JOANNE STUBBE: This recitation on mass spec is supposedly associated with reactive oxygen species. So [INAUDIBLE], which happens all the time in this course, because we can't describe all the techniques as we go along. So what I'm going to do is just give you a two second overview of what you need to think about to put the paper you read into the big picture.

I don't think the paper-- the paper also explains it. And this week we're going to focus on the mass spec paper, which is mostly sort of trying to figure out the technology, and then the next week is focused on the biology. And so the major unsolved problem-- so everybody and his brother is using mass spectrometry as a tool nowadays. There has been a revolution in mass spectrometry. [INAUDIBLE]

The instrumentation is cheaper. The mass spectrometric methods have just really taken off. And people didn't even know who mass spectrometrists were, but they're starting to win major prizes, because it's revolutionized what we can do. I was talking to somebody yesterday, and they just got a mass on a protein that's 3.3 million. How do you get a protein into the gas phase that's 3.3 million? Right. Doesn't that sort of blow your mind? Anyhow, it's been a revolution.

And we're going to be looking at-- this module seven, which is on reactive oxygen species, and we've been talking about the question of homeostasis. And so one of the things with these reactive oxygen species is they are used by us to kill bacteria, viruses, or parasites. But now, in the last five years or so, everybody's focusing on the fact that here are these reactive oxygen species that play a key role in signaling, which is everywhere, and the signaling process we're going to be looking at next time and is alluded to in this particular paper is epidermal growth factor receptor and epidermal growth factor.

There are hundreds of these proteins that have receptors that are involved in growth and epidermal growth factor receptor [INAUDIBLE] of successful cancer therapeutics. So it's interesting what happens up here, what happens down here, how do you control all of that,

and people are studying this. So we've already seen cystine is unique. And if you have a reactive oxygen species, and we'll see that the reactive oxygen species we'll be looking at, when we're going to be looking at a number is actually superoxide.

So that's one electron reduced oxygen, which in the presence of protons can rapidly disproportionally give[?] oxygen gas to hydrogen peroxide. And hydrogen peroxide can react with cystines to form sulfenic acids, which is the subject of the paper you had to read.

And so the question is how prevalent is this, and the question is, is this important and interesting in terms of regulation inside the cell? And so the key issue is-- even cystines aren't all that stable, you know, if you have proteins with cystines, and you let it sit around for a long time, you could form-- and the protein's concentrated, you could form disulfides. It's not a straightforward reaction, but you can form disulfides.

The question is if you had hydrogen peroxide inside the cell, which you do, can you form sulfenic acids, and do they have a consequence biologically? OK, and that's the question we're going to address next time. And so the issue is this is unstable. So if you want to develop a method to look for this species, and you start cracking open cells, and you start working it up, what happens is this falls apart and reacts and gets destroyed.

And an example of this is the area of DNA therapeutics and DNA drug interactions, therapeutics that interact with DNA. For decades, you see lesions on your DNA. How do you determine what the lesions are? Mass spec has been a major method to look at that. Almost all the lesions in the early days were complete artifacts of the analytical chemistry to work them up.

They had to get them into some form that you could stabilize the lesion and then analyze it. And what was happening because they weren't careful enough and quantitative enough, they changed it to something else. And so the data in the early years was all completely misinterpreted.

So the issue in this paper is that other people had developed this, and Kate Caroll has taken this on. Can we have a way of derivativizing this [? mentally ?] inside the cell, because if you disrupt-- if you disrupt this by cracking open the cells and trying to purify things, it undergoes further reaction, and what this can undergo further reaction to is SO2 minus, sulfonic acids or SO3 minus. Sulfinic acids and sulfonic acids.

OK. And it turns out this reaction is also reversible with hydrogen peroxide a lot of people are looking at that at this stage is irreversible. Anyhow. So the question is can you develop methods to look at all of these things. And in fact Tannenbaum, who was in the chemistry department, but also [INAUDIBLE], he is looking at nitrosation of SHs, again forming a reactive species, and he's developed new methods sort of like Carroll has to try to specifically look at these modifications.

And in the end, what you want to do, and this is the key, you might be able to detect this-- the question is, is this interesting? So you have to have a way to connect this back to the biology inside the cell. And that's what the second paper is focused on. So what we're doing today is simply looking at the technology that's been developed to try to get a handle, how do you look at sulfenylation, you're not really focusing on the biology of the consequences.

And so what we're using is mass spec. And we're using a method of mass-- how many of you have done mass spec? So if you know something and I say something wrong, you should speak up, because I'm not a mass spec expert. And actually, I've got a whole bunch of information from, say, the Broad, and I thought it was not very good. So we need a way of trying to figure out that you're going to see-- there's hundreds of variations on the theme. I'm going to give you a very simplified overview of what things you need to think about.

And so if I say something that you don't agree with, tell me. OK, so when looking at mass spec-- this didn't exist when I was your age-- using soft ionization methods, and what does that mean? It means that you don't want your molecules to crack. So the issue is that what mass spec is about-- so really looking at mass spec, and the key issue of what you wind up looking at is mass to charge. OK. So m over z.

OK, so the problem is how do we get something charged enough so that the mass is small enough so that you can see it, taking a look at the mass analyzer, which is going to be part of all mass spectrometers. OK. So there are two different ways you could change the mass to charge. You could dump an electron in. And if you dump an electron in, that produces radical species, which can then fragment. We want to avoid that. That's not soft ionization methods.

But how can we control this? The way we can control this is dumping in protons. So what we do is we can control it by adding protons or by subtracting protons. And we'll see that the different methods we're going to be looking at, we'll see there are two main methods that most of you have probably heard about your classes. One is electrode spray ionization, so ESI. And

I think, if you're in Brad's lab, they have a lot of these.

Yesterday's class had people that had used these, but really didn't know much about what's inside the machine. So this is the kind of thing I think your generation, if you're going to use this as a tool, need to roll up your sleeves and understand a lot more about what's going on, and MALDI, maser MALDI. Matrix Assisted Laser Desorption-- it will become clear why it's called that in a minute.

So these are the two methods. And what we do is we can protonate, so that we can move this into the analyzer range, where we can actually read it. So what we'll see is the analyzer-- I'm going to show you sort of what the three parts of a mass spectrometer are-- can only read 1,000 to 2,000 daltons. OK, so if you look at your protein, much, much bigger. So you're going to have to stick a lot of charges on there to be able to see anything. So that's the whole thing, and the question is, how do you do it by one method or by using the other method?

OK, so all mass specs have sort of the same components. And you can go to websites. The Broad does have a website, and what the Broad will tell you is what all these spectrometers are, but I don't think they do a particularly good job telling you what's useful for what, and why it's useful, which is, I think, what you need to use if you're only going to use it fleetingly and then move out.

So you have a source. So you have an inlet. How do you get your sample from the liquid phase or the solid phase into the gas phase? OK, so that's going to be that. And so what is the distinct ionization method? And we will see that there are many ways that you can ionize, and we're just going to briefly look at in a cartoon overview of how this happens. And then so once you ionize it, it needs to move from the source.

So you need to have ion movement into the analyzer. So this is the mass analyzer. And this becomes important. And we will see in a second that there are many methods to do the mass analysis, mass to charge analysis, and then after you do this, you have a detector. And then, furthermore-- and I think this is a big part of it now, if you're doing wholesale anything, you have to have a really sophisticated method of data analysis.

And so that's the other thing that I get frustrated about all the time. So you see people-- I mean, people do experiments where they've spent-- last year, somebody spent three months trying to get all the proteins out of a cell, 10,000 proteins out of the cell by mass spectrometry right. Now, because the technology is changing, they can do it in four days.

But what do you do with all this information? And how do you use this information in a constructive way, and how do you know if it's correct or not? So those are the kinds of things. I think if you're going to use this-- I think everybody is going to be using this technology. You need to educate yourself about how to look at this. OK, so that's what the issue is. And so we have a source, an analyzer, and a detector.

OK, so this is just a cartoon of that, which describes this in more detail. And I think he put this on the web. I think he put the PowerPoint on the web. I was doing this at the last minute yesterday. So it's different from the handout I gave you that's written out. This is a PowerPoint. OK, so you can go back and look at this, but one of the other things I wanted to say is that sometimes when you analyze your mass, you want to analyze it further, and that was true-- many of you might not have caught it, but that was true in the analysis that was carried out in this paper. Did anybody recognize that you had to analyze this using more than one mass spec? Did you look at the data carefully enough?

So also you probably didn't read the supplementary information, which also is critical to think about. I mean, if you want to look at the methods, you need to get in there and roll up your sleeves and look at them. So we're going to see that the methods that people often use is they don't look at the whole protein, but they degrade it down into pieces. So then you can find here a whole bunch of pieces, OK. But that doesn't tell you anything.

The mass does tell you something. It might tell you whether it's sulfenylated or hopefully, you can distinguish between any other modification, but it doesn't tell you the location of the sulfenylation. And so you can do a second method. So you could have some other gas. There are many ways to do this that you bring this in to now take a peptide. So you pick one mass charge. You throw in something that's going to degrade it by fragmentation, and then I'll show you in a minute we understand what kind of-- using certain methods, we understand the fragmentation patterns, which actually allow you to sequence the amino acids.

And the reason I'm bringing that in is when I first got to MIT, Klaus Biemann was in the lab, and I did many experiments with him. And these are the first experiments that were done to sequence peptides by mass spec as opposed to doing Edman sequencing, which the mass spec was actually better, and there are pluses and minuses, but I noticed from looking at the literature, people were still using the same method that he developed.

So this is just a cartoon. And it just shows you that there are many ionization methods. We're

focusing on these two, FAB, fast atom bombardment. We didn't have any of these when I was your age. Fast atom bombardment was something I used a lot because I've worked on DNA drug interactions, and it allows you to look at nucleic acids. And a lot of these other methods don't. I mean, we're focused on proteomics in this particular paper, and then mass analyzer.

So you have time of flight. I think Brad's lab has MALDI time of flight. So what does that mean? You've got a long tube in here, and what happens is you have mass to charge, and they're different sizes. And so the smaller ones fly faster. They don't want to keep away from the walls, but the smaller ones fly faster than the bigger ones. So that helps you differentiate between all the ions you're actually looking at.

I guess somebody just told me you guys just got a new quadrupole ion trap. Anyhow, if you want to look at this, I have notes on all these things. But I think this is something you'd have to study in detail. And so while I have pictures of them all and how you can differentiate one from the other, I think it doesn't really mean that much to me, because I don't know enough about the physics of how they were designed. I mean, this really has revolutionized what you can do.

OK. So that's the components of all mass spectrometry. What I want to do is very just briefly look at the ESI and then look at the MALDI and then show you what the issues are in general, and then we'll focus right in on the paper, and the recitation I did on Thursday, we didn't quite get through all of it. We got through most of it, but then we'll continue next week and also attach this to the biology, which is the second paper, the nature chemical biology paper also written by the Carroll group.

ESI. So that's the one we want to look at next. And so that's up there. This is a cartoon of how this works. So what do you do, and how do you do this? So the first thing is you have your protein of interest, which I'll call the analyte, because we want to charge. Lots of times you put it under more acidic conditions, pH 6 or something, 6 1/2, depends on the protein. So you get more charge states.

And if you're trying to look at something big, you need a lot of charges on there to get it into this mass range of 1 to 2,000 to be able to see it using this method, and apparently what you do here-- can you see this? Have you done this? Can you see this capillary? Can you look at what's going on?

AUDIENCE: I don't think so.

JOANNE STUBBE: OK. So I was just wondering, because I haven't ever. So it's all closed off. It's in a box, and so you can't-- there's not like a thing where you can watch what's going on?

AUDIENCE: Not that I've seen.

JOANNE STUBBE: OK, because I think it's sort of amazing. How do you get this huge protein and solution into the gas phase? Right. I mean, that, to me, is like mind boggling, OK? I mean, these guys were geniuses. And you know, there's been a number of Nobel Prizes for this, but I wouldn't have a clue how to do something like that. So what you do is apparently, you put it down a capillary and then you spray it out, and then you have to-- so what you get at the end of this, this plume of spray, apparently you've got a lot of the analytes and a lot of solvent molecules, and then the goal is during this process, to get into the analyzer is to get rid of-- to separate all the analytes mixed together into a single analyte and remove all the solvent.

OK, so that's the goal. And apparently, according to the people that were here yesterday, this is taken from, I think, sort of one of the papers that was first out. This is the way they did it in the old days. I don't know if they still do it this way, but the goal is really, to get a single analyte with no solvent on it. OK, and so the question is, how do you do this, and the chamber they had was at atmospheric pressure, and then they had a potential and pressure gradient, which allowed it to get into the mass, before the mass analyzer.

So you start here with the initial spray, and then as you go farther, you remove some water molecules. You finally get to the place where you've removed enough water molecules that all these positively charged species come together, they're incredibly unhappy. And then they fragment apart. I mean, that's the way they describe it. It sounds reasonable. So you get smaller and smaller till eventually, you get to a place where you have an analyte that you can look at specifically and the water has been removed, and that's what you look at.

OK. So again, we need to be in the range of 1 to 2,000. So that's the way these things work. Although, I think, again, how you get to looking at the single ions I think in different mass spectrometers. And so what the issues are, I think, are shown here, and this is the beauty of this methodology. So if you have a protein of 10,000 molecular weight, you couldn't see it, because the mass analyzer is limited.

So you have to go all the way down to eight charges on it to be able to see it. And then, you divide that by that, and you get-- what do you have? You have to do some corrections, but you get something that's this size. OK, but you can see it now because of all the charges on it, but

the beauty is if you add more charges, you get another peak. And you get another peak.

And it all has the same information, and it just differs by the number of charge. So you have all this information. You can use that-- all these informations together to give you a very accurate mass on this system. So this method by analyzing all the data, and now the computers do this, I guess, routinely can give you a very accurate mass. So if you look at this printout, it doesn't look like that. This is what it looks like.

And what do you think's going on there? So we look at mass charge, and we're in the range of 1,000 to 2,000 daltons. And then what is this all-- what is all of these peaks associated with? Anybody got a clue?

AUDIENCE: Isotopes.

JOANNE STUBBE: Yeah, so isotopes. So where are we seeing isotopes before? So these are mostly stable isotopes. We spent recitation two and three looking at radio isotopes. OK. I would say, you know, radioactivity is pretty important. Stable isotopes are extremely important to mass spectrometry. So if you get into this, you're going to be able-- you'll see that being able to label things with different kinds of stable isotopes is key to really deconvoluting the complexity when you're looking at a whole proteome and thousands of peptides.

We're getting down-- it becomes very complicated, and you have to be able to compute what you expect based on the normal natural abundance isotopic distribution. So that's the key thing. So we look at the normal isotopic distribution. And if you look at that, I think in the next one, I show you an example of that. So what are the isotopes-- you probably can't read this here, but if you pull out your computer, you'll see this.

So we have C12, C13. OK, we have hundreds of amino acids with carbons. So you have C12 and C13. C12 is 99%. C13 is 1%. That's an actual abundance. OK, so every one of these has different natural abundance. We know what they are. In fact, if you're an organic chemist, you can measure isotope of x using a mass spectrometer, if you have something that's really accurate, which we do. I've measured a lot of C13 isotope effects, using a mass spectrometer, based on differences in natural abundance and changes. Yeah.

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: The what?

AUDIENCE: The natural abundance of deuterium?

JOANNE STUBBE: Yeah, I think it's up here. So it's up here. I think it's-- let's see, 3%. Yeah, protons deuterium 3%.

AUDIENCE: Would you expect a huge distribution from that?

JOANNE STUBBE: You see isotope effects on everything. You see-- if you do mass spec, I mean, this is something I think that's not appreciated, and you have a linker with deuteriums in it, and even if you chromatograph it, you change the chromatographic properties based on the deuterium, and so you might think it's migrating here, and it doesn't. It has an isotope effect on how it migrates.

So yeah, you need to pay attention to all of this stuff. OK, and it seems like a small amount, but the beauty is that it is a small amount, but it's incredibly informative, and we have very powerful computers that can allow us to do the analysis. So we do have protons. You see deuterium used. You saw deuterium used in this paper you read today. They did CD3 and CH3's. OK, you can also see the tritium. OK, that's much smaller.

I don't know what the ratio is, but you can look at it. But you also-- this one is also incredibly important and is widely used in proteomics-- N14 and 15, and people do isotopic labeling. So they might see N15 labeled lysine or arginine or deuterated lysine or arginine. And why do you think they would deuterate the lysine or the arginine or N15 label it? What do we know about lysine and arginine in terms of thinking about proteins and analysis of proteins? What do you think about lysine and arginine?

You've seen it several times over the course of this semester, and you probably saw it in 5.07.

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: What?

AUDIENCE: The protons will exchange?

JOANNE STUBBE: Well, now as you put it-- no. So that that's true if it was on a hydrogen and a nitrogen, it would exchange, but they put the deuteriums in on carbon, so they're not exchanging. OK, so why that would happen in any amino acid, why lysine and arginine? And the reason is that almost all-- and this was also done in this paper, you don't work on the huge protein. You cleave it to

pieces. And you cleave it into pieces, and where you cleave is with trypsin, which is the major-- you've seen this used now over and over again. That's a major thing you use because it cleaves next to basic amino acids.

So these become really important in labeling experiments, if you read much mass spec data, or if you look at Alice Ting's work, everything is N15 and deuterium labeled, and lysine and arginine to try to make sure they have coverage of the whole proteome, which is what her lab actually looks at. OK. So we have isotopic labels, and we can take advantage of these, and we can calculate what the distribution should look like, OK, of the isotopes should be, depending on what the-- we know what the sequence is. We know what the abundance is. And so you can calculate the whole mass spec.

So let's see. So there's going to be a number of things that we want to do, and what we're going to be describing today and the next time is a "workflow." These are the words that people use all the time, and "platform." And what we're trying to do in the case of the Carroll papers is simply look at whether the protein is modified or not. But as with most post-translational modifications, do you think this is going to be 100% modified? No.

In fact, it's only partially modified. That adds to the complexity of understanding whether the biology is interesting or not, so what you have then is something that's modified and something that's more non-modified. So then the question is, how do you tell how much is modified and how much is non-modified? If this enhances the rate only a factor of two, and this is 99.8%, of this, are you ever going to be able to see an effect of this modification? That's the question that you have to focus on, and everybody and his brother is doing experiments like this. We will see in a second, hundreds of post translational modifications, and the question is what are they doing in terms of thinking about the biology of the system.

OK, so what's the platform? What's the platform we're going to use? So there are two ways you can look at this. So we have a protein that has been modified. You're going to-- if you had a huge protein, and you only had a single OH on it, even if it was 100%, and the protein was, say, 300,000 molecular weight, you might not be able to see it. You need to do a calculation to see whether you could see it or not. If you have a small protein of molecular weight 30,000, or whatever-- I think the 22,000 or 23,000 like glutathione peroxidase, used in this paper, you could see it. So you could look at the protein directly.

But how else could you do this? You would enrich. If you were doing this in the whole cell, you

would want to separate this away from everything else. OK, so to do that, you want to be able to have a way to stabilize this, OK, and that's what this paper is all about, and then not only to stabilize it, but to separate the stabilized form out. So where does this happen? And in this particular cartoon, where do you see post translational modifications?

Probably the most popular one is phosphorylation. So we have signaling cascades in kinases. And in fact, if you look at the epidermal growth factor receptor, it's a tyrosine kinase, and it gets phosphorylated and is regulated. And this sulfenylation is supposed to be on top of the phosphorylation. So you have multiple post-translational modifications that can affect activity.

So Forest White, for example, in BE, works on kinase signaling cascades. And so he's developed a method, as have others, to be able to pull phosphorylated proteins out of a crude gemisch. OK. So, you know, if you look at this, here he's got iron bound to a phosphate and bound to some bead. So the iron's bound to some chelate around the bead, just like your nickel affinity column, which then binds to the protein.

But this raises the issue that I was discussing in class, which I spent a lot of time on over and over again, but you need to think about, do you think these bonds are tight, how tight do you think those bonds are? What do you need to think about for this kind of analysis to work? It's the same thing with nickel affinity column that you talked about when you were looking at purification of proteins.

AUDIENCE: It has to be stable enough.

JOANNE STUBBE: It has to be stable enough. That's the key. So you have to undergo ligand exchange. It's got to-- if you didn't have-- when you start, you don't have phosphorylated form of your protein around. You have nothing. You have water there. OK, so the waters have to undergo exchange, so the phosphate can then bind, but it's an equilibrium, and so up and down the column is coming off and on. Yeah.

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: It could. I mean, so it's a question of what out competes what. It's a question of relative Kds. So what you have to do is study all of this to figure out how to optimize this, how did they arrive at this? Probably somebody did a lot of studies. OK. This is a new method. I don't know how new it is, but it's a method I don't know that much about, again, of pulling phosphates out. So that's one way. So you have-- so you usually have an affinity purification.

And if we look at the Carroll paper, what she does in the next paper is she's going to figure out a way-- she's derivatized, she's made a dimedone derivative, which stabilizes the sulfenic acid, and then she attaches something to it that's going to allow us to affinity purify that. We'll come back and talk about that later.

So what are they using over here? They're using-- this is-- if you look at histones that get acetylated or methylated, they have an antibody that's specific for the acetylated lysine, so they use antibodies to pull something out. So that's a method-- the second way of pulling things out are using antibodies. That's quite frequently used. And what did they use in this paper? Did it detect the modified sulfenic acid? Does anybody remember? Did you read the paper carefully enough?

AUDIENCE: Like, a anti-dimedone antibody?

JOANNE STUBBE: Yeah, so they use an antidimedone antibody. OK, so that becomes really critical that you know that your antibodies are actually working effectively. So we have antibodies, and then, another thing that people are interested in this department, the Imperiali lab, is sugars. We have sugars everywhere. OK, we don't really understand the function of these sugars. We understand some of them, but it's amazingly complex.

And what we have are proteins called lectins, and any of you heard Laura Kiessling talk, maybe undergraduates wouldn't have done this, but she discovered a new lectin and discovered the basis, the structure the sugar that binds to this lectin. And so you can selectively move that type of sugar. Again, it's an equilibrium. So they're coming off and on, but it binds, hopefully, enough so that the other stuff washes through, and you enrich in the protein of interest.

So these are sort of some of the tricks that are actually used. We're going to see, in the case of the Carroll paper, next time we use click chemistry to make something with a biotin on it, because biotin you all know can bind to streptavidin, which has pluses, and it has minuses, but it allows you to pull things out more easily, because the interaction is so tight.

So you could do this-- the workflow could be on the intact protein, or it could be on peptides. OK. And so the bottom half of this graph shows what happens after you treat this with trypsin. So with trypsin, and you're always cleaving next to lysine or arginine. So the C terminus of your protein is always a lysine or an arginine. And you can find that more easily if you

deuterate or N15 label it. That's what people routinely do in the [? Broad. ?]

And then you have, I think this is the most amazing thing, so you have a protein. And then you have an HPLC column. Have any of you done HPLC? And so do you think-- you could have a protein of 300,000 molecular weight, and look at the separation of your peptides. But if you look at any one of these things, do you think it's pure? So it's not pure. So every one of these peaks, if it's 300,000 molecular weight, you can calculate-- the reason people use trypsin is-- does anybody know why use trypsin, besides that cleaves at lysines in its specifics? Why do people use trypsin as a thing to cleave a big thing down into a little thing?

AUDIENCE: What's the rationale for cleaving it? [INAUDIBLE]

JOANNE STUBBE: So the rationale for cleaving it is just to make it smaller and easier to analyze. That's the rationale for cleaving it. So a peptide, a small peptide. But the question is, how big is the small peptide that's easy to analyze? And so that's the rationale. It gives you a distribution of peptides that's pretty good, that are all accessible to mass spec methods. So I don't know what the distribution is, but you know, people have done that calculation.

And so almost always the peptides fly, whereas if you use other things, and you have something much bigger, it might not get ionized in the appropriate way or in a quantitative way, and you completely miss it. So the trypsin has been most successful. But each one of these little peaks is not one peak. You'll see when you put it into the mass analyzer, and if you read this paper carefully, you will see they got multiple mass charge species, which then they associated with specific peptides, OK. They know the sequence of their protein. And then they always use tosyl phenyl chloro ketone. Why do they use that? Anybody have an any idea?

So in the experiments where they're doing the trypsin cleavage, they put in tosyl phenyl chloro ketone. Anybody know why? OK. No good. This is something that-- so tosyl phenyl chloro ketone is an alpha halo ketone. So it's activated for nucleophilic attack, and what you do is you have an acylated N terminus and an aromatic, and that's specific for chymotrypsin, like proteases. And so what this does is that covalently modifies the active site of chymotrypsin, and kills chymotrypsin.

If you choose the wrong time to cleave with trypsin, you don't start getting cleavage next to hydrophobics, which then makes the analysis of the peptides much more complex. So the analysis of the peptide, a lot of people have done a lot of peptide chemistry, and I was telling this story before. I always go off on tangents. But Stein and Moore won the Nobel Prize.

Maybe this is what you do when you get old, but Stein and Moore won the Nobel Prize, you know, in the 1950s, the 1950s, for separating amino acids. Do you know that they had a three story column of Dowex that was composed of anion exchange Dowex and cations? It was all polystyrene backbones of anion and cation polystyrenes, to be able to separate the amino acids.

OK. And when you do that, of course, it gets stuck on the resin. Your recovery's out of the bottom of this chromatography. You need tons of stuff to put on the column in the first place. And this is what's happened. I mean, you have a little tiny HPLC column that has huge number of theoretical place that allows you amazing separations. I mean, again, the technology is sort of mind boggling, what you can do now.

OK, so what you're doing here is then you're just asking the question, if you have a post-translational modification, x, you can either look at the entire protein. And so you could probably tell it was modified, but telling the location of the modified location, you can't, or you can treat it with trypsin. And then you get, again, with trypsin, you have little pieces. And one of these little pieces will have an x on it.

And then you can define it. And then if you want to do sophisticated analysis, you can hit it-- use a second mass spectrometer, and actually sequence this. OK. So I think the next one just briefly goes to MALDI. And MALDI-- so Matrix Assistant Laser Disorption-- have any of you ever done that? OK. So where do you do that? Do you do that in [INAUDIBLE] lab?

AUDIENCE: No, in the undergrad lab.

JOANNE STUBBE: Oh, OK, because this is Brad's new thing. OK. OK, so you're looking at peptides. OK, so what do you use as the matrix?

AUDIENCE: We used some aromatic acid. I don't remember.

JOANNE STUBBE: OK. So you probably used sinapinic acid.

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: OK. So this is so-- you're using a different one still from this one-- this is-- I don't know. I got this idea somewhere. I don't know. So when I've done this-- I did do this maybe 10 years ago-- I've looked at a lot of peptides. We went through five or six of them before we found one that really worked well. So I don't know how state of the art has become, you know what it is. But

the other one in the book that I got this from was, again, an acid. And so what is the idea?

So the first thing you have to do is you have to ionize. So the way you do that is you mix your matrix and solution with your protein of interest, your analyte, then you evaporate it. So you have a solid on a little plate. And then you use a laser beam at 337 nanometers. And the light is absorbed by whatever the matrix is and causes you to have a plume of material. This is, again, amazing to me that the protein goes into the gas phase.

And then, you have to go through this, go into the analyzer. Did you do time of flight? OK, so you have time of flight. So you guys know what it is then. And in the end, you do detection. So, again, the protocol is the same, but the method is different, and this is widely used and easy really easy to use nowadays. So the issue then is this is what you face when you're looking at a whole proteome. So you just can't calculate the mass of all the proteins from the gene sequences.

Why? Because almost every single amino acid in your proteins are modified. So that adds complexity to all of this. So de-convoluting the mass spec becomes more complicated. So this just shows you, you don't need to look at this, but if you look at cystine, you could form disulfides. You can attach a prenyl group, an isoprene group on it. You can attatch palmitic acid on it. You can sulfenylate it. You can nitrosate it.

So you have many, many modifications of the amino acids that are chemically reactive and involved on catalysis, and then not only involving catalysis, they are involved in regulation. So that then adds to the complexity of trying to deconvolute what the mass spec, I think, is actually telling you.

And then, sorry, it went backwards. And so then what that does is tells you-- whoops. I'm just completely discombobulated here. OK, so what that does is that, again, you're just adding different masses on to all of these amino acids. The problem is that you have modified, and you have unmodified. And the question is what's the distribution? OK, and so if you have a very non-abundant protein, and most of it's unmodified, it's going to be much harder to find. So these are just things you need to think about, and your technology to look needs to be extremely well worked out, so that when you look and you don't find something, you know what the lower limits of detection are.

So here we are at our system. Now we're into the Carroll paper, and so what we're looking at is sulfenic acids, degenerated by hydrogen peroxide. We'll see-- do you think that's a fast

reaction, hydrogen peroxide with a cystine? Anybody have any intuition? I think these reactive oxygen species you're going to find are not so intuitive about the chemical reactivity. I'll give you a table with what we think we know in general. But I think it's not so intuitive.

If you look at the rate constants for reaction of a hydrogen peroxide with a cystine it's 1 per molar per second, really slow. OK. So then the question you have to ask yourself, so this was something that was debated in the literature for 15 years. Is this so slow that this could never happen inside the cell? Because I just gave you a second order rate concept. So we have two molecules interacting at the concentration, this could be high. This is really low. You can calculate the rate constant for the actual reaction.

It's really, really slow. OK, so we'll see that there are some proteins, peroxiredoxins that are in humans, are there in quite high levels that can increase this rate to 10 to the fourth per molar per second. So there's a huge rate increase but you need to think about all this kinetic stuff to really understand if this modification can happen inside the cell. Otherwise, well, if it can't happen, why are you wasting your time looking for it? Which is what a lot of people are doing scientifically.

OK, so let me see what the next-- OK. So now we're into making a reagent that can specifically modify this, or specifically modify this. OK. So the reagent that they chose-- she didn't invent this reagent-- was dimedone. And this reagent specifically interacts with sulfenic acids. It doesn't react with the free cystine. So you've got to study all of this.

And if you're going to use this as a reagent inside the cell, you want it to be fast. You don't want to take 30 hours to do the reaction. You want it to be over fast, and you want it to happen at pH 7. So how do you think this reaction works? Where's the most reactive part of this molecule?

AUDIENCE: Those two protons?

JOANNE STUBBE: So two protons. So this these have low pKas, so you can easily form the enolate. Depends on the details, the experimental details. And now you have this, and what you end up with is this molecule. And so the question is, does this go in 5%? You need something that goes in quantitative yield at pH 7, rapidly.

OK, we're going to come back and talk about what the issues are, because the issues are even harder if you want this region to work inside the cell. OK, we're doing this on glutathione

peroxidase, which is what he's using as a model to see if all of this stuff works. OK. So what you really want to do if you're thinking about regulation in the end, is you want to know how much is in each form, and you know, if you read hundreds of papers published on methods trying to figure this all out, but what she did in this case, was she developed a second reagent with an iodo group.

OK. And as you can see, what is the product of the reaction? The product of the reaction is the same as the product of this reagent. But this reagent does not react with sulfenic acid. OK, so you get no reaction. So how does this reaction work? What do you think? The what?

AUDIENCE: SN2.

JOANNE STUBBE: So it could work by an SN2, but the way probably works is it attacks the iodine. So you form-- this is probably the mechanism from what's been done in the literature. So you attack this, and you form this, which then gets attacked by the enolate.

So it doesn't really matter what the mechanism is, but the key thing is for this to react-- if you're interested in a mechanism, which I am, it does matter what it is. So the key thing is now you have the same reagents. So how could you ever use it attached? How could you ever use it to distinguish sulfenylation from a cystine. So what did they do in this paper?

AUDIENCE: [INAUDIBLE]

JOANNE STUBBE: Yeah, so they put the deuterated form on this. So what they did then was in this paper, so you got to keep these straight, if they see deuteriums present, so they made this deuterium label, and this protonated so now you have a mass difference of 6. OK.

And in the system, they're using glutathione peroxidase, which has three cystines in it. And one of the cystines is more reactive than the other two, but for proof of concept they mutated two of these cystines into serine initially, so you only had a single reactive cystine, but then they went back and studied the whole protein.

OK, so let me just introduce you to this, and then we'll come back and talk about this next time. Let me just do one more thing. OK, so here is the difference in mass between these two species. So this is what you're looking at. If they start out with deuterium labeled dimedone, the peak that they observe is going to be associated with sulfenylation, and if they start out with the protonated material, the peak they observe is going to be associated with the

[INAUDIBLE] group.

OK, so that's the idea. And then what they did was they simply took their protein, and they have, in this case, 50 micromolar of their protein, and then they increase the concentration of hydrogen peroxide. They don't really talk very much about how they design the timing, but they use, you know, two equivalents. So they use variable amounts of hydrogen peroxide.

And what you can see is the maximum amount. So now what you're using, we talked about this before, but we're using anti dimedone antibodies for the detection. And here, they're starting with no hydrogen peroxide. So you don't see any dimedone derivative, and then you increase the concentration. But you get to the highest concentration here that they looked at.

So it's 100 micromolar versus 50 micromolar in the protein they used. But what did this immediately tell you? Did any of you look at this data very carefully? What is this? This guy here is associated with a [INAUDIBLE] group that is only reacted with iododimedone, so if you got 100% yield, what does that tell you? This tells you the maximum amount of material you're going to observe.

So if you look at this peak, and you look at that peak, you can't do this by eyeball. You need to do this quantitatively. The phosphor images or methods that allow us to do this quantitatively. What do you see?

AUDIENCE: It's not at the max.

JOANNE STUBBE: Yeah, it's not at the max. And so what we'll do next time-- so these are sort of controls, and the question is how effective is this reagent, and if you start hanging stuff off of your dimedone over here, are you going to change the rate of modification? Can it get into the active site where this SOH actually is, these are the kinds of things we're going to talk about next time when we look a little bit more at the details of the reaction with this, and you should look at the reaction with gap dehydrogenase, which is another control enzyme they ended up looking at it, because what they do is address what the issues are that you're going to encounter when you get into something real that you care about. And that's much more complicated. OK so that's it.