

Chapter 2

High-Throughput Biophysical Approaches to Therapeutic Protein Development

Feng He, Vladimir I. Razinkov, C. Russell Middaugh, and Gerald W. Becker

2.1 Introduction

Protein therapeutic products typically experience many development cycles, in which decisions are empirically derived concerning the identity, manufacturing process, final product presentation, and administration methods. To obtain a quality therapeutic product, significant resources are spent on the search for appropriate development parameters throughout a product's life cycle. Due to the length and complexity of such development in the pharmaceutical industry, any process that can reduce the amount of time and resources while still reaching acceptable outcomes is highly preferred. One prominent area of such improvement is to increase the throughput capability of existing technologies. The focus of this chapter is on providing an introduction to the biophysical methods and techniques possessing high-throughput capabilities. We will especially focus on those techniques that are frequently used in the development of protein therapeutics. Though the definition of high throughput can be quite broad, the technologies and methodologies highlighted in this chapter all possess multi-sample throughput capability and/or automation-enabled measurements and analysis.

During the development cycle of protein therapeutics, it is critical to understand the physical integrity and other structural characteristics of the product. In the early stages of product development, preferred physical properties are often used as

F. He (✉) • V.I. Razinkov • G.W. Becker
Drug Product Development, Amgen Inc., 1201 Amgen Court West,
Seattle, WA 98119, USA
e-mail: fhe@amgen.com; razinkov@amgen.com; gerald@amgen.com

C.R. Middaugh
Department of Pharmaceutical Chemistry, University of Kansas,
2030 Becker Dr., Lawrence, KS 66047, USA
e-mail: middaugh@ku.edu

criteria to select protein candidates (described in Chap. 6). The selection philosophy can be based on an established relationship between a particular biophysical property and product quality, or simply follow the general assumption that better biophysical characteristics can lead to more stable products if other methods cannot differentiate the drug candidates. Typically, early stage development is conducted at small scale employing *in vivo* and *in vitro* systems because protein availability is limited. Thus, methods that consume less material but generate useful information are the most attractive. Once a protein drug candidate is selected, development is primarily focused on optimizing the parameters that effectively enable the manufacturing, packaging, storage, and delivery of the final product. Before these parameters can be finalized for commercial processes, however, numerous analyses need to be performed to define the space and limitations of physical conditions that best fit the product. High-throughput biophysical tools often play an important role in these efforts by providing a faster readout when the protein product is subjected to a range of experimental conditions.

Two areas during protein therapeutic development frequently employing high-throughput biophysical analyses are downstream and formulation development. The goal of downstream development is to find the most suitable purification process so that it can be properly scaled to deliver commercial product. Although the final optimization usually takes place at scales similar to the commercial settings, small-scale and high-throughput approaches are often utilized to predict protein behavior. For example, multiwell plate-based chromatographic techniques combined with high-throughput liquid handling instruments can provide a tremendous amount of information on protein–resin interaction and therefore help guide purification design with very affordable material input (Coffman et al. 2008). Although the ability of a process to perform adequately at a large scale remains to be derived empirically, small-scale development is critical because it provides opportunities to test the potential manufacturing steps while varying physical parameters. In fact, many regulatory authorities, when evaluating new drug product applications, require such small-scale data that can demonstrate the robustness of the processes as well as the edges of failure. Besides purification, the formulation development of protein therapeutics also requires a large number of experimental trials to select suitable conditions for final product presentation. The general aim is to find a formulation that will best retain the physical, chemical, and biological properties of the product while meeting the desired shelf life requirements. More recently, patient convenience and comfort have become major factors that influence formulation development. For instance, product formulations compatible with self-administration and above-freezing storage are preferred when treating chronic diseases. To derive suitable formulations, large arrays of factors are typically screened during the development phase for their ability to stabilize the drug product. Since it is well established that physical instability may negatively impact protein therapeutics, biophysical techniques offer relevant tools to monitor protein conformation in response to formulation and storage conditions. An important area of focus during formulation development is the evaluation of protein aggregation propensity. Protein aggregates are thought to often impose detrimental effects on the therapeutic potency and side

effect profile of the drug which may even lead to significant clinical safety concerns (Jiskoot et al. 2012; Rosenberg 2006). As a result, protein aggregation is frequently used to differentiate formulation candidates. Over the last decade, it has been well documented in the literature that high-throughput biophysical methods are very capable of detecting the presence of protein aggregates over a wide size range (Mach and Arvinte 2011). The feasibility and performance of a protein therapeutic product can also be dependent on other key properties of the molecule. An emerging example is protein viscosity. To reach the desired bioavailability, protein drugs delivered via the subcutaneous route typically require high concentrations. This often leads to high solution viscosity that may cause significant difficulties during product manufacturing and administration of the protein (Shire et al. 2004). Traditional analytical methods have limitations in material consumption and throughput (Jezek et al. 2011), but newly developed biophysical techniques offer significant advantages, including a reduction of sample volume as well as increases in throughput capability (He et al. 2010a; Wagner et al. 2012).

In the following sections, the role of high-throughput biophysical analysis in the development of protein therapeutics is discussed. Technical background for selected biophysical methods is reviewed as well as their high-throughput utility. In addition, a general introduction to empirical phase diagram (EPD) is presented as an example of the application of high-throughput biophysical characterization and data interpretation in the development of protein therapeutics.

2.2 High-Throughput Biophysical Techniques That Can Be Applied to Protein Therapeutic Development

Due to increasing demands in sensitivity, diverse sample applicability and throughput, biophysical instrumentation has been transformed significantly over the past decade to implement multiwell measurements and automation modules. Such advancement has accelerated the research and development of protein-based therapeutic entities by decreasing the resource requirement and widening the experimental design space. In this section, a brief introduction is provided, concerning the background and utility of selected high-throughput techniques.

2.2.1 Surface Plasmon Resonance

Characterization of target binding is an essential component during the development of protein therapeutics. Since the majority of therapeutic targets bind to specific targets, the ability of protein therapeutics to interact with their respective sites of interaction is often used to differentiate among drug candidates. Many high-throughput biophysical tools are available to characterize the binding constant, K_d , and stoichiometry, (n), between protein product and therapeutic targets.

Protein–protein interactions can be characterized with the help of surface plasmon resonance (SPR) (Rich and Myszka 2000). This method is based on the measurement of changes in refractive index near a sensor surface caused by the binding or dissociation of a protein with its target (receptor). SPR instruments evaluate protein binding in real time without labeling, but surface immobilization of at least one component is required. SPR methods can be both qualitative and quantitative (Karlsson 2004). In the standard setup, measurements are performed sequentially with two flow cells: one that holds the sample and the second a reference solution. Recent microfluidic and automation technologies have created new opportunities for development of high-throughput instrumentation based on the SPR principle. The Biacore A100 biosensor can process multiple samples (1,000 samples/day) and allows high-quality data sampling. This instrument has been used to develop an in vitro high-throughput kinase assay (Takeda et al. 2006). Another SPR instrument, the Biacore Flexchip microarray device, has been used for rapid identification of high-affinity human antibodies from a phage display screen. Fab fragment analysis with surface plasmon resonance microarrays in a high-throughput format permits the determination of kinetic constants for 96 different Fab fragments in a single experiment (Wassaf et al. 2006). High-throughput antibody affinity characterization accelerates early discovery of lead candidates. The ProteOn™ XPR36 multiplexed SPR instrument from Bio-Rad Laboratories (Hercules, CA) (Bravman et al. 2006) employs microfluidics integrated into 6×6 interaction array. The ProteOn™ XPR36 has also been used for determination of antibody affinity (Bravman et al. 2006). Plexera® Bioscience (Woodinville, WA) offers a PlexArray™ HT instrument which can test thousands of protein interactions in only 30 min. This system is based on a high-density array with the capacity to evaluate more than 1,000 spots with a spot size as small as 100 μm. Microarray technology has been widely applied in SPR-based settings to miniaturize spot size and sample volume to increase throughput (Otsuki and Ishikawa 2010). A wide variety of other instruments based on SPR and related phenomena are available as well.

2.2.2 Liquid Chromatography

Liquid chromatography (LC) is perhaps the most heavily used tool in the field of biotechnology. LC methods are the main components for the purification and analysis of protein therapeutics during their development cycle (Ahrer and Jungbauer 2006; Andrew and Titus 2001). Understandably, most LC technologies have adopted the high-throughput scheme by automating and streamlining the sampling mechanism. The principle of protein LC is based on the nature of protein–resin interactions in a given liquid mobile phase. An initial major step towards high-throughput LC technology is the implementation of high-performance liquid chromatography (HPLC) (Swadesh 2001). This technique uses a fast flow rate under high enclosed pressure, instead of gravity force, to drive the mobile phase through

the column. This permits faster sampling throughput and improves the resolution of sample partition. A recent advancement has been categorized as ultra-performance liquid chromatography (UPLC) where smaller diameter particles and subsequently higher pressures enable even faster separation times (Xiang et al. 2006). UPLC technology has clearly shown its advantage in reversed phase (RP) chromatography and has become the primary separation method for mass spectrometry analysis (Stackhouse et al. 2011; Szapacs et al. 2010).

Another application of high-throughput LC technology is the development of protein purification methods. The goal is to obtain the largest amount of the therapeutic protein in its active form while reaching the highest purity possible. The range of such methods is currently extremely wide, and the specific purification steps often need to be derived empirically. Primary methods include affinity, size-exclusion, ion-exchange, and hydrophobic interaction chromatographies. Multiwell plates or miniaturized columns are frequently used to screen a large number of parameters including type of resin, protein loading and elution conditions, as well as efficiency for the product of interest (Fahrner et al. 2001). The quantity of column resin and protein material needed is often measured in microliters, and the experimental steps are simply executed by gravity, centrifugal force, or a pump. This type of high-throughput approach offers an opportunity to evaluate as many variables as possible early in development with a minimum of time and protein. The outcome of the screening can often help to select product-specific processes for further development and may even allow companies to bypass intellectual property restricted common practices. The latest improvement in this field highlights the use of automated liquid handling systems coupled to positive displacement liquid transfer technology (Susanto et al. 2008; Wiendahl et al. 2008). Such a system has greater similarity to large-scale chromatographic instruments used in manufacturing by generating a pressurized liquid flow through the microcolumns. The results obtained using such high-throughput techniques are believed to be more representative of a protein's behavior during large batch purification.

2.2.3 *Light Absorption Spectroscopy*

Protein concentration measurements are essential during purification and formulation development, and high-throughput compatible UV spectroscopy is a well-utilized tool (Zhao et al. 2010). Besides protein concentration measurements, second derivative UV spectroscopy has been frequently used to characterize protein tertiary structure (Kuelto et al. 2003; Mach and Middaugh 2011). Many absorption spectrometers are compatible with a multiwell plate format and/or automation modules. Typically, light passes through the plate vertically while the light source and detector move from sample to sample. Alternatively, the plate itself can be moved. Since the signal from proteins is typically strong, UV detection and absorbance integration are generally quick, allowing fast analysis of a large number of samples. The disadvantages of this technique are also very well understood.

The path length of the vertical light absorbance is poorly controlled and highly dependent on the amount of sample present in the well. In addition, proteins with high extinction coefficients, such as monoclonal antibodies, can easily saturate the light detector. New adjustable path length spectrophotometers have recently become available, although their throughput is not as high and light scattering frequently complicates the use of this method.

Compared to UV absorbance, high-throughput optical density (OD), or turbidity, assessment is more widely applied to the protein therapeutic development. Turbidity usually refers to the obscuration of a sample at wavelengths near the visible light range where proteins in solution do not manifest significant absorbance. The most widely employed wavelength range for this purpose is 350–400 nm, which avoids any specific absorbance arising from amino acid side chains or common color pigments. Turbidity is proportional to the amount of light blocked or scattered by the solution components. In a protein sample, aggregates and precipitates are known to give rise to solution turbidity as measured by OD, making it a quick method to assess the quality of protein samples with respect to the presence of protein aggregates. High-throughput turbidity assessment is frequently used during protein formulation development to evaluate a large number of samples that are put on storage or under environmental stresses (Zhao et al. 2010; Capelle et al. 2009). Though quantitative determination of protein degradation is not usually possible with turbidity measurements, the information obtained is sufficient to discriminate or rank order formulations. Turbidity assessment is generally noninvasive and can be applied using a variety of spectroscopic instrumentation and sample cells, including microtiter plate readers and pharmaceutically relevant containers. The latter provide a unique opportunity to assess sample quality of protein therapeutics in their actual storage units, and permit a real-time monitoring of aggregation during the manufacturing and distribution of a protein commercial product.

Another common tool for protein analysis is circular dichroism (CD). CD is a technique that measures the difference in absorbance of left- and right-handed circularly polarized light. CD is generally divided into near-UV (250–350 nm) and far-UV (190–250 nm) measurements (Li et al. 2011). Near-UV CD is sensitive to the tertiary structure of proteins, due to the presence of optically active chromophores including the aromatic amino acid side chains and disulfide bonds. Far-UV CD, on the other hand, is used to study the secondary structure of proteins. Alpha helix, beta sheet, turns, and disordered structure all display unique CD spectra. Expanding the high-throughput capabilities of CD instruments is achieved either by increasing the number of cuvettes that an instrument can employ or the use of autosamplers. In addition, it has recently become possible to obtain data from the near- and far-UV regions in a single scan. CD is typically more time-consuming than other light absorbance measurements and can be significantly affected by both light scattering and absorption flattening phenomena. In order to minimize the interference, high-concentration protein samples are often measured via the use of short path length (μm range) cells (Harn et al. 2007).

2.2.4 *Vibrational Spectroscopy*

Vibrational spectroscopy includes Raman, Fourier transform infrared (FTIR), and near-infrared (NIR) spectroscopy. With respect to protein therapeutics development, various Raman techniques and FTIR are commonly used to analyze protein secondary structure, while NIR is often applied to the analysis of the sample components other than protein, such as organic compounds (Siebert and Hildebrandt 2008). Because most chemical materials have identifiable spectral patterns, these techniques are also frequently used for raw material and forensics analysis (described in Chap. 10). Another common advantage of these techniques with protein therapeutics development is that they can be used to analyze lyophilized protein samples directly, a task at which many other biophysical techniques fail. Near-IR instrumentation has successfully incorporated an automated sample presentation unit that permits higher throughput analysis. The high-throughput development of FTIR, however, is challenging due to geometrical problems. The only currently available high-throughput option for FTIR involves the drying of liquid samples, which might produce structural changes in the protein. Novel solutions to this problem are known to be under investigation. In contrast, incorporation of multiwell plate and microarray technologies instrumentation has been demonstrated with Raman spectroscopy (Anquetil et al. 2003). The advantage of Raman-based technologies is that they are typically compatible with a wide range of transparent container types and suffer less from interference by water. The major disadvantage of Raman spectroscopy is that signals from proteins are normally weak. Resonance and surface-enhanced Raman technologies can partially overcome this problem but suffer from limited applicability for a variety of reasons. In summary, vibrational spectroscopic techniques possess unique abilities to provide quality and conformational information on protein products, including lyophilized samples. High-throughput applications of these techniques, however, clearly require further development.

2.2.5 *Fluorescence Spectroscopy*

The aromatic side chains in proteins serve as fluorophores which emit photons at higher wavelengths once excited with UV range light. The excitation and emission profile is highly dependent on the polarity of the side chain environment and, therefore, is sensitive to the local tertiary structure of the protein (Lakowicz 2006). This fluorescent property is referred to as protein intrinsic fluorescence. Since most large proteins have widely distributed aromatic amino acids, the overall intrinsic fluorescence provides a good measure of protein folding. Upon conformational changes, the intrinsic fluorescence emission peak generally shifts in wavelength. If present, tryptophan (Trp) emission dominates the fluorescence of proteins with tyrosine (Tyr) contributing indirectly. This method is often applied to protein formulation studies to detect changes in protein conformation as a result of stresses such as temperature, pH, and solute.

The high-throughput capability of fluorescence techniques also typically relies on a multi-sample cuvette holder, a multiwell plate compartment, or an autosampler. Generally, fluorescence intensity is detected at a 90° angle to the light source to minimize scattering. In the case of multiwell plates, fluorescence signal is usually acquired via a top or bottom reading method. Because of the noninvasive nature of the intrinsic fluorescence measurements, it can be applied to analyze protein conformation in samples pulled from long-term studies. The utility of intrinsic fluorescence can be extended by fluorescence lifetime and anisotropy measurements, through which further information concerning protein conformation is obtained (Fowler et al. 2002; Owicki 2000). Use of front surface geometry permits the fluorescence from highly scattering samples to also be obtained. Recently, a high-throughput, microtiter plate-based fluorometer (the Avacta Optim 1000) has been developed, which measures fluorescence spectra and light scattering simultaneously. This instrument is capable of performing rapid thermal melts with a resultant overall increase in throughput of better than a factor of 10.

Useful fluorescence signals can also be obtained from extrinsic fluorescent probes such as small molecule dyes. There is a wide variety of fluorescent dyes commercially available for protein research and development (Hawe et al. 2008a). Different dyes can be employed to reveal different properties of protein solutions. For example, hydrophobic dyes are typically used to probe the presence of apolar sites on a protein. Anilinonaphthalene sulfate (ANS)-based dyes are the most common hydrophobic probe choices. An increase in surface hydrophobicity is believed to be an indication of protein unfolding. This usually results in enhancement of fluorescence intensity by the associated dye. Another widely used dye is SYPRO Orange, a probe originally developed as a gel stain. Recently, SYPRO Orange has been used to detect protein aggregation in monoclonal antibody product samples and has been shown to display specificity to structurally perturbed protein aggregates (He et al. 2010b; Mach et al. 2011). Similarly, a number of other dyes have been reported to be sensitive to the aggregated protein species (Hawe et al. 2010a; Hawe et al. 2010b). SYPRO Orange has also been employed to study protein unfolding (described in Sect. 2.7). Other extrinsic probes have been employed to measure solution viscosity (Schäfer and Schmidt 2006; Haidekker et al. 2005; Kung and Reed 1989). Though the application of these viscosity-sensitive dyes has been extensively demonstrated in biologically relevant samples, their utility in probing therapeutic protein viscosity remains to be fully tested. One major concern is that these dyes can interact with proteins in high-viscosity samples, which typically correlates with high protein concentration (Hawe et al. 2010a). In addition to probing the protein itself, other extrinsic fluorescent tools are available to study formulation buffers. For instance, 1-N-phenylnaphthylamine (NPN) and 9-anthryldiazomethane (ADAM) have been shown to detect polysorbate, a common surfactant used in protein formulation, and its degraded form in solution (Khossravi et al. 2002). Fluorescence spectroscopy is also compatible with HPLC instrumentation as an on-line detection method to monitor elution (Hawe et al. 2008b), offering yet another high-throughput analytical option. The combination of chromatography and fluorescence is particularly attractive, since real-time information can be obtained on specific protein species as they fractionate and elute from the column.

2.2.6 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is another sensitive method to evaluate interactions in solution (Torres et al. 2010). Understanding how proteins interact with other solution components, such as surfactants and excipients, could be informative and beneficial for the development of protein therapeutics. Unlike SPR, ITC does not require immobilization or chemical modifications of the interacting compounds (Pierce et al. 1999). This enables ITC to be applied to more types of interactions that occur with proteins. In its standard format, this technique requires a significant amount of protein and the experiment includes long-duration titrations. The high-throughput development of ITC has been aimed at reducing the sample consumption as well as improving the measurement speed. Recently ITC instruments with reduced sample volumes have become commercially available, including iTC200 and Auto-iTC200 calorimeters from MicroCal (GE Healthcare, Waukesha, WI), as well as Nano ITC instrument (TA Instruments, New Castle, DE) which offers higher sensitivity and titration speed and employs a significantly smaller sample cell compared to the two-cell MicroCal VP-ITC instrument (Peters et al. 2009). The Auto-iTC200 can run up to 384 titrations automatically through the use of a temperature-controlled autosampler. A miniaturized ITC microcalorimeter can reduce the protein requirement up to seven times (Verhaegen et al. 2000). Forty-eight samples in each array can be measured with a reaction volume of 10–20 μ L. In addition, closed-chamber microfluidic calorimeters with thermopile heat sensors have also been described in the literature (Lee et al. 2009). These calorimeters achieve enhanced sensitivity by surrounding the measurement chamber with a vacuum. Flow microfluidic devices can use as little as 10–20 μ L of sample volume (Lerchner et al. 2008). In addition to the single-chamber settings, further advancements utilizing array technologies might be possible.

2.2.7 Differential Scanning Calorimetry/Differential Scanning Fluorometry

Stability of protein therapeutics under thermal stress is believed to be critical and often assessed during development (Bruylants et al. 2005). A low temperature of unfolding can lead not only to protein instability but also a decreased energy barrier for unfolding events caused by protein interactions such as protein–surface (Chang et al. 1996), protein–solvent, (Schiffer and Dotsch 1996) or other interactions. Screening approaches focused on protein thermostability can reveal undesired protein therapeutic candidates or manufacturing and storage conditions early during the development. Differential scanning calorimetry (DSC) is a quantitative method widely used for determination of protein thermal stability (Privalov and Privalov 2000). However, a low-throughput DSC takes a significant amount of sample and time to complete a typical experiment. Automated autosampling instruments such

as the VP-Capillary DSC Platform from GE Healthcare (Piscataway, NJ), the PYRIS Diamond™ DSC Autosampler from PerkinElmer (Waltham, MA), or the Nano DSC Autosampler System™ from TA Instruments (New Castle, DE) significantly improve sample preparation, but analysis time is still lengthy and the significant amounts of protein required make analysis difficult for the early screening of hundreds of drug candidates and formulation excipients. Improvements in microfluidic technology, creation of array-based calorimetry microchips, and the use of microplates will enable higher-throughput calorimetry (Vermeir et al. 2007; Lerchner et al. 2006). Microelectromechanical system (MEMS)-based calorimetry requires only 1.2 μL of solution, and the sensitivity is about 5 mg/mL of protein concentration (Wang et al. 2008). Since the dimensions of this device are approximately 5×5 mm, there are opportunities for microarray fabrication.

DSC can detect the unfolding transitions of several domains in a multidomain protein such as an immunoglobulin. It is, however, often sufficient to screen the thermal stability of proteins based only on their lowest melting temperature. Single-domain proteins can, of course, also be characterized by determination of their single transition. In cases such as this, a high-throughput method, known as differential scanning fluorimetry (DSF), based on the extrinsic fluorescence of probes sensitive to the polarity of their environment can be used (Pantoliano et al. 2001a). Unfolding of proteins usually exposes hydrophobic regions to the solution resulting in a significant increase in the fluorescence of these probes when bound. The method was originally used for screening of small molecule interactions with proteins and can therefore be used to identify some types of excipients (Pantoliano et al. 2001b). The unfolding temperature of a protein shifts after binding of a ligand and binding parameters can be determined by this shift in the melting temperature. In other studies this method has been applied to the evaluation of crystallization and general stability (Malawski et al. 2006; Ericsson et al. 2006). Because of low background fluorescence in the presence of native antibodies, DSF has been successfully used for mAb formulation development (He et al. 2010c). Examples of DSF scans obtained during formulation screening for a therapeutic protein are shown in Fig. 2.1. A 96-well plate with different formulations was screened to determine the most thermally stable formulation. The midpoint of the transition determined by the increase in fluorescence intensity was found to correlate well with the unfolding transition measured by DSC. One significant disadvantage of DSF in applications for protein formulation development is the high-fluorescence background in the presence of detergents commonly used in protein formulations (see high-fluorescence background in Fig. 2.1). New probes, such as the thiol-specific fluorochrome N-[4-(7-diethylamino-4-methyl-3-coumarinyl) phenyl] maleimide (CPM), seem to overcome this detergent problem and have been used for stability profiling of membrane proteins under different solution and ligand conditions (Alexandrov et al. 2008). Fluorescent plate readers with a thermostat are necessary to make these measurements. The maximal temperature of a typical scan is high and standard plate-based fluorometers usually cannot provide the necessary temperature range. Instead real-time polymerase chain reaction (RT-PCR) instruments equipped with 96, 384 and even 1024-well plates are well suited for DSF measurements.

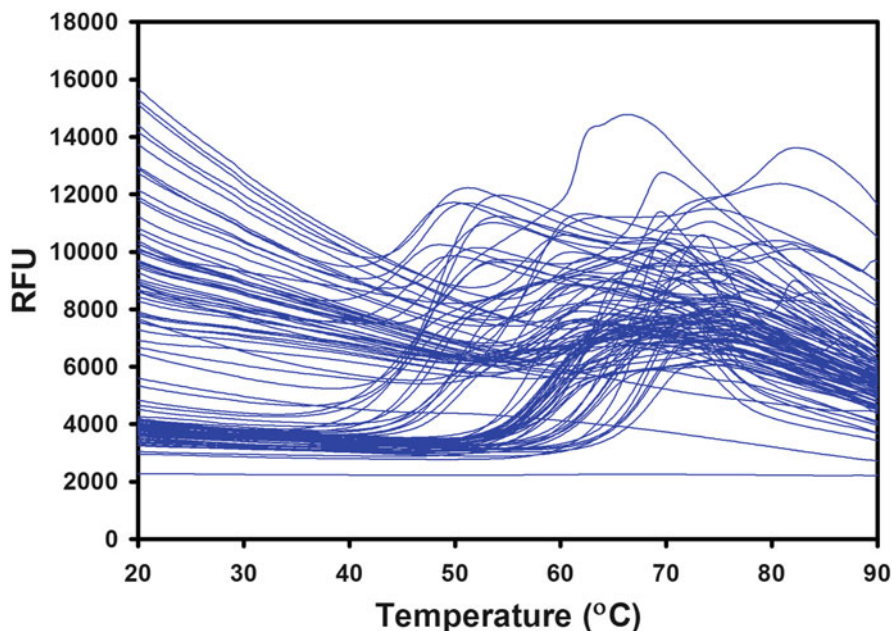


Fig. 2.1 Representative differential scanning fluorometry spectra obtained with a therapeutic protein on 96-well plate filled with different formulations for each well. Fluorescence intensity in relative fluorescence units (RFU) is shown as a function of temperature. Protein concentration is 1 g/L. The sample volume is 30 μ L. Data were obtained using the Bio-Rad CFX96 RT-PCR plate reader

Many companies that manufacture these instruments have realized the popularity of this application and have included software options to obtain and analyze DSF data. Another advantage of DSF is the wide range of protein concentration that can be used. Depending on protein properties, transitions can be detected at as low as 0.05 mg/mL or as high as at 100 mg/mL. Another high-throughput method, the ProteoStat™ assay from Enzo Life Sciences (Farmingdale, NY), provides an improved thermal shift approach based on extrinsic fluorescence for assessment of protein stability through monitoring protein aggregation, rather than protein unfolding. These methods, which employ high-throughput technologies, further expand the application of thermal analysis to modern pharmaceuticals. While it is not guaranteed that thermal stability correlates with a protein's physical stability during storage, better thermal stability usually indicates a greater energy requirement to unfold the protein. When executed under similar experimental conditions, high-throughput thermal analysis offers useful information that can be used to rank protein or formulation candidates. It seems safe to state that when all other properties are comparable, the protein constructs or formulations which lead to better thermal stability will always be more desirable.

2.2.8 *Light Scattering*

Aggregation is one of the major problems in protein pharmaceutical development. The presence of aggregated protein can compromise the purity, safety, and efficacy of a drug product. Several separation and detection techniques are used to monitor protein aggregation. Light scattering methods have the advantage of high sensitivity due to the size of scatterers, which makes it possible to detect small amounts of large protein aggregates in pharmaceutically relevant samples. There are two basic types of such methods used in protein therapeutics development: static light scattering (SLS) and dynamic light scattering (DLS). SLS can be applied to determine the protein's molecular mass and the mean square radius of gyration. SLS is often used with separation methods such as SEC or field-flow fractionation (FFF) for the purpose of obtaining a more accurate estimate of the size of the components separated by these techniques (Tarazona and Saiz 2003; McEvoy et al. 2011). Such online scattering method is commonly known as multi-angle light scattering (MALS). The throughput of SLS analysis used in this fashion often depends on the throughput of the separation technique. Though significant advancements have been achieved in numerous types of separation technologies, a variety of physical parameters, such as high pressure and short equilibration times, can be problematic for the coupling of light scattering detectors. Light scattering can also be coupled to plate readers. Simple monitoring of the scattered light at fixed wavelengths and angles can provide sensitive detection of aggregates formation. The Stargazer-384™ system (Harbinger Biotechnology and Engineering Corporation, Toronto, Canada), a 384-well microplate reader, was used in a study to monitor colloidal stability of mAbs at elevated temperatures (Goldberg et al. 2010). As previously discussed, the high-throughput Avacta Optim® 1000 (Pall Corporation, Port Washington, NY) is available for light scattering as well as fluorescence measurements with a volume as low as 1 μ L. In these instruments the light scattering signal is used to monitor aggregation by detecting the increased intensity, similar to turbidity measurements but with higher sensitivity. It should be noted, however, that if the aggregates formed are less dense than the monomeric protein, decreases in scattering can also be seen.

Dynamic light scattering is based on the measurement of the fluctuations in intensity of scattered light. An autocorrelation function, derived from fluctuation analysis, can reveal the distribution of the hydrodynamic radii of protein molecules present in solution (Schmidt 2010). Separation is not necessary, but resolution of species depends on their size difference and concentration. Because of the exponential form of the autocorrelation function, no more than two to four components can be comfortably resolved in the same solution. DLS plate readers have become very popular in the biopharmaceutical industry for protein and vaccine characterization (Vincentelli et al. 2004). Multiwell plate formats, small volumes, and automated procedures for measurement and data analysis make the DLS method high throughput and easy to apply. Antibody self-association has been studied with the help of gold nanoparticles and their characterization by dynamic and static light scattering. Nanoparticle–antibody conjugates displayed complex aggregation behavior dependent on pH and

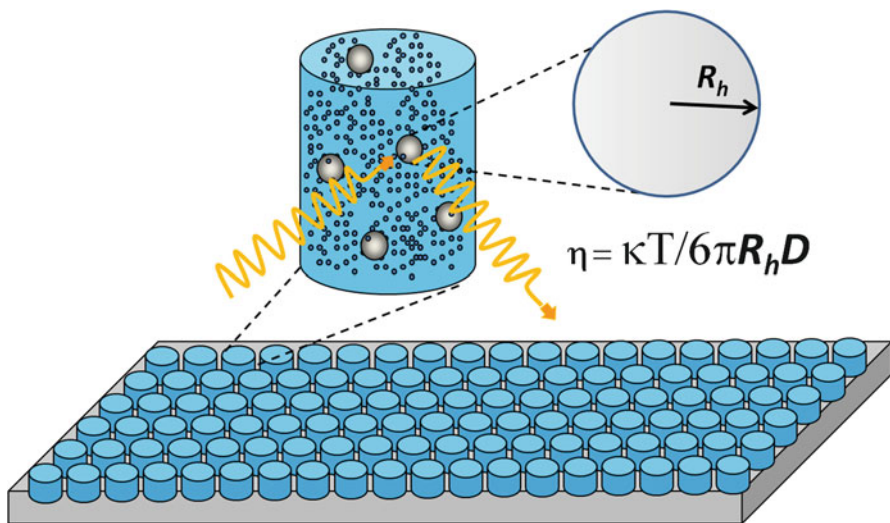


Fig. 2.2 High-throughput method for viscosity measurements based on dynamic light scattering determination of the diffusion coefficient of polystyrene beads externally added into a protein solution

ionic strength of the solution. Use of a DLS plate reader was a significant part of the high-throughput analytical development (Sule et al. 2011).

In biopharmaceutical drug development DLS has been used not only for direct detection and characterization of aggregation but also for the study of large colloidal structures. Certain large colloid-like aggregates have been shown to inhibit enzymes leading to false-positive HTS leads. These so-called promiscuous inhibitors were detected and screened by DLS using a plate reader (Feng et al. 2005). Results from such high-throughput assays for promiscuous inhibitory aggregates have been used to develop new computational models of this phenomenon. A method for quantitative characterization of macromolecular interactions using DLS has been introduced in a temperature-controlled plate reader format (Hanlon et al. 2010). This technique enabled determination of equilibrium dissociation constants and thermodynamic parameters. The low volume of plate-based DLS reduced the sample amount to a few microliters per experiment, with detection limits in the femtomolar range.

Biopharmaceutical products are often formulated at high concentrations to maximize delivery dosage and efficiency, and solutions of some proteins become very viscous at high concentrations (Yadav et al. 2010), creating significant problems for processes like purification, filtration, and injection through syringes. Standard methods for viscosity measurements have low throughput and require large quantities of protein. Thus, there is increasing demand for higher-throughput viscosity screening. A DLS assay based on measurement of the diffusion coefficient of beads added directly to the protein solution is high throughput and run in a multiwell plate format (He et al. 2010a). As shown in Fig. 2.2 the Stokes–Einstein

equation can be used to calculate the viscosity of a protein solution using the known radius of the added beads and the measured diffusion coefficient. Furthermore, DLS measurements of diffusion coefficients as a function of protein concentration can be used to derive the interaction parameter, k_D , which has been shown to correlate with protein properties such as viscosity and particulation propensity (Yadav et al. 2010; He et al. 2011). It is widely accepted that the second virial coefficient, B_{22} , obtained by SLS measurements contains information on protein–protein interaction (Printz et al. 2012). The k_D parameter derived from DLS measurements offers a simple way to compare samples under similar conditions (discussed in Chap. 3).

2.2.9 Design of Experiment and Data Analysis

All high-throughput methodologies mentioned above share a common ability to generate a large amount of biophysical information on therapeutic proteins. This enables more complex experimental designs at various stages of pharmaceutical development. The Quality by Design (QbD) concept has gained popularity in recent years among the biopharmaceutical industry and regulatory agencies (Rathore and Winkle 2009; Rathore and Devine 2008). Design of product quality is built on comprehensive understanding of a well-defined process and product space where the protein therapeutic is in its most desired form. The principle of design of experiment (DOE) is often applied to systematically evaluate the protein of interest under a variety of conditions, which are often selected based on the types of stresses that a protein therapeutic is subjected to during manufacturing, storage, and administration. The degree of these stresses often exceeds reality, and the experimental results can be used to predict the protein behavior when failures occur during the product life cycle. The combination of high-throughput measurement and DOE can also help enhance the statistical power when interpreting the results. More importantly, the implementation of high-throughput methodology offers opportunities to simultaneously assess a large number of samples. This is particularly valuable when employing methods that are only used to qualitatively rank order protein samples.

Application of high-throughput techniques results in large data sets, often requiring mathematical tools for rigorous analysis. Statistics helps to establish correlations among measured properties of a molecule. Commonly used statistical analyses that are frequently applied to the development of protein therapeutics include Gaussian modeling, analyses of variance (ANOVA), and the t -test. In addition, biophysical characterization often involves spectroscopic methods such as CD, FTIR, and fluorescence, and the results usually include measured values as a function of wavelength of the optic source. Such data can be analyzed by methods of chemometrics including the singular value decomposition technique which has been used to determine the maximum changes in protein properties caused by

particular factors like pH or ionic strength. This methodology has been applied to the interpretation of CD and FTIR spectra obtained during the production of antibodies (Greenfield 2006; Sellick et al. 2010). Another useful mathematical tool is polynomial-based data fitting. This approach involves fitting an arbitrary polynomial model to a limited data set and then using the same model to predict protein behavior outside of the tested range. For example, discrete data at pH 5, 5.5, and 6 can be used to generate a polynomial model that best fit the experimental results. The continuous mathematical model can then be used to predict results at pH 5.8, and even at pH values outside of 5–6, if the assumption holds. The polynomial method is especially effective when the source data set is large. Even more predictive information can be obtained while considering multiple variables simultaneously (Sall et al. 2007).

2.3 Empirical Phase Diagrams as Tools to Interpret Results from High-Throughput Biophysical Approaches

2.3.1 *Combination of Biophysical Techniques and Data Analysis*

As discussed above and in other chapters in this text, high-throughput screening (HTS) is usually performed with only one or two low-resolution techniques of the type previously considered. The selection of the particular technique is typically based on what is known about degradation pathways of the target, convenience, and availability of appropriate instrumentation as well as speed. It has recently become possible to combine the results from multiple techniques with the goal of providing a more comprehensive picture of a protein's structure and its response to various environmental perturbations. This can be used to select optimal methods for HTS as well as for various forms of comparative analysis. A number of methods are available for this purpose. We will consider here only the one that has been most thoroughly described in the literature [reviewed in Maddux et al. (2011)], but other approaches such as Chernoff faces and star charts (Yau 2011) in which information is encoded in facial features or abstract geometric shapes are under consideration. The former method is known as the EPD. The word empirical is inserted in front of the phrase “phase diagram” to differentiate it from the well-known thermodynamic or equilibrium phase diagram since equilibrium conditions are not implied in the former.

The basic idea behind the EPD is to represent the protein (application has also been made to peptides, nucleic acids, virus-like particles, viruses, and bacteria cells) as a vector in which the components of the vector are experimental values obtained from the various methods employed as a function of solution variables. Preparation of EPDs generally involves buffer subtraction of the data, peak

selection for data analysis (entire spectra can also be used), averaging of multiple data acquisitions, normalization, input matrix synthesis, singular value decomposition, and finally color mapping of the most significant data using an RGB color scheme. A detailed description of the method including the mathematics involved is presented in Maddux et al. (2011). In general, far-UV circular dichroism (CD) is used to monitor secondary structure although FTIR and Raman spectroscopies can also be employed. Tertiary structure is most commonly analyzed with intrinsic fluorescence, near-UV CD, or high-resolution derivative absorption spectroscopy. Dye binding using compounds such as 8-anilino naphthalene sulfonic acid (ANS) is frequently used to probe the exposure of apolar regions in the protein. Dissociation and association (including aggregation) are typically probed with static and/or DLS (although see below). Overall thermal stability is often studied with differential scanning calorimetry. The most common independent variables (i.e., forms of stress that have previously been employed) are temperature and pH although a wide variety of other variables have been used as described below. Perhaps the major limitation of the EPD approach has been the time and instrumentation necessary to prepare an EPD. This has recently changed with the advent of equipment capable of performing multiple different types of measurements simultaneously. Originally an EPD typically required a fluorometer, CD spectropolarimeter, light scattering system, and perhaps a DSC or FTIR spectrometer. Several newly developed instruments now at least partially overcome this limitation. For example, recent improvements in CD instruments now permit both near- and far-UV spectra, near-UV absorption spectra, fluorescence, and SLS (turbidity or scattering at the fluorescence emission wavelength) to all be acquired simultaneously in a four-position sample chamber under variable temperature conditions (Hu et al. 2011). This permits EPDs to be generated in less than a day. A similar “protein machine” with a six-position sample chamber has also been recently described (Maddux et al. 2012). Perhaps the simplest version of a system with rapid EPD generation capability is a UV absorption spectrometer, typically of the diode array variety, to permit sufficient resolution of derivative peaks (Kueltoz et al. 2003). At high resolution (usually second), the derivative spectrum of a protein will usually manifest distinct peaks for phenylalanine, tyrosine, and tryptophan (if present). Since the residues are usually buried, Tyr is often interfacial, and Trp present in highly variable environments, temperature, and pH-induced peaks shifts can frequently provide a fairly detailed picture of a protein’s structural response to various perturbations. Such data can easily be represented in the form of an EPD (Kueltoz et al. 2003). Similarly, fluorescence microtiter plate-based fluorometers have been developed which employ multiple fluorescence and light scattering measurements as a function of temperature, as also described in the fluorescence section. The latter is of especially high throughput, permitting the generation of many EPDs in a single day. EPDs for four model proteins obtained from multiple instruments, two CD-based spectrometers, and a high-throughput fluorometer are shown in Fig. 2.3, where it can be seen that all produce similar EPDs although small differences are apparent due to the various types of measurements used to construct each EPD.

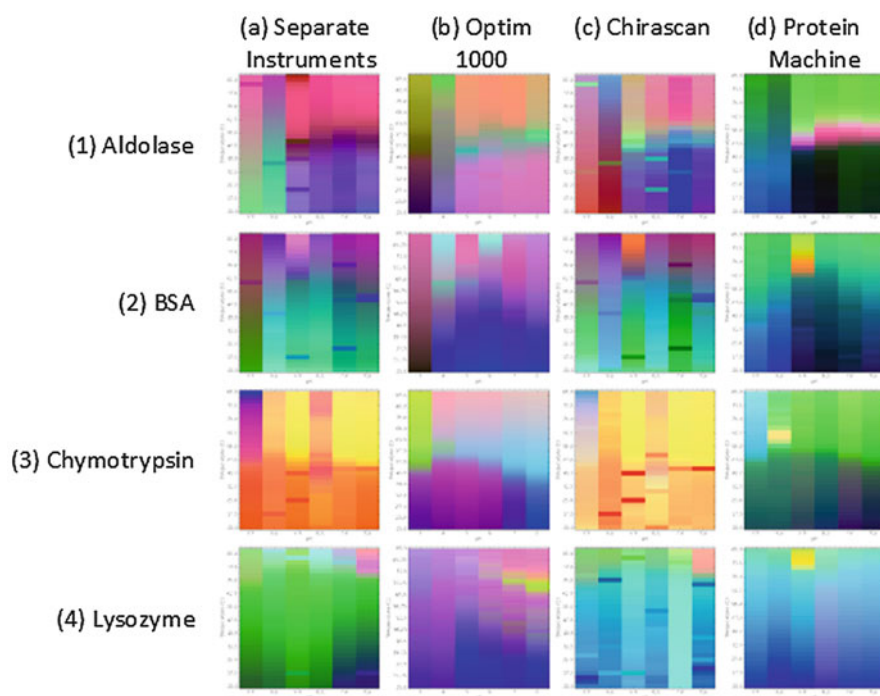


Fig. 2.3 Empirical phase diagrams (EPDs) of four model proteins (1) aldolase, (2) BSA, (3) chymotrypsin, and (4) lysozyme constructed using data collected from various instruments: (a) intrinsic fluorescence (FL) and static light scattering (SLS) data from a Photon Technology International (PTI) fluorometer, and circular dichroism (CD) from an Applied Photophysics Chirascan, (b) FL and SLS by an Avacta Optim 1000 microtiter plate fluorometer, (c) FL and CD Applied Photophysics Chirascan, and (d) FL, SLS, CD, and UV absorbance by an Olis Protein Machine

2.3.2 High-Throughput Characterization and Preformulation Development

The EPD method provides a comprehensive overview of how a protein responds to environmental alteration in the form of a colored diagram in which regions of different color correspond to different structural states of the target molecules. By reference to the original data, native partially folded and molten globule, extensively unfolded, dissociated, oligomerized, and various aggregated states can all be identified. This provides the scientist with clues to trouble spots in a protein and a basis with which to select assays with which to screen for potential stabilizers. For example, if aggregation or a particular structural change occurs under moderate temperature and/or pH conditions, one can select a less stable condition and one or more techniques sensitive to selected degradation events for screening purposes. Typically, a supplemental GRAS (generally regarded as safe) library containing a selection of

buffers, sugars, sugar alcohols, amino acids, polymers, detergents, and osmolytes is used. In the initial screen relatively high concentrations of compounds are used with their concentration dependence and use in combination later optimized. It is usually wise to employ at least two methods: one sensitive to aggregation (e.g., light scattering) and one to structural change (e.g., fluorescence, CD) for this purpose. DSC is also commonly employed especially due to the recent availability of highly sensitive high-throughput instruments. It is also possible to prepare EPDs in the presence of selected stabilizers to permit a more detailed analysis/comparison of their effects on a protein. The information thus obtained by a temperature/pH EPD thus provides a basis for buffer and excipient selection at an early stage of pharmaceutical development. Although not yet published, a new version of the EPD has been developed in which the colors have actual physical measuring in contrast to the arbitrary assignment of color in the original EPD.

2.3.3 Additional Application of High-Throughput Methods and the EPD

High-throughput methods and EPDs can also be applied to a wide variety of different situations, some of which will be briefly described here. Two commonly encountered forms of stress in the protein therapeutic area are freeze/thaw and shear. It is often necessary to freeze and then thaw both during development and manufacturing situations. An EPD can be created using the number of freeze/thaw cycles under defined conditions as an independent variable accompanied by temperature and pH stress. All three variables (temperature, pH, freeze/thaw cycles) can be combined into a three-dimensional representation in which the EPD is presented as a colored surface. Shear stress is also often encountered in the development, manufacture (especially filling), and shipping of protein pharmaceuticals. To explore this potential degrading stress, the intensity of the shear can be varied by a mechanical process such as stirring, shaking, or some other forms of agitation, and this is used as a variable in EPD production.

Protein concentration is another important variable that has assumed increasing importance with the use of high-concentration formulations. This variable can be typically evaluated over the range of 0.05–300 g/L depending on the solubility of the protein and the methods employed in the analysis. Proteins usually alter their structure to little or no extent as a function of protein concentration, but aggregation and surface adsorption are both highly concentration dependent. Thus, aggregation-sensitive techniques such as light scattering are often of special importance in protein concentration-dependent studies. Another common variable of particular importance is ionic strength. In the Debye–Huckel charge shielding regime (0–0.15 ionic strength), a number of intermediate concentrations should be evaluated to probe electrostatic effects. At higher salt concentrations both preferential hydration and binding effects usually dominate with salt concentration into the molar range appropriately examined.

It is also possible to create EPDs based on phenomena such as aggregation. A variety of different types of aggregates have been identified in protein solutions based on their relative size and the nature of a protein's conformation (altered or native) within the aggregate. A number of methods are available that are sensitive to these features (see Chap. 9), permitting an aggregation-based EPD to be created. Again, using variables like temperature, pH, ionic strength, freeze/thaw, and shear, soluble protein aggregates can be detected by methods such as size-exclusion HPLC, sedimentation velocity analytical ultracentrifugation, FFF, and DLS. Complimentary structural data can be obtained by the methods described above with FTIR and Raman spectroscopy especially useful because of the particulate nature of such samples. Larger (i.e., submicron) aggregates can be characterized by DLS including single-particle microscope-based approaches (nanoparticle tracking analysis) and classic microscopy-based techniques (atomic force microscopy, scanning electron microscopy, and transmission electron microscopy) although they are difficult to quantitate and new methods such as quartz crystal microbalances and nanomechanical resonators are seeing increasing use. Subvisible and visible particles can be analyzed by methods such as coulter counting, light obscuration, micro-flow digital imaging, and various visual procedures. Using parameters such as size, composition, structure, and particle number as dependent variables, an EPD can be generated that provides a comprehensive picture of the nature of protein aggregates that form under a wide variety of stress conditions.

Although not yet described in the scientific literature, chemical degradation can also be analyzed in various high-throughput modes and be summarized in EPDs. The application of EPDs described above has all been to various physical processes in which covalent bonds are not broken. Of equal importance to protein degradation, however, are chemical changes such as oxidation and deamidation events. Chemical changes are usually quantitatively determined by peptide mapping combined with mass spectrometry (MS) in the form of HPLC-MS experiments which permit both the amount and location of a residue modification within a protein's amino acid sequence to be determined. To increase throughput, once the nature of any changes has been identified, HPLC-MS analysis may be replaced by methods such as RP-HPLC and capillary electrophoresis or isoelectric focusing. A convenient way to present and analyze such data is in the form of rate constants for the individual, for example, deamidation and oxidation events. This of course requires time-dependent measurements. The rate constants can be used as the dependent variables in an EPD since they are typically determined as function-independent variables such as pH and temperature. Of special interest is the comparative use of physical and chemical EPDs which permits an exploration of the relationship between structural changes and chemical events (and vice versa).

As a final example, EPDs can be used in a strictly comparative mode. Structural comparisons between both similar and assumed identical proteins are often a very important element of pharmaceutical analysis. For example, when manufacturing changes are made, during the development of biosimilars or when investigating mutant proteins, a detailed comparison of the various species is a critical part of the analysis. A direct comparison of EPDs of the target molecules provides a convenient

and sensitive way to see if structural identity has been obtained. As an example, second-generation functional mutants of fibroblast growth factor one (FGF-1) have been compared using EPDs and used to select molecules that are not dependent on heparin for their activity (Alsenaidy et al. 2012). Such EPD-dependent comparisons have even been performed with different rotavirus serotypes despite their individual complexity (Esfandiary et al. 2010). Various mathematical (difference) methods exist to facilitate such comparisons.

2.4 Advantages and Challenges of Implementing High-Throughput Technology in Therapeutic Protein Development: An Industry Perspective

High-throughput technologies can be applied across all aspects of biopharmaceutical discovery and development from the early stages of candidate screening and selection to the later stages of formulation development. The benefits of a successful high-throughput screening strategy in this environment are many. The most obvious one is speed. Faster assays mean that sufficient data to drive a decision can be collected within a shorter timeframe leading to more rapid decisions and ultimately an accelerated development timeline. In the pharmaceutical industry where product development is a protracted process, any acceleration of the timeline can mean getting a promising drug candidate into clinical testing sooner and ultimately to market faster, potentially providing a competitive advantage and leading to an earlier revenue stream. A second advantage is that high-throughput assays typically require smaller volumes and fewer samples than standard assay formats. This sample sparing feature is especially important early in development in which the purification process is an early stage of development and consequently the quantities of the candidates being tested may be in short supply. Another advantage is that high-throughput procedures permit many more drug candidates to be tested than would be possible using a standard approach. In the early stages of discovery research where many candidates are being evaluated based on screening assays, a good high-throughput screen for binding permits many more candidates to be tested and allows this to be accomplished within a shorter period of time. Finally, high-throughput assays permit additional molecular features to be evaluated. A good example of this advantage is found in formulation development where the influence of a variety of solution conditions and potential excipients will need to be tested. The solution pH, buffer salts used, excipients, surfactants, and the presence or absence of salt all need to be evaluated, and this can be accomplished much more easily using high-throughput approaches.

High-throughput assays have been a standard approach to the discovery of novel small molecule drugs for several years. These assays were typically designed using an isolated drug target, often the soluble domain of a membrane bound receptor, and a library of small molecules, often containing tens of thousands of compounds,

would be screened with a readout indicating a binding event or inhibition of binding. With the birth of the biotechnology industry approximately three decades ago and the beginning of the development of recombinant proteins as therapeutic entities, the need for high-throughput approaches for biotherapeutic drug discovery and development has grown. The greater chemical complexity of proteins and the need to maintain native three-dimensional structure during both processing and storage of the drug substance and drug product have contributed to the application of the various biophysical methods discussed in this chapter. Another property of many proteins, the tendency to self-associate to form dimers and higher oligomers, in some cases an undesirable property, has also demanded the use of highly selective biophysical techniques. The development of high-throughput approaches to the application of these biophysical methods has lagged behind other methods, but the burgeoning biotechnology industry with its unique analytical requirements has helped to recently accelerate this development.

The implementation of a high-throughput screen, however, is not a simple matter of just miniaturizing an assay and running it in a 96-well plate. High-throughput assays must be carefully developed and validated before use in drug development. Special equipment is often necessary to successfully perform a high-throughput assay. Depending on the readout of the assay, a special instrument with the capability to measure signals from a plate may be needed. Special devices for heating or cooling a plate are often required to maintain the temperature required for the assay, and, in some cases, liquid handling equipment is necessary for setting up the multiple conditions to be examined. One of the biggest challenges to implement a high-throughput strategy is the handling of the data produced. High-throughput methods have the potential to generate vast quantities of data which not only must be collected, stored, and archived but also must be analyzed and then presented in a form that can be understood by others. The generation of these very large data sets has perhaps had an unintended consequence. As described above, statistical methods are an absolute requirement for the analysis of these data. This has led to a much greater understanding of the relationships among the variables in an experiment. At the extremes of an experiment are experimental design and presentation of results. DOE has been critical to the success of high-throughput experimental design. When working with multiple molecules or a large number of variables, it is a physical impossibility to design and execute experiments that examine all possibilities in a meaningful way, even with the use of high-throughput methods. DOE provides a way of designing experiments that provide the necessary data while significantly decreasing the number of experiments. This approach is expected to grow within the biopharmaceutical industry as more high-throughput assays are brought on line. Equally demanding is the presentation of the processed data from high-throughput experiments. One approach described above which draws on the statistical analysis of large multiple data sets is the EPD in which a protein is represented as a vector in which the components of the vector are experimental values obtained from the various methods employed as a function of solution variables. The concept and preparation of EPDs have been discussed earlier in this chapter, but the outcome is a presentation using color mapping to denote the most significant data.

This approach as well as others allows a very complex data set to be presented in a way that is readily understandable.

Although high-throughput biophysical methods become increasingly important in the development of therapeutic proteins, there are still biophysical methods that have not been adapted to a high-throughput format. Examples include circular dichroism and mass spectrometry which are still in their infancy but which, no doubt, will have high-throughput adaptations in the near future. It is clear that there is a need for new and better high-throughput biophysical assays and that the needs of the biopharmaceutical industry will play a role in their development.

References

- Ahrer K, Jungbauer A (2006) Chromatographic and electrophoretic characterization of protein variants. *J Chromatogr B Analyt Technol Biomed Life Sci* 841(1–2):110–122
- Alexandrov AI, Mileni M, Chien EY, Hanson MA, Stevens RC (2008) Microscale fluorescent thermal stability assay for membrane proteins. *Structure* 16(3):351–359
- Alsenaidy MA, Wang T, Kim JH et al (2012) An empirical phase diagram approach to investigate conformational stability of “second-generation” functional mutants of acidic fibroblast growth factor-1. *Protein Sci* 21(3):418–432
- Andrew SM, Titus JA (2001) Purification of immunoglobulin G. *Curr Protoc Immunol*. Chapter 2:Unit 2.7
- Anquetil PA, Brenan CJ, Marcolli C, Hunter IW (2003) Laser Raman spectroscopic analysis of polymorphic forms in microliter fluid volumes. *J Pharm Sci* 92(1):149–160
- Bravman T, Bronner V, Lavie K, Notcovich A, Papalia GA, Myszkowski DG (2006) Exploring “one-shot” kinetics and small molecule analysis using the ProteOn XPR36 array biosensor. *Anal Biochem* 358(2):281–288
- Bruylants G, Wouters J, Michaux C (2005) Differential scanning calorimetry in life sciences: thermodynamics, stability, molecular recognition and applications in drug design. *Curr Med Chem* 12:2011–2020
- Capelle MA, Gurny R, Arvinte T (2009) A high throughput protein formulation platform: case study of salmon calcitonin. *Pharm Res* 26(1):118–128
- Chang BS, Kendrick BS, Carpenter JF (1996) Surface-induced denaturation of proteins during freezing and its inhibition by surfactants. *J Pharm Sci* 85(12):1325–1330
- Coffman JL, Kramarczyk JF, Kelley BD (2008) High-throughput screening of chromatographic separations: I. Method development and column modeling. *Biotechnol Bioeng* 100(4):605–618
- Ericsson UB, Hallberg BM, Detitta GT, Dekker N, Nordlund P (2006) Thermofluor-based high-throughput stability optimization of proteins for structural studies. *Anal Biochem* 357(2):289–298
- Esfandiary R, Yee L, Ohtake S et al (2010) Biophysical characterization of rotavirus serotypes G1, G3 and G4. *Hum Vaccin* 6(5):390–398
- Fahrner RL, Knudsen HL, Basey CD et al (2001) Industrial purification of pharmaceutical antibodies: development, operation, and validation of chromatography processes. *Biotechnol Bioeng* 74:301–327
- Feng BY, Shelat A, Doman TN, Guy RK, Shoichet BK (2005) High-throughput assays for promiscuous inhibitors. *Nat Chem Biol* 1(3):146–148
- Fowler A, Swift D, Longman E et al (2002) An evaluation of fluorescence polarization and lifetime discriminated polarization for high throughput screening of serine/threonine kinases. *Anal Biochem* 308(2):223–231

- Goldberg DS, Bishop SM, Shah AU, Sathish HA (2010) Formulation development of therapeutic monoclonal antibodies using high-throughput fluorescence and static light scattering techniques: role of conformational and colloidal stability. *J Pharm Sci* 100(4):1306–1315
- Greenfield NJ (2006) Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 1(6):2876–2890
- Haidekker MA, Brady TP, Lichlyter D, Theodorakis EA (2005) Effects of solvent polarity and solvent viscosity on the fluorescent properties of molecular rotors and related probes. *Bioorg Chem* 33(6):415–425
- Hanlon AD, Larkin MI, Reddick RM (2010) Free-solution, label-free protein-protein interactions characterized by dynamic light scattering. *Biophys J* 98(2):297–304
- Harn N, Allan C, Oliver C, Middaugh CR (2007) Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. *J Pharm Sci* 96(3):532–546
- Hawe A, Sutter M, Jiskoot W (2008a) Extrinsic fluorescent dyes as tools for protein characterization. *Pharm Res* 25(7):1487–1499
- Hawe A, Friess W, Sutter M, Jiskoot W (2008b) Online fluorescent dye detection method for the characterization of immunoglobulin G aggregation by size exclusion chromatography and asymmetrical flow field flow fractionation. *Anal Biochem* 378(2):115–122
- Hawe A, Filipe V, Jiskoot W (2010a) Fluorescent molecular rotors as dyes to characterize polysorbate-containing IgG formulations. *Pharm Res* 27(2):314–326
- Hawe A, Rispiens T, Herron JN, Jiskoot W (2010b) Probing bis-ANS binding sites of different affinity on aggregated IgG by steady-state fluorescence, time-resolved fluorescence and isothermal titration calorimetry. *J Pharm Sci* 100(4):1294–1305
- He F, Becker GW, Litowski JR, Narhi LO, Brems DN, Razinkov VI (2010a) High-throughput dynamic light scattering method for measuring viscosity of concentrated protein solutions. *Anal Biochem* 399(1):141–143
- He F, Hogan S, Latypov RF, Narhi LO, Razinkov VI (2010b) High throughput thermostability screening of monoclonal antibody formulations. *J Pharm Sci* 99(4):1707–1720
- He F, Phan DH, Hogan S et al (2010c) Detection of IgG aggregation by a high throughput method based on extrinsic fluorescence. *J Pharm Sci* 99(6):2598–2608
- He F, Woods CE, Becker GW, Narhi LO, Razinkov VI (2011) High-throughput assessment of thermal and colloidal stability parameters for monoclonal antibody formulations. *J Pharm Sci* 100(12):5126–5141
- Hu L, Olsen C, Maddux N, Joshi SB, Volkin DB, Middaugh CR (2011) Investigation of protein conformational stability employing a multimodal spectrometer. *Anal Chem* 83(24):9399–9405
- Jezek J, Rides M, Derham B et al (2011) Viscosity of concentrated therapeutic protein compositions. *Adv Drug Deliv Rev* 63(13):1107–1117
- Jiskoot W, Randolph TW, Volkin DB et al (2012) Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. *J Pharm Sci* 101(3):946–954
- Karlsson R (2004) SPR for molecular interaction analysis: a review of emerging application areas. *J Mol Recognit* 17(3):151–161
- Khosravi M, Kao YH, Mrsny RJ, Sweeney TD (2002) Analysis methods of polysorbate 20: a new method to assess the stability of polysorbate 20 and established methods that may overlook degraded polysorbate 20. *Pharm Res* 19(5):634–639
- Kueltzo LA, Ersoy B, Ralston JP, Middaugh CR (2003) Derivative absorbance spectroscopy and protein phase diagrams as tools for comprehensive protein characterization: a bGCSF case study. *J Pharm Sci* 92(9):1805–1820
- Kung CE, Reed JK (1989) Fluorescent molecular rotors: a new class of probes for tubulin structure and assembly. *Biochemistry* 28(16):6678–6686
- Lakowicz JR (2006) Principles of fluorescence spectroscopy, 3rd edn. Springer, New York
- Lee W, Fon W, Axelrod BW, Roukes ML (2009) High-sensitivity microfluidic calorimeters for biological and chemical applications. *Proc Natl Acad Sci USA* 106(36):15225–15230

- Lerchner J, Wolf A, Wolf G et al (2006) A new micro-fluid chip calorimeter for biochemical applications. *Thermochim Acta* 445:144–150
- Lerchner J, Maskow T, Wolf G (2008) A new micro-fluid chip calorimeter for biochemical applications. *Thermochim Acta* 47:991–999
- Li CH, Nguyen X, Narhi L et al (2011) Applications of circular dichroism (CD) for structural analysis of proteins: qualification of near- and far-UV CD for protein higher order structural analysis. *J Pharm Sci* 100(11):4642–4654
- Mach H, Arvinte T (2011) Addressing new analytical challenges in protein formulation development. *Eur J Pharm Biopharm* 78(2):196–207
- Mach H, Middaugh CR (2011) Ultraviolet spectroscopy as a tool in therapeutic protein development. *J Pharm Sci* 100(4):1214–1227
- Mach H, Bhambhani A, Meyer BK et al (2011) The use of flow cytometry for the detection of subvisible particles in therapeutic protein formulations. *J Pharm Sci* 100(5):1671–1678
- Maddux NR, Joshi SB, Volkin DB, Ralston JP, Middaugh CR (2011) Multidimensional methods for the formulation of biopharmaceuticals and vaccines. *J Pharm Sci* 100(10):4171–4197
- Maddux NR, Rosen IT, Hu L, Olsen CM, Volkin DB, Middaugh CR (2012) An improved methodology for multidimensional high-throughput preformulation characterization of protein conformational stability. *J Pharm Sci* 101(6):2017–2024
- Malawski GA, Hillig RC, Montecarlo F et al (2006) Identifying protein construct variants with increased crystallization propensity – a case study. *Protein Sci* 15(12):2718–2728
- McEvoy M, Razinkov V, Wei Z, Casas-Finet JR, Tous GI, Schenerman MA (2011) Improved particle counting and size distribution determination of aggregated virus populations by asymmetric flow field-flow fractionation and multiangle light scattering techniques. *Biotechnol Prog* 27(2):547–554
- Otsuki S, Ishikawa M (2010) Wavelength-scanning surface plasmon resonance imaging for label-free multiplexed protein microarray assay. *Biosens Bioelectron* 26(1):202–206
- Owicki JC (2000) Fluorescence polarization and anisotropy in high throughput screening: perspectives and primer. *J Biomol Screen* 5(5):297–306
- Pantoliano MW, Petrella EC, Kwasnoski JD et al (2001) High-density miniaturized thermal shift assays as a general strategy for drug discovery. *J Biomol Screen* 6:429–440
- Peters WB, Frasca V, Brown RK (2009) Recent developments in isothermal titration calorimetry label free screening. *Comb Chem High Throughput Screen* 12(8):772–790
- Pierce MM, Raman CS, Nall BT (1999) Isothermal titration calorimetry of protein–protein interactions. *Methods* 19(2):213–221
- Printz M, Kalonia DS, Friess W (2012) Individual second virial coefficient determination of monomer and oligomers in heat-stressed protein samples using size-exclusion chromatography-light scattering. *J Pharm Sci* 101:363–372
- Privalov GP, Privalov PL (2000) Problems and perspectives in microcalorimetry of biological macromolecules. *Methods Enzymol* 323:31–62
- Rathore AS, Devine R (2008) PDA workshop on “Quality by Design for Biopharmaceuticals: Concepts and Implementation”, May 21–22, 2007, Bethesda, Maryland. *PDA J Pharm Sci Technol* 62(5):380–390
- Rathore AS, Winkle H (2009) Quality by design for biopharmaceuticals. *Nat Biotechnol* 27(1):26–34
- Rich RL, Myszkka DG (2000) Advances in surface plasmon resonance biosensor analysis. *Curr Opin Biotechnol* 11(1):54–61
- Rosenberg AS (2006) Effects of protein aggregates: an immunologic perspective. *AAPS J* 8(3):E501–E507
- Sall J, Creighton L, Lehman A (2007) JMP start statistics: a guide to statistics and data analysis using JMP, 4th edn. SAS Press, Cary
- Schäfer G, Schmidt H (2006) High-throughput spectroscopic viscosity measurement of nanocomposite sols with ETC-effect. *J Sol–Gel Sci Technol* 38(3):241–244
- Schiffer CA, Dotsch V (1996) The role of protein-solvent interactions in protein unfolding. *Curr Opin Biotechnol* 7(4):428–432

- Schmidt R (2010) Dynamic light scattering for protein characterization. *Encyclopedia of Analytical Chemistry*, 20–24
- Sellick CA, Hansen R, Jarvis RM et al (2010) Rapid monitoring of recombinant antibody production by mammalian cell cultures using Fourier transform infrared spectroscopy and chemometrics. *Biotechnol Bioeng* 106(3):432–442
- Shire SJ, Shahrokh Z, Liu J (2004) Challenges in the development of high protein concentration formulations. *J Pharm Sci* 93(6):1390–1402
- Siebert F, Hildebrandt P (2008) *Vibrational spectroscopy in life science*. Wiley-VCH, Weinheim
- Stackhouse N, Miller AK, Gadgil HS (2011) A high-throughput UPLC method for the characterization of chemical modifications in monoclonal antibody molecules. *J Pharm Sci* 100(12):5115–5125
- Sule SV, Sukumar M, Weiss WF, Marcelino-Cruz AM, Sample T, Tessier PM (2011) High-throughput analysis of concentration-dependent antibody self-association. *Biophys J* 101(7):1749–1757
- Susanto A, Knieps-Grünhagen E, von Lieres E, Hubbuch J (2008) High throughput screening for the design and optimization of chromatographic processes: assessment of model parameter determination from high throughput compatible data. *Chem Eng Technol* 31(12):1846–1855
- Swadesh J (2001) *HPLC: practical and industrial applications*, 2nd edn. CRC Press, Boca Raton
- Szapacs ME, Urbanski JJ, Kehler JR et al (2010) Absolute quantification of a therapeutic domain antibody using ultra-performance liquid chromatography-mass spectrometry and immunoassay. *Bioanalysis* 2(9):1597–1608
- Takeda H, Fukumoto A, Miura A, Goshima N, Nomura N (2006) High-throughput kinase assay based on surface plasmon resonance suitable for native protein substrates. *Anal Biochem* 357(2):262–271
- Tarazona MP, Saiz E (2003) Combination of SEC/MALS experimental procedures and theoretical analysis for studying the solution properties of macromolecules. *J Biochem Biophys Methods* 56(1–3):95–116
- Torres FE, Recht MI, Coyle JE, Bruce RH, Williams G (2010) Higher throughput calorimetry: opportunities, approaches and challenges. *Curr Opin Struct Biol* 20(5):598–605
- Verhaegen K, Baert K, Simaels J, Van Driessche W (2000) A high-throughput silicon microphysiometer. *Sens Actuators A Phys* 82(1):186–190
- Vermeir S, Nicolai BM, Verboven P et al (2007) Microplate differential calorimetric biosensor for ascorbic acid analysis in food and pharmaceuticals. *Anal Chem* 79(16):6119–6127
- Vincentelli R, Canaan S, Campanacci V et al (2004) High-throughput automated refolding screening of inclusion bodies. *Protein Sci* 13(10):2782–2792
- Wagner M, Reiche K, Blume A, Garidel P (2012) Viscosity measurements of antibody solutions by photon correlation spectroscopy: an indirect approach – limitations and applicability for high-concentration liquid protein solutions. *Pharm Dev Technol*. doi:10.3109/10837450.2011.649851
- Wang L, Wang B, Lin Q (2008) Demonstration of MEMS-based differential scanning calorimetry for determining thermodynamic properties of biomolecules. *Sens Actuators B Chem* 134:953–958
- Wassaf D, Kuang G, Kopacz K et al (2006) High-throughput affinity ranking of antibodies using surface plasmon resonance microarrays. *Anal Biochem* 351(2):241–253
- Wiendahl M, Schulze Wierling P, Nielsen J et al (2008) High throughput screening for the design and optimization of chromatographic processes – miniaturization, automation and parallelization of breakthrough and elution studies. *Chem Eng Technol* 31(6):893–903
- Xiang Y, Liu Y, Lee ML (2006) Ultrahigh pressure liquid chromatography using elevated temperature. *J Chromatogr A* 1104(1–2):198–202
- Yadav S, Liu J, Shire SJ, Kalonia DS (2010) Specific interactions in high concentration antibody solutions resulting in high viscosity. *J Pharm Sci* 99(3):1152–1168
- Yau N (2011) *Visualize this: the FlowingData guide to design, visualization, and statistics*. Wiley, Indianapolis
- Zhao H, Graf O, Milovic N et al (2010) Formulation development of antibodies using robotic system and high-throughput laboratory (HTL). *J Pharm Sci* 99(5):2279–2294

Biophysics for Therapeutic Protein Development

Narhi, L.O. (Ed.)

2013, IX, 293 p. 73 illus., 31 illus. in color., Hardcover

ISBN: 978-1-4614-4315-5