Biophysical Characterization of Therapeutic Nucleic Acid Modalities

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INTRODUCTION

- Biochemical assays are essential when establishing critical quality attributes. These platforms like liquid chromatography coupled with mass spectrometry excel at detection and quantification of chemical modifications, impurities, and fundamental chemical characterization.
- The biochemical "fingerprint" is complemented by biophysical analysis for an overall physicochemical approach to system understanding. Biologics have a strong structure-function relationship and the function of the biologic is thought to be retained as long as the structure is conserved.
- Biophysical techniques can be applied to a variety of systems and the simplicity of the data output enables quick adoption into current workflows of an analytical lab.

- The following data focus on a label-free in-solution approach to biologic characterization of nucleic acids and their delivery vessels.

METHODS

Typical Differential Scanning Calorimetry (DSC) Data Collection:

- Volumes for manual runs were 550 µL for sample and buffer. Current volumes required for automated runs are 490 µL of sample and 800 µL of buffer
- Typical nucleic acid concentrations required are 0.25-2 mg/mL and AAV particles are 0.1-0.5 mg/mL
- A background and negative control is buffer loaded into both sample and reference cells.

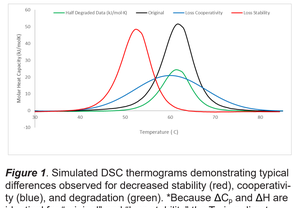


Figure 1. Simulated DSC thermograms demonstrating typical differences observed for decreased stability (red), cooperativity (blue), and degradation (green). *Because ΔC_p and ΔH are identical for "original" and "loss stability" the T_m is a direct comparison of stability, ΔG.

Auto Nano DSC

Typical Isothermal Titration Calorimetry Data Collection:

- Volumes for manual runs for a low volume (LV) Nano ITC or Affinity ITC are 300 µL min the cell and 50 µL in the syringe (100 µL required to load)
- Ideal concentrations are 5x-500x the K_d of the interaction in the cell and the syringe concentration is 8x greater than the cell concentration.
- A background and negative control is the titrant in the syringe and buffer in the cell.

Auto Affinity ITC

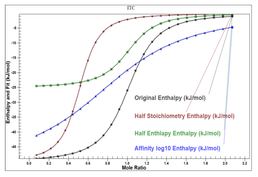


Figure 2. Simulated ITC thermograms demonstrating differences in enthalpy (specificity), affinity, and stoichiometry

Data Acquisition:

- DSCRun©
- ITCRun©

Analysis solution:

- NanoAnalyze©.

NanoAnalyze© is a compliance-ready software that can be used as part of 21CFR pt 11 compliance.

Virus Characterization

- Identifying empty or full capsids is a critical drug efficacy question that is typically answered by the biophysical technique analytical ultracentrifugation. DSC can also address this question as often the stability of empty and full capsids are different.

Figure 3. Cartoon of an Adenovirus.

- In this analogous example, the thermal stability of the Adenovirus coat proteins in solution, is compared with the stability of the corresponding highly structured viral protein shell.

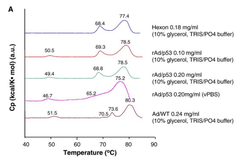


Figure 4. DSC scans of capsid protein alone (hexon), mutant virus (rAd/p53) at two concentrations and in different buffers, and wildtype virus (Ad/WT). Samples were scanned at 1 °C/min in a Nano-DSC. Figure from App note, data initially published by Ihnat et al., 2005 note, data initially published by Ihnat et al., 2005

- The coat protein provided two endothermic unfolding events but the lower transition at 50 °C that was present in the intact virus was absent (Figure 4A).

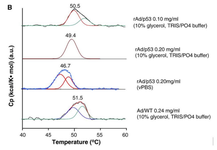


Figure 5. DSC data deconvolution of ~ 50 °C peak. Figure from Choma, C. 2009. Data initially published by Ihnat et al., 2005.

- Comments from original study (Ihant):
- Higher temperature transitions arise from changes in the hexon protein.
- 50 °C transition represents the disruption of the virus particle into protein and nucleic acid.
- The 50 °C transition is not reversible and its profile is dependent on concentration and environment. This lower temperature transition could be used to distinguish between adenovirus mutants, and to screen and optimize stabilizing formulations.

Other examples of virus investigations completed via biophysical methods

Serotype Identification

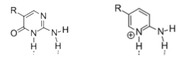
- For this example, Differential Scanning Fluorimetry was used. A group at Allergan was able to identify 10 different AAV serotypes based on their thermal stability differences.
- Rodrigues, G. et al. "Pharmaceutical Development of AAV-Based Gene Therapy Products for the Eye" *Pharmaceutical Research*. **2019**. 36(29).

Formulation Development

- An MMR vaccine and other antibiotics were prepared with the additive, silk fibroin, a protein polymer that can form films, hydrogels or microspheres. The silk has amphiphilic properties and is hypothesized to create a nanoscale pocket that stabilized the particles as seen by a change in the solution stability temperature (T_p) by 51.5 ºC.
- Zhang et al. "Stabilization of vaccines and antibiotics in silk and eliminating the cold chain" *PNAS*. **2016**. 113(26)

Complex Formation with Chemical Modification: PNA

- Peptide nucleic acids (PNA) are a class of compounds that are a synthetic mimic for DNA. Their affinity and specificity for complementary base pairs can be greater than RNA or DNA, which is hypothesized to be related to their neutral and flexible backbone.



- Chemically modified nucleobases can alleviate pH dependency and improve binding affinity. The bases investigated were pseudoisocytosine (J), a neutral base with H-bonding and 2-aminopyridine (M).
- The target was a hairpin, (HRP), a mimic for microRNA 215, which is implicated in cancer development and drug resistance (Zengeya, 2012)

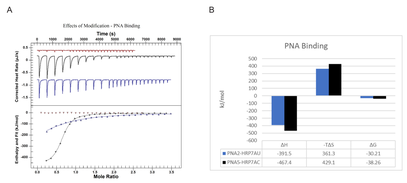


Figure 6: A. 100 µM modified PNA binding to a base-modified 10 µM HRP. No binding result, shown in red. **B**. Thermodynamic profile of the binding. Color identification is consistent in A and B.

- PNA5 (M modified) had a greater affinity and specificity than PNA2 (J modified). Both PNA ligands are able to form multiple H-bonds, which explains that large and favorable enthalpy as well as the unfavorable entropy, which arises from the specific orientation of the bases for H-bonding.
- The charged M-base (PNA5) can also benefit from favorable electrostatic interactions thereby increasing its affinity over PNA2 for the same target.

Complex Formation: siRNA

- DSC can be used as a confirmation screen for siRNA binding to its protein target. Controls for this type of study includes first scanning the unbound materials.
- For the sample below, the siRNA-protein complex shows decreased stability compared to the unbound material. It The exotherm at 66.2 C indicates that aggregation is occurring following unfolding

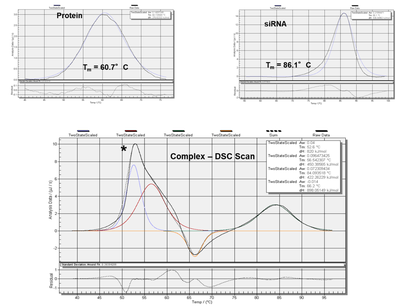


Figure 7: DSC thermograms for the unbound protein and siRNA (top) , the bottom thermogram is the complex with excess unbound protein and siRNA.

- RNA therapeutics such as siRNA and mRNA have been plagued with stability issues to overcome.
- Conjugation, chemical modification (ie. GalNAc), and delivery encapsulation (Hu, 2019), are all strategies for improving stability.

Encapsulation

- Liposomes are a class of a promising delivery systems for nucleic acid therapies prone to degradation.
- Two well-known delivery systems already approved by the FDA are Doxil ™ and DaunoXome™ In the liposome cartoon, the core is shown as hollow and empty. When used for drug delivery, either the core encapsulates the drug (most common) or the drug is conjugated to the external phospholipid head. Another conjugation mechanism employed is the addition of a synthetic PEG (polyethylene glycol) molecule to increase lifetime in the body (Cattel. et al.).

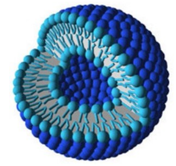


Figure 8: Cartoon of a liposome. (Image, Barte)

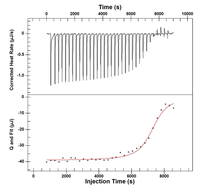


Figure 9: ITC thermogram of plasma-DaunoXome™

- Liposomes composition, size and modifications can all be studies via calorimetry. For example, when cholesterol is added to the structure the transition broadens as the complex loses cooperativity of unfolding and additional configurations and degeneracy levels are available.

- Additionally, Liposome encapsulation or interactions with target complex targets such as plasma (Figure) can also be investigated with titration calorimetry.

Metastable states

- Prior to addition of the drug the liposome on its own should be characterized. In the figure below, the shift to a lower temperature indicates that the liposome was prepared in a manner resulting in a metastable state. This means that the liposome is not in its most stable configuration when formed and lifetime questions arise.

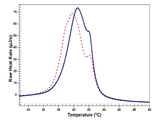


Figure 10: The figures shows the first heat scan, blue, and the 2nd heat scan, red-dash of a liposome.

FINAL COMMENTS

- Biophysical instruments can detect and quantify changes that are ultimately related to the function of a biologic. Due to its universal signal, heat, all types of modalities and delivery vehicles can be studied without modification immobilization using calorimetry.

- The universal detector, heat, is especially useful in nucleic acid modalities where intrinsic signals employed by other techniques are limited.

- The information from a DSC and ITC complements other techniques that provide biochemical information, like LC MS. DSC will provide a simple and quick view of change where MS data will provide the detail of the change.

REFERENCES

Original data was collected in the TA-Waters Microcalorimetry lab by N. Demarse, M. Mathews, and C. Quinn.

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Virus figure. Royalty free download. https://www.pngfly.com/png-dwt0yy/download.html

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