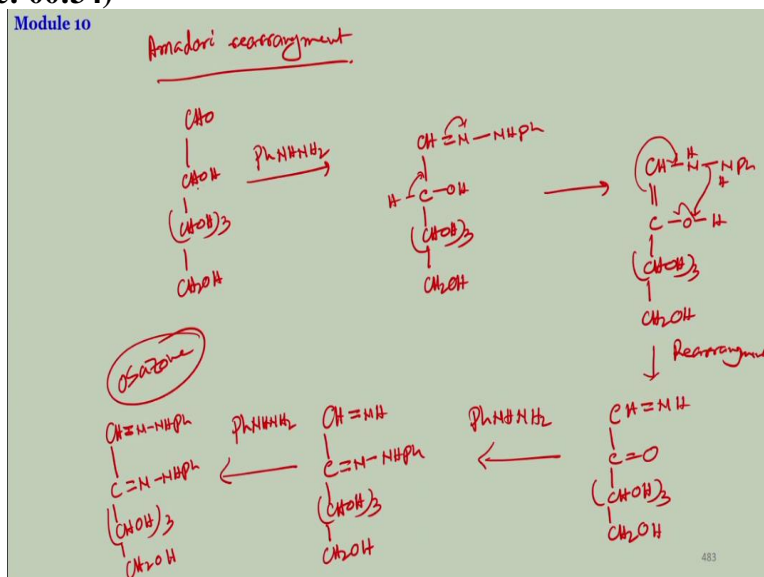


Essentials of Biomolecules: Nucleic Acids, Peptides and Carbohydrates
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Lecture-34
Carbohydrate Chemistry-II: Polysaccharides and Its Nanoparticles

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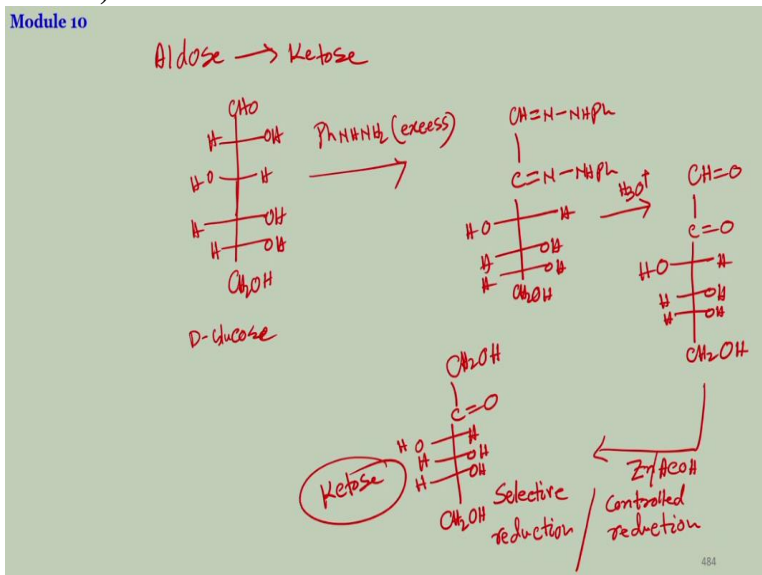


Hello everybody and welcome to the lectures. So, we are discussing module 10 about the carbohydrates, the various applications as well as the chemistry of different carbohydrates that occur in nature. Now, we have seen how the smaller the carbohydrates can be of different types. One is the small molecules that are basically we call monosaccharides and then little bit larger molecules or disaccharides and then can be the polymorphic of the monosaccharides that we call polysaccharides.

They are typically large molecules which are present in different organisms as a major component. And then we have started discussing some chemistry of the monosaccharides mainly, last we have discussed about a rearrangement known as a Amadori rearrangement, where we have seen that if you take an aldose, in this case we have taken aldohexose aldose means, you have the CHO at the terminal. And then if you treat it with phenol hydrazone, then it goes all the way here and gives you Osazone formation which is basically this.

So, both CHO and next CH OH gets converted into our double bonded nitrogen gives you hydrazone derivative actually, so phenol hydrazone derivative basically, and we call it Osazone. So today I will discuss how we can convert from one type of sugar to the other type.

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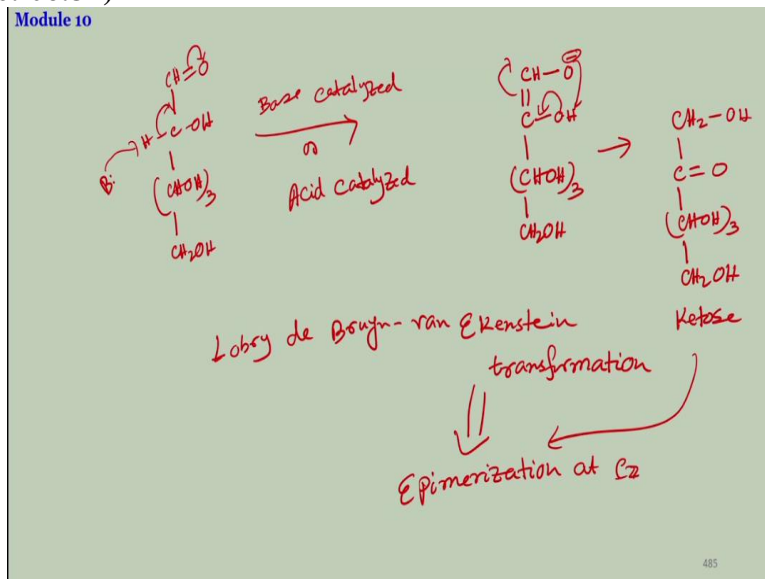
That is all aldose to ketose. For example, if you start with glucose, how can you convert it into kind of fructose? So, in general, aldose to ketose. So, if you start with OH H H OH OH H OH H CH₂ OH. This is your glucose D glucose. So, if you take glucose and if you treat first with an excess of phenol hydrazine in excess quantity then we have seen that it will form this these 2 on now H OH rest would be intact CH₂ OH.

Now, if you hydrolyze it fully in acid, acid catalyzed hydrolysis basically. So, this will be converted into CHO this should be converted into keto plus to be intact. So, you have aldehyde and keto both. Now our aim is to get only the keto. So you have to do a selective reduction of the aldehyde that can be done obviously you know, how to do as selective conversion or selective reduction of aldehyde and not touching the keto part. So, if you do the controlled reduction zinc acetate basically controlled reduction.

Nowadays there are other modern reagents that are more selective towards aldehyde and not to ketone, you can use those also. So, selective reduction or control reduction you can call it control reduction or selective reduction that will reduce the aldehyde into the corresponding alcohol it

will leave you the keto and other parts as it is. So now you have a ketose so you can make a conversion from an aldose to a ketose.

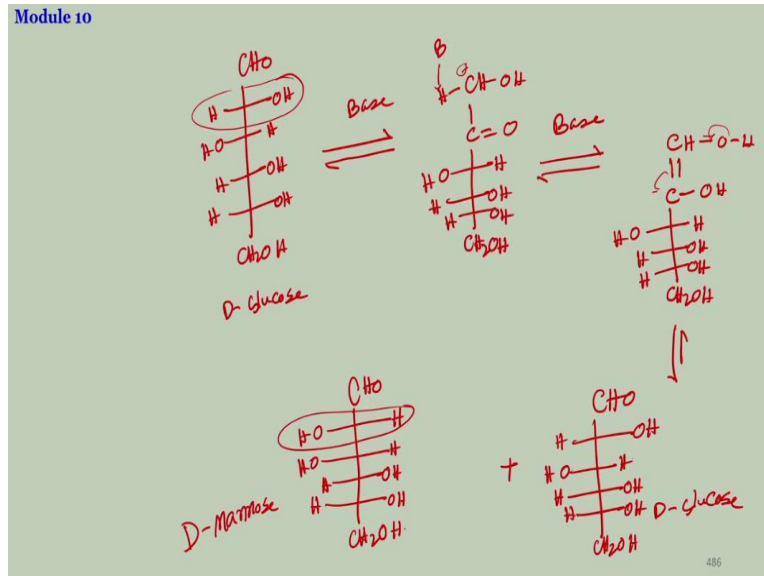
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Another one is CHO CH OH CH OH whole 3 is basically the same glucose and then CH₂ OH so this is acid catalyzed or base catalyzed treatment you can treat it with a base it can be base catalyzed or it can also be acid catalyzed either of the 2. So for example, if you use base either always base or maybe hydroxyl group OH minus. So nevertheless, if it is a base, it will take up the proton here. This will go and you will have the regions here will be like this OH whole 3 CH₂ OH. And you have OH here this would be your minus actually O minus.

Now this will take up the proton it will come here, this will go there. So basically this will become now CH₂ - H + OH and then this will be your keto CH OH whole 3 and CH₂ OH. So, this is again your ketose. This has a name, this is a very famous reaction called Lobry de Bruyn van Ekenstein transformation and this method is used for this transformation is used for epimerization at if you consider this is C₁, then we will be at C₂ position. The next one epimerization means changing its stereochemistry at that specific position.

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So if you start with D glucose again and you want to change the stereochemistry of this carbon specifically you can use this reaction to do that. So, one thing I forgot to mention that in the previous reaction, be it based catalyzed be it acid catalyzed this reaction is actually a reversible reactions. So all the stuffs here are reversible stuffs. So they all have these structures, they exist in equilibrium. This which means if you treat it with a base then you are forming the ketose.

Similarly, based, so, if you do the next reaction it can be something like this that this is as H here. So, instead of CH₂ you write CH OH. So, base will take up this it becomes minus, would come here it will go there. So, CH O H C OH it will go on as you have done in the previous transformations in the backward way and it will give you ultimately again your CHO back. So, when you are getting back your CHO this one will again come back it will take up a proton. So, it will come this one will happen.

So, it will take up a proton again and that take up the proton can come either from this side or can come also from the other side both are possible. So, here you will have 2 structures now. One is this that gives your D glucose is plus this can be other way around because this is now SP² hybridized. So proton abstraction can happen in either direction this is your I guess if I am not wrong mannose so the stereochemistry of the epimer here is changed from this to the other one deposit 1.

So epimerization can be done at the C 2 position, using the acid catalyzed reactions it can be acid catalyzed, it can also be base catalyzed. So there are many other reactions involving carbohydrates. There are many, of course, and mostly on the monosaccharides. So, far I have been talking about the monosaccharides the small molecules, the sugars basically. And now, I will move on from the monosaccharides to higher ones that are present as biomolecules polymeric biomolecules or we call it biological macromolecules.

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Module 10

Polysaccharides → Biological macromolecules

Heparin → obtained from animal tissue
→ Polysaccharide (two monomeric units)
→ important biomolecule
→ anticoagulant in medicinal chemistry
→ clinically used

→ conjugation of uronic acid and glucosamine (1→4 conjugation) through glycosidic bond.

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So, poly saccharides now we will talk on that are basically biological macromolecules large molecules some of the structures I have talked about like cellulose, lignin, and glycogen, they are of course polysaccharides and very common part in present in living organisms and they constitute sometimes a majority of top of the living constitutes a living body. So, many of those molecules, why I am talking here is they have a lot of applications, we use them for many different applications in a lot of different kinds of industries.

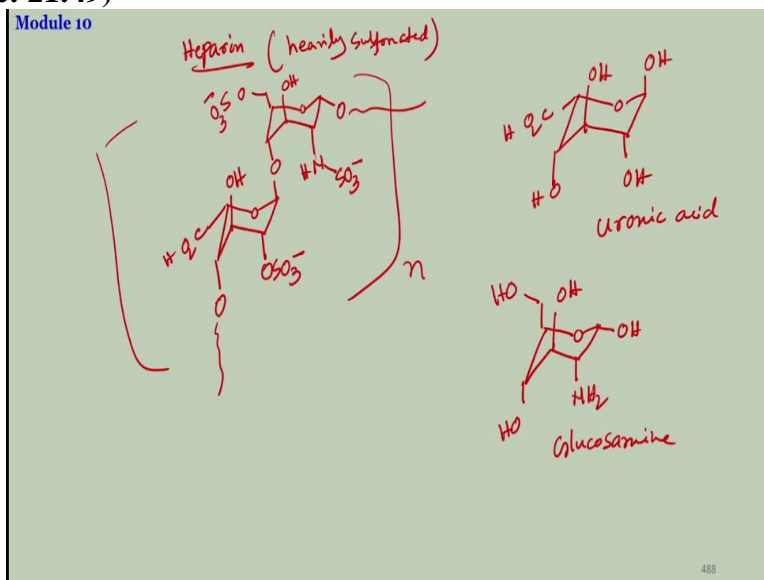
And especially our interest is as chemical biologists our interest is the application of such molecules in chemical biology or in medicines. So, biological applications basically. So, since most of these molecules are isolated from living organisms, and they are non toxic in nature, so, that is one specific criteria that enables them to have multiple number of utilities in biology because that is one of the major aspects that you require, whenever you synthesize something artificially, that always you have to deal with the problem of its toxicity.

Most of the molecules are toxic to the living cells. And therefore, their applications are very restricted. If you really have to find the molecules there are only a handful of molecules so most of the medicines that are in the market, many of them show toxicity to the other cells apart from the diseased cells. So, a good application is a molecule that will have no side effects on any cells, any other normal cells. And saccharides are the carbohydrates since they belong to nature.

Since they are isolated from all natural sources, most of them have the biocompatibility in them. So, I will start with some of the examples heparin, heparin is a polysaccharide. I will show you the structure that has shown many utilities. So, this is actually so it is obtained from animal tissue. Obtained from animal tissue. This is a very important polysaccharide, polysaccharide that have 2 monomeric units basically 2 monomeric units.

So the polymer is constituted of 2 different monomers attached together in a series. I will show you this structure. It is an important biomolecule and it is widely used as an anticoagulant in medicinal chemistry. Heparin is in the market it is used as an anticoagulant, so clinically used I will show you the structure. This is actually a conjugation of uronic acid and glucosamine in a 1 4 link or 1 4 conjugation that forms a glycosidic bond through glycosidic bond.

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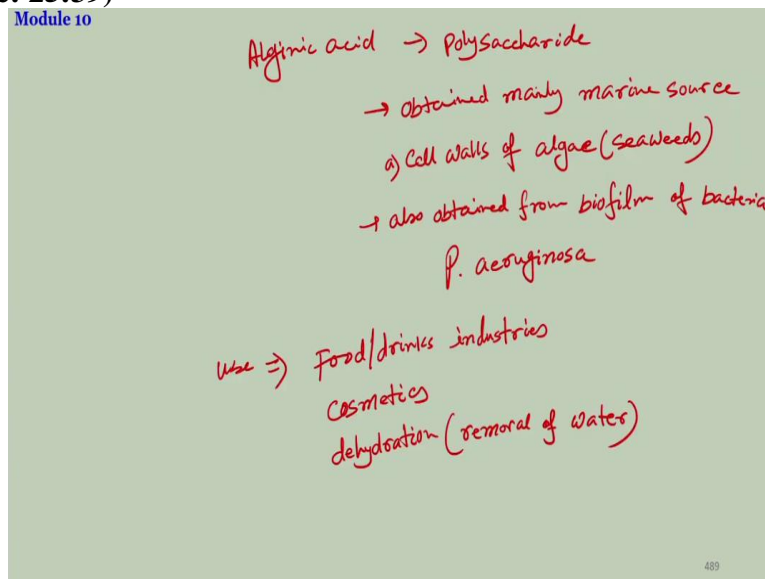
I will show you the structure of heparin first and then I will show you the structure of individual monomer, this oxygen, it can move. So I am just drawing to disaccharide part and it can be the multiple of in this carboxylic acid in the alpha position. There is an OH here in the beta position.

And heparin is actually heavily sulfonated there is a sulphate group in each. So here is an alpha position there is OSO₃⁻.

And here the hydroxyl group is present in the beta position of the plane, which forms another bond. So this is the glycosidic bond with the next carbon. So basically this molecule, this condition here is this and here, CH₂ OSO₃⁻ some other sulfonation. This hydroxyl is up the plane OH here this is down the plane, there is one NH SO₃⁻ and then it has again O in the alpha position, this can go on.

So, this is basically whole n. This is the basic skeleton of heparin OH down OH OH CO₂ H OH this is your uronic acid OH OH CH₂ OH has been sulfonated here basically, this is the basic unit OH NH₂, NH₂ is also sulfonated here substituted by sulfonation. So, this is your glucosamine these 2 units have been polymerized and sulfonated it gives rise to heparin structure. So this is heparin is one of the polysaccharide which is quite oil used. Other one is alginate, there are many I will just include 2 more of them 2 or 3.

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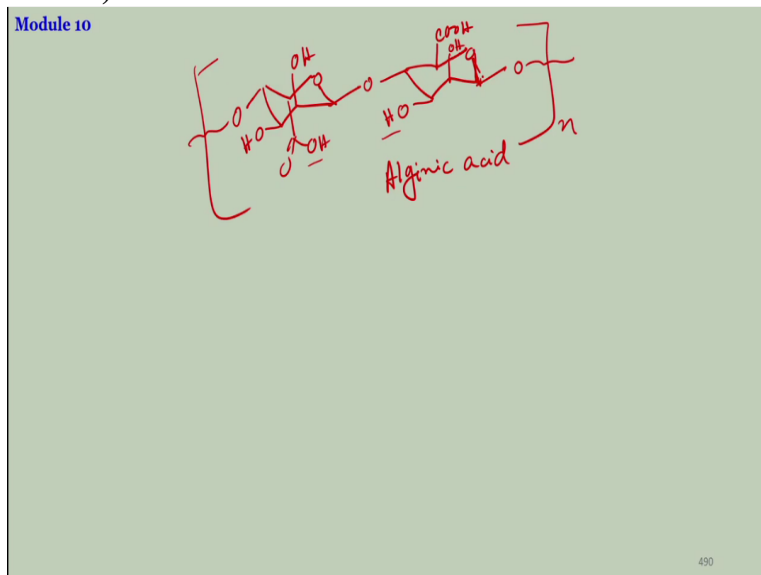
Alginate acid or sodium alginate or calcium alginate actually depends upon the application. The salts are made alginate acid is also a polysaccharide this is obtained mainly from marine source. So, it is often from CH₂ actually and from where cell walls of algae especially certain kinds of seaweeds or that is source one that is from the marine source second, also obtained from certain bacteria, those bacteria forms biofilms.

So biofilm of the bacteria, the term biofilm may be very unknown to at present and I do not have the scope in this course to discuss about biofilm. Biofilm is a kind of a film production that bacteria makes to protect themselves from antibiotics. So biofilms are basically resistant to the applied antibiotics. We usually use antibiotics to kill the bacteria, bacteria has to survive. So they construct a biofilm certain field on the surface of the bacteria.

And that does not allow the antibiotic to come to the bacteria and this biofilm contents alginic acid sometimes certain biofilms. Biofilm forming, obtained from biofilm of bacteria especially pseudomonas aeruginosa those biofilms contents, alginic acid is really widely use because of its long structure. It is used as a thickening agent in food industry drinks industry. So it is used in food, drinks foods or beverage industries this is used in cosmetics.

The calcium salt of alginic acid is used for dehydration of water from certain sources. So, dehydration means removal of water. So water proofing is also and this is being used. Nowadays, alginates nets are also used for medical purposes as a drug delivery systems. So I will show you the structure of alginic acid.

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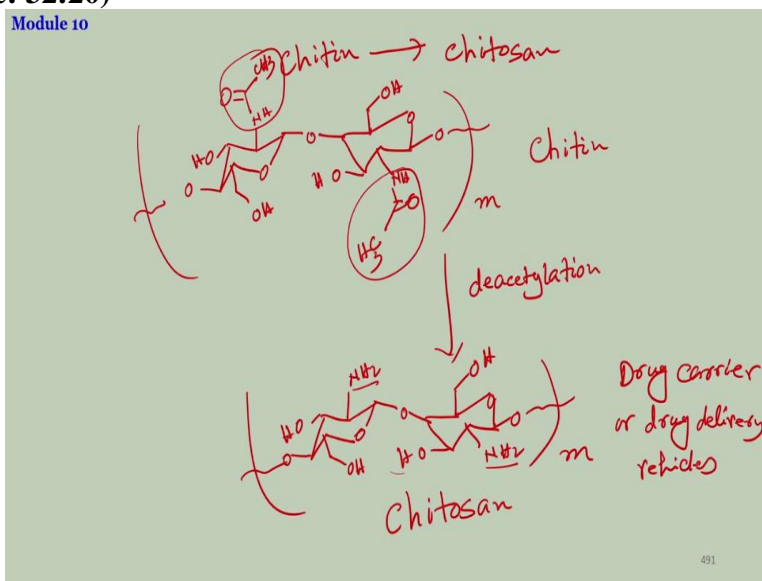
So this is your hydroxyl group here. This has acid group carboxylic acid here we have O which can go on that is the point of polymerization there is a free hydroxyl group here. It this form glycosidic bond O. So, this is from here. So, this is our basically mixture of the same unit both

the monomers are almost as isomers. Only thing is that in this case the carboxylic acid is down the plane here carboxylic acid is up the plane OH here this OH is up and this will go on like this whole n.

So, this is the structure of alginic acid. So, as you can see and the polymeric structure of the polysaccharides, they have many functional groups, they have hydroxyl groups they have in this case it has carboxylic acid groups. For chemists or biologists these are quite important and we can make use of these functional groups. If we want to modify them, I will show you certain modifications just a little later. How a lot of research is going on, innumerable amount of work is being going on if you if you check the latest publications.

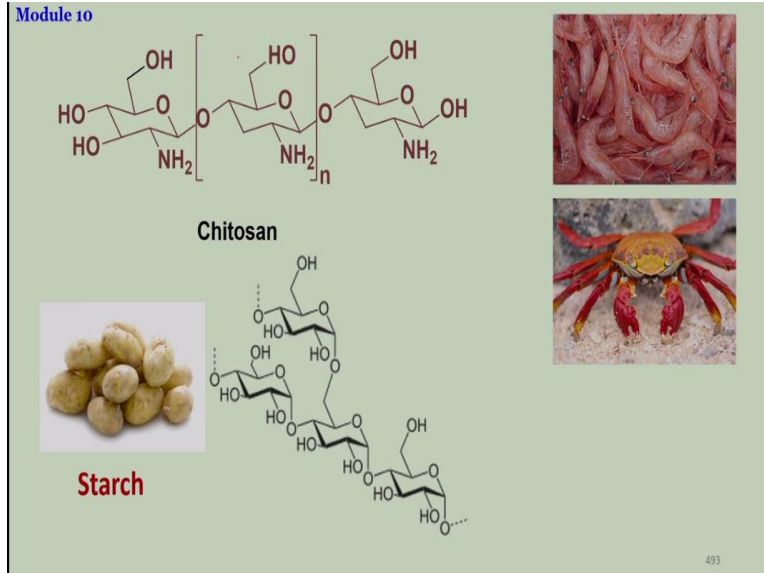
There will be every month they will plane to your publications and that you can find on the various chemical changes on the polysaccharides. And for those chemical changes are made to obtain different kinds of properties, different applications another important one is which has a lot of use in medicinally research in chemical biological research as well as for other kinds of utilities is the another carbohydrate containing molecule which is known as chitosan or chitin.

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Chitin is a polysaccharide from chitin comes the molecule, so biological macromolecules chitosan or you can call chitosan. So this is obtained.

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I will show you the source here. This is chitosan structure of chitosan. And this is mostly obtained from the marines as well and mostly from the shells, hard shells of streams and crabs. Plenty of them you can isolate own sub quantities of nitrogen and they are actually sold in bulk amount as well as the very purified ones for biological work. So, this is the structure of chitosan why this is attractive for us is because it has the functional group amines along with the hydroxyl groups.

So, all the carbohydrates all the polysaccharides have hydroxyl groups of course, you can target those hydroxyl groups for certain chemical modifications. But apart from that, you can be very specific if you have other kinds of functional groups which are unique in nature. So, in this case, as a chemist the amine groups are very attractive for us, because you can do a lot of reactions on these things.

And therefore, modify them to change their property, their physical properties that chemical properties and then you can make use of them for various aspects. So since I am showing also you also the structure of starch is another polysaccharide which is basically a branched polysaccharide that is obtained in many foods such as potato starch is a major component. So, as you have seen, this is the structure of chitin NH CO CH 3 OH and this is your CH 2 OH here is OH NH CO CH 3 O.

And so on polymerization them, this is your chitin basically which exist in N acetyl formation and that makes it very hard. So, all the cells of the stream or the crabs are quite tough actually. And those cells majority of those cells contain a large amount of chitin. Now, if you do the deacetylation which means you remove the acetyl protections then what you have is basically the chitosan.

So you have free amine group now beach that you can extend CH₂ OH this and then you have here is OH goes on here comes the amine and here it is OH whole m this is your chitosan so many of these polysaccharides such as hydrogen, such as heparin, they are used of course, as I have talked about, because of their unique functional groups, you can make use of them to change their properties to make other structures out of it, but uniquely, you can use these molecules itself for biological purpose.

So, most of these molecules show very little toxicity to the cells and they are very much biocompatible because they are obtained from natural source. So, if you consume them, it does not harm much. So, in other words, they are not harmful for the cells. So, that is one advantage, a major advantage actually, that gives you some of these molecules themselves show biological activities such as heparin I have talked about hydrozoan and also to some extent, so, antibacterial property.

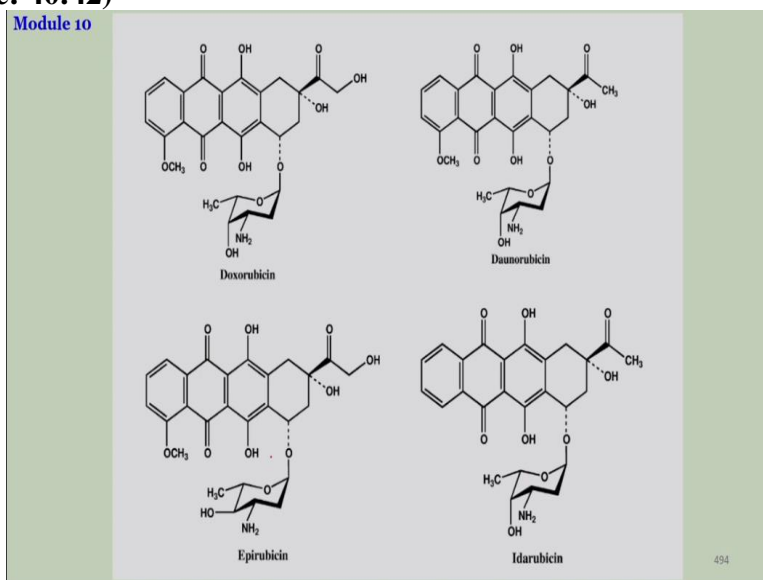
Moreover, you can use these molecules to encapsulate drugs. These are polymorphic molecules, they have hydrophilic part here hydrophilic part this is electrostatic interactions that can give you and they have a long carbon chains that has hydrophobic environment. So, overall it can give you a mixed kind of geometry and you can interact the small molecules into it. Drug delivery I have talked before.

So, these kinds of molecules also are widely applied for delivery of drugs. In other words they are used as drug carrier for drug delivery vehicles. So you can entrap small molecules such as drugs into them. Because drugs of course, are foreign particles and body are the cells does not allow them to penetrate into the cells, the cell penetration ability, or the bioavailability of most of the drugs are very, low.

So, only a little bit amount of the drugs that you inject or that you inhale that you consume, actually reach the targets or actually it is actually reach inside the cells. So, in order to increase their cell viability or bioavailability, you have to camouflage the drugs into something that will be compatible for the cell. So, these things most of these materials we consume in our food supplements also. So, if you can take drugs along with them, they can be easily taken up by the cells.

So, that is one second application is a majority application nowadays a lot of research, you will find they are using these kinds of molecules is to, forming the nanomaterials. So, first I will show you some of the examples of the drugs. That contents carbohydrates that I have already shown you.

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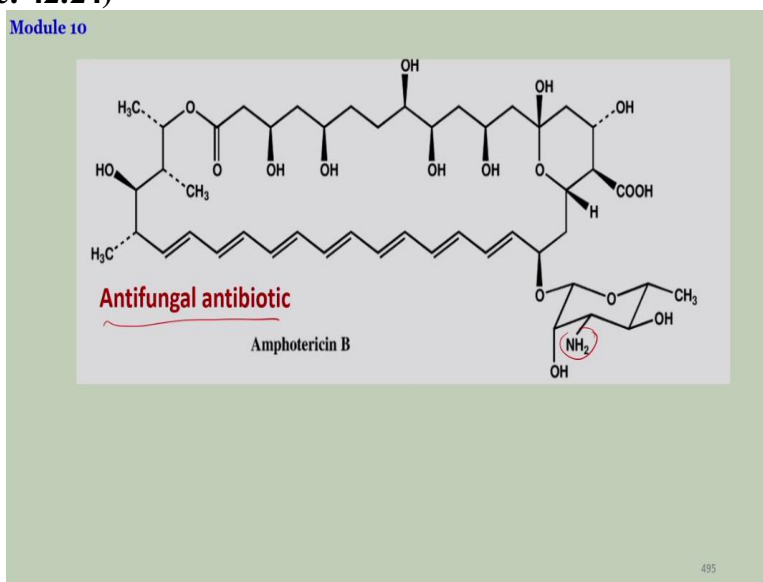


That doxorubicin, daunorubicin, epiubicin, idarubicin all of them contain a certain carbohydrate unit and all of them are very important drugs. They have if you look at their structures, large carbon chains, planar Systems aromatic carbons, which are not soluble, which are very hydrophobic in nature, not soluble. So it is very hard to take them inside the cells because they are not soluble at all. So many most of them get rejected by the cells or when you try to inject them into it.

So all this carbohydrate macromolecules, such as hydrogen is widely used, heparin is being used. There are other apart from carbohydrates since I am talking about drugs. Apart from carbohydrates, there are many other kinds of drug delivery vehicles. I have talked about peptides a little bit, but most of the drug delivery vehicles are actually polymorphic in nature, and they are long, very long chain long carbon chains such as polyethylene in glycol.

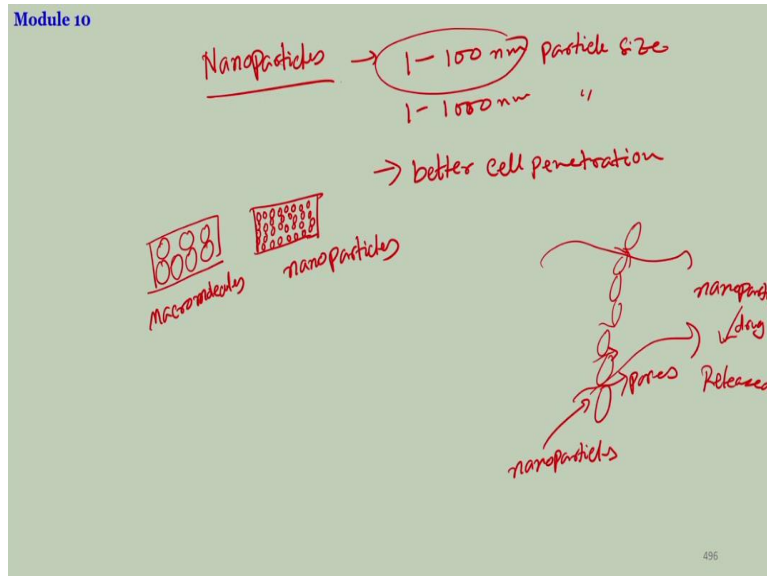
There are other kinds, many, many different types. A large section of them are carbohydrates. So, this drugs because of the hydrophobic nature, they can be entrapped also inside the certain types of polysaccharides or certain types of carbohydrates to trap it encapsulation at it and then they behave as a unit thing, common thing and then you can easily take them into the cells.

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This is another molecule that I probably have not talked about amphotericin is also has a carbohydrate unit has an amine group in here and the rest is long chain with the multiple double bond and this is widely used antifungal antibiotic. This is basically an antibiotic, which is used to kill the fungus.

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So first, I will now start a brief on the nanoparticles a little bit. Because modern days a lot of research is going on 1 nanoparticles and they have huge applications. For example, most of the pain relief gels that you use and the ointment that are muscle injury or sports injury, for quick relief of pain, the gels or the ointment that you use nowadays comes in nano gels. And those basically are nanoparticles in the gel form, and the nanoparticles, they can act very quickly.

Why I will just tell you, so in general if you have nanoparticles. Now, I will give you a small introduction on the use, and application of nanomaterials and the nanoparticles in chemical biology and in medicinal chemistry. Nanoparticles are basically the particles that have size between 1 to 100 nanometer particle sizes. They are in the lower range, it can go from up to at least 100 to 1000 even 1000 nanometers obviously.

So, this means they have very small size and small size I have tremendous advantages number 1 advantages if you have normal molecules for normal particles with a large size large macromolecules so, they have this kind of shape. So, within this small box, it can accommodate only a few of the large molecules, the same molecules. If you convert into the nanoparticles that can be done, the same molecules can be reduced to nanoparticles which means their sizes can be reduced into the nanometer scale.

Then the size becomes very small and within the same space it can contain or it can accommodate many, of those same molecules. So, these are macromolecules these are nanoparticles. So, high surface area basically, in a small space you can accommodate a large number of molecules or a large quantity of those particles. So, that gives you certain advantages obviously, for chemical biologists we have even better advantage, very important advantage. That is, it gives a better cell penetration ability.

So, as our major concern, obviously I am talking about is to send the active molecules into the cells, be drugs, bait other probe molecules that you want to study the certain activities, certain chemical transformations, certain binding properties that is happening inside a living cell. For all cases, you need to send your stops into the cell and that itself is a tremendous task, because cell simply rejects them.

So, now, if you reduce the size of the material, the same materials if you reduce the size into very small particles, then what happens to our cell walls or the cell walls of other organisms also, obviously, we have a pretty good cell walls that is why they are reject most of the molecules. Now, they have little pores also. So, there are small gaps in between very small gaps pores. So, those gaps they allow salts to water salt to go and come out I mean to invade into it or come across it.

So basically kinds of semi permeable membrane sometimes; so this gaps they allow this very small molecules to get into it such as water or the other medium or such as the salt, but little bit larger molecules, they will be blocked because they are foreign particles most of the times. Now, if you reduce them, if you reduce those large particles into the nanomaterial, the sizes of those nanoparticles are even smaller than the pores.

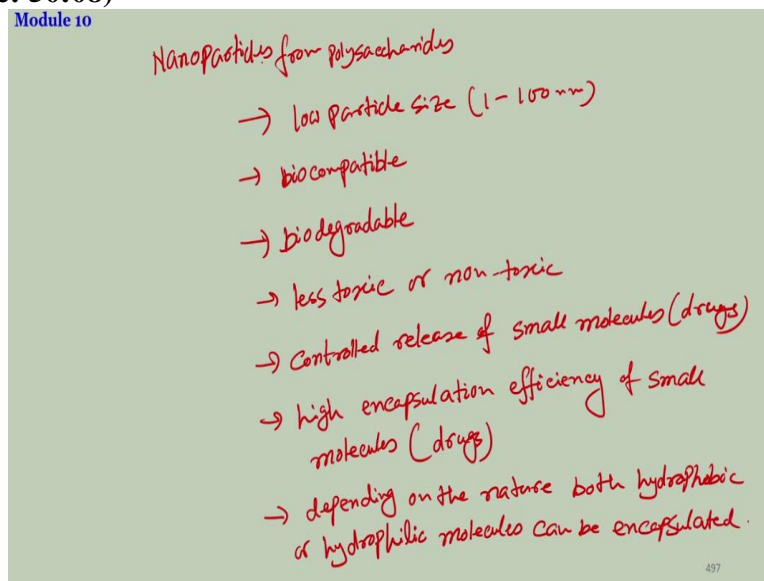
So, now, the nanomaterials or that I call them nanoparticles now, they can go into go through the pores, they can make ways through the force into the cells, because they are so small that the cells cannot see that something is getting into it. So, you can take the nanoparticles inside the cells quite easily. If the size is less than 100 nanometers. Ideally, we call them that it should be

with less than 100 or at most less than 200 nanometer, then you can have a better opportunity to innovate into the cell walls.

So once you take the nanoparticles in it, those nanoparticles are made up of basically your probes basically your targets can be drugs can be other molecules can be carbohydrates, and they can be released here. If this is drug, drugs can be released inside and that is one way of taking the drugs into the cells. And that is also the reason why most of the pain relief gels are made off of nano gels.

Nowadays, because once you apply on your skin, or on the muscles, where there is a pain, so it can penetrate the skin. It can print it to those muscle cells very easily, very fast. And that is why we get quick relief. So there are a lot of advantages of nanoparticles in chemical biology. So this is one of them better self-nutrition.

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I will mention all of them are some of them at least. So particularly nanoparticles from made up of the carbohydrates list, let us say polysaccharides such as hydrogen such as heparin, such as alginate. So, they have obviously, low particle size let us say 1 200 nanometer. They are highly biocompatible in nature. Because as I have said since they are isolated from natural sources, most of them they are biocompatible and many of them are biodegradable also.

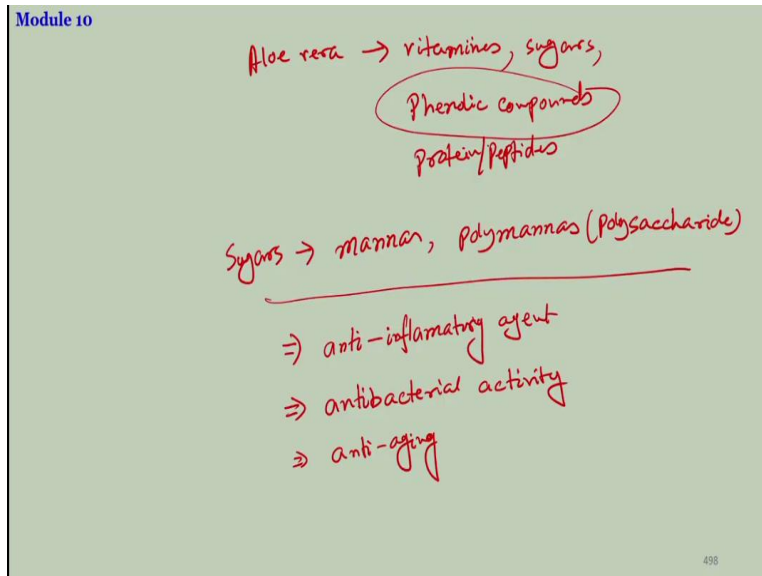
So, they do not stay much inside the cells, once they get inside the cell, they can be part of the metabolic system and therefore, be degraded and that is one of the very good advantage for them to enable them to act as a drug delivery carrier, because your carrier you do not want much the carrier itself does not have much activity most of the times, so, you do not want them to remain in those cells.

So, if those carriers are biodegradable so they can be immediately degraded by the metabolic system and be released from the body and that in through that way also your drug can come out less toxic, I think the most important property less toxic or nontoxic. Since they are food supplements, basically most of them are not toxic for the cells, that is one of the highest advantage because what you want to carry is your drug and that has certain activity, apart from that the associated things should not have adverse effects on the cells.

So, if they are nontoxic in nature, they do not have adverse effects on the cells. So, that is what should be your criteria. They allow controlled release of small molecules such as drugs, they have high in the capsulation efficiency which means that because of their large macro molecular structure, they can encapsulate they can trap a large quantity of the small molecules, which can be drugs. High encapsulation efficiency of small molecules such as drugs apart from drugs it can be other materials also.

Sometimes they are also used to transport ions different kinds of ions calcium transport potassium transport, also being used by such kinds of stuffs. Depending on the nature both hydrophilic drug as well as hydrophilic molecule as well as hydrophobic molecule can be encapsulated and we encapsulated and all of these things are reduced into a very small particle size. So those are the kinds of use of polysaccharides into forming of the nanoparticles. So another important I should mention about is the source of the carbohydrates that have that are used in medicinal industry a lot that you know also is Aloe Vera.

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Aloe Vera contains a lot of different molecules, such as vitamins. It has different vitamins actually. It has sugars, means the carbohydrate. Very importantly, it has phenolic compounds. Phenolic compounds are they are very good antioxidants as proteins peptides among there are many others also. So when we are talking about sugars, it has a high amount of manners. The structure you can find out or polymanner which is basically a polysaccharide.

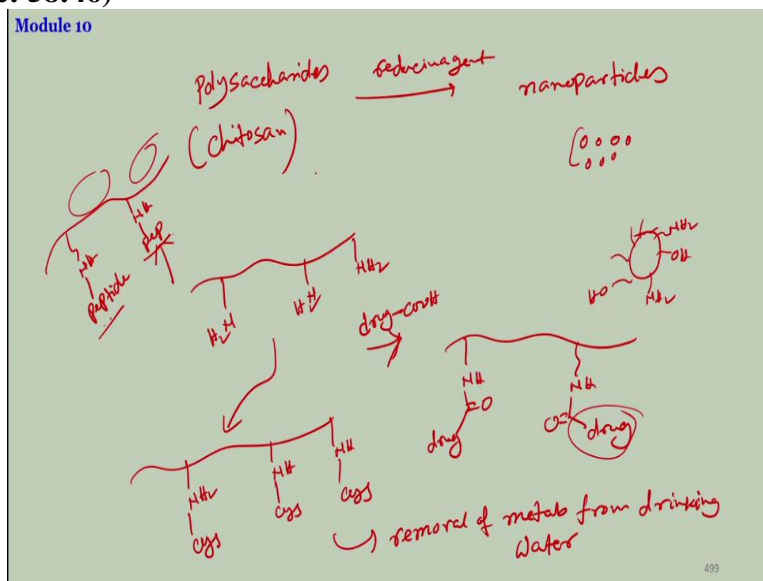
And because of all this competition in Aloe Vera it shows lots of good biomedical activities this is a very nice anti-inflammatory agent. It is antibacterial activity, anti-aging. I think that is the most positive property of Aloe Vera that you know, because Aloe Vera is used in lots of cosmetics to give it anti-aging property for the skins. And that is the reason is this phenolic compounds because they are antioxidants, antioxidants or anti-aging also.

So and nowadays, people have started thinking that it might have some anti-tumor activities also what not you are though. So these are some of the property of Aloe Vera that are well known. And why I am talking about this is these sugars because of these polysaccharides are present there. You can synthesize a lot of different kinds of nanoparticles or nanomaterials using the Aloe Vera extract.

And they are also used to trap in or encapsulate drugs for the carrier, because Aloe Vera is very much biocompatible, as well as by degradable in nature the extract because they have the small

compounds and the large compounds in mixture, which are all basically nontoxic compounds very quickly.

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Now, I will start how can you synthesize the nanoparticles? So, all these large molecules I have talked about, can themselves be converted into nanoparticles? What do you need is a reducing agent. So, if you have polysaccharides such as chitosan and you add a reducing agent certain reducing environment is required. Then you get nanoparticles. So small particles basically, those nanoparticles still retain their surface.

If you have a nanoparticle in their surface, all the functional groups will be there. So hydroxyl it can be I mean, hydroxyl amine hydroxyl on. So, they still retain their properties to some properties, but the size gets smaller. So, that is number 1. That gives you a lot of advantages also, you can change a lot of properties, if you have nanoparticles that has hydrogen in it, as I was saying that it has amine you can use them for a whole lot of stuffs.

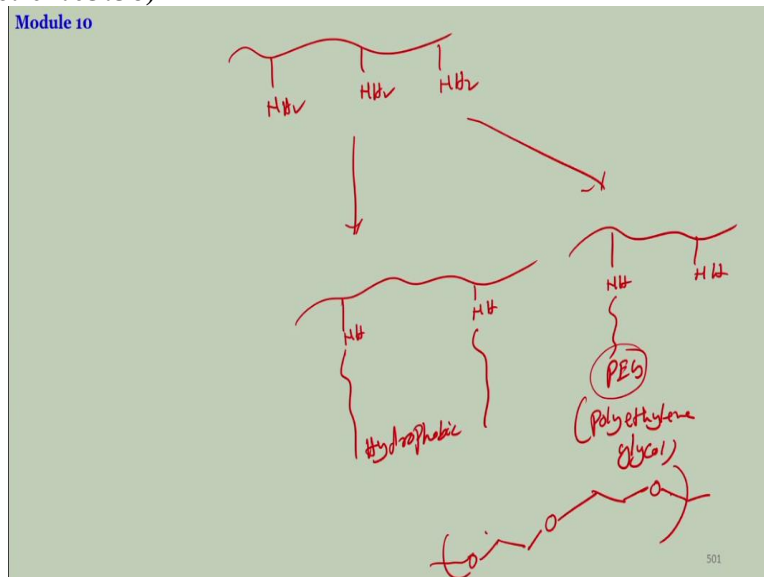
You can conjugate other molecules or other functional groups, even other polymers to entirely change its property. Such as, for example, you can attach covalently attached drug here using the peptide chemistry if you have a drug that has a free carboxylic ligand. You can attach those unsynthesized amide bond using the peptide chemistry and can be your drug shown. So you can attach a drug in your chitosan and then your drug will be taken into the cell. If you can make the whole thing internet nanoparticles, you can take more quantity into the cells.

And because of his low size, you can be assured, if the size is less than 100 nanometer, you can be assured that your drug would be taken into the cells. Otherwise, usually, these drugs might have had the difficulty to get into the cells. So this is one way you can take the drug in inside the cells. You can make other kinds of modifications such as you can connect it to for example; cysteine amino acid system has sulfur side chain SH.

That can be used to remove heavy metals from water from drinking water purifications removal of metals from drinking water you can even it use a peptide here cell getting peptide for example, to motor getting peptide for example, if you take amine peptide all by the same reaction that is peptide coupling reaction. We are talking about pep. So if these peptides are targeting peptide with the shortened sequence, you can take drugs here, drugs would be encapsulated into your macro molecule.

This one will make sure that this whole cyst systems gets into the tumor specific cells. If you have that peptide, for example, tumor homing peptide if you use that will take your whole stuff into the tumor cells specifically and you have drugs in here. If the whole thing is nanoparticle, it can go very easily. And to change its other properties, sometimes it is been cross linked to other polymers also.

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There are many things that goes on. One thing at least I should add another thing I should mention otherwise it will not be complete. So, here I am taking only the hydrogen you can change it into a poly the carbon chain hydrophobic. So, you can have long carbon chain that will change its property entirely, because now you have more hydrophobic nature into the molecule. So therefore, it can capture the hydrophobic drugs, such as drugs so revisions which are other ways, a little bit difficult to carry.

So that needs a hydrophobic environment. So you can have that you can use a little bit if you want to increase its solubility and its target specificity also. Polyethylene glycol is another thing, PEG poly ethylene glycol. So, that is basically ethylene group. This is OH here OH, polymer of this. So, it has also it has little bit increases the solubility a little bit gives you a little bit more hydrophilic nature, it can trap hydrophilic drugs in it and carry it.

Moreover, PEG itself has biocompatible property and a little bit bio medicinal property as well. So, there are many different things, many conjugation chemical modifications that you can try to do. So, the next lecture I will give you a little bit introduction of initially the how the nanoparticles are synthesized and then we will conclude. Thank you.