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

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Lecture 37

3D extrusion (Bio)printing of GelMA hydrogels for hard tissue

Let me continue with the scientific case study on the 3D extrusion bioprinting of Gelma hydrogel and here I will be showing you that how we can use the viscosity modifier and then hydroxyapatite addition and also very carefully you know both these addition can be tailored to develop osteochondral tissue or either bone tissue or cartilage tissue like tissue mimicking structures. As you know this hydrogel is defined as a macromolecular network structure of hydrophilic polymers with the ability to retain the significant amount of water molecules, this was the hydrogel definitions that I have also defined it in the last class. now one of the things that I ended in the last lecture is that how one can develop this low concentration GelMa. Low concentration GelMa means this is 5% GelMa Low concentration GelMa based scaffolds by adding viscosity modifier, by adding inorganic filler like hydroxyapatite if you look at this particular slide in the top part, this is the uniaxial compression properties of the 5% GelMa based hybrid hydrogel scaffolds. your attention should be brought to this particular point that if you look at this Y scale is same up to 800 kilopascal of the stress and strain also is up to 80%, strain to failure. here if you see this is like 5G5P1mC Now these are like viscosity modifiers which has been added.

Now when you add this hydroxyapatite, so strength little bit reduced and then strain to failure it is now 50%, maximum strain. this particular scaffold has high strength of around 600 kilopascal. Now this comparison is much valid here and what you can see in this particular bottom plot, this is a lot of data is being plotted here. One is the strength, one is the modulus.

modulus is typically measured by the most linear part in the initial loading of this particular hydrogen scaffolds and then also elongation percentage strain, maximum strain. percentage strain wise there is not much difference. What you see typically the strength value remains moderate when you use that 5GxCyH strength increased little bit. 5G,5G1H, 5G0.5C very small increase.

And then also modulus increased little bit. But the substantial improvement what you can notice here 5% GelMa, now if you this 5% GelMa with 0.5% modified carboxymethyl cellulose, it increases from almost like 150 to roughly around 300, the strength. Elastic modulus also increased quite substantially to close to 50 or 60 kilopascal. Now on this particular baseline matrix once you start adding 1% hydroxyapatite and then you can see this 1% hydroxyapatite addition strength increased.

Now you change the matrix from 5% gelatin to 1% modified carboxymethyl cellulose and there if you add hydroxyapatite the strength further increase, elastic modulus also further increase. Now, this particular group of scaffolds, so suppose this is a group 1, this is group 2 and this is group 3, so group 2 scaffolds you can certainly appreciate that strength improvement is quite significant compared to that of the group 1 scaffolds. In the group 3 scaffolds, when you see that 5% gelatin, 1% modified carboxymethyl cellulose and 2% hydroxyapatite, so when 2% hydroxyapatite addition is there, then you can essentially achieve strength value of almost like 700 kilopascal. Modulus value close to 650 kilopascal, so this is like 700 kilopascal. and this is like almost like 650 kilo Pascal elastic modulus.

this is E and this is sigma C compressive strain, this is compression elastic modulus. Now so what I am trying to address here by Changing this hydrogel scaffold composition by carefully adding the inorganic fillers like hydroxyapatite, both the elastic stiffness and strength can be modulated And the use of modified carboxymethyl cellulose led to a better combination strength and modulus due to increase in the crosslinking density versus vis-a-vis that CMC. And PEGDA is used as the secondary crosslinker here for improving the scaffold stiffness. this was the designing of the hydrogel composition that was pursued and that has a very positive impact as far as the uniaxial compression properties is concerned. Now, biophysical properties, there is two biophysical properties that is very regularly analyzed in the hydrogel community.

One is the swelling properties, another one is that enzymatic biodegradation and enzymatic biodegradation typically people evaluate in the collagen solution. enzymatic biodegradation, so first one is the swelling. Now swelling typically people do almost up to more than a day or around 24 hours. at different time point the swelling ratio is calculated and swelling kinetics can be essentially analyzed by this specific equation where lnF is equal to ln log natural ratio of Q_t/Q_v is equal to lnk plus nlnt, at different time point, you know you can get the kinetic parameters by following the simple kinetic rule. as per the composition and hydrogel composition is concerned, this is the 5% gelatin, It can have swelling ratio as high as 1000%.

But with modification of the hydrogel composition is 5% Gelma, 5% Pegda, 1% carboxymethylcelulose, 1% hydroxyapatite, this been reduced to 550% or so on. baseline hydrogel that is the 5% GelMa, when you add this inorganic component and then secondary crosslinker and also viscosity modifier, then you know it is reduced to almost like half. I repeat. P stands for PEGDA. that is the secondary crosslinker.

Modified carboxymethyl cellulose is the viscosity modifier, And hydroxyapatite addition is essentially to improve the stiffness or strength properties because this is inorganic constituents. But all these constituents were tailored very very small amount in the 5% Gelma Hydrogel and this has a very positive impact. Now, enzymatic degradation. was carried out, this was assessed essentially up to 5 days like up to 120 hours in collagen solution and their kinetics is actually analyzed by this specific equation M_t by M_0 to the power 1 by 2 the square is equal to 1 - kdt. Kd is the enzymatic degradation constant and that has been also shown here as how this composition dependence in degradation kinetics can be evaluated.

You can see more values in terms of quantitative estimates of this n value that is swelling kinetics and then also lnk and then get the diffusivity. diffusivity is nothing but pi r^2, k by 4 to the power 1 by n and if you look at this diffusivity, it is also reduced by changing the hydrogel composition or by modifying the hydrogel composition. And particular experimental data when fitted with this particular equation lnf is equal to lnk + nlnt, it shows good statistical relevance in terms of the coefficient of variation like you know. Correlation coefficient is close to 0.

99. Now degradation kinetics wise in the first day degradation kinetics was essentially Kd X 10^{-2}. it also reduced with the hydrogen modification and 1 to 5 days there is a transition after 24 hours. what you can see again degradation kinetics is essentially reduced by Kd formula. by Kd parameter and it is 0.

27 to 0.08 once again this particular fitting of the experimental data with this degradation equations shows good R^2 value correlation coefficient. experimental results together with quantitative analysis clearly showed positive impact of modified carboxy methylcellulose addition as a viscosity modifier on biophysical properties, less swelling and degradation rate induced, rate indicated improved scaffold stability and then however decreased diffusion coefficient can have a negative impact on the cellular viability. Now correlation of the scaffold microstructure and biophysical properties, if you look at this particularly scanning electron microscopic images, SEM images of the hydrogels which are synthesized like you know 5% GelMa, 2, 5% GelMa, 5% Pegda, 1% modified carboxymethyl cellulose, 1% hydroxyapatite. area porosity that is the green one, you see that it is very drastically reduced. Whereas volumetric porosity, it goes almost like a linear decrease to around 30% in the scaffolds which also shows very good combination of strength and modulus.

and that must be related to volumetric porosity, if you look at this strength property, so it shows this kind of trend. If you look at this elastic modulus properties, it shows almost this kind of trend that you know that it is very close to 0 when volumetric porosity is very high, 3D porous architecture is particularly Qualitative and quantitative evaluation was very important because that allows you to correlate the microstructure or correlate the co-compressive strength modulus and diffusion rate degradation rate with the microstructure. And these effects of cross-linking density and inorganic nanofiller are strongly reflected in the scaffold morphology. Now, for all this composition 5% GelMa, 5% Pegda to 1% carboxymethyl cellulose to hydroxyapatite, we have done rheological analysis using the parallel plate rheometer. This parallel plate rheometer, their basic functioning has been covered in one of the earlier lecture when I have covered the fundamentals of rheology.

what you see here, both the G prime and G double prime. This shows through some transition and this is the 23 to 26 degree Celsius has been identified as the window for carrying out the 3D extrusion printing experiments. And if you clearly see that 5% gelatin the viscosity was very low, viscosity is improved with the addition of this carboxymethyl cellulose and hydroxyapatite and also there is no significant differences between among all the compositions and it shows qualitatively similar kind of trend with temperature. Shear thinning behavior was established. irrespective of the hydrogel composition and these particular values can be computed n value because it shows the similar kind of slope and when we have plotted eta versus shear rate gamma.

G prime and G double prime, G prime is the storage modulus, G double prime is a loss modulus, the ratio between G double prime to G prime will give you tan delta values, both the shear stress and then angle frequency sweep of the G prime and G double prime has been shown at the 3D extrusion printing temperature here and this is the different type of different composition scaffolds that we have done. When G prime and G double prime has been plotted with time let us say up to 30 minutes, half an hour, we wanted to see that at 21 degree Celsius whether this particular hydrogel remains stable in terms of their physical properties particularly storage modulus and loss modulus. Indeed you see that there is no variation with temperature. There is no significant variation and you can see this is the different group of data that has been shown like G prime, G double prime and this is like different group of data G prime and G double prime for different hydrogel matrices. Gelation temperature, viscoelastic yield stress can provide stable extrudability and improved buildability of low concentration of GelMa that is 5% GelMa Now when I have taught you about this process

science of the 3D extrusion printing, I have mentioned very clearly that filament extrudability is one of the main thing and then also filament strength.

filament extrudability and filament strength was carefully analyzed and then extrusion rate was essentially plotted against the extrusion pressure up to 2 bar, extrusion pressure. we have varied between 0.5 to 2 bar with 22g nozzle, plastic nozzle. And then what you see here that 5% gelatin, 5% Gelma, this is higher extrusion rate with but then this particular extrusion rate is lowered as you modify the hydrogel composition. Now when it goes to that extrusion rate microliter per second versus pressure and then 25 gauge this is plastic nozzle.

Here again you get different data and essentially this black one essentially is 5% GelMa, you can see there is a reduction in the extrusion rate. Now, when you use this 27 gauge, this is the plastic nozzle, then you can see this particular extrusion rate is subsequently lowered because this scale is here 300 microliter per second, here it is 200 microliter per second, it is 75 microliter per second. This filament fusion test or filament collapse test, whatever way you can call it, it is all the same. Now, when you see that 5% GelMa to this particular composition, 5% GelMa, 5% PEGDA, 1% modified carboxymethycellulose1% hydroxyapatite, filament strength is very good.

It is very stable. Whereas, there is sagging here without hydroxyapatite. And without PEGD there is also sagging and 5% GelMa does not pass through this filament collapse test at all. And this is the collapse area fraction with the distance you can see that you know for all the other composition it is close in the range of 80 to 90%. Extrudability and buildability, these are the two major things that drive the different biomaterial ink composition. If an extrudability was measured, then different sizes of the nozzles, both the plastic as metallic nozzles were used and then for all the composition.

Now if you see what is the optimum one, that it gives a very stable filament. with relatively good strength that is 5% gelatin with 1% carboxymethyl cellulose. When you add the hydroxyapatite even at this 22g plastic nozzle itself is good. Now when you add this hydroxyapatite with this 5% Gelma, 5% Pegda, 1% modified carboxymethyl cellulose and 1% hydroxyapatite. So, this is good, this is good, this is good.

essentially the selection of the nozzle size, selection of the nozzle type whether go for plastic or metal. That depends on what is the extrudability of the specific hydrogel composition, that is very clear. Printing resolution. Printing resolution wise this is that as printed scaffolds and you can see that what is the scale bar is 5 millimeter. And you see that printing resolution it is like 45 layers that is almost like 15 millimeter you can essentially build the construct.

And you can see structural buildability, you will agree with me that at printing speed of 10 millimeter per second at 21 degree Celsius, print bed temperature and print head temperature with 45 layers, this is the best. where this is no good, it is a 5% GelMa alone and this is 5% GelMa, 5% Pegda, 1% methyl carboxymethyl cellulose and 1% hydroxyapatite, this is the scaffold. when we carefully measure all these printability determining properties and subsequently when you print these materials it essentially correlates perfectly. that is the point that I wanted to make here that science is important because without understanding the science you cannot develop the technology particularly in the 3D printing and bioprinting arena. major message here, buildability of low concentration of gelma can be significantly improved.

It is not only simply improved, it is significantly improved by viscosity modification and without in situ curing,

the large volumetric 3D scaffolds were printed for the first time using the low concentration of gelma without collapse. this is the major message from these results. Now you can see that how this 5% gelatin, 1% carboxymethyl cellulose and 1% hydroxyapatite is printed to make this construct 45 layers long. And then when you have cross-linked it, you can see good printing resolution also, Whereas 5% GelMa, you do not see any safe fidelity it gives you direct evidences like hydrogel modifications essentially allows you to manufacture safe fidelity compliant constructs in this low concentration GelMa system. three types of cell culture test we have done.

essentially we have used the biomaterial inks on a cover slip and then after that we have done UV curing, then we have grown the cells and then check it at day 3 to day 21 using and after the cell culture is over we have used WST-1 live/dead assay and fluorescence microscopy. The second set of experiments, this is experiment 1, this is second set of experiment, this is the third set of experiments, second set of experiments we encapsulate the cells in the GelMa that you know different compositional GelMa and that one that you put it on a cover slip then you do UV curing and then on that we grow cells then as user live/dead and histochemistry was done. Third one is a bioprinting like these encapsulated gel were extrusion printed and then it was cultured and then we have done live dead staining for D_0 to D_7. interestingly, when human mesenchymal stem cells were grown on these 2D scaffolds, what you see day 3 to day 7 to day 10, you do notice there is a morphological changes, if you look at to this. And if you see that there is a very nice orientational changes, it is like vortex like formation of the cells that you can notice here.

But that is noticed only on the 5% Gelma, 5% Gelma,1% modified carboxymethyl cellulose. Now, when you go to other compositions, then you can see that there is a kind of random orientations of the cells on these other compositions, when you see this very blue kind of nucleuses, this DAPI strain nucleuses and then you see the phalloidin strain cytoskeleton. green is all cytoskeleton, blue dots are all DAPI strain nucleus in the fluorescence microscope. clearly when you do this encapsulated HMSCs, if you go to these particular things encapsulated HMSCs and then you grow them for different days from day 3 to day 14, this is a live dead stain images, you do see there is a red dots here that means cells are not alive. it is as I said that in 3D bioprinting it is always a challenge.

to maintain the self-viability and that was also largely related to the scaffold stiffness and lower diffusion coefficient. In terms of matrix mineralization, in the 2 weeks in culture, we have done alkaline phosphatase and also Alcian Blue for the GAG. You do notice for specific compositions this ALP expression is much higher particularly when you add this hydroxyapatite into this scaffold. Whereas, for other compositions you do notice that without hydroxyapatite that GAG expression and collagen expression is particularly better, expression specific predict the formation of tissue specific extracellular matrix. scaffold stiffness hydroxyapatite play critical roles in developing cartilage and bone specific matrix.

Now, when we have done the 3D bioprinted scaffolds which is HMSE, so essentially HMSEs are incorporated in the scaffold before 3D extrusion experiments and then they were essentially printed and then cell viability was assessed immediately after printing after day 3 and day 7 of this printing and you do notice there is a large number of green fluorescently tagged cells are available here. The shear thinning behavior of bio ink essentially allowed uncompromised cell viability. So this entire work was published in ACS Applied Biomaterials and this was the work done by Soumitra who was that time PhD student currently researcher at Tata Steel Medical Materials and then two other project assistant, one is Remya contributed to this work and Praneeth was a visiting student from University of Colombo, Sri Lanka who also contributed to this work. Thank you.