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

Prof. Bikramjit Basu

Materials Research Center, Indian Institute of Science, Bangalore

Lecture 56

Welcome back to this last series of lectures on challenges and opportunities in additive manufacturing. What I am going to do in today's lecture is to continue our discussion on the yet another scientific case study on biocompatibility of 3D extrusion printed alginate gelatin carbon nanofiber hydrogens. Now, why I have selected this particular hydrogel first of all these particular materials scaffolds were developed in our laboratory and second things I put a question mark on carbon nanofiber the reason for putting this question mark is that in the scientific community people researchers still not yet over perhaps have not yet overwhelmingly accepted carbon nanofibers or several carbonaceous materials for biomedical applications. Often there is concerns particularly they mentioned that the reason for this widespread apprehension is that if these nanofibers they are degraded in the biological system let us say in any organism or something how they will be transported to different vital organs and whether this carbon nanofiber based materials will have any toxicity at the tissue level and so on. against this particular backdrop we wanted to see that whether this particular hydrogels when it contains carbon nanofibre will have clinically acceptable biocompatibility in animal model. Before I do that you know because every lecture stands on its own.

therefore, I would like to reiterate some of the common definitions or common concepts which I have explained to you as part of the previous lectures. But I thought it is important to revisit those concepts so that you are extremely thorough with those kind of concepts and ideas. And then also I will explain to you that very briefly the interactions with the biological system. And apart from this biocompatibility study, I will be also showing extensive characterization of these scaffolds in terms of 3D microstructure using one of the state of the art technique called microcomputed tomography, micro CT that we have used very extensively, Let us first start with this clinically relevant interactions between the implanted scaffold and biological systems.

you see this is that extrusion printing. This is the 3D extrusion printing. You apply the pressure on the top. depending on what is the architecture that you want to print, you get a design scaffold, let us say this is a 3D printed scaffolds. Now, these 3D printed scaffolds, we can put it into that animal model.

This animal can be mouse model or rat model and so on. Now we also know that many of these scaffolds that we have investigated in our lab is gelatin based. Either gelma based or alginate gelatin based and this one has been emphasized that this Gelatin based materials they have a specific RGD sequence which allows the cells to recognize very easily and also adhere. essentially it will favor more interactions with the one of the key components of the biological system that is cells. the moment you put these scaffolds into the organism let us say any small animal model.

we do not want the bacteria to grow, we want biofilm, this is the schematic of the biofilm and this entire schematic is being created by BioRender. we do not want either gram positive or gram negative bacteria to grow on the scaffolds and there should not be any biofilm formation. Second thing is that we want the cells to grow and these cells, these are like tightly spaced cells and that means these are like endothelial cells. We want neuron

to also be activated and then that is called the innervation that is important. We want blood vessels to grow and these blood vessels if they will grow then it will cause the vascularization in the scaffolds.

And at the same time, there is number of inflammatory cells which will attack the scaffolds and these scaffolds is always considered as a foreign body or foreign object because this is not part of the animal system. This is something which is fabricated outside that animal body using some of the one of the manufacturing technical 3D extrusion printing. we have to see that how these different inflammatory cells actually react to this particular scaffolds and then what is the inflammatory response and how that will subside over time. here at this point I can tell you. So far today no researcher can claim that they have developed a specific scaffold which will not cause any inflammation when they implant in the animal body.

what I am saying what I am trying to say and what I am trying to emphasize here. Inflammation is the natural process which one cannot avoid whenever they will implant synthetic scaffolds of non-biological origin into a biological into a full organism right so into an animal model. but then question is that one inflammation sets in to what extent inflammation is subsided over time and what is the kind of a length of time period that we are talking about. let us revisit some of the things that I have already mentioned multiple times but as I said these are some of the important concepts that one cannot afford to forget. therefore, I keep on showing it not very repeatedly but at some point of time after 2 or 3 lectures in this NPTEL course.

hydrogels. hydrogels is a 3D network. of hydrophilic polymers which can be cross-linked either by chemical cross-linker, physical cross-linker or something physical cross-linker and also which have significant water retention capacity. Hydro means something to do with this gel, similar water retention capacity. and this has a viscoelastic property.

these are some of the key words which defines hydrogel. it has a viscoelastic property and the viscoelastic property is largely attributed to the 3D network of the interpenetrating structure of hydrophilic polymers and it also has the ability to retain significant amount of water. Now, if you look at this hydrogel structure and then these are the cells and these cells can essentially adhere to the different sites of this hydrogel structure. Now, what are the polymers that to formulate the gel? There can be natural polymer like chitosan, alginate, collagen, gelatin and there is synthetic monomer and then synthetic monomer can be vinyl acetate, methacrylic acid, acrylic acid and so on. as I said certain specific attributes which make hydrogel a very special class of biomaterials and perhaps one of the most widely investigated biomaterials in the global scientific community and these specific attributes number 1 is a viscoelasticity that is if you look at the stress versus strain.

this can have 2 different kind of response. it can go up and down and then it can also undergo stress reversal. it provides mechanical signaling to the cells like it actually allows cellular crosstalk to take place like when it is a soft hydrogel then go to stiffer hydrogel then you can see what it happened. You can have a specific architecture and then it can degrade. once degrade then this kind of polymer chains get loosen, they will lose the structural rigidity and then the question is that once it degrades in in vivo like in animal model what would be the consequences of this degradation.

Now as I said this viscoelasticity and cross-linkability. I mentioned four keywords to define hydrogel. One is the 3D network of hydrophilic polymers, one is the viscoelastic property, one is the cross-linkability and one is significant water retention capacity. these four keywords are very important to describe and define any hydrogel. And this particular cross-linking case, one can have physical cross-linking like you can add certain photo cross-

linker like Igacure for example.

You expose to UV, then it can be photo cross-linked. It can be in the chemical cross-linker like calcium chloride for example. calcium 2 plus 2 ions actually helps in cross-linking and it can be also dual cross-linking. Dual cross-linking mean it has certain sequence of physical cross-linking followed by chemical cross-linking. before I start with the biocompatibility analysis, I would like to remind you about the 3R principle which I have briefly explained in one of the future previous lecture, but I thought this is important to show this particular slide.

aim is to protect animals used for scientific and educational purpose and scope is that all live non-human vertebrate animals and invertebrates likely to feel pain, these kind of all the live animals whenever you do some surgery, animals will certainly suffer pain. Then the question is that according to the globally accepted norms, one has to follow 3R principle, , reduce means number of animals used in the study must be reduced to a minimum number without compromising the objectives of the project, Replace means the use of animals for experimental purposes authorized in cases where no satisfactory substitute method exists. For example, any ex vivo technique like you take some particular tissue and then test it outside the human body with the materials. You can use simply very complex 3D culture methods to show that whether this 3D bioprinted or 4D bioprinting that kind of experiments can in principle replace animal model. For example, you want to do drug testing.

Drug testing typically is done using animal models but in today in some of the countries they are exploring the opportunity to see whether 3D bioprinted models can be used for primary drug screening so that so many different drug molecules they are being discovered in different labs. Now, according to the ethical guidelines, if those drugs can be screened using 3D bioprinted models, for example, liver models. or pancreas models and so on and then if you construct the 3D liver model and you can use some of the competitive drugs then you can see that you know which drug at what dose actually performs better than other competing drugs. this is another example where in principle you can avoid extensive animal study but perhaps I am not sure whether you can 100% remove the animal study in the drug discovery process. Perhaps in a limited number one has to still do the drug testing in animals before one can even try for the human clinical trials.

But certainly it can be reduced and I strongly believe that it can be reduced if you use the 3D bioprinted organs. Third one is the refine. you refine the living conditions and methods used in these procedures should not cause any unnecessary pain, suffering or distress to the animals. I think that I have explained to you enough to just to explain that what is the 3R principle. Now as I said that carbon nanofibers these are used as one of the electroactive fillers to that sodium alginate and gelatin hydrogels.

And this particular case you know we use this animal models and this particular animal models we have essentially implanted into this animal model. Then we expect the neural network, neuronal network in the scaffold is expected so that you know if we implant it you know in a cylindrical conduit structure then whether it will aid in bridging the gap between the proximal and distal end this nerve conduit structures, So whether innervation is possible. And when you add this carbon nanofibers as expected it will produce homogeneous black colored solution. And you can see this is one of the work from one of my PhD students Sulob Roy Chowdhury who just is submitting the PhD thesis at IISC Bangalore. he has added different percentage of 0.

25% carbon to 0.75% carbon nanofiber in 3A5G that is 3% sodium alginate, 5% gelatin. And when you add this carbon nanofiber you can see this gel and if you do invert this gel you will see this gel is completely stable and solidifies. this is that gel and then once we start this microcomputer tomography again I would like to reiterate

here that microcomputer tomography use 70 to 80 kilo volt. X-ray beam, this is a high energy beam and why I am saying 70 to 80 because it is much larger than X-ray diffraction. X-ray diffraction people use typically 20 kilo volt X-ray beam.

here 70 to 80 kilo volt electron beam, X-ray beam that is through collimator that is passed on this is your 3D printed scaffolds. and this essentially this is rotated so that you get capital N number of large number of 2D ortho slices, these ortho slices they are stacked together. you get one ortho slices like this, another ortho slices like this and then they are stacked along the z-axis to get the complete 3-dimensional structure of these 3D printed scaffolds. You will see some of the examples here like this is for example this is the 3D volumetric porosity analysis for the synthesized hydrogel ink. these are 3D porous structures and this is the 3D porous structure and this is the 2D, this is the 2D orthoslices.

Now, you can do lot of different kind of quantitative approaches like for example 3D pore network analysis. essentially that allows in the Avizo computational software platform that allows how the pores are distributed in the 3-dimensional space. And these are the set of combination of parameters which are used for the micro CT scanning like what is the objectives, pixel size, exposure, power as well as the voltage as I said 80 kilo volt here. Now 2D orthoslices are acquired from different locations when these scaffolds were being scanned. And you can see this is a small video, it shows that how that asynthesized hydrogel biomaterial ink, They have very much fibrous kind of a structure and you can see this kind of macromolecular structure are oriented in different manner and you can see this is the small crystalline domain here.

These are like small crystalline domain which are essentially distributed randomly within these hydrogel scaffolds. there are 3 things that one can effectively quantify but there are more things one can do also but I am just showing these 3 aspects in relation to the 3D porous microarchitecture. One is a pore volume fraction. Second one is a pore diameter and third one is a pore tortuosity. pore volume fraction it provide adequate space in the scaffold for the diffusion of oxygen and nutrients and it provide desired mechanical support and regulate the vascularization.

Pore diameter is very important because it also allows the formation of blood vessels, so that is like vascularization. which is also promoted by pore tortuosity. pore tortuosity essentially means whether a single pores this actually is continuous through the structure or the single pores can go through a kind of a curved kind of a path, more the curved kind of a path that a pore follows that means more is the tortuosity. And this tortuosity values have been calculated using the standard formulation and then we got this tortuosity for all the different tissues. This is the natural tissues which are reported in this advanced healthcare materials this 2022 paper and you can see this tortuosity value somewhere varies between 1.

2 to 1.6. Pore volume fraction in all those tissues like vascular nerve and all it is somewhere around 0.6 to 0.8. And pore diameter it is varying between 5 to 20 or 30 to 200 or 100 to 600 micrometer. Now, this is like a 50 layer constructs, this particular structures you can see, this is like 50 layer and you can see through and through the pores can be seen.

For example, you can see this particular pores, you can see through and through this pores is continuous. this is like a very continuous cylindrical pore channel that are being developed and this one can take the different volume of interest VOI and find out that what is the pore size and pore interconnectivity. Here, pore tortuosity we found at 1.09 and pore volume fraction we found out 74% or 0.

74. If you go back to these particular things, 1.09 means 1.1. it is kind of in the lower range of this pore tortuosity value of the different tissues like nerve, skin, muscle, cartilage.

And pore volume fraction is around 0.79, that is 0.8, so which is like upper limit of all the different pore, of all the different natural tissues like skin, muscle, cartilage or liver. when we have done this particular in vivo study in small animals, what we have done first, we have shaved that here at the implanted site. We make an incision. On the incision sites in the subcutaneous level, we have put the implants, the scaffolds in black in color, which essentially means that these particular scaffolds contains carbon nanofiber.

2 dorsal sides diagonally opposite, 2 sides you know independently we have placed the implants. Then after 30 days you can see fully hair has been grown and then you can if you see this particular video when the animal has received this particular scaffolds they actually show normal healthy movement or regular movement. essentially full recovery of hair growth and wound closure that is the major message this from this particular slide. One of the things that we regularly monitor is the animal body weight and over the time after 0 to 30 days you can see then you know that at regular intervals when you have taken the animal body weight it shows a progressively linear increase in the animal body weight leading to almost like 450 gram animal body weight when you start from around 250 or 300 gram of the initial weight. this actually at the implanted site and if you look at this particular scaffolds here, it shows some of the signature of the vascularization.

This is a degraded scaffold. This is a carbon nanofiber based scaffold and you can see it is degraded in the expanded tissue and then you can see that in other expanded tissue, the degraded scaffold very clearly can be seen after 30 days of implantation. Now, like one of the scientific case study I have shown you before, as part of the scientific case study, we have also analyzed the serum biochemistry as well as different blood parameters and in the 7 days, this animal 1 and then on 30 days animal 3, for example total protein is 5.5, it is reduced, animal 1 to 3 because normal is 0 days is 5.7, 7 days it increased to 8.

2, so total protein is increased. Then, random blood sugar that is reduced, then serum creatinine that is increased initially at day 7. Some of these for example, SGPT, ALT that is also increased, but what you notice that after 7 days when it goes to 30 days, it essentially reduced. many of these blood parameters, for example, BUN value it was 15.7, then it is increased to 21.

5 bun, blood urea nitrogen. in the rat and then but it gets reduced again to 15.5 normal values. CPK values like 169.1 it is significantly reduced to 64.

1 but it recovers it goes to 132 and 229. at 7 days some of the serum biochemistry parameters are either elevated or reduced very significantly but then at 30 days it actually comes back to normalcy. what it means that? This all this degradation product from this scaffolds when this bio distributed then serum biochemistry parameters essentially indicate the animals again regain their normal physiological serum biochemistry parameters at the end of 30 days of the total implantation study. analysis of host response, you can see the histology analysis, 2 different type of staining agents we have used, one is the H&E staining and another one is the mason's trichrome staining. This is at 10 times and inset figure is 40 times.

this is like 40 times and this is at 10 times. And what you can see, blood vessels formation at different sites, it is very clearly see this blood vessels formation. And then also you can see multinucleated giant cells at different

location, multinucleated giant cells that also one can see. inflammatory response TNF alpha expression, tumor necrosis factor alpha. when you do the subcutaneous implantation, it is expected that this TNF alpha is secreted from the macrophages. Now, macrophages can be at the M1 level and M4 level and they get activated.

The receptor binding and signal transduction gene expression essentially shows how TNF alpha can activate downstream signaling processes and in some of the cases if certain proteins are activated, it can go to inflammation and survival or can essentially trigger the process of apoptosis, process of apoptosis is programmed cell death. what are the things that we are trying to understand? We are trying to see that what are the different type of inflammatory cells, This inflammatory cells when it will attack or when it will try to reside on this particular scaffold surfaces, we will see that how this number and distribution of inflammatory cells like neutrophils, monocytes, macrophages and fibroblasts, these kind of different type of inflammatory cells, these are actually they are distributed and what is the nature of inflammatory reactions. And second one, in case of fibrous tissue formations, then what is the indication of the degree of acceptance of the implanted material? Another staining that we use called VWF staining that is Von Willebrand factor. Von Willebrand factor actually plays a critical role in hemostasis and thrombosis process. Biologically, this can be described as a large multimeric glycoprotein that is synthesized and stored in vascular endothelial cells.

You can see more references which are mentioned here on VWF staining. And what is the important thing that for you to remember VWF, this will be small, is released when endothelial cells are activated by various agnoists. What is agonists? These chemicals or synthetic substances like hydrogen scaffold materials that activate receptors to produce a biological response. as part of this, we have done not only TNF alpha expression and you can see this inflammation is very significant at 15 days. But at 30 days, you can see this inflammation is subdued quite large extent.

VWF, you can see more and more expression in the VWF at this many of the indicated sites here at the end of 30 days of the implantation. this brings us to the end of this another scientific case study where I have shown how 3D extrusion printed scaffolds can be very safe for the animal health and their safety and efficacy has been demonstrated in specific animal models. I have shown you that how the entire study is conducted, how different serum biochemistry parameters as well as the immunohistochemical staining was performed in this particular case. these are the first steps that one has to establish that in vivo biocompatibility before one can do more clinically relevant studies in the higher animal models and so on. Thank you.