

## **Experimental Nanobiotechnology**

**Prof. P. Gopinath**

**Department of Biosciences and Bioengineering,**

**Indian Institute of Technology Roorkee**

### **Lecture 20: *In vitro* Hemocompatibility Test**

Hello everyone, today we are going to learn *in vitro* hemocompatibility test. In today's lecture, we'll be learning about what is biomaterial and biocompatibility. We'll also be learning about various hemocompatibility tests. Under the hemocompatibility test, we'll be learning about the hemolysis assay in more detail, both theoretically as well as practically through demonstration.

Let us see what biomaterial is. Biomaterial means any material that has been designed to interact with a biological system for medical purposes. It can be for therapeutic or diagnostic applications. Biomaterial is used to replace a part or function of the body in a safe, reliable, economical, and physiologically acceptable manner.

So these are some of the examples of biomaterials: dental implants, joint replacements, hip replacements. These are some examples of biomaterials. Whenever we develop biomaterials, we have to understand biocompatibility before we implant these biomaterials into the human body.

Let us see what biocompatibility is. Biocompatibility means a material's ability to function properly with a suitable response from the body in a given application. The material should be compatible with the biological system. It should not induce any adverse effects. That is called biocompatibility.

The biocompatibility of biomaterials can be assessed and tested by the following criteria. The first one is genotoxicity. Genotoxicity means the particular material should not induce any damage to DNA. Next one is cytotoxicity. The particular material should not induce any toxicity to the cells.

The third one is immunogenicity. The particular material should not induce any immune responses, including allergic responses. The fourth one is hemocompatibility. The

particular biomaterial should be compatible with our blood. That is called hemocompatibility.

Let us see why to do hemocompatibility test. Hemocompatibility test is very important for biomedical devices and implants that interact with the blood because it focuses on unique risks such as thrombosis, hemolysis and immune reactions. So, that is why the hemocompatibility test is very important to understand the safety and efficacy of the blood contacting materials.

The other biocompatibility test mainly focus on the general safety of the particular material, but the hemocompatibility mainly focus on the interaction between the materials and the circulatory system. So, that is why this hemocompatibility test is very very important for implantable biomedical devices. That is the reason the hemocompatibility is one of the important factor in deciding the application of implantable biomaterials such as artificial blood vessels and orthopedic implants.

Whenever we develop biomedical implants, we have to study the hemocompatibility and if the hemocompatibility is not good, we have to improve the hemocompatibility by surface modification or redesigning the particular material before we implant into the body. According to International Organization for Standardization, the hemocompatibility tests are conducted in the following model. First one is static.

Under the static model, we have blood coagulation assay, hemolysis assay, and partial thromboplastin time. Under the dynamic model, we have the Chandler loop system and chronic shunting. In today's lecture, we are going to learn about the hemolysis assay in more detail. Before we learn about hemolysis assay more in detail, let us see what are the various hemocompatibility tests. Under the hemocompatibility test, there are various assays.

Let us see them one by one in detail. The first one is the hemolysis assay. This assay evaluates the damage to red blood cells after exposure to the test material. The test material can be a biomaterial, a nanomaterial, or any kind of test substance. The next one is anticoagulants.

This assay measures the concentration or activity of anticoagulant drugs in the blood. The third one is platelet adhesion and activation. This assay evaluates the impact of test materials on blood platelet adhesion and activation. Through this assay, we can understand whether the platelets are attaching to the biomaterial and if there is any

change in their shape. So, if there is a change in the shape and if the platelets are attached to the particular material, that material is not suitable for

Biomedical implants or clinical applications. Next is blood coagulation time. This assay measures how long it takes for blood to clot in the presence of test material. And the last one is the blood protein adsorption assay. This assay quantifies the amount of blood proteins adsorbed to a surface.

The surface can be a biomedical implant or medical device. Let us learn about the hemolysis assay in more detail. When RBCs, that is, red blood cells, come in contact with the test material, that is, your nanomaterial. This nanomaterial disrupts the membrane integrity of your RBCs.

Once it damages the RBC membrane, hemoglobin in the RBCs will leak out of the cells. That is called hemolysis. The degree of hemoglobin release is calculated in terms of percentage to understand the hemocompatibility of nanomaterials. And this is a simple and cost-effective technique. That is why most labs use this hemolysis assay to understand the hemocompatibility of their biomaterial.

Let us learn how to perform hemolysis assay. The first step in the hemolysis assay is procurement and processing of blood. We can collect the blood from human or goat. We have to collect the blood in a container which contains tripotassium EDTA which is an anticoagulant and very important point is we have to take the approval

from the human ethics committee or animal ethics committee or institute biosafety committee for handling certain samples before you handle some of the blood samples or any sample which is related to human or animal, we have to take approval from the particular committee. Once you collect the blood, the next step is you have to centrifuge the blood at 2000 rpm for 10 minutes, then isolate the RBC. Once you isolate the RBC, we have to add the colloidal nanomaterial of different concentration

for understanding at what concentration the nanomaterial is inducing the hemolysis. Once you add the nanomaterials, incubate the RBC with nanomaterials for 2 to 3 hours. After incubation, we can do the centrifugation. Once you do the centrifugation, if the particular nanomaterial is inducing hemolysis,

You will get this kind of result, and if the particular nanomaterial is not inducing any hemolysis, you will get this kind of intact pellet. Then, you collect the supernatant and

measure the absorbance using a spectrophotometer. The absorbance can be measured at 541 nanometers using the plate reader.

Then, the percentage of hemolysis can be calculated by

$$\text{Hemolysis (\%)} = \frac{A_{\text{test}} - A_{\text{negative}}}{A_{\text{positive}} - A_{\text{negative}}}$$

$A_{\text{test}}$  means this is the absorbance of the supernatant from the test samples, and  $A_{\text{negative}}$  is the absorbance of the supernatant from the negative control tubes, and  $A_{\text{positive}}$  is the absorbance of the supernatant from the positive control tube.

Here, this negative tube control is where we will be using only saline, and in the positive control, we will be using water or some kind of detergent which can completely damage the RBC membrane and release all the hemoglobin. That is your positive control tube. Once you plot the hemolysis percentage, you get this kind of data.

From this data, we can understand whether the particular material is hemolytic or not. For example, if the hemolytic percentage is between 0 to 2 percentage, it is non-hemolytic. If it is between 2 to 5 percentage, it is slightly hemolytic. If it is more than 5 percentage, the particular material is hemolytic. From this data, you can understand, so almost this first sample 1 and 2, these are

less than or equal to 1, that means the sample 1 and 2 are non-hemolytic and sample 3 we can see it is slightly above the 2, that means it is slightly hemolytic. Let us see how to study the hemocompatibility of nanofibrous scaffold using hemolysis assay. In this study, we made nanofibrous scaffold using silk protein, silk sericin and silk fibroin and this nanofibrous scaffold are loaded with SST, that is a silver sulfadiazine drug.

And this nanofiber scaffold is mainly useful for wound dressing application. To study the hemocompatibility of this nanofiber scaffold, we incubated this nanofiber scaffold in presence of RBC. And after incubation, we measured the absorbance at 541 nanometer. You can see the percentage of hemolysis. Our material is highly biocompatible.

You can see the hemolysis percentage is less than 2 percentage that means the particular material is compatible whereas in case of water you can see that complete hemolysis is there. This is your positive control where you can observe the complete hemolysis

whereas in case of the test material where we use different nanofibers you can see that there is no hemolysis

that means this material is hemocompatible for biomedical application. The human assay protocols may vary with difference in blood sources. For example, if you are collecting the blood from human or rat or goat or it may be also vary with respect to the erythrocyte concentration and depends on whether you are using whole blood or you are using isolated RBC and also depends on the wavelength which was used for

hemoglobin measurement and the detergents which you use for your positive control. For example, in our study, we used water as a positive control for hemolysis. In some of the studies, we can use Triton-X or Tween-20 detergent as a positive control for hemolysis. I hope you understood what is hemolysis assay. Let us go to lab and learn this technique more in detail.

Today, we are going to learn how to perform a hemolysis assay for the synthesized BSA nanoparticles. To perform the experiment, we require a BSA nanoparticle stock solution, trisodium citrate-treated blood, here you can see it is not coagulated, 0.9% saline, deionized water, centrifuge tubes, microcentrifuge tubes for carrying out the experiment, centrifuge microtips, a micropipette, and a vortex mixer. To proceed with the experiment, we require diluted blood. For that, we already have trisodium citrate-treated blood,

saline to dilute the blood, and an empty centrifuge tube to prepare the aliquot solution, a vortex mixer, a 1 mL micropipette, and microtips. Add 100 microliters of blood to 9.9 mL of saline solution. Once we add 9.9 mL of saline, we have to vortex it to ensure complete mixing of blood with saline. We have prepared the blood dilution, and now we will prepare the test material.

Here, we will prepare a one-tenth concentration of the BSA nanoparticle stock solution. For that, we will add 1 mL of stock solution to 9 mL of saline. Vortex it to mix well. We have prepared the solutions, and now we are going to perform the experiment. For that, we require nine microcentrifuge tubes: three sets of triplicates for each sample. One is for negative controls, the second is for the test sample, and the third is for the positive control.

In the negative control tube, we are going to add saline with blood. In the test sample tube, we are going to add our test material. Whereas, in the positive control, we are going to add the deionized water. Now, we are going to add 100 microliters of blood into all

nine tubes. After adding the diluted blood, we are going to add 0.9 mL of saline into the first set of three tubes. Then, we are going to add 0.9 mL of

prepared test material into the second set of three tubes. Finally, we will add 0.9 mL of deionized water into the last three tubes. Then, we have to incubate all the tubes for 2 hours at 37 degrees Celsius. After incubation, we have to centrifuge all the tubes at 2000 rpm for 10 minutes. First, we are going to place the negative control tubes.

Here, we can observe the pellet formation for the negative control. Similarly, we have to do this for the other two samples as well. Now, we are going to place the test sample tubes. Here, we will observe whether the test sample is lysing the blood or not.

If lysis is happening, it is not advisable to use it in blood-related applications. Here you can see the pellet formation at the bottom of the tube. Indicating that it is not lysing the blood. Finally, we are going to place the positive control tubes for centrifugation. Here you can see the lysis of blood due to deionized water.

We can observe that there is no pellet formation for the positive control, which confirms the lysis of blood. In the negative control and sample tubes, you can see the pellet formation, whereas in the positive control, there is no pellet formation. Now we will take 100 microliters of supernatant from all the tubes, and add it to a 96-well plate for measuring the absorbance. Here we should carefully remove 100 microliters of supernatant from all the tubes and add that to the 96-well plate.

We have to use separate tips for each tube. Now we have added 100 microliters in three columns. The first column is for the negative control, the second column is for the test sample, and the third column is for the positive control, all in triplicates. Finally, we will measure the absorbance at 541 nanometers and calculate the value to find out the hemolysis percentage. The hemolysis percentage, which is less than 2%, indicates that the BSA nanoparticles

are highly hemocompatible and suitable for use in drug delivery or blood-related applications. As a summary, in today's lecture, we learned about what biomaterials and biocompatibility are and also we learned about various hemocompatibility tests. Under the hemocompatibility tests, we learned about the hemolysis assay in more detail, both theoretically and practically through a demonstration. Thank you for your kind attention.

I will see you in another interesting lecture.