ELIZABETH NOLAN: ...going to do today is some kind of superficial overview and focusing on things that are important to consider when either designing experiments that probe binding, or also when reading about experiments done by others, and thinking about their data and how the data was fit.

So the readings this week were some excerpts from two different types of review articles-- so the Wedd paper and the Giedroc paper. And I guess, just to start, what did you think about these readings and the reviews? What kind of impressions? Did you like them or not like them? How are they different, and all that?

AUDIENCE: I liked when they went into certain considerations you need to keep in mind. That was kind of helpful with the longer one.

ELIZABETH NOLAN: All right, in the Wedd.

AUDIENCE: The longer one?

ELIZABETH NOLAN: So in the Wedd one.

AUDIENCE: It was like there was a 15-page one. Yeah, yeah, this one.

ELIZABETH NOLAN: Yeah, this one.

AUDIENCE: Yeah.

ELIZABETH NOLAN: Great. Challenges of different-- determining metal-protein affinities.

AUDIENCE: Mhm. Understanding, like, pH effects, which wasn't something I'd thought about it in a while, I

guess.

ELIZABETH NOLAN: Mhm. Mhm.

AUDIENCE: I really enjoyed the manganese review because I haven't been introduced much to metals in biology. That's kind of like where I want to go, so I really liked this review.

ELIZABETH NOLAN: OK, so that's good, a good introduction to one aspect of the field of metal homeostasis. Any other thoughts? OK, so what I would say in terms of why we selected excerpts from these two papers, one, as Alex mentioned, this review by Wedd, it's extremely comprehensive.

And the introductory parts give some really good, just brief and clear summary about considerations and pitfalls that happen when people are studying metal-protein interactions. And so right off the bat, there's an emphasis on some important things to think about when either designing your own experiment or reading about experiments done by others.

And then we didn't assign this whole paper, but one of the great things about this review article is that there's this systematic consideration of many different types of binding problems. And the considerations are applicable to more than just a metal-protein interaction. But if you think about biochemistry in broad terms, there's many different types of binding problems. So it's just something to keep in mind for a resource if you ever need that down the road there.

And then the other one is very much looking at the biological system and competition between host and microbe for metal nutrients. And so there's a lot of questions involving metal-protein thermodynamics, so what are relative affinities? There's also questions about kinetics there that aren't-- they're not really addressed in this.

But a lot of effort these days is going towards trying to understand these metal transport systems and also host defense factors that are involved in this tug-of-war. And also it relates to topics that will come up in lecture. Joanne will be focusing on iron homeostasis and heme, but many of the concepts are similar.

And another nice thing about this Giedroc review, and it's something that will come up as we talk about binding experiments more, is this figure 5. So they're talking about a technique called isothermal titration calorimetry and using this method to determine binding affinities.

And they've done a lot of simulations. And so if you ever end up thinking about binding

problems or doing experiments to look at binding, you can begin to have a qualitative appreciation for what data mean by studying simulations like this one. Or in the packet I've made, there's one looking at, say, optical absorption spectroscopy and what a titration curve will look at for different systems that have different affinities between a metal and a ligand there.

So basically, what we'll do is consider just a simple one-to-one bimolecular complex in recitation today and talk about, thinking about, determining a dissociation constant value, which is often how biochemists measure Kd, different methods, and a lot of the things one needs to consider experimentally when studying metal-protein equilibria.

And again, many of these aspects apply to other types of binding problems. So it could be protein-small molecule, protein-protein interaction, protein-DNA. Some are specific to metals because they have their unique characteristics and behavior.

OK, so if we think about a simple, bimolecular, one-to-one complex, all right, and have a metal, M. And often we think about a metal as being a Lewis acid. And then we have some ligand, and we can think of that as a Lewis base forming a complex, so ML.

Often we talk about free versus bound, so free metal or free ligand as metal or ligand that's not complexed versus bound because it's in a complex. And today we'll think about the ligand as being some protein that has a site for a metal, but it could also be a small molecule, for instance.

So in introductory chemistry, typically talk about affinity constants for equilibria. In biochemical experiments, people often report affinity as a Kd, so dissociation constant. So if we think about the equation for Kd, we have the concentration of the complex. I'm sorry. That's the Ka.

Concentration of the metal ligand over the concentration of the complex, so the Kd also equals 1 over the Ka. And you can also think about the Kd in terms of a ratio of the rate constants for dissociation and association here. K off over K on, here, just as different ways to show this.

So if we just look at this equation here, the units for a dissociation constant, our concentration, so units, it could be anything from millimolar, micromolar, nanomolar, et cetera, here. And if we think about a system having increased affinity, so let's say the protein affinity is high. That is a lower Kd. So a protein with a nanomolar Kd value for a metal, that's higher affinity than a protein, say, with a micromolar or millimolar affinity for that metal here-- so lower Kd, higher

affinity.

So what is the common data fitting that we might see in a textbook or in some experiments? We think about it as very similar to thinking about steady-state kinetics in terms of the plot and the equations. So imagine we have some protein, so we have our ligand. And we titrate in some metal, let's say plus 2.

And we have some measure of response to see formation of that complex. So maybe it has a color, like it's a protein that binds cobalt. And cobalt gives some new d-to-d transitions that we can monitor, or maybe it's some other method here.

OK, so we can have a response that tells us about formation of the complex versus the concentration of free metal, here. And say we get something that looks like this. What we can say is that the response equals a constant times the concentration of free metal over the Kd plus the concentration of free metal here. So effectively, we get the Kd. So similar to thinking about KM in steady-state kinetics, but keep in mind the Kd and KM are two different things here for that.

So if we think about this type of plot and we think about setting up an experiment, so say we have a protein and we want to determine its affinity for some metal. What do we need to know?

AUDIENCE: A different concentration for what you're putting in.

ELIZABETH NOLAN: Yeah, well, that's for sure, right? So you need to know the concentration, one, of the protein in your cuvette, or in whatever sample hold you're using, and then the concentration of the metal you're titrating in. But beyond that, based on this equation, what do we need to know?

AUDIENCE: You're trying to determine Kd.

ELIZABETH NOLAN: Yeah.

AUDIENCE: Then we need to know M-free. I'm not sure what B is.

ELIZABETH NOLAN: Yeah, so this is just-- I mean, think back to steady-state kinetics, right there. So I'm just putting it in as that because we don't know what this response is. But imagine you normalize the data to 1 such that your maximum response is 1. B would be 1.

Yeah, so metal-free, so you just mentioned the concentration of metal you're adding in. So let's say you add in 1 micromolar of a metal, and you have 10 micromolar of protein. What is your free metal concentration?

AUDIENCE: But you subtract the balance from the total metal.

ELIZABETH NOLAN: Yeah, right. So the total is the metal going in, and then you have free and bound. Is it easy, always, to know what this is?

AUDIENCE: Probably not.

ELIZABETH NOLAN: Yeah, not always, right? And is there always free metal available, right? So this is something we're going to talk about a little bit moving forward.

And so what we'll see is that this equation's great. In many instances, it can't be used because we don't know what the free metal concentration is, or we're in a regime where we don't have any free metal concentration. OK?

AUDIENCE: Is this response [INAUDIBLE]?

ELIZABETH NOLAN: No. No, these are thermodynamic measurements, all right? So this-- let's say, for instance, that there is a system where, in the absence of metal, it's colorless. And one of the wonderful things about many transition metal ions is that they give us color. So imagine you add in a metal and you end up getting some transition.

So perhaps this response is Amax at each addition of metal. So some sort of colorimetric titration. That's one example. You could also imagine using some sort of spectroscopy. And say there's some specific signal for your metal-bound protein that differs from the free metal there, and then you could use that and quantify it. So for instance, EPR NMR, any method like that, MCD, here.

So no, this is not a rate here. This a response versus the concentration of free metal. And you see as the concentration of free metal increases, we're seeing an increase in whatever this observable is about the system here.

So let's just consider a case. Let's just say we have something like this-- some UV this titration. And we have our ligand. And we titrate in some metal.

And how do we often plot this? Let's say we have the ratio of metal over the ligand. And here,

let's say we have some change in absorbance at some wavelength, like what we have here. And we'll take one extreme case.

So here, let's say you get data that looks like this. OK. So you've done some titration. You've added some aliquot of metal. You let the solution equilibrate. And then you read the optical absorption spectrum.

And so what do we learn from something like this? So what do we see in these data?

AUDIENCE: There's a point of saturation.

ELIZABETH NOLAN: Yeah. So something's happening here, right? So what we see is that over this regime-- which as I've drawn this, we have a ratio of metal to ligand of one. We see that this change in absorption occurs. It's quite linear.

And then once we had a ratio of one to one, we see that there's no more increase in absorption observance at that wavelength. It plateaus. So what does that tell us about the interaction between this protein and the metal?

AUDIENCE: Probably that one binds to one.

ELIZABETH NOLAN: Yeah, right? This tells us something about stoichiometry, first of all-- that for whatever is causing this particular change in the spectrum, we see that change happens to one equivalent of metal and stops, which gives indication of a one to one stoichiometry here. What else does this tell us? So if you see something like this.

What's happening in terms of the free metal concentration over this regime? So when there's less than one equivalent of metal added, where is that metal?

AUDIENCE: It's probably with the protein.

ELIZABETH NOLAN: Yeah, right? It's with the protein. So it's bound effectively. This is evidence for some sort of high-affinity complex, because what you see is that the absorbent change occurs up to one equivalent, and then it stops. Right?

So we can contrast that to something like a case where it's more of a curve, like what we see up here-- where it takes more than one equivalent of metal to saturate that site. In this case, maybe it's one to one stoichiometry. Maybe it's something else. You need to do some more experiments to see.

I say this is some high-affinity complex. So we have no or negligible concentration of free metal. Question one is, what does high affinity mean in terms of a range of Kd? And secondly, if there's no free metal, what are we going to do in terms of determining a Kd value?

So what do we think of as high-affinity binding?

AUDIENCE: Nanomolar?

ELIZABETH NOLAN: Yeah. So that's pretty good, right? Nanomolar or lower Kd. So something like this, what happens if you see data like this is that typically, you'll say, OK, this indicates we have a one to one complex. And the dissociation constant has an upper limit that's typically in the regime of 10 nanomolars. So that sets the upper limit, right? It could be orders of magnitude lower, but we can't see that in these data here.

And so that's something to watch out for when looking at how people analyze binding data, because sometimes, a Kd is reported as an absolute value from a direct titration. So this is what I would call a direct titration, meaning that we only have the ligand here, and the metal is titrated in, or whatever the binding partner is.

OK, but if you're in a regime where you're just getting an upper limit, that value is just an upper limit. And it could be one nanomolar. It could be 10 picomolar. It could be femtomolar. There's some more experiments that need to be done to sort that out here.

So let's just say we have a case where this Kd is one nanomolar. Thinking about this and what we know from steady state discussions earlier in this course-- and again, this isn't the same thing, but some of the same ideas apply. What concentration regime would you want to set up the experiment?

So say you think your protein has a Kd up for a metal of one nanomolar. What concentration of protein do you want to use in the titration?

AUDIENCE: Maybe high picomolar?

ELIZABETH NOLAN: High picomolar. So why would you want high picomolar, and what does high picomolar mean?

AUDIENCE: Because I think otherwise, you wouldn't be able to resolve the dissociation? Like, it'll basically-- if you're above that, it's just going to continue to look linear. There's going to be no curvature

for you to observe what the dissociation would be.

ELIZABETH NOLAN: So typically, you want to be around your Kd. So if the Kd is one nanomolar, you want to be a bit below or a bit above. And if you're really being rigorous, try a few different concentrations. Because at the end of the day, this response should be independent of that within a range of error.

So what's the issue? Let's say your Kd is one nanomolar, or for that matter, one picomolar. And you'd like to set up an experiment. And you need an observable for this response. So this gets back to some of what JoAnne talked about in recitations two and three, and needing a detectable signal in the pre-steady state kinetic experiments, that you have to work with a high concentration of protein to see something. And so that becomes the same issue here.

If your system would allow you to work at one nanomolar or one picomolar to have an observable, you would be in a range where you can see something other than this. But often, whatever we're observing, we need to work at a high-protein concentration, because the extinction coefficient is weak, or we just need a high concentration for whatever that type of signal is, which is what can put us in this regime here. So that's something to think about.

So what can be done in order to get more information than what's shown here for a high-affinity site? So let's say you're not able to work at a concentration that's appropriate, based on the Kd of this high-affinity site, that you need to work at a higher concentration. What can be done?

So effectively, what is often done is what I'll call an indirect approach. Another way this is described is to set up a competition titration, where you take your ligand or protein of interest, you take a competitor, and you titrate in the metal. OK, so what is this competitor? Typically, it's a small molecule with a known affinity, so a known Kd, for the metal of interest under the experimental conditions you're using.

And so there's different flavors of using a competitor. And I'll just highlight a few in passing. So one way to use the competitor is to use some small molecule ligand that allows you to buffer the free metal concentration. So in these cases, it's some sort of system that will not affect the readout of, say, metal binding to your protein.

So you can imagine, for instance, using EDTA, EGTA, NTA, like what's on the nickel NTA columns for affinity chromatography. And there are published affinity constants for these small

molecules for different metals. And so you can set up a metal ion buffering system.

And so the idea is that in addition to your normal buffer-- and we'll talk more about buffers in a minute-- you have a very high total concentration of metal and a high total concentration of a chelator. And you can make these buffers such that the buffer will buffer the free metal concentration. So you can buffer free metal, say, in the nanomolar or subnanomolar regime.

So what does this mean? Your total metal concentration and total concentration of this competitor is much higher than the concentration of your protein. And so when you introduce-- you set up your titration, you have the protein in this buffer system, the protein will bind some of the metal. And then the buffer will adjust such that the free metal ion concentration you've set it at remains the same. So that gives you a way to get free metal concentrations.

Another approach that's often used-- it's also controlling your overall metal concentration, but in a bit of a different way-- is to take a competitor that is also some sort of colorimetric or fluorescent indicator of the metal. And so in effect, what you do is you use the competitor as a readout for competition in the assay. And so what you can do is ask, OK, under these conditions, when the metal bind to the protein, there is no change in absorbance or fluorescence at some wavelengths. But there will be a change from the competitor at that wavelength.

So if you put these together, you can ask, OK, as the metal is titrated in, where does the metal go? Do we see a response from the competitor or not? If not, it tells you that the protein won. If yes, and it's the same as the competitor in the absence of the protein, the competitor won. Right?

So those are two cases of out competition where either the protein out-competes this competitor or the competitor out-competes the protein. That's not very helpful for actually determining an apparent dissociation constant value. It will give you information about limits here. But what you really want to have happen, and as this name suggests, is that you want the protein and this competitor to compete.

So effectively, you see the response of the competitor attenuated, compared to the response in the absence of protein. So some metals here, some metals there. And then what you can do is a mathematical analysis to fit that data, based on knowing the affinity of the competitor for the metal, and knowing the concentrations of the competitor in the ligand here.

So this is something that Wedd talks about quite a bit in the review that was assigned, in terms of setting up these competition titrations here. And so when done well, that can really be quite powerful here for that. And there's many other themes and variations about how to do that. But just to keep in mind, if your binding event is too tight to measure by a direct titration, you want to think about a way to do a competition titration here.

So in the packet, I put in an excerpt from a paper that was published in 2003 showing some titration curves like what I sketched here, where there's some response to indicate how much is bound versus some concentration of metal. And one of the reasons I really like this plot is that it gives a qualitative sense for Kd values over a range of magnitudes and what that curve would look like here. And just having a sense of this qualitatively gives you a lot of leverage in terms of just looking at data and analyzing it, whether it's your own or someone else's in terms of, is this a high-affinity site? Is this a low-affinity site? Likewise in the Giedroc review with a different type of method called EITC here.

So what we're going to do is talk a little bit about some general concepts and then some general considerations for, say, setting up these types of experiments. And so some of this relates to concepts in class. So JoAnne talked about the Irving Williams series.

So based on that series, if you're, say, looking at some protein, and you're interested, say, in the Kd for binding of manganese versus zinc, what would you expect qualitatively? So imagine each of these metals is bound at the same site. And today in class, we talked about the different types of ligands that proteins use. So histidines or carboxylates, or maybe a cystine. We'll leave tyrosine out for the moment. But what would we expect? Which metal will bind with higher affinity based on Irving Williams?

AUDIENCE: The zinc.

ELIZABETH NOLAN: The zinc, right? So as we march along the first row for manganese, we see that the affinity increases and copper combined with higher affinity than zinc. So there's a swap at the end. So that's what we would expect.

So what does that mean, just in terms of reading something in the literature? Right. If someone's reporting binding affinities for a protein, and you see that the values are of a similar order of magnitude for manganese and zinc, you might want to scratch your head a little bit and ask what's going on. Right?

So is it a case where both metals are bound tightly and the titration didn't resolve a difference because you're just in an upper limit? Is there something unusual about this site that is causing the selectivity to be contrary to what we expect based on the Irving Williams series there? So the point is you can use those generalities as a guide. And there's always exceptions to the rule.

I missed class on Wednesday. Did you go over hard-soft acid base? So have any of you heard about this hard-soft acid base concept. No. No. Yes. So, like, what's the hard-soft acid base theory?

AUDIENCE: So smaller or electronegative things will associate those are, like, hard things--[INAUDIBLE] larger and fluffier atoms than--

ELIZABETH NOLAN: How is an atom fluffy? No. Right. So think about how polarizable it is. But that's along the right track. So basically, we can classify different metals and different ligands as being relatively hard or relatively soft. And then there can be the gray area in the middle, which is called borderline.

So if we think about, say, a metal ion that's a hard Lewis acid that's something like calcium, for instance-- iron(III)-- these types of metals, like oxygen donors, which are hard bases, for instance-- often it's metal in a high oxidation state if that's an option. So iron(III) versus iron(II). Iron(III) is more hard. They're not very polarizable.

And so, often hard metals are bound by hard acids. So an example like JoAnne brought up and Tara backed in today in class, and if you remember the structure from when we talked about siderophore biosynthesis, it uses six oxygen donors to bind iron(III). So from hard-soft acid base theory, that's a sensible ligand set.

On the other extreme, what's soft? So that's a soft acid-- some metal with a large ionic radius. So if we think about to the right in the periodic table-- mercury, cadmium, copper one. And they like soft ligands, like cystine. So sulfur, that's quite polarizable. So soft, typically lower oxidation state. More to the right in the periodic table.

And then you get metals that are in the middle, like zinc, iron(II), cobalt(II). There. So this gives you some indication of a guide, and why I bring this up is we've talked about the Irving Williams series, but depending on the ligand set, that series might not make sense. Right? So something like an EF-hand domain that binds calcium ions, it uses many oxygen donors. It's

going to prefer calcium, say, over copper, even though calcium is in another place in the periodic table and also not defined by that-- formally defined by the Irving Williams series there.

OK. So that's something you can keep in mind when analyzing the data just qualitatively, right? And so in the Giedroc review, if you look at those data, it's the case in many of the systems where what's currently reported or reported at that time are Kd values that are similar for certain metals that are separated along the first row. So then the question is, what's really going on? And some of it is an issue related to methods and experimental design, in terms of finding conditions that allow high-affinity binding to be studied here.

So let's consider just some practical considerations in terms of experiments as we go forward. So in the beginning of this Wedd paper, he talks about a bunch of pitfalls that can come up in terms of experimental design. Do any of you recall what some of these problems are? You know, when he brings up on page two, "reliable evaluation and comparison of metal binding affinities is important for quantitative understanding of medal selection and speciation. "

So that's central to everything that JoAnne has been talking about in terms of homeostasis the past few days in lecture. And then what does he say? "However, estimation of these metal binding constants is problematic at the moment, as disparate values have been reported in the literature." And then he highlights a few examples that are illustrative of this wider problem here.

And so what's striking about some of these issues he shows in that page two of this review? Did these things concern you when reading the review? So what do these highlight in general? Yeah.

AUDIENCE: Wait. What was the exact question?

ELIZABETH NOLAN: So in terms of in Wedd's paper, he begins this paper by citing a number of examples of problems in the literature. And I guess I'm asking, were these problems striking to you? And if so, why? And really, what is generally the issue here?

AUDIENCE: I feel like there's such a wide range of magnitude of the Kds that kind of points to an inconsistency in experimental set-up.

ELIZABETH NOLAN: Yeah.

AUDIENCE: To where maybe somebody could give something else--

ELIZABETH NOLAN: Right. So these values are hugely different that he's citing here. Right? I mean, 10 orders of magnitude different-- you know, reported Kds that vary by six orders of magnitude. These are huge differences. This isn't one nanomolar versus 10 nanomolar. This is hugely different, and depending on what number you come up with, there's huge implications for what that means in a biological system.

So what are some of the reasons for why there may be so many discrepancies? And in each case, we don't really know, but what we're going to do now is just think about some of the aspects of experimental setup that might be affecting determination of one of these values and how to think about these things. So in terms of pitfalls, I'll begin with one, which is just fitting the data in an inappropriate manner.

So there are so many programs out there that will fit data. But the end of the day, you need to ask, what does this fit mean? Is it meaningful for the system that's being studied? So did it take into account all parameters? Is it the correct stoichiometry? Do the numbers that come out make sense? What other experiments can be done to try to test that there?

So that's a general issue. And then, as I've mentioned here in passing, often direct titrations are fit inappropriately because this is concluded to mean some absolute Kd when it doesn't. It just gives you a limit here.

So let's just think about taking a protein and titrating it with a metal. That experiment will happen in a buffer. So do we need to think about the buffer?

AUDIENCE: Yeah, but then it could be, like, a cuvette here for metal that you're interested in.

ELIZABETH NOLAN: So that's the first question. Does the buffer influence metal speciation in the cuvette by having some affinity for the metal of interest? So from that perspective, what buffers could be classified as problematic? So you need to think about the chemical composition, the chemical structure of the buffer.

AUDIENCE: EDTA or something?

ELIZABETH NOLAN: OK. So EDTA could be in your buffer for some reason, but that's not your buffer, right? So the buffer is what's going to control the pH there. So Tris is an example. What are other examples

of common buffers?

AUDIENCE: Bis-Tris?

ELIZABETH NOLAN: Yeah, bis-Tris. Others?

AUDIENCE: PBS?

ELIZABETH NOLAN: Yeah, PBS. So a phosphate buffer. That's often used in tissue culture experiments and other experiments. So let's start with the Tris buffer. Is it a good idea to do a metal binding titration where you want to get a Kd in Tris buffer? Shaking head no. So why?

AUDIENCE: Because if you're going for metal being bound with protein, if the Tris is poured into the middle, then it might alter your readout.

ELIZABETH NOLAN: Yeah. OK. So let's break that down. So one, Tris-- that has an affinity for certain metals. You have an amine-based buffer. So that's one issue. And then the other thing you need to think about in this are, what are the relative concentrations of the buffer to your protein of interest? So what's a typical Tris buffer concentration used, say, in protein purification or some type of experiment?

AUDIENCE: Like normally?

ELIZABETH NOLAN: Yeah. Typically higher than one million molar, too. Right? So maybe 20, 75 million molar. Maybe even higher than that. So you have this substantial concentration of your Tris buffer, compared to a protein concentration, which if you have a micromolar Kd you'd like to look at a micromolar range of protein.

So that will influence the metal binding equilibria in the experiment. So then the question is, if you're doing that titration under that type of condition, are you taking that Tris metal interaction into account in the data analysis?

Are there other buffers that are arguably more appropriate? And the answer is yes. So there's buffers like HEPES. These are buffers that are called good buffers-- zwitterionic buffers that in general have lower metal affinities, and are often used for titrations.

What about, say, metal contamination from the buffer or from the water? So what's important to think about there? Is that an issue?

AUDIENCE: If you're using hard water or something, there's calcium that would bind to your protein.

ELIZABETH NOLAN: Yeah, right. So you need to think about the water. You know, where did this water come from? Where did your Tris come from, or whatever other buffer? Because again, if you have 100 million molar buffer, it's not only the molecules of, say, HEPES, but it's whatever other contaminants are in there. And there's a lot more of that than your protein, which gets into this issue of Irving Williams' series and zinc.

So zinc contamination is everywhere. Zinc is everywhere. So are you getting a zinc contamination, say? And your metal binding protein, some portion of it is complexing zinc and you can't see that, because zinc is spectroscopically silent. That's going to be a problem. So that's something to think about and keep in mind.

So for rigorous work, high-purity buffers can be used. Or there are tricks out there to demetalate buffers. Those tricks often have a few caveats as well for that. But I think contamination is something to keep in mind, and can be a bit of a nuisance. But you just need to know how to look for it and deal with it.

And also, these contaminations-- it becomes an issue, too, in terms of what is your protein concentration? So if you have a one micromolar metal contamination, and you're working with one millimolar protein, it's probably OK. But if you're working with one or 10 micromolar protein, then there's a problem, because you're going to have more of that complexed there.

So why are we using the buffer? We're using the buffer to control pH. So how do we want to think about pH from the standpoint of these titrations?

AUDIENCE: You don't want to make something that you're trying to coordinate the metal with, so like the proteins [INAUDIBLE].

ELIZABETH NOLAN: Or even histidine that has a pKa that isn't in the regime. And cystine, right? That has a pKa.

So often, we think about the pH of the buffer used in protein purification that will make the protein stay in a happy state. But then the question is, is that pH appropriate for the metal binding study? What is the effect of that pH on the ligands and the primary coordination sphere?

So are they protonated or deprotonated or a mixture of the two? And then how does that affect the affinity itself? So these Kds will have a pH dependence based on pKas of the side chains here. And I mean, also, are there pH requirements for the metal? And is your experimental setup such that the pH remains constant throughout the titration?

So an example-- iron(III). So JoAnne talked about iron(III) in class today, and this ridiculously low Ksp at pH 7 of 10 to the minus 18. You can't just have your iron(III) stock solution at pH 7 and have much of anything soluble.

So what do people do about that? Often, the stock solution is stored in acid because it's soluble there. Can you titrate that acidic solution directly into your protein? These are just things to think about here.

What else can be in the buffer? So thinking about anyone who has purified protein. So you brought up EDTA, right? And that certainly would be something that would need to be taken into account. Hopefully you would only have it present if you wanted to do something like a competition. Otherwise, that's going to be a major issue in terms of sorting things out.

But what else might be in the buffer? So what if your protein, say, is a cytoplasmic protein and it has a lot of cystines? Are those cystines likely to be reduced or oxidized in the native form if it's a cytoplasm protein?

AUDIENCE: Reduced.

ELIZABETH NOLAN: Yeah. Reduced, right? Because that's a reducing environment. And then you go into the periplasm or the ER, which is where you find proteins that have more disulfide bonds. So let's say your protein likes to have a bunch of reduced cystines in it. Chances are you have a reducing agent in the buffer you use for protein purification. And maybe you need to keep that reducing agent around during an experiment, or maybe you can work in an anaerobic chamber and get rid of it.

But let's just say the reducing agent's present. Is that something we need to think about from the standpoint of a metal-protein interaction? So what are examples of these reducing agents?

AUDIENCE: TCEP.

ELIZABETH NOLAN: TCEP's one, yeah. And we'll come back to that one in a minute. What are some others?

AUDIENCE: [INAUDIBLE]

ELIZABETH NOLAN: Yep. And what else? Another thiol-based reducing agent commonly used in protein purification.

AUDIENCE: DDT?

ELIZABETH NOLAN: DTT. Yeah.

AUDIENCE: Oh, DTT.

ELIZABETH NOLAN: DTT, right. So let's just consider, say, DTT and BME together. Is there something we need to consider there? Yes, because depending on your metal, these reducing agents will have some affinity. And often, they're in very large excess over the concentration of protein. So it's a similar issue to the Tris buffer issue, in terms of how are these reducing agents affecting metals speciation and metal binding equilibria in the experiment.

So TCEP. This is Tris-carboxyethel phosphine. So not as commonly used in protein purification. But it is a reducing agent that you commonly see used in certain metal binding titration. And that's because it's thought to cause less interference. So the affinity-- that equilibrium constant is much weaker.

So what is one of the pitfalls of using TCEP that people often run into? Do you know? So if you just have TCEP and aqueous solution

AUDIENCE: It's going to start--

ELIZABETH NOLAN: What?

AUDIENCE: Reducing, just if you leave it there.

ELIZABETH NOLAN: Well, it needs something to reduce. So if you just have TCEP and water, is that neutral? Basic? Acidic? So it's acidic. And the manufacturer instructions say this pretty explicitly. But oftentimes they go unread, right? So if you end up working with quite a bit of TCEP in your experimental conditions, the first thing you need to ask is the buffer adequate to buffer the pH when TCEP's added.

You don't want the TCEP acidifying your buffer and then you're not working at the pH you think you're working at. So what does that mean? You may want to pH adjust your TCEP solution before starting the experiment there. That's just something to keep in mind. I've seen that happen many, many times, in terms of the TCEP there.

Temperature control. The equilibrium constant is temperature dependent. So what is the temperature control throughout one experiment, and then also if you're repeating this experiment over multiple days, because you want to get error analysis and show that it's reproducible, is that temperature good for that? So those are some key things.

And then what do we need to think about in terms of using a competitor when setting up the experiment? So one, we need to know the Kd value of the competitor for the metal of interest. And hopefully, we know something about this system so we can make an appropriate choice, because as I said before, we want to see competition there.

What could go wrong? And again, this isn't meant to be all gloom and doom. This is just, you know, you need to be aware of certain things that can happen in your experiments and know to look out for them so you can fix things as necessary. So here, we have the protein, we have the competitor, we have the metal.

And as I've described it, we want the protein and the competitor to operate effectively, independent of one another. So they can both bind the metal, and somehow this metal is going to be distributed between the two based on the relative concentrations and the relative Kds. So what could muck that up? That's the ideal scenario.

AUDIENCE: Could they both bind the metal?

ELIZABETH NOLAN: Well, we definitely know they both can, right?

AUDIENCE: Simultaneously.

ELIZABETH NOLAN: Simultaneously. So what would that be called? So that this can be a major headache. What happens is that you get what's called a ternary complex. So you have the ligand, the competitor, and the metal as one. So imagine that your protein has a metal site that's not coordinatively saturated. And so as a result, maybe you have the metal in this site but then the competitor also binds.

That's not good from the standpoint of setting up this competition, right? Because how do you parameterize for that? So that can be a big issue, and something that you need to watch out for when designing the experiments. Could something happen between the competitor and the protein itself in the absence of metal?

AUDIENCE: Perhaps they could interact, and then, in their interactions, block the metal.

ELIZABETH NOLAN: Yeah. It could block or perturb. So what might happen? I mean, we can just imagine a scenario where this protein has some hydrophobic patch. And maybe this competitor has a fluorophore for that's relatively hydrophobic. Or maybe part of the ligand is hydrophobic. And so you end up getting the competitor sticking to the protein.

That doesn't necessarily mean the competitor won't bind the metal, but it will perturb how that competitor behaves. That could perturb the optical readout. It could perturb the metal affinity of the competitor. So that's something to also watch out for.

So we talked about the buffer and contaminations in the buffer. What about the competitor here? So typically, these small molecules are coming from some commercial source. Right? And so you have similar issues, even though you're using a much smaller concentration.

And so don't always assume what you're getting is as pure as they tell you. And that could be organic impurity, or it could be a metal contamination, because these competitors are ligands. And they could have picked up some metal along the way.

So what can be problematic from the standpoint of, say, organic impurity here? One common example is that if you're using something that's fluorescent or brightly colored, to have an optical readout. Maybe there's an impurity that wasn't removed in the synthesis and purification that's also very bright. So you have something that's compromising the optical signal of the probe.

And then there's also the possibility, since these are ligands, that there's a contaminant that can also bind a metal. So if there was some byproduct that wasn't fully removed during purification. If that's the case, it will influence speciation as well there.

So what does one do in terms of gold standard and testing? You need to know what the primary literature is about this competitor molecule, and then effectively test your sample and make sure it has the expected optical properties and the expected behavior when binding the metal of interest. And if that all looks good, then can move forward. Also, just typical tests of

purity, LCMS, HPLC. Even with many of these, if they're highly colored, a simple TLC will give you a lot of information there.

So I'll close with that, and just would reiterate broadly that a lot of the topics discussed in the Wedd review and in the packet, although from the perspective of metals and proteins, it's more general to any type of binding problem. And if you need more resources in terms of binding problems related to metals, I highly recommend reviews by Wilcox and Giedroc, in addition to this review by Wedd there. So they talk a lot about aspects of experimental design and certain methodologies there.