

Biomaterials For Bone Tissue Engineering Applications
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Lecture number 12

So we will continue our discussion on the scaffold, so scaffold, if I may recall, this is a three dimensional interconnected porous structure.

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Scaffold

Cells are often 'seeded' or cultivated into a three dimensional synthetic porous structure to facilitate tissue formation *in vitro*. These structures, typically called scaffolds, are often critical, both *ex vivo* as well as *in vivo*, for recapitulating the *in vivo* milieu and allowing cells to influence their own microenvironments. Scaffolds usually serve at least one or most of the following purposes:

- a) Allow cell attachment and migration
- b) Deliver and retain cells and biochemical factors
- c) Enable diffusion of vital cell nutrients and expressed products
- d) Exert certain mechanical and biological influences to modify the cell behavior.

Handwritten notes in red ink:

- A circle around item (a) "Allow cell attachment and migration".
- A large circle on the right containing the text "interconnected porosity in 3D space".
- A circle at the bottom left containing the text "microporosity 1-10 μm".
- A circle at the bottom right containing the text "macroporosity > 40 μm".

Mitra J, Tripathi G, Sharma A, Basu B: Scaffolds for bone tissue engineering: role of surface patterning on osteoblast response. RSC Advances 2013, 3(28):11073-11094.

So one of the things that I have (explained) I have mentioned a couple of times in the during the last module is the interconnected porosity. So this is interconnected porosity in 3 D space. So that is one of the one of the important, one of the important aspects of a biomaterial scaffold.

So the there are two type of things that I have mentioned in this scaffold definition, one is that micro porosity and one is that macro porosity. So micro porosity is like 1 to 10 micron and macro porosity is like more than 40 to 100 micron. So there is no distinct distinct demarcation in terms of the length scale of the porosity, but it is quite widely accepted in the biomaterials literature that micro porosity is like you know 1 to 10 micron and macro porosity is like more than 40 micron, 100 micron and so on.

So micro porosity is useful for initial cell attachment and cell attachment and cell adhesion and migration. Macro porosity is more useful for bone ingrowth as well as angiogenesis, vascularization for all these things that you need little bit so that bunch of blood vessels can grow

inside this tissue structure and so on. so it also exerts certain mechanical and biological influences to modify cell behaviour.

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Scaffold

Typically, the term scaffold implies porous constructs with interconnected pores of typically 10-100 μm , that facilitate tissue ingrowth, reduce limitations due to diffusion of nutrients and oxygen on account of its high porosity.

While the morphology and porosity are of primary importance in scaffolds, mechanical properties are of secondary importance.

Mitra J, Tripathi G, Sharma A, Basu B: Scaffolds for bone tissue engineering: role of surface patterning on osteoblast response. *RSC Advances* 2013, 3(28):11073-11094.

So typically the term scaffold is restricted to three dimensional porous constructs with interconnected porosity and the here it is like 10 to 100 micron and facilitate tissue ingrowth and reduce limitations due to diffusion of nucleus and so on. So morphology and porosity are of primary importance to scaffolds for a mechanical properties of secondary importance.

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Tissue engineering

Tissue engineering can be conceptualized as the means of orchestrating cells, engineering materials and suitable biological factors to enable relevant biological functions.

In a nutshell, *"Tissue engineering is the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals"*.

Morais JM, Papadimitrakopoulos F, Burgess DJ: Biomaterials/tissue interactions: possible solutions to overcome foreign body response. *The AAPS journal* 2010, 12(2):188-196.

So tissue engineering, this is also this is very established field currently and tissue engineering is defined as a creation of new tissue for the therapeutic reconstruction of the human body by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals. So molecular signals means you can add growth factors along with the cells for the target in a target specific biomedical applications. Mechanical signals means that in a scaffold itself has a certain elastic stiffness property; so that elastic stiffness can also helps in the mechanical signal transduction mechanisms.

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Implant

An implant is a general term used to describe any object that may be placed in direct contact with living tissues. The Food and Drug Administration (FDA, USA) defines medical implants as devices or tissues that are placed inside or on the surface of the body.

Many implants are prosthetics, intended to replace missing body parts. Other implants deliver medication, monitor body functions, or provide support to organs and tissues.

An implant essentially conveys a foreign body that is not essentially porous, and whose main function lies in providing mechanical support to the osseous structure, while exhibiting good osseointegration properties. For instance, the total hip prosthesis is an implant and not a scaffold. Hence, load bearing properties, like strength, elastic modulus, fracture toughness and fatigue resistance of implant together with acceptable biocompatibility property are of prime considerations from a materials perspective.

Handwritten notes: Tib Al 4V, SS 316L, strength, E-modulus

So implant has also been sufficiently mentioned in the last module and it is a general term used to for describe any object that may be placed in direct in direct contact with living tissue. And for implants that mechanical properties like strength properties, like elastic modulus properties, these are like more of importance. But one thing I (must) I have mentioned and I must reiterate here, that implants must have uncompromised biocompatibility property, that means that biocompatibility property is equally important as important is the strength and elastic modulus.

Most of the metallic materials for structural biomedical implant applications are used, like Ti6CL 4T, titanium 6 percent, aluminium 4 percent vanadium, and stainless steel 3C316L, like that austenitic stainless steel grade with 18:8 chromium nickel ratio and also it is low carbon grade, L stands for low carbon grade; so this low carbon grade stainless steel 316 also has reasonably good corrosion resistance, those are like popular example of the implants; whereas these scaffolds that more of the polymeric scaffolds are quite widely used, polymeric as well as

metallic scaffolds they are quite (widely) sorry; polymeric and ceramic scaffolds are quite widely used because you can make some foamy structure or porous constructs of this polymeric. And many of the polymeric materials, many of them are biodegradable in nature, so that they can degrade inside the body during this long term exposure to physiological medium or physiological environment and as a result these biodegradable polymers have attracted lot of attention in the biomedical community.

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Implant

While scaffolds too may be considered as implants, all implants are not scaffolds.

Conceptually, a porous scaffold and a nonporous implant would have different level of interactions with the host tissues. For example, a porous scaffold will promote tissue in-growth and tissue regeneration, while an implant is expected to augment lost tissue function.

So while scaffolds too may be considered as implants, but all implants are not scaffolds. So this is one of the important concept that you must remember and conceptually a porous scaffold and non porous implant would have different level of interactions with the host tissues. For example a porous scaffold will promote tissue ingrowth and tissue regeneration; while an implant is expected to augment lost tissue function. So although they have they are expected to have very good biocompatibility property for their use in medical applications; but however they have different level of (bio) interactions with the living systems.

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Vascularization

Vascularization is a medical term used to describe the presence of vessels in a tissue. In animals, this generally denotes the presence of blood vessels. Vascularization is one of the key challenges in tissue engineering. Above a critical pore size, mass and oxygen transport in scaffolds is severely limited by diffusion, leading to the formation of a necrotic core in scaffolds that consists of dead cells. The pore architecture of a scaffold therefore determines and facilitates the vascularisation *in vivo*.

Handwritten annotations in red ink include: a box around "the presence of vessels in a tissue", a box around "necrotic core", a box around "pore architecture of a scaffold", and a box labeled "micro-CT" with an arrow pointing to "pore architecture of a scaffold".

So in this context; another term which is quite relevant and that is why I am introducing this term that is called 'vascularization'. So it is a medical term used to describe the presence of vessels in tissue in a tissue and in animals this generally denotes the presence of blood vessels. So vascularization is one of the key challenges in tissue engineering, so any scaffold that you want to develop or any scaffold that you fabricate in laboratory, it must have vascularization property.

And this is essentially introduced by the fact that above a critical pore size, mass and oxygen transport in scaffolds is severely limited by diffusion, and leading to the formation of necrotic core in the scaffold, that actually facilitates the dead cell formation. So though pore architecture in three dimension and this pore architecture in three dimension it is mostly determined, it is mostly used, it mostly revealed by a technique which is known as micro computed tomography. So micro computed tomography gives you both qualitative and quantitative results of the porous architecture in scaffold materials.

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Angiogenesis

Angiogenesis or neoangiogenesis is a term used to describe the formation of new blood vessels. Neoangiogenesis is common in tumours or to a lesser extent in tissues surrounding implants. While efforts are directed at reducing angiogenesis in tumours, more angiogenesis around an implant is a positive feature, suggesting better acceptance of the implant. Here again, the pore architecture significantly influences the angiogenesis property of a scaffold, when implanted *in vivo*.

Another important thing like vascularization is angiogenesis or new angiogenesis; it is the term used to describe the formation of new blood vessels. And new blood vessels is very important and this is very common in tumors and to a lesser extent tissues around implants. So, this, here again the pore architecture significantly influences the angiogenesis. So therefore both the vascularization property as well as angiogenesis property, these two important property are determined by the three dimensional pore architecture in a tissue engineering scaffolds.

Okay. So, having defined both the implant as well as tissue, because these two terms are as I said in the last module, they are used interchangeably and quite many of the people, they think that they are synonymous words, implant and scaffolds, but they are not actually. That is why I have spent so much effort to explain you that why the scaffolds is different from implant.

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Haemocompatibility

Haemocompatibility involves the study of the compatibility of a synthetic material with blood and blood cells. This is an important property to be evaluated for blood-contacting devices, like cardiovascular stents, pacemakers, cardiac patches, etc. It comprises a study of different factors such as:

- (a) **haematology**: A study of red and white blood cells and their quantification in blood, haemolysis and products of leukocyte activation.
- (b) **coagulation**- Indicated by platelet adhesion, leukocyte adhesion and fibrinogen adsorption. Thrombin generation also indicates coagulation.

An ideal haemocompatible material should not cause platelet adhesion and should be non-thrombogenic. It should not disturb the natural delicate haemolytic balance between coagulation and fibrinolysis. It should be pro-healing and should not be pro-inflammatory.

Handwritten red note: blood compatibility
↓
tissue

Now let me discuss in little bit more detail on another important thing is the blood compatibility. So which has been mentioned earlier, but let me spend some more time just to explain that blood compatibility property. Now why blood compatibility is so important or why I am emphasizing so much in blood compatibility; because irrespective of whether it is a scaffold or it is a implant material, each of the synthetic material when implanted in an animal; they will interact with the blood, with the with the blood which is actually a tissue. So blood is actually tissue and because it contains lot of cells I will explain to you in soon. And therefore, the compatibility of blood with a synthetic material is equally important.

So definitionwise it involves a study of compatibility of synthetic material with blood and blood cells. And this is this two terms is there I have mentioned one is haematology and one is coagulation. Haematology is actually study of red and white blood cells and their quantification in blood, haemolysis and products of lymphocyte activation; whereas coagulation is indicated by platelet adhesion. So platelet adhesion is important aspect that one is to worry about while while evaluating the blood compatibility and fibrinogen absorption on a material substance.

So an ideal haemocompatible material, which is may be very difficult to find out; an ideal haemocompatible material should not cause platelet adhesion, should not cause platelet adhesion, and should be non-thrombogenic in nature. So this is one of the things that in terms of haemocompatibility, material should not facilitate the platelet adhesion and also, should be non-

thrombogenic in nature; or it should not disturb the delicate and natural balance of RBC and WBC, all these factors there.

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Hemocompatibility

- Hemocompatibility evaluation determines whether materials used in the device and the device itself, when operated at its maximum conditions, do not cause excessive damage to red blood cells (i.e., hemolysis).

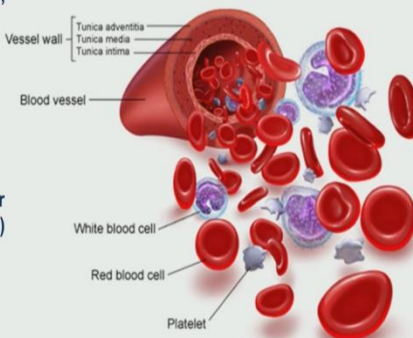
RBC

So this other definition of haemocompatibility is that it determines whether materials used in the device and the device itself, when operated at its maximum conditions does not cause excessive damage to the red blood cells. So this is that RBC; so it should, that RBC should not be damaged or because of the interaction with the synthetic material.

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Blood Composition

- Suspension of cells in a solute of water, proteins, and electrolytes.
 - Red blood cells(RBCs)
 - White blood cells(WBCs)
 - Platelets
- Plasma-yellow coloured fluid
 - Blood from which the cellular components (RBCs, WBCs, platelets) have been removed by centrifuge

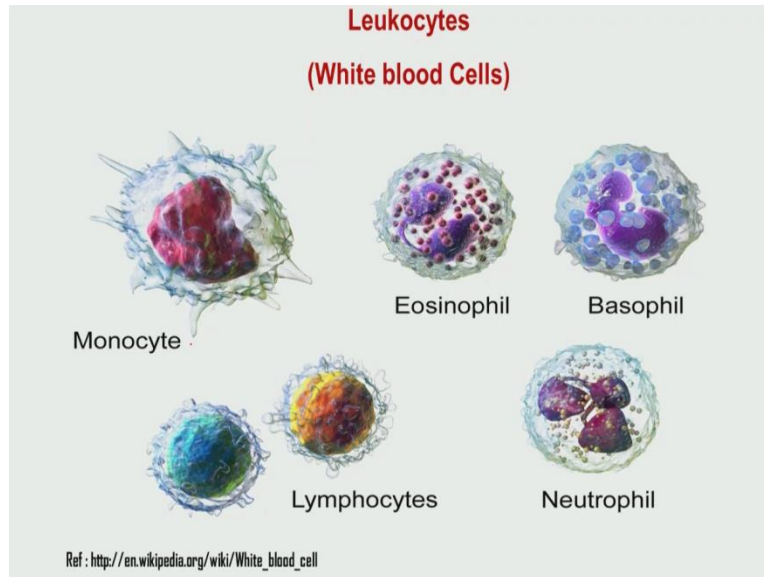


The diagram illustrates the components of blood. A cross-section of a blood vessel is shown with its wall layers: Tunica adventitia, Tunica media, and Tunica intima. Inside the vessel, various blood components are depicted: red blood cells (RBCs), white blood cells (WBCs), and platelets. Labels point to these components: 'Vessel wall' (with sub-labels for Tunica layers), 'Blood vessel', 'White blood cell', 'Red blood cell', and 'Platelet'.

Ref: <http://www.myvmc.com/anatomy/blood-function-and-composition>

So little bit introduction of the blood as a tissue. So this is, this in in case of blood you have this yellow colour fluid, this is called plasma, blood plasma, and in the blood plasma you have different type of cells. These are like RBC, red blood cells, white blood cells and platelets. So, this plasma actually you can consider it as a extra cellular matrix in case of this blood cells, like red blood cells or white blood cells and platelets.

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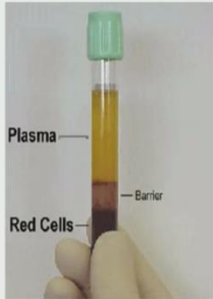


And these are the different morphological features of these leukocytes or white blood cells. So you have monocyte, you have eosinophil; like many times if you see the blood test report you have eosinophil count also, basophil, neutrophil and lymphocytes. So these are like different morphology and then certainly this morphology is quite different from that of the other animal cells that you have seen briefly in some of the other modules when I discussed in terms of the cell material interaction so on.

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Blood plasma

- Plasma is the liquid component of blood.
- It is mainly composed of
 - water (92%)
 - blood proteins 7% (albumin, globulins, and fibrinogen)
 - inorganic electrolytes

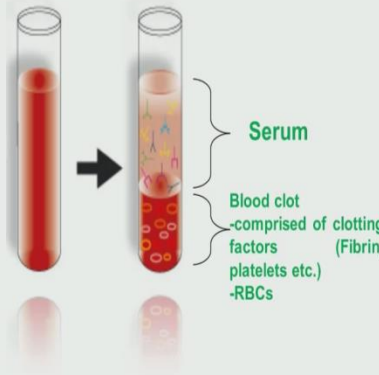


So content wise (blood) blood plasma is the liquid component and it has 92 percent water, and it has other proteins like 7 percent protein like albumin, globulins and fibrinogen and other inorganic electrolytes.

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Blood serum

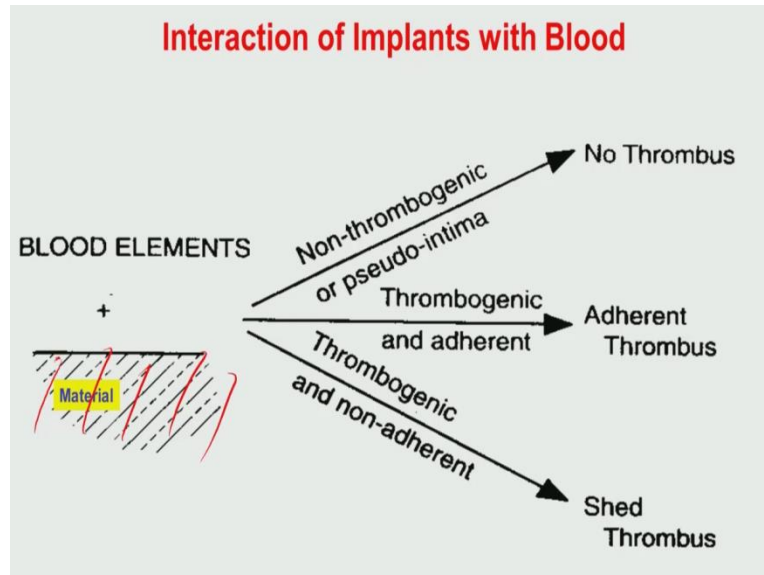
- Serum is the same as plasma except that clotting factors have been removed.
- It is obtained by letting a blood specimen clot prior to centrifugation.



So if you see this normal test tube, if you take your human blood, then you see the top part that is the serum, and in the bottom part where lot of cells, they will concentrate and they will try to settle down in the bottom part of the test tube. They are mostly comprised of the clotting factors when the blood clots, like platelets and RBCs. Normally in human blood the platelet counts

should be 1 point 5 lakhs or above, if platelet count drops down quite a bit; then in case of bleeding this bleeding will not stop in case of a patient.

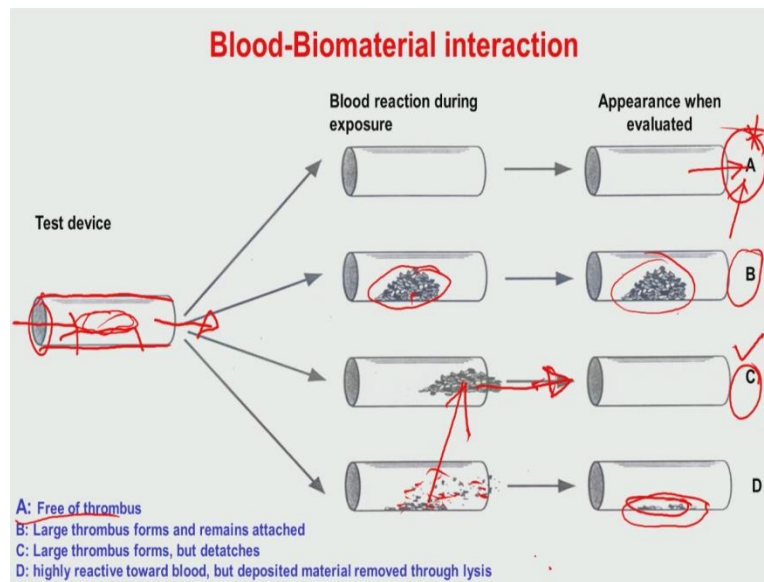
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Okay, now coming to the application of haematology in the biomaterial science, so what I will just explain to you, that this is a biomaterial and this biomaterial if it is exposed to the different blood elements like you know that is that, you have the blood plasma; blood element means this is you have RBC, WBC, platelets and blood plasma. So plasma is a (liquid) fluid in this blood.

So there are three kind of scenario that you can immediately expect. One is that this material can be non thrombogenic in nature, that means there will be no thrombus formation. Second scenario is that if material can be thrombogenic in nature, so it can have adherent thrombus and third one, the material is thrombogenic and non adherent, that means one thrombus will form but it will be actually shedded away.

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So let me just show you that these scenarios little bit more with little bit more clarity. So this is a test tube here. In the test tube you just put your sample somewhere here, okay. One side you make your, you make the test blood sample to flow and get it out of the other side. Now when these blood elements will interact in this interaction zone with the material, so there are a couple of scenarios that I have mentioned in the last slide, in the last slide is that, this is the blood reaction during exposure. So once, in one case there will be no thrombus formation, this blood will simply pass through; so that is the free of thrombus. So that is the most ideal scenario. That is why I put a star there.

Another case, second case there is a thrombus formation, very distinct thrombus formation. Even if when the blood flow is stopped, this thrombus will remain adherent to the material substrate. So this is I put a circle here, this is B; so this is what is not at all (expected) this is what is not at all acceptable in terms of biomedical applications.

The third one is that the thrombus may form but thrombus will be taken away along with the blood. Fourth one is that thrombus will form but it is not stable, as stable like these. However there will be lot of distributed shedded thrombus; so some part of the thrombus will still get stuck after the entire blood flow is stopped and then you can see some signs of the thrombus formation.

So while nothing is ideal in our world, so this A situation is the most ideal scenario, and as long as this thrombus form is shedded away, that is also it is okay; and third, fourth one also can be accepted to some extent but may not be in an ideal scenario.

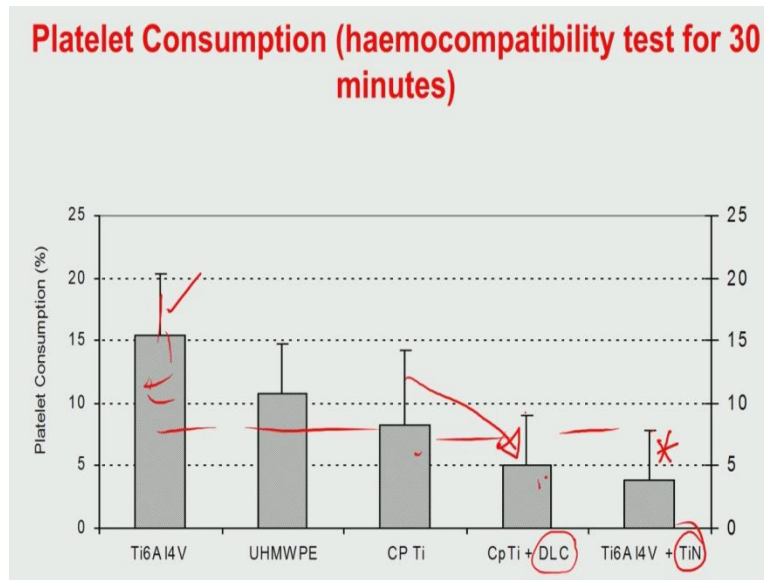
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So this is just an example of the thrombus formation; there is two material which are used or which are which are potential use in the hard held applications. One is commercial pure titanium; CP stands for commercially pure titanium, and another one, you have a diamond like carbon coating; DLC stands for diamond like carbon coating, and diamond like carbon coated commercially pure titanium.

After the standard haemocompatibility testing in commercial pure titanium, you can see large huge significant thrombus formation. But in the DLC coating, this thrombus formation is significantly reduced. Now if you do quantification, some kind of quantification, then you can see quantitatively also this thrombus formation A would should be statistically much less significant as compared to commercially pure titanium without any diamond like carbon coating. So the conclusion is that, diamond like carbon coating like ceramic coating is helpful to improve the haemocompatibility of this titanium based materials.

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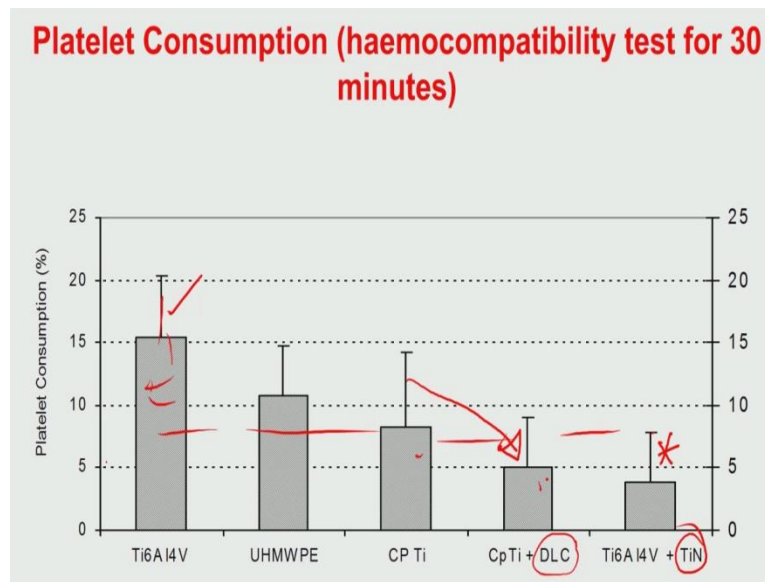


Now in terms of quantification people have done this haemocompatibility test for thirty minutes, and they have quantified that what is a platelet composition, platelet consumption after this thirty minutes test, and they have used different type of materials, titanium 6 percent, aluminium 4 percent, vanadium, ultra high molecular poly ethylene, commercially pure titanium, commercially pure titanium plus diamond like carbon coating and titanium 6 percent aluminium, 4 percent vanadium, on the top of it they have used titanium nitride coating. In one case you have diamond like carbon coating, in one case you have titanium nitride coating.

Now after this platelet after this haemocompatibility test, now if you do some statistical analysis, and you take Ti 6 Al4 V as your baseline material, then immediately you can notice that once you put titanium and nitride coating it is significantly much less than what you have obtained with the bare material or uncoated material. Similarly if you put titanium, commercially pure titanium and DLC coating, again DLC coating has reduced but may not be statistically significant because this error buds may overlap as you can see quite clearly here.

So titanium nitride coating is certainly quite useful, DLC coating also reduces the platelet consumption. So as per haemocompatibility is concerned, that is the reason people are trying quite consistently to use ceramic coatings on a metallic material to reduce the haemocompatibility, to get a better hemocompatibility property.

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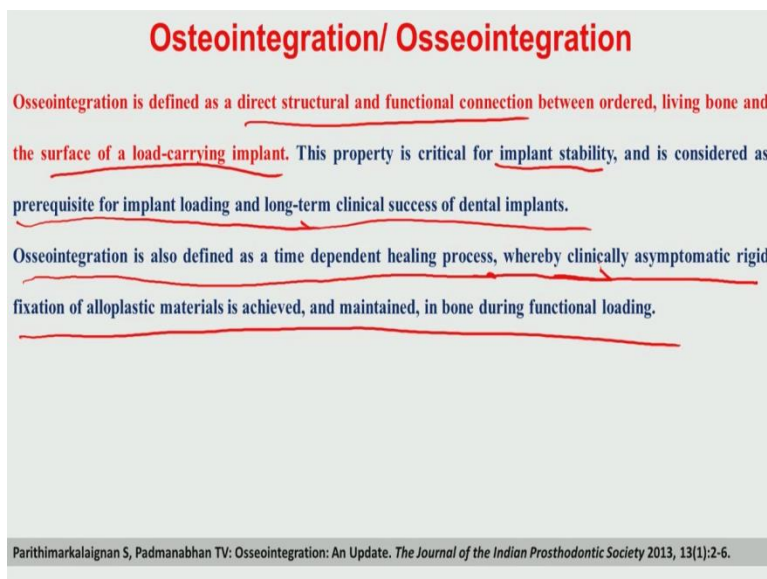


Now as far as white blood cell consumption is concerned, this WBC consumption, and here again with respect to titanium 6 aluminium 4 vanadium people have done with the titanium nitride coating, the white blood cell consumption, there is not much significant difference in terms of diamond like carbon coating as well as the titanium nitride coating with respect to their with respect to their uncoated substrate.

So in a way that ceramic coatings has shown to be quite useful they do not disturb that white blood cell contents in the blood, at the same time they significantly reduce the platelet consumption after the standard haemocompatibility test for thirty minutes as recommended by standard guidelines.

Now, so once this cell level compatibility like cytocompatibility and blood level compatibility is established, next stage is certainly that In vivo biocompatibility or the pre-clinical testing in animal models. And there we have to understand that what the scientific information we would like to obtain from, particularly for bone tissue engineering applications.

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Osteointegration/ Osseointegration

Osseointegration is defined as a direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant. This property is critical for implant stability, and is considered as prerequisite for implant loading and long-term clinical success of dental implants.

Osseointegration is also defined as a time dependent healing process, whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved, and maintained, in bone during functional loading.

Parithimarkalaignan S, Padmanabhan TV: Osseointegration: An Update, *The Journal of the Indian Prosthodontic Society* 2013, 13(1):2-6.

Now these are the three four definitions that is quite useful before we get into the more details of this In vivo testing. First one is the osteointegration or osseointegration. Osteo; so anything osteo means bone, or anything osteo means that osteo structure in the body. So osteointegration is defined as the direct structural and functional connection between ordered living bone and the surface of a load being implant, which is essentially non-living material.

And this property is critical for the implant stability that is a long term implant stability, and certainly it is a prerequisite for load bearing, load bearing implant and long term clinical success. So therefore osseointegration is quite useful, it is quite important process as far as the pre-clinical assessment is concerned.

More text book type of definition has been given here; it is defined as a time dependent healing process. That means it is a kinetic process and therefore when you have to do this kind of testing in the animal models, you have to do also at a different time point. So you have to sacrifice the animals at a different time point during your study, so that you can understand that what is the time dependent healing process that takes place when this materials, when this materials are placed in an in an animal.

Whereby clinically asymptotic rigid fixation, of alloplastic materials is achieved and maintained in bone during functional loading. So therefore that osseointegration is to be tested when the material is to be placed at some loaded bone part, and then time dependent healing process how

this material is helping to achieve this time dependent healing process, that is kind of a central theme of this osseointegration.

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Osteointegration/ Osseointegration

Osseointegration may be divided into three stages:

- (a) Incorporation by woven bone formation
- (b) Adaptation of bone mass to load (lamellar and parallel-fibered bone deposition)
- (c) Adaptation of bone structure to load (bone remodeling).

Osteointegration may be qualitatively assessed by a clinical mobility test or from radiographs and qualitatively determines usage of micro-computed tomography (micro-CT) analysis of explanted bone (e.g. bone volume/total volume ratio).

histology
micro-CT

Parithimarkalaignan S, Padmanabhan TV: Osseointegration: An Update. *The Journal of the Indian Prosthodontic Society* 2013, 13(1):2-6.

And it may be divided into three stages. One is the incorporation of woven bone formation, second one is adaptation of bone mass to load, like it can support the loading and the biomechanical loading, and third one is adaptation to bone or bone structure to load, that is bone remodelling. And qualitatively assessed by clinical morbidity test from radiographs and qualitatively using the micro computed tomography test and analysis of the explanted bone.

So there are two things that one can see, that one that one can do that histology, just to see that how this how this bone formation has taken place, and in three dimensional you have to do microCT. So microCT will give you more quantitative determination, while histology is


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Osteoinduction

This is the process to induce *osteogenesis*, which is qualitatively described as bone cell functionality in the surrounding region of an implant, similar to their activity in the host bone structure.

It is a phenomenon regularly seen in any type of bone healing process.

Osteoinduction indicates recruitment of immature cells and their simultaneous stimulation into preosteoblasts. Bone healing processes, as in fractures, are primarily dependent on osteoinduction.



Albrektsson T, Johansson C: Osteoinduction, osteoconduction and osseointegration. *Eur Spine J* 2001, 10(2):S96-S101.

The second definition is osteoinduction; this is the process to induce osteogenesis. Osteogenesis essentially means that is qualitatively described as bone cell functionality in the surrounding region of an implant. So if you place an implant, suppose you have a long bone structure, you cut this part of the bone, then you put some implant here, so this is the shaded region implant. So now you see that how this cellular activity is there in the neighbourhood of this synthetic material. And it is a phenomenon regularly seen in any type of bone healing process. And osteoinduction indicates essentially recruitment of immature cells and their simultaneous stimulation stimulation to preosteoblast.

So therefore, to prove the osteoinduction process, you have to also see that whether the certain stages of differentiation process is taking place or not. Bone healing process, as in fractures, are primarily dependent on the osteoinduction process. So there are two terms I have defined now, one is osteointegration or osseointegration, and another one is osteoinduction. So induction means, so you recruit immature cells and then you simultaneously stimulate them to preosteoblasts.

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Osteoconduction


This is a phenomenon regularly encountered in bone implants and this signifies bone growth on a biomaterial surface. Implant materials of low biocompatibility such as copper, silver and bone cement show little or no osteoconduction.

And third one is the osteoconduction; induction is this one and osteoconduction. So this is a phenomena, it is again with respect to the bone implant, and this signifies the bone growth on a biomaterial surface and implant materials of low biocompatibility like copper, silver and bone cement essentially show little or no osteoconduction.

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In vivo biocompatibility assessment

- Selection of animal models
- " of defect model
- design a study & qualitative/quantitative determination of biocompatibility



Okay. So, after this term, we will now start with this In vivo biocompatibility assessment and In vivo biocompatibility assessment, certain things I would like to stress upon; that is the selection of animal models, then selection of a particular defect in the animal, and how to design a study

and qualitative or quantitative determination of biocompatibility. Okay so this, so as I said before that In vivo and In vitro may be let me just recapitulate what I have taught you quite few modules back.

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Importance of *in vivo* experiments

- Right animal model to approximate human environment
- Second step prior to clinical use
- Critical for development of clinical devices

In vitro tests cannot replace *in vivo* tests, as there is:

- no inflammation
- no immune response
- single cell type
- no tissue remodeling

In vivo tests provide:

- interactions of different cell types
- effects of hormonal factors
- interactions with extracellular matrix

In contrast, interactions with blood-borne cells, proteins, etc. can be studied via *in vivo* tests

So In vivo is kind of one step ahead than In vitro in terms of the biocompatibility assessment; and particularly you have to then select the right animal model to approximate human environment, and this is a second step prior to clinical use and this is certainly clinical for development, critical for development of clinical devices. In vitro test cannot replace In vivo test simply because in vitro you cannot kind of stimulate the inflammation process, immune response and mostly In vitro tests are single cell type. As I said that unless you do core culture you cannot grow two cells, two different type of cells in a single In vitro experiment on a material. And then fourth one is a no tissue remodelling kind of can be assessed in the In vitro.

Therefore In vivo tests are crucial and it provides certain interactions of different cell types, like different cell types are present. It also allows you to understand the effect of hormonal factors. Third it also allows you to understand the defects of extra cellular matrix. So these, this is that important distinction that In vivo tests, and during the In vivo long term experiments like, you know 26 weeks or 52 weeks or 78 weeks, the physiological environment also dynamically changes. So this dynamical (change) dynamically changing physiological environment cannot be simulated in a simple In vitro testing in case of the the simple In vitro testing in the lab scale.

And that places a significant importance that why In vivo test is really required, is really required to get more clinically relevant results; and after the In vitro tests I said that one has to select a few samples for In vivo tests, because In vivo tests you need to go through animal ethical committee approval, so therefore you cannot use significantly large number of animals for your In vivo test in view of the animal welfare is concerned and in view of the strict ethical committee regulations are involved.

So therefore you have to thoughtfully select one or two samples which can go to the In vivo tests. Along with the control sample or along with the baseline sample one has to further conduct the In vivo tests, which I will focus in the next module.