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

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Lecture 36

Scientific case study-3D extrusion bioprinting of GelMA hydrogels for hard tissue

In last few lectures, I have introduced you to 3D extrusion printing as one of the most promising additive manufacturing route to manufacture scaffolds of soft materials. in this lecture I will share with you one of the scientific case study on GelMa hydrogels for hard tissue applications. and the manufacturing route employed here is 3D extrusion bioprinting. You may recall the hydrogel by definition it is a macromolecular network structure of hydrophilic polymer with the ability to retain significant amount of water molecules this was the basic definition of hydrogels. GelMa by far is one of the most widely investigated hydrophilic polymers which are used for the hydrogel fabrication in academic setting or in the research laboratory. And hopefully you know the clinical translation of this GelMa hydrogels will be for patient care will be realized in a much much larger extent in the years to come.

the process science is very important and I have emphasized the process science for metal 3D printing. Now this slide you have seen it before but to recapitulate I thought that I will show and explain very very briefly this slide once again. your hydrogel is loaded here in this nozzle and it will experience pressure. when you use biomaterial ink, the biomaterial ink will not contain cells, but when you will use bio ink as you can see here, this is the bio ink formulation, when you will use bio ink that will contain cells.

one of the major challenges in the formulation of bio inks is that the cells which are contained in this particular soft hydrogel matrices. should not undergo cell death so that the cell viability is not compromised when encapsulated by hydrogels. this is the major things and therefore biomaterial ink formulation should have desired rheological properties in terms of viscosity, gel strength, shear thinning behavior and post-printing recovery like thixotropic properties and all. It should have good extrudability and this extrudability while it is extruded under pressure P that one has to have a very stable, long, filament structure and without any breakage, without any shape distortion of the filaments and this can constantly and consistently will be printed on a support structure. And then third one is the buildability, buildability is like you know how this structure can

be built with different infill design and also layer heights, line width, line spacing but most importantly I have mentioned multiple times and I am mentioning is again is the safe fidelity compliance.

that is very important safe fidelity compliance. And as I said that cell fate processes it is not only viability but also functionality of the cells when encapsulated within the hydrogel that is also equally important and that has to be assessed or analyzed at different time scale it is up to few weeks in culture and one of the major thing in the 3D bioprinting is the tissue maturation. And tissue maturation that length scale of which you need to do the cell culture or 3D cell culture of the 3D printed hydrogels that length scale can vary like you know it can go up to few weeks in culture. selection of biomaterials, so these are like bioprintable materials, biomaterials you have additive polymers like natural or synthetic and out of this natural synthetic you have a PEG, polyethylene glycol, PVA, polyvinyl alcohol, polycaprolactone, PCL, then this is you have PEG, then this is you have PVA. And then you have a polylactic polyglycolic acid copolymer PLGA.

Then you have polyurethane PU and there is pleuronic F127. These are like different synthetic polymers, which can be 3D bioprinted. Then from the natural polymers you can have a decellularized extracellular matrix that is DECM, you have polysaccharide based natural hydrogels, you have a protein based natural hydrogels and there you will have collagen, gelatin, silk, fibrin and gelatin and matrigel. In the polysaccharide case, you have cellulose, agarose and then I will show you also in one of the case study where nanocellulose particles are added to one of the hydrogel ink or one of the biometallic that is alginate hydrogel. Then you have gellan gum, these are like carrageenan base, chitosan, alginate, hyaluronic acid, so these are like some of the prominent polysaccharide based biopolymers.

Then, in the case of these other materials like in the mineral based inorganic fillers, you can have silicate based like bioactive glass, 45S5, nanoclay, mesoporous silica nanoparticles. Then you will have calcium phosphate based materials like hydroxyapatite, tricalcium phosphate and so on. In carbonaceous materials, these are like carbon nanofibers. graphene, oxide, carbon nanotube, this one is mineral based inorganic materials, these are like additives to the hydrogel bio ink which will impart specific properties to this biomaterial ink, Now, this side of this part, it is like used either as a matrix component or as viscosity modifier and so on. that you will see in one of the scientific case study under the broad theme of the 3D extrusion printing.

it should be cleared from this slide let me clear it off what I have written. It should be clear from this slide, in one side you have the major matrix components like collagen, gelatin, matrigel, silk fibroin. these are like protein based matrix component of the

hydrogel. to recap what I have. just discussed about the bioprintable biomaterials.

I will put a star here just to emphasize that this protein-based biomaterial ink is most commonly used like collagen, silk fibroin or gelatin and it is all natural derived materials. then decellularized extracellular matrix, what is decellularized? If you recall tissue is nothing but cell plus ECM, I mean it is not a mathematics but in a biological context tissue essentially combines the group of cells with similar functionality which are dispersed or which are embedded in an extracellular matrix. what is the meaning of decellularized matrix? Decellularized matrix essentially means by certain chemical or biochemical processes if you adapt to remove the cells from a tissue like there is no cell then what will remain from a tissue it is decellularized ECM. if you grow cells over time, you allow cells to synthesize extracellular matrix, then intelligently if you remove the cells from the medium, then you will be left with that decellularized extracellular matrix. Either way, you start with the tissue.

in you adapt biochemical process or you grow cells, you allow cells to synthesize extracellular matrix proteins then when it gets matured then you intelligently remove the cells to get only decellularized matrix. this is also another scaffolding material, then you have polysaccharide based materials, there is like nano cellulose, like chitosan, gellan gum, alginate and then some of them are also can be used as a matrix component of the hydrogel biomaterial these are the natural side and synthetic side as I said PEG, PVA, PLGA, PCL they are all used here and these part are essentially used for the additives like inorganic additives either carbonic acid fillers or calcium phosphates or silicate based like bioactive glasses and so on. as I said that gelma is perhaps the most widely investigated matrix component of the hydrogel ink. I should introduce you in more details about the overall GelMa synthesis. synthesis and chemical crosslinking of GelMa.

what I was talking to you that there are three ways that GelMa can be synthesized starting from gelatin. One is a conventional method, one is a sequential method and third one is a facile one pot method. Now what you remember from the earlier discussion is that in the gelatin you have NH2 amide side group. Now you want to treat them with methacrylic anhydride MA and as a result that you produce gelma and methacrylic acid. Now these all these synthesis technique if you see their reaction environment is different in terms of the conventional method people use phosphate buffer saline at 50 degrees Celsius 1 to 6 hours and in the sequential method researchers use carbonate bicarbonate buffer 0.

1 molar concentration 50 degrees Celsius for 3 hours and there is a sequential pH control and then temperature Then third one is that facile one per synthesis that pH adjustment and methacrylic anhydride addition that is carefully controlled and again carbonate

bicarbonate buffer is used. Now question is that why Gelma is so widely investigated? Now if you look at this backbone chain of the Gelma, in this backbone chain if you look at this red part that which has been zoomed here, it has the RGD motif. What is RGD? arginlyglycylasparic acid. this RGD is a very common peptide motif which is responsible for cell adhesion to ECM. Now what is ECM that I have already explained to you earlier, extracellular matrix.

because of the presence of RGD motif which is the 3 amino acids together. And this is a particular arginlyglycylasparic acid. this is a common peptide motif which is responsible for cell adhesion to extracellular matrix. Now there are three ways you can do this physical gelation. One is the physical gelation.

You can essentially treat them at temperature less than the gel formation temperature. Then you can use the chemical cross-linking essentially you can add that irgacure or some of the photo cross-linker expose them to UV. Then third one is the coordinated gelation and cross-linking, so essentially you give both or you can mention is the dual cross-linking like you can do both UV based linking as well as the physical gelation. So, the storage modulus that you will see that G prime when you measure the rheological properties of these materials, the storage modulus keeps on increasing when you have more and more crosslink density. certainly you can see if you go from here to here that molar crosslink density is expected to increase and what you see that is a kind of a cell.

this is a kind of fibroblast cells and fibroblast cells are attached to this gelma and this is also another type of cell. this cell adhesion and cell spreading what we are trying to show you here that also increases because of the larger storage modulus of the GelMa. Now key points to remember, so I think I have been discussing on this collagen and gelatin and GelMa for quite some time now. at this point it is instructive me to summarize some of the key points so that you can remember. gelatin is the denatured form of collagen that you should always remember and what is collagen? Collagen is the organic matrix of the natural bone.

Now, GelMa is essentially derivative of gelatin which has specific RGD motifs to attach biological cells that also I have explained. GelMa as a primary matrix constituent for hydrogels because of the better gelation property Gelma as a primary matrix has been most widely investigated for tissue engineering and 3D bioprint. Fourth one is that high concentration based GelMa as you will see later that has been more widely explored in the additive manufacturing community. In the additive manufacturing community and collagen is sourced from cartilages of fish, pig, pig means porcine or cow means bovine. Fish scale collagen or gelatin, fish scale collagen or gelatin is much less investigated in the scientific community.

Now if you look at that historical evolution of GelMa over last 2 decades, if you start with this 2000, so GelMa was first introduced as a photocross-linkable polymer. Then subsequently in 2010, this is the first used, so first used as the cell encapsulated GelMa microgel Then you can, then 2011, scientists have shown that GelMa can be also micropatterned of cell laden GelMa bioink. Then cartilaginous matrix bioprinting was reported in 2013, then goes on series. as you see that from 2010 onwards, The intensity of research on GelMa has also increased And if you see that more number of papers started coming like for bone tissue engineering, GelMa was shown to promote vascularization, then dental bioprinting was reported in 2015, then goes 2016 several papers like GelMa has been started, GelMa has been investigated for wide spectrum of artificial organs, for example, nerve tissue, cardiac tissue bioprinting, liver constructs, cancer and drug delivery, then GelMa started used for the microfluidics and stereolithography, skin bioprinting and so on. Then 2022, scientists have reported also for 3D bioprinting of liver constructs using GelMa.

2021, there is a paper for GelMa of the bioprinting of artificial ovaries. as you see that you know the scientific research on GelMa in different research groups around the world has really intensified over last 2 decades. for example that Gelma foam and hydroxyapatite, this combination was used for bone tissue engineering, I will give such an example. if you look the natural bone, natural bone has the outer cover that is the cortical bone, this is the harder part of the bone. Then you have a cancellous structure which is the inner part, this is spongy and porous and then you have the trabecula.

natural bone you have lamella and this lamella is made up of the collagen fibres. And if you go down to the structural details, so collagen fibre contains tropocollagen and hydroxyapatite crystals. Now hydroxyapatite can be synthesized by different methods. one of the methods which uses the nitrate precursor for example $Ca(No_3)_24H_2O$ this where calcium to phosphorus ratio is typically maintained at 1.67 for the stoichiometric HA, this is the autoclave based synthesis route.

this autoclave-based synthesis route, one of my former student, Soumitra during his Ph.D. at IISC, he has prepared and showed that how hydroxyapatite, needle shaped can be formed by these methods. Now, gelma synthesis as I have shown you before, you start with the PBS buffer 53 hours, the conventional synthesis technique that we follow in our group, then dialysis, deionized water for 7 days, then lyophilization, then gelma foam is prepared. Now degree of substitutions in the case of gelma is typically determined by NMR spectroscopy and NMR spectroscopy you have when the gelma is formed then you will get the signature of NMR bands H and Hb and Hy and Hx which normally you do not see in the gelatin.

now if you look at this particular thing, so it is the what is the lysine-methylene peak area in GelMa and what is the lysine-methylene peak area in-gelatin, that ratio that you have to deduct, subtract from 1, then you get this degree of substitution. As I said this autoclave based synthesis route which was pursued in my group and then they found that what is the morphological analysis, size distribution, anisotropy of gelma. Now what you can notice here that if the reaction time, so in this particular process we have changed the both the reaction time and then reaction temperature. reaction temperature at 200 degree Celsius you can see very nice nice needles and this needles aspect ratio C by A has been at least 8 to 10 and you can see here that what is the particle diameter. Particle diameter is roughly very small it is like nanoscale particle diameter like 30 to 40 nanometer.

phase pure anisotropic needle shaped hydroxyapatite, these were utilized as nanofillers in Gelma in regulating biophysical properties and printability. Now, what you see here that scaffolds buildability. first I will show that 15% GelMa which was used initially as part of Soumitra's PhD thesis. Now, if you see that 15% GelMa when it is being printed, you cannot have a good structure or structural fidelity or shape fidelity certainly is not maintained, Now, if you go to 15% GelMa with 1% hydroxyapatite, that nanoscale hydroxyapatite, you do see that structural resolution and structure is being built. And when you add this 3% hydroxyapatite to 15% GelMa and you can see that this particular structure is being built you know very nicely, you can essentially see very nice structure.

for the moment if you just stop it, if you stop this movie and just if you concentrate So, on this movie only, on this video only, you can see that how this 3% hydroxyapatite addition improves the buildability of this particular GelMa construct, Now, limitation of the high concentration GelMa , for example, people have reported that NIH3T3 cell viability is actually goes on reduction with higher percentage of the GelMa . If you look at embryonic stem cell proliferations like and if you look at this 3, 5 and 10% GelMa , so this cell viability also reduced particularly after 6 days of implantation. Encapsulated, when you encapsulate MSC mesenchymal stem cells and then you grow them day 2, day 4 and day 6, then day 6 also this particular cell number decreases. Now with GelMa , with larger concentration of GelMa , there are reports from a Chinese group in bioelectrochemistry, there also you see that it goes below 74% or below 80% at 15% GelMa . therefore, high concentration gelma, there are certain limitations and that was the reason that why we have started working on the lower concentration gelma.

a lower concentration GelMa is preferable for maintaining optimal encapsulated cellular functionalities. And besides higher matrix stiffness cross-linking density can potentially impose physical stress on embedded cells while reduced matrix porosity limit nutrient supply. you need to have a perfect balance between the matrix stiffness as well as the

porosity. if your matrix stiffness is higher and crosslinking density is higher then what would happen that can impose physical stresses on the embedded cells that I am going to discuss with you at some time point in this course. While reduced matrix porosity, suppose matrix porosity is reduced to improve the mechanical properties, then that will certainly reduce the nutrient supply and then also vascularization process will be impaired.

we have used in the low concentration GelMa . And low concentration GelMa we have used, but you have to modify the hydrogel composition, we have used carboxymethyl cellulose as one of the viscosity modifier. And if you see GelMa with carboxymethyl cellulose and you cannot use straightway carboxymethyl cellulose. For example, if you do GelMa solution, if you do physical gelation, then you get uncrosslinked GelMa gel, then if you do UV crosslinking. UV cross-linking in the presence of photoinitiator then you get UV cross-linked GelMa hydrogels.

Now there is other way. Suppose if you use the carboxymethyl cellulose but you modify so that it gets attached to this GelMa chain then if you add this GelMa CMC solution physical gelation almost less than 21 degree Celsius, then you get cross-linked GelMa-CMC hydrogel. As I said that we are concentrating on the low concentration of GelMa, that was a challenge that we took. The 5% GelMa concentration that you can see in this particular data. If you modify it with 0.5% carboxymethyl cellulose, 1% carboxymethyl cellulose, 2% carboxymethyl cellulose, then suddenly you see the degree of cross-linking almost linearly reduced.

degree of cross-linking goes down to almost like 60% at the 2% carboxymethyl cellulose modified or cross-linked GelMa hydrogel. there is also degree of crosslinking that is measured at PBS 37 degree Celsius. We have also measured degree of crosslinking at 2 days and then with different concentration. there are 3 approaches that we have used in this UV crosslinked modified carboxymethyl cellulose. what we have shown in this particular case when we have done modification of carboxymethyl cellulose and then we have used as a viscosity modifier to GelMa, the degree of cross linking almost remains same, it is like almost like 95% or so.

how this methacrylic modifications were done, so you take this carboxymethyl cellulose, Like you remember gelatin to GelMa, you added this methacrylate anhydride, Now you start with the carboxymethyl cellulose, you add this methacrylate anhydride and deionized water pH 8, 9 and in this ice conditions 0 degree Celsius, 48 hours, you essentially add this particular group to this chain. then you make this methacrylate carboxymethyl cellulose, now if you see that 5% GelMa with carboxymethyl cellulose without any modification to CMC, you get this shape is certainly not maintained, when

you add this 1% carboxymethyl cellulose, so you can see it is a good gel strength. Now you add the 2% modified carboxymethyl cellulose to 5% GelMa, it is perfect you can see the evidence as that what this methacrylic modifications to CMC can do to improve the good gel strength and good gel properties particularly in the case of this GelMa. And you can see the signatures of this methacrylation to carboxymethyl cellulose by NMR spectroscopy analysis. as you can see this particular technique is quite extensively used to find out the signatures of any chemical modifications in this macromolecular structure.

This is the FTIR, so FTIR essentially see that again you can see this particular area which is like OH- but you can see there are also signatures of this other IR bands which essentially confirm carboxymethylcellulose modifications is done. this is like more chemistry based approaches, so essentially what you see that when you start with this methacrylate modified carboxymethyl cellulose, then if you do UV cross linking, So, what would happen if you look at this particular bonds, you will see that this becomes more like a single bond, you get two modified carboxymethyl cellulose being bonded. Now, if you add that two UV cross linked. and then expose this gel to UV crosslinking then essentially mCMC is now added to this backbone chain this has been confirmed using chemical spectroscopic techniques as well. In the next class I will be essentially describing more on the properties as well as the cell compatibility of this particular gel mass system. Thank you.