MECHANICAL AND BIOLOGICAL PROPERTIES OF CHITOSAN/PURECOL COLLAGEN HYDROGELS

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SUMMARY

Combinations of chitosan and collagen gels were prepared to 1) improve the mechanical properties of Advanced Biomatrix collagen gels and/or 2) improve the cell-matrix interactions in Lerouge's chitosan thermosensitive hydrogels. The aim of this study was to evaluate the mechanical properties and the cytocompatibility of chitosan/PureCol 0.5% hydrogels by adding different volume ratios of collagen PureCol 0.5% in our chitosan hydrogels.

HYDROGELS PREPARATION:

Shrimp shell chitosan (Kitomer, Mw 250 kDa, DDA ≈ 90%) was purchased from Marinard Biotech (QC, Canada). An acidic solution of 3.33% (w/v) of chitosan was prepared with hydrochloric acid(HCl 0.1M). The usual hydrogel formulation used in our lab is made of chitosan solubilized in 0.1M HCland mixed with a gelling agent solution (phosphate buffer + sodium hydrogen carbonate)at a volume ratio of 60/40.
PureCol 0.5% (Advanced Biomatrix) was used as collagen source.
It is important to remind that chitosan dissolved in acetic acid was previously discarded since it led to gels with slow gelation and pH under physiological value (see previous report).

Here are the different chitosan/collagen volume ratios, the volumes and concentrations of chitosan and collagen to make 1ml of chitosan/collagen hydrogel.

TABLE 1: VOLUME AND CONCENTRATIONS OF CHITOSAN AND PURECOL TO PREPARE DIFFERENT VOLUME RATIOS OF 1ML GEL

Chi/Coll Volratio	Chit gel volume	PureCol volume	[Chi gel]i (% w/v)	[Coll]i (% w/v)	[Chi gel]f (% w/v)	[Coll]f (% w/v)
100/0	1	0	2	0.5	2	0
75/25	0.75	0.25	2	0.5	1.5	0.125
50/50	0.5	0.5	2	0.5	1	0.25
25/75	0.25	0.75	2	0.5	0.5	0.375
0/100	0	1	2	0.5	0	0.5

The gels containing cells were prepared in 3 steps:

a) Mixing cell suspension within PureCol (at physiological pH)
b) Mixing acidic chitosan solution with gelling agent to form hydrogel solution at neutral pH at room temperature
c) Mixing the two solutions prepared in a) and b)

Mixing cells within the collagen prior to mixing with Chitosan hydrogel solution was chosen to reduce risks of damage on cells, since we aim to develop gels for cell therapy and tissue engineering. Each time, the two solutions were mixed using a LuerLock connecting two syringes. It is important to note that this mixing method can generate important shear stress on cells and was not optimized in the present study.

pH measurements :

Protocol: The pH of the different chitosan/PureCol hydrogels was evaluated after 24h of gelation at 37°C.
Results: The pHof chitosan/PureCol gels are very close to physiological values.

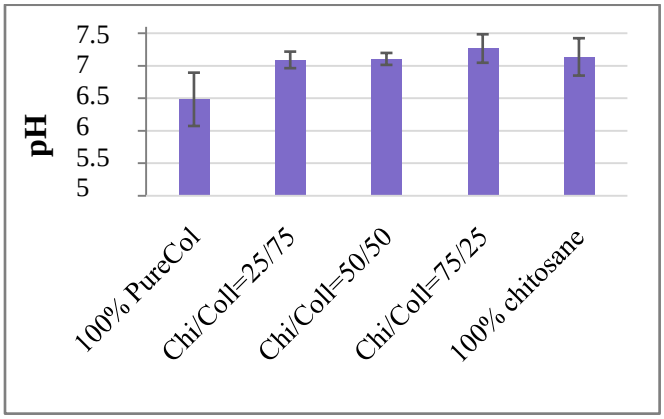


FIGURE 1 : PH OF CHITOSAN AND PURECOL GELS (MEAN OF N=3 SAMPLE)

Gelation kinetics

Protocol:

- The rheological properties of the chitosan-PureColgels were studied using an Anton Paar instrument (Physica MCR 301) equipped with a co-axial cylinder geometry.
- Immediately after mixing, the variation with time of the storage modulus (G') and loss modulus (G'') was measured at 37°C during 1h. Gelling time (t_{gel}) is the time for which G'=G'' according to the approach by Winter and Chambon[1]

Results : t_{gel} are presented in Table 2 while Figure 1 presents the evolution of the storage modulus G' as a function of time at 37°C for all hydrogels.

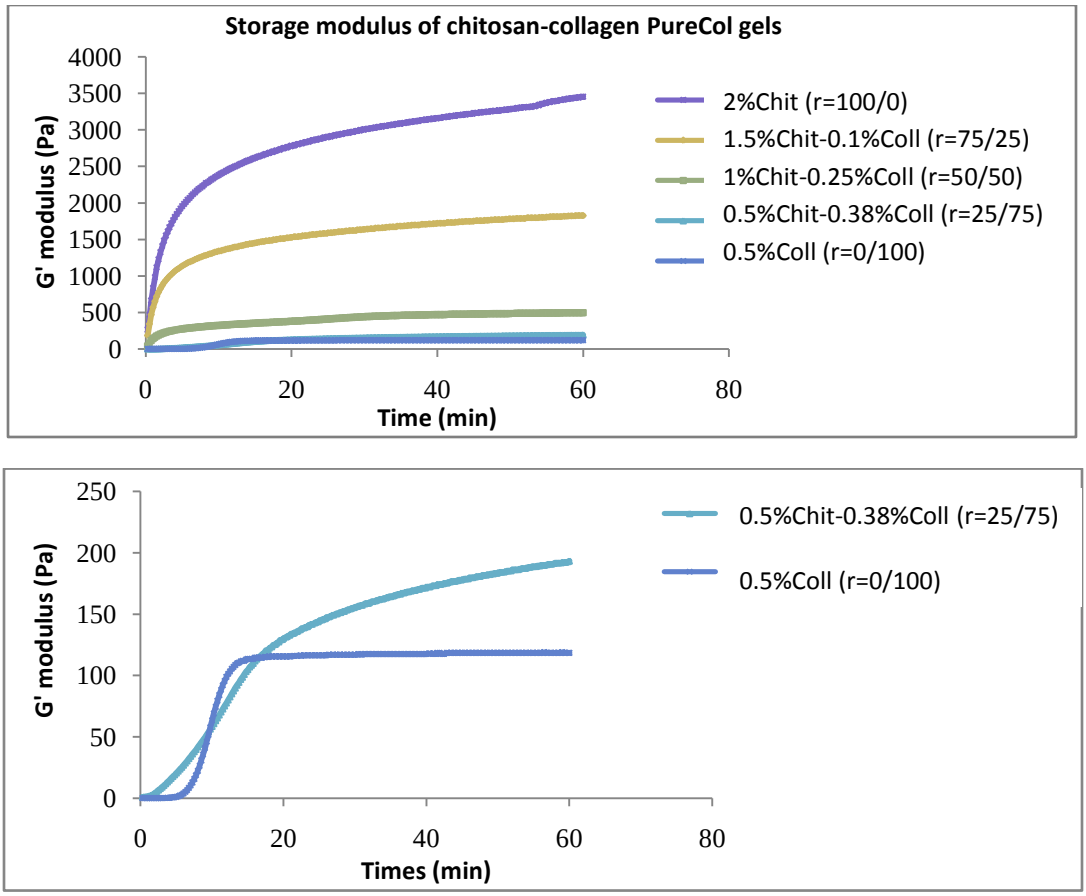


FIGURE 2: STORAGE MODULUS OF CHITOSAN AND PURECOL GELS DURING 1H (MEAN OF N=3 SAMPLE)

TABLE 2 : GELLING TIME (TGEL) OF CHITOSAN AND PURECOL GELS

Chi/Coll (volume ratio)	t_{gel} (s)
100/0	< 15 ± 0
75/25	<15 ± 0
50/50	< 15 ± 0
25/75	127.5 ± 110
0/100	330 ± 21

Conclusions:

- The addition of PureCol 0.5% slows down gelation time as t_{gel} increases with the addition of collagen
- The shape of the gelation curve is influenced by gel composition. G' value of 100% PureCol gel is stable during 10 min, suddenly increases and then plateaued at around 100 Pa). On the contrary, G' of 100% chitosan gels increases rapidly as soon as put at 37C. It doesn't reach a plateau even after 1 hour (G' >3500 Pa after 1 hour).
- Chitosan-Collagen mixture present an intermediate behavior, G' increases more slowly and reached lower values when increasing the collagen content. This shows that the addition of collagen decreases the mechanical properties of chitosan gel but do not impair gelation.

COMPRESSIVE STRENGTH:

Protocol:

- Unconfined compression tests on chitosan/PureCol hydrogels were performed using the Physica MCR 301 equipped with a parallel plate.
- 2.0 mL of hydrogel was introduced into cylindrical molds (14 mm diameter, 12mm height) and incubated at 37°C during 24h before compression testing betweentwo parallel plates until 50% deformation. The secant modulus at 30 and 50% deformation was calculated.

Results: E30 and E50% values are presented in Figure 3, as a function of gel composition. In addition to the formulations described above, chitosan gels with reduced chitosan content were studied to evaluate the role of chitosan reduction versus collagen addition in the changes of mechanical properties. Figure 4 presents the 3 stress-strain curves obtained for CH-Collagen r=50/50, as example.
Statistical analysis (ANOVA + Post hoc Bonferroni) was performed to compare all CH2% with all CH+Coll formulations only.

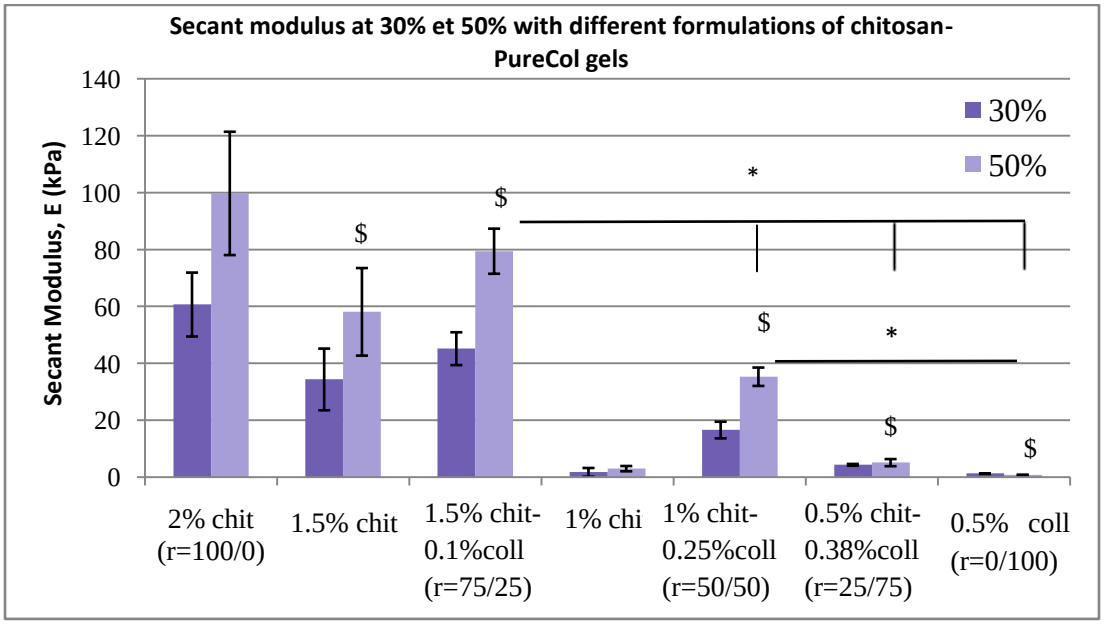


FIGURE 3: SECANT MODULUS AT 30% AND 50% OF DIFFERENT FORMULATIONS OF CHITOSAN-PURECOL GELS (MEAN+SD, N=3)
$: p<0.05 compared to 2% chit gel. * : p<0.05 between formulations (post hoc Bonferroni)

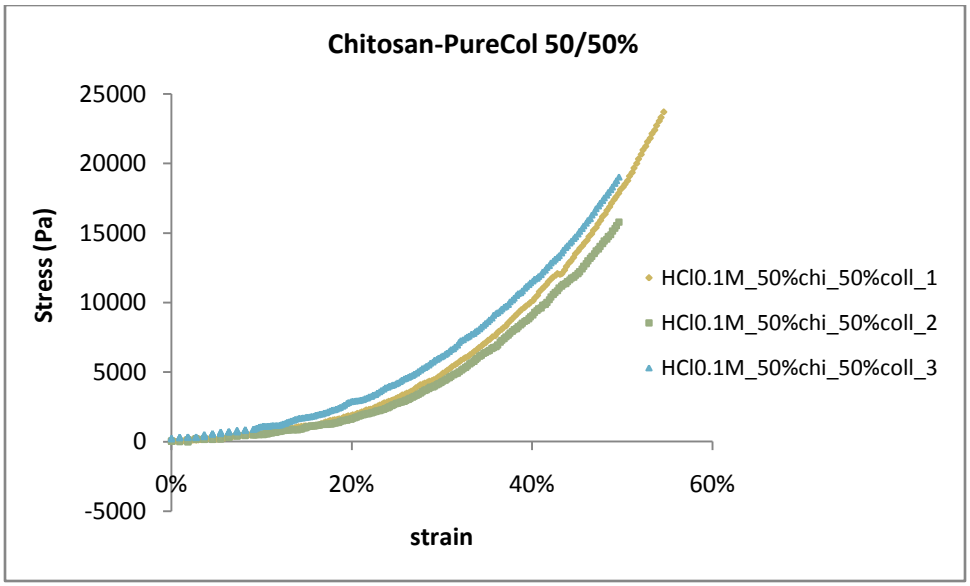


FIGURE 4 : STRESS—STRAIN CURVES OF CH-COL50/50% (THREE SAMPLES)

Conclusions:

- PureCol presents very poor mechanical properties. Adding chitosan to collagen gels strongly increases their mechanical properties.
- Adding collagen to our chitosan hydrogels seems to decrease their stiffness. But in fact the drop in mechanical properties is explained by the reduction of the final concentration of chitosan in the gel.
- For the same final concentration of chitosan, the gels with collagen seem to have better mechanical properties.

GEL POROSITY :

Protocol:

- Immediately after mixing the two solutions, the samples are poured in a cylindrical mold and left to gel for 24h at 37°C. After 3 days of freezedrying, we observed the gels with a SEM (Hitachi S-3600).

Conclusions:

- Although the porosity of freeze-dried gels is not the same as hydrated one, SEM images suggests that the addition of collagen increases the number of pores and decreases their size. Moreover, when 50% of collagen, or more, is added, we observed fibrillar structures which are probably collagen fibers.

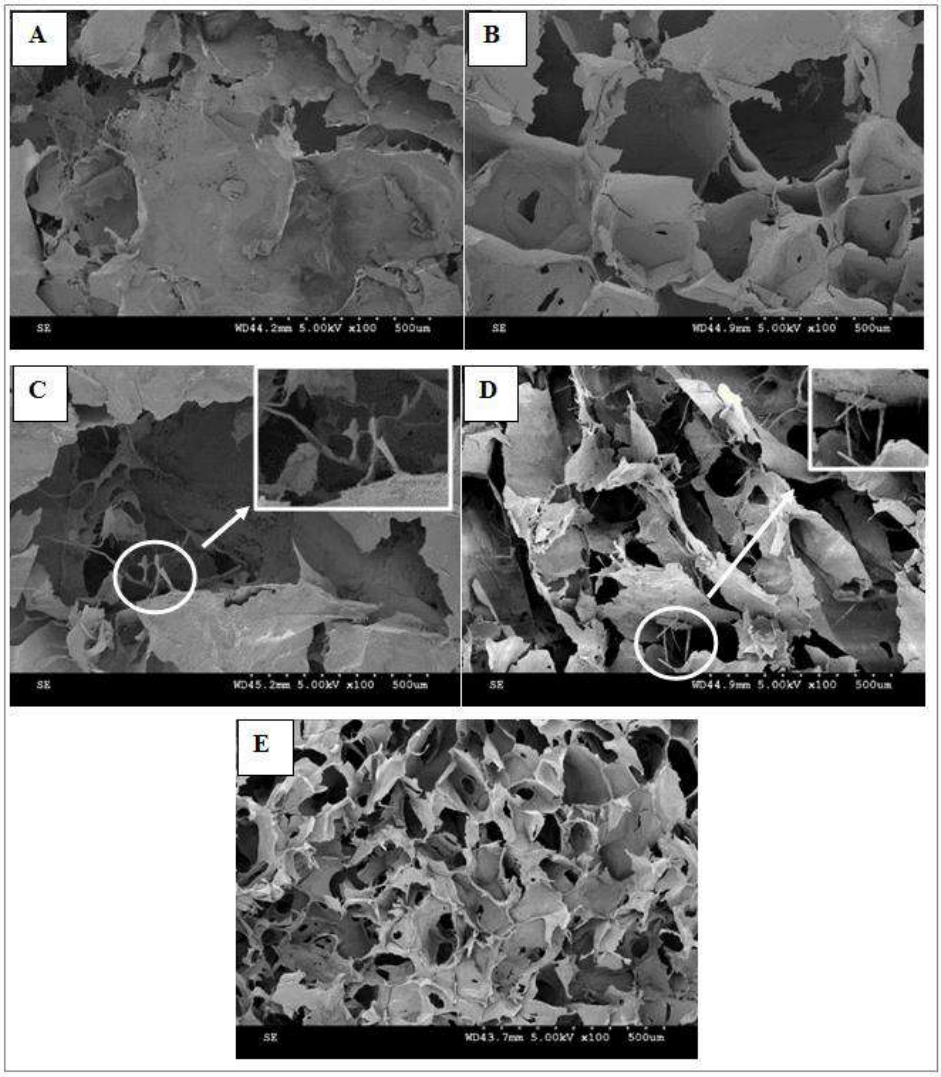


FIGURE 5:SEM PICTURES A) R = 100/0 (CHIT/COLL). B) R = 75/25 (CHIT/COLL). C) R = 50/50 (CHIT/COLL). D) R = 25/75 (CHIT/COLL). E) R = 0/100 (CHIT/COLL)

VIABILITY OF ENCAPSULATED CELLS :

Protocol:

- For collagen-free gels, L929 fibroblasts are encapsulated in the chitosan and gelling agent mix (11 million cells per ml)
- For the chitosan-PureCol gels, the cells are first added to the collagen solution and then mixed with the chitosan gels
- The metabolic activity of encapsulated cells was evaluated by Alamar Blue assays at days 1, 4 and 7.
- Cell viability and repartition of live and dead cells in hydrogels were evaluated by Live-Dead assay with a viability/cytotoxicity kit, and observed in fluorescent microscopy

Results: Figure 6 presents the metabolic activity of cells entrapped in the various formulations according to AlamarBlue assay (quantitative results). Figure 7presents pictures in fluorescent microscopy after Live/dead staining (qualitative assessment of the amount of living cells).

Conclusions:

- There is no clear trend in AlamarBlue results, with large variability. Even if the experiment was done 3 times, big standard deviations were observed. The method to destroy the gels is hard to reproduce homogeneously. Piece of gel may interfere with fluorescence measurements. Moreover collagen gels were more difficult to detroyed so the fluorescence was probably underestimated.
- At day 7, the 25% Chit-75%Coll gel showed a statistically higher fluorescence emission than the other formulations ($p<0.01$). However, there is still doubt on the validity of these results.
- Live/Dead assays show that the addition of collagen in hydrogel leads to a clear decrease of the number of dead cells and an increase of live cells.However these are only qualitative results. It is difficult to compare the various CH-Coll formulations, yet formulations with 50% PureColl or more seem better than the other formulations.

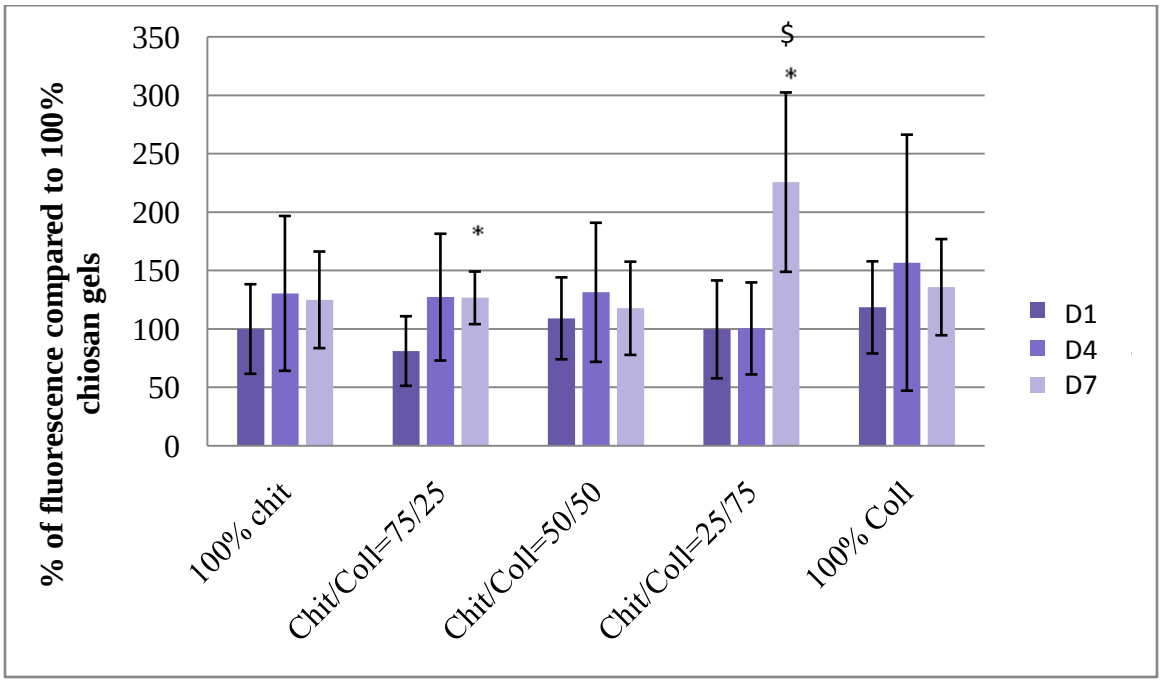
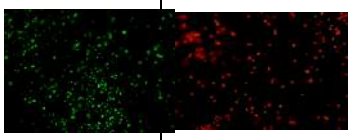
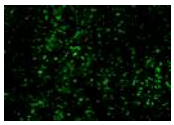
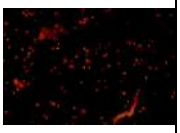
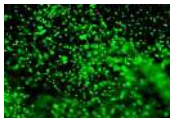
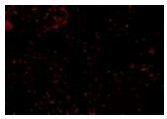
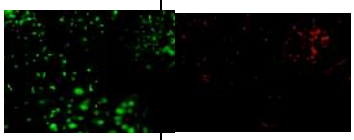
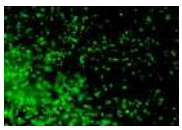
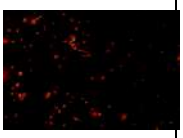
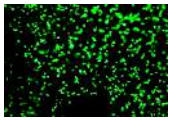
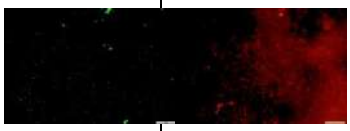
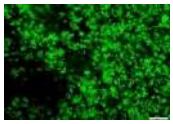
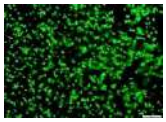
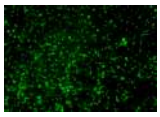
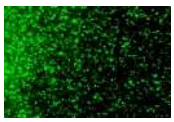
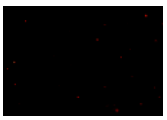
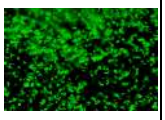
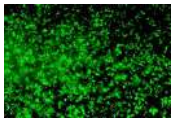
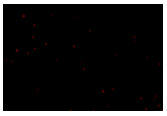
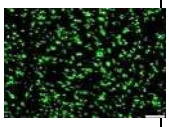
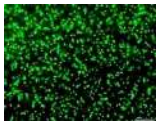


FIGURE 6 : METABOLIC ACTIVITY OF ENCAPSULATED FIBROBLASTS FOR 7 DAYS. ANOVA ANALYSIS WAS PERFORMED TO COMPARED THE METABOLIC ACTIIVTY BETWEEN DAY 1 AND DAY 7 (*) AND BETWEEN DAY 7 OF 100% CHITOSAN AND THE OTHER GELS ($). P<0.05

	100% Chit		75%Chit-25%Coll		50%Chit-50%Coll	
	Live	Dead	Live	Dead	Live	Dead
J1						
J4						
J7						

	25%Chit-75%Coll		100%Coll	
	Live	Dead	Live	Dead
J1				
J4				
J7				

CONCLUSIONS:

- These results confirm that **addition of chitosan to PureCol strongly enhances its mechanical properties (rigidity and mechanical resistance (maximal stress)) and shorten gelationtime at 37C.No damage on cell viability was observed on entrapped L929 fibroblasts, according to Live/dead assays.**
- The increase of the Coll/Chit ratio within our chitosan hydrogels slows down the gelation kinetics and leads to a decrease in mechanical properties, but this is attributed to the reduction in chitosan concentration. For a similar concentration of chitosan, the collagen-containing gels appear to be stiffer.
- Cell viability testing by Alamar Blue and Live / Dead staining led to contradictory results. The method to quantify the cell viability with Alamar Blue within these gels needs to be optimized.
- According to the Live / Dead colorations, the 100% Chit formulation appears to be less cytocompatible than the gels containing collagen.
- While chitosan25/collagen75% seems to lead to the best AlamarBlue results, it is difficult to conclude that it really presents an advantage in terms of cell viability compared to other Chitosan-collagen mixtures.Moreover, this gel is relatively weak (secant Young modulus of 5 kPa compared to 100 kPa of the chitosan gel without collagen) but still stronger than PureCol.
- The best formulation may depend on the targeted application since a compromise must be chosen between mechanical properties and cell viability.
- An interesting candidate is 75% Chitosan-25% Collagen formulation which appears to be more cytocompatible than the 100% Chitosan gel and also has very interesting mechanical properties (secant modulus reaches 80kPa in compression).

TABLEAU 3: SUMMARY OF ADVAMTAGES/LIMITATIONS OF THE VARIOUS FORMULATIONS.

Formulation ratio Chit/Coll	Gelationkinetics	Mechanicalproperties	Cellviability
100/0	**++**	**++**	**-**
75/25	**++**	**++**	**+ -**
50/50	**++**	**+**	**+**
25/75	**-**	**-**	**++**
0/100	**- -**	**- -**	**++**

1. Winter, H.H. and F. Chambon, *Analysis of linear viscoelasticity of a crosslinking polymer at the gel point.* Journal of Rheology, 1986. **30**(2): p. 367-382.