# Lab Report



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## 1. Preparation of the Chitosan (CS) solutions

**Preparation of CS HA solution:**

The incoming procedure was followed to prepare the CS/HA solution in order to get the hydrogel. A CS solution and a β-Glycerol Phosphate (β-GP) + Hyaluronic Acid (HA) solution are prepared separately and mixed at the end of the procedure.
For the CS solution, a 3,75% w/v solution of chitosan is prepared. We are using chitosan 95/100 where 95 is the degree of deacetylation and 100 the molecular weight. Both factors are demonstrated to affect the physiochemical and biological properties (1) and it is been demonstrated to be the best option for us based on pH measurements, degradation tests, rheology tests and printing characteristic.

We dissolve 3.75 g of the cited chitosan in 100mL of a 0.2 M HCl solution. As we do not have a 0.2 M HCl solution, we prepare it by mixing 98.34 mL of ultrapure water and 1.66 mL of a 37%w/w $HCl_{aq}$ solution. Once the chitosan powder is added to the HCl solution, the mixture is stirred for 48 hours at environmental temperature at 80 RPM until a homogeneous solution is obtained. The resulting solution is then stored in the fridge at ~5 $^{o}$C.

For the β-GP + Hyaluronic Acid solution, DMEM, which is a cell culture medium, was used as a solvent instead of water in order to favor the living and growth of the cells that the hydrogel is designed to encapsulate.

The incoming procedure was followed. 1ml of media is added to 1104.5 mg of β-GP powder and then mixed for 5 minutes with the help of a magnetic stirrer. When the solution is homogeneous, media is added until we reach an amount of 2.2 ml. As we want to obtain a 1% w/v solution of hyaluronic acid in the final solution and we are going to use 5 ml of the CS solution, the amount of hyaluronic acid will be 72 mg. The hyaluronic acid will be added very slowly to the β-GP solution while it is mixing in the magnetic stirrer so that it does not agglutinate and can be dissolved more easily. Once all the powder is added to the solution, we leave it in the magnetic stirrer for 20 minutes.

Main problem found during the preparation of the β-GP + Hyaluronic Acid solution were the appearance of bubbles when mixing the powder on the media with the help of a Vortex. This was solved by using a magnetic stirrer instead of a vortex.

The final step is to mix both solutions to obtain the CS HA solution. We put the bijou containing the chitosan solution in an ice bath in order to maintain the temperature between 4$^{o}$C and 6$^{o}$C while it is mixing in the magnetic stirrer. Then, drop by drop, we

add the β-GP + HA solution to it very slowly in order to avoid gelation. Once all the solution is added, we leave it mixing in the stirrer for 15 minutes.

**Preparation of the CS HA+DA solution**

The incoming procedure was followed to prepare the CS HA + DA solution in order to get the hydrogel. A HA (Dopa) powder is synthesized first and then a CS solution and a β-GP + HA (Dopa) solution are prepared separately and then mixed at the end of the procedure.

Before preparing the hydrogel, the HA + DA powder that will be added to the β-GP solution needs to be synthesized by carbodiimide coupling chemistry. First of all, a pH 5.5 MES buffer solution is prepared. In order to do so, we prepare 250 mL of a 0.1 M KHP (Potassium Hydrogen Phthalate) solution by mixing 5.1 g of KHP and water until reaching a volume of 250 mL and then we mixed the solution in the magnetic stirrer until a homogeneous solution is achieved. Then we add 183 mL of a 0.1 M NaOH solution. As we only have a 1M NaOH solution, we prepare it by mixing 18.3 mL of 1M NaOH solution and 164.7 mL of ultrapure water and stirring it for 5 minutes. Finally, we put the solution in the magnetic stirrer for a few minutes to obtain an homogeneous buffer solution.

Once the buffer solution is prepared, we continue with the synthesis of the HA + DA powder. 500 mg of HA are fully dissolved in 50 mL of the buffer solution and then 288 mg of EDC and 173 mg of NHS are gradually added into the HA solution with a 15 minutes interval after the addition of each substance for mixing in the magnetic stirrer. Then, 95.7 mg of 2-(3,4-Dihydroxyphenyl)ethylamina (Dopa or DA) are added, the recipient is covered with a holed aluminum foil and the reaction mixtures are stirred at room temperature for at least 4 hours. The solution is purified by dialysis for 3 days against the buffer solution prepared previously to inhibit oxidation of catechol groups and then 1 day against neutral water for pH adjustment. The solution is lyophilized and a white cotton-look like mass is obtained.

A β-GP + HA (Dopa) solution is prepared following a similar procedure as the one described in the preparation of CS HA solution section. We start by preparing the 2.2 mL β-GP solution by dissolving 1104.5mg of β-GP in media. We put it in the magnetic stirrer and while the solution is mixing, we weight 72 mg of the HA (Dopa) cotton we synthesized and we slowly add small pieces of it to the solution. When all the HA (Dopa) is added, we leave the mixture stirring for an hour until a homogeneous solution is reached.

The final step is to add the β-GP + HA (Dopa) solution to the CS solution following the same procedure as the previous section. We put the CS solution in an ice bath, we add the β-GP + HA (Dopa) solution drop by drop and we leave it mixing for about 15 minutes or until we clearly see a homogeneous solution.

## 2. Characterization of hydrogels

### FTIR Analysis

An FTIR analysis has been made on the Dopamine-conjugated Hyaluronic Acid in order to check if we have synthesized it successfully.

### Gelation time

Gelation time is defined as the time it takes the solution to become a hydrogel at a certain temperature. Test Tube Inversion Method was followed in order to characterize it by measuring the time at which a sample of the solution does not flow anymore at 37$^{o}$C.

The incoming procedure was followed. A sample of 0.5 mL of the solution is introduced in a 1.5 mL Eppendorf and this, placed inside the incubator at 37$^{o}$C. We wait 2 minutes and then we check if the solution has gelled by turning the Eppendorf upside down. If the solution still flows, we introduce the sample in the incubator again and every 30 seconds we repeat the procedure to check if the solution flows. Once it doesn't, we consider the solution has gelled and we take the total time as the gelation time.

### pH measurement

As the resulting gel must encapsulate NPs and MSCs, the pH of the solution before and after gelling must be adequate for cell viability. This pH is set in a range from 7 to 7.4.
In order to characterize the pH values, two measurements were taken on each sample, before and after becoming a gel. These measurements were taken on solutions CS Beta GP+HA and CS Beta GP+HA (Dopa) to check the cell viability of each of them.
The incoming procedure was followed. The first pH measurement was taken on each solution right after being prepared and in a liquid state with a pH-meter and a glass electrode. The glass electrode was placed so the tip of it was in contact with the solution and we waited until the pH value in the pH-meter was stable. Then a second pH value was measured after the solution was introduced in the incubator at 37$^{o}$C and turned into gel.

### Degradation test

A degradation test was realized to have an approximation of the time needed for the hydrogels to degrade and release the cells encapsulated in it in an environment similar to the human body. To do that, we use samples of the hydrogels and we leave them in contact with media in order to simulate an in-body environment. We set three

different periods of time for degradation, 18, 30 and 55 days and we used three different samples for each period.

We name the tubes first with the number of degradation days and then A, B or C for each of the tubes with the same number of degradation days, so we have 18A, 18B, 18C, 30A, 30B… In order to do the test, we followed the incoming procedure.

In each tube, we put a sample of approximately 400 mg, we weight it, we close the tubes and we introduce them in the incubator at 37ºC. When the solutions are turned into hydrogels, we take them outside the incubator and we add 0.5 mL of media in each tube. Then we close and seal the tubes, so the media doesn't evaporate, put all the tubes with the hydrogel and the media back into the incubator and we wait the correspondent number of days to take them out.

Once the degradation time has passed, we take the samples out of the incubator, we take out the media with the help of a micropipette and we weight them. Then we put the samples in the freeze dryer and, again, we wait for two days and weight the dried resultant sample.

| | CS + HA | CS + HA (Dopa) |
|---|---|---|
| t= 18 | 3 tubes | 3 tubes |
| t= 30 | 3 tubes | 3 tubes |
| t= 55 | 3 tubes | 3 tubes |
| | **9 tubes** | **9 tubes** |

Degradation test couldn't be performed on the CS + HA (Dopa) samples with the higher values of time due to the lack of time to finish the Thesis but first results can be compared with the results in the CS + HA test for the values of 15 and 21 days of degradation.

**Rheological measurements**

Rheological measurements were taken on the different solutions in order to determine the mechanical properties related to their flow behavior and compare them with each other. The following tests were performed on a CS solution, a CS + HA solution and a CS + HA(Dopa) solution with the rheometer.

Three parameters are controlled in any given single test: frequency of oscillation, amplitude of oscillation, and test temperature. A typical test, like the following ones performed in our samples, holds two of these parameters constant while varying the third.

Strain sweep test

All oscillatory experiments have to be carried out at small strain or stress amplitude in order to remain within the so-called linear viscoelastic region. Inside this region, which is limited by the critical stress (or strain), the material's structure is in equilibrium and the relation between the applied stress and the measured quantities is linear; this means they are only a function of time or frequency at constant temperature.

In hydrogels, G' and G'' are approximately linear. Gels show this behavior as long as the structure is undisturbed, so we can identify this point on the end of the linear region where a decrease in viscosity or elastic modulus (G'' and G' respectively) and an increase of the phase shift happens.

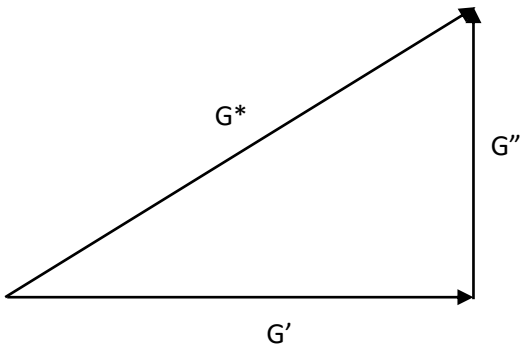
A shear strain test is usually the first test carried out on an unknown material. All subsequent tests have to be carried out at strains below the critical strain value in order to stay in the viscoelastic behavior.

For this test, we set the temperature at 37$^{o}$C and wait 30 minutes to ensure the gelation of the solution that we are analyzing and then we maintain the temperature for the rest of the test. We also set while shear strain increases progressively.

Ramp Sweep Test

This test measures viscosity against temperature increasing at a certain rate. This allows us to know at which temperature the solution turns into gel by identifying the abrupt change of viscosity values in the results.

We are going to use complex viscosity which is the frequency-dependent viscosity function determined during forced harmonic oscillation of shear stress and the value given by the rheometer. Complex viscosity is defined as $|\eta^*| = |G^*| / \omega$ where $G^*$ is the complex shear modulus and $\omega$ the angular frequency. The complex shear modulus is the ratio of the shear stress to the shear strain. It follows from the complex relationship $G^* = G' + iG''$



Frequency sweep test

A frequency sweep test has been performed on both hydrogels in order to establish the frequency dependence of a material. In order to do it, we have measured G' and G" against an increase of the oscillatory frequency (rad/s) from 0 to 100.

Time sweep test

The Time Sweep Test was done in order to know at which exact time the solution becomes a hydrogel. This value is known when G' and G'' cross each other. The temperature was set at 37oC to simulate the in-body environment.

Temperature sweep test

The Time Sweep Test was done in order to know at which exact time the solution becomes a hydrogel. This value is known when G' and G'' cross each other. For this test, we set the range of temperatures from 0$^{o}$C to 45$^{o}$C increasing at a rate of 1.5$^{o}$C/min and we set the continuous rotational frequency at 1Hz.

# 3. Results

## 3.1. FTIR analysis

The following results correspond to the FTIR analysis of the Dopamine-conjugated Hyaluronic Acid. We can see a clear peek in three of the four samples around the 1630 $cm^{-1}$ which correspond to the amide bond between HA and DA. So we can affirm that the HA-DA has been synthesized successfully.

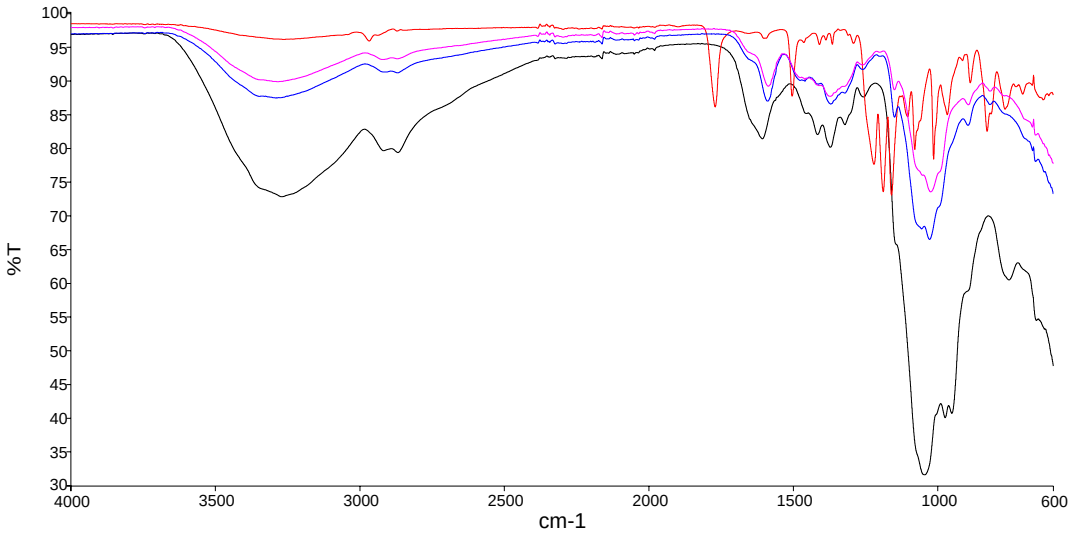



*Figure 1. FTIR results of samples A, B, C and D*

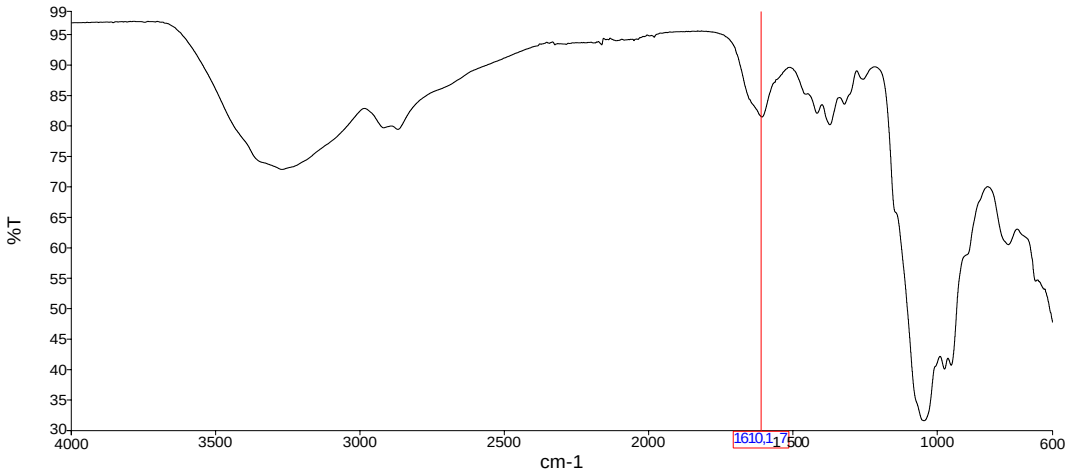



*Figure 2. FTIR result of sample A*

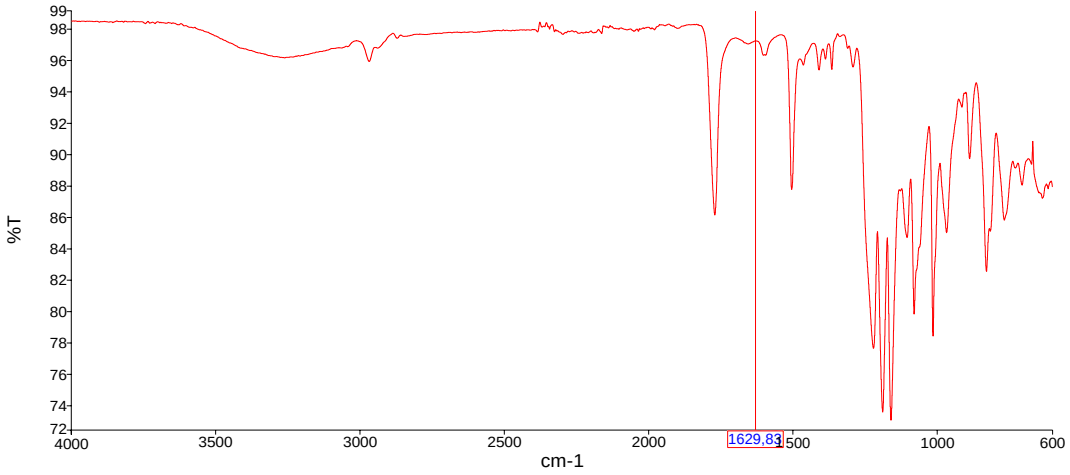



*Figure 3. FTIR result of sample B*

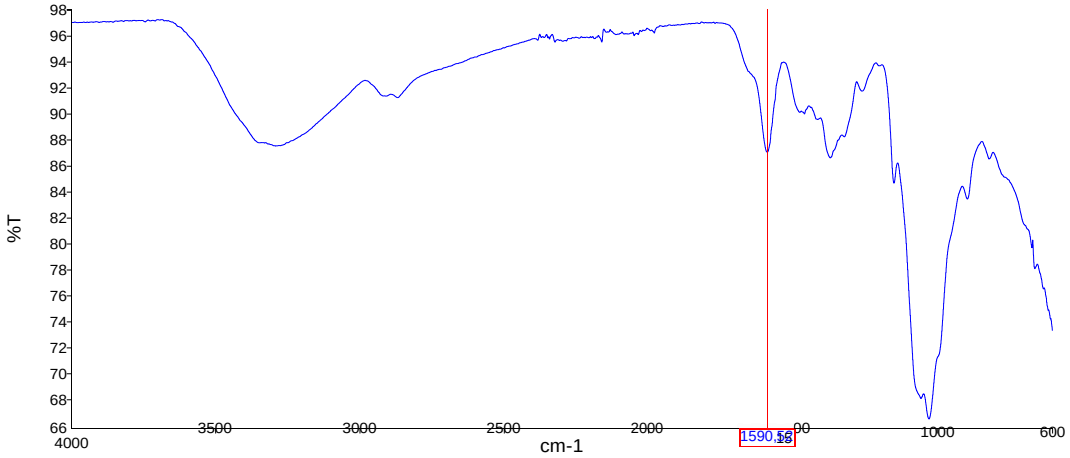



*Figure 4. FTIR result of sample C*

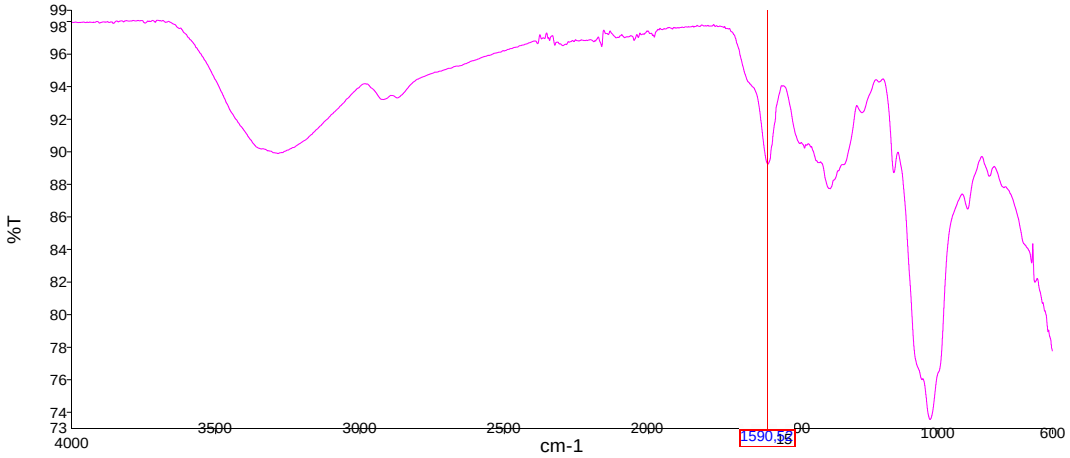



*Figure 5. FTIR result of sample D*

### 3.2. Gelation time and pH measurements

| | CS + HA | CS + HA (Dopa) |
|---|---|---|
| pH before gelation | 7.07 | 7.05 |
| pH after gelation | 6.9 | 6.91 |
| Gelation time | 5’ 00” | 8’ 30” |

### 3.3. Rheology tests

Strain sweep test

Figure 6 shows values of storage modulus (G’) and loss modulus (G”) at different values of strain.

We can see that both hydrogels, CS HA and CS HA(Dopa), have almost the same values of G’ and G” at the different values of strain. We can see that the values of strain where G’ and G” are linear and therefore the hydrogels are in the viscoelastic region which goes from 0,01 to 10.

Taking all of this into account, an intermediate value of 1% strain was set to perform the other tests.

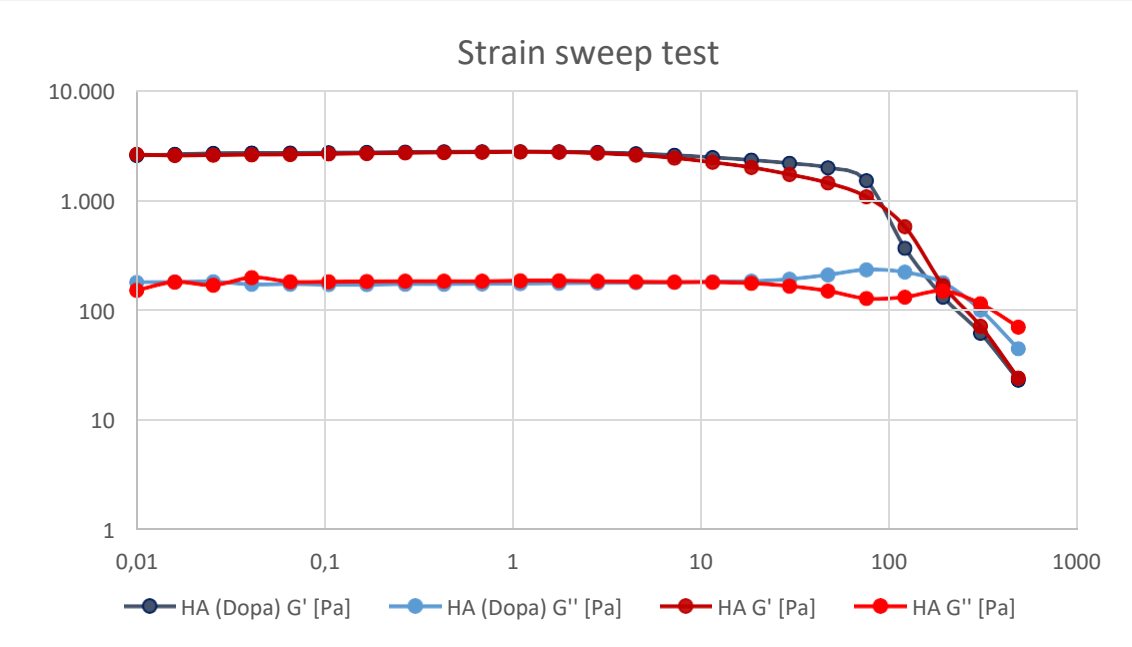



*Figure 6. Strain sweep test for CS HA and CS HA-DA*

Frequency sweep test

Figures 7 and 8 show values of storage modulus (G') and loss modulus (G") at different values of oscillation frequency (rad/s) at 37 ºC.

Typical for gels is that the storage and loss modulus are approximately parallel. Gels show this behavior as long as the structure is undisturbed. We can see that our both hydrogels present this behavior.

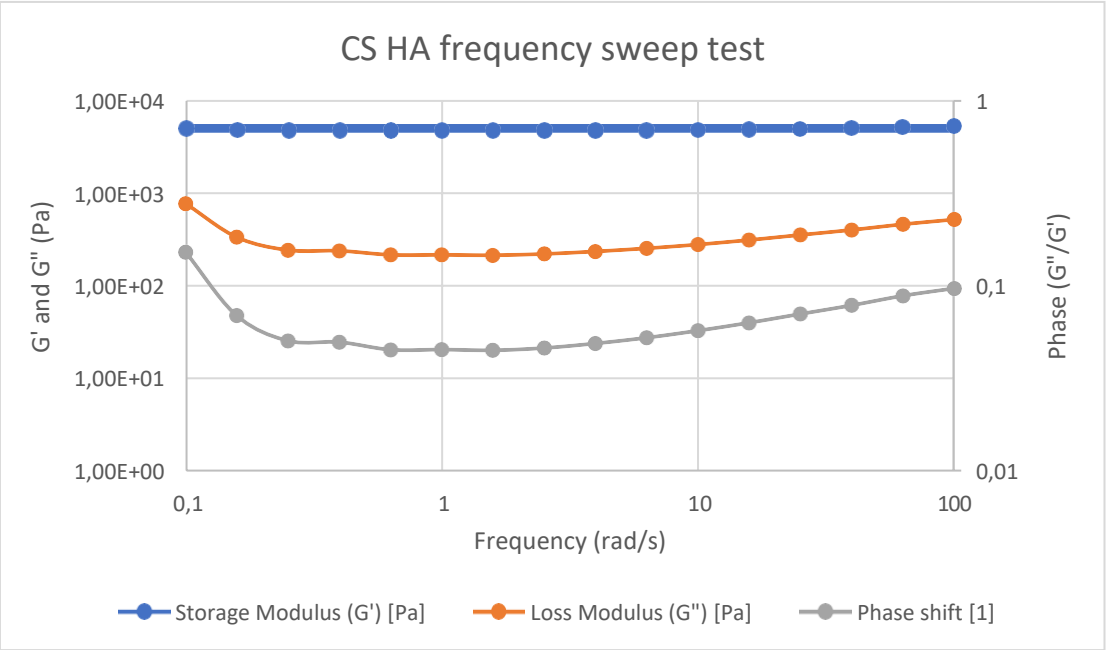



*Figure 7. CS HA frequency sweep test*

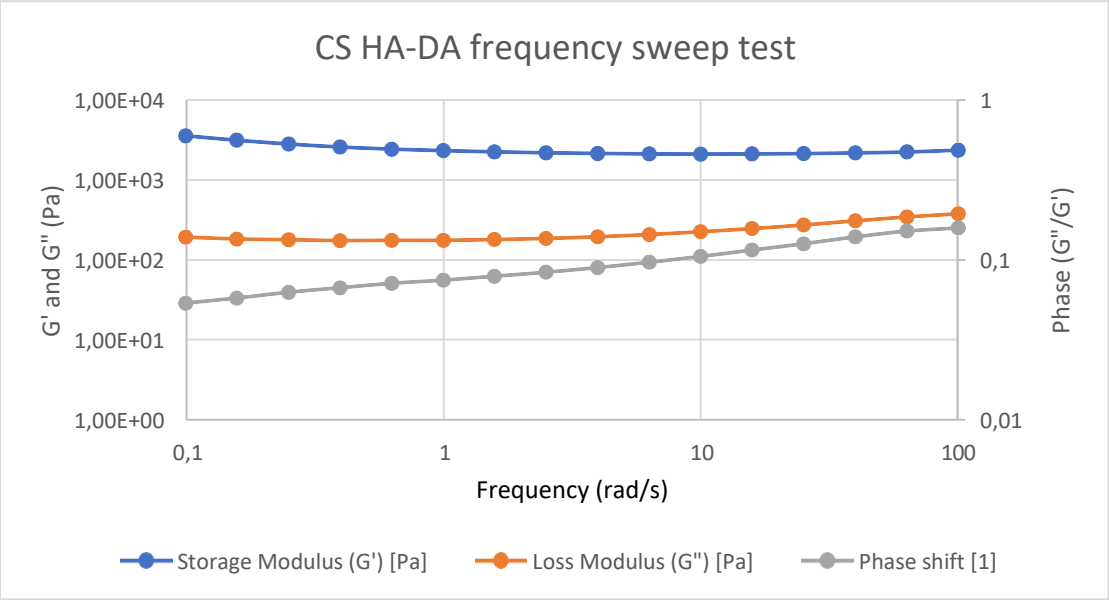



*Figure 8. CS HA-DA frequency sweep test*

It's remarkable that neither G' or G" are significantly affected by the variation of oscillatory frequency and that the phase shift is nearly constant during all the test. This means that the hydrogel maintains its mechanical properties and remains more elastic than viscous independently of the frequency value.

The following graphs show the values of complex viscosity at different frequencies. As we defined previously, complex viscosity is $|\eta^*| = |G^*| / \omega$ where $|G^*|$ is the complex shear modulus $|G^*| = \sqrt{(G'^2 + G''^2)}$. G' and G" are approximately linear and that's why $|\eta^*|$ is inversely proportional to ω. Both hydrogels present parallel and similar values of viscosity.

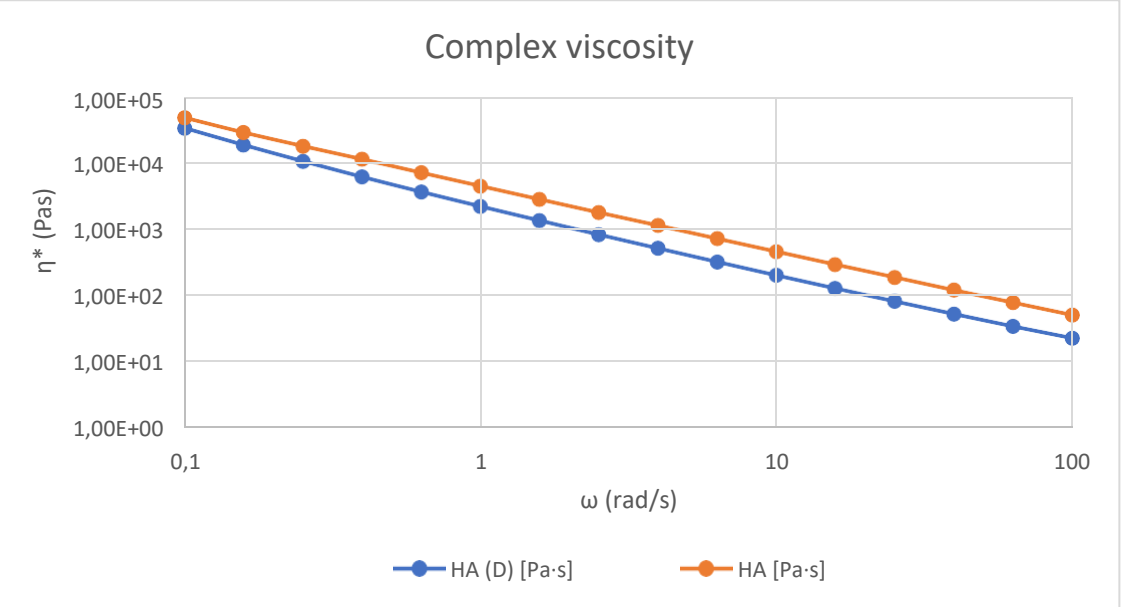



*Figure 9. Complex viscosity against oscillation frequency*

Time sweep test

In the following figures, values of Storage Modulus (G') and Loss Modulus (G'') depending on time at constant rotational oscillation and temperature are shown. The gelation time was determined at the crossing over of storage moduli (G') and loss moduli (G") following the Winter-Chambon power law.

We can observe from the results that the gelation point is quite similar in both samples so the presence or absence of dopamine doesn't make a big difference. In the CS HA solution, the gelation happens after 5' 47'' at 37ºC while in the CS HA(Dopa) solution, happens after 5'37". We should see if this difference is relevant at the moment of printing.

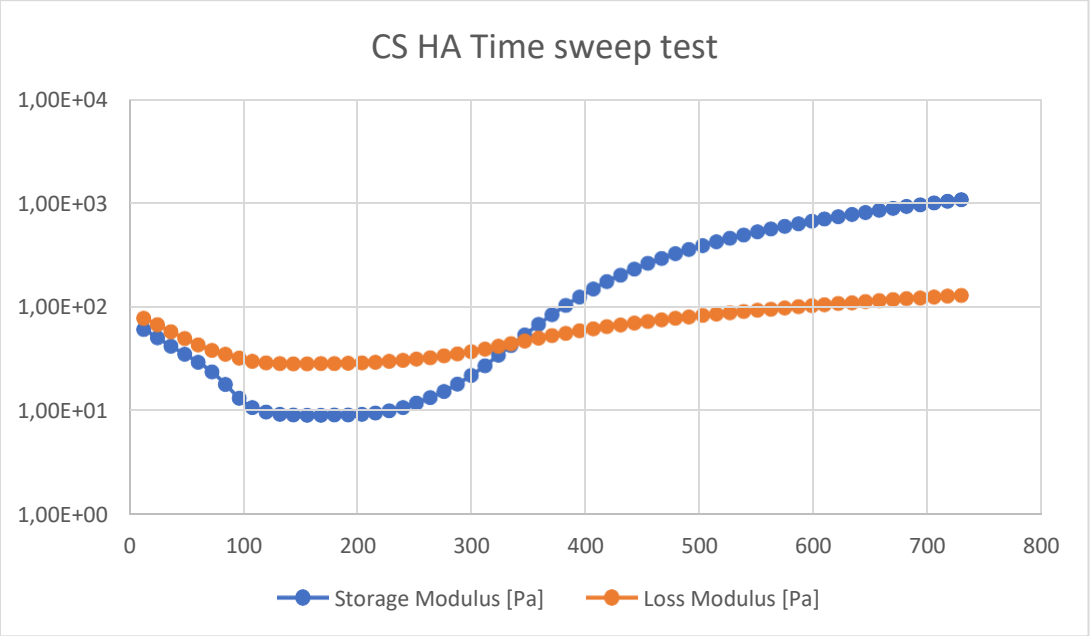



*Figure 10. CS HA Time sweep test*

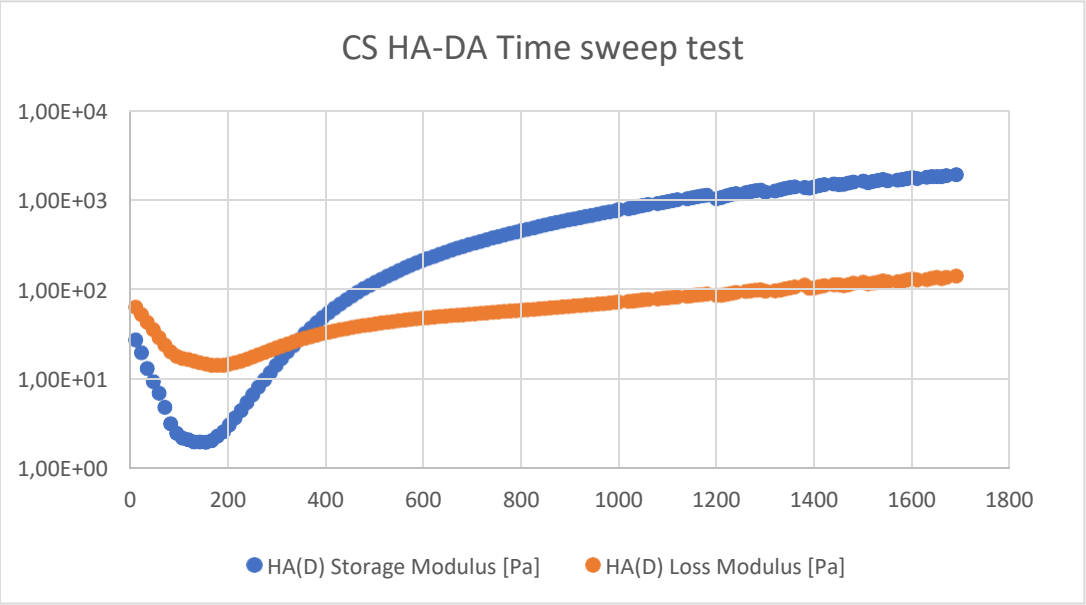



*Figure 11. CS HA-DA Time sweep test*

Temperature sweep test

In the following figure, values of Storage Modulus (G') and Loss Modulus (G'') while temperature increases are shown for CS HA-DA. We can set the gelation temperature when both lines cross, which corresponds to a temperature of 33.4 ºC.

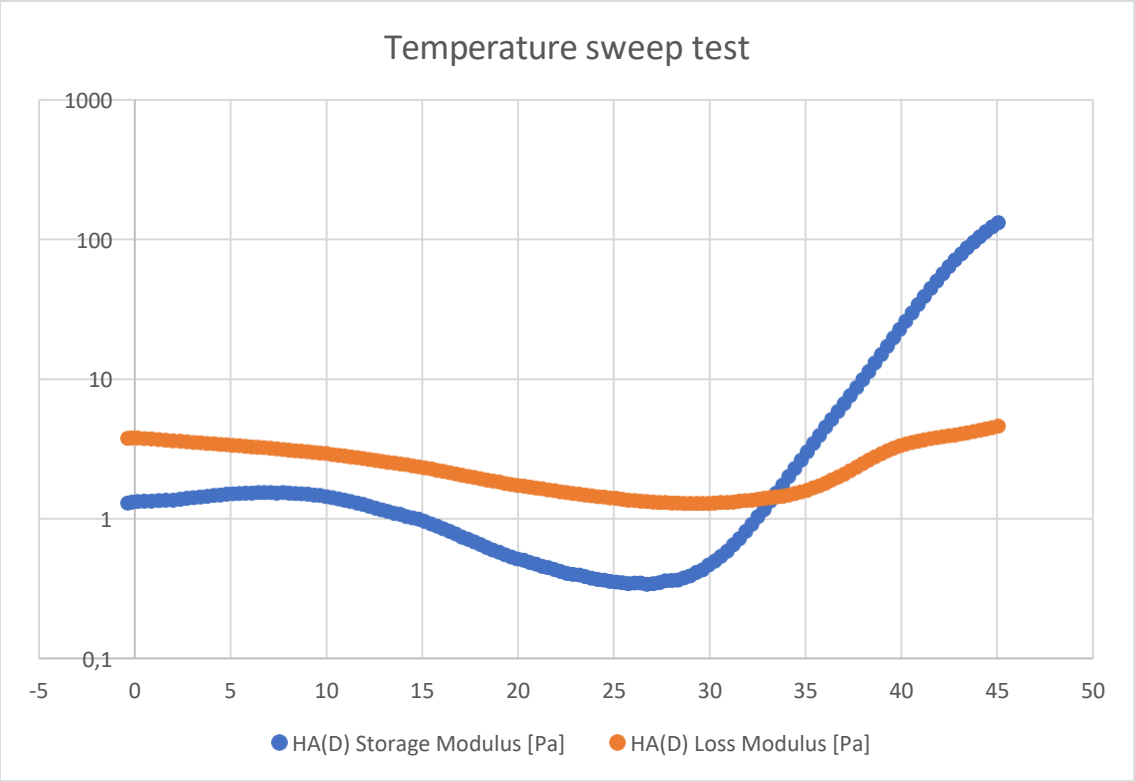



*Figure 12. Temperature sweep test*

### 3.4. Degradation time test

The following diagrams show the results of degradation test on 1mL of CS HA and CS HA-DA samples in contact with 1 mL of DMEM. More detailed results of the tests are included in the attached Excel.

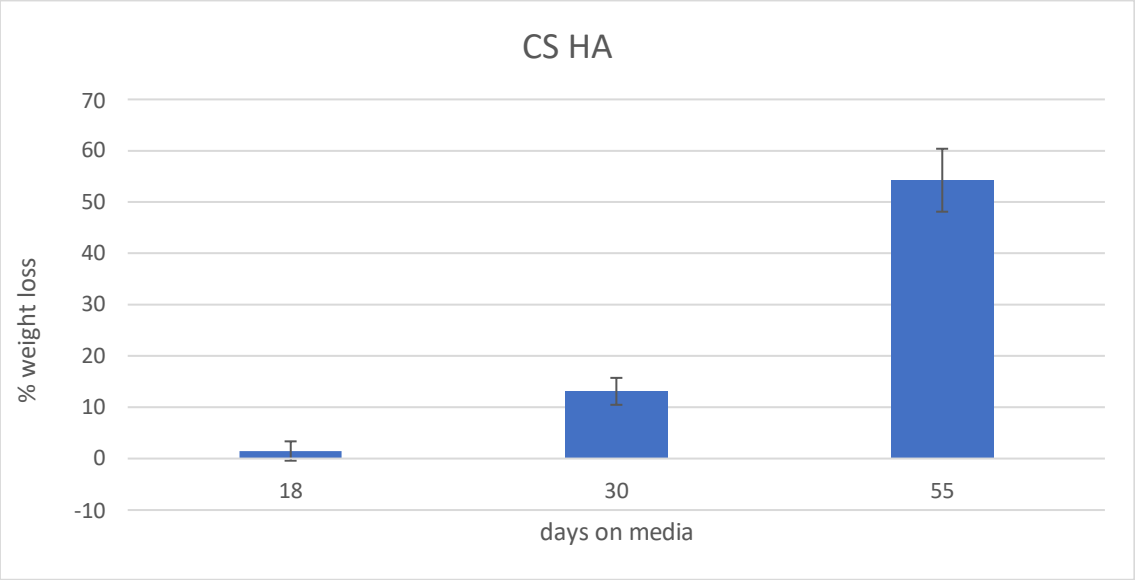



*Figure 13. CS HA % weight loss on different periods in contact with DMEM*

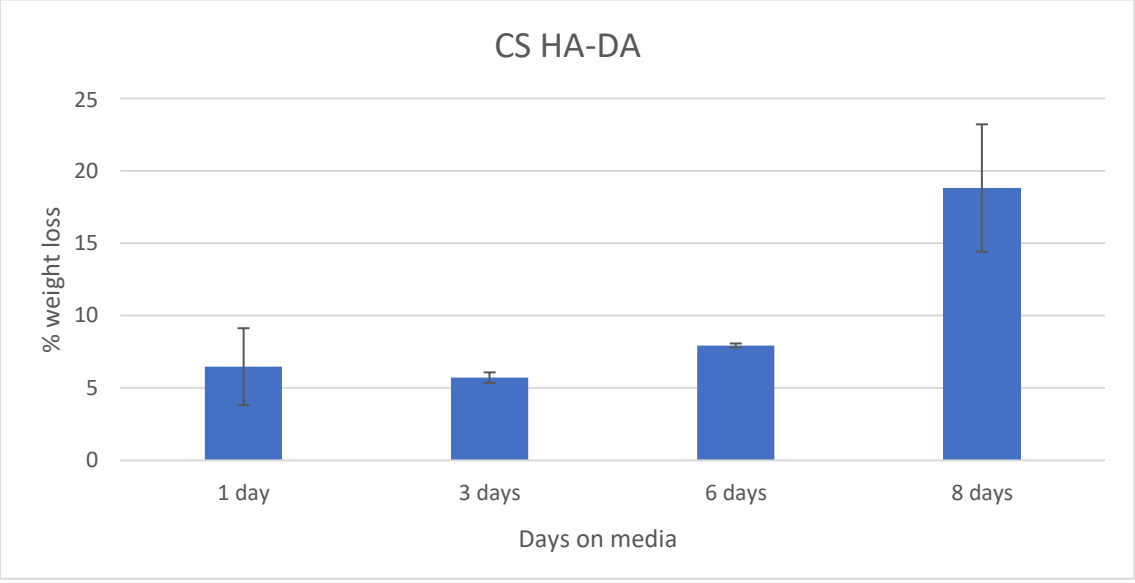



*Figure 14. CS HA-DA % weight loss on different periods in contact with DMEM*

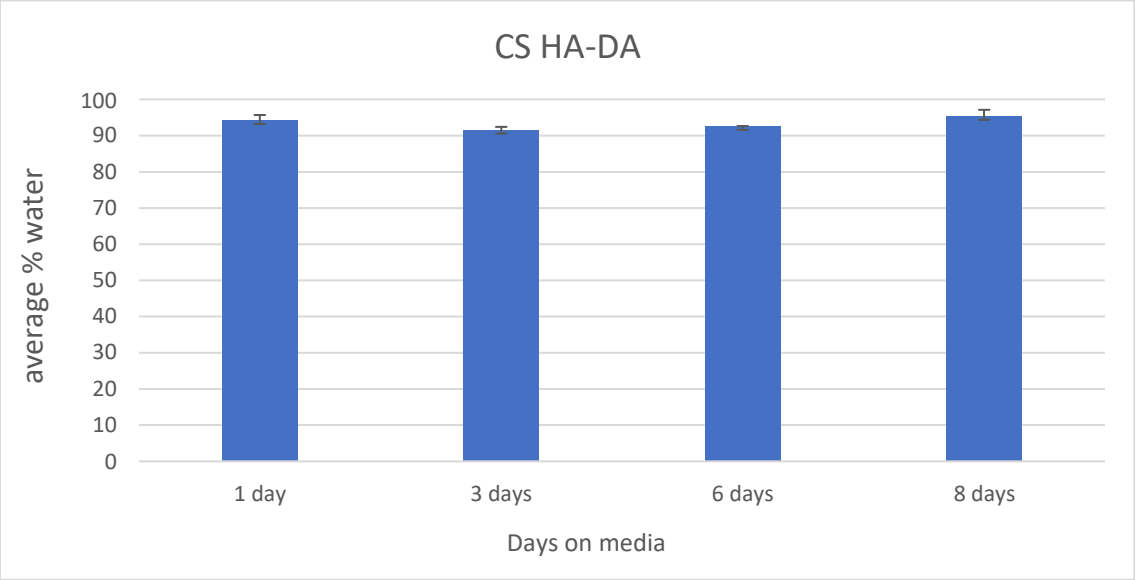



*Figure 15. . CS HA-DA % water on different periods in contact with DMEM*