Advances in Additive Manufacturing of Materials: Current status and emerging opportunities

Prof. Bikramjit Basu

Materials Research Center, Indian Institute of Science, Bangalore

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In this lecture, I will be discussing mostly the clinical translation challenges and also largely the scientific case study particularly biocompatibility of 3D extrusion printed alginate gelatin hydrogels. now clinical translation of the tissue specific bioprinted scaffolds, One of the major demand by orthopedic surgeons for this artificial cartilage, this articular cartilage is required for many orthopedic surgeries and then if this 3D bioprinted cartilage can be regenerated can function as the articular cartilage the orthopedic surgeons will appreciate very deeply. Now let us take that example that Gelma -mCmC and NanoHAP that is the biometal ink that I have shown sufficient results and in one side you have the Gelma CMC hydroxyapatite another side would be just simple Gelma mCmC. So one side would have more bone response, another side will be more chondral response. And this is the classic case of osteochondral scaffolds. And what one can do further, then the researchers can do animal studies like in vivo study.

and trying to see that whether you know, articular cartilage can be regenerated and what is the functionality of this cartilage that is being regenerated at the joints. The second one is the nerve graft conduits. Now, remember this kind of examples that what I have shown you before, let us say alginate, gelatin, carbon nanofiber. The purpose of adding carbon nanofiber is to increase the electrical conductivity of the scaffolds to make the bio ink more electroactive or electroconductive and when you do this 3D extrusion printing of this particular biometrial ink or bio ink then you can essentially get this particular nerve conduit structures and this is like 10 to 15 millimeter in length and this is like 3 to 4 mm kind of a inner diameter.

And then these scaffolds can not only be printed as a nerve conduit like cylindrical conduit structure but also it can have different shape fidelity compliant structures. You can squeeze it you know this particular grid structure it can be squeezed. this is like different compositions that we have developed 3A, 5G. 3% alginate, 5% gelatin, 0.25% carbon, 0.

5% carbon, 0.75% carbon based electroactive, electroconductive hydrogel scaffolds. Now, this is the pseudo 4D printed conduits and mechanical property analysis. why we call pseudo 4D printed? Because you have the 3D printed sheet. And then if you put it in let us

say calcium chloride solution here, then you can roll it and then you make it a cylindrical graph.

this is a permanent change in these materials. But it is not exactly like 4D bioprinted because essentially you have pseudo 4D printed, we do not say even 4D bioprinted because it does not contain the cells. The second thing is that this particular pseudo 4D printed because you are giving just the chemical changes by cross linker additions, you can see this is that micro city video of this particular scaffolds, particular conduits and this micro city things you can very clearly see this is a through and through hollow cylindrical conduits and you can see that this wall thickness is also it is kind of more uniform. this is kind of prepared in the laboratory and These kind of materials when you do tensile testing you can see this is a 2.5 mega Pascal kind of maximum strength that you can essentially achieve the tension.

And here you can see the compressibility this is you can see this is the tension. And the compression also it can go very high up to 3 megapascal. certainly around 2 to 3 megapascal or 2.5 to 3 megapascal of strength can be obtained under tension or compression for this pseudo 4D printed structures. Now, this particular nerve conduits, now what would be the next step? Next step is that for example, if you cut the sciatic nerve of let us say rat model, And then if you give this kind of electrical stimulation of the scaffolds, then we would like to know this kind of functional electrical stimulation.

We would like to know whether the neural tissue regeneration can be essentially done during the 3D bioprinted scaffold. this is something that we need to do in the years to come and then scientists should do this kind of work to make sure that you know that it does not stop at the lab scale. It is a in vivo study and then to take it further. Now, I will just show you that one such examples of the bioscientific case study that is the biocompatibility. by the very basic definition of the biocompatibility as I mentioned before that in vitro study that means which is the physiologically simulated conditions by testing in the glassware.

that study if you do then we can call it as a cyto-compatibility if it is cell level study. If it is using that animal blood then we can call it as a hemo-compatibility. If it is using this bacteria then we call it as antibacterial property, in vitro antibacterial property. But whenever you do this study in the whole organism like in an animal like rat model or mouse model then we call it as a biocompatibility of these hydrogels. then you can confirm that yes indeed the biocompatibility study has been conducted.

Now, this is again an example that I have described in much more details. It is that alginate gelatin. there are two types of hydrogels that I have discussed in this particular NPTEL course. One is the Gelma. One is called alginate gelatin.

both are essentially gelatin-based biomaterial inks but in one case you have a gelatin methacrylate and then another case is the alginate gelatin. Now, sodium alginate and gelatin this is also another system that is very very widely investigated. What is relatively less explored when you add these nano fillers like carbon nano cellulose or nano cellulose as a particle. these are the chemical composition of the different constituents of the biomaterial ink. Then this part actually I have just explained in the earlier lecture that you know that materials, viscoelastic properties play an important role during the 3D extrusion printing.

Then using these particular compositions we have essentially manufactured shape fidelity compliant structures and this is like a urinary patch, this is like a patch and this is like a tubes. This is like a tube, this is a hollow 3D printed tubular grafts and then the main thing is that we have to address the biocompatibility of these scaffolds because it also contains a nanocellulose. And these particular hydrogel structures can be chemically cross-linked by putting them in the calcium chloride solution and that has been mentioned here. there are different compositions which has been mentioned here 3A5G, 3A5G1CP, 3A5G1C, 3A5G2C. in all these cases, one can add either 1% or 2% of the nanocellulose particles, Now, when you have done biocompatibility study, essentially we have done subcutaneous implantation that means under the skin, these 3D printed scaffolds were placed and then we have sacrificed the animal at different time points, let us say 7 days and 30 days.

And this subcutaneous implantation was carried out at the dorsal side of the rat model, Wistar rat model. in that bilateral implantation was done at two sites you know that two implants were placed so that we have essentially followed the 3R model. in the 3R model which essentially means it is replace, reduce, refine. This is the 3R principles, these are 3R principles which are followed in the in vivo study. this 3R principle replace means if in vitro study can provide clinically relevant scientific information for any biomaterial or biomaterial structures or any nanoparticles or any biological materials for intended clinical applications, so then the study should be restricted to in vitro only.

If in vitro study is not good enough, you need to do in vivo study. Then we have to define the study plan in a manner that animals will experience minimum pain. and animals will receive the highest possible healthcare during the complete timeframe of the study and they should not be neither tortured nor should be given excessive pain for this kind of scientific study that is the refine your animal study protocol. Reduce means you need to reduce the total number of animals that need to be sacrificed as part of this in vivo study. you can do these experiments or you can do this study the way I have been describing here.

Instead of putting two scaffolds in one animal, you can put one scaffold in one animal, another scaffold in another animal. you can use two animals, but then you will be

sacrificing the two animals. we have carefully thought about it and then we are also cautious that by placing 2 scaffolds in 1 animal, we are not giving the animal significant pain during the implantation process we could have placed 3 implants or 4 scaffolds but we did not put 2 is the maximum and that we have discussed with veterinary surgeons. at Sri Chitra Tirunal Institute of Medical Science and Technology as well as other veterinary surgeons at the time of conceiving this study plan. And thereafter, we have got the approval of the Institution Animal Ethics Committee of Indian Institute of Science Bangalore and then our research group has conducted this particular study which is still unpublished.

therefore, I will quickly go through the basic study plan. and then how we have conducted the study, what are the parameters we have investigated. The detailed experimental results we are not going to explain into a much much deep finer details, but suddenly show some of the representative results for you to understand that how we have performed these experiments and how we have analyzed the data. Now at the 7 days and 30 days, so one animal has been, so for each scaffold essentially 4 scaffolds are used and 2 different animals. one is sacrificed at 7 days, one is sacrificed at 30 days, And then thereafter we have done several of the experiments, several of the analysis.

We have taken the blood sample from retro orbital plexa and then we have analyzed the blood parameters like RBC, platelet count, WBC, leukocytes, hemoglobin. We have also analyzed the serum biochemistry like serum creatinine, bilirubin, BUN, ALP, AST, CPK. Now, why this analysis is important? Because these hydrogels, they are biodegradable in nature. inside the animal body, it is quite expected by the turn of 30 days, these hydrogels will degrade, will disintegrate because we are not restricting the animal's regular movement in terms of walking inside the animal cage and so on. during the degradation process when these biomaterial in constituents are distributed biologically into the different organs, we would like to see or particularly distribute in the blood, we would like to see how the different blood parameters and serum biochemistry parameters, they are being affected and whether that will have a negative effect or negative influence.

Second one, we have taken the tissue samples from the hydrogel scaffolds and then did that histology analysis and then we have found out that what is the neoblood vessels and in the neoblood vessels we have analyzed the CD31 receptor analysis and TNFR complexes as well by and as well as in the DAB staining. in terms of the histology analysis, just to see that whether there is any change in the local tissue architecture and whether there is any signature of the inflammation, necrosis due to this degradation products being transported to different organs or tissues. We have done both H and E staining as well as MTS staining, masson's trichrome staining and hematoxylin and eosin staining. these are the two different ways you can essentially stain the tissue samples. And then once these stains are being done, so first you have to do tissue embedding, then you have to do the staining and then

you can observe, you can analyze the tissue structure under the optical microscope.

Now this is how these particular animals were monitored. You can see that you know these animals were being weighed very regularly every day and see that whether there is a gradual increase in the weight of the animals. This is how these hairs are being shaved before the surgery. Then you can see this is the 3D printed scaffolds are being placed here in the peritoneal cavity in this particular area, dorsal area.

This is one implantation site. This is another implantation site after the suture. This post-operative 7 days, there is some signatures of the swelling. But at 30 days, you can see that hairs are fully grown into the rat model at the end of the study period. Now, how the body weight is increased? You can see 3A5G scaffold, the body weight is increased from 200 gram to 350 gram. The same case in the 3A5G1C body weight is increased almost like 200 to 350 gram.

quite a regular increase of the body. this is an uneventful animal study with 3D printed scaffolds. In terms of the blood parameters and CBC count and monocytes, neutrophil, platelet count and WBC, these all increases at 7 days and goes down at 30 days. And you have this blood urea nitrogen serum (bun) biochemistry results. Again, when it goes from acute stage to sub-acute stage of inflammation, this actually drops.

Now foreign body response, so like you know any 3D printed scaffolds are being treated as a foreign body to any animals because it is not naturally available, it is synthetically made outside the animal body and now it is being implanted inside the animal body. therefore, this response of the host or host response or the animal's response or the organism response to this particular biomaterial implant said biomaterial it is known as the foreign body response. And histological evaluation as I said that it has been made using the hematoxylin and eosin staining and MTS staining, masson's trichrome staining 3A5G and 3A5G1C, one is a nano cellulose content and one is without nano cellulose 3% alginate, 5% gel mass. Now, we can clearly see this is the signature of the MNGC that is multinucleated giant cells and then you have also macrophages and these macrophages are very clearly shown at different places, you can see that these macrophages, this is that MTA stained images and this is the H and E stained images. And MTA stained images essentially shows this materials does not have very very significant inflammation behavior.

it demonstrated gradual healing response acute to sub-acute. Now, when the degradation products, they are biologically distributed or bio-distributed at different vital organs like kidney, brain, heart, liver, lungs, intestine, adrenal, spleen, stomach and testes, we wanted to know or we wanted to analyze whether they will have any signature of the toxicity to the local tissues. And at both the 7 days and 30 days, we have found out that these tissue

sections exhibit normal tissue architecture without any significant alterations at any spatial locations in all the analyzed tissue sections. essentially they all the tissues demonstrated healthy morphology without any noticeable alterations. Now, we wanted to know that whether these materials or these scaffolds will have any inflammatory response in terms of TNF alpha, tumor necrogenesis factor alpha expression.

So, after the subcutaneous implantation and accumulation of macrophage, then they will essentially secrete TNF alpha from the macrophage activation and receptor binding and signal translation and gene expression can be characterized. Now, in this particular case, you can see this is the hydrogel scaffolds and within the hydrogel scaffolds, there are different type of cellular structures that has been shown. It can be all the multinuclear nucleus cell, multi-multinucleotide giant cells, macrophages and these macrophages also can undergo from M1 to M4 transitions where both are pro-inflammatory. And in the pro-inflammatory case, one is that actually reduce the phagocytosis, iron recycling and foam cell formation. Another one is phagocytosis decrease and CD163 will decrease and plaque will be instability.

So, TNF alpha, IL-6, IL-12 and IL-1 beta these are essentially with the pro-inflammatory markers whereas the IL-6, TNF alpha, MMP7 these are also pro-inflammatory markers in the M4 stage. what we analyzed? We analyzed the number and distribution of the inflammatory cells like neutrophils, monocytes, macrophages and fibroblasts. And then quantification and timeframe occurrence essentially indicates the nature of inflammatory reaction. Another one what we have analyzed thickness and quality of the fibrous tissue surrounding the material and that is the indication of the degree of acceptance of the implanted material. Now this is the TNF alpha stained images and you can see it is a normal physiological reactions.

If you see this is the zoomed up version, this is also zoomed up version and you can see very clearly this nucleus in the blue stain that you can see and then you can see this is the vascularized living tissue to trauma and invasion of the scaffolds that has been taking place, These are like different type of cells that is very clearly shown here. essentially what we have concluded from these responses is that for both the materials or independent of the addition of the nano cellulose, both the scaffolds 3A5G and 3A5G1C they exhibit comparable TNF alpha expression that is a normal inflammatory response. Now, the other things that we are also interested to know that whether this will have angiogenesis or this will essentially form the vascularization. this kind of pictures that what you see that we have utilized the BioRender, this is the commercial site BioRender.

com. These sites we have used and then we have recreated this kind of images using that particular website. So, you have a blood vessels and then these blood vessels they can get

matured. These are primary vessels, this is the secondary and these are tertiary. in terms of angiogenesis, blood vessel branching and their distribution and extension or maturation of the blood vessels. These are very important and that one we have used to analyze that particular aspects we have analyzed CD31 expression.

What is CD31? VEGF is one of the growth factor in case of endothelial cells, it will trigger both downstream signaling proteins and that will essentially determine cell viability, cell survival, vascular cell permeability, cytoskeleton rearrangement, cell migration, proliferation, NO production, nitric oxide production and essentially that will lead to this angiogenesis that blood vessel formation. nature and distribution of vasculature that is important vital for the host to establish new vasculature with the material matrix for sustenance and metabolic function of the infiltrating tissue. what I am trying to explain here is that if vascularization is promoted which can be quantitatively analyzed by angiogenesis CD31 expression, then what would happen that this kind of hydrogel even when it is biodegraded, it promotes the vascularization locally at the site of implantation. And what it means that if blood vessels they get matured, so that oxygen and nutrients can be supplied to the cells and tissues which are growing at the implanted site and that is good and that is important for the vitality or survival of the biological cells and tissues.

That is the point I was trying to make out here. this is that angiogenesis or CD31 expression, this is the 3A5G scaffolds. And these are 3A5G1C scaffolds is a post implantation 7 days and post implantation 30 days. And one can clearly see there is an extremely good CD31 stained sites are very clearly visible here in the 7 days as well as the 31 days. And these were essentially quantified using this vascularization density in percentage and you can see 3A5G at the 7 days it is around 10 to 12% because the modest is 7.

5 the mean value. Here it is little bit higher let us say 9%, 8% and so. In 30 days it is suddenly reduced but when it has nano cellulose particles, so it still maintain that vascularization is around 7.5%. 7.5% to 10% nano cellulose incorporation certainly lead to better vascularization at 30 days implantation sites.

Before I close I would like to mention that you know this in vivo study is very important and then whenever researchers they essentially try to assess that how 3D printed scaffolds will promote vascularization or new tissue formation as part of the biocompatibility or host response or foreign body response, one has to carefully design the in vivo study protocol following 3R principle that is reduce, refine and replace. that principle and also there are two ways one can do. One is the general biocompatibility response can be studied the way I have explained in this particular lecture. Another one is the more application specific biocompatibility evaluation. For example, if you want to do the neural nerve tissue

regeneration applications, then this is the model that you have to follow.

Then you have to cut the part of the sciatic nerve, you have to place this your tubular contact or the cylindrical contact and since one of the primary criteria for any neural tissue is to conduct the electrical impulse, electrical signals across the end-to-end nerve. whenever you are putting this graft, you have to ensure that if you apply the external electrical stimulation by using some of these tissue healing stimulator or tissue healing stimulator, then whether this neurological functionality can be regained and also what is the functionality of the regenerated neural tissues that will be formed at the site of the implantation. So, it is not only the tissue maturation but also one has to provide the evidences that this tissue maturation leads to clinically acceptable functionality of the regenerated tissue. If that is not done, then we have to see, we have to strategize that why it is not being done, what is the reason and how to regenerate the tissues with clinically acceptable functionality. I thought that I will explain to you in this particular lecture that how to conduct the biocompatibility in vivo study or animal study.

And then how these 3D printed scaffolds can be utilized in this particular biocompatibility study.