I. Proteomics by Mass Spectrometry

1. **What is an internal standard and what does it accomplish analytically?**

   Internal standards are standards added intentionally to all samples, standards and blanks. The ideal internal standard is completely resolved from the unknown analyte by the measuring instrument but is chemically ‘identical’ to the analyte. Identical means that it experiences the same losses during sample preparation, the same matrix effects and responds to changes in instrument sensitivity in the same way as the analyte.

2. **Discuss isotope dilution from the perspective of the ‘perfect internal standard’ in mass spectrometry**

   Mass spec is unique in its ability to distinguish identical chemicals of different mass (isotopomers). Most isotopomers have chemical and physical properties that are indistinguishable. For example, CH$_3^{37}$Cl and CH$_3^{35}$Cl have indistinguishable boiling points, electronic spectra, dielectric constants, reactivities, solubilities etc., but are completely resolved in even inexpensive mass spectrometers.

3. **No two proteins are likely to have the same exact mass, yet even very high resolution mass spectrometry of protein mixtures rely crucially on pre-separation of mixtures by chromatography. Why is this?**

   The basic idea then is this – the multi-kDa protein peak will have many sidebands comprising small and variable amounts of $^{13}$C and $^2$H. So, for example, if the resolving power of an instrument is 10,000 at 5kDa, then while it is conceivable that one might resolve the centers of the distributions formed by two different proteins of nominal mass 5000 Da and a 4,998 Da proteins, their individual isotoper masses will not be resolved. Take home message: Proteins will produce isotopic distributions around their nominal masses that are normally unresolved. Even if an instrument has the resolving power to distinguish two proteins, they actual resolution may be difficult to these distribution functions.

4. **How is product ion scanning superior to single quadrupole analysis of a protein mixture for the purpose of identifying or quantifying a given component of a complex biological sample?**

   In the product ion scan, one can screen the mixture for the mass of the component of interest in Q1, fragment it in Q2 and then scan for the fingerprint of the fragmentation products in Q3. This is powerful if you know what you are looking for! Even if Q1 admits some interfering species, the fingerprint in Q3 is likely to be interpretable.
5. By coupling ion-exchange and reverse-phase columns in series one may obtain a quasi-2-D separation, and this can be followed by a mass spectrometric detection mode.

a. A strong cation exchange resin contains sulfonic acid (a strong acid) residues. Assume the solution pH is 7.5. Will all proteins bind to this resin? Yes [ ] or No [x]? ( / 2)

b. Why would increasing ionic strength of, e.g. NaCl decrease the strength of this binding interaction? ( / 3)

c. On what basis does the subsequent reverse-phase column retain analytes. ( / 5)

Polarity – in reverse-phase less polar analytes will be more retained, and more polar analytes will be less retained.

d. Will changes in ionic strength make large changes in retention in the reverse-phase column? Yes [ ] or No [x]? ( / 3)

e. How does the combination of strong cation exchange followed by reverse-phase LC equate to a 2-D separation? Describe this briefly in words ( / 2)

The protein-SP interactions in the ion exchange column are strong and elution is nil until an appropriate ionic strength buffer triggers it – at that point, a reverse phase column can be used to relatively quickly separate these components. An increase in buffer ionic strength then triggers this again.

f. Sketch the 2-D chromatogram ( / 5)

![2-D Chromatogram Sketch]
II. Protein Folding Kinetics

1. Describe the functions of the four light beams indicated in the figure.
   a. IR ( / 2)
      The IR beam **heats the sample** using two counterpropagating nanosecond duration pulses.
   b. UV ( / 2)
      The UV beam **stimulates the fluorescence of tryptophan residues in the protein** using a series of picosecond duration pulses.
   c. DL ( / 2)
      The diode laser beam monitors the solution temperature on a long timescale.
   d. to PMT ( / 2)
      This is the beam of fluorescent light emitted by the tryptophan residues on the protein sample.

2. Describe the relevance of the information contained in the figure.
   a. Top curve 2A: What does ‘ellipticity’ measure in the h-apoMb protein? ( / 3)
      Ellipticity measures the extent of folding of the protein.
   b. Why is it plotted against T? ( / 3)
      Ellipticity is plotted against temperature in order to characterize the equilibrium temperature dependence of the folding state.
   c. Bottom curve 2B: How is curve-B data derived from curve A data? ( / 3)
      The linear baselines on 2A are used to represent the linear temperature dependence of the ellipticity function in the absence of a folding-state change. Curve B is the fraction of distance between points in curve A.
   d. How was curve B data used to design the experiment? ( / 3)
      Curve B allowed Gruebele et al. to plan the temperature limits of the experiment – a jump from -10 to 20 degrees should span 60% to 100% folded.
Regarding Figure 4.

a. Which of the metrics, short or long lifetime, or short or long amplitude was used in this experiment? ( / 1)

**Long amplitude**

b. Why? ( / 4)

The long amplitude is the only fluorescence metric that is actually well correlated with the folding state as inferred from the ellipticity data. Other metrics such as lifetimes do not change between -10 and 0 degrees, where average folding extent is expected to change by nearly 40%.

c. What was the function of guanidinium – how did it help the authors choose the metric? ( / 3)

Guanidinium is a denaturant that provided a control for the 4B data - if the change in lifetime were unrelated to folding, then they would likely be invariant to guanidinium. But they do change as a function of guanidinium.

4. Why is cold-denaturation required for this experiment? ( / 4)

The rate of folding is more interesting to researchers than the rate of unfolding. For temperature induced changes in folding state, only increases in temperature can be made quickly – so the only proteins that can be made to fold quickly by temperature change are those that fold upon heating – i.e. cold denatured ones.

5. Regarding the optics

a. What is the IR beam diameter? **2 mm** ( / 1)

b. What is the UV beam diameter? **0.2 mm** ( / 1)

c. Why are they different? ( / 2)

The IR beam diameter defines the region of solution that is heated. The most uniform heating is expected to be at the center of the IR beam. The UV beam is small enough to probe just within this relatively uniformly heated volume.

6. For the protein and in the context of this paper, define:

a. folded ( / 2)

The protein is in its native 3-dimensional configuration – that is, the tertiary structure is native.

b. ‘molten globule’ ( / 2)

The protein is in a tertiary structure that is intermediate between denatured and folded – this is an intermediate, non-equilibrium structure that is achieved almost instantly.

( / 20)
III. Surface Enhanced Raman Spectroscopy

1. Why do we capitalize Raman spectroscopy? ( / 1)
   Raman is the name of the discoverer.

2. Distinguish between scattering and fluorescence phenomena. What are the differences and similarities? ( / 2)
   Scattering is nearly instantaneous, and normally does not involve an upper electronic state.

3. Distinguish between elastic and inelastic scattering. ( / 2)
   Elastic means the scattered photon has the identical energy of the incident photon.

4. How much stronger is fluorescence than normal Raman scattering? ( / 2)
   A good fluorophore converts $10^{14}$ (100,000,000,000,000) times more photons into fluorescent radiation than unenhanced Raman scattering.

5. List two of the technological advances that have made Raman measurements more commonplace. ( / 2)
   CCD detection, laser excitation, confocal microscopy, Holographic notch filters (Rayleigh rejection filters) (Emission filters)

6. Why is Raman considered a ‘selective’ measurement relative, compared, for example, to UV absorption or possibly to fluorescence spectroscopy? ( / 3)
   Raman produces a vibrational spectrum that is more of a ‘fingerprint’ – i.e. is more unique to the analyte than is fluorescence (normally a relatively featureless and broadband emission phenomenon).

7. Approximately how much stronger is fluorescence from a strong fluorophore than unenhanced Raman scattering? ( / 2)
   A good fluorophore converts $10^{14}$ (100,000,000,000,000) times more photons into fluorescent radiation than unenhanced Raman scattering.

8. Describe, in general terms, the setting (place) where surface enhanced Raman is observed. ( / 2)
   Surface enhanced Raman spectroscopy occurs at asperities on rough Ag surfaces, or on the surfaces of Au or Ag nanoparticles.

9. What two mechanisms are believed to underlie the SERS phenomenon? ( / 2)
   Electromagnetic and chemical enhancement.

10. Which of these two is usually the larger contributor? ( / 1)
    Electromagnetic

11. What is the approximate penetration depth of the SERS effect? ( / 1)
    2-4 nm
III. Single Molecule Studies of the Chromatographic Interface

1. Why do the fluorescent signals appear as ‘bursts’ in Figure 2? Use a few sentences (5 pts) and a sketch of the interface, illustrating the excitation beam and the molecules (5 pts) to illustrate your point.

Molecules diffuse briefly into and out of the focal point of the excitation laser. During their transits, they ‘light up’ and produce many fluorescent photons – bursts from the perspective of the detector.

2. What are the relative types of information derived from the CCD images and the APD signals?

The CCD provides an image illustrating ‘hot spots’ where DiI accumulation occurs. Time resolution at these points (1s) illustrates that bursts are appearing on the 2.6 and 26 s timescales. The APD, more sensitive and faster than the CCD gives time resolved signals showing bursts on the 0.07 and 2.6 s timescales.

3. Why do we care about adsorption lifetimes in this setting?

Adsorption is the retention mechanism in HPLC. This work clearly shows that octadecyl silica exhibits mixed-mode retention. This is bad for separation because there is not a single K (partition coefficient) so some molecules are anomalously retained. This phenomenon leads to peak broadening.
4. What is the function of the autocorrelation (what information does it yield)? Answer in the context of the following equation:

\[ G(T) = \frac{\epsilon_{\text{diff}}}{1 + \frac{T}{\Gamma_{\text{diff}}}} + \sum_{i} \frac{\Gamma_{\text{ads},i}}{\Gamma_{\text{diff}}} \exp\left(-\frac{T}{\tau_i}\right) \]  

A larger number of adsorption events can be studied by fluorescence correlation spectroscopy, which employs autocorrelation of single-molecule bursts. It has recently been shown that autocorrelation of single-molecule bursts reveals quantitative information about adsorption equilibria: the relative concentrations of adsorbing and diffusing species, \( \Gamma_{\text{ads}}/\Gamma_{\text{diff}} \), the rate constant for desorption, \( 1/\tau \), and the diffusion coefficient, \( D \), of the fluorescing species.\(^{24}\) If there are multiple specific adsorption sites, \( i \), the contributions are additive, as shown in eq 1.

The molar adsorptivities and quantum efficiencies, \( \epsilon \) and \( \gamma \), could differ for the adsorbed (ads) and diffusing (diff) species. It was shown that the beam variance, \( \tilde{s}^2 \), affects how easy it is to observe the exponential contribution from specific adsorption.\(^{24}\)

The autocorrelation is helpful in distinguishing signal and noise especially in large data sets. Random noise will result in a single abrupt decay followed by a small residual value in \( G(T) \). If signals are mixed in with the noise, \( G(T) \) will exhibit a decay that is characteristic of the signal. In this case, the \( G(T) \) function had a predicted form that distinguished between diffusing and adsorbing molecules. Diffusing molecules have a \( \sim D^{-1}T^{-1} \) decay and adsorbing molecules have a \( \sim e^{-T/\tau_{\text{ADS}}} \) form. Each different adsorption site gives a different \( \tau_{\text{ADS}} \), and these add up.
5. Discuss the 'subtracted' autocorrelations in Figure 4. Identify the parts of the traces that correlate to the important physical phenomena that the paper is describing.

The subtracted autocorrelations are plotted as \( \log(G-G_{\text{Diff}}) \) vs \( T \) (time-shift). This presentation allows the two adsorptive components (hypothesized to have exponential appearances in the autocorrelation domain) to be clearly resolved since \( \log(e^x) = x / 2.303 \).

In this case, both the 60% ACN and water data clearly have these two linear regimes corresponding to 2.6 and 26 s adsorption times.

This is strongly supportive of the overall theory that the autocorrelations can be resolved into diffusing and adsorbing components.

This supports the overall hypothesis of the paper that strong adsorption sites on octadecyl modified silica exist and can be observed using single molecule spectroscopy.

| Table 1. Parameters Recovered from Best Fit to the Autocorrelations of Figure 6 |
|---------------------------------|-----------|----------|----------|-----------|----------|
| \( D \) (cm\(^2\)/s) | \( \Gamma_1 / \Gamma_{\text{diff}} \) | \( \tau_1 \) (s) | \( \Gamma_2 / \Gamma_{\text{diff}} \) | \( \tau_2 \) (s) |
| 0% ACN | \( 1.0 \times 10^{-6} \) | 0.11 | 0.068 | 0.04 | 2.6 |
| 60% ACN | \( 6.5 \times 10^{-6} \) | 0.11 | 0.068 | 0.17 | 2.6 |

Figure 3. Normalized autocorrelation decays using the full data sets of bursts for water (C) and 60% acetonitrile/water (C). The solid curves are the best fit to eq 1 in each case, with the fitting parameters summarized in Table 1.

Figure 4. Same autocorrelation decays as in Figure 3, but with the contribution from diffusion subtracted out. The \( \log G \) scale helps illustrate that there are two components of the decay and they are exponential.