# SECTION 1

# EARLY DISCOVERY STAGES AND BIOTHERAPEUTIC CANDIDATE SELECTION

# 1

# BIOPHYSICAL METHODS APPLIED IN EARLY DISCOVERY OF A BIOTHERAPEUTIC: CASE STUDY OF AN EGFR-IGF1R BISPECIFIC ADNECTIN

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## 1.1 INTRODUCTION

Biophysical characterization of protein therapeutics and associated reagents in drug discovery is critical to selection and optimization of molecules that have the desired biological activity and to selection of drug candidates that can be efficiently developed and manufactured. Protein therapeutic molecules are larger and more complex than small-molecule drugs. Consequently, analytical strategies for determining whether a protein therapeutic is pure, stable, and homogeneous require that a larger number of physical properties be investigated, including characterization of tertiary and quaternary structures. Furthermore, several physical properties of protein therapeutics, for example, aggregation state, require multiple, orthogonal methods to confidently define them (Table 1.1).

In addition to production and characterization of hundreds or thousands of drug candidates during drug discovery, a large number and diversity of protein reagents must also be produced and characterized. To begin with, the biological target must be produced in a form that is well behaved and representative of the functional form to be targeted *in vivo*. There are a multitude of other protein reagents needed to run the program as well (e.g., multiple affinity-tagged forms of the target for use in a variety of assays, truncated forms of the target for structural studies, counter-targets, co-targets, and nonhuman species ortholog variants of the target; Figure 1.1; see also Kim and Doyle [1] for a detailed listing). Target reagents that are aggregated or misfolded confound the drug discovery process during hit identification and downstream assays. The famous admonition "garbage in, garbage out" is often cited as a reminder that biophysically well-behaved reagents generally lead to higher success rates during lead identification and optimization of protein therapeutics. Biophysical methods thus play a wide variety of roles in the characterization of biotherapeutic candidates and protein reagents during the early discovery stages of biotherapeutics.

Biophysical characterization is a central part of the selection and optimization process. But how much biophysical characterization is optimal for each type of reagent or biotherapeutic candidate molecule, and how does the extent of biophysical characterization change during each stage of the discovery process? The goals of this chapter are to describe the types of biophysical methods that are used in a stage-dependent manner throughout discovery for reagent and drug candidate production of protein therapeutics and to discuss how the application of these methods in discovery help to de-risk the potential costly challenges later in the development and manufacturing phases.

TABLE 1.1. Biophysical and biochemical methods used to characterize targets, reagents, and drug candidates for protein therapeutic discovery programs in terms of identity, purity, stability, oligomeric status, binding activity, and molecular binding mechanism

| Method[a] | Molecular information | Targets | Reagents | Hits | Leads | Final candidates |
|---|---|---|---|---|---|---|
| Analytical SEC | Self-association | 1 | 1 | 1 | 1 | 1 |
| Thermal melt | Thermal stability | | | 1 | | |
| Biosensor | Confirm binding | 1 | 1 | 1 | | |
| SDS PAGE | Purity, approximate mass | 1 | 1 | | 1 | 1 |
| LC-MS | Identity, primary structure, purity | 1 | 1 | 2 | 1 | 1 |
| SEC-MALS | Self-association, absolute mass | 1 | 2 | | 1 | 1 |
| DSC | Thermal stability | 2 | 2 | | 1 | 1 |
| Biosensor | Binding affinity, kinetics, epitope discrimination | 1 | 1 | | 1 | 1 |
| AUC—sedimentation equilibrium | Self-association, absolute mass, dimerization constant | 2 | | | 2 | 2 |
| X-ray crystallography | Define epitope, define atomic binding interactions | 2 | | | 1 | 1 |
| ITC | Solution binding affinity, molar ratio | 2 | | | 2 | 1 |
| KinExA | Very tight solution binding affinity | | | | | 1 |
| Thermal stability profiling | Thermal stability over diverse set of conditions | 2 | 2 | | 2 | 1 |
| Accelerated degradation | Indicator of manufacturability | | | | 2 | 1 |

Cases where a method is frequently used are designated by 1, and cases where the method is less frequently used but recommended are designated by 2.

[a]SEC, size-exclusion chromatography; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DSC, differential scanning calorimetry; MALS, multiple angle light scattering; ITC, isothermal titration calorimetry; KinExA, kinetic exclusion assay. Accelerated degradation refers to a set of biophysical methods (see text).

The discovery process is described in this chapter by several stages: target generation, hit evaluation, lead selection, lead optimization, lead formatting, and final lead candidate selection of a molecule to progress into development. We note that the types and extent of biophysical characterization will depend to some degree on the molecular class of the protein therapeutic (monoclonal antibody, Adnectin, antibody fragments,

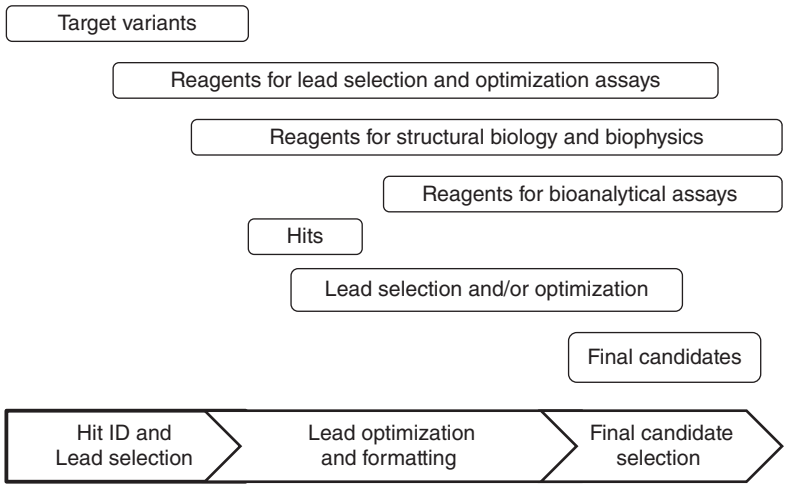


Figure 1.1. Scheme showing the different classes of protein reagents and drug candidates produced and characterized by biophysical methods from the initiation of a drug discovery program through selection of a final molecule for subsequent progression into development. Initially the protein production and biophysical characterization efforts are focused on the target(s) and reagents. As the program progresses, the amount of protein chemistry increases and shifts toward the production and characterization of protein therapeutic candidate molecules. The type and extent of biophysical characterization done for each class of protein and for each stage of discovery is different as described in Table 1.1.

non-antibody fragments, etc.) and the technology used for selecting lead candidates (immunizations, phage display, RNA display, etc.). The purpose here is to present a case study of biophysical applications during the discovery of a bispecific Adnectin against epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF1R). Many of the details for this system have been reported elsewhere [2].

## 1.2 TARGET IDENTIFICATION

Identification of a drug's biological target is a critically important part of a biotherapeutic discovery program. One of the expanding areas in biotherapeutics research is the design of bispecific biotherapeutics that bind to two different, already validated biological targets. The proposed benefits for the bispecific-targeting approach include improved efficacy and lower cost of goods than developing two drugs independently.

Drug targets may also be identified from genetic validation studies (correlation between mutation of target and disease state) or pharmacological validation studies (utilizing a surrogate molecule such as a natural ligand to demonstrate efficacy in a non-clinical setting). The Holy Grail for identification of completely novel targets is to utilize the growing information from genomic, proteomic, and interactomic studies to

draw correlations between specific drug targets, or sets of drug targets, and treatment of disease.

This chapter describes a case study for discovery of an Adnectin [3] bispecific biotherapeutic that targets inhibition of both EGFR and IGF1R (Emanuel et al. [2]). EGFR is a clinically validated target for cancer therapy, and there are both small-molecule kinase inhibitors and biotherapeutic inhibitors of the extracellular domains presently available as marketed drugs. IGF1R is also an attractive target for cancer therapy and there are several small-molecule and biotherapeutic inhibitors in preclinical and clinical studies [4].

## 1.3 TARGET GENERATION

Once a target has been identified, it is usually produced recombinantly to provide sufficient material to enable selection of biotherapeutic candidate "hits" through a screening or selection process. There are several technologies commonly used for generating biotherapeutics hits, including in vivo immunization, phage display, mRNA display, and yeast display [5, 6]. All of these technologies rely on the production of biophysically well-behaved target molecule. Biophysical methods thus play a critical role as "gatekeeper" at this phase of discovery, to ensure the quality of the target being used for screening or selections is suitable for generating the best candidates.

The first step in producing the target reagent is to engineer a form of the target molecule that will be expressed well and has acceptable biophysical behavior when purified. Sometimes the design is fairly straightforward. For instance, the construct design, expression, and purification for some targets may be well described in the literature. Construct design may also be straightforward if the protein target itself is structurally small and simple. An example would be a soluble target such as a cytokine. The construct design of a simple small protein could be as straightforward as expressing the entire native protein. On the other hand, construct design of large membrane-spanning protein targets can be much more challenging since the membrane-spanning and intracellular regions usually need to be deleted in order to make well-behaved soluble extracellular fragment(s) of the target. Whether or not some or all of the extracellular domains extracted from the full-length protein can be expressed, purified, and well behaved biophysically is often not known in advance.

### 1.3.1 Multiple Constructs Strategy

Given significant uncertainties and risk surrounding the production of critical target molecules, it is prudent to approach the problem with the design of multiple constructs in parallel, at least through DNA expression vector or small-scale expression screening stages. There are several reasons for designing multiple constructs up front for a target molecule. First, most target molecules need to be produced as fusions with a variety of affinity tags (e.g., His tags, Flag tag) to facilitate purification and development of different types of downstream assays. These non-native sequences may in turn alter the native functional or biophysical behavior of the target. Thus, different types of tags, each having

different linker sequences joining them to the target molecule, may need to be made and tested for suitable functional and biophysical behaviors by trial and error. Second, different domain regions, or fragments, of a target protein will have different intrinsic expression and biophysical properties, some of which will have acceptable biophysical and functional behaviors and others will not. As a general rule, the more novel is the target, the less is known about its expression and biophysical and functional properties and the greater the risk is of making it in useable form. Novel targets thus deserve more upfront engineering of multiple constructs. Finally, different forms of a target protein may generate different types of epitope families of lead drug candidates from the high-throughput screening or selection process, for reasons that may not be obvious. In order to obtain a sufficient diversity of initial drug candidates to evaluate during discovery, it is therefore useful to screen against multiple forms of the target molecule. For all these reasons, it is prudent to carefully plan out the target design strategy and backup strategies at the beginning of the target generation process, since the cycle time from construct engineering through biophysical and functional assessments is measured in weeks to months.

In the cases of EGFR and IGF1R, there are extensive precedences in the literature for making a variety of extracellular fragments. Moreover, there are three-dimensional crystal structures for some of these fragments, showing where the self-contained domain regions are at atomic resolution. We designed multiple variants of the extracellular regions of EGFR and IGF1R target proteins. The variants included different purification tags, different expression hosts, and different length variants of the extracellular regions. A subset of the constructs designed were expressed, purified, and characterized with biochemical and biophysical methods as described in Table 1.1.

Production of the target molecule, and multiple variants thereof, is only a subset of the total number of reagents needed to support a protein therapeutics drug discovery program. The scheme in Figure 1.1 describes the various classes of additional reagents needed, as well as protein therapeutic drug candidates, that must be produced and characterized during the discovery phase. Ideally one would like to have all the variants of the target, co-targets, counter-targets, and species ortholog targets upfront in the early phase of a discovery program in order to facilitate selection of leads with the optimal diversity and cross-reactivity profiles. However, producing all these reagents upfront is very time consuming and it is not uncommon for a program to move forward as soon as an adequate amount of the human target protein is available, and then to produce the other reagents for optimizing cross-reactivity and potency later in the program.

## 1.4 HIT EVALUATION

In the earliest stage of drug candidate biophysical assessment, many potential lead candidate molecules need to be evaluated in high-throughput mode (typically on the order of hundreds or thousands, or more, depending on the hit identification technology being used). The purification methods used at this stage are high throughput and must be robust and simple enough to generate large numbers of candidates within a reasonable period of time, but do not need to yield proteins that are as high in purity or quantity

as will be needed in the later stages of discovery. The biophysical assessment at this stage must also be rapid and simple and be able to distinguish the higher-quality lead candidates from the lower-quality leads. Some of the key biophysical methods used for hit identification include analytical size-exclusion chromatography (SEC), biosensor analysis, and thermal stability fluorescence (TSF; Table 1.1). These methods provide information about the self-association, binding affinity, and conformational stability properties of the hit molecules, respectively, and can be conducted in high-throughput mode using small quantities (sub-milligram) of protein sample.

### 1.4.1 Qualitative and Rapid Self-Association Check

Figure 1.2 shows example analytical SEC data [7, 8] for a well-behaved homogeneous candidate protein therapeutic in comparison to one that is heterogeneous and contains high molecular weight (HMW) species. Here we assume the homogeneous profile reflects a monomeric drug candidate. This assumption will be more rigorously tested at later stages of discovery using the more rigorous methods in Table 1.1. The presence of aggregates or HMW species suggests that production and storability of the molecule will likely involve more challenges during discovery than the molecule that exhibits homogeneous, monomeric behavior. Furthermore, the heterogeneity observed at the hit stage signals a risk that the poorer behavior might be retained during the later stages of

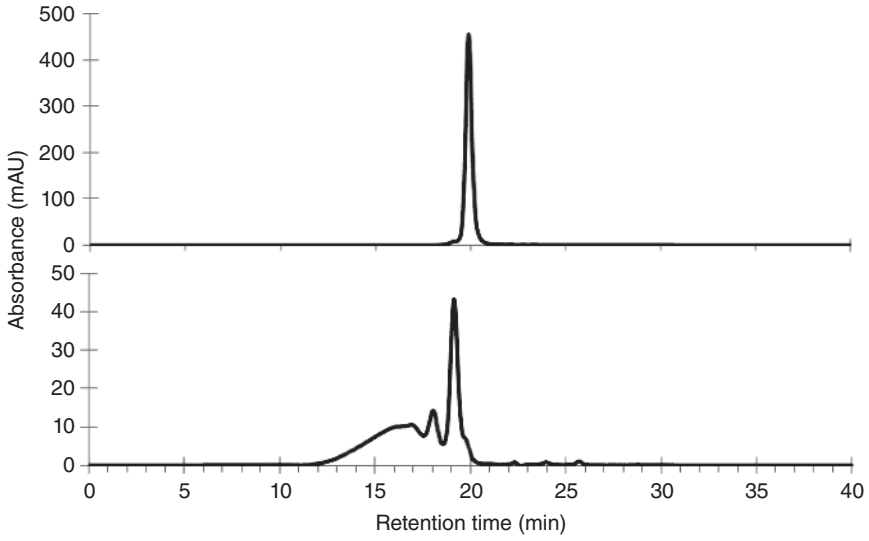


Figure 1.2. Analytical size-exclusion chromatography data showing examples of the elution profiles of early-stage Adnectin drug candidates. The top panel shows a homogeneous, monomeric drug candidate, and the bottom panel shows a candidate that has high molecular weight (HMW). Data of this type is used to select the most promising drug candidates for advancement.

discovery and during development. Barring any other exceptionally redeeming properties of the candidate having the HMW species present (such as being one of the very few hits having unique cellular activity or potency), one would normally select the homogeneous molecule to progress into the subsequent stages of discovery.

### 1.4.2 Qualitative and Rapid Thermal Stability Check

The conformational (or folding) stability of a protein is broadly used as a general measure of stability. This is because the partially or fully unfolded species of proteins are usually more prone to physical and chemical mechanisms of degradation (e.g., aggregation, proteolytic clipping, deamidation) than are the natively folded species. Thermal denaturation of proteins can be measured by many different technologies. One commonly used method that is rapid and requires only microgram amounts of protein is TSF [9]. This method goes by several different names such as thermofluor, thermal stability perturbation, and thermal shift assay. Here we refer to it as thermal stability fluorescence or TSF. Figure 1.3 shows an example of thermal stability for an Adnectin as measured by TSF. In this experiment, the temperature of the protein sample in the presence of an extrinsic fluorophore is increased, while the fluorescence of the sample is monitored. When the protein unfolds, there is an increase in exposed hydrophobic surface area which then binds to the extrinsic fluorophore and causes an increase in fluorescence. In principle, one can monitor the extent of unfolding from the extent of the change in fluorescence shown in the figure. A convenient measure of the thermal stability that can be used to rank-order the relative thermal stabilities of a series of closely related

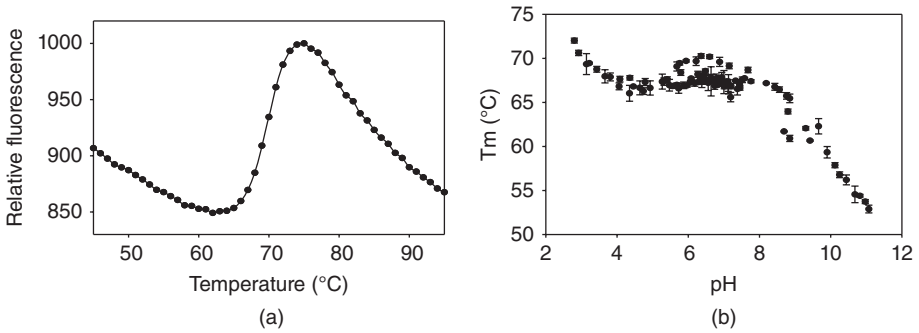


Figure 1.3. Example of thermal stability of a biotherapeutic candidate molecule as measured by thermal stability fluorescence. (a) Fluorescence of an Adnectin candidate in the presence of the extrinsic fluorophore anilinonaphthalene sulfonic acid (ANS) as a function of temperature. As the protein unfolds, hydrophobic regions are exposed to solvent, bind ANS, and cause an increase in fluorescence. The midpoint of the transition (Tm) is obtained by curve fitting and is used as a qualitative measure of thermal stability. The Tm for the curve shown is 70.2°C. (b) Tm values measured in high-throughput mode for the same Adnectin in many different buffer pH conditions. The experiment was done in 384-well format and demonstrates the ability to rapidly screen buffer conditions that may influence the thermal stability of the drug candidate.

protein drug candidates is the temperature at which half the protein is unfolded, also called the midpoint temperature and denoted by Tm [10]. Generally speaking, a higher Tm is preferred, as it implies the conformational stability is higher. All other parameters being equal, one would prefer to progress drug candidates that have higher thermal stability, with the anticipation that they may be easier to produce, handle, and store. However, it is also important to recognize that the Tm by itself does not always predict shelf life or manufacturability of a protein therapeutic. In some cases, aggregation can be initiated by the solubility limit of the natively folded protein or a chemically modified folded form of the protein [11].

### 1.4.3 Confirmation of Binding

One of the most important factors used to evaluate hit candidates is to determine whether or not they bind the target molecule, and if so, how tight the interaction is. Biosensor is a biophysical method often used at the hit evaluation stage because they can be run in higher throughput mode, while consuming very little of the hit molecules [12–14]. Biosensor is a workhorse technology for all phases of protein therapeutics drug discovery and more will be described about this technology later in this chapter and throughout the book. Because the purity values of the hit molecules may not be accurately understood, analysis of the association kinetics is difficult to interpret quantitatively (the association kinetics are dependent on an accurate knowledge of the active concentration of reactant in solution phase which is usually the drug candidate). Instead, the main goal for biosensor work at the hit evaluation stage is to confirm the hit molecules bind to the target. This would normally be done at concentrations of reactants high enough to allow detection of binders that have an acceptable affinity, but low enough to reduce potential nonspecific interactions with the surface. For example, the hits could be tested at a single concentration of 1 μM to discern if they bind with equilibrium dissociation constants of at least approximately 1 μM. If binding is not detected at 1 μM, then the hit molecule either does not bind the target or its affinity is much weaker than 1 μM and perhaps of little interest as a lead molecule. The rate of a hit dissociating from the target may also provide useful information for comparing between hits. Hits having unusually long dissociation rates likely indicate they are binding either with higher affinity or by distinct binding modes compared to hits with much faster dissociation rates.

## 1.5 LEAD SELECTION

The next stage of discovery is the selection of lead families of candidates for optimization and progression into the later stages of discovery. The decisions about which candidate molecules to advance have long-term consequences for the success and challenges that will be encountered by the program, including whether the binding epitopes are able to elicit suitable biological efficacy from the target and whether there are any chemical or physical liabilities associated with the lead candidate or family. Ideally one would like to select multiple lead families that bind to a diversity of epitopes, to maximize likelihood of favorable biological activity, and have favorable biophysical properties, to

increase the chances of ultimately producing candidates that have superior stability and manufacturability attributes.

The biophysical properties that are used as part of the selection criteria include self-association, conformational stability, binding affinity, and binding epitope. In order to measure these biophysical properties rigorously, it is necessary to produce the potential lead molecules at the milligram scale and to purify them to a higher purity standard (e.g., sample is at least 95% molecule of interest). The biophysical methods themselves are also more rigorous at this stage. Prior to biophysical analysis the candidates usually undergo an evaluation of purity and identity by SDS PAGE and LC/MS. SDS PAGE and the LC part of LC/MS provide information about purity, and the mass spectrometry data provide mass information of sufficient accuracy to confirm the identity of the protein candidate to its expected amino acid sequence.

### 1.5.1 Self-Association

Prior to selecting a lead candidate it is important to obtain an accurate understanding of the self-association properties in a standard biological buffer system such as phosphate buffered saline (PBS) or histidine buffer. Ideally, one would like to evaluate the self-association properties in more than one buffer in order to minimize the risk of buffer-specific anomalous behavior. The analytical SEC assessment done at the hit identification stage provides a qualitative measure of self-assessment but can sometimes be obscured by interactions with the column matrix or non-candidate impurities [8]. These obstacles can be overcome to a large extent by coupling the SEC method with multiple angle light scattering (SEC/MALS) [15]. The MALS detector system allows one to measure the absolute mass of the protein sample across the elution peak(s), irrespective of elution time. Figure 1.4 shows SEC/MALS data for an Adnectin lead molecule. In this case the protein elutes with a homogeneous profile as measured by absorbance at 280 nm. The dotted curve drawn across the elution peak represents the weight-average MW of the sample measured at many individual time points during peak elution. The average value of the measurements across the main peak is 11 kDa and is within error equal to the mass of a homogeneous monomer of the protein (theoretical mass = 10.9 kDa). At time points earlier than the main peak elution, the light-scattering signal detects MW species for very small amounts (1% or less) of HMW material that are of a size approximately that of a dimer.

Another method for rigorous analysis of protein self-association behavior is sedimentation equilibrium analytical ultracentrifugation (SE-AUC). Like SEC/MALS, SE-AUC is a method that measures the absolute mass of the protein sample [16]. It is significantly more time consuming than SEC/MALS but has the advantage that it can measure self-association equilibrium constants for simple equilibrium systems such as monomer–dimer equilibria. This is an important advantage for lead molecules against targets that are influenced by dimerization. In such cases, the dimerization constants for a series of lead molecules can be used as a criterion for selection of progressible candidates and provide insight into the final format of the drug molecule (e.g., monomeric or dimeric). Figure 1.4c shows AUC data for the same lead Adnectin in Figures 1.4a and 1.4b. The data are from a sedimentation equilibrium experiment and provide strong confirmation that the protein is a homogeneous monomer over the

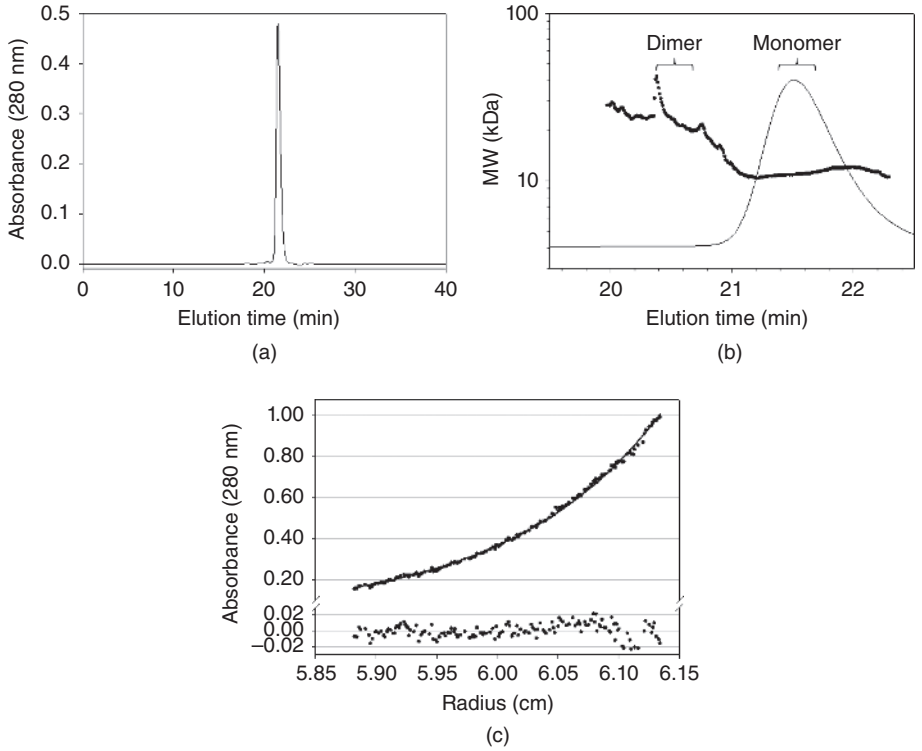


Figure 1.4. Self-association analysis of an Adnectin candidate as determined by size-exclusion chromatography combined with multiple angle light scattering shown in (a) and (b) and analytical ultracentrifugation shown in (c). The Adnectin eluted from a size-exclusion column (a) with a major peak (99% of 280 nm signal) at 21.6 min and a minor peak (1%) at 20.5 min. From light-scattering data collected during the run, the MW versus elution time plot (b) shows that the main peak eluted with a MW consistent with monomeric protein, and the shoulder likely contained dimer. (c) shows the sedimentation equilibrium analysis of the absolute mass of the Adnectin as measured by analytical ultracentrifugation. The best-fit curve shown is the one representative from a set of multiple centrifugation speeds fit globally to a single mass species. The best-fit from the global analysis yielded a mass of 10.3 kDa. This agrees well with the theoretical mass of 10.9 kDa.

concentration range shown (A280 from about 0.1 up to 1) based on the goodness of fit to the single-exponential curve-fitting analysis.

### 1.5.2 Thermal Stability

At the lead selection stage, the preferred method for measuring thermal stability of the lead candidates is by differential scanning calorimetry (DSC). DSC is the gold standard method for measuring thermal unfolding. It measures the excess heat capacity of the protein as temperature is scanned and directly monitors unfolding from the change in

heat for the reaction. One reason why DSC is the preferred method is that it is not susceptible to fluorescence or other optical artifacts that sometimes occur with TSF or other optical methods. Another reason is that DSC instrumentation offers high-precision and high-accuracy temperature control.

DSC is in principle a rigorous way to also measure the thermodynamics of the unfolding–folding equilibrium, including the free energy, enthalpy, and heat capacity changes. These parameters describe the conformational energy of the protein in detail. That said, DSC is oftentimes subject to artifacts such as scan-rate dependence of the unfolding curve, lack of unfolding–folding reversibility, or artifactual heats originating from side reactions such as aggregation. Thus, in practice, DSC data are mainly used in drug discovery as a semi-quantitative measure of stability. Even so, it is more direct than optical methods and can be controlled more precisely.

Figure 1.5 shows DSC data for two Adnectin candidates. The one shown in the lower part of the figure is superior in two ways. First, the unfolding begins at a higher temperature and the Tm is several degrees higher. This indicates the candidate has higher conformational (or folding) stability. Second, the unfolding reaction of the lower one is reversible. That is, after thermally unfolding, it can be cooled, refold in the calorimeter,

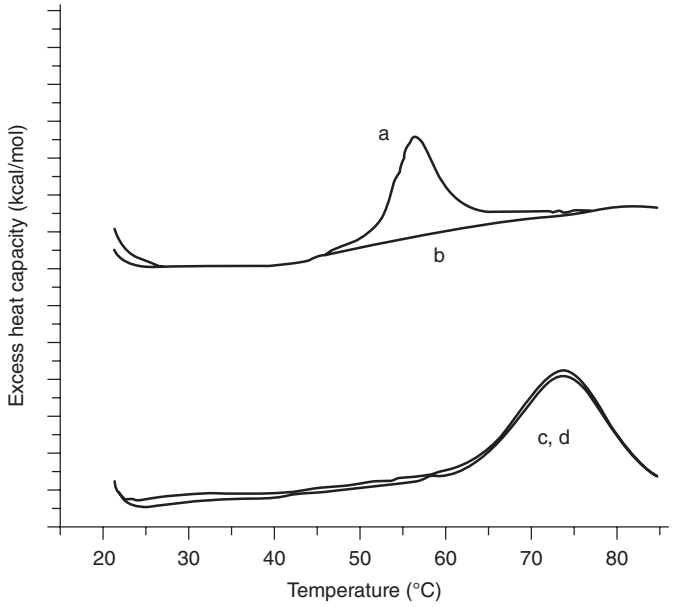


Figure 1.5. Differential scanning calorimetry (DSC) of two different Adnectin drug candidate molecules. The top two traces are indicative of a thermally irreversible protein system. The Adnectin in the top portion of the panel denatured in the first thermal scan (a) shows no evidence of regaining structural integrity in the time frame allotted for the second thermal scan (b). The lower half of the panel displays a different Adnectin molecule that displays essentially complete thermal reversibility under the conditions tested. The first thermal scan (c) and second scan (d) are observed to overlay, indicating the protein melted in the first scan has recovered structural integrity and behaves identically when thermally scanned a second time.

and be thermally unfolded again. The ability to unfold–fold reversibly is in some cases an indicator for improved expression levels [17] and reduced aggregation tendency [18]. This is presumably due to the ability of the refolding reaction to compete against aggregation side reactions of the unfolded form(s).

### 1.5.3 Binding Affinity, Kinetics, and Epitope

The primary objective of most protein therapeutic programs is to identify a molecule that binds to a preferred epitope on the target and with high affinity. A preferred epitope is one that yields one or more of the following outcomes: strong or partial antagonism of target function, strong or partial agonism, selective modulation of some target functions but not others, presence or absence of target degradation, cross-reactivity to the same epitope on the target from nonhuman species used in critical program assays, cross-reactivity to closely related human co-targets, lack of cross-reactivity to human liability targets, and so on.

Unfortunately, it is usually not possible to know if a hit molecule binds to a preferred epitope, and it is therefore necessary to select a diversity of lead candidates that bind to different epitopes for further studies and affinity optimization. A common method for selecting lead molecules that bind to distinct epitopes on a target is biosensor technology. To accomplish this, lead molecules are examined in pairs to determine if they can bind to the target molecule simultaneously or not. Ideally one should have a sound understanding of the binding affinities, kinetics, and concentration ranges used in such studies, as described by Yamniuk et al. [19]. Figure 1.6 depicts an example

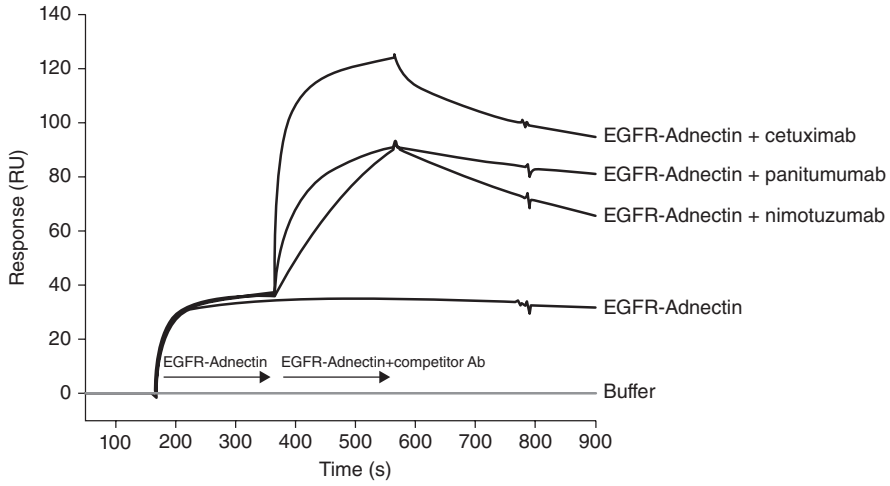


Figure 1.6. Biosensor data, measured on a Biacore T100 surface plasmon resonance instrument, showing an EGFR Adnectin does not compete with clinically approved mAbs for binding to EGFR. EGFR was immobilized by amine coupling and experiments were conducted as described elsewhere [2]. Briefly, the EGFR Adnectin was flowed over the EGFR surface alone at 450 nM, and then either alone at 450 nM or together with 450 nM mAb as shown. Reproduced with permission from Reference 2.

epitope discrimination study with a lead EGFR Adnectin in comparison to three anti-EGFR monoclonal antibodies. The studies were conducted using biosensor technology and demonstrate that the EGFR Adnectin binds to the target molecule EGFR at an epitope that is sterically distinct from EGFR antibodies cetuximab, panitumumab, and nimotuzumab. Details about these experiments are provided by Emanuel et al. [2].

At the lead selection stage, the protein candidates should be purified to high standards and in hundreds of microgram to milligram amounts. They are therefore suitable for enabling a more rigorous analysis of binding affinity and kinetics using biosensor technology. Generally speaking, the higher the affinity the more attractive the lead molecule is for advancing further into the later stages of discovery. The kinetics may also provide clues for discriminating between different modes of binding to target. Slower association kinetics may be due to rate-limiting conformational change in the target (or the drug candidate) and thus could reflect a novel mode of binding the target that could correspond to a novel biological outcome. Example biosensor data showing the association and dissociation curves for EGFR and IGF1R Adnectins binding their targets are discussed in Sections 1.6 and 1.8.

## 1.6 LEAD OPTIMIZATION

Once lead candidates have been selected they are optimized in terms of their binding affinity, cross-reactivity, potency, and biophysical stability attributes. Again, biophysical technologies play a central role in guiding the optimization to generate advanced lead candidates that have the desired biological activity and are likely to be manufacturable. The biophysical methods used at this stage are very similar to those used in the lead selection stage, but the extent of characterization is increased.

During lead optimization, assays are often performed that require the drug candidates to bind to the target from nonhuman species such as mouse or rat. In order to help validate these types of assays biosensor technology is often used to demonstrate that the leads bind to the nonhuman targets and that upon affinity optimization to the human target, the cross-reactivity toward the nonhuman targets is maintained (or not). For the EGFR part of the EGFR–IGF1R bispecific molecule, we produced extracellular fragments of EGFR from multiple species to verify with biosensor technology that the leads bind to EGFR from species relevant to preclinical efficacy and toxicology studies. Similarly, for the IGF1R part of the molecule, we also conducted biosensor studies to assess the nonhuman species cross-reactivity. Figure 1.7 shows biosensor data for binding the leads to human, monkey, mouse, and rat IGF1R. The results demonstrated very tight and nearly indistinguishable affinities for human and monkey IGF1R. Binding to rat and mouse was also observed, and in an affinity range acceptable for downstream studies involving those targets. This is a case study with a favorable outcome. However, not all programs are as fortunate. Some programs could require a parallel discovery effort to create a species-specific surrogate biologic in order to conduct critical studies needed to progress the program.

Structural biology methods such as X-ray crystallography and NMR are also important biophysical tools for lead optimization. Solving the three-dimensional structure of a

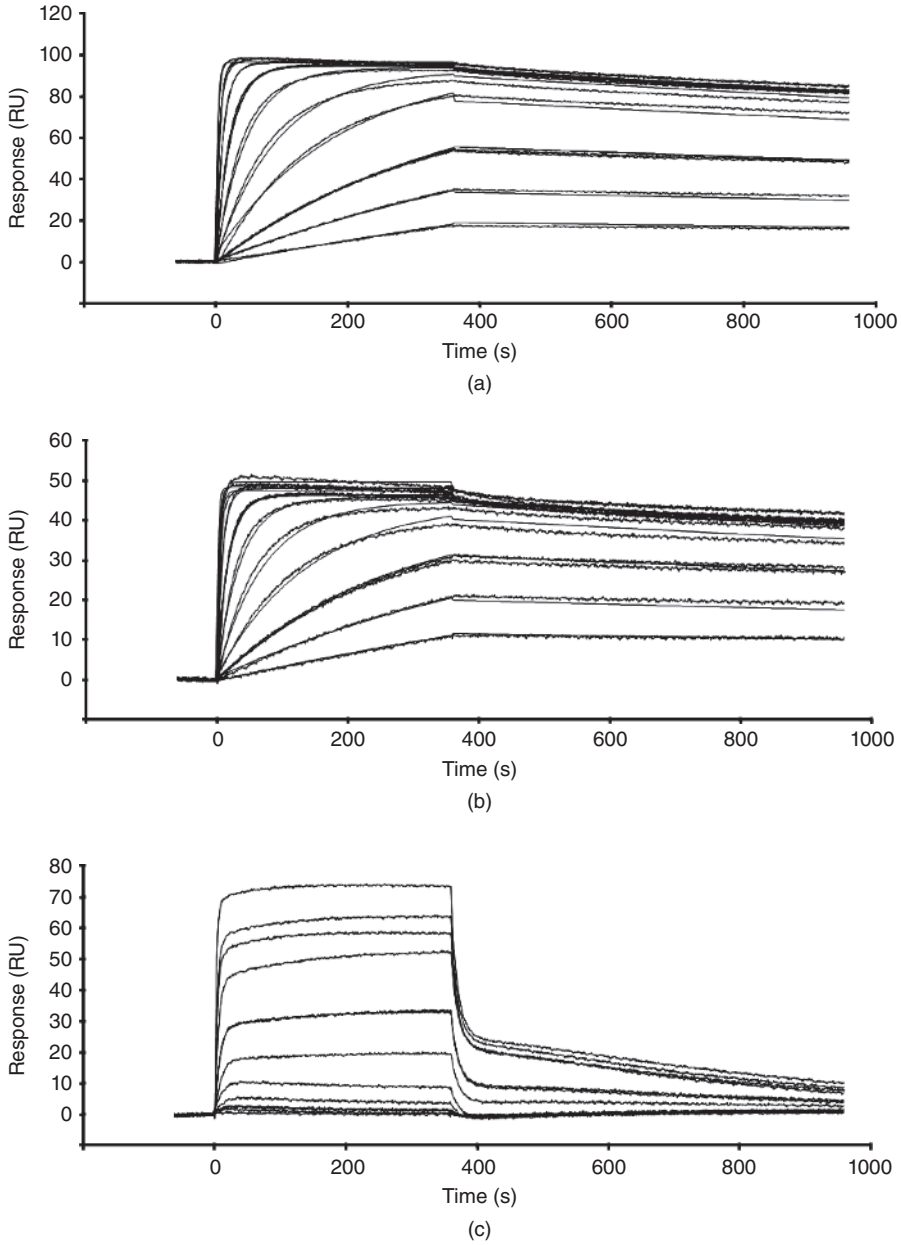


Figure 1.7. Biosensor data for anti-IGF1R Adnectin binding to IGF1R-Fc from different species: human (a), monkey (b), mouse (c and d) and rat (e and f) IGF1R. In each panel, the IGF1R-Fc target was captured on the surface by protein A and the Adnectin was flowed across the surface at multiple concentrations from 1 to 500 nM. The data for human and monkey IGF1R were well described by a simple Langmuir fitting model. In contrast, the kinetic data for mouse and rat (c, e) could not be well described with a simple model.

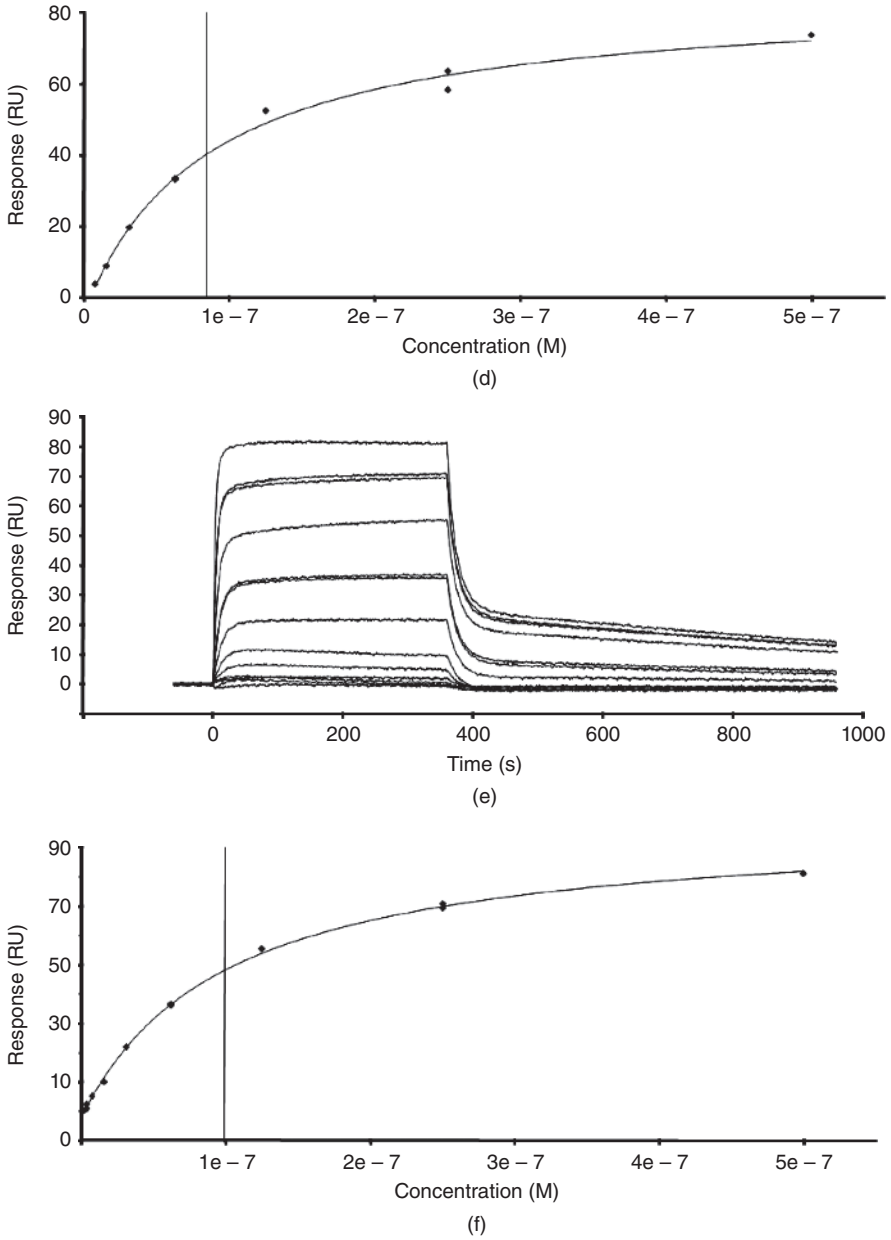


Figure 1.7. (*Continued*) Instead, the affinities for mouse and rat IGF1R were obtained from a steady-state equilibrium analysis (d, f) by fitting to a simple equilibrium model [20] based upon the amount of Adnectin bound versus total concentration of Adnectin in solution. Binding equilibrium dissociation constants for the four interactions were thus determined as 0.3 nM (a, human), 0.2 nM (b, monkey), 84 nM (d, mouse), and 99 nM (e, rat).

lead molecule in complex with its biological target provides an atomic-level understanding for designing improved binding affinity or cross-reactivity to species ortholog forms of the target or co-targets. An example where crystallography was used to determine the three-dimensional structure of an Adnectin lead bound to its biological target is reported by Ramamurthy et al. [21]. In their report the authors describe in molecular detail how the Adnectin lead molecule binds the target. The unique ways the Adnectin binds offer unexpected opportunities to optimize the lead molecule–target interaction.

## 1.7 LEAD FORMATTING

Most of the so-called next-generation antibody fragment and non-antibody fragment discovery programs offer novel ways to format the drug candidates to provide multivalent and/or multispecific target binding [22,23]. Many of these programs also include molecular formatting, such as fusion to the Fc fragment of an antibody or by covalent conjugation to large inert moieties such as PEG, to create extended pharmacokinetics. Any of these formatting modifications may alter the biophysical stability or target-binding ability of the lead molecules, and biophysical methods are required to determine which formats yield final candidates with acceptable stability and binding profiles. In the present case, mono-Adnectin leads against EGFR were formatted into single-chain bispecific leads by in-line fusion with a high-affinity, stable mono-Adnectin against IGF1R. Furthermore, in order to extend the pharmacokinetics of the candidates, they were conjugated to 40 kDa branched PEG. A full analysis of the biochemical, cellular, and biophysical properties of the component mono-Adnectins and the formatted versions is given by Emanuel et al. [2]. In some cases formatting by these or other mechanisms can decrease (or increase) binding affinity and/or change self-association, aggregation, solubility, or thermal stability of the lead. Biophysical analysis of formatted lead candidates is critical to guide optimization of the formatting itself (size or location of PEG, orientation and linkers of in-line fusions, etc.), and selection of the preferred combination of lead candidate and format. In developing a bispecific candidate, for example, the physiological mechanism might require that a single bispecific candidate molecule be able to bind simultaneously and with high affinity to two different targets. This would be tested at the lead optimization and formatting stages by biosensor or perhaps ITC or AUC studies.

### 1.7.1 Solubility

As the discovery program moves into the later stages of discovery, more emphasis is placed on selecting candidates that have a better chance of having cost-effective manufacturability. A critical part of manufacturability assessment is the determination of solubilities of the drug candidates. In some programs an estimate for the dosing amount and concentration is known at this stage of discovery, and in other programs the dosing information may not be well understood yet. In either case, selecting candidates with better solubility profiles will facilitate the downstream process development and formulation work.

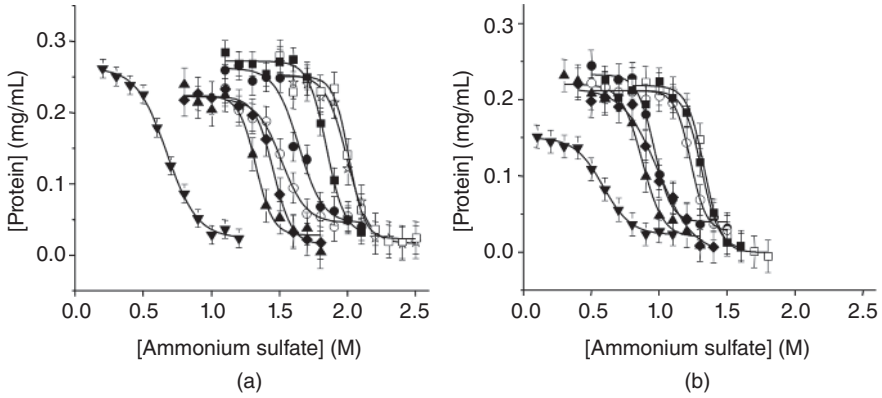


Figure 1.8. Determination of relative solubility values for seven EGFR mono-Adnectins and an anti-IGF1R Adnectin (a), and the same seven when formatted as a bispecific Adnectin with the single anti-IGF1R Adnectin (b). The IGF1R Adnectin in (a) is shown as stars and is the furthest curve to the right. Relative solubility values were measured by an adaptation of the ammonium sulfate precipitation method reported by Trevino et al. [24]. The data in this figure show the concentration of drug candidate remaining soluble as a function of molarity of ammonium sulfate. The higher the solubility of a protein, the more ammonium sulfate required to cause precipitation. Thus, the more left-shifted the curve, the less soluble the protein therapeutic candidate. The results provide a way to rank-order the solubilities of the drug candidate molecules.

Normally the dosing concentration is quite high for a typical protein, and reaching the high concentrations can be a substantial challenge for many programs, as described in the other chapters of this book. There are several commonly used methods for measuring protein solubility. Typically they require larger amounts of protein and can be time consuming to conduct. A method that has become popular recently for rapid assessment of the solubilities of several candidates at a time, and thus suitable for use during discovery, is PEG precipitation or ammonium sulfate salting-out studies [24, 25]. The method is conducted at conditions where the protein is in the native form, and the objective is to gain information about the solubility of the native form. We conducted ammonium sulfate salting-out studies on the EGFR mono-Adnectins to help select the candidates with the most favorable solubility behavior. The results are shown in Figure 1.8. Most of the candidates had similar solubility behavior, but one lead candidate in particular had much lower solubility. The rank-order of estimated solubilities obtained by this higher throughput method was in good agreement with solubility limits measured by centrifugal spin concentration (data not shown), although the most soluble molecules could not be fully characterized by the spin method due to a lack of sufficient amounts of the candidate molecules to reach their upper limit solubility values. We also tested the same EGFR candidates after formatting to the bispecific format using a common IGF1R mono-Adnectin with all of them. The rank-order of solubilities was in good agreement with the individual EGFR mono-Adnectins, indicating that for this system

the mono-Adnectin behavior was predictive of the behavior in the bispecific format. The same trends were also observed after PEGylation (not shown).

### 1.7.2 Thermal Unfolding Behavior

Although the solubility of the natively folded protein is an important property to understand, oftentimes aggregation of a drug candidate is related to the insolubility of partially or fully unfolded forms of the protein [11, 26]. Thus it is important to conduct careful thermal unfolding studies on late-stage protein therapeutic candidates. As discussed above in the Section 1.5, the DSC analysis should include an assessment of the onset temperature for unfolding (the temperature when unfolding can first be detected), the Tm or midpoint temperature for unfolding, and the reversibility of unfolding. In general higher unfolding temperatures and greater reversibility are desirable properties to select for, since the less exposure that a protein has for its unfolded form(s), the less likelihood for reacting along unfolding-based aggregation pathways.

## 1.8 FINAL DEVELOPMENT CANDIDATE SELECTION

In the final stage of discovery a candidate molecule is chosen to progress into development. At this stage, there is an increased emphasis on the use of biophysical methods to select lead candidates that will have favorable manufacturability. That is, how stable are the candidates? Can a process be developed to make the final candidate at larger manufacturing scale? Will the final candidate have a long shelf life? How concentrated can the candidate be made and remain stable? Answering these questions directly in the discovery stages is impractical due to the large number of candidates that must be made and characterized and the small scale of protein production typically done in discovery. However, there are biophysical approaches that allow for the selection of a final candidate based on its broad biophysical behavior in many conditions and by multiple methodologies. The assumption made is that a candidate that exhibits superior biophysical properties under a broad range of conditions will stand the greatest chance for performing well in development.

To address these issues most of the biophysical methods in Table 1.1 are needed. The number of biophysical methods used during final candidate selection is expanded to increase confidence in understanding the biophysical properties of each of the candidates. For example, to determine the binding affinity with high confidence, orthogonal methods such as ITC [27] or KinExA [28] are used. These methods measure affinity in solution and avoid potential immobilization-dependent or complex kinetics artifacts that are sometimes present in biosensor studies. Similarly, a thorough understanding of the aggregation properties of a protein requires multiple methods [29].

Additionally, the number of solution conditions studied must also be expanded to understand the generality of the drug candidate's stability profile. Studies at this stage include broad screening of each candidate's thermal stability against pH, salt, and excipients. Figure 1.3b shows the Tm value obtained by TSF over a wide range of pH. The results show that the Adnectin candidate is quite stable from low pH up to around

pH 8, but exhibits a drop in thermal stability at pH values above pH 8. Knowledge about specific conditions that stabilize or destabilize a candidate is useful for production and handling during discovery phases and also serves to inform the design of production and formulation work done later in development. These data provide a rapid and insightful view of the general stability behavior [30] of the protein candidate and can identify conditions or excipients that may facilitate or obstruct the manufacture or storage of the candidate. This type of data is useful for selecting the final candidate for development and it also provides the development teams with fundamental thermal stability information that can be used to jump-start formulation and process development.

In order to rapidly assess the chemical and physical liabilities of the candidates, accelerated degradation studies are conducted. The candidates are thus exposed to conditions of pH, temperature, protein candidate concentration, time, and so on that challenge a protein's stability to reveal any degradation reactions that may exist [31, 32]. Many of the biophysical methods listed in Table 1.1 are used as part of the accelerated studies, and several others are used, such as ion exchange chromatography or hydrophobic interaction chromatography, to investigate for the production of charge or conformational species.

## 1.9 CONCLUDING REMARKS

The discovery phase of a protein therapeutic is a protein chemistry-intensive environment that includes the production of a large number of drug candidates and a large number of diverse protein reagents. Biophysical methods are necessary to ensure the fidelity of the drug discovery process. Protein reagents that are made in discovery and relied upon for critical assays must be characterized to ensure that they are properly folded and active. Biophysical methods also play a prominent role in characterizing the lead candidates and for selecting well-behaved leads for progression through the later stages of discovery. Finally, biophysical methods play a central role in the selection of biotherapeutics that will be cost-effective to manufacture and safe as drugs.

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