UNCHAINED LABS

Application Note

Stability of sub-unit vaccines measured with the UNit label-free stability platform

Introduction

Sub-unit vaccines and the need for adjuvants

Of the many classes of vaccines that have been developed since the pioneering work of Edward Jenner at the end of the 16th century, attenuated viral and bacterial vaccines were for many years the most common variant in circulation. However, these bacterial vaccines often targeted polysaccharides that are commonly found on the surfaces of the target bacteria but do not provoke sufficient immune responses in children to stimulate immunity. Modern bacterial vaccines often constitute a polysaccharide (that the vaccine is required to stimulate protection against) that does not necessarily promote an immune response, conjugated with a toxoid that readily raises an immune response. The immune system will then readily form antibodies against both the polysaccharide and the toxoid efficiently protecting the individual.

Recent advances have led to an increase in the prevalence of sub-unit vaccines that constitute an antigen that is characteristic of the surface of, or a toxin produced by, the bacterium or virus against which one wishes to protect an individual. In order to raise a sufficiently large immune response to protect the individual, the antigen protein needs to be adsorbed onto a chemically inert carrier called an 'adjuvant'. Typically this adjuvant is an aluminium salt or gel. Adjuvants are required for subunit vaccines as they aid presentation of antigen to cells (Jones, 2005). Whilst they are required for such vaccines, adjuvant absorption has been shown to result in some changes to the antigen T_m. In the case of bound antigens reduced T_m is often observed, which may be due to conformational perturbations or simply changes in the tryptophan microenvironment upon adsorption (Clapp, 2010). The protection provided by the individual's immune response to the vaccine will depend on the conformation of the antigen protein whilst adsorbed to the adjuvant. It is therefore critical that this conformation is maintained sufficiently closely to that of the characteristic protein from the bacterium or virus such that the immune system will be able to recognize it. Attempts to stabilize the antigen protein through protein engineering or formulation must take into account how the protein behaves whilst adsorbed onto the adjuvant (Clapp, 2010) (Peek, 2006).

Measuring the thermal stability of vaccines

Typical measurements to determine the stability of adjuvant bound and unbound antigens include front face fluorescence, Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). However these methods are hindered by having low sample throughput and require relatively large volumes of solution. These systems also require cleaning between measurements when used in conjunction with adjuvant containing samples, which are known to stick to surfaces. The cleaning needs to be rigorous and is time consuming (Jones, 2005). Differential scanning fluorimetry (DSF) has also been used previously and requires the addition of an extrinsic probe dye which binds to the protein and may therefore affect the stability of the protein, the interaction of excipients with the protein (Ausar, 2010) and the interaction between the antigen and the adjuvant in solution.

The intrinsic fluorescence of a protein provides a convenient, label-free method to monitor protein conformation. The method is compatible with rapid, high throughput measurements from small sample volumes, and since no probe dyes are added to the protein sample there is no risk that these might affect the behavior of the protein or added excipients (Braun, 2011). The UNit platform from Unchained Labs is capable of obtaining intrinsic protein fluorescence and static light scattering (SLS) data simultaneously from up to forty-eight small volume (9 µL) samples, rapidly and

automatically. Protein intrinsic fluorescence provides a sensitive, label-free way to monitor the higher order structure of proteins in solution while the simultaneously recorded static light scattering provides a means to monitor protein aggregation.

The UNit is able to determine metrics predictive of protein stability including thermal melting temperature (T_m), aggregation onset temperature (T_{agg}), rates of unfolding and aggregation and thermodynamic parameters such as the free energy change of unfolding (ΔG_{UN}) and the enthalpy of unfolding (ΔH_{UN}) without the addition of a fluorescent label. With the addition of a wide range of dyes a plethora of other stability-indicating metrics can be determined and analyzed rapidly and with ease.
In this application note we investigate the ability of the UNit platform to determine changes in the conformational stability of model proteins that are both 'free' in solution and when adsorbed to an aluminium hydroxide adjuvant (Dong, 2006).

Using intrinsic protein fluorescence to monitor protein conformation

The aromatic amino acid residues tryptophan and tyrosine found in most proteins fluoresce when illuminated with ultra-violet light. The intensity and wavelength of this emission is affected by the environment in which the fluorescing residues find themselves. These residues are hydrophobic and as such tend to be buried within a correctly folded protein and shielded from the solvent environment. If, however, the protein unfolds fully or partially the residues may become exposed to the solvent environment or find themselves in a different environment within the protein and a change in the intrinsic fluorescence is observed. This can be used to detect changes in the conformation of the protein in response to changes in solvent composition or to monitor the protein conformation as a function of temperature and in that way to determine its T_m of a protein in a given solvent environment.

The UNit high throughput protein stability screening platform

The UNit instrument from Unchained Labs is designed for high throughput, low sample volume investigations into protein stability. The instrument uses proprietary multi well sample plates with each well requiring only 9 μL of sample and allowing 48 samples to be investigated in a single experiment. The instrument uses thermo-electric heating and cooling for precise control of sample temperature allowing thermal ramps or isothermal type experiments to be performed.
The sample is simultaneously illuminated with multiple wavelengths of laser light allowing simultaneous acquisition of both fluorescence and static light scattering data, the latter being only of use for the measurement of free antigen solutions as addition of the large Alhydrogel® particles results in the dramatic increase in intensity of the SLS signal and renders any small changes due to antigen aggregation undetectable.

Investigation into antigen protein stability on the UNit

To determine whether the UNit could be used to investigate the stability of antigen proteins, both free in solution and also when adsorbed to an adjuvant a series of experiments with model proteins were performed.
There are a number of commonly used methods of comparing fluorescence spectra data for protein unfolding experiments including calculation of a barycentric mean fluorescence wavelength (BCM) and determining a ratio between intensities at 350 and 330 nm – characteristic wavelengths at which tryptophan fluoresces when fully exposed to water and when buried in the core of a protein. Generally the BCM method results in smoother data as it is an averaging method using information from the whole spectral range, whereas the ratio relies on information derived around two specific wavelengths.

Whilst the BCM does give smoother data, its determination from the complete spectrum can lead to artefactual skewing to higher wavelengths when the light scattering signal (that is present in the same spectrum) increases significantly. Normally this only occurs rarely as protein aggregates do not often reach sufficient weight average molecular mass. However, the presence of adjuvants here may lead to the presence of such artefacts in this calculation. As such, the data presented here is derived from the ratio of the fluorescence data as described in the tech note: Fluorescence Data Analysis

Experimental Details

Materials

The model proteins used as antigens were ovalbumin (albumin from chicken egg white, Sigma, A5503) and BSA (albumin from bovine serum, Sigma, A7030), the buffer used in all cases was MOPS (10 mM, pH 7.4) (Sigma, M1254). The adjuvant used was Aluminium hydroxide gel (Alhydrogel®, Brenntag Biosector, Frederikssund, Denmark).

Experimental

In the case of both BSA and ovalbumin the antigen proteins were adsorbed to the adjuvant by mixing equal volumes of the stock antigens (8 mg/mL) and adjuvant solution (4 mg/mL, gently mixing and incubating at room temperature for 2 hours. In order to remove any free antigen the mixture was centrifuged after incubation (1 minute, 3000 rpm), the supernatant removed and the pellet re-suspended to the original volume in the buffer of interest to the original volume. This procedure was repeated 3 times. Jones *et al*. (Jones, 2005) reported a maximum loading onto the adjuvant of 1.6 mg/mL for ovalbumin and 2.2 mg/mL for BSA (Jones, 2005) under the same conditions. The antigens free in solution were prepared at 2 mg/mL.

UNit settings

9 μL of each sample was loaded into the wells of the UNi consumable and placed in the UNit instrument. A thermal ramp was carried out between 15 and 90 °C with the protein intrinsic fluorescence and static light scattering signals being recorded every 1 °C, with a slit width of 50 µm, exposure time of 1000 ms and a hold time of 60 seconds. Eight replicate samples of each of the free antigen samples (BSA and Ovalbumin) and each of the adjuvant (Alhydrogel®) bound antigen samples were measured.

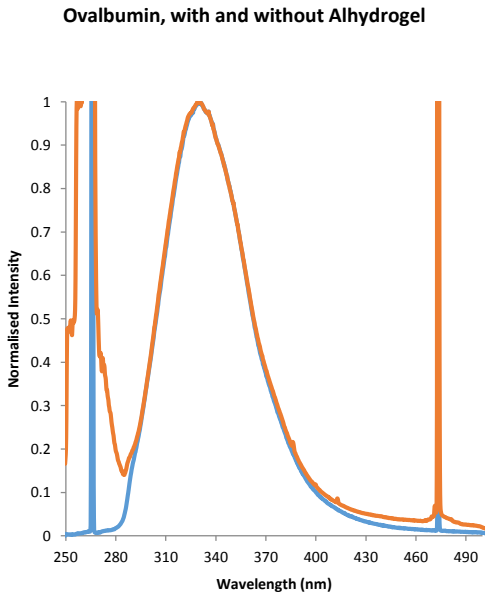

Figure 1: Ovalbumin free in solution (blue) and bound to Alhydrogel® (red) showing no change in spectral characteristics, measured at room temperature (20 °C)

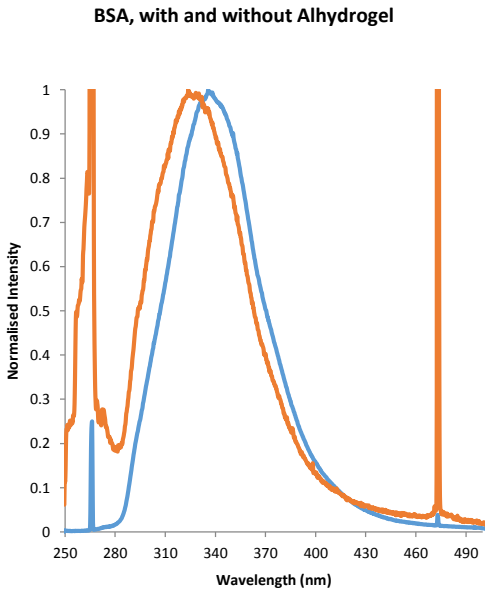

Figure 2: BSA free in solution (blue) and bound to Alhydrogel® (red) showing a small shift in peak position to shorter wavelengths when bound, measured at room temperature (20 °C)

Results and discussion

Comparison of conformation of free and adjuvant bound protein

Fluorescence spectra for ovalbumin and BSA are shown in Figure 1 and Figure 2 respectively. In each case the figure shows an overlay of the intrinsic fluorescence spectra obtained (as an average of 8 replicates for each sample) in which the data has been normalized to ease comparison. Spectra in red show data for antigens that are bound to an adjuvant and those in blue are from free antigens.

The fluorescence intensity peak maxima are almost identical for ovalbumin, with λ_{max} seen at 329.8 nm for free ovalbumin and 329.3 nm for bound. Both have a peak wavelength close to ~330 nm which would indicate that the aromatic residues are well protected from the solvent environment and that the protein is folded in both cases. The differences between the fluorescence peak shape are very small, indicating very close conformational similarity.

The substantial light scattering signals centered at 266 nm and 473 nm for the adjuvant bound protein samples occurs due to the presence of the comparatively large mass adjuvant particles.

In Figure 2 the fluorescence spectra of free and adjuvant bound BSA are compared. It is important to note that BSA behaves atypically for a protein in solution, with the center wavelength shifting to *shorter* wavelengths upon unfolding (Peek, 2006) (Moriyama, 1996). Indeed, a shift to shorter wavelength is observed upon adjuvant binding, with λ_{max} at 335.7 nm for free BSA, typical of protein in the native state, and bound BSA seen at 323.7 nm, a difference of 12 nm, which is typical of unfolded BSA (Jones, 2005) (Moriyama, 1996). A closer look at the fluorescence spectral shapes shows a broadening of the peak in the bound state, implying a number of different conformations and a change in the environment of the protein's tryptophan residues.

In general for both ovalbumin and BSA, the changes in the fluorescence ratio upon binding to Alhydrogel® with temperature appear much broader than when the proteins are free in solution. This would suggest that the antigens are bound in a variety of different conformations leading to a number of slightly different T_m values. An example of the ratio is given for ovalbumin in Figure 3 and for BSA in Figure 4.

If the ratio data between 350 and 330 nm for free ovalbumin and BSA, and for both when bound to Alhydrogel®, is plotted versus temperature, it is possible to calculate an approximate T_m value for each. Further, if the first differential of this data is plotted it gives a clearer representation of the unfolding information, analogous to that of typical DSC data (as presented in Figure 3). In the case of ovalbumin, both bound and unbound, the data show very broad transitions, an effect that is also more pronounced in the adjuvant bound BSA. This is indicative of multiple different conformational states.T_m values, calculated using the UNit Analysis software, that are the averages

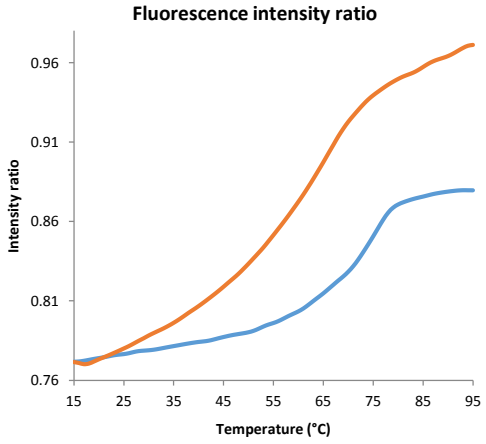

Figure 3: Fluorescence intensity ratio (350/330 nm) of ovalbumin, bound (red) and unbound (blue) to Alhydrogel®

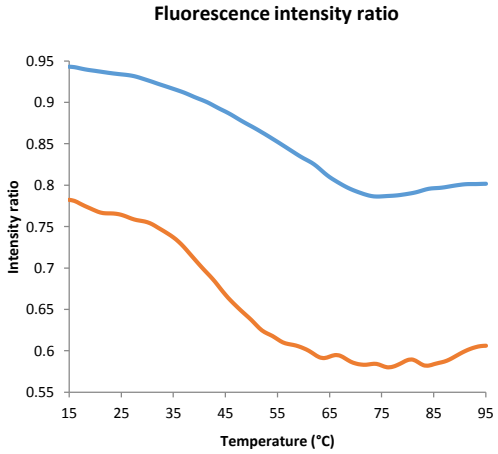

Figure 4: Fluorescence intensity ratio (350/330 nm) of BSA, bound (red) and unbound (blue) to Alhydrogel®

from 8 replicates of each of the four samples, are presented in Table 1.

The peak for free ovalbumin was found to be centered around 74.0 ± 0.6 °C and when bound centered around 64.7 ± 0.8 °C. This shift of approximately 9 degrees indicates that the Alhydrogel® binding does indeed affect the thermal conformational stability. This effect is of particular interest when compared to data presented in Figure **1** which shows that upon binding at 20 °C, there appeared to be no significant differences in the tertiary structure of the ovalbumin.

The process of binding to Alhydrogel®, which is largely governed by electrostatic effects, has in some way affected the thermal stability of the protein while appearing to have little impact on the structure of the protein. It may be that the presence of the adjuvant sterically hinders the protein and prevents it embarking on the unfolding pathway usually followed in free solution. This could lead to the population of species that are usually not observed in solution.

Sample Name	Mean Tm (°C)	Standard Deviation (°C)
BSA	62.5	2.1
BSA-Alhydrogel®	40.6	2.0
Ovalbumin	74.0	0.6
Ovalbumin-Alhydrogel®	64.7	0.8

Table 1: Table of average T_m values for free and Alhydrogel® bound antigens

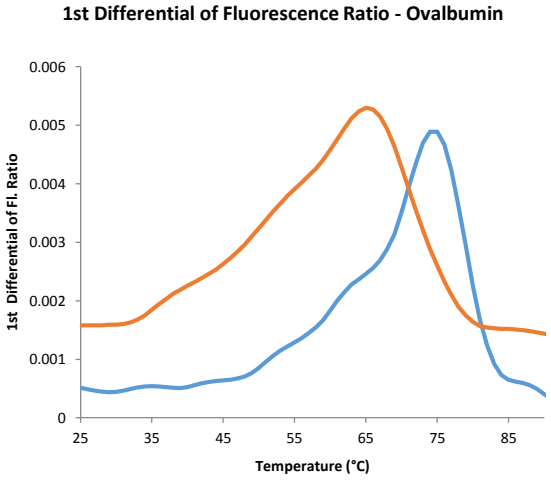

Figure 5: First differential of the fluorescence intensity ratio for adjuvant bound (red) and free (blue) ovalbumin

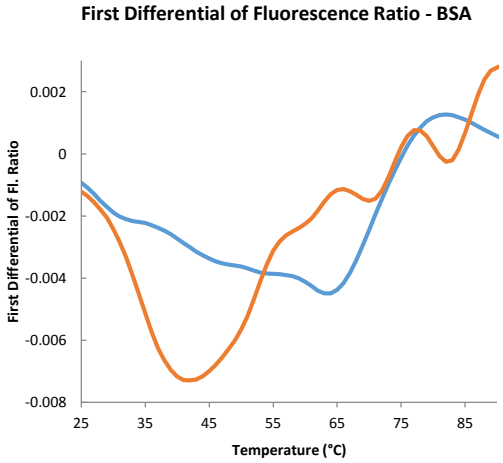

Figure 5; First differential of the fluorescence intensity ratio for adjuvant bound (red) and free (blue) BSA

Conclusion

Fluorescence data presented in this application note suggest that there may be a slight change in conformation of antigens initially following adsorption to an adjuvant, as shown here with two model proteins. This was seen particularly clearly for BSA by comparing the fluorescence peak maximum wavelength at room temperature. However, there was little significant difference observed in the case of ovalbumin following adjuvant binding.

The thermal unfolding profile of the adsorbed proteins indicated a lower unfolding midpoint temperature and a broader transition region than observed for the 'free' protein. This may indicate that following adsorption of the protein onto the adjuvant there was a heterogeneous distribution of protein molecules with different melting temperatures. The observed reduction of 10 - 20 °C, was antigen dependent and agrees with the literature (Jones, 2005).

The static light scattering data is valid for the antigen 'free' in solution and shows characteristic increases in signal upon heating, indicative of thermally induced aggregation (data not shown here). However, in the case of samples bound to the Alhydrogel® it is not possible to follow any changes in the sample aggregation because at the onset of the experiment, the static light scattering signal saturates the detector due to the presence of the large aluminium hydroxide particles.

It has been shown that the UNit is an ideal platform to perform measurements of simultaneous SLS and intrinsic fluorescence on free antigen samples, and of intrinsic fluorescence on antigen samples bound to Alhydrogel® adjuvants in a thermal ramp experiment. This has allowed accurate T_m measurements to be determined and compared for both bound and unbound antigens. The data clearly shows that Alhydrogel® binding decreases the thermal stability of ovalbumin and BSA.

These measurements are high throughput in nature, require only small (9 μL) volumes of sample and the disposable UNi consumable sample holder eliminates the time consuming cleaning step associated with alternative label-free technologies. In this application note a thermal ramp was carried out, but this may be extended to isothermal experiments and a whole range of other adjuvant-antigen combinations.

References

Ausar, S. F. (2010). Application of Extrinsic Fluorescence Spectroscopy for the High Throughput Formulation Screening of Aluminum-Adjuvanted Vaccines. Biotechnology, 431-440.

Braun, L. J. (2011). The Role of Adjuvant in Mediating Antigen Structure and Stability. Biotechnology, 1391-1399.

Clapp, T. e. (2010). Vaccines with Aluminum-containing Adjuvants: Optimizing Vaccine Efficacy and Thermal Stability. JOURNAL OF PHARMACEUTICAL SCIENCES, 388-401.

Dong, A. e. (2006). Secondary structures of proteins adsorbed onto aluminium hydroxide: Infrared spectroscopic analysis of proteins from low solution concentrations. Analytical Biochemistry, 282-289.

Jones, L. e. (2005). Effects of Adsorption to Aluminium Salt Adjuvants on the Structure and Stability of Model Protein Antigens. The Journal of Biological Chemistry, 13406-13414.

Moriyama, Y. e. (1996). Fluorescence Behavior of Tryptophan Residues of Bovine and Human Serum Albumins in Ionic Surfactant Solutions: A Comparative Study of the Two and One Tryptophan(s) of Bovine and Human Albumins. Journal of Protein Chemistry, 265-272.

Peek, L. e. (2006). Effects of Stabilizers on the Destabilization of Proteins upon Adsorption to Aluminum Salt Adjuvants. JOURNAL OF PHARMACEUTICAL SCIENCES, 547-557.

Toll-free: (800) 815-6384
Tel: (925) 587-9800
orders@unchainedlabs.com
unchainedlabs.com