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Review article

High throughput screening of protein formulation stability: Practical considerations

Martinus A.H. Capelle, Robert Gurny, Tudor Arvinte *

Department of Pharmaceutics and Biopharmaceutics, University of Geneva, University of Lausanne, Geneva, Switzerland

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Abstract

The formulation of protein drugs is a difficult and time-consuming process, mainly due to the complexity of protein structure and the very specific physical and chemical properties involved. Understanding protein degradation pathways is essential for the success of a biopharmaceutical drug. The present review concerns the application of high throughput screening techniques in protein formulation development. A protein high throughput formulation (HTF) platform is based on the use of microplates. Basically, the HTF platform consists of two parts: (i) sample preparation and (ii) sample analysis. Sample preparation involves automated systems for dispensing the drug and the formulation ingredients in both liquid and powder form. The sample analysis involves specific methods developed for each protein to investigate physical and chemical properties of the formulations in microplates. Examples are presented of the use of protein intrinsic fluorescence for the analysis of protein aqueous properties (e.g., conformation and aggregation). Different techniques suitable for HTF analysis are discussed and some of the issues concerning implementation are presented with reference to the use of microplates.

Keywords: High throughput screening; Protein formulation; Spectroscopy; Physical and chemical characterization; Fluorescence; Aggregation; Stability

1. Introduction

Many protein targets and protein drugs have recently been discovered and it is expected that about a dozen therapeutic proteins per year will gain regulatory approval in the coming decade. Between 2003 and 2006, a total of 32 biopharmaceuticals were awarded marketing approval within the European Union and the USA, making a total of 165 currently approved biopharmaceuticals [1]. In 2006, the first biosimilar, recombinant human growth hormone (Omnitrope® from Sandoz) and the inhaled recombinant insulin (Exubera® from Pfizer) have been approved

Abbreviations: ADME, absorption distribution metabolism excretion; ANS, 1-anilino-naphthalene-8-sulfonate; ATR, attenuated total reflection; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CCD, charged-coupled device; CD, circular dichroism; CE, capillary electrophoresis; DANS, 5-dimethylaminonaphtalene-1-sulfonate; DMSO, dimethyl sulfoxide; DSC, differential scanning calorimetry; ESI, electrospray ionization; FDA, food and drug administration; FIDA, fluorescence intensity distribution analysis; FILDA, fluorescence intensity and lifetime distribution analysis; FLARe, fluorescence lifetime assay repertoire; FLIM, fluorescence lifetime imaging microscopy; FTIR, Fourier-transform infrared spectroscopy; FRET, fluorescence resonance energy transfer; HTS, high throughput screening; HTF, high throughput formulation; ICH, International Conference on Harmonisation; LC, liquid chromatography; MALDI, matrix assisted laser desorption ionization; MS, mass spectrometry; NDA, new drug application; sCT, salmon calcitonin; SR, synchrotron radiation; TCSPC, time-correlated single-photon counting; Tm, melting temperature; TRA, time-resolved anisotropy; uHTS, ultra high throughput screening.

* Corresponding author. Department of Pharmaceutics and Biopharmaceutics, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, CH-1211 Geneva 4, Switzerland. Tel.: +41 22 3796339; fax: +41 22 3796567.
E-mail address: Tudor.Arvinte@pharm.unige.ch (T. Arvinte).

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[1]. Biopharmaceuticals have generated in excess of $33 billion in sales annually with an expectation to reach $70 billion by 2010 [1]. The expected increase in the number of protein drugs highlights the need for improved methods to optimize the development of protein formulations.

During the Human Genome Project [2], a sustained development of high throughput screening (HTS) occurred: automated preparation of a large number of samples and screening of their properties in multi-well plates. Also powder dispensing [3,4] and weighing [5] in submilligram quantities have recently become available. At the moment, the focus in the field of HTS is on drug discovery, functional genomics and combinatorial chemistry. The pharmaceutical industry has invested billions of dollars in the discovery of new targets and leads during the last 10–15 years. New techniques, for example, the development of ultra high throughput screening methods (uHTS), have increased the number of assays per day from several thousands to 100.000 or more [6,7]. Unfortunately, these efforts have led to only a few promising candidates for further development [8]. The yearly reports of Fox [9–11] give a good appreciation of how the pharmaceutical industry is evolving and learning. A shift can be detected from increasing the throughput to a maximum amount of assays per day to a lower throughput with higher quality screening data [12].

Extensive studies are required to fully characterize the physical and chemical properties of a new biopharmaceutical drug. Biological malfunctions and hence many different forms of diseases can be induced by the failure of proteins to fold correctly, or to remain correctly folded [13]. In several *in vitro* experiments, protein aggregates have been found to be particularly cytotoxic. Pre-fibrillar aggregates, rather than mature fibrils, are probably responsible due to an intrinsic ability to impair fundamental cellular processes by interacting with the cell membrane [13].

In an optimized protein formulation, the protein should remain stable for several years, maintaining the active conformation, even under unfavorable conditions that may occur during transport or storage. The optimal combination of excipients can be found employing a high throughput formulation (HTF) platform designed to allow invasive and non-invasive screening in well plates. An HTF platform consists of two parts: (i) sample preparation and (ii) sample analysis. Several high throughput techniques already exist to characterize the stability and activity of protein formulations. For example, a high throughput formulation method was implemented by members of the group of Arvinte in 1998 for the characterization of TGF-β3 in aqueous solutions [14]. In 2002 Nayar and Manning described aspects of the HTF concept [15]. Formulation is crucially important in biotechnology and formulation activities need to be brought forward early in the pharmaceutical development process [16].

Thus, protein formulation screening needs to be performed before the assessment of safety, toxicity, ADME (absorption distribution metabolism excretion), pharmacology and the testing of biological activity in animals. At present, protein formulation in the pharmaceutical industry is generally a slow process and will benefit from a fast formulation screening approach. In this review, the chemical and physical degradation pathways of proteins are outlined, followed by a discussion of the most important HTS techniques and their applicability in protein formulation.

2. Protein formulation

2.1. Introduction

To better comprehend the use of high throughput screening methods for protein formulation, the following issues are discussed: (a) chemical degradation, (b) physical degradation, (c) basics on protein formulation and (d) accelerated stability tests. Practical experience has shown that there are no general stabilization approaches for proteins [17] and that for each protein a customized formulation needs to be developed. The shelf-life for an economic viable protein drug is between 18 and 24 months [18], which is difficult to maintain due to both chemical and physical degradation of the protein. The protein in the formulation has to be stabilized in its active form with minimized degradation.

2.2. Chemical degradation

Chemical degradation of proteins refers to modifications involving covalent bonds, such as deamidation, oxidation, hydrolysis and disulfide bond shuffling [19,20]. The most common chemical degradation pathways are deamidation and oxidation. Deamidation of Asn and Gln residues is an acid and base catalyzed hydrolysis reaction, which results in the naturally occurring amino acid residues Asp and Glu. Deamidation is much reduced at pHs approaching neutral. The sequence, conformation and flexibility of the protein is important for the catalytic effect of appropriate residues or side chains in close proximity to Asn or Gln [21]. Asn residues deamidate via the formation of cyclic imide intermediates. Imide formation is in principle reversible [21]. Degradation by oxidation most likely occurs at the side chains involving histidine, tryptophan, tyrosine, methionine and cysteine groups.

The pH of a protein formulation is an important factor that influences the rate of oxidation by changing the oxidation potential of oxidants, the binding affinity of catalytic metal ions and ionizable amino acids and the stability of oxidation intermediates [22]. Tryptophan can undergo photooxidation, resulting in the formation of kynurenine and *N*-formylkynurenine [23]; methionine is oxidized by atmospheric oxygen [19]. Disulfide bond exchange between cysteines can lead to an altered three-dimensional structure resulting in a loss of activity [24]. Other chemical degradation pathways described in the literature [21,23,24] include glycation, carbamylation, cleavage of peptide bonds and β-elimination.

Characterization of chemical degradation products is often performed with liquid chromatography, electrophore-

sis or mass spectrometry. Raman and Fourier-transform infrared (FTIR) are two spectroscopic methods that can detect chemical degradation of proteins. Other methods, such as fluorescence spectroscopy, could also be applied if the chemical degradation resulted in conformational changes, or if the degradation site is in the proximity of a fluorophore.

2.3. Physical degradation

Proteins are complex molecules and especially smaller proteins and peptides can have a large number of possible conformations. The three-dimensional folded state of a protein may have several thermodynamic favorable conformations, where the native state may or may not involve the energetically most favored conformation. A small fraction of protein molecules may occupy higher energy states [22]. Physical degradation of proteins includes conformation changes, undesirable adsorption to surfaces, denaturation, precipitation and aggregation [20,24]. Protein aggregation is a major hurdle in almost all phases of the drug development. Pathologic protein aggregates are the cause of >20 different diseases in humans, e.g., Alzheimer's disease, Parkinson disease and prion diseases [13]. Accordingly, the presence of a large percentage of aggregates in a protein medicine is unacceptable [25]. Avoiding protein aggregation contributes to the preservation of the required biological function [26]. Several mechanisms of physical aggregation have been proposed, e.g., double nucleation (for reviews, see Refs. [20,21,24,25]). Temperature, pH of solution, protein concentration, freeze–thawing, freeze–drying, spray–drying, reconstitution and agitation are some of the main external factors that influence the aggregation of proteins. For example, the blood coagulation cofactor rFVIII aggregates as a function of the pH and a correlation between the disappearance of monomeric rFVIII and the loss of its activity has been found [27]. Another example is the comparison of the aggregation/activity relationships of human and salmon calcitonin. An enhanced potency for human calcitonin was found when peptide fibrillation was avoided [28]. Protein adsorption to container surfaces and at air/water interfaces depends on the specific properties of the protein, concentration, ionic strength, pH, surface composition and hydrophobicity [29]. A transition from α-helix to β-sheet conformation has been reported for hen egg lysozyme adsorbed to homogeneous self-assembled monolayer surfaces deposited on polycarbonate membranes [30]. Physical degradation can be characterized by a variety of analytical methods, for example, UV–Vis transmission and fluorescence spectroscopy, light scatter and circular dichroism. These techniques are further explained in the corresponding sections.

2.4. Protein formulation

Protein physical and chemical stability are important. However, formulations to address these issues also need to be considered with reference to other aspects such as: (a) the interactions with biological components, (b) cellular mechanism of action, (c) delivery and targeting in relevant animal models (d) human clinical results (e) the route of application (f) technical feasibility and (g) user friendliness. The following factors need to be studied when producing a stable protein formulation: protein concentration, pH range and buffer type, excipient type and concentration, storage temperature, lyophilized or in solution, multi- or single dose. The protein concentration is dictated by the therapeutic dose, dosage form and stability.

A reported study of the selection of the appropriate excipient and the amount needed for a particular monoclonal antibody has shown that a specific sugar to protein molar ratio was sufficient to provide storage stability for up to 33 months [18]. For multi-dose liquid protein formulations preservatives are needed. Preservatives are known to accelerate aggregation and chemical decomposition of proteins. This is the case for human growth hormone in the presence of phenolic compounds [31]. A high throughput formulation screening can help for fast finding the most suitable excipients, such as sugars or preservatives.

Several approaches have been applied for the formulation of biopharmaceutical drugs including the trial-and-error approach, the formulation decision trees [32] and the use of combinatorial techniques [33]. Whichever technique is chosen, knowledge at the molecular level of the protein is beneficial. Protein stabilization can be achieved by internal and external stabilization [22]. Internal protein stabilization is a modification in the amino acid sequence that results in an increase of the stabilizing forces without changing the overall conformation [17].

The current review focuses on screening methods for finding the optimal ingredients that stabilize protein drugs in aqueous solutions. The addition of excipients may alter the environment of the protein, resulting in either an increase or decrease in protein stability. A summary of around 60 commonly used excipients in protein formulations is listed in Table 1 [15,19,21,34–36]. Excipients can have different effects on the chemical and physical degradation of a protein. For example, freeze–drying of human growth hormone in the presence of sodium chloride causes chemical degradation by oxidation and deamidation processes [37]. Studies on the biochemical composition of animals surviving in a dry environment and anhydrobiotic organisms led to the discovery of trehalose, a disaccharide, involved in stabilizing membranes and proteins [38,39]. Trehalose is a good protein drug stabilizer and was introduced in 1998 as a pharmaceutical ingredient by Genentech for Herceptin®.

2.5. Accelerated stability tests

A particular formulation may have no immediately apparent effect on physical or chemical stability. Therefore, accelerated stability tests can help in facilitating the determination of the most suitable excipients and concentrations

Table 1
A list of commonly used excipients in protein formulations

Excipient	Examples
Salts	Ammonium sulfate, calcium chloride, magnesium sulfate, magnesium chloride, potassium chloride, sodium chloride, sodium gluconate, sodium sulfate, zinc chloride
Buffers	Acetate, carbonate, citrate, citrate–phosphate, glycine, HEPES, histidine, maleate, phosphate, succinate, tartrate, triethanolamine (Tris)
Sugars and polyols	Cyclodextrins, fructose, glucose, glycerol, inositol, lactose, maltose, mannitol, sorbitol, sucrose, trehalose
Amino acids	Alanine, arginine, aspartic acid, glycine, lysine, proline
Surfactants	Poloxamer 188/407, polysorbate 20/40/80, sodium lauryl sulfate
Antioxidants and preservatives	Ascorbic acid, benzyl alcohol, benzoic acid, citric acid, chlorobutanol, *m*-cresol, glutathione, methionine, methylparaben, phenol, propylparaben, sodium sulfite
Polymers	Dextran, polyethylene glycol
Other	Albumin, dimethyl sulfoxide, EDTA, ethanol, thioglycolic acid

The included excipients are FDA approved for parenteral administration and part of the inactive ingredients list or part of FDA approved biopharmaceuticals [15,19,21,34–36].

[40]. Storage at different temperatures (0–50 °C), illumination of samples, mechanical stress, multiple freeze–thaw cycles, oxygen purge, increased humidity and seeding are different ways to accelerate protein degradation [20,40]. An example of an high throughput accelerated stress study, which could distinguish between protein stabilizing excipients, was the induction of aggregate formation by mechanical stress in combination with a HTS turbidity assay [41].

The International Conference on Harmonisation (ICH) Guideline Q5C on stability testing of biotechnological products advises that the drug manufacturer should provide data on the stability of the biopharmaceutical drug, incorporating information on the many external conditions that can affect the potency, purity and quality. Accelerated stability tests are not a guarantee of reduced degradation under normal storage conditions, since the degradation measured at higher temperatures may be different to those at lower temperatures. Proteins in particular can unfold at higher temperatures. Stability data of the drug during a long-term, real-time, real-condition stability study remain necessary [42].

3. High throughput screening methods

3.1. Introduction

The development of high throughput screening equipment and assays has increased over the last 10 years. HTS is used in many scientific areas, e.g., catalyst screening, food processing, chemical synthesis, drug discovery, ADME toxicological and cell based screening. In this section, several analytical techniques are discussed with potential use in high throughput protein formulation studies. In 1998, Auer [43] wrote that HTS within most pharmaceutical companies involves performing several million assays per year. The development of ultra high throughput screening (uHTS) has increased the throughput to >100.000 assays per day [6]. The term high throughput is relative in respect to the number of samples tested and depends on the type of information needed. Even with relatively few assays performed per day the concept of: "high throughput protein formulation" (Fig. 1) remains.

A HTF platform can be subdivided in two parts: (i) sample preparation and (ii) sample analysis. First, the preparation of the protein formulation consists of mixing excipients with the protein using liquid and powder handling systems. These automated systems can be integrated with e.g., robotic arms, barcode readers, stackers, shakers, cooling or heating systems, incubators, centrifuges, filtration stations, plate sealers, washers and a variety of detection systems. A well plate is prepared using one or more of the techniques and can be given a unique barcode. The sample preparation should not induce stress on the protein, e.g., shear forces due to fast pipetting.

After preparation the well plate will be analyzed and/or stored for future analysis. Drug specific detection methods need to be developed to characterize the physical and chemical properties of the protein formulation. A continuous evaluation of the data, followed by designing new protein formulations, has to be done manually, or with the aid of specialized data analysis software. All these processes can be adapted for high throughput protein formulation (summarized in Fig. 1). The important factors in

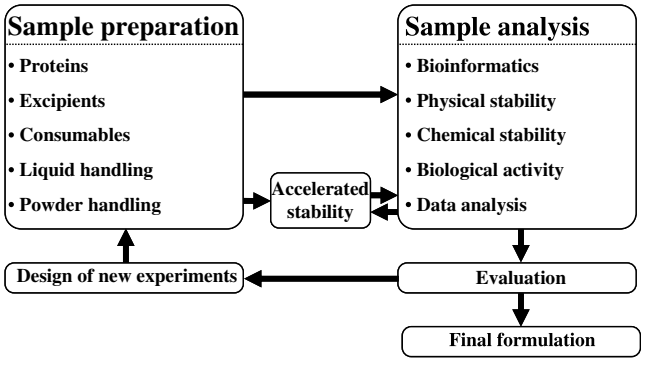

Fig. 1. A flow-diagram of the high throughput formulation concept is shown. Each protein formulation starts with the sample preparation followed by the analysis, with or without an accelerated stability test. After data analysis and interpretation a new experiment can be designed.

the field of high throughput protein formulation are: (a) minimizing the amount of protein needed, (b) selecting the correct assay(s) and consumables, (c) preparing formulations using an excipient library, (d) integrating multiple automated techniques, (e) continuous data evaluation and (f) flexibility in designing new experiments. Assay miniaturization can reduce the amount of protein and excipients. However, the possibilities of screening many parameters often increase the amount of protein used in the HTF studies; the simultaneous measurement of different parameters (e.g., absorbance and fluorescence) offers benefits here.

Techniques typically applied to analyze a protein and its degradation products include: column chromatography, electrophoresis, spectroscopy, thermal analysis, light scattering and mass spectrometry [40]. It is important that these techniques be complemented with methods to assess the biological activity (outside the focus of this review article) of the protein formulation. With respect to the possibility of recovering the protein formulation after the measurement, HTS techniques can be classified into two groups: non-invasive and invasive techniques. The non-invasive methods, such as UV–Vis absorbance, fluorescence spectroscopy and light scatter, can be used consecutively to characterize the protein formulation saving consumables and products. Afterwards, depending on the volumes required, one or more invasive techniques can be applied, such as calorimetry, capillary electrophoresis (CE), liquid chromatography (LC) and mass spectrometry (e.g., coupled to either CE or LC). Methods with potential use in high throughput formulation screening are listed in Table 2.

Table 2
Methods with potential use in high throughput formulation screening and their references

	Methods	References
1	Bioinformatics	[152,153]
2	UV–Vis absorbance	[14,52–54,58,59,102]
3	Fluorescence intensity	[6,7,80,155]
4	Resonance energy transfer	[82–84]
5	Fluorescence lifetime	[95–97]
6	Fluorescence microscopy	[159,160]
7	Fluorescence anisotropy	[88,92,93]
8	Raman spectroscopy	[107,108]
9	Light scatter	[56,103,105]
10	CD	[61–63,68]
11	FTIR	[116–118]
12	Calorimetry	[123,124]
13	X-ray crystallography	[143,145,147–149,149]
14	HPLC	[125,127,128,137,156,157]
15	Size-exclusion	[158]
16	Mass spectrometry	[138–140,157,158]
17	Capillary electrophoresis	[125,131–135]
18	NMR	[161,162]
19	Surface plasmon resonance	[163,164]
20	Ultracentrifugation	[165,166]
21	Off-gel electrophoresis	[129,130]

3.2. Non-invasive high throughput screening for protein formulation

3.2.1. High throughput spectroscopy

In our experience, high throughput spectroscopy is one of the most powerful and versatile HTS techniques available for the characterization of protein formulations. The three most common detection techniques in high throughput screening are UV–Vis absorbance, fluorescence and luminescence. In luminescence assays no external energy source is required, as the emission of light is caused by e.g., (bio)chemical reactions. A review of the available spectroscopic microplate readers was written in 2004 by Malik [44].

Many different types of well plates are available. They can contain a different number of wells and are available as 6-, 24- to 96-, 384- and 1536-well plates. Black well plates are suited for fluorescence assays because of the prevention of light interference between wells. White well plates are used in luminescence measurements due to the dispersion of light and the resulting increase in light intensity. Transparent microplates are used mainly for UV–Vis absorbance assays. The microplates are made from polypropylene, polystyrene, glass or special proprietary acrylic UV transparent plastics. The bottom of the wells can have different shapes: round or "U" bottoms for mixing and retrieving material from the wells; conical or "V" bottoms for concentrating material on the bottom and flat or "F" bottoms optimized for spectroscopic detection. For liquid handling deep well plates have been developed that contain up to 2.2 ml per 96 wells. The choice of microplate depends on the assay, volume, costs and available equipment and compatibility. For protein characterization a minimum auto-fluorescence and UV absorbance of the microplates and seals is required in order to determine the intrinsic spectroscopic properties of the protein. The trend of equipment manufacturers is to miniaturize the wells and retain the sensitivity of the assay to save on reagents and materials. The 3456-well plate is already used in ultra high throughput screening; each assay requires only 1 μl. Affymax is reported to be working on a microplate that consists of 20,000 wells in which each well holds a volume of 25 nl [45]. Protein adsorption to surfaces, sample homogeneity, evaporation and detection are only some of the problems that arise when working with 25–1000 nl.

In a microplate the light beam passes through a liquid–air interface. The type of plastic, excipients (e.g., surfactants) and protein influence the contact angle, and thus the radius of the meniscus and pathlength of the light in the solutions in the well plate. A Beer–Lambert Law linear correlation exists between the optical pathlength (volumes) and the near infrared absorbance of water at a wavelength around 977 nm [46]. This pathlength control assay can be used not only for the comparison of absorbance units (AU) of measurements that have different pathlengths (microplate versus cuvette) but also for controlling

multichannel pipettors and the liquid levels in the microplates after prolonged storage [46]. Another approach to resolve the problem of the differences in the meniscus is the use of well plate inlets that have been developed to generate a homogeneous pathlength [47].

In protein formulation, different excipients can be screened that might have different effects on the surface tension. The linear correlation between optical pathlength (volume) and absorbance for each tested mixture of excipients needs to be optimized. To give an example, consider the dispensing of different volumes of water and a 200 mg/ml bovine serum albumin (BSA) solution in a 96-well plate. The absorbance of water at 975 nm minus the absorbance of empty wells plotted against the volumes resulted in two linear correlations (Fig. 2). Four samples per volume were prepared and the average values were calculated. The water solutions have a longer pathlength than the BSA solution, caused by the differences in surface tension. The linear correlations for both solutions are:

For water(▲) :

$$\text{Absorbance} = 6.31 \times 10^{-4} \times \text{Volume}(\mu\text{l}); \quad R^2 = 0.9968 \tag{1}$$

For BSA(●) :

$$\text{Absorbance} = 5.58 \times 10^{-4} \times \text{Volume}(\mu\text{l}); \quad R^2 = 0.9991 \tag{2}$$

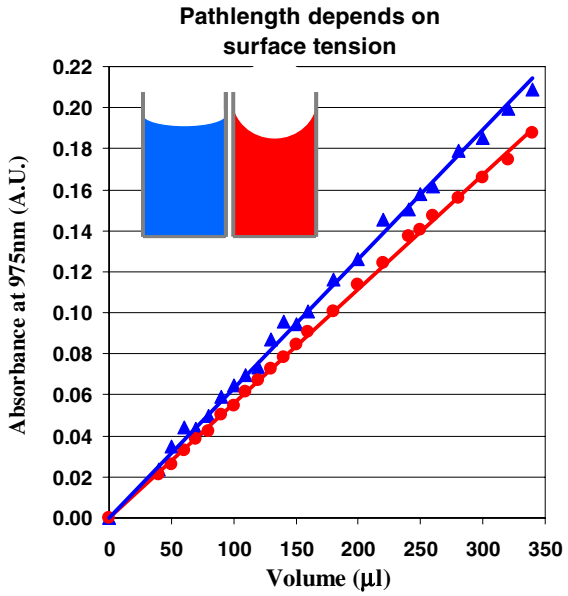

Fig. 2. The pathlength in a Corning Costar UV transparent 96-well plate was measured for water and a 200 mg/ml BSA solution. The average absorbance of four empty wells was deducted from each absorbance measurement at 975 nm. The wells filled with water (blue, ▲) have a longer pathlength than the wells filled with the same volume of a 200 mg/ml BSA solution (red, ●). The origin of the variation in pathlength is the difference in contact angles between the two solutions and the microplate, resulting in changes in surface tension and meniscus curvature. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

where R^2 is the correlation coefficient. If an absorbance is measured of 0.189 AU, the calculated volume when using Eq. (1) will be 300 µl and when using Eq. (2) 339 µl. A precision of less than 3% in the volumes dispensed was obtained with a Sciclone ALH500 from Caliper Life Sciences. These data show the importance of correcting for the pathlength of protein solutions in microplates.

Microplates cannot always be measured immediately after preparation and are stored in libraries, storage chambers, stackers or on the bench top. The prevention of evaporation requires that the microplates be properly sealed. Even when a seal was applied, depending on its characteristics, the solutions could evaporate with time [48]. The water evaporation of a fluorescein solution in 96-, 384- and 1536-well plates with and without a seal will be stronger at the corners and borders of the well plate than in the centre [49]. That water evaporation increases with an increase in number of wells has been attributed to changes in surface–water interface and the meniscus curvature [49]. Already after 5 h up to 25% of water can evaporate when no seal is applied. Automated application and removal of tight-fitted seals or covers [50] offers the possibility to reduce evaporation and to measure the samples without the interference of a seal or cover. Changes in the temperature and humidity of the laboratory should be avoided in order to reduce the evaporation and eliminate possible condensation. UV light transparent seals are available to study protein absorbance and fluorescence in microplates. From our experience, the UV transparent pressure sensitive seals were better than the glue containing seals, since the glue may interact with the protein solutions.

3.2.2. UV–Vis transmission spectroscopy

UV–Vis transmission measurements are used in several HTS assays for the determination of protein concentration [51,52], colorimetry [53,54], solubility and turbidity [14,55,56]. In a study of the determination of haemoglobin concentration a comparison between the use of cuvettes and microplates has been made. The reported advantages of using microplates were the increased speed and improved statistical accuracy due to the possibilities of measuring replicates [57]. Most microplate readers are capable of measuring UV–Vis absorbance in transparent 96- and 384-well plates. Lavery has shown the feasibility of measuring absorbance in white 1536-well plates by using fluorescent properties [58]. A colored product can quench the fluorescence signal from a microplate by absorbing either the excitation or emission light. The decrease in light intensity can be measured as an absorbance signal [59]. The advantage of miniaturizing UV–Vis absorbance assays are the reduction of reagent costs, consumables and increasing throughput, which is especially evident when running kinetic measurements. Protein aggregation can be characterized by measuring the turbidity (apparent absorbance) at wavelengths >350 nm, since most proteins do not absorb around these

wavelengths [60]. This assay will be explained in more detail in Section 3.2.8.

3.2.3. Circular dichroism (CD)

CD is based on the differential absorption of left and right circularly polarized light as a function of wavelength. CD and UV absorbance detection at a fixed wavelength have been used in a high throughput HPLC flow through assay to distinguish between enantiomers of 1-phenylethanol with a throughput of 700–900 assays per day [61]. Such a throughput is not feasible for the characterization of protein solutions, since the measurement of an entire CD spectrum is required to characterize conformational changes. Far UV measurements (160–250 nm) are used to assess the secondary structure content of a protein/peptide. Near UV measurements (230–320 nm) provide conformational information about aromatic amino acid side chains and disulfides. Measurements at longer wavelengths can be used to monitor prosthetic groups (haems) and colored additives such as dyes. The potential for CD monitoring at wavelengths longer than 220 nm is similar to ordinary absorption although the presence of the meniscus in a well plate may seriously affect CD measurement.

It has been claimed that replacing the Xe lamp in a CD spectrometer with a synchrotron radiation (SR) source enables greater penetration in the UV to 160 nm. However, concentrations of ~10 mg/ml are required with pathlengths of ~50 μm. This implies large amounts of protein for screening although ~1 mg quantities in 0.1 ml may keep the quantities down. It has been claimed that the synchrotron radiation source enables measurements down to 160 nm, with a higher signal-to-noise ratio particularly in the presence of buffers and absorbing components [62,63]. Spectral differences were observed after repeating SR circular dichroism measurements more than twice on the same protein sample due to the high flux of photons [64]. However, a modern conventional laboratory-based CD spectrometer can match SR-based CD spectrometers in most cases certainly down to 170 nm.

Several articles [65–67] describing high throughput screening methods monitoring biophysical properties of proteins have used CD as a complementary tool for secondary structure analysis. Sample throughputs for measuring CD protein spectra were reported as between 1 and 5 samples per hour, depending on the measurement parameters. In practice, a single CD spectrum over the 250–190 nm range can take as little as ~3 min.

A CD spectrometer can operate as a multi-mode polarization modulation spectrometer that measures simultaneously the UV absorbance, turbidity and optical activity spectra [68]. This provides another option to expedite sample throughput. The main challenge in the use of CD spectroscopy for high throughput screening lies in the sample presentation to the spectrometer. The need for a meniscus-free solution with sub-millimeter pathlengths suggests that sampling methods based upon flow rather than well plates are likely to be the methods of choice.

3.2.4. Fluorescence intensity

Fluorescence measurements have been used extensively in protein formulation and characterization studies and are the method of choice in both HTS and uHTS due to its high sensitivity and speed. A detailed description of how to apply fluorescence spectroscopy for protein characterization has been written by Jiskoot et al. [69]. Proteins that contain the amino acids tryptophan, tyrosine or phenylalanine are used in intrinsic fluorescence assays. The use of specific fluorescent probes can give additional information about the protein conformation. The measurement of the fluorescence intensity provides information on the local conformation and environment of fluorophores. Spectroscopic parameters like quantum yield, extinction coefficient, emission wavelength and spectral shape of the fluorophores depend on their local environment [70]. In protein formulation studies the addition of excipients or changes in pH can induce a conformational change, which might result in a red- or blue shift and decrease or increase in fluorescence intensity. For example, we characterized the unfolding of BSA, from the globular heart-shaped conformation at pH 6 to the elongated unfolded form at acidic

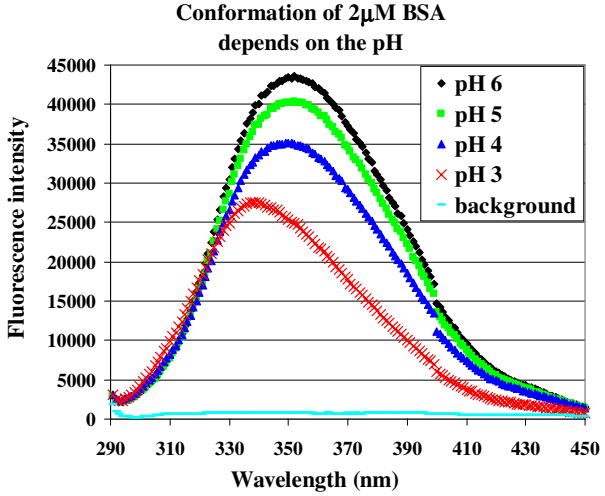

Fig. 3. The BSA fluorescence emission was measured in a 10 mM sodium citrate buffer after excitation at 280 nm. The average spectrum for each pH value was calculated from four fluorescence emission spectra. A measurement was made at each nanometer using the Tecan Safire microplate reader. The variation between the fluorescence values of the four replicates was less than 3%. The red (×), blue (▲), green (■) and black (◆) fluorescence spectra correspond with the BSA at pH value 3, 4, 5 and 6, respectively. The background signal of the buffer solution is shown in turquoise. The decrease in pH resulted in a decrease in fluorescence intensity, indicating that the two tryptophan amino acids in BSA are in a more hydrophilic environment as the protein unfolds. The blue shift of 12 nm, observed when lowering the pH value from 6 to 3, suggests that the tryptophan groups are more rigid at pH3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

pH, by measuring the intrinsic Trp fluorescence emission spectra in 10 mM sodium citrate buffer. The unfolding of BSA from pH 6 to pH 3 resulted in a 37% decrease in fluorescence intensity and a blue shift of 12 nm (Fig. 3). Protein unfolding studies that were based on titration with denaturants, such as urea or guanidium hydrochloride, have been performed in high throughput using the change in intrinsic protein fluorescence for the determination of conformational transitions [71]. The resistance of proteins towards unfolding was correlated with an increased stability [71].

Fluorescence intensity measurements have also been applied for screening of protein interactions. The binding of a ligand to a protein can result in a large decrease in tryptophan fluorescence intensity due to dynamic or static quenching [70], examples in Refs. [72,73]. The throughput of intrinsic fluorescence is hindered due to the measurement of the entire fluorescence emission spectrum. According to Tecan, the time to measure the fluorescence emission with a 1 nm increment between 300 and 450 nm using a Safire^2 microplate reader, including the plate in and out movement, is for a 96-well plate 6 min and for a 384-well plate 22 min. The UV transparent 96-well plates are about three times more expensive than 96-well black microplates. The advantages of UV transparent microplates include the possibility to measure consecutively the UV absorbance, fluorescence and light scatter. Our experience is that it is of advantage to measure the fluorescence from the bottom of the plate, since there is less optical interference from the liquid meniscus.

Other fluorescence assays can be designed when different dyes are added to the protein solutions. For example, fluorescein or fluorescein derivatives are frequently used to increase sensitivity in fluorescence microscopy. For fluorescence polarization UV absorbing dyes such as pyrene and isomers of 5-dimethylaminonaphthalene-1-sulfonate (DANS) are often employed because of their long fluorescence lifetimes [69]. A dye frequently used in protein characterization studies is 1-anilino-naphthalene-8-sulfonate (ANS). Binding of ANS to hydrophobic regions of the protein results in an increase in fluorescence intensity, which has been applied in the characterization of the molten globule state of fetuin as a function of pH [74]. The advantages of using fluorescent probes are the high throughput when the intensity is measured at a single wavelength. Filter-based plate readers, which are less expensive than monochromator-based readers, can be used. One effect to be investigated is the influence of fluorescent probes on the conformation of the protein, since it was reported that ANS induced, as a result of electrostatic interactions, folding of unfolded horse cytochrome *c* at acidic pH [75]. The intrinsic and extrinsic fluorescence measurements are complementary and are both necessary for interpreting possible changes in protein conformation and stability.

Fluorescence intensity distribution analysis (FIDA) and related single molecule detection systems are other fluorescence techniques that have been reviewed extensively for HTS [76,77]. FIDA allows the characterization of labeled molecules with respect to their molecular brightness and concentration at the single molecule level and can distinguish between bound and non-bound molecules. This technique uses a highly focused laser beam with a detection volume of around 1 fl. The signal is not affected by miniaturization and can be used for uHTS. The molar concentrations of bound and free ligand are quantitatively determined from a single measurement [78,79]. In 2D-FIDA, two detectors monitor the fluorescence intensity of either different wavelengths or different directions of polarization. The main advantage of this method is the concomitant information of two detectors, which allows additional detection modes, improves the statistics, and hence makes shorter measurement times possible [80]. The problems with the single molecule detection methods are sample evaporation, liquid handling and surface adsorption and less so the detection efficiency. Another drawback of using these methods for protein formulation studies is that the fluorophore concentration is limited to less than 50 nM if fluorescence fluctuation techniques are used. To our knowledge, the single molecule detection techniques have not yet been applied for the formulation of proteins, protein (un)folding and protein–protein interaction assays.

Fluorescence detection in HTS can be performed with a photomultiplier tube, as is used in conventional fluorescence microplate readers, or with a charge-coupled device (CCD) camera. CCD imaging has been applied in microscopes, fluorescence confocal detectors and fluorometric imaging plate readers. CCD detection devices are used to image entire microplates at once, which is inherently faster than moving a detector from well-to-well, which makes CCD imaging useful for high throughput kinetic studies [81]. Applying different magnifications in CCD enables the discrimination between entire populations, general structures within wells and when using a high magnification can resolve specific structures inside cells [81]. Image-based screening might be beneficial during protein formulation for the structural characterization of protein aggregates, protein crystals and specific drug delivery systems.

3.2.5. Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) has been used in high throughput screening especially for studying protein–protein interactions, quantification of enzyme activity and cell based assays [82–85]. In resonance energy transfer, the excitation light absorbed by a molecule (energy donor) is transferred to a neighboring molecule (energy acceptor) if the distance between the two molecules is less than 50 Å [70]. For FRET an overlap is required between the emission spectrum of the donor and the excitation spectrum of the acceptor molecule. The effectiveness of the energy transfer depends on the distance between both chromophores and the alignment of the transition electric

dipoles. FRET can therefore be used to measure the distances between the donor and acceptor molecules. For a transmembrane protein the intramolecular distance between two helices has been determined using the energy transfer from tyrosine placed in one helix to tryptophan placed in the other helix [86]. Human serum albumin has been titrated with three dyes to determine the intramolecular distance between the tryptophan and the three binding sites [87].

For the moment, energy transfer studies for characterizing protein formulation are not common practice, but could offer valuable information for formulations consisting of two proteins (e.g., albumin as excipient). Tyrosine or tryptophan residues can be used in FRET as energy donor molecules and dyes as energy acceptor molecules. Knowledge of the intermolecular distance between the donor and acceptor could help to distinguish between stable and unstable protein formulations. For example, Trp in the excited state can transfer its energy to an ANS molecule. The fluorescence emission of ANS increases with an increase in local hydrophobicity and can therefore simultaneously be used as a probe for protein aggregation and as an energy acceptor molecule. Similar resonance energy transfer studies could be designed in high throughput to study protein formulations.

3.2.6. Fluorescence anisotropy

Fluorescence anisotropy (FA) has been reviewed for HTS by Owicki [88]. The anisotropy assay is based on the principle that the fluorescence anisotropy of a chromophore is low when the chromophore is attached to a small structure and high when attached to a large structure [88]. Some examples on the use of FA are the study of protein–protein interactions [89,90] and the determination of equilibrium dissociation constants [91]. An excellent performance of an uHTS fluorescence anisotropy-based assay in the 1536-well format has been demonstrated by Turconi et al. [92]. The fluorescence anisotropy screening was shown to be a low-cost alternative to radioligand binding assays (scintillation proximity assay) [93]. Although high throughput FA has potential use in protein formulation characterization, no microplate assays have, to our knowledge, been reported. Tryptophan or tyrosine fluorescence anisotropy, in combination with fluorescence lifetime measurements, can give valuable information on the local environment, such as the rotational correlation coefficient and local viscosity [69,70,94].

Fluorescence anisotropy high throughput screening has a few associated problems. The polarizers on the excitation and emission sides attenuate much of the light, which results in a decrease in sensitivity. Linearly polarized light will excite only those transition electric dipoles of fluorophores that are oriented in the plane of polarization. Subsequent fluorescence is constrained to polarization in the same plane as the excitation. A relatively high fluorophore concentration is required to obtain a good signal. The anisotropy measurement has to be from the top, since the plastic bottoms of the microplates affect the polarization of light, introducing possible interference caused by changes in meniscus curvature. The microplate readers available on the market use either special filters, lasers, LEDs and the monochromator-based readers are based only on wavelengths in the visible range. Therefore for now, fluorescence anisotropy screening can only be used with fluorescence-labeled proteins.

3.2.7. Fluorescence lifetime

Fluorescence lifetime is the average time of the exponential fluorescence decay of a population of molecules from the excited state to the ground state [70]. The decay in fluorescence intensity is measured in the nanosecond to microsecond range. Fluorescence lifetime measurements can give insight into the physical properties of the fluorophore, binding characteristics, molecular motions, hydrodynamic volume, inter- and intra-molecular distances, energy transfer and fluorescence quenching. Two techniques exist for measuring fluorescence lifetime: time domain and frequency domain. More detailed information on fluorescence lifetime can be found in the book of Lakowicz [70].

Between 1998 and 2002 several articles reviewed the use of a frequency domain-based fluorescence lifetime microplate reader (FLARe = fluorescence lifetime assay repertoire) for high throughput screening of 96- and 384-well plates [95–98]. The disadvantage of the FLARe reader was that only fluorescence lifetimes of several hundred nanoseconds could be measured. The FLARe microplate reader was abandoned by Molecular Devices. At the moment two time-correlated single-photon counting (TCSPC) fluorescence lifetime microplate readers are available on the market (Tecan ULTRA Evolution™ and CyBi®-NanoScan) that can measure fluorescence lifetimes of 1–20 ns with a 0.1 ns resolution. Both microplate readers use pulsed lasers as the light source, which limits the wavelength range. During the protein formulation process the screening of fluorescence lifetime can help in elucidating the local environment of tryptophan or tyrosine residues [69,99], or the local environment of a specific label or dye. The fluorescence lifetime together with fluorescence anisotropy data enables the calculation of the local viscosity and the rotational correlation coefficient of the fluorophores using the Perrin equation [94]. Unfortunately unlike intrinsic fluorescence, anisotropy intrinsic fluorescence lifetime cannot be measured yet with the available microplate readers. For more information on fluorescence lifetime imaging microscopy (FLIM) [98], time-resolved anisotropy (TRA) [98], fluorescence intensity and lifetime distribution analysis (FILDA) [100] and confocal HTS systems [77,80] see cited reviews.

3.2.8. Light scatter

The scattering of light in solution is dependent on different factors, such as the wavelength of incident light, refractive indices, the protein concentration, and particle size and shape [101]. The two main light scatter assays used in high

throughput screening are based on nephelometry and turbidimetry. Nephelometry is the measurement of the intensity of the scattered light at e.g., a 90° angle to the direction of the incident light, as a function of the concentration of the dispersed phase. The light source is in many cases a laser (wavelength ~635 nm) which passes vertically through the microplates. The forward scattered light is detected after elimination of the non-scattered incident light beam by a light trap. Nephelometry can determine in high throughput the solubility of compounds [56]. The optical quality of the microplate, pathlength and meniscus, dust, air bubbles and the color of the solution (depending on the laser used) can influence nephelometry.

A UV–Vis absorbance plate reader can measure the molecular absorbance of compounds as well as the turbidity due to particles present in solution (apparent absorbance). The latter is used in turbidimetry where the intensity of the light beam transmitted by a solution or medium is measured. Outside the UV/Vis wavelength range for electronic transitions, proteins or excipients do not absorb light and the absorbance is zero. At these wavelengths (normally >340 nm) an absorbance signal is observed only when the solution becomes turbid. High throughput aqueous solubility determinations of pharmaceutical compounds have been successfully measured using UV absorbance [102]. The UV absorbance data have been shown to be in good agreement with data obtained with a nephelometer and with a HPLC coupled to a UV detector [103]. High throughput turbidimetry has been applied for the characterization and formulation of TGF-β3 [14]. The conformation, self-association and aggregation were monitored in 96-well plates as a function of pH, NaCl and protein concentration. Another high throughput assay monitored the aggregation of reduced insulin chains at 650 nm in 384-well plates. H_2O_2 was added to stop the enzymatic reaction, which converted the turbidimetric assay to an end-point high throughput method [104].

An example from our work on the characterization of the aggregation properties of salmon calcitonin (sCT) in time is shown in Figs. 4 and 5. We obtained different sCT aggregation profiles depending on the pH of the buffer solution. One 384-well plate was measured continuously at 25 °C for over more than 2 days (Fig. 4). The number of microplates that are measured can be increased by using an automated robotic liquid handling nephelometry system that is composed of robotic transportation, a multichannel liquid dispenser, microplate reader, barcode reader and associated data management software that allows user-free runs [105]. The use of an automated system for protein formulation will increase the throughput by measuring, preparing and analyzing multiple microplates at different time intervals. Another option to increase the throughput is the use of accelerated stability tests to decrease the fibrillation time (t_f) by determining the turbidity at an elevated temperature (e.g., 40 °C) or with continuous agitation. The forced generation of immunoglobulin aggregates after shaking horizontally has been demonstrated by Mahler and coworkers, who concluded that the induction of protein aggregates and absorbance analysis was a good combination for high throughput formulation studies [41]. The advantage of turbidimetry over nephelometry is that no specialized microplate reader is required. Nephelometry

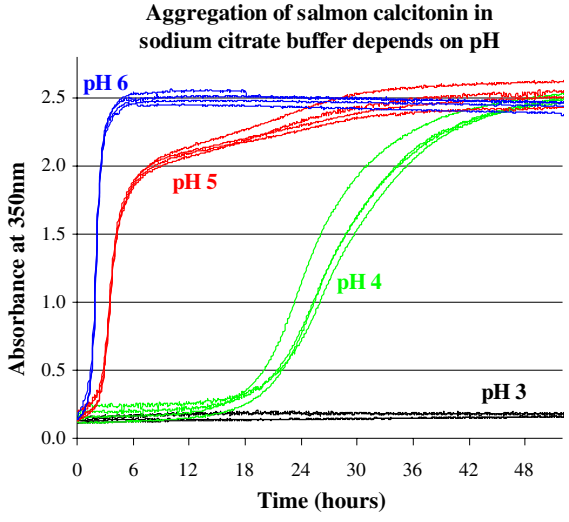

Fig. 4. The aggregation of salmon calcitonin (sCT) was monitored by measuring the turbidity at 350 nm at 25 °C. The formulations (four replicates) consisted of 10 mg/ml sCT in a 10 mM sodium citrate buffer at pH 3 (black), 4 (green), 5 (red) and 6 (blue). The sCT remains stable at pH 3 for at least 2 days, but aggregation occurred for the sCT at pH 4, 5 and 6. The protein formulation was prepared and pipetted in a Costar Corning UV transparent 384-well plate using a Sciclone ALH500 liquid handling station from Caliper Life Sciences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

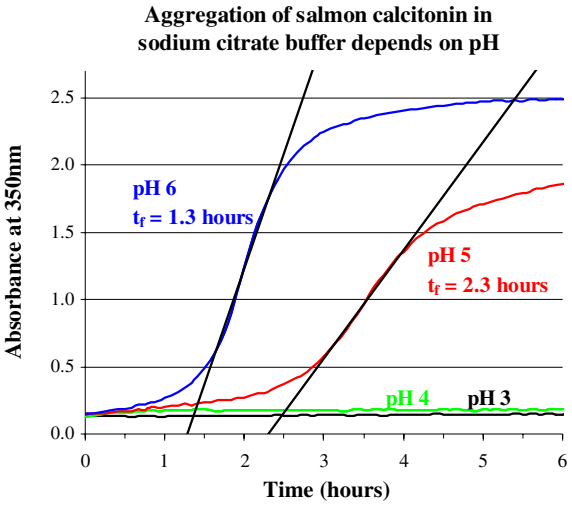

Fig. 5. The fibrillation time was calculated for the average aggregation of sCT in 10 mM sodium citrate buffer. The fibrillation time (t_f) was defined by Arvinte and coworkers [154] as the time corresponding to the intersection with the time axis of the linear increase in turbidity. The fibrillation time calculated for sCT at pH 4, pH 5 and pH 6 was 18 (see Fig. 4), 2.3 and 1.3 h, respectively.

detects the scattered light at a wavelength and angle depending on the microplate reader; in turbidimetry the user can adapt the wavelength of measurement to the expected particle size and turbidity. High throughput screening of turbidity or nephelometry of protein formulations helps in selecting the excipients that do not induce aggregation and promote protein stability. Data analysis of the light scatter assays is based on a yes or no response in regard to protein aggregation or on the interpretation of a kinetic protein aggregation curve (Fig. 5). The light scatter assays can achieve a throughput of the order of thousand microplates per day when measuring at a fixed wavelength, which is similar to throughput of the fluorescence intensity assays [6].

Dynamic light scatter measurements in microplates can be performed using the DynaPro™ Titan from Wyatt Technology (www.wyatt.com). This instrument can determine the size and presence of protein aggregates with a read time of 1 min per well and for protein samples with a minimum concentration of 2 mg/ml. The technique can be used for formulation studies, especially for thorough characterization of the stability in final formulations.

3.2.9. Raman spectroscopy

Raman spectroscopy is a non-invasive analysis technique that can characterize liquid as well as solid protein formulations [106]. Quantification of the secondary protein structure and changes in conformation after application of stress have been reported by several research groups [107–112]. Recently, a Raman microscope was optimized for measuring inside a wide variety of multi-well plates and arrays [107]. A first step towards the use of microfluidics incorporated in a Raman screening platform for polymorphic analysis has been reported by Anquetil et al. [108]. Polarized Raman spectroscopy has determined the β-sheet conformation and orientation of fibroin in silk monofilaments [111]. Fourier-transform Raman spectroscopy and self-deconvolution were used to analyze in solution β-lactoglobulin and ovalbumin. The amounts of α-helix, β-sheet, random coil and β-turn were measured before and after heating the protein formulations. Different bands were distinguished for the β-sheet that were attributed to intra- or inter-molecular β-sheet structures and β-sheet structures exposed to water. After heating and aggregation of proteins an increase in β-sheets and a decrease in α-helices were observed [110]. A formulation study on a therapeutic antibody monitored the amide I Raman band to screen for stabilizing excipients. The antibody β-sheet content decreased during lyophilization. Almost complete structural conservation was observed in the presence of sugar-to-protein molar ratios >360 [112]. Raman High throughput screening systems are produced by several companies, such as Avalon Instruments, Thermo Electron Corporation, Horiba Jobin Yvon and Kaiser Optical Systems. Raman spectroscopy in well plates offers the possibility to analyze in high throughput liquids, gels and solids and there is no signal interference from the seals or microplates. Raman spectroscopy is acceptable in water with glass optics unlike infrared (IR). However, the Raman signal is inherently weaker and more difficult to quantify than its IR counterpart.

3.2.10. Fourier-transform infrared spectroscopy

Infrared spectroscopy can give information on the secondary structure of a protein in the aqueous and solid state. The minimum protein concentration needed to obtain a high quality infrared spectra in water is around 3–5 mg/ml and as little as 5 μl of solution is required for loading the sample cell [113]. These requirements make a high throughput based approach feasible. To our knowledge no high throughput Fourier-transform infrared (FTIR) system has been reported for protein characterization. FTIR is especially suited for the determination of chemical degradation in high concentrated protein formulations. High throughput FTIR spectroscopy started recently to appear in the domain of catalysis research. FTIR imaging detectors based on arrays were used, which contain several thousand elements on an area of only a few square millimeters, where each of these pixels can record a full IR spectrum [114]. A similar high throughput approach described by Kazarian and coworkers, called macro-attenuated total reflection (ATR)-imaging, revealed the mechanism of drug release [115] and characterized different polymer/drug formulations [116] with simultaneous analysis of 100 samples in a controlled environment. Conventional ATR infrared spectroscopy has been applied for structural analysis of protein solutions, such as hen egg white lysozyme [117]. One of the advantages of ATR-FTIR spectroscopy is the possibility to measure in solids, solutions, gels and creams.

High throughput FTIR measurements are difficult due to the presentation of the samples to the FTIR apparatus, but the development of a plate extension device (HTS-XT) from Bruker Optics enabled the coupling of microplates with their FTIR spectrometers. An example of such a microplate-based high throughput FTIR system has been described by Tielmann [118]. Three times 3 μl of a mixture of enantiomers of *N*-1-phenylethylacetamide in 0.02 M DMSO and culture supernatant was transferred into a silicon 384-well plate, followed by an invasive drying step in a desiccator before measuring the FTIR spectra of the samples. A throughput of 9700 samples per day with an accuracy amounting to ±7% of the true values, as measured by gas chromatography, was obtained [118]. A disadvantage of FTIR for protein formulation studies is the interference of the large absorption peak of water. FTIR and macro-ATR-imaging have the potential to become useful HTS techniques in protein formulation, but are at the moment still in the development stage. Conventional infrared spectroscopy measurements can be performed for complementary information on the protein structure. For now, Raman spectroscopy is preferred for high throughput screening of secondary structures and structural perturbations of proteins in solution.

3.3. Invasive high throughput screening for protein formulation

3.3.1. Calorimetry

In contrast to the non-invasive techniques described above, differential scanning calorimetry (DSC) is an invasive method, since the integrity of the protein is generally damaged during the measurement. In DSC, the protein formulation is placed in the sample cell and the reference cell contains the same solution except for the protein. Both cells are heated at a constant rate and the protein in the sample cell undergoes thermal unfolding from a low temperature native state to a high temperature denatured state. The data are analyzed by plotting the differential power input versus the temperature. The temperature midpoint of the transition of the enthalpy change between the native and denatured form is the melting temperature, T_m. The more thermostable proteins need larger amounts of energy to unfold, resulting in a high T_m. The use of DSC in assessing the stability of protein formulations has been reported by several research groups [119–122]. The latest differential scanning calorimeter of MicroCal is adapted for the 96-well plate and is capable of measuring unattended up to 50 samples in 24 h [123].

Another high throughput assay based on calorimetry is called the ThermoFluor®, which can be used for the screening of protein interactions [124]. Proteins that show a high affinity for ligands or excipients can become more thermally stable resulting in unfolding at elevated temperatures. The unfolding of proteins can be screened using a fluorescent dye that binds to the accessible hydrophobic interior of a protein (e.g., ANS) depending on the increase in temperature. Here, T_m is the transition midpoint of the measured increase in fluorescence intensity. The assays were performed in volumes of 4 μl in 384-well thermocycler microplates. In Ref. [124], carbonic anhydrase solutions were overlaid with 1 μl silicone oil in order to prevent possible evaporation. The temperature increase was at a rate of 1 °C per minute and one or more images were collected at each temperature. The typical imaging measurement time of a 384-well plate was 2–30 s using a charge coupled device (CCD) camera [124]. A throughput of one 384-well plate every 50 min was achieved when measuring the thermal stability of the protein formulation between 30 and 80 °C. The ThermoFluor® technique has potential for screening protein formulations, especially regarding the simple readout, data analysis and similar throughput as the spectroscopy screening. The disadvantages of calorimetry are: (i) the samples are destroyed during the measurement, (ii) the relatively low throughput of DSC compared to fluorescence or UV–Vis absorbance spectroscopy, (iii) the difficulty of detecting subtle changes in protein conformation, (iv) in the ThermoFluor® assay the possibility of interference by silicon oil with the proteins in solution and (v) unlike spectroscopy no direct interpretation of the results is possible in terms of molecular events.

3.3.2. Electrophoresis and liquid chromatography

The two main techniques to separate proteins, peptides and their degradation products prior to analysis are electrophoresis and liquid chromatography. Miniaturization of the separation process to capillary columns has enabled a direct coupling of these systems to a mass spectrometer, resulting in automation of analysis with high reproducibility and sensitivity [125]. Other frequently used detection modes for capillary-based separations are UV absorbance and (laser induced) fluorescence. Depending on the mode of electrophoresis the separation is based on electrophoretic mobility, size, charge, hydrophobicity, affinity (specific interactions with other molecules) or iso-electric point. The chromatographic modes used for the separation of biomolecules are size-exclusion, ion-exchange, reversed-phase, ion-pair reversed-phase, hydrophobic interaction and affinity chromatography [125]. All these separation modes have been performed in capillaries. Self-interaction chromatography, an adaptation of affinity chromatography, and the measurement of the osmotic second virial coefficient were successfully applied in a formulation screening of a commercial amylase [126].

Another approach to increase sample throughput is by reducing separation time without reducing the quality. High temperature LC [127] and ultra-high pressure LC [128] have been described with run times of a few minutes. However, characterization of protein formulations might be compromised due to chemical degradation inflicted by the separation under extreme conditions. Several instrument manufacturers have incorporated autosamplers supporting the 96- and 384-well plate format, e.g., Agilent Technologies, Beckman, Waters, Gilson, Varian and Dionex.

Capillary electrophoresis and liquid chromatography have several advantages for high throughput screening of protein formulations, such as high separation efficiency, fast separation time, low reagent consumption and low sample volume. Possible issues with capillaries are protein adsorption to the capillary walls, which can lead to variable migration times, band broadening and peak tailing. An advantage of capillary electrophoresis over liquid chromatography (LC) is that CE can be used to analyze proteins and peptides with different sizes, whereas LC is less suited for the analysis of large protein molecules. Another advantage of CE over LC is the possibility of analyzing the protein formulations in an aqueous environment without adding organic co-solvents. More information on electrophoretic and chromatographic separation techniques for the high throughput analysis of proteins can be found in the review by Huck and coworkers [125].

Besides electrophoresis inside capillaries and on gels, off-gel electrophoresis techniques [129] have been developed. Separation of proteins according to their iso-electric points has been reported in a multi-well off-gel electrophoresis device allowing the direct recovery of the proteins in solution to facilitate further analysis [130]. The resolution of

fractionation in either 96- or 384-well off-gel electrophoresis devices has been shown to be 0.1 pH unit for the separation of complex biological mixtures [130].

Multiplexed capillary electrophoresis systems have been described with 48 [131], 96 [131,132] and 384 [131,133] capillaries for high throughput protein analysis. A summary of the available multiplexed capillary electrophoresis systems is given by Pang et al. [134]. A tryptic digest of bovine β-lactoglobulin resulted in 17 different peptide fragments, which were separated using a 96-capillary array and six different separation conditions within 45 min [135]. Differences in migration times and peak intensities between capillaries have been reported, which can be resolved by the application of an internal standard [135]. Addition of an internal standard alters the protein formulation and should therefore be avoided. No internal standard is needed for the identification of protein degradation products. The presence of many capillaries, one near to another, generates a high temperature with identical separation conditions, resulting in the formation of bubbles and loss of current in some of the capillaries [135]. In order to avoid these high temperatures a lower current can be applied, which results in an increased analysis time and lower sample throughput.

3.3.3. Mass spectrometry

Mass spectrometry and tandem mass spectrometry detection are used in protein formulation for the analysis of chemical and enzymatic degradation products. The degradation products, separated by LC or electrophoresis, can be analyzed and identified by MS. Automated sampling from microplates and the coupling of mass spectrometry with different separation techniques have been performed in a high throughput manner [136–138]. Electrospray ionization (ESI) mass spectrometry is more sensitive to the presence of salts in the samples than matrix assisted laser desorption ionization (MALDI) MS. Protein formulation often consists of salts and possible metal ions that have to be removed prior to MS analysis by desalting or specialized sample preparation techniques. Automated on-line sample preparation and desalting of oligonucleotides in combination with ESI-MS analysis has been reported having a throughput of 1000 samples per day [139]. The sample preparation prior to ESI-MS analysis can induce protein physical or chemical degradation and the incorporation in a matrix may be more suitable. An example of sample preparation is the use of a robotics platform for mass spectrometry immunoassays, comprising e.g., sample incubation, repetitive aspiration, dispensing, rinsing and mixing with the matrix before dispensing the matrix/analyte mixture onto a specialized 96-well format MALDI-MS plate [140]. Direct injection from a vertically placed 96-well V-bottom plate in a microfabricated capillary electrophoresis device coupled to a subatmospheric ESI-MS interface allowed high throughput separation and identification of peptides and protein digests [138].

Conventional mass spectrometry characterized the excipient-induced oxidation of a peptide lyophilized formulation. In another study of accelerated stability, it has been shown that an increase in mannitol concentration and the presence of reducing sugar impurities resulted in an increase in degradation product [141]. Another example is the identification of disulfide bond formation in a recombinant botulinum neurotoxin protein as a function of pH and temperature, which enhanced the thermal stability without affecting the potency [142]. The identification of e.g., the oxidation, deamidation or glycation by-products during the formulation of proteins helps to understand the mechanisms and can contribute in improving the chemical stability. Screening of chemical degradation by e.g., capillary electrophoresis is sufficient in an early stage of protein formulation optimization. In-depth understanding of the different degradation pathways and products can be obtained at a later phase using mass spectrometry.

3.3.4. X-ray crystallography

This method is reviewed due to the similarities between X-ray solution screening platforms and high throughput screening of protein formulations. Although the goal of the two techniques differs: stable protein solution or protein lyophilisate versus protein growing and detection of crystals, both techniques use purified protein solutions and screen for excipients that either stabilize (formulation) or crystallize the proteins by varying pH, buffer, temperature, salts, metals or solvents. Slight modifications in existing automated high throughput crystal growth platforms could make them suitable for high throughput protein formulation screening. Image analysis, as used in protein crystallography, could be applied in protein formulation studies for characterization of protein gels and aggregates in microplates.

A purified, homogeneous and monodisperse protein solution is needed for growing crystals. The presence of protein aggregates in the solution inhibits crystal growth. Screening for the optimum protein solubility by testing 24 buffer solutions and 14 additives resulted in high quality protein crystals [143]. It has been suggested that random screening approaches were more efficient than using a predefined set of buffer solutions and sampling protocols [144]. The volume used in crystallography is nanoliters, which seems difficult to use in protein formulation studies. The detection and scoring of crystals, crystal growth, morphology and size is performed with automated imaging capture and processing systems [145]. Neural network algorithms were used to predict future conditions that are likely to yield crystals, improving the efficiency and success rate of protein crystallization [146]. Several high throughput methods e.g., cloning, purification, crystallization and detection have been developed to automatize the entire X-ray crystallography process [147,148]. For an extensive review on high throughput protein crystallography the article of Pusey et al. [149] is recommended. Note that protein crystals

can be used as protein slow release formulations, as was shown with TGF-β3 crystals [150].

4. Conclusion

Several practical considerations on developing high throughput protein formulation platforms and the corresponding analysis techniques are described. A high throughput platform for protein formulation typically consists of: a liquid and powder handling system, integrated barcode readers and sealing units, a robotic system for plate transport, storage chambers, incubators and microplate readers. High throughput systems are expensive, but compared to a conventional approach for protein formulation, cost savings would include less reagents' use, a shorter time to market and an increased chance of success. Failure to develop a biopharmaceutical drug can be due to lack of efficacy, but also to a poor formulation [16].

Ideally, immediately after preparing and dispensing of a protein formulation in a well plate using liquid and/or powder handling systems, a physical and chemical analysis is performed. The intrinsic protein fluorescence properties of tryptophan and tyrosine and the extrinsic fluorescence properties of fluorescent probes can be analyzed within one UV transparent microplate. The use of multiple non-invasive complementary analytical techniques will result in a high quality characterization of the protein formulation. UV–Vis absorbance spectroscopy is performed mainly for determining protein concentration, turbidity and solution pathlength. Fluorescence intensity and fluorescence resonance energy transfer are the most sensitive high throughput screening techniques and can give information on the local environment of the fluorophore, intra- and inter-molecular distances, (un)folding, hydrophobicity, conformation, protein interactions and aggregation. Fluorescence lifetime and fluorescence anisotropy in microplates are at present limited to the measurement of fluorescently labeled proteins. More technical and assay development is needed before these two techniques can be incorporated into high throughput protein formulation studies. Nephelometry and turbidimetry are two high throughput methods used for characterizing protein aggregation. Turbidimetry has the advantage that the measurements can be done with a standard UV–Vis absorbance microplate reader. Circular dichroism, Raman and infrared spectroscopy all have potential for the analysis of protein secondary structure, but are still expensive and have a low sample throughput. The most promising high throughput results on protein secondary structure analysis were reported with Raman spectroscopy.

In one possible high throughput experimental design the stability of proteins in solution was first measured with non-invasive methods, followed by one or more destructive analytical techniques, such as calorimetry, chromatography, capillary electrophoresis and mass spectrometry. Aspiration of several microliters from the well plate containing the protein formulations is sufficient for a proper measurement. Capillary electrophoresis coupled with UV or fluorescence detection is the most adapted to high throughput measurements and does not require specialized sample preparation, as is the case with mass spectrometry analysis. MS is useful for the identification of the separated degradation products. Calorimetric measurements that use capillaries to aspirate solutions from microplates have a throughput of about 50 samples per day. These HTS DSC measurements are as precise and sensitive as those performed with classical DSC equipment. The calorimetric analyses that are performed directly in thermocycler 96- or 384-well plates can achieve a throughput of one plate every 30–50 min making this technique suitable for HTS.

The similarities between the methods used in X-ray crystal screening and protein formulation screening can help in designing high throughput formulation platforms. Bioinformatics programs (visit www.expasy.org), such as neural network algorithms, calculations of iso-electric point and molar mass, prediction of protein folding patterns [151] and the analysis of protein sequence similarities, might help in finding optimum protein formulation conditions, similar to the software developed for finding optimum crystallization conditions. In the near future, virtual screening [152,153] of protein structures and "docking" experiments could be developed to study the influence of excipients on the protein drug. Other possible developments that would further enhance high throughput formulation screening are technical improvements in microplate-based CD, fluorescence lifetime, Raman and FTIR equipment.

When screening for the optimal formulation, the physical and chemical degradation products, the desired dosage form and concentration should be established. Buffer type, pH range and ionic strength are other parameters that need to be determined in the early phase of the high throughput screening. In a next step to improve the stability of the protein drug, other excipients can be added to the formulation or the concentration of excipients found to stabilize the protein can be optimized. Performing accelerated stability studies in microplates will strengthen the data on shelf-life, thermal stability and aggregation kinetics. Consecutive use of non-invasive and invasive high throughput techniques plus assay miniaturization will reduce, depending on the number of parameters tested, the costs of protein, reagents and consumables. In summary, many high throughput techniques are available for the preparation and analysis of protein formulations. It is our conviction that high throughput screening techniques, similar to those presented in this review, will become an important asset for the development of stable pharmaceutical drug formulations.

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