiling crude cell lysate, which denatures and precipitates most cellular proteins and leaves a soluble fraction that is highly enriched in the desired protein.[26] On the other hand, many IDPs undergo thermoresponsive phase transitions which may require the use of mild temperatures around 25 °C throughout the protein preparation,[7] a characteristic that is counter-intuitive to those who are used to maintaining temperatures closer to 4 °C throughout the purification of a globular protein. Tolerance to higher temperatures may be beneficial for chemical labeling approaches of IDPs as it can speed up the relevant reactions.

For IDPs that are especially aggregation-prone, chemical denaturants are often necessary to achieve reasonable yields from a recombinant protein preparation. In many IDP preparations, chaotropes such as urea or guanidinium hydrochloride can be used to redissolve aggregated protein or to maintain solubility at a pH or temperature that would otherwise induce aggregation or LLPS.[27] Chemical denaturants may also be required to keep IDPs soluble during size-exclusion or ion-exchange chromatography purification steps. In some cases, the IDP will be sequestered into inclusion bodies during expression, and urea or guanidinium hydrochloride may be required for extracting proteins from the inclusion body and for mitigating the risk of aggregation in subsequent steps.[28] Chemical denaturants are often compatible with cysteine chemistry and even intein-based segmental labeling approaches, and can therefore be useful in the preparation of modified and labeled IDPs.[29]

Introduction of chemical probes for imaging and spectroscopic studies

Understanding the structure, dynamics, interactions, and functions of IDPs often relies on fluorescence-based approaches such as fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET), and/or spectroscopic studies by nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR).[30–32] A common requirement for these techniques is the site-specific installation of chemical probes that report on the properties of the IDP or its environment. A wide range of technologies for site-specific labeling have been demonstrated, and many of these approaches are applicable to IDPs.[33–35] Here we review the applications of cysteine chemistry, unnatural amino acid incorporation through genetic means (amber suppression), inteins, and sortase, with a particular emphasis on the unique challenges presented by IDPs and LLPS. We focus primarily on the installation of small chemical probes such as fluorescent labels, rather than the use of large fusion fluorescent proteins, as these approaches may be less familiar to the reader and may have distinct advantages in certain applications.

Cysteine is the chemical handle of choice for facile labeling of IDPs

A wide range of small-molecule chemical tools are available for the site-selective modification of proteins under aqueous conditions.[36,37] Of these, the most robust and practical reactions take place at nucleophilic cysteine residues. Cysteines are particularly rare in IDPs and are therefore valuable reactive handles that can be targeted selectively if present or added into the recombinantly-produced proteins if needed. The potent nucleophilicity of the cysteine thiol allows for chemoselectivity even in the presence of other nucleophiles such as the primary amines on lysines and protein N-termini.

Covalent labeling at cysteine residues is a popular strategy for introducing small-molecule fluorescent probes in proteins including IDPs (Fig. 1A). This is most often achieved through maleimide or iodoacetamide functionalized dyes and there are many commercially available options covering a wide range of absorbance and emission properties, including cyanine-based probes, the Alexa Fluor® series, and the BODIPY family of dyes.[38] Labelling at a cysteine residue with a maleimide-based probe is often as simple as incubating the dye with the protein for less than an hour and subsequently removing the unreacted dye *via* gel filtration or reverse-phase chromatography.[39–41] In the case of IDPs, it may be beneficial to perform the labeling step while the protein is still fused to the solubility tag or under denaturing conditions to avoid issues with aggregation or premature LLPS. The straightforward and robust nature of cysteine chemistry make this the preferred method of choice for labeling IDPs, especially if the purification protocol is challenging and delivers relatively low yields. Sometimes, it may also be possible to use the primary amines of lysine residues to attach fluorescent probes through *N*-hydroxysuccinimide ester chemistry.[42] However, there are typically many more lysine residues in a protein compared to cysteine and it is difficult to control the specificity of these reactions, especially in the context of IDPs where all side-chains are solvent exposed and accessible. The unique reactivity of the α-amine of a protein's N-terminus can also be exploited to attach imaging or spectroscopic probes through chemical or enzymatic means. Interested readers are referred to the comprehensive review of these methods by Rosen & Francis.[43]

In addition to fluorescent labeling, cysteine is a useful handle for introducing spin labels for NMR- and EPR-based methods. A common NMR approach for IDP studies is the paramagnetic relaxation enhancement (PRE) experiment.[44] PREs are particularly powerful in detecting weak intra- and inter-molecular interactions at residue-specific resolution in dynamic biological systems and are often applied to characterize the molecular basis of LLPS.[18,23,24,45,46] In a PRE experiment, the protein of interest is labeled with a paramagnetic relaxation enhancement probe, typically a nitroxide-based stable radical moiety such as *S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-yl)methyl methanesulfonothioate

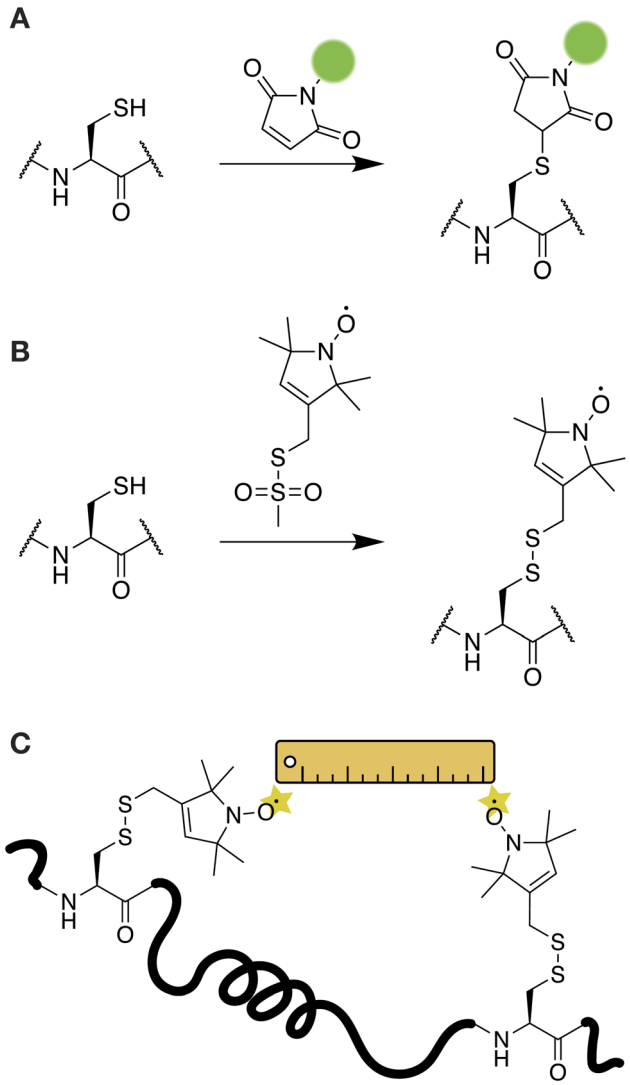

Fig. 1 Cysteine approaches for introducing imaging and spectroscopic probes in IDPs. (A) Alkylation reactions with maleimides are often used to attach fluorescent probes. (B) The EPR probe MTSL can be introduced through a disulfide oxidation reaction. (C) The distance between two MTSL probes can be measured through an experiment called double electron–electron resonance (DEER).

(MTSL).[44] The addition of MTSL results in an oxidation reaction and the formation of a disulfide bond with the targeted cysteine residue (Fig. 1B). The nitroxide moiety on the probe can induce distance dependent relaxation effects that reduce the peak intensity for residues within a 10–25 Å radius.[13] This information can be used to construct a map of the residues that participate in intra- or intermolecular interactions for IDPs. For example, PRE-based NMR experiments have been used to describe the transient interactions formed by the low complexity domains of FUS, TDP-43, hnRNPA1, and hnRNPA2 in LLPS environments.[23,24,45,47,48]

The MTSL probe can also enable the characterization of IDP behavior by EPR. For example, MTSL-labeled tau was used to characterize tau dynamics within liquid–liquid droplets and to report on tau–water interactions.[42] In this case, the native cysteine residues of tau were mutated to serine, and a new cysteine was introduced at a different position in the protein sequence for labeling so that the relevant interactions could be captured. A commonly used EPR experiment is double electron–electron resonance (DEER), which measures up to 10 nm distances between two electron spin probes.[49] DEER is conceptually similar to FRET experiments and can be used to build a structural model of the protein of interest and to characterize protein–protein interactions (Fig. 1C).[50,51] If intramolecular DEER is performed, then two spin labels need to be introduced in the protein. As DEER can probe distances between two identical spin probes, labeling can be easily achieved by introducing two cysteine residues at the relevant positions in the IDP. For example, in a recent study, DEER of a doubly-labeled construct of the FUS LC domain was used to interrogate the dynamics and conformational distributions of the protein within a phase separated sample.[52] EPR and DEER can also be performed with Gd^{3+}-based spin probes attached through cysteine chemistry.[51,53]

Despite their popularity, some important considerations need to be taken into account when working with cysteine-based labeling approaches. When oxidation-based reactions are used for labeling (*e.g.* with MTSL), the protein cysteines need to be reduced and available before the labeling reaction takes place.[54] Once the disulfide bond between the protein and the probe is formed, care must be taken to avoid reducing conditions or agents in the sample buffer. Even the more chemically resilient maleimide labeling reactions can be susceptible to hydrolysis and thiol exchange, processes that can be exacerbated by higher pH and long storage in aqueous solutions.[55] Cysteine-based reactions are also not bioorthogonal and are therefore not suitable for in-cell applications. Finally, using cysteine labeling approaches, it is difficult to introduce two different probes on the same IDP as may be required for intramolecular FRET experiments. In this case, cysteine chemistry may be combined with unnatural amino acid incorporation by genetic means and bioorthogonal labeling reactions, as discussed below.

Unnatural amino acids provide flexibility for specific and multiple labeling

In cases where labeling at a cysteine is not an option, or a second distinct chemical probe needs to be site-specifically introduced to an IDP of interest, the incorporation of an unnatural amino acid (UAA) can help expand the reactive scope of the target protein.[56,57] The introduction of an entirely unique chemistry into the IDP with a UAA precludes any cross-reactivity with other nucleophilic residues or issues with multiple labeling that come along with the classical thiol-reactive chemical probes described in the previous section. Unnatural amino acids can be introduced by genetic means through a technique often referred to as amber suppression (Fig. 2A).[56,58,59] In this case, the amber stop codon, UAG/TAG, is assigned to the UAA and cloned at the desired position in the protein sequence. At the same time, the cells are transformed or transfected with a second plasmid that encodes an engineered

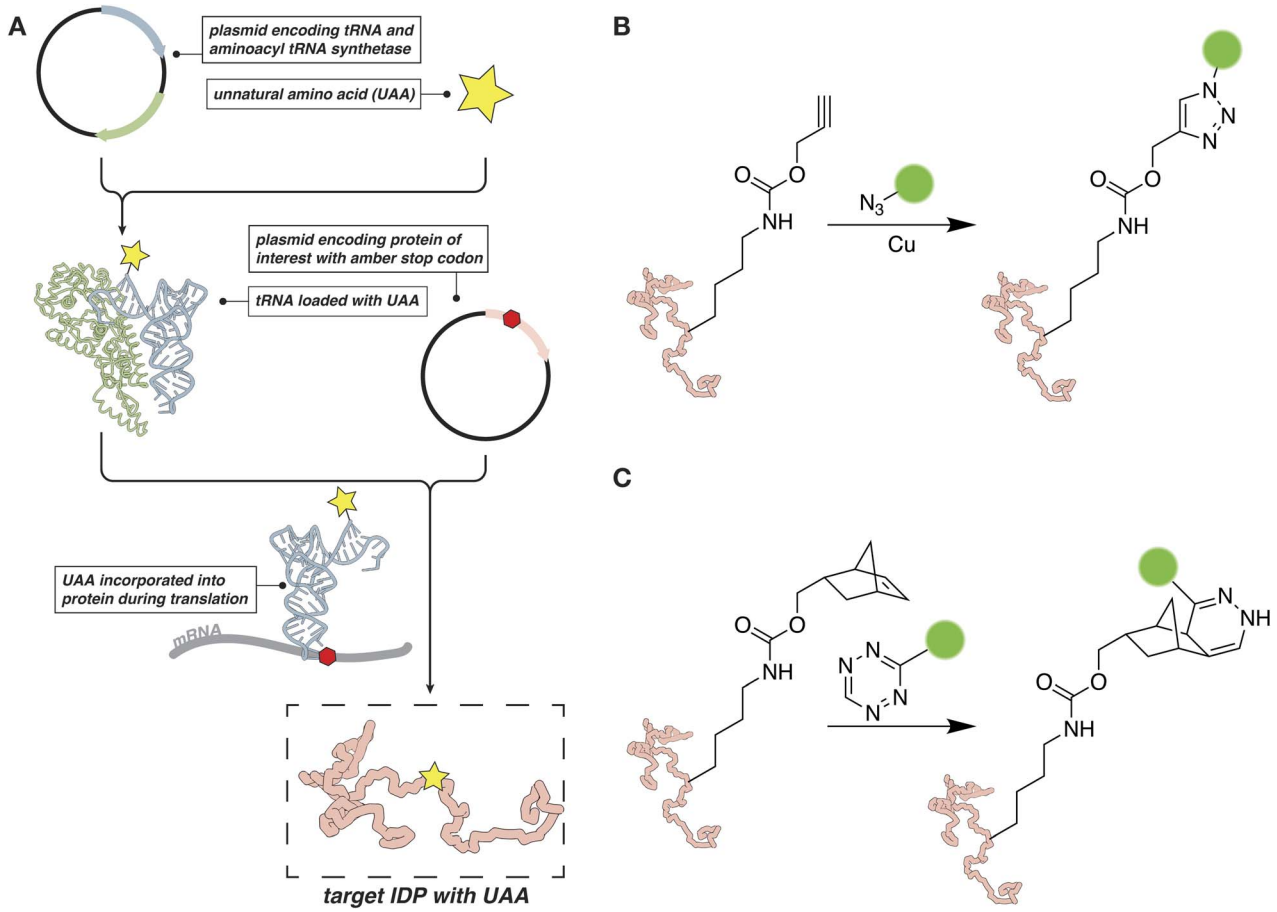

Fig. 2 Amber suppression as a tool for studying IDPs. (A) An overview of the amber suppression strategy. (B) Bioorthogonal CuAAC reaction for protein labeling. (C) Bioorthogonal labeling reaction based on tetrazine–norbornene chemistry.

tRNA/tRNA-synthetase pair. The tRNA synthetase can recognize the UAA and load it onto the cognate tRNA. The loaded tRNA, in turn, recognizes the amber stop codon and delivers the UAA to the ribosome for incorporation into the growing protein sequence.

Amber suppression is often used to introduce a chemical handle for bioorthogonal chemical reactions, such as an azide, tetrazine, strained alkene or alkyne.[60–65] These chemical moieties can then be targeted with a suitable "warhead" carrying the fluorescent or spectroscopic probe of interest. The bioorthogonal reactions can be performed both *in vitro* on the purified protein and in cells. For example, copper-catalyzed [3 + 2] azide–alkyne cycloaddition (CuAAC) reactions have been used to attach EPR probes to proteins in mammalian cells, while tetrazine-based reagents have been used to tether fluorescent probes (Fig. 2B and C).[62,65] If a suitable engineered tRNA/tRNA-synthetase pair has been developed, fluorescent probes, spin labels, or other suitable moieties, can be introduced directly as the UAA.[56–58,66] For example, several reports from Schmidt *et al.*, describe the evolution and subsequent application of a $tRNA^{Pyl}$/pyrrolysyl-tRNA-synthetase pair capable of installing a spin-labeled nitroxide-based amino acid.[67,68] This allows for the direct introduction of a probe without the need for performing chemistry on the target protein after expression, a capability that could prove especially useful for IDPs that are sensitive to abiotic conditions.

From a conceptual point of view, amber suppression is relatively straightforward to implement as it requires only the addition of two plasmids and the UAA before protein expression. In practice, it can often severely reduce the yield of the desired protein as truncation products are very common. This problem is even more pronounced if two or more UAAs need to be installed.[69] Despite these shortcomings, amber suppression has great potential for the biophysical studies of IDPs. For example, it can be used in combination with cysteine chemistry or alone to install two distinct fluorescent and spectroscopic probes on the same protein. More importantly, as these reactions can be performed in cells, bioorthogonal chemistry and amber suppression can provide an alternative to fluorescent proteins for LLPS studies in the cellular milieu. In the context of spectroscopic probes, these strategies can enable the structural characterization of IDPs by EPR or PRE NMR in a native environment.[65,70] While there are currently few examples in the literature of successful applications to IDP and LLPS studies,[71] we expect that continuing developments to improve efficiency of UAA incorporation and speed of bioorthogonal labeling will

make these approaches a reliable and useful option for biophysical analysis both *in vitro* and in cells.[72,73]

Inteins and sortase are versatile tools for segmental labeling of IDPs

While IDPs are often studied by NMR, their repetitive sequences and low chemical shift dispersion can make resonance assignments challenging.[74] In such cases, it is helpful to segmentally label the protein, so that only a portion of the sequence is visible by NMR while the properties of the full-length polypeptide are preserved.[75,76] Segmental labeling is often performed with split inteins, protein engineering tools that can connect two separate protein segments through a native peptide bond in a process called *trans*-splicing (Fig. 3A).[77] To perform segmental labeling, the protein of interest (*i.e.* the extein) is divided into two fragments called the N- and C-exteins, respectively. Each fragment is fused with the corresponding N- or C-intein and the fusions are expressed and purified separately, so that each fusion construct can be labeled as desired (*e.g.* ^{15}N and natural abundance, or ^{15}N and ^{13}C respectively). After purification, the two constructs are mixed and the N- and C-inteins come together through non-covalent electrostatic interactions and adopt the functional intein horseshoe-like fold.[78] Upon folding, the assembled intein performs *trans*-splicing of the extein fragments and releases the newly ligated native protein of interest. Since most optimized split inteins use cysteine chemistry to carry out the *trans*-splicing reaction, segmental labeling requires that the extein is split at a native cysteine residue or that a cysteine is introduced at the desired location. Some inteins may also require a few additional residues beyond the junction cysteine, although the most efficient engineered intein so far, Cfa GEP, is quite tolerant to variations in the extein sequence.[29,79,80] Some inteins can also carry out splicing reactions at serine or threonine junctions, although their full extein dependency is less known.[81,82]

While inteins are gaining traction as segmental labeling tools for a variety of proteins, the biggest challenge for their application to most IDPs is the requirement that the *trans*-splicing reaction is performed under denaturing conditions to keep the reactants and final product soluble. The presence of urea or guanidinium can interfere with the folding of the intein and severely reduce the efficiency of splicing. The best intein to use in such cases is the Cfa GEP intein, which was engineered to withstand harsh conditions and can carry out splicing in buffers that contain up to 4 M guanidinium hydrochloride and 6 M urea.[29,79] There are also inteins that are tolerant to very high salt concentrations and can in principle be used for splicing in such conditions that often prevent phase separation of the protein of interest.[83] Extein–intein fusions may also have increased expression levels and higher solubility when compared to the full-length IDP construct, although this may be highly protein dependent. In cases where the solubility and purification of the extein–intein needs to be improved, solubility or affinity tags such as MBP or His_6 can be fused on the intein-side of the construct.[29,84] At this position, the tags do not interfere with the splicing reaction and are automatically removed from the extein when splicing takes place.

In addition to segmental labeling, inteins have other useful applications. For example, they can be adapted to attach C-terminal small molecule fluorophores to proteins of interest.[77,79,85] In this case, an engineered or native contiguous intein is fused to the C-terminal side of the extein. The fused intein can be cleaved in the presence of a thiol which generates a C-terminal thioester on the extein. The thioester can then be reacted with a small peptide bearing the desired fluorophore, thus generating the labeled protein. This strategy may be useful when fluorescent proteins such as GFP are not compatible with LLPS studies (*i.e.* if they interfere with LLPS) and when cysteine labeling is not an option. In a different adaptation of this strategy, the intein can be hydrolyzed from the extein at slightly basic pH.[86–89] If a suitable affinity tag is attached on the intein side, *e.g.* a His_6 or chitin, then the intein can be hydrolyzed directly during affinity purification. In this case, the intein and the tag remain on the column while the pure protein of interest

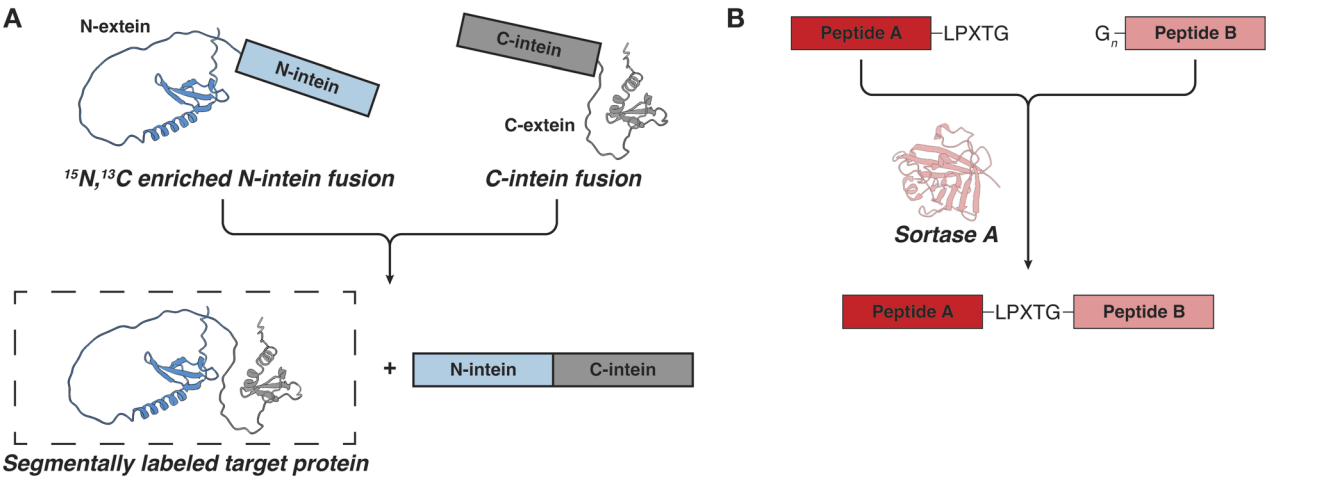

Fig. 3 Approaches for segmental labeling of IDPs for NMR studies. (A) Intein mediated protein *trans*-splicing. (B) Ligation of recombinant or synthetic polypeptides by sortase.

is released in solution. This strategy circumvents the need for a protease cleavage step to remove the affinity tag and may be a useful option when such steps are problematic in the IDP purification protocols.

An alternative tool for segmental labeling and protein modification is the transpeptidase sortase (Fig. 3B). This protein can stitch together two polypeptides in *trans*, provided that they carry an appropriate signal peptide.[90–92] The recognition signal typically consists of the LPXTG sequence (where X is any amino acid) on one peptide, and one to five glycine residues on the other peptide.[90] The two peptides are mixed and sortase is added in *trans* to carry out the ligation reaction, resulting in a minimum six residue "scar" in the newly formed protein. If one of the peptides is prepared in minimal isotopically labeled media, the resulting full-length protein will be segmentally labeled. In the context of IDPs and LLPS, there are several important considerations that need to be kept in mind.[93,94] First, to carry out the ligation reaction, sortase needs to be folded, which precludes the use of high concentrations of denaturants. Second, the reaction requires the presence of Ca^{2+} ions in the buffer, which may be incompatible with LLPS studies for some proteins.[92] And third, as the product polypeptide also contains the LPXTG recognition motif, the sortase reaction may be reversible and care must be taken to remove the product quickly and to minimize the generation of undesired products.[93] Nevertheless, the sortase-based ligations are conceptually elegant and may provide a useful alternative when the protein of interest is incompatible with intein splicing (*e.g.* if the protein charge interferes with the binding and folding of the intein fragments which proceeds through electrostatic interactions). Sortase has also been used extensively to attach proteins to surfaces and to add IDRs to the folded regions of proteins.[95,96] Interestingly, sortase has an IDR which undergoes a disorder-to-order transition upon binding of the signal peptide and Ca^{2+} ions.[97]

Introduction of post-translational modifications

Innate biological LLPS is often controlled by post-translational modifications (PTMs) including phosphorylation, acetylation, methylation, and ubiquitination.[98,99] Faithfully recapitulating PTMs *in vitro* is therefore crucial for constructing experiments that interrogate LLPS. While there are a variety of chemical and biochemical tools for installing or modelling PTMs after a protein has been recombinantly purified, IDPs present unique challenges for the applications of these tools.[98] Here, we review enzymatic approaches, bioisostere substitutions, cysteine chemistry and native chemical ligation, with the goal to give a range of options to biophysicists and biochemists interested in understanding the role of PTMs in IDPs and LLPS mechanisms and interactions.

The most common strategy to introduce PTMs in recombinantly produced IDPs is enzymatic modification. This approach is flexible as it can generally be applied to any substrate protein if the appropriate enzyme can be purchased or expressed and purified in house. Enzymes also introduce the same chemistries that are seen in cells, avoiding issues that may arise with PTM isosteres or PTM mimetics that result from other methods for PTM installation. The chemical accuracy and ease of use of this approach has made it the method of choice for many studies involving a variety of combinations of IDPs and PTMs, including phosphorylation of tau,[100] acetylation of FUS,[101] and mono-, di-, and trimethylation of histone tails[102] (among many others). The major drawback with enzymatic PTMs is that the activity of the enzyme can be difficult to control, and the resulting protein can be either overmodified (with the PTM present at multiple sites on the protein) or undermodified where the desired PTM is not installed efficiently. Controlling the installation site is also an issue, especially in unfolded IDPs whose residues are entirely solvent exposed. The proteins that result from mixtures of sites and distributions of PTMs that are generated by enzymes are often hard to separate from each other. These factors limit the enzymatic approach to cases where an appropriately active enzyme is available and there is either only one specific substrate residue in the protein or a distribution of modifications is desired, similar to the cases illustrated above.

A second straightforward strategy to incorporate PTMs is to genetically encode a bioisostere into the protein of interest. Bioisosteres, commonly used in medicinal chemistry and chemical biology, are atoms or functional groups with similar chemical and physical properties.[103] In the context of PTMs, perhaps the best known example is the substitution of phosphorylated serine or threonine residues with glutamic acid. Similar to enzymatic PTM incorporation, ease of application is a major benefit with this approach: bioisosteric residues can be encoded in the protein with cloning, and no further modification is required after protein purification. The drawback is that few good bioisosteres for PTMs exist among the canonical amino acids. Despite this limitation, this strategy has been commonly used in the literature, especially in the context of phosphorylation.[104] This includes a number of studies by the Fawzi group that reveal the influence of site-specific phosphorylation on the structural distributions and LLPS propensities of both FUS and TDP-43.[23,46] As more tRNA/tRNA synthetase pairs that encode pre-modified amino acids are developed, we expect that the incorporation of chemically accurate PTMs *via* genetic encoding by amber suppression will become a valuable strategy for incorporating PTMs into IDPs. It is already possible to encode phosphotyrosine and acetyllysine, for example, and new tRNA/tRNA synthetases are actively being developed.[105,106]

Cysteine chemistry presents a convenient way to introduce PTM mimics that are inaccessible with genetically encoded

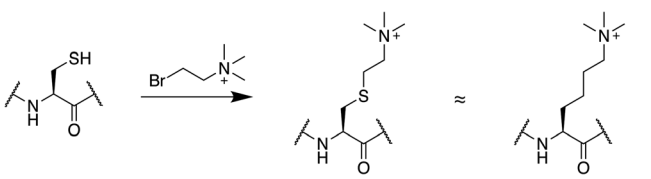

Fig. 4 Cysteine alkylation can be used to prepare methyl lysine mimics.

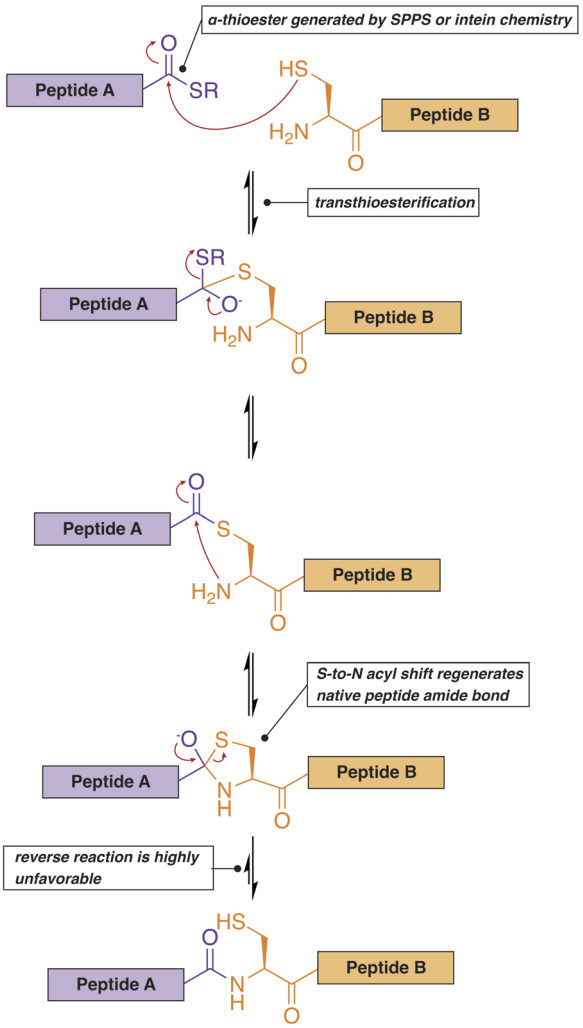

Fig. 5 Native chemical ligation can be used to ligate two synthetic or recombinant polypeptides. One of the peptides ends with a C-terminal thioester while the other peptide contains an N-terminal cysteine residue.

bioisosteres. Mimics of lysine methylation are common targets for this approach (Fig. 4). To use this strategy, a lysine residue is substituted with a cysteine, which is then alkylated with appropriate reagents to generate a mono-, di-, or trimethyllysine as desired.[107] This produces a sidechain that is the same length as lysine but bearing a sulfur instead of a carbon atom at the γ position. The reaction is compatible with denaturing conditions and is very popular in chromatin studies as it presents a relatively straightforward way to generate methylated histones.[107–110] It is important to note that in binding studies, alkylated cysteines display slightly higher K_d values compared to native methylated lysine residues.[109] They are, however, a good option when large amounts of methylated protein are needed, and are especially useful in the context of isotopic labeling for NMR studies.[108] Similarly, cysteine-based chemistries have been leveraged to introduce acetyl-lysine and methyl-arginine mimics into histone tails after cysteine mutations at endogenous lysine or arginine residues.[111–113] Asymmetric disulfide linkages can also be used to attach ubiquitin at well-defined positions in a protein sequence.[114,115]

Native chemical ligation (NCL) represents another versatile option for the site-selective introduction of PTMs in IDPs (Fig. 5).[116,117] Unlike most of the methods discussed above, this technique can generate proteins with multiple well-defined PTMs, enabling the construction of highly accurate models of post-translationally modified systems *in vitro*. In this case, a synthetic peptide bearing the necessary modifications is prepared through Fmoc- or Boc-mediated solid-phase peptide synthesis. The peptide is then ligated to another synthetic or recombinant fragment to build the full-length protein of interest. To perform NCL, the N-terminal fragment must contain a C-terminal α-thioester, while the C-terminal fragment needs to start with a nucleophilic amino acid such as cysteine. The thioester can be generated synthetically (native chemical ligation) or *via* the use of an intein that is fused to a recombinant protein or peptide fragment (expressed protein ligation, EPL).[116,118] The α-thioester serves as a reactive handle for the formation of a native amide protein backbone *via* a *trans*-thioesterification reaction with the cysteine sidechain of the second peptide fragment. Since the development of NCL, however, the range of chemistries that can be formed at this ligation site has extended far beyond cysteine to include many other natural and unnatural amino acids,[119] with recent methodologies expanding the scope to extremely challenging residues such as proline.[120] Another notable improvement in the NCL methodology is the development of the C-terminal hydrazide as a more stable and flexible replacement for the C-terminal α-thioester.[121] It should also be noted that the ligation reaction can proceed in the presence of urea or guanidinium hydrochloride and is therefore compatible with the production of IDPs that are prone to aggregation or premature LLPS.[122]

The primary advantage of using NCL or EPL to introduce PTMs into IDPs is that these techniques offer the capability to site-specifically introduce multiple distinct PTMs if desired. An excellent illustration of the value that this capability can offer to IDP research is provided by recent work by Ge *et al.*, in which a semisynthetic construct of the partially disordered JARID2 protein was produced using NCL.[123] In this report, multiple NCL reactions were utilized to generate a protein construct containing two distinct post translational modifications on individual residues in a site-specific manner. NCL and EPL have also been used extensively to produce α-synuclein and tau bearing a wide variety of PTMs, sometimes in combination with segmental isotopic labeling for NMR studies.[124–128]

Noncovalent small-molecule chemical tools for modulating LLPS and IDP behavior

One of the most exciting frontiers for IDP chemical biology is the development of noncovalent modulators of IDPs and LLPS. Disordered proteins do not offer hydrophobic pockets or other defined structural features that can serve as targets for small molecules. IDPs are therefore often avoided in high-throughput

screens for small-molecule effectors due to the high potential for failure. Despite this, successful screens for high-value protein targets such as Aβ, Myc, and α-synuclein are present in the literature,[129–134] suggesting that screening is a viable strategy for the discovery of chemical tools and drug leads that target IDPs directly.[134] Rational structure-based design has also been challenging although there is now at least one example of a successful structure–activity relationship (SAR)-based campaign against IDPs in the literature.[135] Given the central roles of IDPs and LLPS in cell biology and disease, the dearth of small-molecules capable of selectively engaging IDPs for use as chemical probes or therapeutic leads is an important problem in the field and makes this area of research especially exciting.[136] In this section, we will outline the precedent and explore the prospects for the discovery and development of such small-molecule modulators of IDPs and LLPS.

The current chemical landscape of LLPS modulators is sparse and lacks specificity

The chemical tool most often used to study LLPS is 1,6-hexanediol, a general disruptor of this process. Akyl alcohols including 1,6-hexanediol were first used to probe interactions between the FG-nucleoporins that gate the nuclear pore complex, which are IDPs that exhibit behavior consistent with LLPS.[137–139] Since its demonstration as a modulator of FG-nucleoporins, 1,6-hexanediol has been used as a convenient probe for assaying the properties of droplet-like structures in cells, for the rapid characterization of LLPS *in vitro*, or for differentiating between LLPS droplets and solids.[140–143] Despite its widespread use, 1,6-hexanediol is typically added at high concentrations of 5–10% w/v. Such high concentrations can interfere with the analysis of *in vitro* LLPS systems that are sensitive to abiotic conditions as well as with studies done in cells where 1,6-hexanediol disrupts a broad spectrum of cellular processes and can be cytotoxic.[140,144,145] Additionally, 1,6-hexanediol works primarily by disrupting hydrophobic interactions, a mechanism that does not address electrostatic, dipolar, and cation-π interactions that are also known to drive LLPS.[5] This suggests that the efficacy of 1,6-hexanediol in disrupting LLPS droplets may vary.[5,146,147] Conclusions drawn from experiments that rely on 1,6-hexanediol must therefore be considered with care and with the limitations of this tool in mind.

In addition to 1,6-hexanediol, a number of alkyl alcohols have been used to characterize LLPS. As a general trend, less hydrophobic alkyl alcohols (2,5-hexanediol and 1,2,3-hexanetriol, for example) are less effective at disrupting LLPS droplets, which is consistent with the proposed mechanism of action.[141] This difference in efficacy can be used as a tool for differentiating between phase-separated structures with different susceptibilities to disruption by alkyl alcohols.[143]

On the other end of the spectrum from 1,6-hexanediol and related disruptors lie small-molecule promoters of LLPS. Promoters include physiologically-relevant small molecule hydrotopes such as ATP, which can enhance the propensity of FUS to undergo LLPS in a concentration-dependent manner.[148,149] A synthetic example of a hydrotope capable of enhancing LLPS is 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS). This molecule, along with a handful of similar but less effective naphthalene sulfonate derivatives, has been shown to promote LLPS for a number of common LLPS-prone

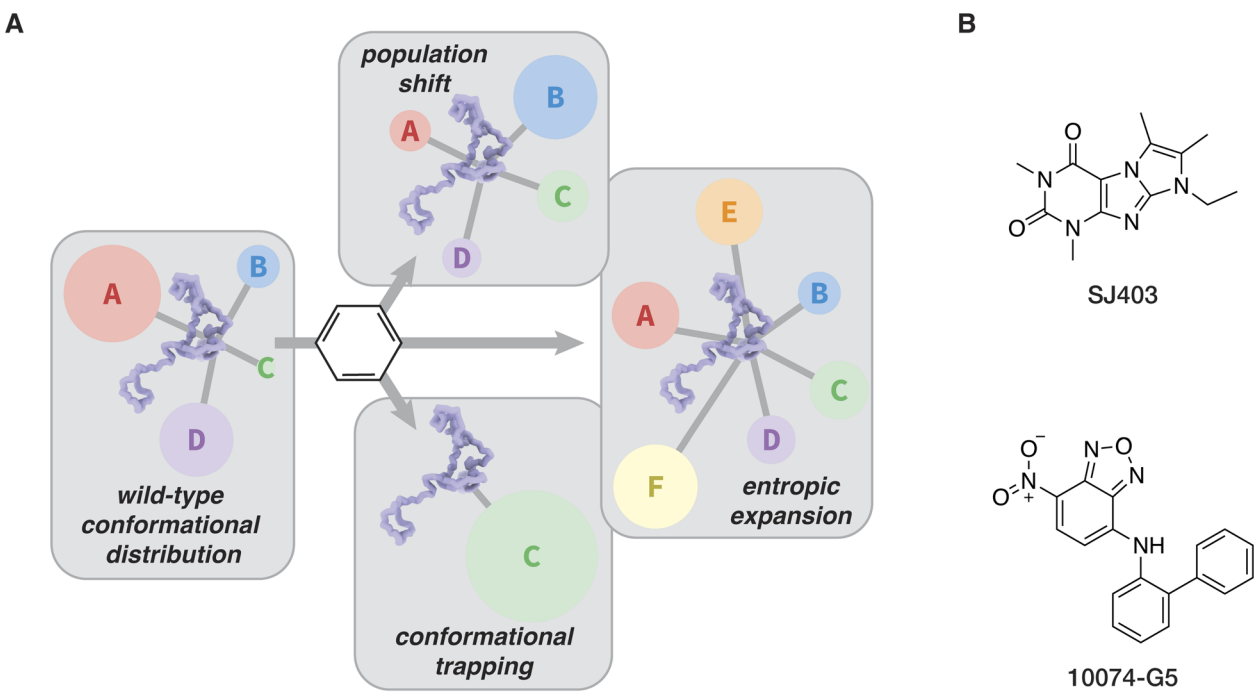

Fig. 6 Noncovalent small molecule modulators of IDPs. (A) Unique modes of action for small molecule modulators of IDPs, with A–F representing arbitrary populations of conformers. (B) Two small molecule modulators discussed in the text.

model proteins such as TDP-43 low complexity domain, tau, and FUS LC.[150] The study that introduces bis-ANS further demonstrates that Congo Red, a small molecule probe that is used as a reporter for amyloid, is capable of promoting LLPS in a similar manner to bis-ANS. This work suggests that bivalent, negatively charged compounds with hydrophobic cores can serve as hydrotopic drivers for LLPS. Some biologically important small molecules have also been shown to be capable of maintaining LLPS and modulating the onset of the liquid-to-solid transition of LLPS droplets. In one of the few systematic studies of its kind, Jonchhe *et al.* found that hydrophobic moieties in small molecules delay the onset of a liquid-to-solid phase transition in tau LLPS droplets.[151] In the same work, TMAO is shown to be an especially potent inhibitor of the liquid-to-solid transition, which is consistent with the observation that amphiphilic compounds like bis-ANS and ATP can drive LLPS.

Although they do not necessarily modulate LLPS directly, LLPS-sensitive probes that fluoresce or otherwise report on LLPS, are valuable tools for characterizing this process and may prove superior to disruptive reporters such as 1,6-hexanediol in many experimental contexts. Molecular rotors such as thioflavin T are sensitive to viscosity and can be used to probe LLPS while also serving as a tool for identifying and visualizing protein aggregation and fibrilization.[152] Recently, a novel aggregation-induced emission fluorogen, sodium 1,2-bis[4-(3-sulfonatopropoxyl)phenyl]-1,2-diphenylethene (BSPOTPE), was demonstrated to be capable of reporting on LLPS by partitioning into droplets *in vitro* and fluorescing in a viscosity-dependent manner.[153] The continued development of chemical tools capable of reporting on LLPS without the need for toxic or otherwise disruptive concentrations of 1,6-hexanediol will allow for less perturbative *in vitro* and in-cell assays. Molecules designed to non-perturbatively probe LLPS characteristics may also be useful for providing SAR data for chemistries capable of selectively partitioning into LLPS droplets. Just like biological LLPS condensates, whose composition is tuned by the chemistry of the client and scaffold proteins, it is feasible that small molecules could be tuned to partition into LLPS droplets with a particular set of chemical properties.[5,154] For example, recently, a handful of cancer therapeutics were shown to selectively partition into biomolecular LLPS condensates both *in vitro* and in cells.[155] It is our hope that further development of selective small-molecule LLPS modulators will lead to better chemical tools for studying LLPS and better therapeutics for addressing clinically-relevant LLPS dysregulation.

Rethinking the physicochemical basis of binding to IDPs

In order to develop more effective small molecule modulators of IDPs and LLPS, it is important to first consider the properties that would make a modulator effective in the first place. In general, three outcomes are available for small molecule binding to an IDP (Fig. 6A). First, the conformational distribution of an IDP may be changed or the conformational space available to an IDP may be reduced, a mechanism referred to as a population shift or conformational restriction.[156,157] Alternatively, the conformational states available to an IDP may increase, a mechanism referred to as entropic expansion.[158,159] Lastly, a small-molecule IDP modulator may bind to and induce a single conformation of an IDP, which we refer to as conformational trapping.

The population shift mechanism relies on transient enthalpy-driven binding of a small molecule to an IDP. Many IDPs adopt transient secondary structure, either due to a predominant low-energy conformation or as the result of a transient interaction with another protein (in the case of LLPS, this interaction is often with another copy of the same protein). As an example, adoption of transient secondary structure by an IDP has been demonstrated by Conicella *et al.*, who showed that transient α-helices in the C-terminal domain of TDP-43 exist both in the dispersed and LLPS phase, and that mutations that enhance the propensity for the formation of α-helices also enhance the propensity for LLPS.[47,160] Molecular dynamics and NMR are particularly powerful for providing insight into the role of transient secondary structure in the behavior of IDPs and IDP interactors. In a recent study, Zhu *et al.* integrated solution NMR data with all-atom molecular dynamics to provide a structure-based explanation for SAR differences between a family of bisphenol A-based modulators of the intrinsically disordered transactivation domain of the androgen receptor.[161] Rational design of IDPs that target transient structure has been achieved by clustering snapshots from molecular dynamics simulations and using favored structural ensembles as targets for docking experiments.[162]

The discovery and optimization of a molecule that confers a population shift in an IDP can also be performed in the absence of a full structural ensemble, as illustrated in work described by the Shelat, Zuo, and Kriwacki groups that shows the discovery and optimization of a small molecule capable of engaging the IDP $p27^{Kip1}$. An initial fragment-based NMR screen against $p27^{Kip1}$ yielded SJ403 (Fig. 6B), which can bind transient clusters of hydrophobic residues.[163] SJ403 is shown to induce a population shift through this binding mode, and further SAR work on this molecule generated a compound capable of sequestering $p27^{Kip1}$ into small soluble oligomers *via* the hydrophobic binding mechanism.[135,156] This effort provides an elegant example of the implementation of well-established drug discovery and optimization approaches to discover a small-molecule modulator of an IDP.

The entropic expansion mechanism introduces a much stronger focus on the entropic contributions to interactions between a small-molecule modulator and IDPs.[158] In the conformational trapping mechanism, and to a lesser degree the population shift mechanism, binding can be optimized through rational design principles against a favored IDP conformation. The strategy mirrors that of lead optimization for a folded protein, where the main goal is to introduce changes to a small molecule binder in order to decrease the enthalpy of binding.[164] In the entropic expansion approach, on the other hand, the entropic benefit of introducing more diversity into the conformational ensemble of a population of IDPs is considered. Enthalpic contributions provide a degree of target specificity through transient, weak interactions with

structural or sequence motifs in IDPs, while the entropic contribution favors interactions with the IDPs and effectively reduces unwanted LLPS or aggregation by expanding the range of conformations that the proteins can adopt.[158] The only example to date of a molecule that works through the entropic expansion mechanism is 10074-G5 (Fig. 6B), a small molecule inhibitor of amyloid-β_{42} aggregation discovered by the Knowles, Dobson, and Vendruscolo groups.[165] Since many IDPs undergo pathological conformational collapse into aggregates and fibrils, it is clear that molecules that exploit the entropic expansion mechanism could be viable therapeutic leads. It is possible that other small molecules that protect against aggregation of IDPs while preserving their wild-type function work through this mechanism.[166,167] Further efforts exploring the entropic expansion mechanism will be valuable for the development of better chemical tools for studies of IDPs and LLPS.

For both the population shift and entropic expansion mechanisms, a framework for initial small-molecule binding and selectivity must exist. For enthalpically driven binding, selectivity can be explained in some cases by the existence of transient binding pockets. Recently, Robustelli *et al.* provided an alternative mechanism for selectivity in small molecule binders of IDPs. Using all-atom molecular dynamics simulations and solution NMR studies of a fragment of α-synuclein and fasudil, a known ligand for α-synuclein, Robustelli and coworkers demonstrated that the spacing of low-affinity binding sites for fasudil along the α-synuclein sequence can compensate for the lack of a single well-conserved or high-affinity binding site. This mechanism for selectivity, called "dynamic shuttling" provides a compelling explanation for small-molecule selectivity towards specific IDP sequences that is compatible with both the population shift and entropic expansion paradigms for small-molecule-IDP interactions.[168]

A final mechanism for IDP interactions with small molecules is conformational trapping. Due to the large entropic cost associated with restricting an IDP to a single conformation, this mode of interaction is challenging to achieve with a small molecule. An example of conformational trapping can be found in the biotinylated 5-aryl-isoxazole-3-carboxyamide molecule (b-isox) described by Kato *et al.* In this case, the microcrystalline form of b-isox is the active modality. Grooves on the crystal surface provide binding sites capable of overcoming the entropic penalty associated with conformational trapping of IDPs, and multiple IDPs associated with RNA granules can be trapped and subsequently isolated using b-isox as a chemical tool.[169]

Future directions

A quarter of a century of IDP research has revealed that unstructured polypeptides carry out a vast number of important and precise functions in the cell.[10,170,171] Despite these advances, there is still much to learn regarding how cells translate a multitude of non-specific interactions into specific biological outcomes, and how the cellular machinery perceives functional and pathological states of IDPs. Further developments in both structural biology and protein chemistry will be crucial to these efforts.

Here, we have reviewed the current state of the art in chemical and biochemical approaches that allow the preparation of IDPs for imaging and structural studies and enable the investigation of PTMs in physiologically relevant contexts (summarized in Table 1). While these tools have become much more efficient and versatile over time, the handling requirements and aggregation propensity of many IDPs still present a tremendous challenge. The development of the Cfa GEP intein, for example, enabled *trans*-splicing and segmental labeling of many new proteins, including IDPs such as FUS.[29,79,172] Yet, more can be done to improve the efficiency of inteins in high concentrations of guanidinium hydrochloride and urea, conditions that are essential for the handling of many aggregation and LLPS prone IDPs. The development of cysteine alkylation approaches to mimic lysine methylation, on the other hand, has allowed NMR spectroscopists to study the role of this modification using specifically methylated, biologically relevant samples.[107,108,110] The design of similarly robust and easy to implement methodologies to create acetylation and phosphorylation mimics or modifications on recombinantly produced templates, would be highly beneficial.

Going forward, we expect that protein engineering efforts on IDPs will shift more and more to the cellular environment. This reflects a recent increase in efforts from imaging, NMR and EPR spectroscopy to understand how cells shape the conformational ensembles, interactions, and dynamics of IDPs and LLPS-based compartments.[51,173,174] While most imaging efforts so far have relied on fusion fluorescent proteins such as GFP, the significant bulk of these tags may not be compatible with all IDPs or LLPS studies.[41] In these situations, alternative labeling approaches with small molecule fluorescent probes are needed. Here, we expect that unnatural amino acid incorporation through amber suppression and bioorthogonal labeling methodologies would be particularly valuable. In addition to the delivery of fluorescent probes in cells, this technology is also gaining traction for EPR and sensitivity-enhanced NMR spectroscopy in cells.[65,70] Recent developments have focused on improving the efficiency of UAA incorporation, and in particular, the installation of two or three UAAs in mammalian cells.[66,72,73] It should also be mentioned that the intein approach can also be adapted to studies in cells, where it can be used to control IDP function and for the installation of PTMs.[175,176]

Perhaps the greatest challenge facing the IDP chemical biology field is the development of specific small molecule modulators that allow regulation of IDP and LLPS functions in cells. These efforts invariably tie back to more detailed understanding of the structural ensemble, dynamics, and interactions of the IDP of interest. These developments will no doubt require close collaborations between synthetic chemists, structural biologists, and computational chemists. A number of NMR studies, often complemented with all atom molecular dynamics simulations, have already provided valuable information regarding the interactions between small molecules and

Table 1 Summary of chemical tools suitable for IDP labeling and modulation, as discussed in the text

Goal	Strategy	Advantages	Disadvantages	References
Purification of IDPs	Purification under denaturing conditions	• In principle, compatible with all IDP/IDRs	• Denaturant must be removed • Refolding required for constructs that contain both folded and IDR domains	27, 28 and 172
	Purification at extreme pH	• Straightforward recovery of target protein	• Applicable to only a small subset of proteins	18 and 22–26
	Purification at extreme temperature	• Straightforward recovery of target protein	• Applicable to only a small subset of proteins	26
Labeling with spectroscopic or imaging probes	Maleimide or iodoacetamide-based probes	• Site-specific at cysteine residues • Compatible with isotopic labeling for NMR	• Not applicable in cells • Potential labeling at multiple sites • Probes may perturb IDP function and LLPS	36–41
	N-Hydroxysuccininimide ester-based chemistry	• Site-specific at lysine residues • Compatible with isotopic labeling for NMR	• Not applicable in cells • Potential labeling at multiple sites • Probes may perturb IDP function and LLPS	42
	Fusion protein-based approaches	• Genetically encoded • Applicable in cells	• Large fusion proteins may perturb IDP function and LLPS	See ref. 41 and 183 for comparison of the effects on LLPS of small molecule labeling *vs.* fusion protein labeling
	Bioorthogonal chemical approaches and amber suppression	• Genetically encoded • Applicable in cells • Site-specific labeling • May be compatible with isotopic labeling for NMR	• UAA incorporation may be inefficient and lead to low protein yields • Probes may perturb IDP function and LLPS	56, 61–67, 70, 184 and 185
	Native chemical ligation and expressed protein ligation	• Site-specific labeling • Multiple probes can be introduced at the same time • Access to a wide range of chemical probes • May be compatible with isotopic labeling for NMR	• Segments must be accessible synthetically • Difficult to label sites away from the N- or C-terminus • Final protein yields may be low • Not applicable in cells • Probes may perturb IDP function and LLPS	77, 79, 116 and 118 Also see relevant references for introducing PTMs through NCL and EPL below
Segmental labeling for NMR spectroscopy	Cfa GEP	• *Trans*-splicing can be achieved under denaturing conditions • High efficiency • Robust to a range of extein sequences • May be applied in cells	• Splicing reaction must take place at a cysteine residue	29, 79 and 172
	MCM2	• Salt-inducible splicing can be used to control intein activity • Robust reactivity under high-salt conditions • Serine-based reaction mechanism provides versatility	• Splicing cannot be performed under denaturing conditions • May not be applicable in cells • Splicing rate and extein sequence compatibility are poor compared to Cfa	83
	Sortase	• A possible alternative to intein-based methods • May be used to attach proteins to cell surfaces	• Requires the insertion of a five/six amino acid scar into the target protein • Continued reaction between reactants and	90–96 and 186

Table 1 (*Contd.*)

Goal	Strategy	Advantages	Disadvantages	References
			desired product may reduce yield	
Introduction of post-translational modifications	Enzymatic methods	• Straightforward introduction of native PTMs	• Lack of control over stoichiometry and location of PTM installation	100–102
	Genetic encoding of a bioisostere	• Straightforward introduction of PTM mimics • Applicable in cells	• Scope of PTMs is limited to those that can be mimicked effectively by a bioisostere	23, 46 and 104
	Amber suppression	• Introduction of native PTMs or PTM mimics • Applicable in cells	• Scope of PTMs is limited to those that have a tRNA synthetase available • UAA incorporation may be inefficient and lead to low protein yields	105 and 106
	Cysteine alkylation	• Efficient and specific introduction of methyl-lysine mimics • Reactions are compatible with denaturing conditions	• Methyl-lysine mimic may not faithfully reproduce the function of the native PTM • Not applicable in cells	41, 107, 109 and 110
	Native chemical ligation and expressed protein ligation	• Site-specific introduction of PTMs • Multiple PTMs can be introduced at the same time • Access to a wide range of modifications	• Segments must be accessible synthetically • Difficult to introduce PTMs away from the N- or C-terminus • Final protein yields may be low • Not applicable in cells	125–128, 187 and 188

IDPs.[135,156,158,163,165,168] There have also been exciting developments in the coarse-grained simulations of LLPS[177–180] and we are looking forward to the extension of these studies to all atom simulations and the incorporation of small molecule modulators.[181,182]

While IDPs present significant challenges to chemical and structural biologists, they also put forward valuable opportunities to hone existing chemical and biophysical methodologies and to develop more efficient and precise tools. Such developments will have far reaching implications for the chemical and biological fields and will undoubtedly enrich our understanding of protein function in health and disease.

Author contributions

R. F. B. and G. T. D. conceived the idea and wrote the manuscript. R. F. B. prepared the figures.

Conflicts of interest

The authors declare no conflicts of interest.

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