High-Throughput Assessment of Antigen Conformational Stability by Ultraviolet Absorption Spectroscopy and Its Application to Excipient Screening

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Received 14 June 2011; revision received 31 August 2011; accepted 6 September 2011
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23336

ABSTRACT: In high-throughput screening (HTS) assays, the use of ultraviolet absorption spectroscopy (UA) is commonly limited to concentration and turbidity measurements. Our aim was to evaluate microplate-based UA and its second-derivative [\(\text{2d-UA}\)] for measuring the conformational stability of two recombinant antigenic proteins in the presence of 44 excipients. Protein conformational stability was assessed by \(\text{2d-UA}\) upon titration with guanidine hydrochloride. \(\text{2d-UA}\) was compared with tryptophan fluorescence spectroscopy (TF) and differential scanning fluorimetry (DSF), both commonly used techniques for measuring protein conformational stability. The HTS data were corrected for plate, row and column effects by applying a median polish procedure. Irrespective of the unfolding method applied, similar stabilizing excipients were identified by all analytical methods for a given antigen. The native forms of both antigens were destabilized by arginine, hydroxypropyl-\(\beta\)-cyclodextrin, and sodium docusate, and were protected by polyols. The median polish correction improved the quality of the prediction models and the screening resolution. The higher sensitivities of TF and DSF compared with \(\text{2d-UA}\) allowed the identification of a larger number of stabilizing excipients. However, similar screening resolutions (\(\zeta\)-factor > 0.8) were observed for \(\text{2d-UA}\), TF, and DSF in a HTS of excipients applied to one of the antigens. Therefore, \(\text{2d-UA}\) deserves more attention in HTS studies focused on protein conformational stability.

Introduction

Various stress conditions may affect vaccine integrity during manufacturing and storage (Shire, 2009). Antigen integrity is a critical factor in the preservation of a vaccine’s immunogenicity profile (Hermeling et al., 2004). Ensuring protein conformational stability may help to limit protein aggregation (Thirumangalathu et al., 2009; Wang, 2005) and oxidation (Thirumangalathu et al., 2007). High-throughput screening (HTS) methods offer the potential for the rapid identification of excipients able to stabilize the conformation of protein-based antigens.

Over recent decades, numerous studies on protein conformation have been performed with spectroscopic methods, for example, fluorescence and circular dichroism. An increasing number of HTS studies report the evaluation and identification of protein-stabilizing conditions by intrinsic (Capelle et al., 2009; Dasnoy et al., 2011) and extrinsic (Capelle et al., 2009) fluorescence, differential static light scattering and differential scanning fluorimetry (DSF; Senisterra and Finerty, 2009). In contrast, ultraviolet absorption spectroscopy (UA), although available in most laboratories, has been mainly relegated to measuring protein concentrations, turbidity, and enzymatic activity (Mach and Middaugh, 2011). In microplates, UA is often used to measure protein concentration at 280 nm (Zhao et al., 2010) and sample turbidity at 350 or 360 nm (Ausar et al., 2007; Capelle et al., 2009; Kissmann et al., 2008a,b).

Protein conformational stability has already been studied by UA and second-derivative UA [(\(\text{2d-UA}\)] in cell-based spectrophotometers (He et al., 2010; Kuelzoz et al., 2000; Peak et al., 2007; Vessely et al., 2009). To our knowledge, \(\text{2d-UA}\) is not commonly used for studying protein conformational stability in microplates.
Chemical (Aucamp et al., 2005; Liu et al., 2009) and thermal (Kuelzio et al., 2000; Peek et al., 2007; Senisterra and Finerty, 2009) unfolding methods have been used for measuring protein conformational stability. Both approaches were reported to have provided similar insights on protein thermodynamic stability (Pace and Scholtz, 1997).

Antigen A and Antigen B are experimental recombinant proteins for vaccination and produced by GlaxoSmithKline Biologicals (Rixensart, Belgium). The objective of this work was to demonstrate that (2d)UA can be used to study the effect of excipients on protein conformational stability in HTS mode. The chemical unfolding of Antigens A and B was monitored by various features extracted from (2d)UA spectra. Tryptophan fluorescence spectroscopy (TF) and DSF were used as benchmark analytical methods. The stabilizing excipients identified by (2d)UA, TF, and DSF were assessed in their ability to enhance the isothermal stability of the antigens.

Materials and Methods

Materials

Antigens A and B were produced by GlaxoSmithKline Biologicals. Both proteins were studied at a concentration of 250 µg/mL. Antigen A was prepared in 10 mM phosphate buffer (NaH₂PO₄/K₂HPO₄), pH 6.8. Antigen B was prepared in 10 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, 10 mM NaCl.

L-Arginine (Arg), L-aspartic acid (Asp), L-histidine (His), L-isoleucine (Ile), L-leucine (Leu), L-proline (Pro), L-serine (Ser), L-threonine (Thr), and L-valine (Val) were obtained from Ajinomoto (Tokyo, Japan). L-Alanine (Ala) was obtained from Amresco (Solon, OH) and L-glycine (Gly) from Evonik Rexim (Ham, France). Polyethylene glycols (PEG) 300, 600, 1,000, 1,500, and 6,000 were obtained from Merck (Darmstadt, Germany). PEGs 400, 3,350, and 4,000 were gifts from Sasol (Johannesburg, South Africa). L-Glutamic acid (Glu), glycylglycine (GlyGly), L-lysine (Lys), myo-inositol, silicon oil (DC200, Fluka), 8-anilino-1-naphtalene-sulfonic acid (ANS), 4',4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt (bis-ANS), SYPRO Orange (SO), dimethylsulfoxide (DMSO), and sodium dihydrogenophosphate were obtained from Sigma–Aldrich (St. Louis, MO). All solutions were prepared with water for injection obtained by triple distillation. Excipient solutions were filtered through a 0.22 µm polyether sulfone membrane (Sartolab, Sartorius Stedim, Aubagne, France). All excipients except GlyGly were of compendial grade or tested following their respective Ph.Eur. monograph prior to use. Excipient concentrations are presented as percentages and were prepared on a weight-to-volume (w/v) basis.

Regular (Costar #3635) and half-area ultraviolet (UV)–transparent (Costar #3679) 96-well acrylic microtiter plates were obtained from Corning (Corning, NY), clear and black 96-well polypropylene microplates (Whatman Uniplate) were obtained from GE Healthcare (Waukesha, WI), and 384-well microplates (4titude Framestar 384) were obtained from Biotek (Leiden, The Netherlands). UV-transparent seals (VIEWseal) were obtained from Greiner Bio-one (Kremsmünster, Austria). MicroAmp optical 96-well reaction plates and optical adhesive films were obtained from Life Technologies (Applied Biosystems, Carlsbad, CA).

Chemical Unfolding

Chemical unfolding was monitored by (2d)UA and TF. The titration method was adapted from Aucamp et al. (2005). Gdn.HCl stock solutions were prepared at a concentration of 5.0 M for Antigen A and 2.5 M for Antigen B. The initial sample volume was 75 µL. During the selection of conditions to monitor unfolding, empty wells were filled with water. Titration by Gdn.HCl was directly performed in a Varioskan Flash microplate reader (ThermoFisher Scientific, Waltham, MA) equipped with an automated dispenser. Various denaturant volumes were added during titration to explore the whole range of Gdn.HCl concentrations, to a final volume of 300 µL. After every addition of denaturant, the microplate was shaken during 30 s at 300 rpm, lef t during 5 min, analyzed by (2d)UA and then by TF. In preliminary experiments, we verified that repeated sample excitation by fluorescence did not affect absorption measurements. Each screening comprised twelve 96-well microplates, 30 additions of Gdn.HCl per well, and thus involved the recording of 34,560 UA spectra, sets of UA values or TF intensity values.

The microplate reader SkanIt software (ThermoFisher Scientific) was only used for data acquisition. Raw data were exported as text files. Data processing was performed by a customized application (Microsoft, Redmond, WA) developed in Visual Basic, with a SigmaPlot (Systat Software, San Jose, CA) embedded module for curve-fitting operations.
Chemical unfolding studies were performed assuming the equilibrium state in spectroscopic measurements and a two-state protein unfolding mechanism. Data points were fitted to a Boltzmann sigmoid function (Eq. 1), where $D$ is the denaturant concentration, $Y$ the signal, $D_m$ the denaturant concentration at mid-transition, $Y_n$ the signal of native protein, $Y_u$ the signal of unfolded protein, and $k$ the transition slope. $Y_n$, $Y_u$, $D_m$, and $k$ were considered as floating parameters in the curve-fitting algorithm.

$$Y = Y_n + \frac{Y_u - Y_n}{1 + e^{\frac{(D - D_m)}{k}}}. \quad (1)$$

The $D_m$ parameter was selected for evaluating the conformational stability of protein antigens (Aucamp et al., 2005).

**UV Absorption Spectroscopy**

Spectroscopic measurements were performed at 25°C in unsealed UV-transparent microplates with a Varioskan Flash microplate reader (ThermoFisher). Spectra were recorded from 220 to 350 nm with a wavelength step of 1 nm, a bandwidth of 5 nm and an integration time of 100 ms. A microplate was scanned in 30 min. The sample turbidity before titration (OD$_{280}$ = 2.1 ± 4.4 mAU, after buffer subtraction) was too low for assessing the effect of excipients on protein stability.

Spectral processing is illustrated in Figure 1a. Since a more concave meniscus is observed in a protein-containing well that is packed with an associated buffer, the pathway length obtained for the protein well ($L$) is shorter than its blank counterpart ($L’$). Absorption values should be measured with the same pathlength to be compared. The Lambert–Beer law can be applied to absorbing species and measured with the same pathlength to be compared. The absorption values at 230 (Liu et al., 2009) and 290 nm (Thomson et al., 1989) were extracted and corrected for the protein concentration as determined by absorption at 280 nm. The wavelength at maximum of absorption in the 260–290 nm range ($\lambda_{\text{max}}$) and the wavelength at minimum of absorption in the 250–270 nm range ($\lambda_{\text{min}}$) were calculated by interpolation: a series of 19 points were added between raw data points, with a fifth-degree polynomial least-square fit. The center of gravity of absorption $\langle \lambda \rangle$ was calculated in the 245–305 nm range by the center of spectral mass position ($\tau_{\text{csm}}$) method (Eq. 4). Bandpass correction was applied when converting intensities ($I$) from wavenumbers ($\tau$) to wavelength units (Lakowicz, 2004).

$$\langle \lambda \rangle = \frac{1}{\tau_{\text{csm}}} = \frac{\sum I(\tau)}{\sum I(\tau)} = \frac{\sum \lambda \cdot I(\lambda)}{\sum I(\lambda)} \quad (4)$$

Second-derivative spectra were obtained by simultaneous numerical smoothing and differentiation of buffer- and light scattering-corrected spectra by a nine-points filter and second-degree Savitzky–Golay polynomial (Savitzky and Golay, 1964). A series of 19 points were added between raw data points, with a sixth-degree polynomial least-square fit (Kueltzo et al., 2000). The spectra were truncated and limited to the 250–305 nm region. The concentration dependence of the second-derivative signal ($z$) was suppressed by vector-normalization (Eq. 5).

$$z_{\text{norm}} = \frac{z}{\sqrt{\sum z^2}} \quad (5)$$

The position of peaks and shoulders was determined based on differentiation of the second-derivative signal (Fig. 1b). A window of 2.5 $10^{-4}$ intensity units and 5 nm wavelength was applied for peak localization. The three main negative peaks were identified near 285 nm (Tyr/Trp, A), 295 nm (Trp, B), and 260 nm (Phe, C). Peak-to-valley intensity ratios were used to determine the relative exposure of aromatic amino acid residues. The $A/B$ ratio has been reported to be an indicator of Tyr exposure (Ragone et al., 1984). The similarity of spectra ($y$) with the non-treated sample ($x$) was monitored by the correlation coefficient ($r$, Eq. 6, after translation of spectra to positive domain; Prestrelski et al., 1993) and the root mean square of differences (RMS, Eq. 7, where $n$ is the number of data...
points; Park et al., 1989) methods.

\[
r = \frac{\sum xy}{\sqrt{\sum x^2 \sum y^2}}
\]  
(6)

\[
\text{RMS} = \sqrt{\frac{\sum (x - y)^2}{n}}
\]  
(7)

In order to limit the time required for analysis, the scan window was shortened in excipient screening studies. For Antigen A, OD values were measured at 280 and 290 nm (normalized Trp absorption by OD_{290} to OD_{280} ratio), 900 and 975 nm (pathlength), and 320 and 350 nm (wavelength dependence of light scattering signal determined on two points, 3.3 min/plate). For Antigen B, absorption scans were recorded between 270 and 300 nm, and both pathlength and...
light scattering were determined as explained for Antigen A (9.8 min/plate).

**Tryptophan Fluorescence Spectroscopy**

Spectroscopic measurements were performed at 25°C in unsealed UV-transparent microplates with a Varioskan Flash microplate reader (ThermoFisher Scientific). Selective excitation of Trp residues was performed at 295 nm (Lakowicz, 2004). Spectra were recorded from 320 to 420 nm, with a wavelength step of 1 nm, an excitation bandwidth of 12 nm and an integration time of 100 ms. A microplate was scanned in 21.4 min. Bottom optics were used because the focus position of the excitation beam was compatible with measurements in the whole volume range evaluated (75–300 µL). The buffer signal was subtracted. The center of gravity of the emission ($\langle \lambda_i \rangle$) of buffer-corrected spectra was calculated from Equation (4). The average emission of all Trp residues was monitored at 340 nm ($F_{340}$; Aucamp et al., 2005). The relative contribution of buried (emission at 330 nm) and exposed (emission at 350 nm) Trp residues was measured by ratio ($F_{350}/F_{330}$) and difference ($F_{330} - F_{350}$). The $\lambda_{\text{max}}$ was extracted from buffer-corrected spectra after application of a 15-points filter and third-order Savitzky–Golay smoothing algorithm (Savitzky and Golay, 1964; Fig. 1c). TF has been used in a HTS study to evaluate the effect of excipients in their ability to prevent Antigen 18A from aggregation at air–water interface (Dasnoy et al., 2011). $F_{340}$ was used in excipient screening for both antigens (1.6 min/plate).

**Thermal Unfolding**

Thermal unfolding was monitored by DSF. Measurements involving Antigen A and B were performed with a ABI Prism 7900HT sequence detection system (Life Technologies) and a LightScanner Pro (Idaho Technologies, Inc., Salt Lake City, UT), respectively. The selection of excrinsic fluorescent probes was based on a preliminary comparison of different dyes (data not shown). For Antigen A, 1 µL SO 250 × in DMSO was added to 49 µL sample in MicroAmp optical 96-well reaction plates sealed with MicroAmp optical adhesive films. For Antigen B, each well of a parent 96-well polypropylene clear microplate was filled with 99 µL sample to which 1 µL of an ethanolic solution of ANS 500 µM was added. All wells of a daughter 384-well microplate were prefilled manually with 3 µL silicone oil for preventing evaporation during thermal unfolding (Antigen B). A 96-channel (TeMo) head (Tecan, Männedorf, Switzerland) was then used to transfer 10 µL from a parent well to each of four daughter wells. The daughter microplate was then sealed. Just before analysis, the microplate was centrifuged for 5 min at 2,500 rpm. Temperature was raised from 25 to 90°C, at a rate of 1°C/min.

Fluorescence intensity measurements were performed at 0.29 ± 0.06°C for Antigen A and 0.076 ± 0.002°C intervals for Antigen B. The software of both DSF systems were used for data acquisition only. Raw data were exported as text files and analyzed with customized Visual Basic macros (Microsoft). No buffer subtraction was performed. Data were automatically truncated to the transition region, based on the first-derivative smoothed with a running average algorithm (Eq. 8), where $T$ is the temperature, $Y$ the signal, $T_m$ the temperature at mid-transition, $Y_n$ the signal of native protein, $Y_u$ the signal of unfolded protein, and $k$ the transition slope. $Y_n$, $Y_u$, $T_m$, and $k$ were considered as floating parameters in the curve-fitting algorithm.

$$Y = Y_n + \frac{Y_u - Y_n}{1 + e^{\left(\frac{m - T}{k}\right)}}$$

The $T_m$ parameter was selected for evaluating the conformational stability of protein antigens. In the case of Antigen B, the values of the four daughter wells were averaged. The measurement was considered as invalid if no transition was observed or a transition was detected in the same temperature range as detected in the placebo formulation.

**High-Throughput Screening of Excipients**

The present HTS design has been used to evaluate excipients in their ability to protect Antigen 18A from aggregation at air–water interface (Dasnoy et al., 2011). Briefly, a series of 44 excipients were selected based on their presence in marketed parenteral drugs. Excipients were tested at six concentrations in triplicate. Replicates were located on different plates. Edge rows were used for placebo formulations. An excipient-free control was present on each row of a plate. The positions of all other test conditions were randomized in a total of 12 plates.

All samples contained 10 mM phosphate buffer (NaH2PO4/K2HPO4), pH 6.8 (Antigen A), or 10 mM Tris buffer, pH 7.5 (Antigen B). The stock solution concentrations were the following: 100 mM phosphate buffer, 0.5% surfactants, 3% polymers, 15% polyols except 12% inositol, 15% carbohydrates and cyclodextrins, 100 mM calcium and magnesium salts, 250 mM sodium chloride, 3 mM aspartic acid, 50 mM glutamic acid, 150 mM leucine, 250 mM isoleucine, and 500 mM other amino acids.

A customized application developed in Visual Basic (Microsoft) randomized the positions of all test conditions and generated XML worklists containing liquid volumes to be added in each well. This application subsequently imported XML worklists into the Gemini software (Tecan). A Genesis liquid handling station equipped with an eight-tip liquid handling (LiHa) arm (Tecan) prepared excipient mixes at a volume of 1,000 µL in deepwell microplates. In parallel, a deepwell plate containing antigen stock solution was prepared by hand. The transfer from deepwell to
microtiter plates was performed with the TeMo equipped with disposable tips (Tecan). Formulations were prepared in stock clear polypropylene microplates (200 µL) obtained by mixing appropriate volumes from excipient and antigen deepwell plates. The maximum final concentrations of excipients were the following: 0.25% surfactants, 2% polymers, 10% polyols, carbohydrates, and cyclodextrins, 50 mM calcium and magnesium salts, 150 mM sodium chloride, 2 mM aspartic acid, 25 mM glutamic acid, 120 mM leucine, 200 mM isoleucine, and 400 mM other amino acids. For a given excipient, the six concentrations studied were 50 mM leucine, 200 mM isoleucine, and 400 mM other amino acids.

For a given excipient, the six concentrations studied were 50 mM leucine, 200 mM isoleucine, and 400 mM other amino acids. For an assigned dye was chosen from preliminary dye-free sample. Extrinsic fluorescence measurements were performed by OD at 350 nm in half-area UV-transparent microplates (Corning) filled with 150 µL of dye-free sample. Extrinsic fluorescence measurements were performed by assigning a dye was chosen from preliminary experiments (data not shown). One microliter of a 500 µM bis-ANS (Antigen B) or a 500 µM SO (Antigen A) stock solution in DMSO was added to 99 µL of sample in a black microplate. The excitation/emission wavelengths of bis-ANS and SO were 385/525 and 490/600 nm, respectively. The dye was excited with a bandwidth of 12 nm, and emission was measured with an integration time of 100 ms.

**Statistical Analysis**

**Data Correction**

The data correction method was adapted from Malo et al. (2010), in an Excel spreadsheet (Microsoft). Briefly, a non-control-based normalization method was applied, aiming to reduce the noise introduced by the repartition of the experimental conditions on the twelve 96-well plates. Indeed, even though the test conditions are randomly located on the plates, without using the first and last rows, intra- and inter-plate effects may occur leading to false negative/positive results. A median polish procedure (Tukey, 1977) was applied. This method, well-suited to gridded data, works by alternately removing row and column medians from each plate, until all medians are nearly null. Because the outcome may vary slightly depending on whether rows or columns are considered first, both starting strategies were applied, with nine corrections cycles, and the obtained results averaged. Finally inter-plate differences were corrected by aligning the medians of all plates. Corrected results can be then calculated from Equation (9), corrected result for row i and column j on the pth plate ( \( \hat{x}_{ijp} \)) being the difference between the observed value ( \( x_{ijp} \)) and the corresponding predicted error ( \( \hat{e}_{ijp} \)) calculated as the sum of row ( \( R_p \)), column ( \( C_{jp} \)), and plate ( \( P_p \)) biases.

\[
\hat{x}_{ijp} = x_{ijp} - \hat{e}_{ijp} = x_{ijp} - (R_p + C_{jp} + P_p) \tag{9}
\]

**HTS Assay Validation**

The z'-factor is a common adimensional parameter used for evaluating the quality of an HTS assay. Based on the mean (\( \mu \)) difference and variability (\( \sigma \)) of positive (\( C_+ \)) and negative (\( C_- \)) controls, a separation band was calculated (Dasnoy et al., 2011), according to Equation (10). The identification of hits by a HTS assay is considered as feasible when its z'-factor \( \geq 0.5 \) (Zhang et al., 1999).

\[
z' = 1 - 3 \frac{(\sigma_{C_+} + \sigma_{C_-})}{|\mu_{C_+} - \mu_{C_-}|} \tag{10}
\]

**Results**

The effect of a series of 44 excipients on the conformational stability of Antigens A and B was studied in HTS mode by (2d)UA, TF, and DSF. Protein unfolding was performed chemically (by using Gdn.HCl) for (2d)UA and TF, and thermally for DSF. Prior to excipient screening, the selection of the conditions for monitoring unfolding were undertaken for (2d)UA and TF.

**Selection of Intrinsic Unfolding Probes**

Proteins denature and unfold in the presence of Gdn.HCl. Various features were computed from (2d)UA and TF
spectra and evaluated to identify the settings to monitor unfolding through the use of (amino acid residue) probes intrinsic to the protein antigens. For Antigens A and B, unfolding patterns were selected to generate a two-step unfolding model into which data were processed to extract $D_m$ for estimating protein stability. It has been shown that when multiple features are extracted from a spectra, each of them can be associated with a different $D_m$ value (Eftink, 2000).

**UV Absorption Spectroscopy**

In UA, a spectral shift to shorter wavelengths is accounted for by an increase in the polarity of the environment of aromatic amino acid residues, which may for instance be due to an increase in exposure to water. The peaks of aromatic amino acid residues contributing to a protein absorption spectrum (Phe/Tyr/Trp) can be resolved in the 2dUA spectrum (Kueltzo et al., 2000).

The evolution of (2d)UA spectra was analyzed upon unfolding by Gdn.HCl (Fig. 2a and b). The $A_{230}/A_{280}$ (Fig. 2c) and $A_{290}/A_{280}$ (Fig. 2d) ratios, $\lambda_{\text{max}}$ (Fig. 2e), $\lambda_{\text{min}}$ (Fig. 2f), and $\langle \lambda \rangle$ (Fig. 2g) positions extracted from UA spectra were monitored. Upon denaturant addition, the similarity of 2dUA spectra with the non-treated sample was assessed by the $r$ (Eq. 6; Prestrelski et al., 1993; Fig. 2h) and the RMS (Eq. 7; Park et al., 1989; Fig. 2i) methods. The position (Fig. 2j–l) and peak-to-valley intensity ratios (Fig. 2m–o) of the three best-resolved aromatic amino acid peaks ($A$, $B$, and $C$) were monitored in 2dUA spectra. Upon unfolding, blue shifts were detected for $A_{290}/A_{280}$ (Fig. 2d), $\lambda_{\text{max}}$ (Fig. 2e), $\lambda_{\text{min}}$ (Fig. 2f), and $A$ position (Fig. 2i). Some changes were detected in the relative exposure of amino acid residues, as evidenced by an increase in $A/B$ (Fig. 2m) and $A/C$ (Fig. 2n). No clear transition was observed for $A_{290}/A_{280}$ (Fig. 2c), $\langle \lambda \rangle$ (Fig. 2g), $B$ (Fig. 2k), and $C$ (Fig. 2l) positions, and $B/C$ (Fig. 2o). A decrease in the similarity of 2dUA spectra was observed upon titration by Gdn.HCl (Fig. 2h,i).

Sigmoid Antigen A unfolding curves and associated $D_m$ values were obtained for $A_{290}/A_{280}$ (2.24 ± 0.05 M), $\lambda_{\text{min}}$ (2.49 ± 0.19 M), $r$ (2.45 ± 0.06 M), RMS (2.35 ± 0.06 M) and $A$ position (2.23 ± 0.06 M). Regarding Antigen B, sigmoid profiles were obtained for $r$ (0.74 ± 0.02 M), RMS (0.67 ± 0.03 M), $A/B$ (0.65 ± 0.03 M) and $A/C$ (0.61 ± 0.03 M).

Among features associated with a sigmoid unfolding profile, priority was given to those that can be obtained from a limited data recording. In this regard, $A_{290}/A_{280}$ was selected as the setting for monitoring unfolding for Antigen A since it does not require a whole scan to be recorded and can be calculated from absorption values obtained at two wavelengths. $A/B$ was selected as the setting for monitoring unfolding for Antigen B since it can be obtained from a narrow scan window of 30 nm width.

**Tryptophan Fluorescence Spectroscopy**

TF was evaluated as a benchmark method to (2d)UA. TF has been reported to be a suitable technique for measuring protein conformational stability in a microplate (Aucamp et al., 2005), and for identify stabilizing excipients (Dasnoy et al., 2011) and ligands (Mahendrarajah et al., 2011).

With TF, a diminution of quantum yield and a red-shift in the wavelength at maximum emission have been reported to occur upon protein unfolding in an aqueous solvent. A fluorescence spectrum results from the contribution of several populations of Trp residues: the higher the exposure of a Trp residue to aqueous solvent, the higher its wavelength of emission (Lakowicz, 2004). The effect of Gdn.HCl on Trp fluorescence emission spectra was analyzed (Fig. 3a,b). The global contribution of Trp residues was assessed by monitoring $F_{340}$ (Fig. 3c; Aucamp et al., 2005), $\langle \lambda \rangle$ (Fig. 3f), and $\lambda_{\text{max}}$ (Fig. 3g) upon denaturant titration. A decrease in intensity and a red-shift were observed in the emission spectra of both antigens. Without any numerical smoothing, $\langle \lambda \rangle$ revealed to be a suitable method for monitoring changes in the shape of a spectrum (Dasnoy et al., 2011).

The relative contribution of buried and exposed Trp residue populations was assessed by difference ($F_{330} - F_{350}$ Fig. 3d) and ratio ($F_{340}/F_{350}$ Fig. 3e) and revealed an increase in the global exposure of Trp residues in the denatured state. Better unfolding profiles were not observed by monitoring $F_{330}$ and $F_{350}$ than by monitoring $F_{340}$ (Fig. 3c).

For Antigen A, sigmoid profiles and associated $D_m$ were obtained for $F_{340}$ (2.15 ± 0.02 M), $F_{330} - F_{350}$ (2.18 ± 0.02 M), and $\langle \lambda \rangle$ (2.24 ± 0.03 M). $F_{340}$ was the only feature showing a sigmoid profile upon unfolding of Antigen B (0.64 ± 0.01 M). Since $F_{340}$ identified sigmoid unfolding profiles for both antigens, $F_{340}$ was selected as the setting for monitoring unfolding. Hence, we confirmed the suitability of monitoring $F_{340}$ as a fast, simple, generic, and reliable fluorescence method for measuring protein stability in microtiter plates (Aucamp et al., 2005).

**High-Throughput Screening of Excipients**

A HTS approach was followed to evaluate (2d)UA in the assessment of 44 excipients on protein conformational stability. Chemical unfolding (Gdn.HCl) was monitored by UA ($A_{290}/A_{280}$) for Antigen A and 2dUA ($A/B$) for Antigen B, and by TF ($F_{340}$) for both antigens. Thermal unfolding was monitored by DSF for Antigens A (with SO) and B (with ANS). Typical unfolding curves are illustrated in Figure 4.

A median polish procedure was applied to correct for row, column, and plate effects (Tukey, 1977). As illustrated in Figure 5 for Antigen B, this procedure corrected for the plate effect observed with 2dUA and TF, and the row effect observed with TF.

The effect of an excipient on the unfolding probe signal was analyzed by regression (Fig. 6). Basically, four different types of concentration-response to an excipient were
observed: nonlinear or linear, and enhanced or diminished. The six excipient-free controls present on each plate gave an indication of inter-plate variability. The median polish correction narrowed the dispersion of the controls and allowed the fitting of stronger regression models, as illustrated by better correlations between observed and predicted values. The maximal (highest or lowest depending on the excipient) predicted concentration-response was determined by regression analysis, with its associated confidence interval at 95% (Fig. 7). The excipient was considered to have a significant effect on antigen stability when the confidence interval did not span zero.

A comparison of the three analytical methods is given in Table I. The number of stabilizing excipients identified by TF and DSF, was higher than by (2d)UA. Most of stabilizing excipients were inorganic salts, sugars/polyols, or amino acids. The ranking of sugars/polyols according to their effects on antigen stability was quite similar for all three methods. For both TF and DSF analyses, the same Antigen B stabilizers were ranked in the top three.

Although CaCl₂ strongly stabilized Antigen B, it destabilized Antigen A. Inositol was the superior stabilizing polyol and was ranked in the top three for all analytical methods. HP-β-CD appeared to have a destabilizing effect on both antigens. Gly, GlyGly, Lys, Ser, and Thr were observed to stabilize Antigen A using at least two analytical methods. Ala and Val were observed to slightly increase Antigen B stability using all analytical methods. His and Glu were observed to stabilize Antigen B using TF, but to destabilize it using DSF.

**Figure 2.** Unfolding probes obtained from (2d)UA spectra. Antigen A (*) and Antigen B (●) were subjected to chemical unfolding by Gdn.HCl. UA and 2dUA spectra were obtained for (a) Antigen A and (b) Antigen B. The following values were extracted from UA spectra: absorption values at (c) 230 and (d) 290 nm normalized for absorption at 280 nm, (e) wavelength at the maximum of absorption in the 260–290 nm range, (f) wavelength at the minimum of absorption in the 250–270 nm range, (g) center of gravity of absorption. The following values were extracted from 2dUA spectra: spectral similarity by the (h) correlation coefficient and (i) root mean square of differences methods, (j) Tyr/Trp peak (A) position in the 285 nm region, (k) Trp peak (B) position in the 295 nm region, (l) Phe peak (C) position in the 260 nm region, peak-to-valley intensity ratios of (m) A/B, (n) A/C, and (o) B/C. Error bars represent the standard deviation from eight replicate wells.
For both antigens, it was not feasible to monitor protein unfolding using DSF with high concentrations of several cyclodextrins, surfactants, and polymers. Nevertheless, surfactants and polymers had no marked effect on antigen stability using the other analytical methods. SBE-b-CD and PEGs were observed to slightly stabilize Antigen A using UA or TF. High Mw PEGs, PVPs, and Myrj52 were observed to destabilize Antigen B using 2dUA and TF.

For Arg concentrations ≥50 mM with Antigen A, a sigmoid transition was not observed; whereas a sigmoid transition was not observed for all Arg concentrations with Antigen B, irrespective of the analytical method. This different antigen-dependent response to excipient concentration was also observed with sodium docusate, where a sigmoid transition was not observed with sodium docusate concentrations ≥0.008% for Antigen A and was not observed with all sodium docusate concentrations for Antigen B.

**Validation of HTS Assays for Antigen B**

The highest predicted effect on Antigen B conformational stabilization was obtained with 50 mM CaCl₂ by all analytical methods. To estimate the width of the screening window, a post-screening z'-factor was calculated (Zhang et al., 1999) by filling a single microplate with randomly distributed controls (Dasnoy et al., 2011). Positive and negative controls were 50 mM CaCl₂ (highest predicted effect) and buffer alone (no effect), respectively. Results are shown in Figure 8. The z'-factors calculated for all assays were greater than 0.5, indicating that all analytical methods were valid for identifying stabilizing excipients (Zhang et al., 1999). The median polish correction applied to each control was found to lower variability and therefore increase z'-factors.

**Confirmation of Stabilizing Excipients**

The excipients predicted to significantly stabilize an antigen according to at least 2 out of the 3 analytical methods were selected for further characterization. The effect of these excipients on the conformational stability of both antigens was evaluated by isothermal stability at 45°C (Fig. 9). A set of extrinsic fluorescent dyes were evaluated for their ability to detect conformational changes (data not shown). The unfolding of Antigens A and B was monitored by SO and bis-ANS, respectively. Sample aggregation state was assessed by OD350. Although an increase in Antigen A unfolding was observed after 30 min (Fig. 9a), no increase in Antigen A aggregation was observed, even after overnight incubation at 45°C (data not shown). For Antigen B, the increase in unfolding over time preceded the increase in aggregation over time (Fig. 9b). Therefore, bis-ANS emission appeared to be a more sensitive method than OD350 for studying the effect of hydrophilic excipients on Antigen B isothermal stability.

All of the studied excipients limited protein unfolding. Moreover, Lys with Antigen A and CaCl₂ and Pro with Antigen B did not increase protein affinity for the fluorescent dye (extrinsic unfolding probe). All polyols stabilized Antigen B but had minimal stabilizing effects on Antigen A. Polyols stabilized Antigen B better than trehalose. The stabilizing effect conferred by the amino acid excipients were ranked as follow: Lys > GlyGly > Ser = Thr > Gly (for Antigen A) and Pro > Ala > Val > Gly (for Antigen B).
The conformational stabilities of two antigenic proteins were evaluated in microplates by two label-free spectroscopic techniques, TF and (2d)UA. Trp was used as an intrinsic probe for monitoring the unfolding of both antigens by fluorescence spectroscopy (Fig. 3c) and allowed the identification of stabilizing excipients. Hence, monitoring $F_{340}$ was confirmed as a suitable setting for assessing protein conformational stability in microplates (Aucamp et al., 2005). Trp absorption, monitored by the decrease of $A_{290}/A_{280}$, was used to measure Antigen A unfolding (Fig. 2d; Lakowicz, 2004).

Although selective excitation of Tyr remains a challenge for fluorescence spectroscopy of Trp-containing proteins (Lakowicz, 2004), Tyr absorption spectroscopy using 2dUA (because Tyr absorption is altered by its exposure to an aqueous environment; Ragone et al., 1984) was shown to be a suitable probe for monitoring Antigen B unfolding (Fig. 2m). In contrast to TF, 2dUA offers the possibility to monitor each aromatic amino acid residue individually as an unfolding probe. Moreover, based on spectral similarity measurements (Park et al., 1989; Prestrelski et al., 1993), we propose a method to monitor simultaneously the global changes occurring in the Phe, Tyr, and Trp amino acid residue environments by comparisons of 2dUA spectra from samples subjected or not subjected to stress conditions (Fig. 2h,i).

The high sensitivity of fluorescence-based methods make them attractive for studying protein antigens at low concentrations and typical of antigen concentrations found in vaccines (Dasnoy et al., 2011). TF is a label-free method by which a global assessment is made of all Trp residues present in the sample, from both folded and unfolded protein molecules. Hence, TF may not be sufficiently sensitive to detect denaturation if only a small proportion of the protein molecules are unfolded. In contrast, DSF should only detect unfolded protein molecules and should therefore be more sensitive than TF. However, this sensitivity may be compromised by the hydrophobic dye used in DSF; because the dye may perturb the conformation of protein molecules. The interaction between the dye and certain cyclodextrins, polymers, and surfactants also impaired DSF measurements (Fig. 7). For the analysis of Antigen B using DSF, silicon oil was added to prevent evaporation in 384-well microplates and may have affected protein conformation at the oil/sample interface. Nevertheless, in terms of rapidity and ease of
use with small hydrophilic excipients, DSF was confirmed as an efficient analytical method (Senisterra and Finerty, 2009).

Improved resolution (Fig. 8) and better regression models (Fig. 6) were obtained by applying the median polish correction in HTS studies. The randomized positional allocation of samples in the microplates meant that distorting plate, row, and column effects were suppressed. For example, the lower $D_m$ values obtained with Plate 10 by UA and TF (Fig. 5) could have been due to a dilution error when preparing the Gdn.HCl stock solution. If raw data from Plate 10 had been used, these data would have been probably discarded from the screening data. Therefore, randomized sample allocation and the median polish correction should increase the probability of identifying stabilizing excipients.

Stabilizing excipients were mainly identified among salts, sugars, and amino acids. These families of compounds are known to protect proteins by preferential exclusion (Arakawa and Timasheff, 1985). In contrast, HP-β-CD was a destabilizing excipient with both antigens, perhaps through its preferential stabilization of unfolded proteins (Tavornvipas et al., 2006).

The absence of unfolding sigmoid transitions when using Arg or sodium docusate suggests that these excipients alter the conformation of the two antigens. Arg has been reported to preferentially interact with most amino acid residues and peptide bonds (Arakawa et al., 2007). Ionic surfactants, such as sodium docusate, are known to denature proteins even at low concentration (Sellers and Maa, 2005).

His and Glu had opposite effects on Antigen A depending on whether the antigen was chemically (stabilization) or

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**Figure 5.** Effect of the median polish correction on the plate, row, and column effects obtained in the Antigen B screening by 2dUA, TF, and DSF. Graph of studentized residuals: standardized residual information obtained after removal of predicted excipients effects, with raw (○) and corrected (□) values. Error bars represent the least-square differences.
Data analysis strategy for studying the effect of excipient concentration on $D_m$ and $T_m$: example of the effect of (a) inositol, (b) HP-$\beta$-CD and (c) Lys on Antigen A stability. Raw data before (●) and after (□) correction with the median polish procedure were analyzed by regression. The regression model was built from corrected data and allowed the prediction of $D_m$ or $T_m$ for a given excipient concentration. The quality of the prediction model was assessed by measuring $R^2$ as an indicator of the correlation between observed and predicted $D_m$ or $T_m$ values.

Figure 6.

thermally induced (destabilization) to unfold. Hence, protein antigens may behave in different way upon chemical and thermal unfolding. No aggregation was detected by OD$_{350}$ during chemical unfolding, but there may have been a higher propensity to aggregate during thermal unfolding reflected by a $T_m$ decrease and a potential manifestation of the shift from native to aggregated species. This $T_m$ decrease may be due to the presence of excipient but also to a pH change, since the presence of His or Glu at their maximal concentration (400 mM) decreased the pH from 6.83 ± 0.06 to 5.70 ± 0.02 and 3.75 ± 0.07, respectively ($n = 3$).

An objective way of comparing analytical methods used in HTS is through the calculation of their respective $z'$-factors. This was performed by analysing the stabilizing effect of CaCl$_2$ on Antigen B (Fig. 8). Despite the higher sensitivity of fluorescence-based methods, all techniques had a comparable screening window. The median polish correction improved the resolution of all screening assays.

The fluorescent dye operates as extrinsic probe to allow monitoring protein unfolding in the isothermal stability assay (Senisterra et al., 2008) and monitoring protein unfolding was more sensitive than monitoring protein aggregation by OD$_{350}$ (Fig. 9). An increase in Antigen B surface hydrophobicity (bis-ANS emission) preceded Antigen B aggregation (OD$_{350}$; Fig. 9b), and confirms that a conformational change is required for aggregation (Dasnoy et al., 2011; Wang, 2005). The more stabilizing excipients found in isothermal stability studies were Lys for Antigen A, and both CaCl$_2$ and Pro for Antigen B (Fig. 9).

All of the analytical methods used during excipient screening predicted the stabilizing effect of these compounds (Fig. 7). Among the three best compounds predicted by HTS studies (Table I), CaCl$_2$ was identified as the best Antigen B stabilizer by all the analytical methods, whereas only DSF ranked Pro for Antigen B, and Lys for Antigen A in the top three. Since both thermal (used in combination with DSF) and isothermal unfolding methods are based on the principle of heating a sample in the presence of a hydrophobic dye, it may not be surprising that the same compounds were ranked high by both approaches. The performance of excipients identified by extrinsic fluorescence techniques should be confirmed by label-free methods. Although inositol was ranked among the three best-stabilizing excipients for both antigens by all analytical methods (Table I), this compound offered limited protein protection in isothermal stability studies (Fig. 9).

Unfortunately, no general rule exists to predict the best-stabilizing excipient for a given protein. The choice of excipients is generally performed on an empirical basis. Measuring protein conformational stability by chemical or thermal unfolding is a powerful approach for the identification of stabilizing excipients. Based on the screening results obtained by (2d)UA, TF, and DSF, as well as on isothermal stability studies, Lys and CaCl$_2$ were found to be the best excipients for stabilizing Antigens A and B, respectively. These protein-specific excipients would probably not have been prioritised for evaluation by classical formulation approaches. Our results highlight the usefulness of HTS methods for rapidly evaluating a broad choice of excipients in samples containing low protein concentrations.

Conclusions

This study demonstrates that both UA spectra recorded in microplate and their calculated 2dUA spectra provide various features that can be used as protein stability indicators. Relevant (2d)UA spectral features were selected for two antigens and evaluated in their ability to identify stabilizing excipients upon chemically induced unfolding. (2d)UA was compared with analytical methods commonly used in protein stability studies: a chemically induced unfolding and screening-based method similar to (2d)UA but monitored by TF, and a thermally induced unfolding method monitored by DSF. The random positional distribution of samples in microplates allowed the use of a median polish procedure to correct for plate, row, and column effects. Applying these corrections improved the quality of prediction models and the screening resolution. Irrespective of the unfolding method applied, similar
Figure 7. High-throughput screening of excipients for stabilizing Antigens A and B. \( D_m \) was calculated from 280 to 280 nm absorption ratio from UA spectra (Antigen A) or by \( A/B \) peak-to-valley intensity ratio from 2dUA spectra (Antigen B), and by emission intensity at 340 nm in TF. \( T_m \) was measured by SO (Antigen A) or ANS (Antigen B) emission intensity in DSF. Horizontal bars represent the highest or lowest value predicted by regression analysis and its confidence interval at 95%. An absence of prediction indicates that no sigmoid unfolding pattern was observed or no significant effect was predicted at any excipient concentration. A \( \times \) symbol indicates that the high concentrations of excipients were discarded from the regression analysis, because of missing values which may lead to inappropriate extrapolations.

Table 1. Comparison of HTS results obtained by (2d)UA, TF, and DSF.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Stabilizing excipients</th>
<th>(2d)UA</th>
<th>TF</th>
<th>DSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Number 3 Superior</td>
<td>Inositol &gt; SBE-β-CD &gt; Mannitol</td>
<td>Glu &gt; His &gt; Inositol</td>
<td>Lys &gt; Inositol &gt; GlyGly</td>
</tr>
<tr>
<td>B</td>
<td>Number 3 Superior</td>
<td>CaCl₂ &gt; Inositol &gt; PEG600</td>
<td>CaCl₂ &gt; Inositol &gt; Sorbitol</td>
<td>CaCl₂ &gt; Inositol &gt; Sorbitol = Pro</td>
</tr>
</tbody>
</table>
stabilizing excipients were identified by all analytical methods. A higher sensitivity of TF and DSF, both fluorescence-based methods, allowed the identification of a larger number of stabilizing excipients than 2dUA. DSF could not be used to study hydrophobic excipients due to hydrophobic excipient interactions with dye molecules. A HTS of excipients tested with Antigen B showed a similar screening resolution ($z'$-factor > 0.8) with the three analytical methods; 2dUA, TF, and DSF. Therefore, (2d)UA deserves more attention in HTS studies focused on protein conformational stability.

This work was supported by GlaxoSmithKline Biologicals. The authors thank BASF, CyDex, Roquette, and Sasol for providing gift samples of excipients. Ulrike Krause, Pascal Cadot, and Matthew Morgan are acknowledged for their support in reviewing the article.

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