Biophysics in drug discovery: impact, challenges and opportunities

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Abstract | Over the past 25 years, biophysical technologies such as X-ray crystallography, nuclear magnetic resonance spectroscopy, surface plasmon resonance spectroscopy and isothermal titration calorimetry have become key components of drug discovery platforms in many pharmaceutical companies and academic laboratories. There have been great improvements in the speed, sensitivity and range of possible measurements, providing high-resolution mechanistic, kinetic, thermodynamic and structural information on compound–target interactions. This Review provides a framework to understand this evolution by describing the key biophysical methods, the information they can provide and the ways in which they can be applied at different stages of the drug discovery process. We also discuss the challenges for current technologies and future opportunities to use biophysical methods to solve drug discovery problems.

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doi:10.1038/nrd.2016.123
Published online 12 Aug 2016

Since the first successes with structure-based drug design using X-ray crystallography in the 1990s, a wide range of biophysical technologies have matured to become key components of drug discovery platforms within pharmaceutical companies and academic laboratories. Areas in which biophysical measurements have had a particular impact include: enabling drug discovery for more challenging targets, such as protein–protein interactions[1,2]; identifying binding kinetics as a crucial factor for efficacy and selectivity[3,4,5]; and providing the foundation for fragment-based drug discovery (FBDD)[6–8]. Such technologies have also enabled studies of the thermodynamics of drug binding[9]. Biophysical data provide an important complement to data on biochemical and cellular activity as well as aggregation, solubility and cell permeability[10], which have traditionally formed the basis of hit and lead discovery, prioritization and optimization. Taken together, these developments have enabled a more rational, rigorous problem-solving approach to the early phases of drug discovery.

The major reason for the rise of biophysical methods within drug discovery is the increased experience in knowing when and how to apply the plethora of methods to answer diverse questions across a breadth of biological targets. With the aim of helping to disseminate this experience, in this Review we provide an overview of the current range, strengths and limitations of biophysical methods used in drug discovery, giving examples of when and how they can have an impact on drug discovery. We first summarize the main techniques and their requirements, and we then describe the information that they can provide and the stages at which they can be applied in the drug discovery process. We conclude with a discussion of the opportunities for new developments in biophysics — for example, methods that are able to operate in more authentic and physiologically complex settings (that is, in a cellular, tissue or organism setting), as is the trend for other assay technologies.

What are the techniques?

A range of biophysical techniques are currently used to analyse the binding of a compound to a target. The available technologies differ in the physical principle that underlies the detection of binding, throughput, information content, sample requirements, sensitivity and robustness of the resulting data. TABLE 1 summarizes the techniques used most frequently in drug discovery, together with the characteristics of typical experiments, and FIG. 1 shows typical data obtained from the different methods described in TABLE 1. Given the large number of biophysical techniques, this Review is inevitably not able to comprehensively cover all existing or developing techniques, and so some techniques are not discussed in detail or not mentioned.

Two main biophysical techniques are currently used to investigate the structure of protein–ligand complexes. The most common technique is X-ray crystallography, which can be used for proteins of any size. X-ray diffraction patterns of either protein–ligand co-crystals or apoprotein crystals soaked with a ligand are used to determine the structure of the complex at atomic resolution and derive the geometry and other details of protein–ligand interactions[11]. Nuclear magnetic resonance

Table 1 | **Selection of established biophysical methods for analysis of protein–ligand interactions**

Technique or method	Strengths	Limitations	Typical material requirements	Affinity range	Throughput per day*	Refs
X-ray crystallography						
Protein–compound co-crystallization or apoprotein crystal soaking	• Identification of binding site for establishment of modes of action • Direct visualization at atomic resolution of target–ligand interactions for structural optimization of lead compounds	• Need for diffraction quality crystals suitable for ligand soaking or co-crystallization • Often requires access to large research infrastructures (synchrotrons) • No quantitative affinity information	• ~2.5–5 nmol crystallizable protein (for example, 15 µl of protein solution at 5–10 mg per ml with a MM of ~30 kDa) per 96-well nanodrop crystallization plate	No lower limit to 1 M	100s	11
NMR						
Ligand-observed NMR	• Confirmation of ligand binding to unlabelled proteins of any size • Integrity of ligand and protein in every experiment	• Considerably large amounts of protein required (typically tens of milligrams for screening) • Limited derived structural information	• ~3–6 nmol protein per compound or cocktail tested for ^{1}H experiments (for example, 0.1–0.2 mg protein with a MM of ~30 kDa) • Amount of protein needed is ~10 times less for ^{19}F experiments • Competitor ligand to confirm specific binding	100 nM–10 mM	100s	12–14
Protein-observed NMR	• Monitor protein integrity upon ligand binding • Titration can reliably determine K_d • Binding epitope resolved from pattern of perturbations • Can determine structure if NMR spectrum is assigned	• A large amount of isotopically labelled protein is required • Screening of large libraries is only possible in multiplexing mode (see REFS 142–144) • Only suitable for small proteins (MM <40 kDa)	• Protein with a MM of <40 kDa • ~30 nmol of an isotopically labelled protein per compound tested (for example, ~1 mg protein with a MM of ~30 kDa)	100 nM–1 mM	100s	13, 142–144
SPR						
Microfluidic surface-based biosensor (immobilized protein)	• Direct time-resolved determination of interactions over a broad range of conditions • High sensitivity allows analysis of fragments	• Requires immobilization of functional target with high stability over time and towards regeneration procedures • Signals affected by solvent effects	• ~15 nmol protein (for example, ~0.5 mg with a MM of ~30 kDa) for assay development and screening of 2,000 compounds • Reference compounds for evaluation of protein functionality	1 nM–500 µM	100s	15–17
Microarray-based biosensor with CCD camera detection (immobilized compound library)	• Very high sensitivity • Ultra-high throughput (12,000 fragment interactions in triplicates per day)	• Requires chemical modification of compounds to immobilize ligands through a covalent linker to a surface, preferably in different orientations • Detergents are not tolerated	• ~100–150 nmol protein (for example, 3–5 mg with a MM of ~30 kDa) to screen 120,000 compounds • Reference compounds to assess binding specificity or to identify allosteric binders	No lower limit to 500 µM	10,000s	18,19
TSA						
• DSF for monitoring of protein thermal unfolding with a fluorescent reporter ligand (typically using qPCR)	• Fast and robust assay development • Functional knowledge of target not necessary	• Requires a fluorescent dye • Artefacts occur owing to fluorescence quenching or aggregation • Not suitable for disordered or hydrophobic proteins	• 80 pmol protein per analysis (40 µl at 2 µM) • Ideally use a protein with intermediate T_m (<50 °C)	1 nM–100µM	1,000s	20,21

Table 1 (cont.) | **Selection of established biophysical methods for analysis of protein–ligand interactions**

Technique or method	Strengths	Limitations	Typical material requirements	Affinity range	Throughput per day*	Refs
Microcalorimetry						
ITC	• Direct determination of thermodynamic parameters for a binary system	• Only useful for binding events with an enthalpic component • Very high protein consumption, requires high solubility of titrated component	• ~6–60 nmol protein per titration (for example, 0.2–2 mg with a MM of~30 kDa) • Concentrations needed are ~10 times the estimated K_d (when K_d is unknown, a 10–100 µM concentration range is used)	1 nM–100 µM	10s	9,22,23
DSC	• Determination of the effect of a ligand on the thermal stability of a protein • Can be used to study ultra-tight binding that cannot be measured by other methods	• Very high protein consumption	• ~1.5–30 nmol protein per scan (for example, ~500 µl at 0.1–2 mg per ml with a MM of~30 kDa) • A K_d measurement requires a series of scans at different concentrations	Dissociation constants down to 10^{-20} M can be estimated	10s	23,145
MS						
NC-MS (also called native MS; usually ESI-MS)	• Direct visualization of complex formation • High sensitivity • Very low protein consumption • Accurate mass measurements • High-content information • No labelling required	• Requires sample desalting • Protein has to be stable in an ESI-MS suitable buffer (usually a low-concentration ammonium buffer) • Detergents are not tolerated • Unspecific binding makes the detection of low-affinity binders more difficult	• 10–100 pmol protein per compound tested (2–5 µl at 5–20 µM)	No lower limit to 500 µM	100s	19,24, 25
AS-MS (also called SEC-MS)	• Ultra-high throughput when compound cocktails used • Can be applied to solubilized membrane proteins • Ligand mass detection enables verification of compound structure	• Low-affinity binders (including fragments) are hard to detect because they tend to dissociate from the protein during the SEC step owing to high off-rates	• 10 pmol protein (~2 µl at 5 µM) per single-binding reaction with a 2,500-compound library	No lower limit to 10 µM	1,000,000s (compound cocktails)	26
HDX-MS	• Direct detection of protein binding site and/or changes in protein conformation or protein dynamics upon binding	• Spatial resolution is limited and depends on peptide lengths and coverage of target sequence after protease cleavage	• ~30 nmol protein (for example, ~1 mg with a MM of ~30 kDa), mostly for assay development	No lower limit to 20 µM	10s	27,28
MST						
Monitoring of changes in the thermophoretic motion of a fluorescently labelled or intrinsically fluorescent protein upon ligand binding	• In-solution measurements • Applicable to solubilized membrane proteins	• Requires labelling or strong intrinsic fluorescence	• Protein can either be fluorescently labelled or have detectable intrinsic fluorescence • ~7 pmol protein per compound tested at 12 serial dilutions (twelve 10 µl aliquots of protein solution at 60 nM)	1 pM–1 mM	100s	29,30

Table 1 (cont.) | **Selection of established biophysical methods for analysis of protein–ligand interactions**

Technique or method	Strengths	Limitations	Typical material requirements	Affinity range	Throughput per day*	Refs
SAXS						
Low-angle elastic scattering of X-rays, bringing information about the shape and size of macromolecules	• Larger-scale structural changes of proteins upon ligand binding can be visualized at low resolution with high throughput	• Low resolution (1–2nm) does not allow for elucidation of atomic details of ligand interactions	• Monodisperse protein material • ~1 nmol protein per sample (for example, ~0.03 mg with a MM of ~30 kDa) • ~100 nmol protein for an entire study (for example, ~3 mg with a MM of ~30 kDa) • Typically, a concentration series is measured for each sample (for example, 1, 2, 5 and 10 mg per ml)	No lower limit to 30 μM	100s	31,32
QCM						
Monitoring of the vibration frequency of a quartz crystal upon interaction between an analyte and an immobilized protein or cell	• Direct time-resolved determination of interactions with proteins and cells	• Requires immobilization of functional target with high stability over time and robustness towards regeneration procedures	• ~15 nmol protein (for example, ~0.5 mg with a MM of ~30 kDa) for assay development and screening campaign	1 nM–500 μM	100s	33,34
Rotating cell-based ligand binding assay using radioactivity or fluorescence						
Monitoring of molecular interactions at the surface of cells using radioactivity or fluorescence	• Direct time-resolved determination of interactions between labelled ligands with surface-associated proteins	• Laboratory requirement to work with radioactivity (not with fluorescent compounds)	• ~10^5 cells per assay • Fluorescent or radioactively labelled analytes (in approximately picomolar amounts)	1 pM–1 μM	10s	35

AS-MS, affinity selection mass spectrometry; CCD, charge-coupled device; DSC, differential scanning calorimetry; DSF, differential scanning fluorimetry; ESI-MS, electrospray ionization mass spectrometry; HDX-MS, hydrogen–deuterium exchange mass spectrometry; ITC, isothermal titration calorimetry; MM, molecular mass; MS, mass spectrometry; MST, microscale thermophoresis; NC-MS, non-covalent mass spectrometry; NMR, nuclear magnetic resonance; qPCR, quantitative PCR; QCM, quartz crystal microbalance; SAXS, small-angle X-ray scattering; SEC-MS, size-exclusion chromatography mass spectrometry; SPR, surface plasmon resonance; T_m, transition midpoint for thermal unfolding; TSA, thermal shift analysis. *Number of protein-ligand experiments per day for a well-behaved system with optimal facilities.

(NMR) spectroscopy can also be used to solve structures of protein–ligand complexes for targets with a molecular mass of less than ~40 kDa.

NMR also has a much wider application in probing ligand binding, through two alternative experimental setups: ligand-observed NMR and protein-observed NMR. In ligand-observed NMR, changes in the NMR parameters (mostly chemical shifts, relaxation rates and diffusion rates) of molecules or cocktails of molecules are measured in the presence of a target protein[12–14]. Typical experiments performed include magnetization transfer experiments (saturation transfer difference (STD) NMR, water-ligand observed via gradient spectroscopy (waterLOGSY), transferred nuclear Overhauser effect (NOE), NOE pumping and other NOE-based methods), relaxation editing (longitudinal, transverse and double-quantum relaxation) and diffusion editing. The information that can be obtained can range from simply determining whether the ligand interacts with a protein or not, to binding specificity and information on the binding mode (such as epitope mapping, pharmacophore mapping and mapping of ligand orientation). With some techniques it is possible to estimate binding affinities. In protein-observed NMR, the chemical shift perturbations in the 2D NMR spectra of isotopically labelled proteins are monitored in the presence of ligands[13]. Such measurements are limited to small proteins (practically, those with a molecular mass <40 kDa), but when resonances have been assigned, the binding epitope and the dissociation constant (K_d) can be inferred from the data. Both methods require relatively large amounts of a protein, which needs to be stable and of high purity.

Surface plasmon resonance (SPR) is a spectroscopic technique that monitors changes in refractive index at the interface of a liquid sample and a surface with an immobilized sensor molecule, typically the target protein[15–17]. The signal is shifted as a result of analyte binding or induced conformational changes. When using microfluidic systems with continuous registration of the signal, it is possible to characterize the binding mechanism and

determine the corresponding kinetic parameters (the association rate constant (k_{on}) and the dissociation rate constant (k_{off})) and the affinity (K_d). Thermodynamic parameters can also be estimated by performing analyses at multiple temperatures. An alternative configuration of SPR involves imaging of a microarray with immobilized small-molecule sensors using a charge-coupled device (CCD) camera, providing information on binding (yes or no) and binding specificity through on-array competition experiments[18,19]. Both of these SPR techniques require immobilization of one of the binding partners, either the protein in the microfluidic system or a ligand library in the microarray technology. In the latter case, the protein is in the solution over the microarray, and thus, as the signal depends on the mass of the analyte (in this case, the protein), the detection of binding in this setup is extremely sensitive.

Thermal shift analysis (TSA), also known as differential scanning fluorimetry (DSF), measures the temperature at which a protein unfolds through binding of a fluorescent probe to the exposed hydrophobic surfaces[20,21]. If a ligand stabilizes or (less frequently) destabilizes the fold upon binding, then there will be a change in the transition midpoint for thermal unfolding (T_m) (positive or negative, respectively). In some cases, it is also possible to determine K_d values. The major advantages of the method are its fast set up, its inexpensiveness and the requirement of only small amounts of protein material.

Two major microcalorimetry techniques are used for studying protein–ligand interactions[9,22,23]. Isothermal titration calorimetry (ITC) measures the generation or consumption of heat following titration of a ligand on to a protein (or the reverse). The data obtained include stoichiometry, K_d, change in enthalpy (ΔH), change in entropy (ΔS) and the heat capacity change (ΔC_p). Differential scanning calorimetry (DSC) measures heat changes (and corresponding thermodynamics) resulting from the thermal unfolding of a protein. An advantage of these techniques is that experiments are performed in solution, but large amounts of protein are typically required.

Mass spectrometry (MS) is also used in different modes. Non-covalent MS (NC-MS), also called native MS, is usually carried out using electrospray ionization MS (ESI-MS), generating data that can confirm binding, assess specificity through competition experiments, define stoichiometry, measure K_d and give an estimate of the enthalpic component of binding[19,24,25]. Affinity-selection MS (AS-MS), also called size-exclusion chromatography MS (SEC-MS), can be used to study protein–ligand interactions[26], but with decreased information content. Typically, a protein is incubated with a library of compounds and passed through a SEC column to isolate any compounds that are bound to the protein and therefore coelute with the protein. The protein complexes are then dissociated and the ligands are identified by LC-MS or ESI-MS. In this case, confirmation of binding and K_d values can be obtained. Finally, hydrogen–deuterium exchange MS (HDX-MS) can be used to measure the altered accessibility to deuterium exchange of amino acids involved in ligand binding. This method has successfully been used for the characterization of protein–protein interactions, such as those between epitope binding regions of therapeutic antibodies and their antigens. Furthermore, small-molecule binding and alterations in protein dynamics can be investigated[27,28]. The method works for soluble and membrane-bound proteins and relies on an appropriate protease cleavage pattern to get accurate sequence coverage.

Microscale thermophoresis (MST) is a relatively new methodology that monitors fluorescence in an infrared laser-heated spot. It is an equilibrium-based method that can detect ligand binding-induced changes in thermophoretic mobility (the motion of protein molecules along a microscopic temperature gradient), which depend on size, charge and hydration shell[29,30]. These changes in thermophoretic mobility can be used to estimate K_d values.

Small-angle X-ray scattering (SAXS) can be used to study protein–protein, protein–DNA, protein–RNA and protein–small molecule interactions in solution, providing information on the folding, oligomerization state and intrinsic flexibility of a protein and its complexes, as well as the shape of an assembly and an envelope structure to a 1–2-nm resolution[31,32]. Although we are not aware of any extensive ligand screening efforts, SAXS can be used to monitor the effect of ligands on the modulation of protein–protein interactions and of the conformation and oligomeric state of intrinsically disordered proteins.

Quartz crystal microbalance is a continuous flow surface biosensor that measures changes in the vibration frequency of a quartz crystal following an interaction between an analyte and an immobilized target protein or cell, quantified as K_d, k_{on}, k_{off} or thermodynamic parameters[33,34]. Finally, the rotating cell-based ligand binding assay is a relatively new method for real-time monitoring of molecular interactions on cells using radioactivity or fluorescence[35]. This method provides data that can be used to characterize the binding mechanism, as well as K_d, k_{on} and k_{off}.

What do these techniques require?

All of these techniques have certain requirements for the compound to be tested. Ideally, the compound must be soluble well above its binding affinity constant, stable and non-aggregating under the given experimental conditions. However, even under the same experimental conditions, different techniques demand different levels of solubility relative to affinity. For instance, in ligand-observed NMR, a low occupancy of the protein binding site by the ligand (<20%) suffices to observe binding, whereas in X-ray crystallography a substantial percentage (>50%) of binding sites must be occupied to unambiguously identify binding and resolve the protein–ligand co-structure.

Similarly, for a robust measurement, all of the techniques require the protein target to be homogenous and well behaved at relatively high concentrations. However, each of the techniques has differing requirements for the amount, characteristics and preparation of the target.

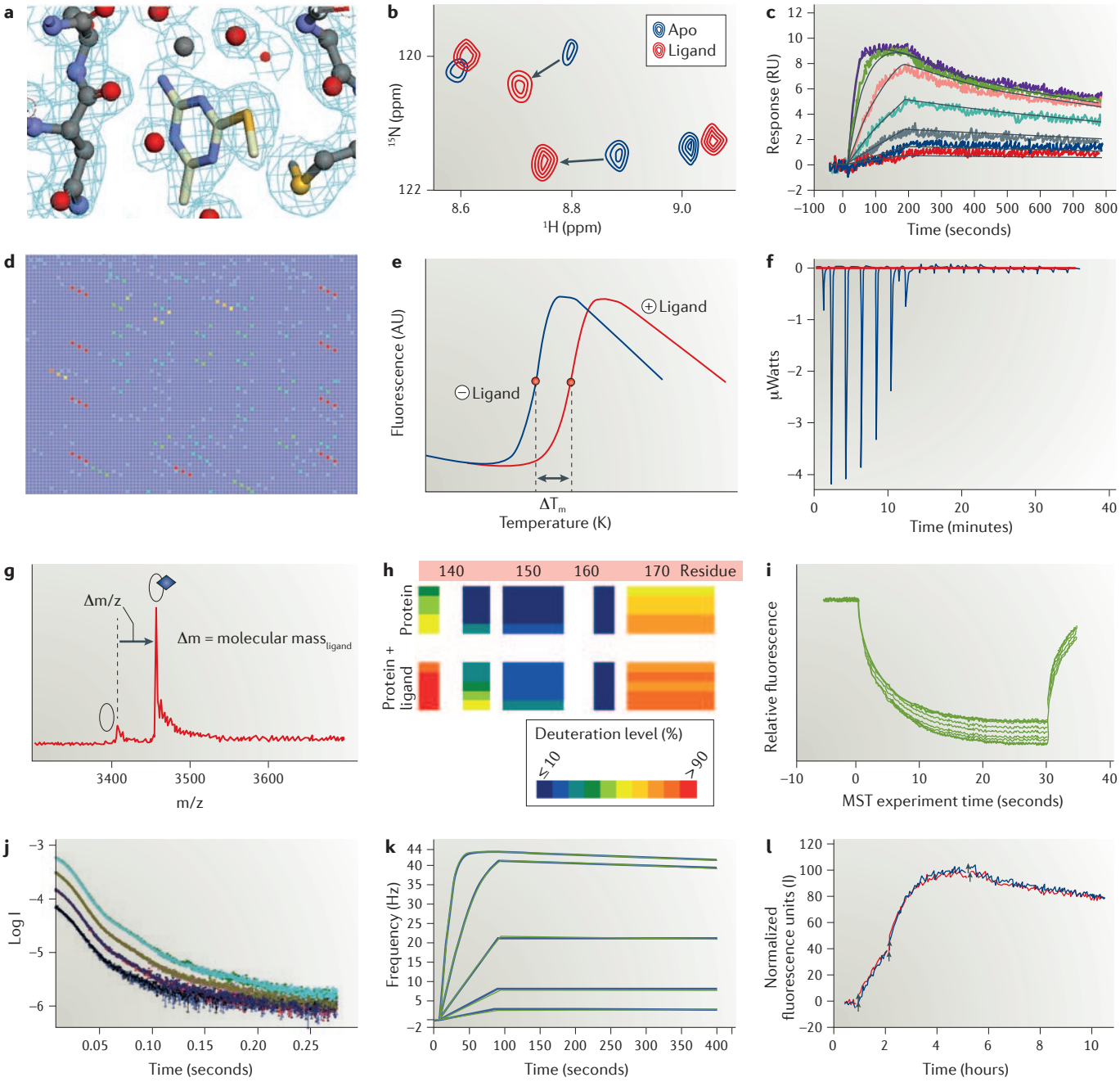


Figure 1 | **Typical data obtained from biophysical methods in drug discovery.** Visual representation of data obtained from methods in TABLE 1. **a** | Detail of an electron density map derived from X-ray diffraction data for a fragment binding to the amino terminus of heat-shock protein 90 (HSP90). **b** | A small portion of a 2D ^{1}H–^{15}N heteronuclear single quantum coherence nuclear magnetic resonance spectrum (protein-observed mode). Resonances in the absence and presence of ligand are coloured blue and red, respectively. **c** | A typical channel-based surface plasmon resonance (SPR) sensorgram at different analyte concentrations. **d** | A fingerprint from a microarray SPR screening, showing the SPR shift on a relative colour scale. The horizontal and vertical axes correspond to the *x* and *y* coordinates on the microarray. Fragment hits are easily identified from triplicate patterns. **e** | A typical recording of relative fluorescence intensity versus temperature for the unfolding of a protein target in the presence of a fluorescent dye. The blue and red curves are the recordings in the absence and presence of test compound, respectively. In the depicted case, the test compound stabilizes the protein (change in transition midpoint for thermal unfolding (ΔT_m) >0). **f** | A thermogram showing heat absorption rates (microjoules per second) upon sequential ligand additions to a target solution (isothermal titration calorimetry; ITC) (raw data). **g** | A portion of a native mass spectrum. Upon ligand addition, the peaks corresponding to the apoprotein decrease and the peaks corresponding to the protein–ligand complex increase. The mass difference can be used to evaluate the binding stoichiometry. **h** | The deuteration level (observed deuterium uptake over the maximum theoretical uptake) for each peptide at increasing time points (from top to bottom) in the absence and presence of a ligand. **i** | Fluorescence monitoring of the thermophoretic motion of a target at different ligand concentrations. **j** | Small-angle X-ray scattering curves for a protein–protein complex at different concentrations. **k** | Typical quartz crystal microbalance (QCM) sensorgram at different analyte concentrations. **l** | Relative intensity versus time showing the interaction of a fluorescent antibody with receptors at the surface of living cells. The red and blue curves depict two independent experiments. Δm/z, change in mass-to-charge ratio; MST, microscale thermophoresis; ppm, parts per million.

Clearly, X-ray crystallography requires crystals, which usually means at least a few milligrams of protein at more than 10 mg per ml concentration. Advances such as cryocooling, microfocus beamlines and new detector technologies have dramatically reduced the number of crystals required to determine 3D structures. New developments — such as serial crystallography done at room temperature in combination with the ultra-high brilliance of X-rays from free-electron lasers — have the potential to work with crystals of sub-micrometre dimension. For NMR, improved electronics and the advent of cryogenic probes have increased sensitivity by more than tenfold over the past 10 years, reducing either the amount of sample required or the measurement time; nevertheless, a protein solubility of around 10 μM or greater is still required. Ligand-observed NMR measurements can be made on unlabelled targets of any molecular mass, whereas protein-observed NMR is mostly limited to proteins of less than 40 kDa and requires isotope labelling (one or more of ^{15}N, ^{13}C and ^{2}H). Routine labelling is only possible by overproduction in engineered bacterial cell lines; the recent innovation of selective labelling of specific amino acid types has made it possible to exceed the 40 kDa limit of protein-observed NMR. Improvements in label-free native ESI-MS have led to greater throughput and the ability to screen hundreds of compounds while still allowing the detailed characterization of the interaction[25].

There is a growing collection of methods in which either the target or the compound is 'tethered' to some aspect of the instrument such that the binding of the other 'free' component can be characterized. The different methods, such as SPR, bio-layer interferometry (BLI) and weak affinity chromatography (WAC), have the common advantage of allowing the binding of many compounds to be measured if a small quantity (less than 1 mg) of the target can be tethered without disturbing target folding, ligand binding properties or function. Such methods are also prone to similar artefacts, such as nonspecific interaction of compounds with the immobilization medium and the possible influence of additives or co-solvents (for example, DMSO) on the binding signals. The assay sensitivity of larger target proteins can be compromised if steric constraints limit the amount that can be tethered, and the binding of small 'free' ligands can lead to small signals when the technique relies on changes in mass or refractive index close to the sensor surface. This is a class of techniques for which increased experience and education has greatly improved application[36,37], and there is now widespread understanding and acceptance of the stringent controls needed to validate the experimental protocol.

The thermal techniques (TSA, ITC and DSC) are all solution-based and do not require immobilization of either the target or ligand. The artefacts in TSA measurements are well documented[38], but TSA benefits from ease of use and requires only micrograms of material. ITC is the gold standard for direct binding measurements. Unfortunately, despite substantial improvements in calorimeter instrument sensitivity, milligrams of protein and high compound solubility are still required to characterize the binding of each compound.

Finally, there are a number of techniques that rely on fluorescence (either intrinsic or extrinsic) of either the target or the compound, such as MST and fluorescence polarization (FP). These solution-based methods bring additional considerations and a need for validation steps if extrinsic labelling is used, as the fluorophore itself may affect some aspect of the binding.

What information can currently be obtained?

One of the challenges for drug discovery researchers who are not familiar with biophysical techniques is to understand what type of questions can be addressed by such techniques and which techniques are the most suitable. The following discussion provides some examples of 'typical' questions posed and the techniques that are currently routinely and robustly used in our laboratories. The discussion uses the phrase 'tool compound' to represent a compound that is known to bind with a particular affinity to a specific site on the target, and is soluble, stable and non-aggregated under the conditions used to make measurements.

Is the target folded? A 1D proton NMR spectrum of an unlabelled protein is relatively quick to obtain. A folded protein will have narrow peaks spread over a large chemical shift range (−0.5 to +10.5 ppm), often with distinct peaks at the extremes of the distribution. An unfolded or partially folded protein will have broader and less well-dispersed peaks, often close to their random chemical shift values. Similarly, a circular dichroism spectrum will reflect folding, particularly for proteins containing substantial numbers of α-helices. A more rapid way of characterizing protein samples is by TSA, through which a clear thermal melt curve is a quick way to characterize folding and homogeneity across samples, particularly if a tool compound is available.

Does a compound bind to the target? All of the techniques in TABLE 1 can be configured to detect the binding of a compound to the target, with the dynamic ranges indicated. Some techniques provide more information than others (as discussed here), but all provide the essential first assessment of whether the compound binds or not — with real confidence if a tool compound is available for assay validation. Measurements at different ligand concentrations and evaluation of dependence on concentration provide additional information on the binding behaviour and an estimation of the affinity.

What is the stoichiometry of binding? Accurate stoichiometry measurements are difficult, as they require accurate knowledge of both the total ligand and macromolecule concentrations and the fraction that is able to form a complex. The absolute errors need to be less than 20% on each of these to be able to confidently distinguish a 1:1 from a 2:1 complex. ITC is probably the most routinely used method to determine stoichiometry, but it consumes large quantities of protein. SPR is a good alternative when a suitable tool compound is available. Single-molecule methods would avoid the need for precise concentration determination and may overcome

sample consumption issues. Currently, the closest technique that matches this is NC-MS, which has many attributes that make it a valuable biophysical technique for drug discovery[24,25]. However, further development of the method is required for more routine use[39].

Where does the compound bind? In the absence of an X-ray structure of the ligand complex, the most robust technique for identifying where a compound binds is heteronuclear single quantum coherence (HSQC) NMR spectroscopy (usually ^{15}N but sometimes ^{13}C). Each peak in the spectrum reports on the local chemical environment of the resonant nuclei, which can be altered by compound binding. If the spectrum is assigned, such that peaks can be linked to individual protein residues, then the actual site of binding can be identified; if not, then a fingerprint of peak movements can determine whether compounds are likely to bind to the same site or different sites. Once a tool compound for a site has been identified, many types of competition displacement assay can be configured using other techniques to more rapidly test and characterize new compounds. However, it is important to remember that these displacement techniques do not directly report the site of binding, unlike NMR and X-ray crystallography, and allosteric site binders may displace probe compounds owing to long-range conformational effects. In any case, the use of site-specific mutants of the target can help to provide information about binding site location.

What is the atomic structure of the target–compound complex? X-ray crystallography is the most powerful, robust and routine method for providing a detailed atomic picture of a compound binding to its target. There are rare examples of discrepant observations between soaked and co-crystallized complexes (for example, when targets require conformational change or when crystal packing interferes with ligand binding) or when the crystallization conditions themselves affect the ligand binding mode (for example, pH). Therefore, as with other experimental methodologies, corroborating data from X-ray structures obtained by alternative crystallization protocols or from orthogonal solution studies such as NMR provide a more complete picture.

What are the thermodynamics of compound binding? ITC is the most direct method used to measure the thermodynamic parameters of binding (K_d, ΔH and ΔS) and, with variation of temperature, ΔC_p. The most straightforward and routinely used experiment is a direct binding titration, in which the heat change upon binding (ΔH) is monitored as small aliquots of a concentrated compound solution are injected into a solution containing the target at constant temperature. This configuration can be reversed when the compound is less soluble and the protein target is highly soluble. Affinity measurements, most often using SPR, at different temperatures can provide an alternative way of determining thermodynamic parameters using a van't Hoff analysis (see below). However, this method involves additional assumptions and has not been widely used so far.

What are the kinetics of compound binding? SPR biosensors with a microfluidic flow system and dextran surfaces are the most extensively used instruments for measuring ligand binding kinetics. With an appropriate configuration, k_{on} and k_{off} can be determined by simply fitting an equation corresponding to a suitable model to the sensorgram that results from the SPR experiment. K_d can also be derived as the ratio of k_{off} to k_{on}. For more complex interaction mechanisms, other kinetic parameters can be estimated. Many compounds have a relatively high k_{on} (limited by the rate of diffusion) with an increasingly low k_{off} as the affinity for the target increases. There is growing evidence from the literature demonstrating that ligand binding kinetics can influence efficacy and safety and therefore therapeutic success for some targets[5]. Attempts to increase our understanding of how to modulate these kinetics parameters by correlating them with X-ray ligand complexes to give structure–kinetic relationships (SKRs) are an area of great interest (see below for current status).

Where are biophysical techniques applied?

Two major developments in drug discovery have been synergistic with the deployment of a wide range of sensitive biophysical methods: the increased focus on drug-like properties during compound optimization[10] and the rise of FBDD[40]. We begin with a brief overview of biophysical methods in FBDD, as this has driven and challenged the development of methods more than any other application in recent years. We then outline how these advances have been exploited in areas such as high-throughput screening (HTS), hit confirmation and more detailed characterization of compound binding.

Fragment screening. The first published example of FBDD was 20 years ago, with the seminal work of the Abbott group, which demonstrated that nanomolar affinity drug candidates could be derived from small fragments that bound with a millimolar K_d[41,42]. This group not only pioneered the use of protein-observed NMR experiments to detect weak interactors but also were the first to conduct fragment screening by crystallography[43]. Since then, many companies have been founded using fragment-based screening (FBS) as their primary hit identification strategy. Several of these are renowned for advancing methodologies for their chosen biophysical approach: for example, X-ray crystallography[44,45], ligand-observed NMR spectroscopy[46], SPR[47], small-molecule microarray SPR[18,19] and NC-MS[19,25]. In both large and small companies, multiple established methods and increasingly emerging biophysical techniques (for example, MST and WAC)[48] are applied concurrently during FBS hit validation. Comparisons of the range of FBS methods, including illustrative successful fragment-to-lead case studies, are well documented in literature reviews[40]. However, faced with a growing array of biophysical methods capable of FBS, two related questions are often posed and debated: why do different techniques result in different hit lists, and which is the 'best' FBS technique to use?

Although many studies report different hits depending on the screening method[49], much of this discrepancy can be explained by variations in assay protocol and

detection method. Techniques have differential sensitivity to the properties of individual compounds within the fragment library (for example, solubility, aggregation potential, stability and ability to interfere with the assay) and assay conditions used (for example, the effect of buffer conditions, including temperature and pH, on both the fragments and the targets). Consequently, weak-affinity fragments may lie just above or below an assay detection threshold as the balance of factors changes.

The goal of FBS is to detect the binding of low-molecular-mass compounds (typically <300 Da) with low affinity to a protein target (in the micromolar to millimolar range). It is widely acknowledged that there is no single 'best' technique; instead, the choice of technique, or often techniques, for primary FBS is frequently dictated by logistical and practical considerations such as equipment and protein availability. More importantly, we believe that success in FBS depends strongly on users' experience and expertise, specifically the ability to establish robust assays, stringent data interpretation protocols and a well-considered screening cascade. In some instances, an inclusive strategy is adopted, progressing all hits from all of the applied methods; in others, only overlapping hits validated by more than one technique are included. Both strategies can be appropriate depending on the circumstances and when they are chosen in light of an understanding of the acquired FBS knowledge, discussed in BOX 1.

The adoption of FBDD in some organizations has required changes in the processes and gate-keeping criteria for progression through early drug discovery. Fragments straight out of FBS often have no measurable target function (either agonism or antagonism). Therefore, it is important to develop robust models of how fragments bind and to understand the opportunities and probability for successful affinity optimization. After the initial investment to improve affinity independently of activity, for fragments that register detectable functional activity, conventional activity coupled with ligand efficacy criteria can be used to prioritize further chemistry efforts towards generating lead molecules. Another barrier is that many larger companies use multiple hit-finding strategies, so it can be difficult to persuade a medicinal chemistry team to undertake the optimization of a 500 μM fragment if a 100 nM hit is available from HTS. For these reasons, structural information on fragment–target complexes, usually obtained by X-ray crystallography, is considered essential in FBDD to provide insight into chemical opportunities for efficient fragment evolution[50].

Most fragment screens are configured to identify compounds that bind to known binding sites (usually the active site of an enzyme). It is often straightforward to find many fragment hits for such 'conventional' targets, provided that the target protein can be readily generated and, in particular, that tool compounds are available to validate the various assays. For other target classes, innovative experimental design may be required to identify and successfully develop these hits. The first example of a difficult target class is represented by proteins with druggable allosteric sites (often initially unknown and without a natural biological function). For this class, there are some striking examples in which careful design of NMR experiments has enabled: detection of hits that inhibit BCR–ABL kinase at a myristoyl binding site[51]; generation of non-bisphosphonate farnesyl pyrophosphate synthase inhibitors[52]; and discovery of compounds that activate enzymes such as glycoside hydrolase[53]. The second example of a difficult target class includes proteins that transiently interact with other proteins or that form multi-protein complexes — two different types of important protein–protein interactions. For such systems, thorough experimental design and assessment with many orthogonal biophysical techniques is needed to validate fragment binding (for instance, see Winter *et al.*[54]).

HTS. Here, HTS is defined as screening a collection of ~1 million compounds or more against a protein target to identify a compound subset that shows activity. Such screens are typically based on biochemical or cellular assays, often with optical detection. Few biophysical methods satisfy the throughput requirements of HTS[55], although many are used at the secondary hit-validation stage[56,57]. TSA[58] and, to some extent, MS[59] are techniques that can be configured to provide the throughput, ease of access and low sample consumption needed for HTS screening. Cocktails of compounds are often used to reduce the number of measurements, material consumption and costs. TSA was initially used for proteins of unknown function or when obtaining reagents required to establish a traditional biochemical displacement or enzymatic assay proved challenging. The simple format of TSA has facilitated its application to other protein classes, especially within academic laboratories.

Hit confirmation and validation. Biophysical methods are now routinely incorporated as an integral component of the HTS hit validation and characterization process of most pharmaceutical companies.

In the past, many HTS triage strategies relied solely on hits progressing through a series of negative selection assays designed to filter out compounds acting through unwanted effects, such as pan-assay interference compounds (PAINS)[60] — for example, compounds that cause target oxidation through their intrinsic redox activity, unstable or reactive compounds and compounds that induce protein aggregation. Unfortunately, this filtering approach is imperfect as not all nuisance mechanisms are known, and problematic compounds for some targets may be true high-quality ligands for others, as evidenced by the finding that 10% of a set of known drugs can exhibit 'promiscuous' activity[61]. This proves especially difficult for challenging targets such as protein–protein interactions, for which the number of authentic hits is often very low.

The use of positive selection assays provides an effective and efficient alternative approach, and many biophysical methodologies have been adopted to serve this purpose[56,62]. The major roles and benefits of biophysical approaches within hit validation fall into three categories. First, some approaches can allow the orthogonal

confirmation of functional activity. For example, many screening assays use optical readouts such as fluorescence and luminescence to confirm activity. For enzyme assays, non-optical detection using RapidFire MS or NMR provides alternative ways to monitor substrate consumption or product build-up. The second major type of positive selection assays is those that verify direct target engagement. For example, a simple biophysical

Box 1 | **Observations from fragment-based screening studies**

After ~15 years of broadly applying the paradigm of fragment-based drug discovery (FBDD), accumulated experience in the industry has led to a common notion of best practices that are summarized here and illustrated in the figure.

X-ray crystallography. An X-ray co-crystal structure of a target-bound fragment is considered the ultimate validation of the binding event. Therefore, screening by X-ray crystallography has the advantage that details of the fragment-binding mode are immediately available. Although the method has long been hampered by the relatively low throughput and high investments, access to synchrotron beamlines and automation in many process steps has substantially lowered the barriers for screening larger libraries. The disadvantages of this technique are that the protein crystal system has to be robust to soaking of ligands at a high concentration and must have solvent channels that allow fragments to easily penetrate the crystal and access the binding site. Success is governed as much by kinetics as by thermodynamics. Often, multiple soaking attempts are needed to obtain a structure of a true ligand to the protein, implying that screening by X-ray crystallography will result in many false negatives[40].

Nuclear magnetic resonance spectroscopy. Nuclear magnetic resonance (NMR) spectroscopy is the most robust method for detecting very weak binding (micromolar affinity), with the added advantage that the spectra obtained report on the integrity of both the target (protein-observed NMR) or the ligand (ligand-observed NMR) with each assay step. Its main limitation is the amount of protein required — typically tens of milligrams for screening a 1,000-member fragment library.

Surface plasmon resonance. Surface plasmon resonance (SPR) provides a robust platform for screening and compound optimization[37] and is one of the most widely used biophysical techniques in fragment-based screening. The main challenges for SPR are retaining full protein functionality after immobilization on a sensor chip, as well as the expertise and experience required to set up a high-quality assay and analysis protocol. The number of successful FBDD lead-generation projects using SPR as one of the main primary screening methods is steadily growing[115,133–135]. New instrumentation and adaptive strategies have led to a broadening of SPR technology applications to difficult target classes, such as membrane-bound proteins, for which novel reconstitution and tethering methods preserve the conformation and activity of the surface-bound protein[115,133,135].

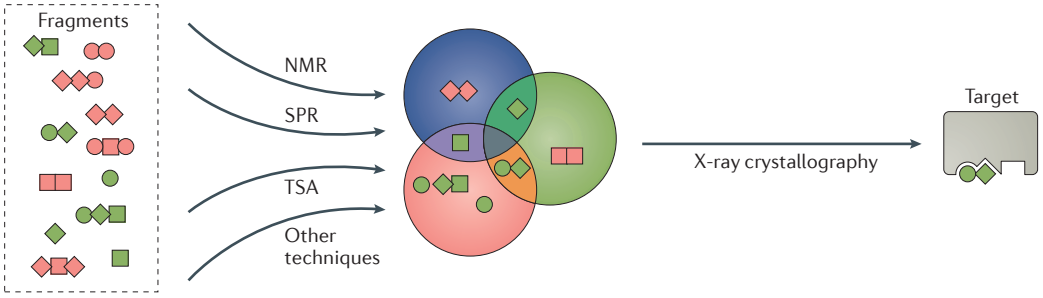
Thermal-shift analysis. Thermal-shift analysis (TSA) methods are particularly attractive to academic groups, given the ease of instrument access. However, the degree of thermal stabilization upon fragment binding to a protein may be too small to be measured, and contaminants or counter ions co-present in samples can be sufficient to cause a thermal shift. In addition, this method can be a source of many false-positive and false-negative hits[38,40].

Isothermal titration calorimetry. There are only a few examples in the literature using isothermal titration calorimetry (ITC) as a primary assay in fragment-screening campaigns, as this method has a very low throughput and equilibrium dissociation constants of fragment interactions are often too weak to be well determined[22,127,136].

Fluorescence-based assays. High-concentration screening using biochemical, often fluorescence-based, binding and functional assays has been reported for fragment screening[40]. The need for a fluorescent readout can result in more complex assay configurations — for example, the introduction of extrinsic fluorophores, coupling systems, antibodies and lanthanide reagents. This complexity increases the probability that, at the high compound concentrations used (typically ~0.5–1mM), one or more of the assay components may be optically or physically perturbed by compound interference.

Orthogonal methods. Given the limitations of individual biophysical methods in identifying a set of *bona fide* binding fragments, validation of hits by orthogonal methods is now commonly applied. This can be done by running multiple biophysical assays on the entire fragment-screening library or by choosing a consecutive assay funnel format (highest sensitivity and throughput assays are run first).

Hit optimization without structural information. FBDD campaigns based on X-ray co-structures still represent the process gold standard. However, as initial biophysically identified fragment hits may turn out to be X-ray false negatives, and sometimes a few rounds of fragment optimization are needed to obtain a favourable ratio of affinity and solubility, researchers are now more encouraged to tackle targets lacking available structural information (termed 'non-structurally enabled targets') and prosecute FBS hits using classical medicinal chemistry strategies.



binding experiment using SPR, NMR, NC-MS or MST can confirm that hits bind directly to the protein target of interest rather than to any other biochemical assay component, such as capturing antibodies or coupling enzymes. Third, positive selection assays can be used to determine whether the mode of action of a compound on a target protein is aligned with the biological understanding of the disease mechanism. This relies on the protein used in the assays being in a functionally relevant state (for example, with regard to post-translational modification and binding partners). Briefly, biophysical assays can yield information on binding specificity and stoichiometry, and ITC and SPR provide additional quantitative data on binding thermodynamics and kinetics that implicitly carry information on the mode of action of the hits (see below). X-ray crystallography (and NMR) can identify the ligand binding site on the target and elucidate the atomic details of the protein–ligand interactions. Taken together, these methods provide a comprehensive data package that adds confidence and optimizes success in the prioritization and selection of HTS hits for the following chemical optimization phase.

In-depth characterization of compound binding. The advent of sensitive biophysical technologies for time-resolved and thermodynamic analysis of protein–ligand interactions that do not require reporter groups such as enzyme substrates has had a tremendous impact on the ability to characterize details of molecular interactions. The kinetics of interactions are typically determined by SPR biosensor technology and the value of kinetic data is well recognized; however, the technology can provide additional data for understanding interactions and optimizing lead compounds. Interpretation of the SPR data is frequently done assuming a simple one-step reversible 1:1 interaction model, but exceptions occur and additional information can be obtained for some systems. For example, interactions between targets and optimized leads are often very tight, resulting in essentially irreversible dissociation rates that are challenging to quantify owing to measurement errors and limited stability of the assay system. Qualitative analyses and ranking of compounds can still provide useful information for prioritization of analogues and further optimization[63]. Another example is dynamic proteins and protein–protein interactions whose function and regulation is dependent on complex interaction mechanisms involving conformational changes of free or bound protein (for example, see Geitmann *et al.*[64] and Seeger *et al.*[65], respectively). In particular, the determination of kinetic parameters requires the use of appropriate mathematical models that take all parameters and the potential mechanism of binding into consideration.

Qualitative details of interactions can also be obtained by analysis under different conditions, termed chemodynamic analysis. Chemodynamic analysis involves a series of identical experiments that only differ in buffer pH, ionic strength or the presence of other substances that influence the interaction. This is valuable information as it provides an improved understanding of the dominating interaction forces, which is important for structural optimization of leads. It also provides a measure of the robustness of the interaction throughout different experimental conditions as well as the relevance of the data for cellular and *in vivo* experiments.

In addition, Eyring analysis of the variation of kinetic rate constants (for example, from SPR) with temperature allows the entropy and enthalpy of the transition state for binding to be explored, supplementing the information on entropy and enthalpy of binding that can be determined from calorimetry. Although it is difficult to predict these thermodynamic parameters with computational methods, the changes that occur during compound optimization can sometimes be rationalized and provide insight into the key features required for high-affinity binding.

Assisting lead optimization. In late-stage hit-to-lead or lead optimization, the focus of drug discovery projects typically moves away from binding affinity and selectivity optimization to include a multitude of additional optimization parameters. These include cellular activity, *in vitro* drug metabolism and pharmacokinetic (DMPK) properties, physicochemical properties, *in vivo* pharmacokinetics and efficacy in disease-relevant animal models. More recently, biophysical parameters have been used as additional optimization criteria. For instance, tailoring the residence time of a compound by extending the dissociation rates has been reported to be a useful complement to affinity optimization and is in some cases the most important parameter[4]. In some projects, the potency of very active compounds is prohibitive for accurate quantification of binding in biochemical assays, whereas biophysical assays are still able to measure activities in the pM range. For *in vivo* experiments with inconclusive pharmacokinetic–pharmacodynamic (PK–PD) relationships, knowledge of the drug residence time on the target derived from SPR may be extremely helpful to explain the observed data.

BOX 2 shows examples of the successful use of biophysics in drug discovery for different purposes: hit identification, hit-to-lead optimization, improvement of absorption, distribution, metabolism and excretion (ADME) properties, attainment of longer residence times and identification of allosteric inhibitors.

Emerging technologies and wider applications

TABLE 2 highlights a selection of emerging biophysical techniques used in drug discovery. These emerging techniques can either provide new information (for example, conformational changes) or the same information (k_{on}, k_{off}, K_d and stoichiometry) in a way that is faster or simpler than established techniques. Many of these techniques use completely new types of physical principles for detection. In addition to the advantages related to the information they can provide, crucial factors for their establishment include material requirements, ease of use and implementation in current drug discovery settings, and cost.

Enabling drug discovery for challenging targets. Numerous targets prove difficult in small-molecule drug discovery, including protein–protein interactions, multi-protein complexes and intrinsically disordered

Box 2 | **Examples of the successful use of biophysical techniques in drug discovery**

Biophysical techniques can be used for various purposes in the drug discovery process, including (among others): hit identification; hit-to-lead optimization; improvement of absorption, distribution, metabolism and excretion (ADME) properties; attainment of longer residence times; and identification of allosteric inhibitors.

Protein-observed nuclear magnetic resonance for hit identification. Fragment screening can identify hits for targets for which high-throughput screening fails. For B cell lymphoma X_L (BCL-X_L), when heteronuclear single quantum coherence (HSQC) measurements can detect when ligands bind to the ^{15}N-labelled protein, the structure by NMR (or crystallography) can determine binding mode, enabling subsequent optimization of the compounds[137]. Two fragments binding at distinct sites (shown in the left-hand side of panel **a** in the figure; Protein Data Bank identifier (PDB ID): 1YSG) provided the basis to develop compound 1 with an increased affinity to BCL-X_L (shown in the right-hand side in panel **a**; PDB ID: 1YSI). Further optimization led to ABT-737. Another high-profile example is the discovery of KRAS inhibitors[69–71].

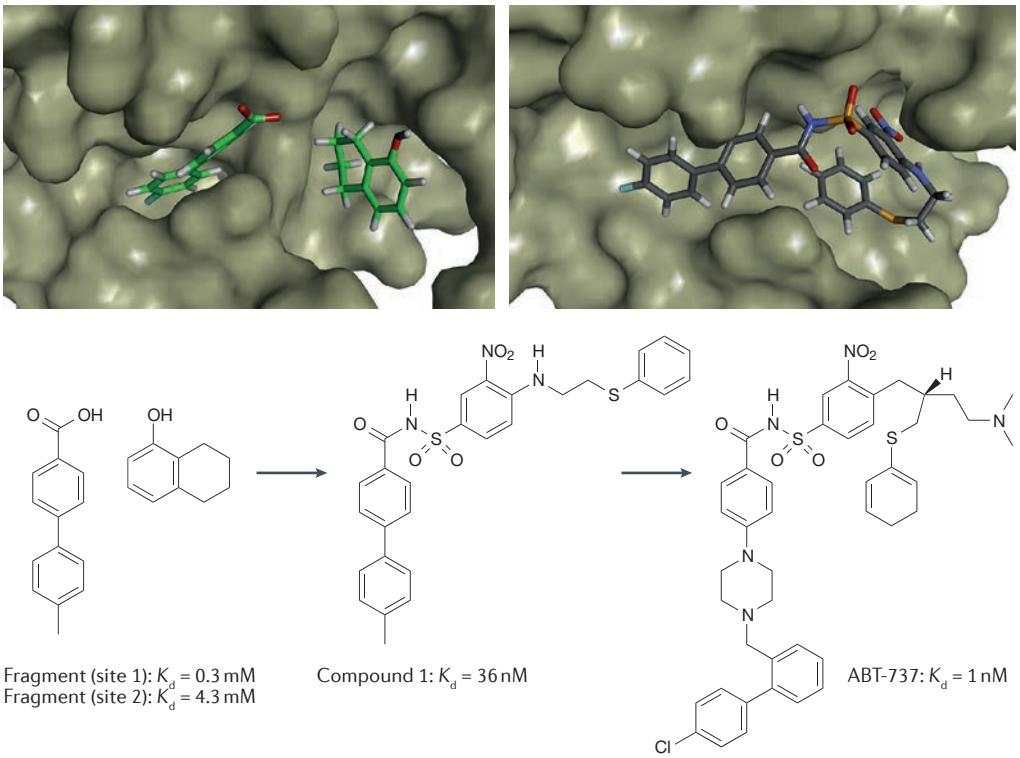
Improving ADME properties from crystal structures of compounds bound to off-target proteins. The BCL-X_L affinity of compound 1 was strongly attenuated in the presence of albumin. In order to identify substituents on compound 1 that reduce binding to albumin without affecting affinity for BCL-X_L, the NMR structure of a thioethylamino-2,4-dimethylphenyl analogue of compound 1 (PDB ID: 1YSX) bound to albumin was determined. The structure was used to guide the optimization (that is, reduction) of plasma protein binding for the BCL-2 and/or BCL-X_L inhibitor ABT-737 (REF. 137), which was subsequently optimized to the recently approved BCL-2-selective inhibitor ABT-199 (venetoclax)[66].

Kinetics to identify lead compounds with a long residence time. One step in the lead optimization of a chemical series inhibiting the molecular chaperone heat-shock protein 90 (HSP90) made a change in the core from a pyrazole to an isoxazole[138]. This gave a large decrease in the off-rate for compound binding that was preserved in the isoxazole clinical candidate AUY922, and this was reflected in the very long residence time on the target.

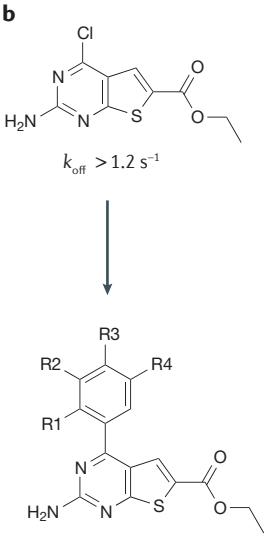
Off-rate screening for hit-to-lead optimization. A decrease in the off-rate (k_{off}) is usually the main contributor to the increase in affinity during compound optimization. This rate constant is concentration independent, which means that improvements in affinity can be monitored without quantification of compounds, provided that the optimized compound dominates the interaction. This enables rapid and efficient exploration of parallel libraries[139]. The example shown in panel **b** of the figure is from a retrospective study demonstrating the potential of the method for a series of HSP90 inhibitors[140]. A set of benzene substituents were introduced on to the thienopyrimidine fragment by a Suzuki reaction performed in a 96-well plate and the resulting reaction mixtures were profiled by surface plasmon resonance (SPR). The initial fragment showed a k_{off} of more than 1 s^{-1}; k_{off} was slower for some of the substitution patterns, most strikingly in the o,p-dichloro variant (for which it was 0.02 s^{-1}). The k_{off} shown by the crude reaction mixture is essentially identical to that of the purified product. Off-rate screening dramatically reduces the time needed to profile compounds and the amount of material (and solvents) used.

Protein NMR to identify allosteric inhibitors. GNF-2 is a highly selective non-ATP-competitive inhibitor of oncogenic BCR–ABL activity. HSQC chemical-shift measurements showed that this compound binds to the myristate binding site of BCR–ABL rather than the ATP-binding site, which is the target of inhibitors such as imatinib. The elucidation of the mode of action revealed that the myristate-binding pocket functions as an allosteric inhibitor site and that small-molecule starting points for inhibitors at this site, such as GNF-2, can be found[51]. Subsequent X-ray crystallography studies and mutational studies were also used during the optimization of GNF-2 to give analogues with improved pharmacokinetic properties suitable for *in vivo* studies[51]. A similar approach has identified allosteric inhibitors of farnesyl pyrophosphate synthase[141].

a



b



R1	R2	R3	R4	k_{off} (s^{-1})
H	F	CN	H	0.6
Cl	H	Cl	H	0.02

proteins. In particular, protein–protein interactions represent a highly challenging area. The binding sites are large and shallow, with binding hotspots separated by long distances. Further challenges include protein conformational flexibility and competition with the endogenous ligand for the binding site. Biochemical assays as used in HTS are frequently not successful in detecting weakly binding compounds that could provide a starting point for a medicinal chemistry programme. In such cases, biophysical methods are instrumental in providing essential ligand binding information. FBDD has made some progress for this class of target, with the selective B cell lymphoma 2 (BCL-2) inhibitor venetoclax (also known as ABT-199)[66] being approved by the US Food and Drug Administration (FDA) in April 2016 and clinical candidates identified for other targets (for example, hepatitis C virus nonstructural protein 3 (HCV NS3))[67]. In addition, early inhibitors have been identified for the first time for another member of the BCL-2 family, induced myeloid leukaemia cell differentiation protein MCL-1 (REF. 68) and for one of the most highly validated (but challenging) drug targets in cancer, KRAS[69–71]. NMR was used to identify fragment hits in most of these examples, exploiting the ability of this technique to robustly identify weak binding compounds. Small-molecule microarray SPR[18,19] has enabled some hits to be identified for protein–protein interactions (by differential screening against the isolated partners and the protein–protein complex) and for intrinsically disordered proteins, such as the Tau protein[72].

Evaluation of target engagement in complex systems. Lack of target engagement at the *in vivo* site of action is believed to be a substantial source of attrition in phase II proof-of-concept studies[73]. This emphasizes the need for novel technologies to assess target engagement in more complex cellular and *in vivo* systems, preclinically as well as in clinical studies, so that the best compounds are chosen and optimized and more reductionist approaches can be validated at an early stage to avoid wasted resources.

The recently reported cellular thermal shift assay (CETSA) is one of a few currently available biophysical methods for this purpose[74]. Although its strengths and weaknesses have not been fully elucidated, the possibility to apply the technology directly in live unmodified cells and with unmodified compounds within *ex vivo* tissue is tantalizing. In a typical CETSA experiment, vehicle- and ligand-treated live cells are heated to different temperatures and the remaining soluble target protein is quantified by affinity reagents; for example, Western gels or quantitative MS technologies[75,76]. In its simplest interpretation, ligand-induced thermal stabilization indicates permeability to a relevant cellular compartment and target binding. Coupling CETSA to a quantitative MS proteomic analysis is a recent development, and the fingerprint of protein changes highlights both the power and complexity of the method, as proteins can be destabilized as well as stabilized, and there can be pathway-dependent as well as direct effects. Nonetheless, CETSA has already demonstrated insights within more physiological systems, especially for selectivity profiling and target identification studies[77].

Study of membrane proteins in a more native-like environment. Studying membrane proteins by biophysical techniques is challenging. This is mainly due to low gene expression, the low overall yield of protein after purification, the typically poor protein stability and the need to work with detergents and lipids. More than 10 years ago, the application of SPR to detergent-solubilized membrane proteins was demonstrated on two chemokine receptors, C-C chemokine receptor 5 (CCR5) and C-X-C chemokine receptor 4 (CXCR4)[78,79]. Both receptors were solubilized directly from cells with a mixture containing detergents and lipids, and captured on a SPR sensor using a specific antibody without prior purification. Further studies on isolated membrane receptors included fragment-screening applications on wild-type β_2-adrenergic receptor that was solubilized and purified in detergent micelles, and finally immobilized with a carboxy-terminal polyhistidine-tag[80]. Furthermore, specific antibodies targeting fusion proteins or tags have enabled biophysical analysis of G protein-coupled receptors (GPCRs)[81].

Most current studies on purified GPCRs use stabilized variants of receptors, and substantial progress in biophysical screening (SPR and target-immobilized NMR screening (TINS)) has been demonstrated by Heptares[82,83] and ZoBio[84]. After careful assessment of ligand binding properties compared to wild-type protein, the engineered receptors can be used in biophysical studies. Examples include SPR screening of low-molecular-mass ligands for stabilized β_1-adrenergic and adenosine A_{2A} receptors that were captured by a polyhistidine tag (His-tag) on the sensor surface. Recently, the discovery of dual inhibitors for orexin receptors (OX_1 and OX_2) was reported[85]. To date, stabilized GPCRs applied in SPR-based fragment screening have been developed by iterative single-point mutagenesis and alanine scanning. Directed molecular evolution represents an alternative approach to stabilize membrane proteins, as shown with the neurotensin 1 receptor[86] (M.H. and S. Huber, unpublished observations). This receptor was used for a fragment screening effort with 6,369 compounds by SPR followed by hit validation by NMR, resulting in 4 confirmed hits. This example represents the first successful fragment screening for a GPCR with a peptidic endogenous ligand (M.H. and S. Huber, unpublished observations). The ability of NMR to detect target–ligand interactions from nanomolar to millimolar affinity with high sensitivity is a fundamental advantage for membrane proteins. For the screening of larger libraries, the TINS approach has been successfully applied to wild-type and stabilized membrane GPCRs[87,88].

Recently, lipid bilayer nanodiscs were developed to enable detergent-free membrane protein preparation. For example, stabilized human A_{2A} receptor with a combination of affinity tag and green fluorescent protein

Table 2 | **Selection of emerging biophysical techniques used in drug discovery**

Name	Type	What is measured?	Derived data	Refs
Biolayer interferometry	Glass fibre-based biosensor	Interaction between white light sent down a glass fibre and the light reflected back up to the instrument. The reflected light comes from interfaces represented by a sensor surface exposed to an analyte and a reference surface with a proprietary bio-compatible layer	K_d, k_{on} and k_{off}	146–148
Backscattering interferometry	Microscale interferometry of HeNe laser light and CCD array detection	Change in specific refractive index signature of target and ligand measured as the mean polarizability of the sample as probed in a microfluidic channel	K_d	149,150
Surface acoustic wave	Surface-based fluidic biosensor	Real-time detection of binding-induced conformational changes through monitoring the shift in the phase of surface acoustic waves that travel along the biosensor	Conformational changes, stoichiometry, K_d, k_{on} and k_{off}	33,151, 152
Second-harmonic generation	Optical prism microplate-based biosensor	Proteins labelled with a second-harmonic-generation active dye are tethered to supported lipid bilayers by a His-tag. An incident femtosecond laser light creates an evanescent wave through total internal reflection when it strikes the sensor surface. Conformational changes that alter the orientational distribution of the label in space or time result in a signal change	Conformational changes	153–155
Waveguide-based grating-coupled interferometry	Integrated microfluidic surface biosensor	Changes in total mass on the biosensor result in a shift of the wave's phase; this phase shift provides information about the on and off rates, as well as the stoichiometry of the interaction	K_d, k_{on} and k_{off}	156,157
Electrically switchable nanolevers	Surface microelectrode biosensor	Change in amplitude of the mobility of DNA nanolevers immobilized on electro-switchable surface and electrically actuated at high frequency. A change in flexibility of molecules immobilized to DNA nanolevers alters the wave's amplitude; this directly reflects changes in the conformation of the molecules — for example, after binding to compounds	Conformational changes, protein size, K_d, k_{on} and k_{off}	158

CCD, charge-coupled device; His-tag, polyhistidine tag; K_d, dissociation constant; k_{off}, dissociation rate constant; k_{on}, association rate constant.

was used for SPR measurements to characterize small-molecule binding in a more native-like environment[89]. Although the off-rates measured this way are similar to k_{off} values derived from detergent-solubilized receptors, one order of magnitude faster on-rates lead to lower observed K_d values. The substantially increased stability of the nanodisc sample holds great promise regarding the efficient use of less-stabilized receptors for biophysical methods.

Besides GPCRs, SPR methods have been developed to characterize ligand binding to ion channels, and binding affinity as well as binding kinetics were investigated for, for example, acid-sensing ion channel 1a[90], γ-aminobutyric acid type A[91], 5-hydroxytryptamine ($5\text{-}HT_3$) [92] and nicotinic receptors[93].

ITC measurements with membrane proteins are challenging because of the special care required for handling of detergents in measurements and analysis of the samples. Reports are limited to monotopic membrane proteins such as carnitine palmitoyltransferase 2 and high-affinity small-molecule characterization in the lead-optimization phase[94].

Single-molecule studies. Single-molecule measurements make use of a diverse range of optical analysis methods, ranging from atomic force microscopy to various optical microscopies with a broad spectrum of light combined with ultra-high-resolution imaging techniques[95–97]. With the invention of novel fluorescent methods in combination with near-field microscopes in the mid-1990s, the application of single-molecule techniques to probe properties at the molecular level started to increase[96–99]. Single-molecule methods represent a potential option for identifying rare as well as transient structural states of a protein target or measuring quantitative kinetic parameters, or monitoring protein dynamics over time trajectories from seconds to minutes while consuming only small amounts of sample material.

One advantage of single-molecule methods is the low sample consumption, as probe volumes only require a femtolitre amount of sample with picomolar protein concentrations. Despite several limitations (such as the long data collection time, high background to noise level and difficulties in analysing low-affinity compounds), the popularity of single-molecule spectroscopy has increased in the past 10–20 years, especially in combination with Förster resonance energy transfer (FRET) to study the complex conformational landscape of proteins, including the determination of kinetic parameters in each conformational state[97,100].

Although the well-known single-molecule patch clamp technique has been used in drug discovery since the 1970s, the application of other single-molecule experiments in drug discovery is scarce owing to the complexity of experiments and the limitations of the methods[101]. Further developments — including higher-throughput detection on microfluidic chips[102],

progress in fluorophore chemistry, and technical improvement of instrumentation, software for data collection and analysis — are still needed to enable wider application of single-molecule technologies in an industrial environment.

Trends in structure-determination technologies. Knowledge about the 3D structures of biomolecules of pharmaceutical relevance and their drug complexes has proven to be of great value for an efficient and successful drug discovery effort. Structural biology provides a detailed view on the mode of binding of functionally active ligands and enables their structure-guided optimization. The generation of structural information in drug discovery has been dominated by X-ray crystallography. In contrast to NMR-based methods, there is no target size limitation, and the determination of multiple structures of target–ligand complexes (an essential step for probing the protein-binding pharmacophore with structurally distinct ligands) is easily facilitated by either co-crystallization or soaking protocols.

Serial femtosecond crystallography (SFX) using ultrashort pulses of coherent and extremely intense radiation generated by X-ray free electron lasers (XFELs) [103,104] and high-resolution single-particle cryo electron microscopy (cryo-EM)[105,106] are two new promising technologies in structural biology. With XFELs, it is now possible to obtain structural information for notoriously difficult targets for which either only very small crystals can be obtained (which are not suitable for diffraction experiments on regular synchrotron beamlines) or radiation damage is a serious problem (for example, see Kern *et al.*[107]). In the context of drug discovery, the enhanced access to novel structures through SFX — in particular, structures of challenging membrane protein targets — is of great value. SFX does not require work at cryogenic temperatures, which helps to derive information on molecular dynamics. Furthermore, SFX enables full automation of sample handling from crystallization to X-ray data collection, as no manual crystal manipulation is required. The use of this method in high-throughput ligand–complex crystallography will need further technological developments. Currently, the high level of effort involved in determining a structure and the relatively poor data quality limit applications to non-routine, low-throughput and high-value projects.

Cryo-EM is used to study large macromolecular complexes (~200–4,000 kDa). Some of these complexes reside in membranes, can adopt multiple conformational states and/or have been refractive to crystallization[105]. Recent examples of targets studied by cryo-EM include transient receptor potential cation channel subfamily V member 1 (TRPV1)[108], γ-secretase[109] and β-galactosidase, for which a 2.2 Å-resolution structure was obtained[110]. For some systems, cryo-EM is able to achieve the resolution needed to elucidate details of protein–ligand interactions, thus enabling structure-based design. Today, the minimal molecular size for high-resolution cryo-EM is about 200 kDa[106,111], leaving a large set of relevant drug targets out of scope. This limitation can probably be reduced by future developments in methodology and instrumentation or by studying molecular targets in the context of larger, functional protein complexes.

Lessons learned and perspectives

The fundamental science of molecular recognition and computational methods for data interpretation have evolved in parallel with the implementation of biophysical methods for drug discovery. The ability to accurately determine kinetic rate constants and thermodynamic parameters for protein–ligand interactions provides the possibility of new insights. For example, for inhibitors of HIV1 protease, it was demonstrated that k_{on} and k_{off} change from one generation of inhibitors to the next. In fact, the fully optimized, latest-generation drug molecules have a particularly long residence time[112]. Furthermore, the widespread assumption that similar ligands have similar association rates was clearly not valid, as small differences in compound structures have a large effect on binding kinetics. Another example is the confirmation that the tight binding of ligands containing moieties such as hydroxamates and sulfonamides is dominated by the interaction with metal ions (for example, Zn^{2+} ions), and the modulating effects of the other parts of the molecule on the kinetics and affinities are not very significant[113,114]. It may thus be difficult to achieve high selectivity with compounds that include such moieties.

As with any *in vitro* assay, it is important to carefully explore how the experimental conditions could lead to false-positive and false-negative results. Ad hoc selection of pH, ionic strength or type of buffer driven by convenience or preliminary protein handling considerations needs to be replaced by more attention to the detailed physiological conditions for the protein of interest. Questions such as 'what is the tissue location of the active drug target?' or 'what are the conditions in a particular cellular compartment?' need to be considered before the study, and an informed decision made for the selection of the experimental conditions to improve the translation of the results to cellular and *in vivo* assays. As an example, a pH shift of two units for the aspartic protease β-secretase 1 (BACE1) substantially reduced the binding of all ligands from some chemotypes with micromolar affinity at physiological pH (W. Huber, personal communication). The pH dependency of inhibitor interactions with BACE1 has been demonstrated to be a unique characteristic of the compounds, influenced not only by the active site aspartic residues, but also by changes in the ligand's pK_a upon binding[115,116]. The implications for drug design and translation of *in vitro* data to *in vivo* effects also requires the development of modelling methods[117].

Use of kinetic and thermodynamic data: expectations and realization. Expectations were raised during the past decade that the application of binding kinetics and thermodynamics in the selection and optimization of leads would improve the success rate of preclinical drug discovery. After the pioneering publication of Copeland *et al.*[5] in 2006, suggesting a correlation of drug–target residence time with drug efficacy and safety, many examples have been published in which binding kinetics

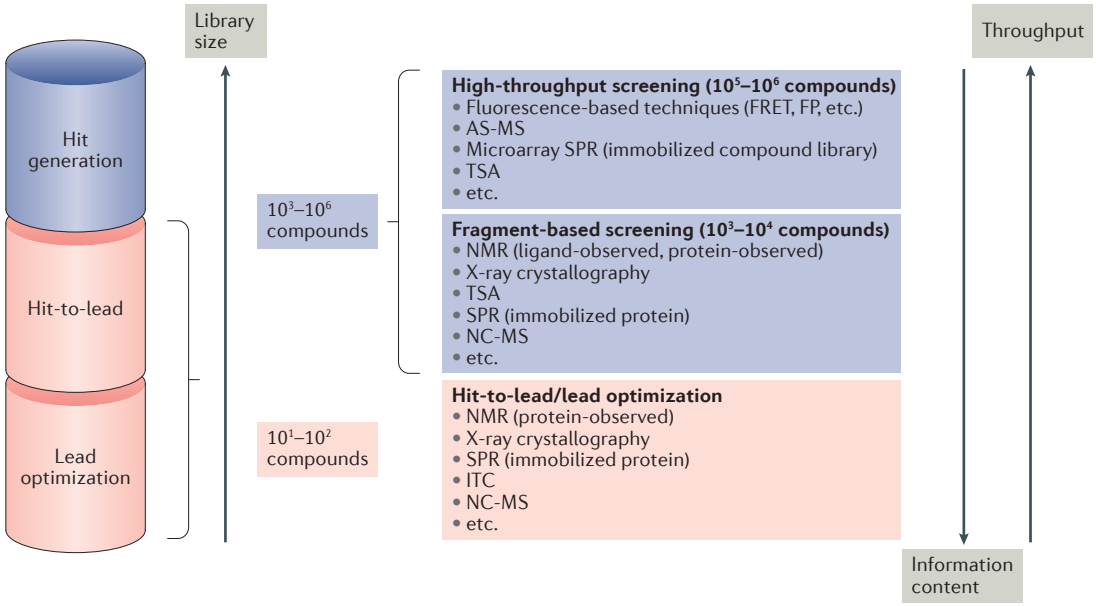


Figure 2 | **Biophysical techniques in drug discovery: throughput versus content.** The typical size of the compound collections involved at the different stages of drug discovery decreases from 10^5–10^6 for high-throughput screening (HTS) to 10^3–10^4 for fragment-based screening (FBS) and then to 10^1–10^2 for the hit-to-lead stage followed by lead optimization. The throughput of the biophysical techniques used at different stages should be adapted to the size of the corresponding compound collections. In general, the information content of the techniques is inversely proportional to their throughput. For instance, HTS provides a high to ultra-high throughput but a low information content (usually just 'yes or no' binding information). Conversely, lead optimization requires the highest information content to guide the choice of the best compounds for the next step (drug candidate selection) and the methods used generally have lower throughput. AS-MS, affinity-selection mass spectrometry; ITC, isothermal calorimetry; FP, fluorescence polarization; FRET, Förster resonance energy transfer; NC-MS, non-covalent mass spectrometry; NMR, nuclear magnetic resonance; SPR, surface plasmon resonance; TSA, thermal shift analysis.

and residence time were incorporated into the design strategies of drug candidates[4,118]. A popular conclusion therefore has been to advocate the use of dissociation rates in addition to or instead of affinity as a key parameter in compound optimization. However, this approach has a number of caveats. First, for many novel targets, the desired kinetic drug profile is not known at the outset of a drug discovery programme, so it is impossible to define a clear goal for off-rate optimization[119]. Second, in the context of drug efficacy, binding kinetics cannot be viewed as being independent from pharmacokinetics; in fact, in many cases, favourable elimination properties of drugs may largely compensate for faster dissociation kinetics[120]. Furthermore, target turnover rates can be faster than dissociation rates, such that beneficial effects of long residence times will not materialize. Finally, as the molecular determinants of binding kinetics are still only incompletely understood[121], the prospective design of kinetic profiles is currently not possible except for rare cases, in particular those in which long residence times are tuned through a covalent mode of inhibition[122]. Therefore, using binding kinetics in compound optimization is currently mainly applied in a monitoring mode, which, for example, can enable retrospective analyses of SKRs or resolve potential cases of PK–PD disconnects.

Similarly, there have been proposals that detailed analysis of the contributions from enthalpy and entropy to the free energy of binding could aid compound selection before lead optimization[22,123,124]. In these proposals there were simplified assumptions that a large negative binding enthalpy is advantageous for target selectivity and ADME properties, because it reflects predominantly specific, direct and polar interactions between the ligand and protein; conversely, a large negative binding entropy is disadvantageous, because it is built mainly on nonspecific van der Waals interactions. However, in recent years, wider analysis has demonstrated that the global parameters ΔH and ΔS: integrate multiple contributions of the complex protein–ligand binding process (most importantly the dominant role of the water structure); are very sensitive to influences of the experimental conditions applied in generating the experimental data[9]; and are subject to the phenomenon of enthalpy–entropy compensation[125]. Retrospective interpretation of differentiated thermodynamic repertoires of ligands is only possible with additional detailed information at hand — most importantly, high-resolution crystal structures of the protein–ligand complex containing information on the water network within the binding site[9,126]. Taken together, these factors currently preclude the prospective use of thermodynamics in compound prioritization. Therefore, current applications focus on deriving mechanistic information on binding modes and protein conformational changes when ITC data are used in concert with structural and computational analyses[127].

Selection of a biophysical method to fit the purpose. The choice of a biophysical method will first depend on the drug discovery stage — hit generation (HTS or FBS), hit-to-lead or lead optimization — in order to find the best compromise between the throughput, the size of the library to be screened, the sample availability and the expected information content (FIG. 2). For instance, ITC will clearly not be the best choice for a primary screening, but it will bring a wealth of valuable information for compound classification and prioritization at a later stage.

In FBS, different screening methods retrieve distinct sets of binders[49]. The choice of which methods to use for which targets is based on a number of factors, some pertaining to the availability and type of target protein. Moreover, skill in the application and choice of a method is as important as the method itself. An often unrecognized factor is that some biophysical technology can be deceptively user-friendly and easily generates large amounts of data. However, without skilled experimentalists the data are not meaningful. The reasons for this vary, but include inappropriate experimental design and conditions, poorly performed experiments, lack of suitable controls and incorrect or inadequate data analysis. For example, in SPR biosensor analysis, the basic art of fast and accurate pipetting and performing appropriate controls is crucial. Moreover, the visualization, presentation and interpretation of data also require experience. Although there is no excuse for poor quality work, each method shows limits and artefacts but, by recognizing and addressing them, a suitable combination of methods can provide the required information.

All in all, the best way to obtain a high rate of valuable hits from a screening campaign seems to be to use a robust, high-throughput, low-sample-consuming technique for primary screening and to confirm hits using lower-throughput but higher-content orthogonal techniques. Contrary to the belief that all drug discovery challenges are best solved through the introduction of new technologies, substantial advances can also be driven by innovative application. The kinetic probe competition assay (kPCA) for the discovery and characterization of GPCR ligands provides such an example of creative experimental design rather than novel instrumentation to extend the toolbox of technologies to progress drug discovery[128,129].

Summary and outlook

The increasing application of biophysical methods, especially over the past 10 years, has led to an improved process for compound prioritization and decision-making in early-stage drug discovery, based on valuable additional experimental information. For conventional soluble target classes, the use of biophysical measurements is well established, as the preceding steps of protein production and enablement of target-specific assays are normally well understood. This enables biophysical primary screening, secondary screening and detailed characterization assays to be developed on timelines that match other screening activities, and thus enables their integration into hit identification approaches. One major benefit of such integration is the ability to reliably and quickly identify true positive hits, thereby reducing wasted resources and increasing return on investment.

Furthermore, biophysical methods are crucial to the success of FBDD. The delivery of clinical candidates for difficult drug targets, such as protein–protein interactions, and an increasing number of marketed drugs derived from FBDD are testimony to the integral role it now has in drug discovery. Finally, biophysical methods are extensively used by medicinal chemists working on the challenge of multi-parameter optimization of compounds.

The future holds considerable promise for the extension of applications of biophysical methods. Improved abilities to produce large amounts of high-quality proteins, including membrane proteins, will increase the percentage of projects in the drug discovery portfolio that can benefit from these methods. Translation of biophysical methods to cellular environments will strengthen the physiological relevance of measurements, with techniques such as isothermal microcalorimetry[130] and in-cell NMR[131] now possible. Single-molecule and cell biophysical measurements are also advancing rapidly and enabling a degree of characterization that was previously inconceivable[132]. All of these developments are substantially enhancing our ability to look at more relevant and complex biological systems. However, for traditional and emerging biophysical techniques, care needs to be taken in the design of the experiments, the evaluation of the experimental conditions and the critical analysis of the results, and qualified personnel are important in all steps of the work.

Overall, methodological improvements in detection sensitivity (better signal-to-noise ratios), protein consumption and throughput, the development of novel technologies investigating new facets of functional behaviour of biomolecules, and an improved understanding of how to interpret results and relate them to existing functional data, will ensure that biophysical methods have a great future in pharmaceutical research.

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Competing interests statement
The authors declare competing interests: see Web version for details.