



**HAL**  
open science

## Extracellular matrix-mimetic composite hydrogels of cross-linked hyaluronan and fibrillar collagen with tunable properties and ultrastructure

Antoine Frayssinet, Dalila Petta, Corinne Illoul, Bernard Haye, Anastasiia Markitantova, David Eglin, Gervaise Mosser, Matteo D'este, Christophe Hélary

### ► To cite this version:

Antoine Frayssinet, Dalila Petta, Corinne Illoul, Bernard Haye, Anastasiia Markitantova, et al.. Extracellular matrix-mimetic composite hydrogels of cross-linked hyaluronan and fibrillar collagen with tunable properties and ultrastructure. *Carbohydrate Polymers*, 2020, 236, pp.116042. 10.1016/j.carbpol.2020.116042 . hal-02862482

**HAL Id: hal-02862482**

<https://hal.sorbonne-universite.fr/hal-02862482v1>

Submitted on 9 Jun 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Manuscript Number: CARBPOL-D-19-04819R1

Title: Extracellular matrix-mimetic composite hydrogels of cross-linked hyaluronan and fibrillar collagen with tunable properties and ultrastructure

Article Type: Research Paper

Keywords: Collagen, hyaluronan, enzymatic cross-linking, composite hydrogels, fibrillogenesis.

Corresponding Author: Dr. Christophe Helary, Ph.D

Corresponding Author's Institution: Sorbonne Universités - University Pierre and Marie Curie

First Author: Antoine Frayssinet, Master

Order of Authors: Antoine Frayssinet, Master; Dalila Petta, PhD; Corinne Illoul; Bernard Haye; Anastasiia Markitantova, Master; David Eglin, PhD; Gervaise Mosser, PhD; Matteo D'Este, PhD; Christophe Helary, Ph.D

Abstract: A platform of enzymatically-crosslinked Collagen/Tyramine hyaluronan derivative (Col/HA-Tyr) hydrogels with tunable compositions and gelation conditions was developed to evaluate the impact of the preparation conditions on their physical, chemical and biological properties. At low HA-Tyr content, hydrogels exhibited a fibrillar structure, with lower mechanical properties compared to pure Col hydrogels. At high HA-Tyr and Horse Radish Peroxydase (HRP) content, a microfibrillar network was formed beside the banded Col fibrils and a synergistic effect of the hybrid structure on mechanical properties was observed. These hydrogels were highly resistant against enzymatic degradation while keeping a high degree of hydration. Unlike HA-Tyr hydrogels, encapsulation of human dermal fibroblasts within Col/HA-Tyr hydrogels allowed for high cell viability. These results show that high HA-Tyr and HRP concentrations are required to positively impact the physical properties of hydrogels while preserving collagen fibrils. Those Col/HA-Tyr hydrogels appear promising for novel tissue engineering applications following a biomimetic approach.

## Highlights

- 1) Biomimetic collagen/hyaluronan hydrogels mimicking the extracellular matrix of tissues.
- 2) Collagen fibrillogenesis not inhibited.
- 3) High HA-Tyr contents and a high [HRP] positively impact the physical properties.
- 4) Synergistic effect of Col and HA-Tyr on hydrogel properties.
- 5) Presence of a microfibrillar network.



Dear Editor,

Please find attached our revised manuscript CARBPOL-D-19-04819 R1 where the reviewers' comments have been taken into account and corrected accordingly. You will find a more detailed response, point-by-point answer for each comment below. We do hope that with these improvements, a final decision will be made by the editorial board and thus our manuscript considered for publication in *Carbohydrate Polymers*.

Yours sincerely,

Christophe Héлары

## RESPONSE TO REVIEWERS

**Reviewer #1:** The topic might be very interesting and the manuscript summarizes a lot of work. I feel sorry for the authors but I can't suggest the publication in the journal. There are two basic arguments supporting my opinion.

1. The manuscript is dealing with protein materials (collagen, tyramine), tyramine slightly modified with hyaluronic acid. It is speaking about many aspects connected with protein materials.

On the website of the journal, there are topics not of interest to the journal:



\* biological, physiological and pharmacological aspects of non-carbohydrate; molecules attached to, or mixed with, carbohydrate polymers, unless the polysaccharide has a relevant and specific role;

\* materials science of biocomposites where there is no mention of any specific carbohydrate polymer, or the role of the carbohydrate polymer is not the major proportion of the study;

**Answer 1:** We claim hyaluronic acid (HA) has a central role in this manuscript. The aim of this study was to improve the properties and correct the drawbacks of collagen hydrogels by the addition of HA. In this manuscript collagen concentration and gelling conditions have been set constant to understand the impact of HA-Tyr (content and gelling conditions) on the physical properties of hydrogels. The purpose of this manuscript was to find the appropriate conditions and the adequate HA-Tyr content to improve the mechanical and physical properties of hydrogels. That's why we wrote in the abstract, the sentences **“At low HA-Tyr content, hydrogels exhibited a fibrillar structure, with lower mechanical properties compared to pure Col hydrogels. At high HA-Tyr and Horse Radish Peroxydase (HRP) content, a microfibrillar network was formed beside the banded Col fibrils and a synergistic effect of the hybrid structure on mechanical properties was observed”** and **“These results showed that high HA-Tyr and HRP concentrations are required to positively impact the physical properties of hydrogels while preserving collagen fibrils”** to focus on the effect of the polysaccharide on hydrogel properties.

**Answer 2:** Actually, tyramine is not a protein. It is a molecule which resembles tyrosine, an amino acid. HA was functionalized with tyramine to allow the enzymatic crosslinking in order to form a HA hydrogel in mild conditions, compatible with cell survival. Therefore, tyramine has not a central role in this study.

**Answer 3:** Carbohydrate Polymers currently publishes articles which describe protein/polysaccharide composites. For instance, we can cite the work from Jitendra Singh et al recently published in Carbohydrate Polymers:

“Protein-polysaccharide based microencapsulated phase change material composites for thermal energy storage”. (2020) <https://doi.org/10.1016/j.carbpol.2019.115531>

2. In a short look into the internet I found an early publication, which seems to be similar to the recent one:

<https://www.ncbi.nlm.nih.gov/pubmed/8126023>

Effects of hyaluronan on collagen fibrillar matrix contraction by fibroblasts.

J Biomed Mater Res. 1994 Jan;28(1):123-32.

Effects of hyaluronan on collagen fibrillar matrix contraction by fibroblasts.

Huang-Lee LL1, Wu JH, Nimni ME.

Therefore, the novelty of the results should be explained in more details.

**Answer 4:** The study published by Huang-Lee et al describes the positive effect of HA to inhibit the collagen hydrogel contraction by fibroblasts. In this article, HA is not functionalized and is not able to form a gel. The impact of HA addition on physical properties of hydrogels (mechanical properties, resistance against degradation and hydration) is not studied. This study is totally different from ours because authors only focus on the effect of HA on fibroblast behavior, *i.e* the ability of HA to inhibit fibroblast contraction. The novelty of our study is to improve physical properties of collagen hydrogels by co-gelling of collagen and HA-Tyr to take advantage of properties of both biopolymers. High mechanical and hydration properties for HA-Tyr and high cell adhesion for collagen. Actually, HA-Tyr does not improve physical properties of hydrogels when it is not cross-linked by HRP and H<sub>2</sub>O<sub>2</sub> (Supporting Information S.I. 6 and Figure 4). Not cross-linked composite hydrogels exhibit mechanical and hydration properties similar to pure collagen hydrogel ones. That's why we wrote **“These results showed that high HA-Tyr and HRP concentrations are required to positively impact the physical properties of hydrogels while preserving collagen fibrils”**. It is worth noticing that the formation of Col/HA-Tyr composite hydrogels also inhibit the contraction by fibroblasts as written line 561: **“Interestingly, no contraction of hydrogels was observed (data not shown). Hence, the addition of HA-Tyr stabilized the structure of Col hydrogels against cell-induced contraction”**.

**Reviewer #2:** The Manuscript Number: CARBPOL-D-19-04819 describes extracellular matrix-mimetic composite hydrogels of cross-linked hyaluronan and fibrillar collagen with tunable properties and ultrastructure. The paper is logically structured and written. Some edition improvements might be helpful.

Specific comments:

-P9 line 213: Was the release of hydrogels components during the swelling experiment investigated?

**Answer 5:**

We have not seen any release of HA-Tyr or Col during the swelling experiment. To illustrate this, we have dried some composites, weighted them (mass M1), make them swell for 24 hours in PBS and freeze-dry them again. Finally, we have weighted them (mass M2). No significant differences of weight were observed. We join the table describing this experiment.

Composite Hydrogels		M1	M2	Difference M2 - M1 [mg]
Col/HA-Tyr	ratio 1:1, HRP 0.05, H <sub>2</sub> O <sub>2</sub> 0.6mM	0,77	0,76	-0,01
Col/HA-Tyr	ratio 1:1, HRP 0.1, H <sub>2</sub> O <sub>2</sub> 0.6mM	1,18	1,28	0,1
Col/HA-Tyr	ratio 1:1, HRP 0.05, H <sub>2</sub> O <sub>2</sub> 1.1mM	0,72	0,85	0,13
Col/HA-Tyr	ratio 1:2, HRP 0.5, H <sub>2</sub> O <sub>2</sub> 1.1mM	1,97	2,19	0,22
Col/HA-Tyr	ratio 1:2, HRP 0.5, H <sub>2</sub> O <sub>2</sub> 0.6mM	3,42	3,93	0,51
Col/HA-Tyr	ratio 1:5, HRP 0.1, H <sub>2</sub> O <sub>2</sub> 0.6mM	2,23	2,56	0,33
Col/HA-Tyr	ratio 1:5, HRP 0.1, H <sub>2</sub> O <sub>2</sub> 1.1mM	2,79	3,12	0,33

- In the manuscript, in Figures 4, 7, and 9, use a period instead of a comma before the decimal fractions.

**Answer 6:** The figures have been corrected by the replacement of commas by points before the decimal fractions. The figure 4 has been replaced by Table 1 and 2 according to the reviewer 4's recommendation.

-In the document, in Figure 4. What does the dotted red line mean?

**Answer 7:** The dotted red line observed in figures 4-7 represents the separation between the two behaviors of HA-Tyr. On the left are the Col/HA-Tyr ratios for which HA is not able to form a hydrogel on its own. On the right, the ratios for which HA—Tyr is able to form a gel. A sentence has been added in the figure caption of figure 4, line 444.

-Regarding rheological analysis. Could you please indicate the G'' value or the tan delta (G''/G') value to have a better idea of the viscoelasticity of these hydrogels?

**Answer 8:** We agree with the reviewer. G'' values have been added to the manuscript in Table 1-3 and S.I. 5. In addition, the paragraph from line 345 has been modified:

**“The influence of increasing HA-Tyr amount at fixed H<sub>2</sub>O<sub>2</sub> concentration and various HRP contents on the storage G' and loss G'' moduli of hydrogels was studied. In all conditions G' was at least 10 times higher than G'', thereby evidencing the physical form of hydrogels was preserved for all Col/HA-Tyr composites (Table 1 and 2, S.I. 5).”**

**Reviewer #3:** The manuscript reports the preparation of enzymatically-crosslinked collagen/tyramine hyaluronan derivative (Col/HA-Tyr) composite hydrogels. The effects of HA-Tyr content, the HRP concentration and the degree of cross-linking on the gel formation, collagen fibrillogenesis, physical properties and cell viability were evaluated. The study is very thorough and systemic. The results from the experiments are well discussed. The manuscript is well written. The content is appropriate for the journal's readership of Carbohydrate Polymers. It is therefore recommended for publication with minor revisions.

\* Highlights. It is suggested to rewrite into short sentences.

**Answer 9:** The highlights have been corrected.

\* Line 169. What are the final concentrations of HA-Tyr in the composite hydrogels?

**Answer 10:** This information is now encompassed in table 1 and 2, and S.I. 5

Ratio 8:1 → HA-Tyr: 0.05%

Ratio 4:1 → HA-Tyr: 0.1%

Ratio 2:1 → HA-Tyr: 0.2%

Ratio 1:1 → HA-Tyr: 0.4%

Ratio 1:2 → HA-Tyr: 0.8%

Ratio 1:5 → HA-Tyr: 2%

\* Page 14-15. Should it be nanofibrils instead of microfibrils based on the size?

**Answer 11:** Actually, all fibrils have a nanometric size, at least in diameter. The term “microfibrils” is used in comparison with regular fibrils. We would like to retain this term in our manuscript.

\* Figure 4. The plots should deliver the data in a clear way and help the reader understand in the short amount of time. It is suggested to make 3D plots so that it can be more readable.

**Answer 12:** Figure 4 has been replaced by Table 1 and 2 to present data in a clearer way.

\* Line 413. Why only minor cross-linking in pure collagen hydrogels formed via tyrosine/tyrosine bonds? Tyramine has similar molecular structure as tyrosine and the formation tyramine-tyrosine bonds was discussed in page 14.

**Answer 13:** Tyrosine residues on the collagen surface represents less than 1% of the total amino acids. Hence, the likelihood to form tyrosine-tyrosine bonds is weak and much lower than that to form tyramine-tyramine or tyrosine-tyramine bonds. Indeed, the functionalization of HA by Tyramine is 6% (degree of substitution) as written line 167. Hence, the di-tyramine bonds formation is more likely than the di-tyrosine ones.

#### **Reviewer #4:**

The manuscript describes the fabrication and development of hydrogels based on crosslinked collagen/tyramine hyaluronan derivative (Col/HA-Tyr) for applications in tissue engineering. It is acceptable for publication after major revision. The following points may help the authors:

General:

(1) The authors should follow the requirements of the Carbohydrate Polymers journal especially the citing of references in the text.

**Answer 14:** The bibliography has been formatted according to the Carbohydrate Polymers journal requirements.

(2) Lines 117-119: The aim of this study was to create a platform of Col/HA-Tyr hydrogels with different compositions and tunable gelling parameters to understand the influence of Col gelling on the HA-Tyr network formation and vice versa. It is enough to say studying the compositions and gelling parameters.

Line 137: please provide the details of "Life Technologies"

**Answer 15:** The sentence line 117 (now 125) has been corrected and the details of "Life Technologies" added (line 144).

(3) It will be much better to provide the text in justified lines.

**Answer 16:** We have followed the journal submission requirements written in the guide for authors: **"Most formatting codes will be removed and replaced on processing the article. In particular, do not use the word processor's options to justify text or to hyphenate words."**

(4) It is difficult to read most of provided figures; the authors may find a simpler method to plot their figures. In Fig.1, The terms crosslinking density and crosslinking speed should be identified.

**Answer 17:**

- 1) The terms cross-linking density and crosslinking speed has been explained in the section 2.4 line 177.
- 2) To make figure 4 clearer, we have replaced it by a table (table 1 and 2, S.I. 5), following the reviewer's recommendation.
- 3) We have simplified the other figures and shortened captions by using different colors only for the different [HRP].

(5) In the layout of the text, you may start with:

3.2 Swelling, degradation and thermal properties of Col/HA-Tyr hydrogels and then 3.3 Rheological properties of Col/HA-Tyr hydrogels for a range of parameters.

**Answer 18:** We think that presenting the ultrastructure of composite hydrogels first is more relevant because we discuss the results in regards to the differences observed in electron microscopy. For example, we make a correlation between the presence of a microfibrillar network and the improved mechanical properties.

(6) The captions of figures are too long for the readers to follow.

**Answer 19:** The captions have been shortened.

Experimental:

(1) Lines 141-142: please correct "Col purity was assessed by SDS-PAGE electrophoresis and the concentration was measured by hydroxyproline titration".

**Answer 20:** This sentence has been corrected (line 148).

(2) Lines 142-145: this sentence is not clear; please revise.

**Answer 21:** This sentence has been clarified (line 172). We use two different collagen concentrations. One for the composites with a low HA-Tyr content and the higher concentration for the 1:5 Col/HA-Tyr ratio as a large amount of HA-Tyr is required to form the composite hydrogel. A S.I. 1 has been added into the supporting information section to show how the hydrogels are fabricated.

(3) Lines 147-148: The Conjugation of Hyaluronan (HA) with tyramine may be proceed via hydrogen bonding between HA carboxylic groups and the amine group of tyramine hydrochloride and not amidation reaction.

**Answer 22:** It is well known that DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride) catalyzes amidation reactions between COOH moieties of HA and NH<sub>2</sub> moieties of tyramine (Petta et al., 2016; Carbohydrate Polymers). So, the hydrogen bonding between HA and Tyramine is unlikely.

(4) Lines 149-150: please revise the sentence "2 g of hyaluronic acid sodium salt (5 mmol carboxylic groups) was dissolved overnight in ultrapure water at a final concentration of 1% (w/v)". How much was the volume of ultrapure water?

**Answer 23:** The volume of water was 200 mL. The sentence has been corrected accordingly (line 156).

(5) Lines 164-176: (Col/HA-Tyr hydrogels synthesis): Please clarify the following points:

\* The concentrations of the initial components to obtain the final Col concentration at 4 mg/ml.

**Answer 24:** We used two different collagen concentrations. One for the composites with a low HA-Tyr content (0.6%) and the higher concentration (0.875%) for the 1:2 and 1:5 Col/HA-Tyr ratio as a large amount of HA-Tyr was required to form the composite hydrogels. The HA-Tyr stock solutions was at 3% or 6% (for the 1:2 and 1:5 ratio) as written line 172 section 2.4. A table has been added in the supporting information section to describe the hydrogels fabrication (S.I.1)

\* The 10X-PBS and 10 x-Phosphates Buffer Saline (PBS).

**Answer 25:** We now use the term 10X-PBS except the first time it appears where we use 10X Phosphate Buffered Saline (10X-PBS) (line 181).

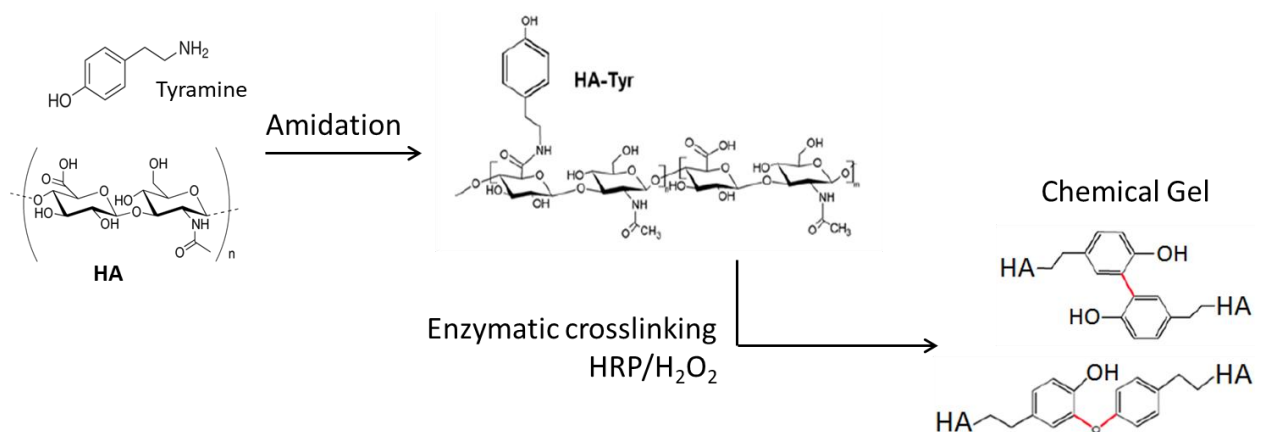
\* In the text it was said that the Col concentration was 4 mg/ml, however in Fig. 1 it was expressed as %.

**Answer 26:** Now the collagen concentration is always expressed as %.

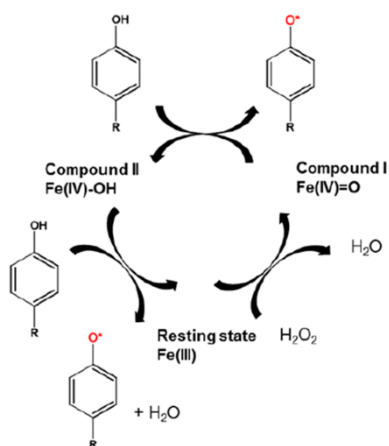
\* Line 172-173: "to explore the effect of HA-Tyr cross-linking densities (dityramine bond formation)". The dityramine bond formation is the type of hydrogen bonding not the cross-linking densities and how possibly dityramine bond can form network structure.

**Answer 27:** The di-Tyramine bonding is the crosslinking between two HA molecules. Here is a drawing to illustrate the reaction. The di-tyramine bonds between HA polymers form a network of HA, *i.e* a hydrogel. It has been published several times by Petta et al (see the bibliography in the manuscript).

General scheme



Crosslinking catalytic cycle H<sub>2</sub>O<sub>2</sub> and HRP vs. Tyramine



## Results and discussion

- (1) The mechanism of the formation (crosslinking) of HA-Tyr network hydrogels should be clarified. How the degree of crosslinking was measured?

**Answer 28:** See above. The degree of substitution by tyramine was measured not the degree of cross-linking.

- (2) Lines 279-282: How the gelation of Col was induced by neutralization with an acidic solution. Please unify the concentration used for Col; is it 4 mg/mL or 4%.

**Answer 29:** See above for the unification of collagen concentrations. The collagen gelation is induced by pH increase. Actually, collagen is soluble in water only below pH 5.5. At this pH collagen is positively charged and soluble. When the pH reaches 7, collagen is neutralized and is not soluble in water anymore. This triggers fibrillogenesis and the formation of a physical hydrogel. This phenomenon is known since the late 70's. The collagen hydrogel formation triggered by NaOH has been described the first time by Bell *et al* in 1979 (PNAS-[doi: 10.1073/pnas.76.3.1274](https://doi.org/10.1073/pnas.76.3.1274)).

- (3) Line 284: The SEM micrograph of pure Col hydrogels (4 mg/mL) (exhibited a typical fibrillar network (S.I 1A), cannot be seen in Fig.2. In general, it was difficult to detect the differences between the structures according to fibrillar network and ultrastructure. Please revise the SEM part. It is clear that the SEM observations were done on the surface of samples; it will more useful to observe the fracture surfaces.

**Answer 30:** The micrograph showing the fibrillar structure of pure collagen hydrogel has been encompassed into the S.I section to avoid the figure 2 to be too heavy and difficult to read. We show in figure 2 that composites exhibit a fibrillar structure similar to that observed for pure collagen hydrogels when the Col/HA-Tyr ratio is below 1:2. From this ratio, composites exhibit a structure in SEM similar to that of pure HA-Tyr hydrogels (consisting of sheets).

**Answer 31:** The observations were not performed on the samples surface. Each sample was torn prior to SEM observation to see its inner structure. A sentence has been added in the section 2.6, line 200.

- (4) Once again, TEM imaging of hydrogels at different compositions does not show differences except a population of banded fibrils. The TEM imaging of hydrogels showed fibrillar structure, while the SEM micrographs do not show this structure.

**Answer 32:** TEM imaging was dedicated to the observation of collagen fibrils as HA is not visible in TEM. The goal was to assess if HA-Tyr addition had an impact on collagen



fibrillogenesis (size of fibrils, banded pattern). It allowed us to see the formation of a microfibrillar network from the 1:2 ratio. From the 1:2 Col/HA ratio, fibrils were not visible in SEM because they were encompassed within HA-Tyr sheets as seen in figure 2. For instance, fibrils are visible on the panel 0.1 U/mL HRP, 1:2 ratio (figure 2).

(5) Line 335: In a first step; there is no second step.

**Answer 33:** "In a first step" has been removed.

(6) The caption of Fig. 4 (A and B) is too long and it is not easy to read and the values of the storage modulus cannot be seen from the figure; it will much easier to formulate the results in Tables. What do you mean by the vertical red line?

**Answer 34:** 2 tables (1 and 2) have been done to replace Figure 4.

**Answer 35:** The dotted red line observed in figures 5-7 represents the separation between the two behaviors of HA-Tyr. On the left are the Col/HA-Tyr ratios for which HA is not able to form a hydrogel on its own. On the right, the ratios for which HA—Tyr is able to form a gel. A sentence has been added in the figure caption of figure 5, line 444.

(7) Fig.5: the black vertical bars representing 0.4 % Collagen and 0.4 % Collagen with 0.5 U mL<sup>-1</sup> 425 HRP activity looks similar for the readers. The Statistical analysis shown in the caption may be stated in text.

**Answer 36:** The swelling properties of 0.4 % collagen hydrogels and those of 0.4 % Collagen with 0.5 U mL<sup>-1</sup> HRP are not significantly different. It is written line 426: "**H<sub>2</sub>O<sub>2</sub> and HRP addition to pure Col did not impact its swelling properties, thereby evidencing absent or minor cross-linking in pure Col hydrogels via tyrosine/tyrosine bonds (Figure 4A and 4B).**" The Statistical analysis has been stated in the text (line 447 and 449).

(8) Lines 488-489: Please revise "The thermal stability of the hydrogel blends was investigated via DSC. The fibril denaturation in pure Col hydrogels was detected at 55-56°C in agreement with literature data. The DSC scans Fig.7 (C and D) are not clear. Once again, the statistical analysis shown in the caption may be stated in text.

**Answer 37:** the sentences line 498 has been revised. The figure is quite heavy because of the standard deviations. But it was the clearer way to present these data. However, it is obvious that a high HA-Tyr content and [HRP] increased the denaturation temperature of collagen.

The Statistical analysis has been stated in the text (line 514).

(9) Lines 533-534: The sentence is not clear.

**Answer 38:** The sentence line 533-534 has been rephrased (now line 543).

(10) In DSC scans, we usually identify glass and melting temperatures and the expression denaturation peak was used. Please clarify this term.

DSC allows detection of T<sub>g</sub> (glass transition) and melting temperature when semi-crystalline synthetic polymers are studied. In this study, we are in presence of HA and Coll. No T<sub>g</sub> or melting temperature are detectable. As denaturation of collagen is an exothermic

phenomenon, it can be detected by DSC. Regarding HA, no denaturation peak was detected because it is a polysaccharide.

1 **Extracellular matrix-mimetic composite hydrogels of cross-**  
2 **linked hyaluronan and fibrillar collagen with tunable**  
3 **properties and ultrastructure**  
4

5  
6 **Antoine Frayssinet<sup>1 †</sup>, Dalila Petta<sup>2 †</sup>, Corinne Illoul<sup>1</sup>, Bernard Haye<sup>1</sup>, Anastasiia**  
7 **Markitantova<sup>1</sup>, David Eglin<sup>2</sup>, Gervaise Mosser<sup>1</sup>, Matteo D'Este<sup>2</sup>, Christophe Héлары<sup>1 \*</sup>**  
8

9  
10 1. Sorbonne Université, CNRS, Laboratoire de Chimie de la Matière Condensée de Paris,  
11 4 Place Jussieu, 75005 Paris, France  
12

13 2. AO Research Institute Davos, Davos Platz, Switzerland  
14

15 †: These authors have contributed equally to this work

16 \*: Corresponding author: Christophe Héлары

17 Email: [christophe.helary@upmc.fr](mailto:christophe.helary@upmc.fr)  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41

## Abstract

42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65  
66

A platform of enzymatically-crosslinked Collagen/Tyramine hyaluronan derivative (Col/HA-Tyr) hydrogels with tunable compositions and gelation conditions was developed to evaluate the impact of the preparation conditions on their physical, chemical and biological properties. At low HA-Tyr content, hydrogels exhibited a fibrillar structure, with lower mechanical properties compared to pure Col hydrogels. At high HA-Tyr and Horse Radish Peroxydase (HRP) content, a microfibrillar network was formed beside the banded Col fibrils and a synergistic effect of the hybrid structure on mechanical properties was observed. These hydrogels were highly resistant against enzymatic degradation while keeping a high degree of hydration. Unlike HA-Tyr hydrogels, encapsulation of human dermal fibroblasts within Col/HA-Tyr hydrogels allowed for high cell viability. These results showed that high HA-Tyr and HRP concentrations are required to positively impact the physical properties of hydrogels while preserving collagen fibrils. Those Col/HA-Tyr hydrogels appear promising for novel tissue engineering applications following a biomimetic approach.

**Key words:** Collagen, hyaluronan, enzymatic cross-linking, composite hydrogels, fibrillogenesis.

67

## 68 **1. Introduction**

69 Hyaluronan (HA) and Collagen (Col) are key components of connective tissues fundamental  
70 towards determining structure and properties of the extracellular matrix (ECM). Owing to  
71 their established biocompatibility, biodegradability, and ability to interact with cells (H. Kim  
72 et al., 2017; Zeltz & Gullberg, 2016a) they are often selected as materials in biomedical  
73 applications. Type I Col is the most abundant protein of connective tissues such as bone, skin  
74 or tendons (Antoine, Vlachos, & Rylander, 2014; Dong & Lv, 2016). It allows for cell adhesion,  
75 proliferation and synthesis of biomolecules favoring tissue repair (Helary, Zarka, & Giraud-  
76 Guille, 2012). Col materials which allow for cell encapsulation are physical fibrillar hydrogels  
77 (Bell, Ivarsson, & Merrill, 1979) where presence of fibrils improves cell adhesion and  
78 orientate phenotype towards physiological behavior (Doyle & Yamada, 2016; Jokinen et al.,  
79 2004). Usually, cellularized Col based hydrogels display poor mechanical properties, rapid  
80 degradation and weak stability due to the cell-mediated hydrogel contraction (Holder et al.,  
81 2018).

82 Hyaluronic acid (HA) is a natural polysaccharide and a non-sulphated glycosaminoglycan  
83 fundamental for hydration and structure of biological tissues; additionally, it plays a  
84 fundamental role in tissue regeneration and other biological processes (Garg, 2004). HA has  
85 a long track record of clinical use as dermal filler (Yeom et al., 2010), viscosupplement in  
86 osteoarthritis (Strauss, Hart, Miller, Altman, & Rosen, 2009), biomaterial to promote wound  
87 healing and in ophthalmic treatments (H. Kim et al., 2017). To form a matrix with  
88 viscoelasticity similar to ECM, this polysaccharide has to be functionalized and cross-linked,  
89 for example through covalent bonds by employing chemical, enzymatic or photochemical  
90 mechanisms (Lopez-Ruiz et al., 2019). Most often chemical cross-linking uses toxic reagents

91 and harsh conditions not suitable for cell encapsulation (Lopez-Ruiz et al., 2019). Photo-  
92 induced cross-linking requires the utilization of UV light which triggers the formation of free  
93 radicals, toxic for cells (Loebel et al., 2017). In contrast, enzymatic cross-linking usually  
94 occurs under conditions compatible with cell encapsulation (Khunmanee, Jeong, & Park,  
95 2017). The Tyramine derivative of HA (HA-Tyr) can be cross-linked by Horse Radish  
96 Peroxidase (HRP) and hydrogen peroxide ( $H_2O_2$ ). HA-Tyr physical properties can be tuned  
97 through the modulation of the degree of substitution, the degree of cross-linking and the HA  
98 gelling kinetic mediated by the  $H_2O_2$  and HRP concentrations (Bell et al., 1979; Loebel,  
99 D'Este, Alini, Zenobi-Wong, & Eglin, 2015; Loebel et al., 2017). Because of its scarce ability to  
100 promote cell adhesion, HA-Tyr hydrogels have been functionalized with RGD moieties or  
101 gelatin (Petta, Grijpna, Alini, Eglin, & D'Este, 2018).

102 Col/HA hybrid hydrogels have been developed to take advantage of the properties of both  
103 polymers and circumvent their respective drawbacks (Raia et al., 2017). Preparation of  
104 hybrid hydrogels require the functionalization of Col and/or HA without negatively affecting  
105 cell encapsulation. Col-hydroxy benzoic acid and HA-Tyr were used to form a hybrid hydrogel  
106 by coupling their phenol moieties (Ying et al., 2019). Here, mechanical and swelling  
107 properties were impaired by the formation of the hybrid network (Ying et al., 2019). A  
108 maleilated Col in combination with thiol-modified HA coupled through Michael addition was  
109 also described (Li et al., 2017). Resulting hydrogels exhibited high mechanical properties but  
110 the process of fabrication inhibited fibrillogenesis (Davidenko, Campbell, Thian, Watson, &  
111 Cameron, 2010; Ying et al., 2019). Hence, they do not mimic the morphology of native ECMs.

112 Cross-linked biomaterials obtained from blended HA and Col were developed, but led to  
113 heterogeneous materials (Z. H. Kim et al., 2015). Composite hydrogels exhibiting an  
114 interpenetrating network of Col and HA were reported. However, when EDC cross-linker was

115 used to form collagen hydrogels, fibrillogenesis was also inhibited (Collin et al., 2011).  
116 Without cross-linkers, it was possible to incorporate a Col fibrillar network within a non-  
117 fibrillar network made of HA-PEG diacrylate. By driving the Col fibrillogenesis faster, the  
118 mechanical properties were increased by Col, but the presence of chemicals to trigger gelling  
119 could be harmful for cells (Walimbe, Calve, Panitch, & Sivasankar, 2019).  
120 To date, a one-pot fabrication of Col/HA hydrogels preserving collagen fibrillogenesis and  
121 the capability of encapsulating cells has not been described. In addition, no systematic study  
122 has been performed to understand the impact of one biopolymer (type and quantity) on the  
123 other during the gelling process to find the best conditions for an optimal composite  
124 hydrogel.

125 **The aim of this study was to evaluate the impact of compositions and gelling parameters on**  
126 **Col/HA-Tyr hydrogels physical properties.** The final goal of this work was to discover the  
127 adequate conditions relevant for specific applications in tissue engineering following a  
128 biomimetic approach and in absence of any synthetic cross-linkers. Tuning the mechanical  
129 properties while preserving the fibrillar form of Col and a high degree of hydration was the  
130 main challenge of this physico-chemical study. Using a one-pot synthesis, the effect of HA-  
131 Tyr content, the HRP concentration and the degree of cross-linking on the gel formation, Col  
132 fibrillogenesis, physical properties and cell viability was evaluated.

133

## 134 **2. Materials and methods**

### 135 **2.1 Materials.**

136 Hyaluronic acid sodium salt from Streptococcus equi with low weight-average molecular  
137 weight  $M_w = 290$  kDa and dispersity index  $\mathcal{D} = M_w/M_n = 1.86$ , where  $M_n$  is the number-  
138 average molecular weight was purchased from Contipro Biotech s.r.o. (Czech Republic). 4-

139 (4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) from TCI  
140 Europe N.V. (Tokyo, Japan), tyramine hydrochloride (Tyr), hydrogen peroxide, horseradish  
141 peroxidase, NaOH and phosphate buffered saline tablets were purchased from Sigma-  
142 Aldrich (St. Louis, U.S.A.). Dulbecco's Modified Eagle's Medium (DMEM), fetal calf serum,  
143 trypsin EDTA, fungizone, penicillin/streptomycin and Alamar Blue reagents were purchased  
144 from Life Technologies (Courtaboeuf, France).

145

## 146 **2.2 Collagen preparation**

147 Type I Collagen (Col) was extracted from young Wistar rat tails and purified as previously  
148 described (Gobeaux et al., 2008). Col purity was assessed by SDS-PAGE electrophoresis and  
149 the concentration was measured by hydroxyproline titration (Bergman & Loxley, 1970).  
150 Stock solutions of type I Col in 17 mM acetic acid concentrated at 0.6% (w/v) or 0.875%  
151 (w/v) were used to fabricate composites.

152

## 153 **2.3 Hyaluronan conjugation with tyramine**

154 Conjugation of Hyaluronan (HA) with tyramine was performed by amidation reaction  
155 between HA carboxylic groups and the amine group of Tyramine Hydrochloride (Tyr).  
156 Hyaluronic acid sodium salt (2 g, 5 mmol carboxylic groups) was dissolved overnight in 200  
157 mL of ultrapure water at a final concentration of 1% (w/v). The following day, the HA  
158 solution was warmed up to 37 °C using a thermostatic oil bath. Five mmol of 4-(4,6-  
159 dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were added to the  
160 HA solution. Subsequently, 5 mmol of Tyr were dissolved in ca. 5 mL dH<sub>2</sub>O and added  
161 dropwise. The whole mixture was stirred at 37 °C for 24 h. Following the addition of 32 mL of

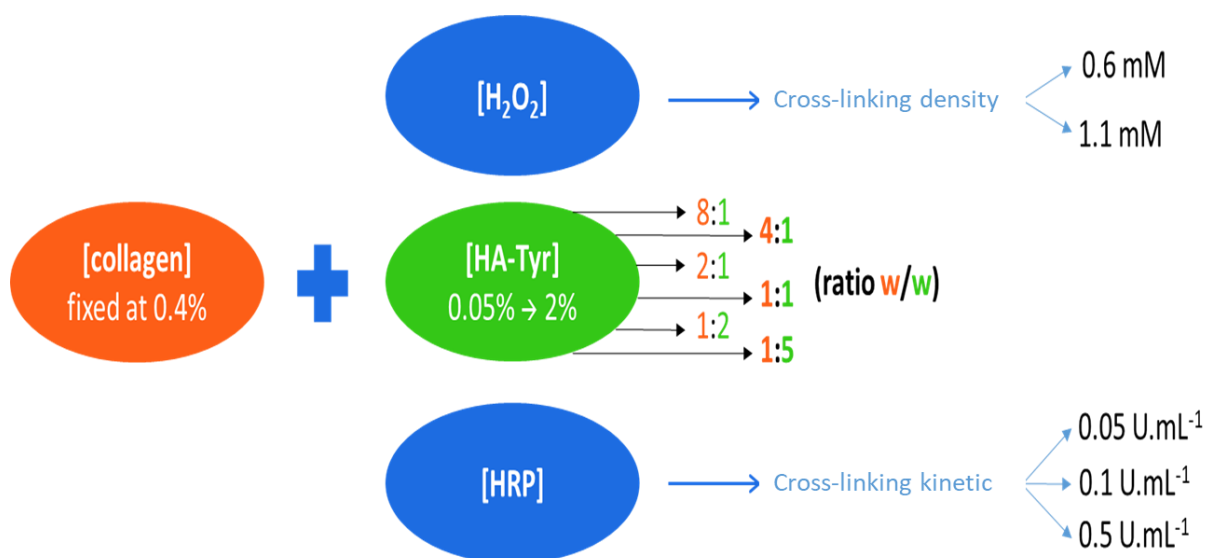


162 a NaCl saturated solution and a 30 min stirring, the newly formed HA-Tyr was precipitated by  
163 adding dropwise 96% alcohol. After several washes, the precipitate was collected by  
164 filtration under vacuum with a Gooch filter P3 and dried at 40 °C for 48h. To detect salt  
165 residues 0.1 M silver nitrate was used. Synthesized HA-Tyr conjugates were characterized  
166 using UV-vis spectroscopy as reported previously (Loebel et al., 2015; Loebel et al., 2017).  
167 The molar degree of substitution ( $DS_{mol}$ , %) was 6%, calculated by measuring the absorbance  
168 at 275 nm of a 0.1% (w/v) HA-Tyr solution in ultrapure water using a Cary 5000 UV-Vis-NIR  
169 Spectrophotometer (Agilent Technologies).

170

#### 171 **2.4 Col/HA-Tyr hydrogels synthesis**

172 Several combinations of Col and HA-Tyr were prepared as follows. Stock HA-Tyr solutions  
173 concentrated at 3% and 6% and Col solutions concentrated at 0.6 and 0.875% were used. For  
174 all hydrogels, the final Col concentration was kept constant at 0.4%. HA-Tyr was mixed with  
175 the Col solution to generate hydrogels with Col/HA-Tyr weight ratio of 8:1, 4:1, 2:1, 1:1, 1:2  
176 and 1: 5 respectively (Figure 1 and S.I 1). For a 1 mL hydrogel, Col gelling was triggered by pH  
177 increase up to 7 using 100  $\mu$ L of 10X-PBS and 40  $\mu$ L of 0.1 M NaOH. The gelling kinetic of HA-  
178 Tyr was tuned using 3 different HRP concentrations: 0.05, 0.1 and 0.5 U mL<sup>-1</sup>. H<sub>2</sub>O<sub>2</sub> at a final  
179 concentration of 0.6 or 1.1 mM was added to explore the effect of HA-Tyr cross-linking  
180 densities (di-tyramine bond formation). Typically, the formation of the Col/HA-Tyr hydrogels  
181 was performed by adding the HA-Tyr with HRP, 10X Phosphate Buffered Saline (10x PBS) and  
182 0.1 M Sodium hydroxide (NaOH). After an overnight incubation at 4 °C, Col was added to the  
183 mixture and HA-Tyr gelling triggered by H<sub>2</sub>O<sub>2</sub> addition. Last, the plates were incubated at 20  
184 °C.



185

186 **Figure 1: Formulations to synthesize Col/HA-Tyr hydrogels.**

187 **2.5 Rheological measurements**

188 Shear oscillatory measurements were performed on hydrogels using an Anton Paar  
 189 rheometer MCR302 fitted with a 25 mm sand-blasted parallel plate upper geometry. All tests  
 190 were performed at 20 °C with frequency sweeps. Mechanical spectra, namely storage  $G'$  and  
 191 loss  $G''$  moduli versus frequency, were recorded at an imposed 1% strain, which  
 192 corresponded to non-destructive conditions, as previously checked with an amplitude sweep  
 193 (data not shown). In order to test all hydrogels in the same conditions, before each run the  
 194 gap between base and geometry was chosen so that a slight positive normal force was  
 195 applied on gels during measurement: respectively 0.04 N and 0.1 N. At least six samples per  
 196 hydrogel type were tested.

197

198 **2.6 Scanning electron microscopy (SEM) analysis**

199 Hydrogels were fixed in 4% paraformaldehyde in PBS (w/v) for 24 h, then washed three  
 200 times with ultrapure water (10 min each) and freeze-dried overnight. **Each sample was torn**

201 prior to SEM observation to see the inner structure of composite hydrogels. Samples were  
202 coated with a 15 nm-gold-layer and imaged using a Hitachi S-3400N scanning electron  
203 microscope operating at 10 kV. For each sample, pictures were acquired at magnification  
204 X10000.

205

## 206 **2.7 Transmission electron microscopy (TEM)**

207 Standard fixation of hydrogels was performed first in PFA 4% (w/v) for 24 h, then in 4%  
208 glutaraldehyde (w/v) for 1 hour at +4 °C. Samples were post fixed using 2% osmium tetra-  
209 oxide (w/v) in cacodylate/saccharose buffer (0.05 M/0.3M, pH 7.4) for 1 hour at 4 °C, then  
210 dehydrated with increasing baths of ethanol and propylene oxide. Last, the hydrogels were  
211 embedded in araldite. Thin araldite transverse ultra-thin sections (70 nm) were performed  
212 using a Leica EM UC7 ultramicrotome and contrasted with 0.5% (w/v) uranyl acetate.  
213 Sections were then observed with a Cryo-microscope Tecnai spirit G2 electron microscope  
214 operating at 120 kV. For each hydrogel, photos were taken at magnification X15000 on a  
215 CCD Camera (Orius Gatan 832 digital) and analyzed.

216

## 217 **2.8 Differential scanning calorimetry (DSC)**

218 10-30 mg of freeze-dried hydrogels were rehydrated with 15  $\mu$ L of PBS and they were  
219 analyzed using a modulated DSC TA Q20. Standby temperature was set at 20 °C.  
220 Temperature was increased from 10 °C to 80 °C at a rate of 5 °C min<sup>-1</sup>. Data were analyzed  
221 using TA Universal Analysis software (n=4).

222

## 223 **2.9 Swelling properties**

224 Hydrogels were freeze-dried overnight and their dried weight (WL) was measured. Dried  
225 samples were then incubated in PBS (10 mM, pH 7.4) under stirring at 37 °C. At different  
226 time points (1 and 14 days), swollen hydrogels were collected, carefully blotted to remove  
227 the excess of surface liquid, and their swelled weight (WS) measured. The corresponding  
228 swelling ratio was calculated using the following formula:

$$229 \text{ Swelling Ratio} = (WS - WL) / WL$$

230

## 231 **2.10. *In vitro* accelerated degradation.**

232 Col/HA-Tyr hydrogels were incubated in PBS for 72 h to reach the swelling equilibrium. Then,  
233 the mass of the samples (WS) was measured. Degradation assay was performed incubating  
234 the pre-swollen hydrogels in 2 mL PBS with 10 Units mL<sup>-1</sup> hyaluronidase and 30 Units mL<sup>-1</sup>  
235 collagenase at 37 °C under stirring. Remaining masses were recorded after 1, 6, 24, 48, 96  
236 hours (n=4 for each time point). For this purpose, hydrogels were removed from wells,  
237 carefully blotted to remove excess surface liquid and the total weight (WDegr) was  
238 measured. The percentage of hydrogels mass remaining was calculated in relation to the  
239 original swollen mass: ((WDegr/WS) x 100). A fresh solution containing both hyaluronidase  
240 and collagenase was added every 24 hours. Hydrogels incubated in PBS without enzymes at  
241 37 °C were used as controls for the degradation kinetic.

242

## 243 **2.11 Fibroblast cell culture**

244 Normal Human Dermal Fibroblasts (NHDF) were cultured in complete cell culture medium  
245 (DMEM supplemented with 10% fetal bovine serum, 100 U mL<sup>-1</sup> penicillin, 100 μg mL<sup>-1</sup>  
246 streptomycin, and 0.25 μg mL<sup>-1</sup> Fungizone) and kept at 37 °C in a 95% air - 5% CO<sub>2</sub>  
247 atmosphere. Cells with 80% confluency were detached with 0.1% trypsin and 0.02% EDTA  
248 and counted using a Malassez Cell.

249

## 250 **2.12 Cell metabolic activity**

251 NHDF were encapsulated within each hydrogel at a final cell density of 200.000  
252 cells/hydrogel. Cells were added to the hydrogel mixture prior to H<sub>2</sub>O<sub>2</sub> addition at 4 °C. After  
253 15 min, 2 mL of complete medium was added and the newly formed hydrogels were then  
254 incubated at 37 °C in a 95% air - 5% CO<sub>2</sub> atmosphere. The 3D cell culture was performed over  
255 7 days using the Col/HA-Tyr ratios 4:1, 1:1 and 1:5. Both cellularized pure Col and HA-Tyr  
256 hydrogels were used as controls.

257 Cell metabolic activity was monitored after 1 and 7 days in culture using Alamar Blue assay.  
258 3D-cellularized Col/HA-Tyr hydrogels were incubated with 300 μL of a resazurin solution at  
259 0.1% (w/v) for 6 hours. The supernatant in each well was then collected, diluted with 700 μL  
260 of fresh medium, and the absorbance measured at λ = 570 nm and λ = 600 nm. The  
261 percentage of resazurin reduction was calculated following the formula provided by the  
262 supplier. Cell metabolic activity of hydrogels was compared to control samples, i.e. cells  
263 cultivated in pure Col hydrogel without HRP and H<sub>2</sub>O<sub>2</sub>. The arbitrary value 100% was given to  
264 control samples.

265

## 266 **2.13 Cell morphology**

267 Morphology of encapsulated cells was observed on histological sections. Hydrogels were  
268 fixed with a PFA 4% (w/v), then dehydrated with ethanol and butanol and embedded in  
269 paraffin. Five micrometer transverse sections were performed using a manual microtome  
270 (Stiassnie, France). Sections were rehydrated and stained with Hemalum for 7 min. Then,  
271 samples were rinsed with dH<sub>2</sub>O and dehydrated again using ethanol and toluene. Last,  
272 sections were mounted between glass and coverslip using an Eukitt mounting medium.  
273 Samples were observed at X400 magnification with a Nikon Eclipse E600 POL equipped with  
274 a Nikon DS-Ri1 camera.

275

## 276 **2.14 Statistical analysis**

277 Results are presented as averages  $\pm$  standard deviation. Statistical significance was assessed  
278 using Mann-Whitney statistical test. The level of significance in all statistical analyses was set  
279 at a probability of  $P < 0.05$ .

280

## 281 **3. Results and discussion**

### 282 **3.1 Composite hydrogels with a high HA-Tyr content exhibit a microfibrillar network and** 283 **banded collagen fibrils**

284 The goal of this work was to obtain Col/HA-Tyr hydrogels with synergistic properties and  
285 mimicking the fibrillar structure of native connective tissues ECM. Earlier studies using  
286 covalently-modified Col and/or chemical cross-linking have highlighted that the interplay

287 between HA-Tyr gel formation and Col fibrillogenesis, under the influence of HA-Tyr/Col  
288 interactions, was a major factor determining the properties of the composite hydrogels.

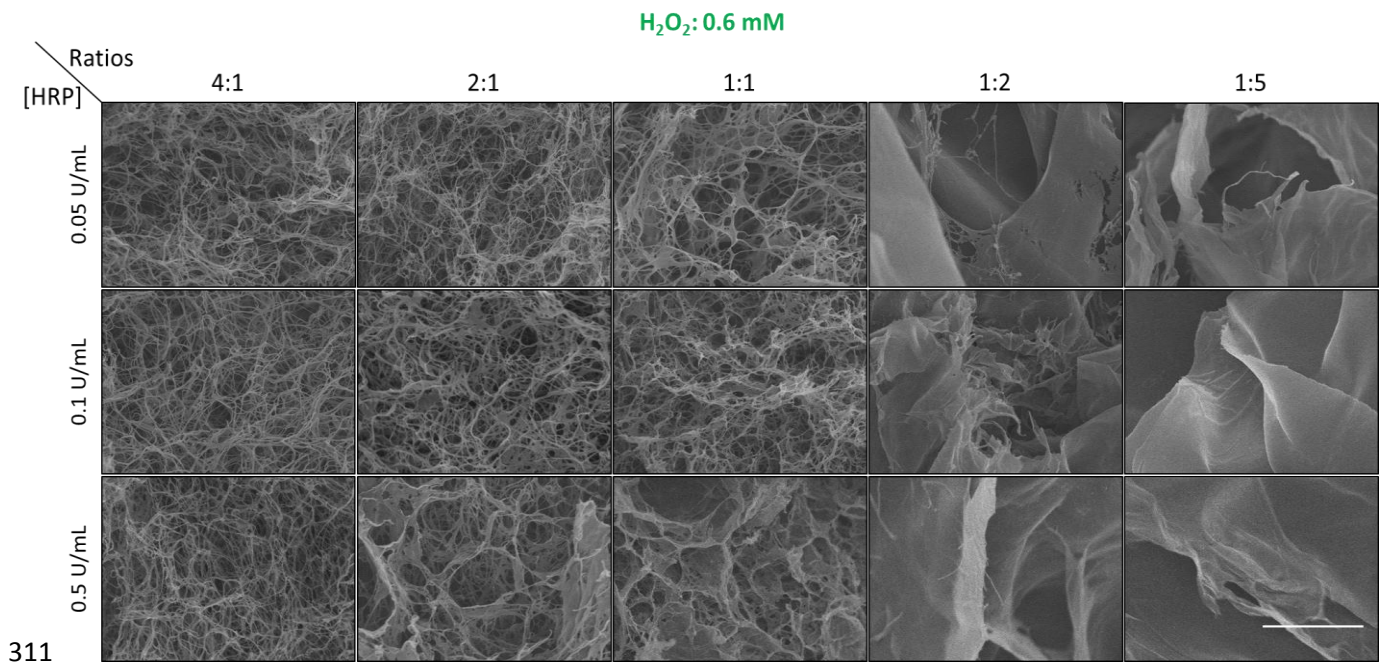
289 In this work, unmodified Col was used, its concentration was fixed at 0.4% (w/v) and its  
290 gelation was induced by neutralization of an acidic solution. HA-Tyr was added in various  
291 amounts and the degree of cross-linking and gelling kinetics, were tuned by the H<sub>2</sub>O<sub>2</sub> and  
292 HRP concentrations, respectively (Loebel et al., 2015).

293 In a first step, the morphology of the hydrogels was examined using scanning electron  
294 microscopy (SEM). Pure Col hydrogels (0.4%) exhibited a typical fibrillar network (S.I. 2A).  
295 When 0.6 mM H<sub>2</sub>O<sub>2</sub> was used, addition of HA-Tyr up to a Col/HA-Tyr 1:1 ratio preserved this  
296 ultrastructure for the lowest HRP concentrations (Figure 2). At higher HA-Tyr content,  
297 sheets-like morphological features reminiscent of the pure HA-Tyr hydrogels were observed  
298 (S.I. 2B). Noticeably, some fibers were still visible at the 1:2 ratio, whereas they were not  
299 visible for higher HA-Tyr content, except for lower HRP concentration, 0.05 U.mL<sup>-1</sup>. For the  
300 highest enzyme concentration, sheet-like structures can be observed from the 1:1 ratio and  
301 remaining fibers were hardly distinguishable at 1:2. Hydrogels formed with 1.1 mM H<sub>2</sub>O<sub>2</sub>  
302 exhibited similar structures and morphological evolution (S.I. 3). Mixtures of Col and HA-Tyr  
303 in the absence of HRP/H<sub>2</sub>O<sub>2</sub> did not exhibit such a fibril-to-sheet transition (S.I. 4).

304 Altogether, the hydrogel morphology as determined via SEM was driven by Col  
305 fibrillogenesis at low ratio and by HA-Tyr gelation for higher polysaccharide content.

306 However, the observation of some Col fibrils in the latter conditions is indicative of partial  
307 Col fibrillogenesis preservation (Kreger et al., 2010). Moreover, the transition from one  
308 morphology to the other shows a strong dependence on the HRP concentration, *i.e.* on the

309 kinetics of the HA-Tyr cross-linking reaction, and, to a lower extent, on H<sub>2</sub>O<sub>2</sub> concentration,  
310 i.e. on the cross-linking density (S.I. 3).



312 **Figure 2. Ultrastructure of Col/HA-Tyr hydrogels observed by scanning electron microscopy.** Cross-  
313 linking performed with 0.6 mM H<sub>2</sub>O<sub>2</sub>. Bar: 5 μm.

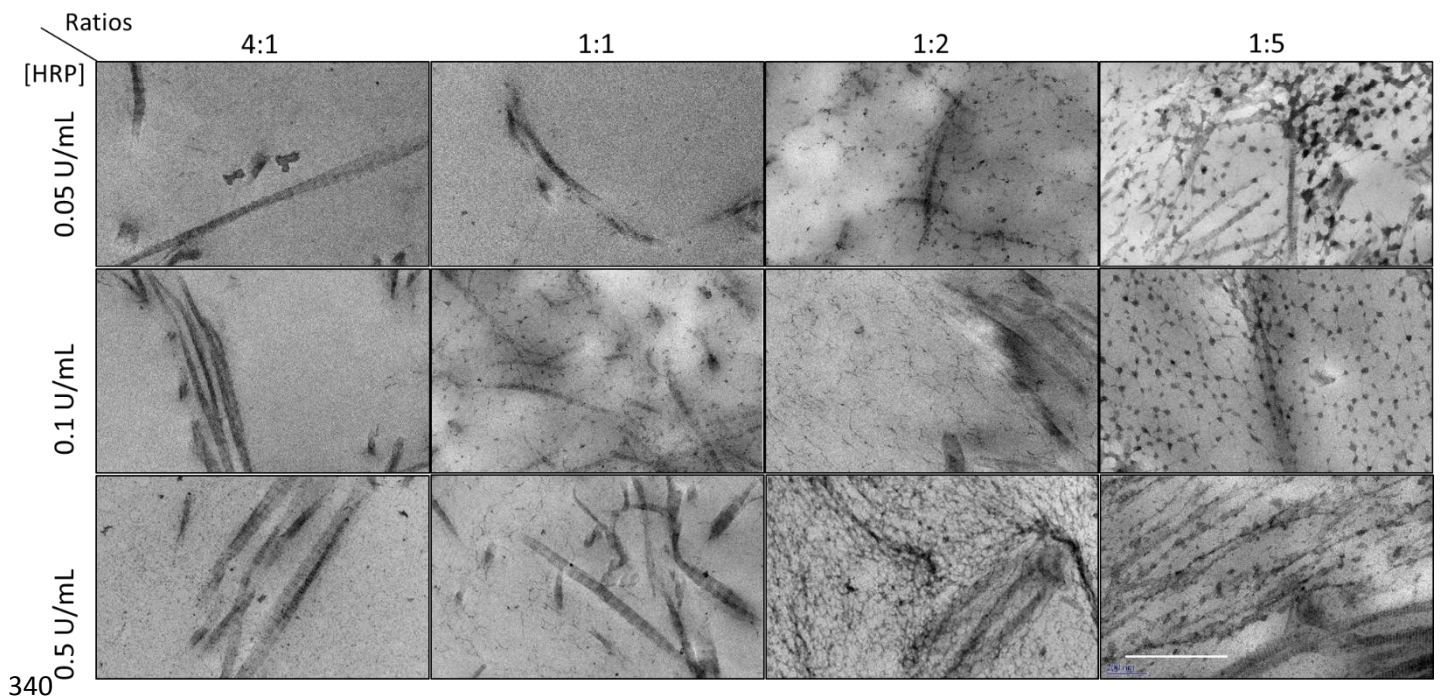
314

315 TEM imaging of hydrogels obtained at 0.6 mM H<sub>2</sub>O<sub>2</sub> confirmed the prevalence of fibrillar  
316 structure of the Col/HA-Tyr hydrogels until the 1:1 ratio, irrespective of the HRP  
317 concentration. Col/HA-Tyr hydrogels exhibited a population of banded fibrils (67 nm) similar  
318 to that of pure Col (Figure 3 and S.I. 2C). From the 1:1 ratio, a microfibrillar network  
319 appeared in hydrogels prepared with HRP 0.1 and 0.5 U.mL<sup>-1</sup>. Above this ratio, microfibrils  
320 were visible in all types of hydrogels and their number increased with HA-Tyr content and  
321 HRP concentration (Figure 3). Despite the presence of the microfibrillar network, typical  
322 collagen banded fibrils of 50 nm in diameter could be observed in all samples, but their  
323 number decreased as the microfibrillar network extended (Figure 3). These microfibrils were  
324 not visible in pure HA-Tyr and Col hydrogels, nor in uncross-linked mixtures, in agreement  
325 with the SEM data (S.I. 2C and 2D). This suggests that the microfibrils originated from the



326 enzymatic cross-linking reaction. At this stage, two mechanisms can be considered in the  
 327 network formation. First, the cross-linking between HA-Tyr chains could create a network  
 328 where Col molecules are confined and fibrillogenesis is limited, leading to smaller fibrils.  
 329 Second, the tyrosine residues in Col could form a covalent bond with tyramine in the  
 330 presence of the HRP/H<sub>2</sub>O<sub>2</sub> catalytic system, thanks to the similarity of the chemical structure  
 331 of the two molecules. The tyramine-tyrosine bonds may hinder fibrillogenesis and again lead  
 332 to microfibrils instead of large banded fibrils (Mazzocchi, Devarasetty, Huntwork, Soker, &  
 333 Skardal, 2018). However, it can be expected that the first mechanism would lead to an  
 334 increase in microfibrils number and a decrease of microfibril size with the HA-Tyr content as  
 335 the network would grow in density. In contrast, in the second mechanism, the number and  
 336 the size of microfibrils are expected to grow at the expense of fibrils, as more HA-Tyr is  
 337 present and more HA-Tyr/Col cross-links can be formed. Eventually, some Col molecules may  
 338 not react with HA-Tyr and could still self-assemble to form fibrils. To further characterize the  
 339 composite, the rheological properties of the hydrogels were studied.

H<sub>2</sub>O<sub>2</sub>: 0.6 mM



341 **Figure 3. Ultrastructure of Col/HA-Tyr hydrogels observed by transmission electron microscopy.**  
 342 Cross-linking performed with 0.6 mM H<sub>2</sub>O<sub>2</sub>. Bar: 500 nm.

343

344 **3.2 Rheological properties of Col/HA-Tyr hydrogels for a range of parameters.**

345 The influence of increasing HA-Tyr amount at fixed H<sub>2</sub>O<sub>2</sub> concentration and various HRP  
 346 contents on the storage G' and loss G'' moduli of hydrogels was studied. In all conditions G'  
 347 was at least 10 times higher than G'', thereby evidencing the physical form of hydrogels was  
 348 preserved in all Col/HA-Tyr composites (Table 1 and 2, S.I. 5).

Composite Hydrogels - [H <sub>2</sub> O <sub>2</sub> ] = 0.6 mM						
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	[H <sub>2</sub> O <sub>2</sub> ] (mM)	[HRP] (U.mL <sup>-1</sup> )	G' (Pa)	G'' (Pa)
Pure Col	0.4%	0%	0	0	252 ± 25	31 ± 2
			0.6	0.05	189 ± 22	24 ± 1
				0.1	209 ± 15	26 ± 1
				0.5	196 ± 32	25 ± 3
8:1	0.4%	0.05%	0.6	0.05	132 ± 14	16 ± 1
				0.1	109 ± 20	15 ± 2
				0.5	110 ± 9	14 ± 1
4:1	0.4%	0.1%	0.6	0.05	124 ± 4	16 ± 1
				0.1	119 ± 13	17 ± 2
				0.5	140 ± 11	15 ± 3
2:1	0.4%	0.2%	0.6	0.05	124 ± 17	16 ± 2
				0.1	121 ± 10	17 ± 2
				0.5	194 ± 19	13 ± 1
1:1	0.4%	0.4%	0.6	0.05	131 ± 48	17 ± 6
				0.1	195 ± 79	18 ± 3
				0.5	369 ± 149	12 ± 5
1:2	0.4%	0.8%	0.6	0.05	45 ± 10	7 ± 2
				0.1	117 ± 66	7 ± 2
				0.5	593 ± 129 *	8 ± 2
1:5	0.4%	2%	0.6	0.05	506 ± 230 *	19 ± 5
				0.1	844 ± 171 *	17 ± 4
				0.5	979 ± 254 *	12 ± 2

349

350

351 **Table 1: Effect of [HRP] on mechanical properties of Col/HA-Tyr composite hydrogels formed with**  
 352 **[H<sub>2</sub>O<sub>2</sub>] at 0.6 mM. (\*: p < 0.05). Comparison between composite hydrogels and pure collagen**  
 353 **hydrogels.**

354

355 Low HA-Tyr content

356 For concentrations below 0.8% (w/v), pure HA-Tyr did not form a stable cross-linked network  
357 regardless of the HRP and H<sub>2</sub>O<sub>2</sub> concentration used (S.I. 5). The storage moduli measured for  
358 Col/HA-Tyr hydrogels formed with a weight ratio below 1:1 were statistically smaller than  
359 those measured for pure Col hydrogels, indicating a slight destabilization of Col hydrogels by  
360 the addition of HA-Tyr, HRP and H<sub>2</sub>O<sub>2</sub> as previously indicated (Docherty, Forrester, Lackie, &  
361 Gregory, 1989).

362 This effect was dependent on HRP concentration and HA-Tyr content (Table 1). For example,  
363 using 0.6 mM H<sub>2</sub>O<sub>2</sub> and 0.5 U.mL<sup>-1</sup> HRP, the elastic modulus decreased to *ca.* 100 Pa with the  
364 8:1 ratio. The storage modulus increased to reach that of pure Col hydrogels (200 Pa) with  
365 the 2:1 ratio and *almost* doubled to reach *ca.* 400 Pa with the 1:1 ratio. At this ratio, the  
366 storage modulus of composites formed with 0.05 U.mL<sup>-1</sup> HRP was still below that of Col.  
367 Additionally, when Col/HA-Tyr mixtures were used without HRP/H<sub>2</sub>O<sub>2</sub>, the generated  
368 hydrogels exhibited rheological properties and an ultrastructure similar to those of pure Col,  
369 thereby evidencing that fibrillogenesis and gel formation occurred (S.I. 4 and S.I. 6).

370 Kuznetsova et al (Kuznetsova, Chi, & Leikin, 1998) showed that polyols can inhibit Col fibril  
371 formation decreasing the mechanical properties of the resulting hydrogels (Christiansen,  
372 Huang, & Silver, 2000; Fratzl et al., 1998). Similar interactions may occur between Col and  
373 HA-Tyr, which is rich in hydroxy groups (Christiansen et al., 2000; Fratzl et al., 1998).

374 However, since the destabilization is affected by the enzymatic cross-linking, tyramine-  
375 tyrosine covalent bonding between HA-Tyr chains and Col triple helices and fibrils cannot be  
376 ruled out (Eastoe, 1955; Gullekson, Lucas, Hewitt, & Kreplak, 2011; Loebel et al., 2015). This  
377 could impair interactions between the fibrils and hinder Col hydrogel formation. This

378 inhibition of Col gel formation diminishes at higher HRP concentration, which can be  
379 attributed to the increase of the HA-Tyr cross-linking kinetics which occurs before collagen  
380 fibrillogenesis.

### 381 High HA-Tyr content

382 At concentrations 0.8% (w/v) and above, pure HA-Tyr forms stable gels. At 1:2 weight ratio,  
383 hybrid hydrogels obtained using HRP at 0.05 and 0.1 U.mL<sup>-1</sup> respectively, exhibited  
384 rheological properties comparable to cross-linked HA-Tyr gels alone and lower than pure Col  
385 hydrogels (Table 1). Again, increasing HRP content increased the shear moduli of the  
386 composite, reaching a storage modulus of 600 Pa for the 1:2 ratio, *i.e* higher than the  
387 addition of the  $G'$  measured in pure Col and pure HA-Tyr. This synergistic effect was also  
388 observed with the 1:5 ratio (Table 1). It is worth noticing that the presence of microfibrils  
389 observed by TEM was correlated to the increase of the storage modulus measured in these  
390 hydrogels. As pointed out above, in such conditions, HA-Tyr hydrogels form before the Col  
391 network. This suggests an interaction between the cross-linked HA-Tyr network and growing  
392 Col fibrils via covalent bond to form a composite hydrogel. This network benefits from both  
393 HA-Tyr/HA-Tyr and HA-Tyr/Col cross-links, which could explain the increase in mechanical  
394 properties.

395 This synergistic effect has not been reported for hybrid gelatin or Col/GAG hydrogels in  
396 which mechanical behavior is driven by the HA hydrogel formation (Lou, Stowers, Nam, Xia,  
397 & Chaudhuri, 2018; Moulisova et al., 2017). The addition of HA was observed to destabilize  
398 the hybrid structure and lower its mechanical properties (Ying et al., 2019). The synergistic  
399 improvement of mechanical properties was not observed in composite HA/Col hydrogels  
400 either. Walimbe *et al.*, showed that a faster Col gelling prior to the HA gel formation was  
401 required to reach high mechanical properties (Walimbe et al., 2019). Here, a slow HA gelling

402 associated with a faster collagen gelling negatively impacted mechanical properties. A  
 403 positive effect of HA is observed only when the HA gelling is fast and is probably due to the  
 404 presence of the microfibrillar network.

Composite Hydrogels - [HRP] = 0.5 U.mL <sup>-1</sup>					
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	[H <sub>2</sub> O <sub>2</sub> ] (mM)	G' (Pa)	G'' (Pa)
Pure Col	0.4%	0%	0.6	196 ± 32	25 ± 3
			1.1	182 ± 44	22 ± 6
8:1	0.4%	0.05%	0.6	110 ± 9	14 ± 1
			1.1	103 ± 3	15 ± 1
4:1	0.4%	0.1%	0.6	140 ± 11	15 ± 3
			1.1	107 ± 7	13 ± 1
2:1	0.4%	0.2%	0.6	194 ± 19	13 ± 1
			1.1	183 ± 22	17 ± 3
1:1	0.4%	0.4%	0.6	369 ± 149	12 ± 5
			1.1	334 ± 30	14 ± 2
1:2	0.4%	0.8%	0.6	593 ± 129	8 ± 2
			1.1	405 ± 37	9 ± 1
1:5	0.4%	2%	0.6	979 ± 254	12 ± 2
			1.1	1540 ± 189 *	17 ± 4

405

406

407 **Table 2: Effect of [H<sub>2</sub>O<sub>2</sub>] on mechanical properties of Col/HA-Tyr Composite Hydrogels formed**  
 408 **with [HRP] at 0.5 U.mL<sup>-1</sup>. (\*: p < 0.05). Comparison between composite hydrogels formed with**  
 409 **0.6 mM and those formed with 1.1 mM.**

410

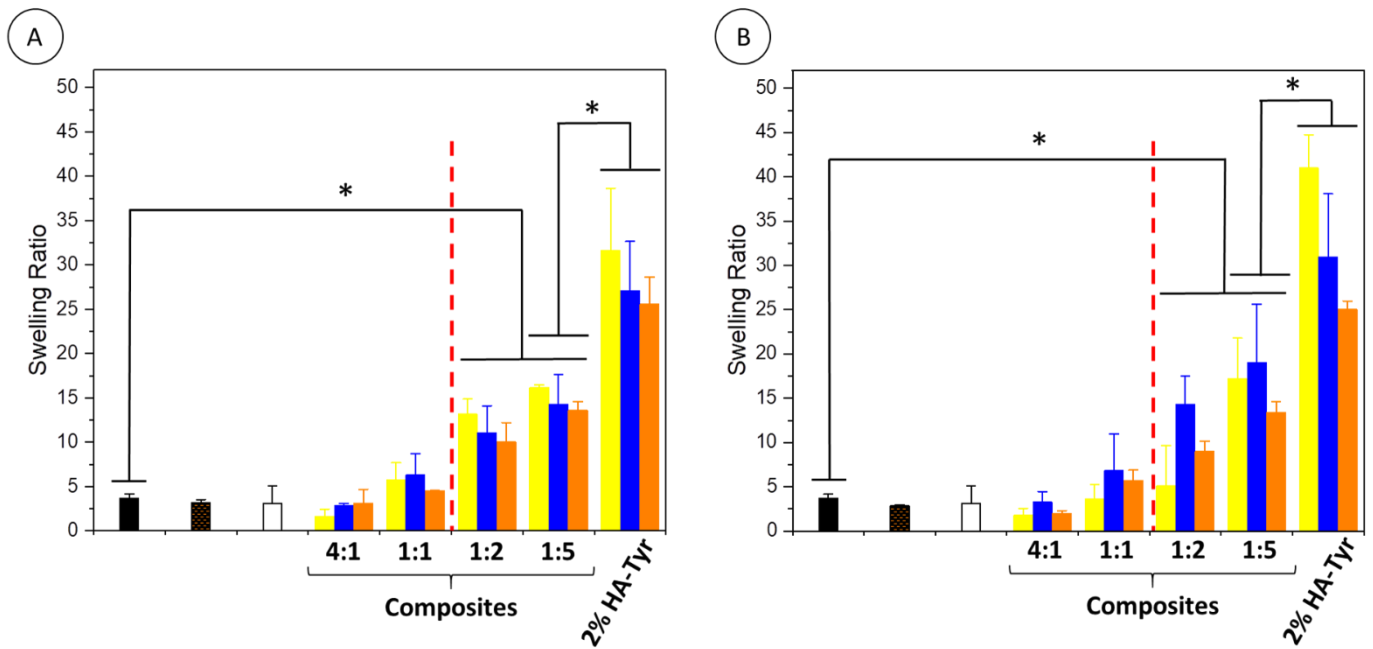
411 When the degree of cross-linking was increased by addition of 1.1 mM H<sub>2</sub>O<sub>2</sub> at 0.5 U.mL<sup>-1</sup>  
 412 HRP, **G' and G'' of the hydrogels did not increased until Col/HA-Tyr 1:2 ratio**, thereby  
 413 suggesting that H<sub>2</sub>O<sub>2</sub> was in stoichiometric excess (**Table 2 and S.I. 5**). In contrast, the storage  
 414 modulus measured in hydrogels with the 1:5 ratio reached 1500 Pa, *i.e* 1.5 times higher than  
 415 that measured in hydrogels formed with 0.6 mM H<sub>2</sub>O<sub>2</sub> (**Table 2**). This shows that the H<sub>2</sub>O<sub>2</sub>  
 416 concentration has an impact on the cross-linking degree and mechanical properties only  
 417 when the highest HA-Tyr content is used (Loebel et al., 2015).

418

### 419 **3.3 Swelling, degradation and thermal properties of Col/HA-Tyr hydrogels**

#### 420 **3.3.1 Swelling properties**

421 The influence of the Col/HA-Tyr ratio on the swelling properties was assessed after 24 hours  
422 of incubation in PBS. The swelling ratio was around 5 for pure Col and 30 for pure HA-Tyr at  
423 2% (w/v), and all composites exhibited values in between. The measured values were in  
424 agreement with previous reports (Helary et al., 2015; Petta, Armiento, et al., 2018; Petta,  
425 Grijpnia, et al., 2018). The higher swelling of HA-Tyr can be attributed to its chemical cross-  
426 linking and the highly hydrophilic nature of HA. H<sub>2</sub>O<sub>2</sub> and HRP addition to pure Col did not  
427 impact its swelling properties, thereby evidencing absent or minor cross-linking in pure Col  
428 hydrogels via tyrosine/tyrosine bonds (Figure 4A and 4B). The swelling ratio measured in the  
429 Col/HA-Tyr hydrogels was similar to that of pure Col until Col/HA-Tyr 1:1 ratio regardless of  
430 the H<sub>2</sub>O<sub>2</sub> concentration and the HRP concentration used (Figure 4A and 4B). HA-Tyr did not  
431 form a gel below the 1:2 ratio, showing that cross-linking is essential to retain water and  
432 increase the swelling properties of hydrogels. These results were confirmed by the Col/HA-  
433 Tyr hydrogels not cross-linked by H<sub>2</sub>O<sub>2</sub> and HRP, which exhibited swelling properties similar  
434 to pure Col. The same swelling behavior was observed after 2 weeks with slightly increased  
435 values (S.I. 7). Thus, surprisingly, swelling and mechanical properties were scarcely  
436 correlated.



437

438 **Figure 4. Swelling properties of Collagen/HA-Tyr hydrogels after one day.** HA-Tyr cross-linking  
 439 performed with 0.6 mM H<sub>2</sub>O<sub>2</sub> (A) and 1.1 mM H<sub>2</sub>O<sub>2</sub> (B). ■ : 0.4 % Col; ■ : 0.4 % Col with 0.5 U mL<sup>-1</sup>  
 440 HRP activity (A and B); □ : 0.4 % Col with 2 % HA-Tyr without any cross-linking agents (H<sub>2</sub>O<sub>2</sub> and  
 441 HRP); ■, ■ and ■ : Col/HA-Tyr hydrogels cross-linked with 0.05, 0.1 and 0.5 U.mL<sup>-1</sup> HRP,  
 442 respectively

443 N = 4. \*: p < 0.05. Mann-Whitney statistical test.

444 **Red dotted line: Minimal Col/HA-Tyr ratio from which HA-Tyr gelling occurs.**

445

446 From the 1:2 ratio, the swelling properties **significantly increased to** reach *ca.* 15 for the

447 Col/HA-Tyr 1:5 (p < 0.05). **This** was due to the addition of large quantities of HA-Tyr and its

448 ability to form hydrogels at these concentrations (Figure 5A and 5B). Nevertheless, Col/HA-

449 Tyr hydrogels formed with the 1:5 ratio exhibit swelling abilities lower than 2% HA-Tyr (p <

450 **0.05**), despite a similar HA-Tyr content. Hence, the presence of Col decreased hydrogel

451 swelling. The microfibrillar network observed in these hydrogels exhibited numerous cross-

452 linking points which could be responsible for the lower swelling properties compared to pure

453 HA. However, these hydrogels are composed of more than 90% water. In paragraph 3.2 we

454 have shown how the [H<sub>2</sub>O<sub>2</sub>] had no impact on the mechanical properties of hydrogels until

455 the 1:2 ratio, this explains the similar abilities of swelling. Mechanical properties increased

456 with the 1:5 ratio when a higher [H<sub>2</sub>O<sub>2</sub>] was used (1.1 mM) but did not impact the swelling  
457 properties. This could be due to the different structure of hydrogels characterized by the  
458 presence of numerous microfibrils in hydrogels formed with 1.1 mM H<sub>2</sub>O<sub>2</sub>. Taken together,  
459 the results show how it is possible to modulate the swelling properties of hydrogels by  
460 tuning the HA-Tyr content (Toh, Lim, Kurisawa, & Spector, 2012).

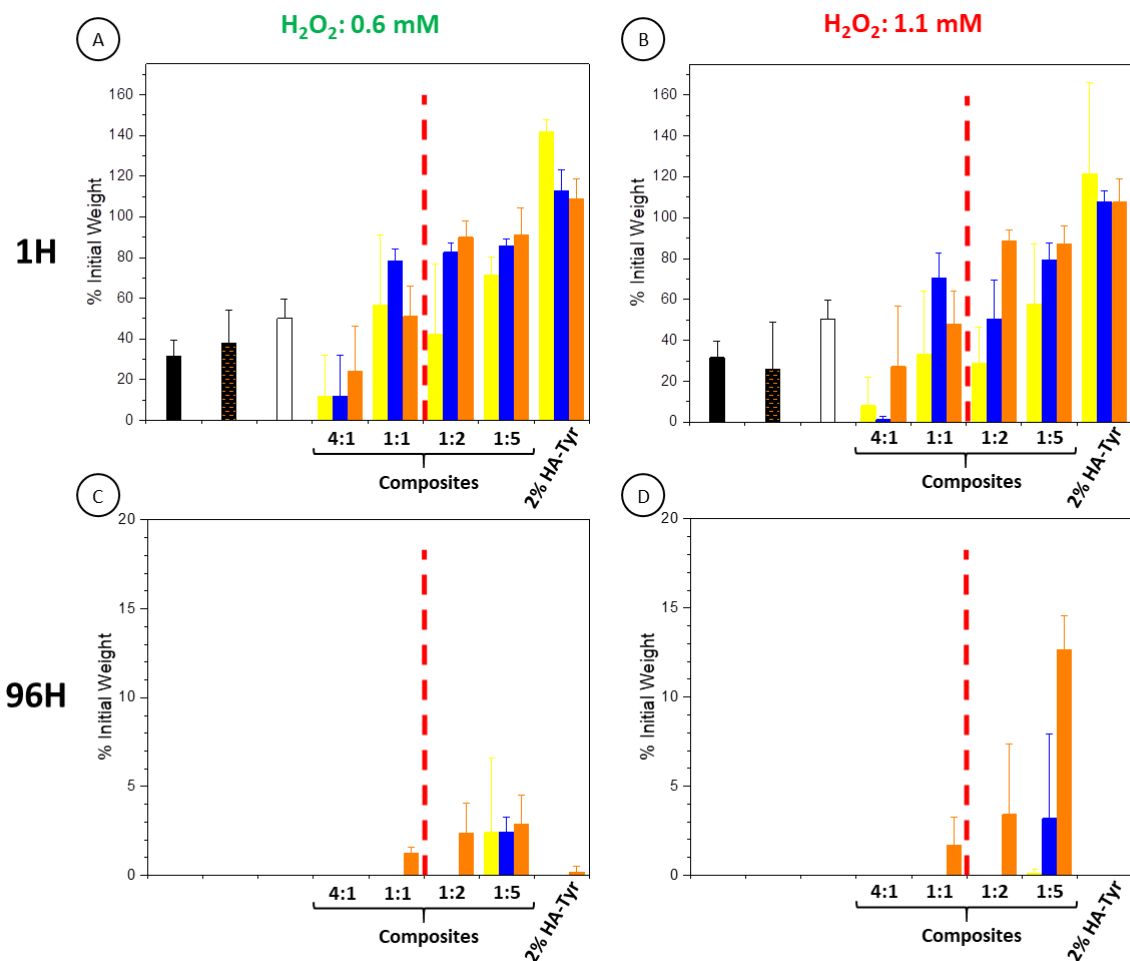
461

### 462 **3.3.2 Enzymatic degradation rate**

463 The degradation by collagenase and hyaluronidase did not reveal any differences between  
464 HRP-cross-linked and not cross-linked pure Col hydrogels regardless of the quantity of H<sub>2</sub>O<sub>2</sub>  
465 (Figure 5A and 5B). After 1 hour of digestion, the remaining mass was around 30%,  
466 confirming once again the absent or minor cross-linking between fibrils via di-tyrosine  
467 bonds. Col/HA-Tyr hydrogels with the 4:1 ratio exhibited a faster degradation than pure Col  
468 except for the 0.5 U.mL<sup>-1</sup> HRP concentration. Hence, hydrogels characterized by weak  
469 mechanical properties were more prone to degradation, as expected. Regarding the other  
470 Col/HA-Tyr hydrogels, the degradation rate was proportional to the HA-Tyr content. In these  
471 cases, the rate was also correlated with the mechanical properties of hydrogels (Table 1 and  
472 3). After six hours of digestion, pure Col hydrogels and not cross-linked Col/HA-Tyr were  
473 completely degraded, which is in agreement with previous studies (Helary et al., 2015).  
474 Hydrogels with the 4:1 ratio were also digested and only hydrogels formed with 0.5 U.mL<sup>-1</sup>  
475 HRP had a residual mass. Hydrogels with the 1:5 ratio (w/w) were more degraded than 2%  
476 HA-Tyr ones despite their identical HA-Tyr content. The Col fibrils could be more prone to  
477 degradation whereas the HA-Tyr network and microfibrils could be more resistant owing to  
478 cross-linking (Figure 3).



479 The long-term analysis of enzymatic degradation revealed that the highest resistance was  
480 observed for Col/HA-Tyr hydrogels with the 1:5 ratio (Figure 5C and 5D). While pure 2% HA-  
481 Tyr and Col hydrogels had completely disappeared after 96 h, hydrogels with the 1: 5 ratio  
482 had a residual mass up to 15%. Besides its positive effect on mechanical properties, the  
483 microfibrillar network seems to protect hydrogels against enzymatic digestion. This network  
484 exhibited numerous cross-linking points which provide a resistance against degradation. In  
485 contrast, pure Col and HA-Tyr hydrogels do not possess comparable mechanical properties  
486 and structure (Helary et al., 2015; Loebel et al., 2017). Composite hydrogels can exhibit  
487 resistance against degradation similar to that of biopolymers on their own because these  
488 materials consist of an interpenetrating network of HA and Col (Walimbe et al., 2019).  
489 Hence, a high [HRP] and HA content to fabricate Col/HA hydrogels overcome these  
490 drawbacks and improve the hydrogel stability thanks to its ultrastructure.



491

492 **Figure 5. Accelerated enzymatic degradation of Col/HA-Tyr hydrogels.** Remaining mass measured  
 493 after 1 hour (A and B) and 96 hours (C and D). ■ : 0.4 % Col; ▨ : 0.4 % Col with 0.5 U mL<sup>-1</sup> HRP  
 494 activity; □ : 0.4 % Col with 2 % HA-Tyr without any cross-linking agents; ■, ■ and ■ : Col/HA-Tyr  
 495 hydrogels cross-linked with 0.6 mM  $H_2O_2$  and 0.05, 0.1 and 0.5 U mL<sup>-1</sup> HRP, respectively.

496

### 497 3.3.3 Differential Scanning Calorimetry

498 The thermal stability of collagen fibrils within composite hydrogels was investigated via DSC.

499 In agreement with literature data, the fibril denaturation in pure Col hydrogels was

500 measured at 55-56 °C (Walton, Brand, & Czernuszka, 2010)(Figure 6C). Until the 2:1 ratio,

501 the Col denaturation peak of the composites was similar to that of pure Col regardless of

502  $H_2O_2$  and HRP concentrations used for HA-Tyr cross-linking (Figure 6A and 6B). For the

503 composites, a shoulder appeared between 45 °C and 52 °C (S.I. 9A) which did not correspond

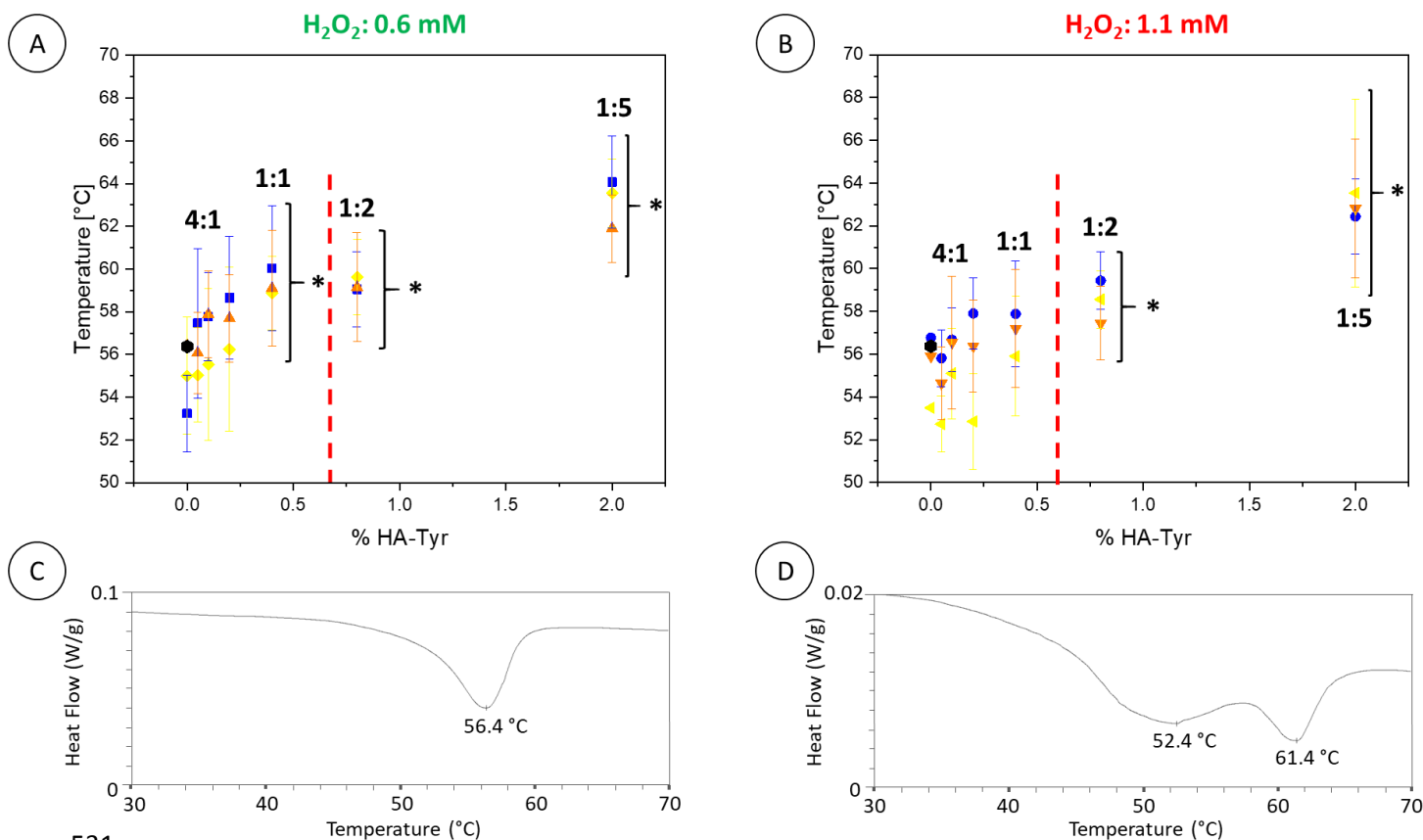
504 to the HRP denaturation temperature (S.I. 9B). As expected, HA-Tyr did not show any

505 denaturation/degradation temperature in this range (S.I. 9C). Rat tail triple helices denature  
506 below 35 °C and chemical cross-linking usually leads to the increase of their denaturation  
507 temperature (Leikina, Mertts, Kuznetsova, & Leikin, 2002). Hence, this shoulder could be  
508 correlated with the stabilization of Col triple helices by cross-linking *via* tyrosine-tyrosine or  
509 tyrosine/tyramine bonds triggered by HRP and H<sub>2</sub>O<sub>2</sub>. The shape of the shoulder suggests the  
510 presence of a heterogeneous population of cross-linked molecules (in number and degrees  
511 of interaction). As the denaturation temperature is below 55 °C, the cross-linking of Col  
512 fibrils cannot be considered. However, the Col cross-linking did not impact the structure,  
513 mechanical and physical properties of these hydrogels. From the 1:2 ratio, two denaturation  
514 peaks appeared: one around 53 °C and another one statistically higher ( $p < 0.05$ ) around 61  
515 °C (Figure 7D).

516 In addition, the thermogram profile did not depend on the H<sub>2</sub>O<sub>2</sub> concentration and the HRP  
517 concentration employed in the enzymatic cross-linking (Figure 7A and 7B). As said above, the  
518 first peak at 52 °C is characteristic of native Col fibril denaturation, thereby evidencing the  
519 absence of cross-linking between fibrils. These results are in agreement with previous  
520 studies which showed that cross-linking of Col fibrils led to their aggregation and a drastic  
521 increase of thermal denaturation, which is not observable in this study (Tian, Liu, & Li, 2016).  
522 The second denaturation temperature can be tentatively associated with the microfibrillar  
523 network. Col triple helices have been stabilized by numerous Col/HA-Tyr covalent bonds,  
524 thereby increasing their denaturation temperature. This temperature depends on the cross-  
525 linking degree of Col (Calderon et al., 2010; Lin & Liu, 2007). Taken together, the results are  
526 suggestive of a microfibrillar network consisting of a hybrid Col/HA-Tyr as this structure was  
527 not visible in pure Col and HA-Tyr hydrogels (S.I. 2B and 2D). In addition, no peak at *ca.* 60-64  
528 °C was observed in these hydrogels (S.I. 9A and 9C).

529

530



531

532 **Figure 6. Denaturation temperature of collagen within Col/HA-Tyr hydrogels assessed by**  
 533 **differential scanning calorimetry.** Peak of denaturation temperature expressed as a function of the  
 534 HA-Tyr content (A and B). Pure 0.4% Col thermogram (C) and thermogram of Col/HA-Tyr hydrogels  
 535 with the 1:5 ratio (w/w) cross-linked with HRP at 0.5 U mL<sup>-1</sup> and 0.6 mM H<sub>2</sub>O<sub>2</sub> (D).  
 536 **●**: 0.4 % Col. **◆**, **■** and **▲**: HA-Tyr cross-linked with 0.6 mM H<sub>2</sub>O<sub>2</sub> and 0.05, 0.1 and 0.5 U.mL<sup>-1</sup> HRP,  
 537 respectively. N = 6. \*: p < 0.05 with a Mann-Whitney statistical test. Comparison between pure Col  
 538 and Col/HA-Tyr Hydrogels.

539

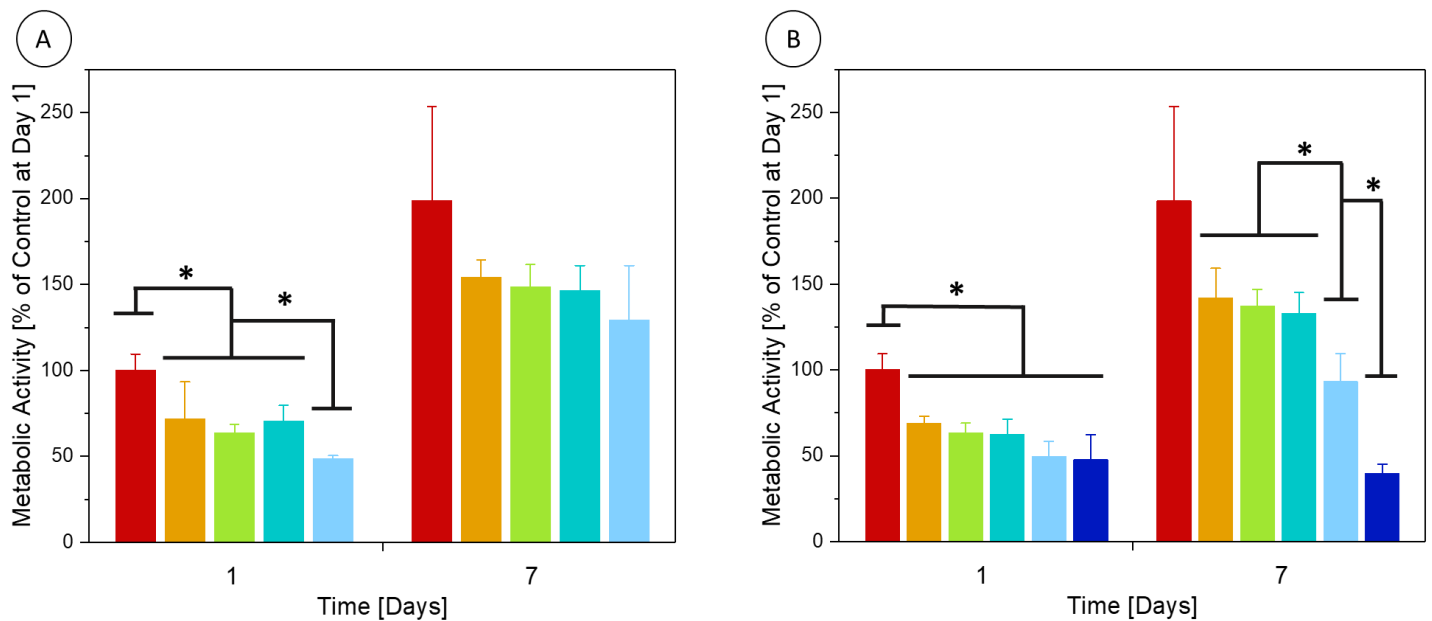
540

### 541 3.4 Behavior of fibroblasts encapsulated within Col/HA-Tyr hydrogels

#### 542 3.4.1 Cell viability

543 Fibroblasts encapsulated within Col hydrogels formed in presence of 0.05 U.mL<sup>-1</sup> HRP and  
 544 0.6 mM H<sub>2</sub>O<sub>2</sub> displayed 75% of the metabolic activity found in pure Col hydrogels without  
 545 cross-linker after 24 h in culture (Figure 7A).

546 Similar cell viability was observed in Col/HA-Tyr hydrogels until the 1:1 ratio. These  
 547 hydrogels have comparable mechanical properties and porosity (Table 1 and Figure 2),  
 548 leading to similar stress during encapsulation. Hydrogels with the 1:5 ratio formed with 0.05  
 549 U.mL<sup>-1</sup> HRP, led to a metabolic activity of 50% compared to that measured in pure Col  
 550 hydrogels (Figure 7A). This could be attributed to scarce cell attachment in the HA-rich  
 551 matrix and to the smaller porosity arising from the microfibrillar network. After one week in  
 552 culture, the fibroblast population doubled regardless of the content of HA-Tyr, thereby  
 553 evidencing all formulations were adequate for cell proliferation (Figure 7A).



554  
 555 **Figure 7. Metabolic activity of Normal Human Dermal Fibroblasts within Col/HA-Tyr hydrogels**  
 556 measured over 7 days. HA-Tyr cross-linking was performed with 0.6 mM H<sub>2</sub>O<sub>2</sub> using a HRP activity at  
 557 0.05 U mL<sup>-1</sup> (A) or 0.5 U.mL<sup>-1</sup> (B). ■: 0.4 % Col; ■: 0.4 % Col with 0.5 U.mL<sup>-1</sup> HRP and H<sub>2</sub>O<sub>2</sub>  
 558 ■: Col/HA-Tyr 4:1 ratio; ■: Col/HA-Tyr 1:1 ratio; ■: Col/HA-Tyr 1:5 ratio (w/w); ■: 2 % HA-Tyr.  
 559 N = 4. \*: p < 0.05: Mann-Whitney test.

560  
 561 Interestingly, no contraction of hydrogels was observed (data not shown). Hence, the  
 562 addition of HA-Tyr stabilized the structure of Col hydrogels against cell-induced contraction.  
 563 It was previously shown that fibroblasts encapsulated within concentrated Col hydrogels

564 (0.3-0.5%) exhibited a synthetic phenotype and high proliferation (Helary et al., 2010; Helary  
565 et al., 2012), promoted by the stiffness of the matrix (Doyle & Yamada, 2016). Fibroblasts  
566 within Col/HA-Tyr hydrogels up to the 1:1 ratio exhibited the same behavior, indicative of  
567 appropriate mechanical properties and adhesion sites. Hydrogels formed with the 1:5 ratio  
568 have higher elastic modulus but less adhesion sites. However, fibroblasts encapsulated in  
569 these hydrogels have the same proliferation ability than that in the other formulations. This  
570 low adhesion to HA-rich hydrogels is generally compensated by the addition of other  
571 molecules (Ghosh, Ren, Shu, Prestwich, & Clark, 2006). Hence the collagen content in our  
572 hydrogels is appropriate to allow for cell adhesion and proliferation.

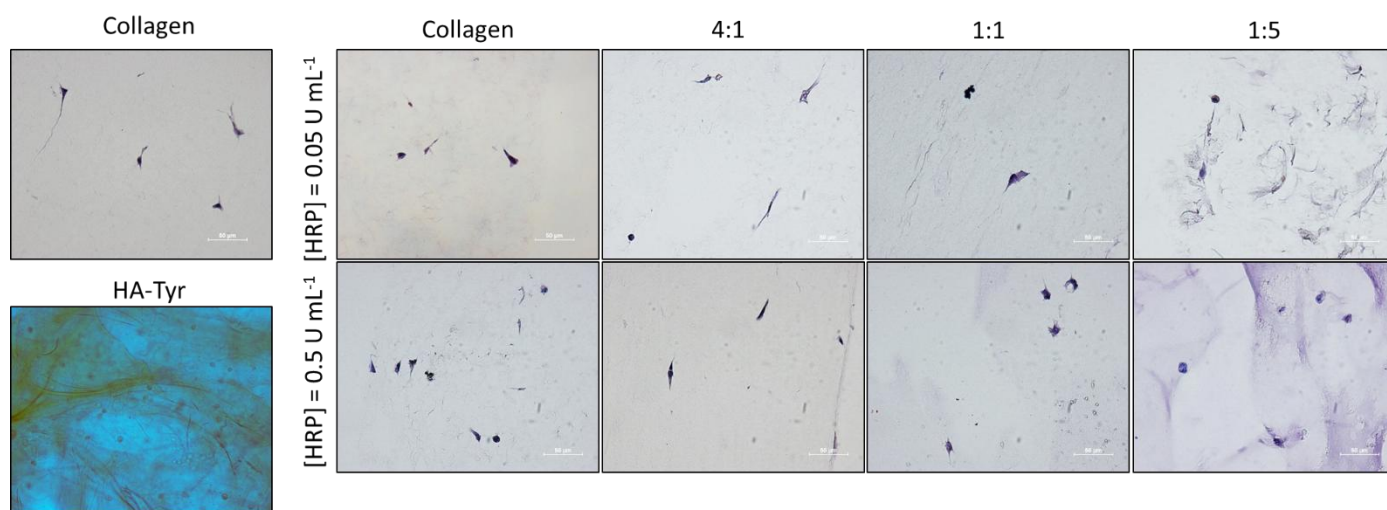
573 Cell viability was not influenced by HRP concentration until the 1:1 ratio (Figure 7B), pointing  
574 out that gel formation kinetic has no impact on cell viability. For the Col/HA-Tyr hydrogels at  
575 1:5 ratio, cells displayed lower metabolic activity at day 1 and 7. These gels are characterized  
576 by elastic modulus around 1000 Pa and a small porosity because of the presence of the  
577 dense microfibrillar network (Figure 3). Hence, porosity and HA content seems to be  
578 determinant for cell adhesion and proliferation for the 1:5 ratio. Interestingly, proliferation  
579 was not observed in pure HA-Tyr hydrogels, evidencing Col is required to allow for cell  
580 adhesion and proliferation (Zeltz & Gullberg, 2016b).

581

### 582 **3.4.2 Fibroblast morphology**

583 Until the 1:1 ratio, fibroblasts within hydrogels exhibited a spindle shape morphology after  
584 one and seven days in culture, characteristic of cells encapsulated within concentrated Col  
585 hydrogels (Figure 8 and S.I. 10) (Helary et al., 2010; Helary et al., 2012). The presence of HA-  
586 Tyr did not impact cell morphology except for the 1:1 ratio using  $0.5 \text{ U.mL}^{-1}$  HRP in which  
587 cells were more rounded (Figure 8). The spindle shape morphology evidenced a strong

588 adhesion to the Col network, allowing for cell spreading. Cells encapsulated in pure HA-Tyr  
 589 and Col/HA-Tyr with the 1:5 ratio did not spread and kept a round shape. This confirms the  
 590 weak adhesion to the HA-Tyr network (Figure 8). These results are in agreement with the  
 591 study of Doyle et al., showing that fibroblast spreading is biopolymer dependent (Doyle &  
 592 Yamada, 2016). In addition, other types of cells such as mesenchymal stem cells do not  
 593 spread in pure HA-Tyr hydrogels (Toh et al., 2012). HA needs to be functionalized with RGD  
 594 peptides or associated with gelatin to allow for cell spreading, proliferation and  
 595 differentiation (Moulisova et al., 2017; Petta, Grijpnia, et al., 2018). However, a strong  
 596 adhesion is not required for stem cells, chondrocytes or NP cells. This is evidenced by their  
 597 rounded shape (Collin et al., 2011). Hence a Col/HA-Tyr 1:5 ratio could be adequate for this  
 598 kind of cells. For fibroblasts, a lower HA content (below 1:1 ratio) is required to get an  
 599 appropriate adhesion and spreading.



600  
 601 **Figure 8. Morphology of normal human dermal fibroblasts encapsulated within Collagen/HA-Tyr**  
 602 **hydrogels after one day.** HA-Tyr cross-linking performed using 0.6 mM H<sub>2</sub>O<sub>2</sub> and HRP concentration  
 603 at 0.05 and 0.5 U mL<sup>-1</sup>. Cells nuclei were stained with Hemalun. Bar: 50 μm.

604

#### 605 4. Conclusions

606 In this study, we introduced a Col/HA composite where fibrillar Col was present within a  
607 continuous cross-linked HA matrix. A range of compositions was explored, identifying that a  
608 minimal Col/HA-Tyr 1:2 ratio and a high [HRP] was required to obtain compositions with  
609 synergistic effect on mechanical properties, thermal stability and resistance against  
610 enzymatic degradation. Ultrastructural analysis confirmed the presence of a bi-phasic  
611 network, where collagen fibrils were present at all ratios. Despite the presence of Col,  
612 hydrogels with a high HA content kept a high degree of hydration. Composition and  
613 structure resemble aspects of natural tissues where HA and Col are present, such as skin,  
614 cartilage, intervertebral disc. As expected, the Col improved cell attachment and survival,  
615 directing toward a more spindle-like cell morphology. Given their cytocompatibility,  
616 structure and physico-chemical properties, the composites here introduced could be used as  
617 starting materials for the fabrication of constructs for tissue engineering applications or in  
618 extrusion-based 3D printing.

619

## 620 **5. Acknowledgments**

621 We thank Dr Francisco Fernandes and Dr Thibaud Coradin for their helpful advices.

622

623

624

625

626

627

628

629



630

## Bibliography

631

- 632 Antoine, E. E., Vlachos, P. P., & Rylander, M. N. (2014). Review of Collagen I Hydrogels for  
633 Bioengineered Tissue Microenvironments: Characterization of Mechanics, Structure, and  
634 Transport. *Tissue Engineering Part B-Reviews*, 20(6), 683-696.
- 635 Bell, E., Ivarsson, B., & Merrill, C. (1979). Production of a tissue-like structure by contraction of  
636 collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl*  
637 *Acad Sci U S A*, 76(3), 1274-1278.
- 638 Bergman, I., & Loxley, R. (1970). The determination of hydroxyproline in urine hydrolysates. *Clin Chim*  
639 *Acta*, 27(2), 347-349.
- 640 Calderon, L., Collin, E., Velasco-Bayon, D., Murphy, M., O'Halloran, D., & Pandit, A. (2010). Type II  
641 Collagen-Hyaluronan Hydrogel - a Step Towards a Scaffold for Intervertebral Disc Tissue  
642 Engineering. *European Cells & Materials*, 20, 134-148.
- 643 Christiansen, D. L., Huang, E. K., & Silver, F. H. (2000). Assembly of type I collagen: fusion of fibril  
644 subunits and the influence of fibril diameter on mechanical properties. *Matrix Biol*, 19(5),  
645 409-420.
- 646 Collin, E. C., Grad, S., Zeugolis, D. I., Vinatier, C. S., Clouet, J. R., Guicheux, J. J., . . . Pandit, A. S. (2011).  
647 An injectable vehicle for nucleus pulposus cell-based therapy. *Biomaterials*, 32(11), 2862-  
648 2870.
- 649 Davidenko, N., Campbell, J. J., Thian, E. S., Watson, C. J., & Cameron, R. E. (2010). Collagen-hyaluronic  
650 acid scaffolds for adipose tissue engineering. *Acta Biomater*, 6(10), 3957-3968.
- 651 Docherty, R., Forrester, J. V., Lackie, J. M., & Gregory, D. W. (1989). Glycosaminoglycans facilitate the  
652 movement of fibroblasts through three-dimensional collagen matrices. *J Cell Sci*, 92 ( Pt 2),  
653 263-270.
- 654 Dong, C., & Lv, Y. (2016). Application of Collagen Scaffold in Tissue Engineering: Recent Advances and  
655 New Perspectives. *Polymers (Basel)*, 8(2).
- 656 Doyle, A. D., & Yamada, K. M. (2016). Mechanosensing via cell-matrix adhesions in 3D  
657 microenvironments. *Experimental Cell Research*, 343(1), 60-66.
- 658 Eastoe, J. E. (1955). The amino acid composition of mammalian collagen and gelatin. *Biochem J*,  
659 61(4), 589-600.
- 660 Fratzl, P., Misof, K., Zizak, I., Rapp, G., Amenitsch, H., & Bernstorff, S. (1998). Fibrillar structure and  
661 mechanical properties of collagen. *J Struct Biol*, 122(1-2), 119-122.
- 662 Garg, H. H., C. & Hale, C. A (2004). *Chemistry and Biology of Hyaluronan* (1st Edition). Elsevier  
663 Science Edition.
- 664 Ghosh, K., Ren, X. D., Shu, X. Z., Prestwich, G. D., & Clark, R. A. F. (2006). Fibronectin functional  
665 domains coupled to hyaluronan stimulate adult human dermal fibroblast responses critical  
666 for wound healing. *Tissue Engineering*, 12(3), 601-613.
- 667 Gobeaux, F., Mosser, G., Anglo, A., Panine, P., Davidson, P., Giraud-Guille, M. M., & Belamie, E.  
668 (2008). Fibrillogenesis in dense collagen solutions: a physicochemical study. *J Mol Biol*,  
669 376(5), 1509-1522.
- 670 Gullekson, C., Lucas, L., Hewitt, K., & Kreplak, L. (2011). Surface-Sensitive Raman Spectroscopy of  
671 Collagen I Fibrils. *Biophysical Journal*, 100(7), 1837-1845.
- 672 Helary, C., Abed, A., Mosser, G., Louedec, L., Letourneur, D., Coradin, T., . . . Meddahi-Pelle, A. (2015).  
673 Evaluation of dense collagen matrices as medicated wound dressing for the treatment of  
674 cutaneous chronic wounds. *Biomater Sci*, 3(2), 373-382.
- 675 Helary, C., Bataille, I., Abed, A., Illoul, C., Anglo, A., Louedec, L., . . . Giraud-Guille, M. M. (2010).  
676 Concentrated collagen hydrogels as dermal substitutes. *Biomaterials*, 31(3), 481-490.
- 677 Helary, C., Zarka, M., & Giraud-Guille, M. M. (2012). Fibroblasts within concentrated collagen  
678 hydrogels favour chronic skin wound healing. *Journal of Tissue Engineering and Regenerative*  
679 *Medicine*, 6(3), 225-237.

680 Holder, A. J., Badiei, N., Hawkins, K., Wright, C., Williams, P. R., & Curtis, D. J. (2018). Control of  
681 collagen gel mechanical properties through manipulation of gelation conditions near the sol-  
682 gel transition. *Soft Matter*, 14(4), 574-580.

683 Jokinen, J., Dadu, E., Nykvist, P., Kapyla, J., White, D. J., Ivaska, J., . . . Heino, J. (2004). Integrin-  
684 mediated cell adhesion to type I collagen fibrils. *J Biol Chem*, 279(30), 31956-31963.

685 Khunmanee, S., Jeong, Y., & Park, H. (2017). Crosslinking method of hyaluronic-based hydrogel for  
686 biomedical applications. *J Tissue Eng*, 8, 2041731417726464.

687 Kim, H., Jeong, H., Han, S., Beack, S., Hwang, B. W., Shin, M., . . . Hahn, S. K. (2017). Hyaluronate and  
688 its derivatives for customized biomedical applications. *Biomaterials*, 123, 155-171.

689 Kim, Z. H., Lee, Y., Kim, S. M., Kim, H., Yun, C. K., & Choi, Y. S. (2015). A composite dermal filler  
690 comprising cross-linked hyaluronic acid and human collagen for tissue reconstruction. *J*  
691 *Microbiol Biotechnol*, 25(3), 399-406.

692 Kreger, S. T., Bell, B. J., Bailey, J., Stites, E., Kuske, J., Waisner, B., & Voytik-Harbin, S. L. (2010).  
693 Polymerization and Matrix Physical Properties as Important Design Considerations for  
694 Soluble Collagen Formulations. *Biopolymers*, 93(8), 690-707.

695 Kuznetsova, N., Chi, S. L., & Leikin, S. (1998). Sugars and polyols inhibit fibrillogenesis of type I  
696 collagen by disrupting hydrogen-bonded water bridges between the helices. *Biochemistry*,  
697 37(34), 11888-11895.

698 Leikina, E., Merts, M. V., Kuznetsova, N., & Leikin, S. (2002). Type I collagen is thermally unstable at  
699 body temperature. *Proc Natl Acad Sci U S A*, 99(3), 1314-1318.

700 Li, R., Cai, Z., Li, Z., Zhang, Q., Zhang, S., Deng, L., . . . Zhou, C. (2017). Synthesis of in-situ formable  
701 hydrogels with collagen and hyaluronan through facile Michael addition. *Mater Sci Eng C*  
702 *Mater Biol Appl*, 77, 1035-1043.

703 Lin, Y. K., & Liu, D. C. (2007). Studies of novel hyaluronic acid-collagen sponge materials composed of  
704 two different species of type I collagen. *Journal of Biomaterials Applications*, 21(3), 265-281.

705 Loebel, C., D'Este, M., Alini, M., Zenobi-Wong, M., & Eglin, D. (2015). Precise tailoring of tyramine-  
706 based hyaluronan hydrogel properties using DMTMM conjugation. *Carbohydrate Polymers*,  
707 115, 325-333.

708 Loebel, C., Stauber, T., D'Este, M., Alini, M., Zenobi-Wong, M., & Eglin, D. (2017). Fabrication of cell-  
709 compatible hyaluronan hydrogels with a wide range of biophysical properties through high  
710 tyramine functionalization. *Journal of Materials Chemistry B*, 5(12), 2355-2363.

711 Lopez-Ruiz, E., Jimenez, G., Alvarez de Cienfuegos, L., Antic, C., Sabata, R., Marchal, J. A., & Galvez-  
712 Martin, P. (2019). Advances of hyaluronic acid in stem cell therapy and tissue engineering,  
713 including current clinical trials. *Eur Cell Mater*, 37, 186-213.

714 Lou, J. Z., Stowers, R., Nam, S. M., Xia, Y., & Chaudhuri, O. (2018). Stress relaxing hyaluronic acid-  
715 collagen hydrogels promote cell spreading, fiber remodeling, and focal adhesion formation in  
716 3D cell culture. *Biomaterials*, 154, 213-222.

717 Mazzocchi, A., Devarasetty, M., Huntwork, R., Soker, S., & Skardal, A. (2018). Optimization of collagen  
718 type I-hyaluronan hybrid bioink for 3D bioprinted liver microenvironments. *Biofabrication*,  
719 11(1), 015003.

720 Moulisova, V., Poveda-Reyes, S., Sanmartin-Masia, E., Quintanilla-Sierra, L., Salmeron-Sanchez, M., &  
721 Ferrer, G. G. (2017). Hybrid Protein-Glycosaminoglycan Hydrogels Promote Chondrogenic  
722 Stem Cell Differentiation. *Acs Omega*, 2(11), 7609-7620.

723 Petta, D., Armiento, A. R., Grijpma, D., Alini, M., Eglin, D., & D'Este, M. (2018). 3D bioprinting of a  
724 hyaluronan bioink through enzymatic-and visible light-crosslinking. *Biofabrication*, 10(4),  
725 044104.

726 Petta, D., Grijpma, D. W., Alini, M., Eglin, D., & D'Este, M. (2018). Three-Dimensional Printing of a  
727 Tyramine Hyaluronan Derivative with Double Gelation Mechanism for Independent Tuning of  
728 Shear Thinning and Postprinting Curing. *Acs Biomaterials Science & Engineering*, 4(8), 3088-  
729 3098.

730 Raia, N. R., Partlow, B. P., McGill, M., Kimmerling, E. P., Ghezzi, C. E., & Kaplan, D. L. (2017).  
731 Enzymatically crosslinked silk-hyaluronic acid hydrogels. *Biomaterials*, 131, 58-67.

732 Strauss, E. J., Hart, J. A., Miller, M. D., Altman, R. D., & Rosen, J. E. (2009). Hyaluronic Acid  
733 Viscosupplementation and Osteoarthritis: Current Uses and Future Directions. *American*  
734 *Journal of Sports Medicine*, 37(8), 1636-1644.

735 Tian, Z. H., Liu, W. T., & Li, G. Y. (2016). The microstructure and stability of collagen hydrogel cross-  
736 linked by glutaraldehyde. *Polymer Degradation and Stability*, 130, 264-270.

737 Toh, W. S., Lim, T. C., Kurisawa, M., & Spector, M. (2012). Modulation of mesenchymal stem cell  
738 chondrogenesis in a tunable hyaluronic acid hydrogel microenvironment. *Biomaterials*,  
739 33(15), 3835-3845.

740 Walimbe, T., Calve, S., Panitch, A., & Sivasankar, M. P. (2019). Incorporation of types I and III collagen  
741 in tunable hyaluronan hydrogels for vocal fold tissue engineering. *Acta Biomater*, 87, 97-107.

742 Walton, R. S., Brand, D. D., & Czernuszka, J. T. (2010). Influence of telopeptides, fibrils and  
743 crosslinking on physicochemical properties of Type I collagen films. *Journal of Materials*  
744 *Science-Materials in Medicine*, 21(2), 451-461.

745 Yeom, J., Bhang, S. H., Kim, B. S., Seo, M. S., Hwang, E. J., Cho, I. H., . . . Hahn, S. K. (2010). Effect of  
746 Cross-Linking Reagents for Hyaluronic Acid Hydrogel Dermal Fillers on Tissue Augmentation  
747 and Regeneration. *Bioconjugate Chemistry*, 21(2), 240-247.

748 Ying, H., Zhou, J., Wang, M., Su, D., Ma, Q., Lv, G., & Chen, J. (2019). In situ formed collagen-  
749 hyaluronic acid hydrogel as biomimetic dressing for promoting spontaneous wound healing.  
750 *Mater Sci Eng C Mater Biol Appl*, 101, 487-498.

751 Zeltz, C., & Gullberg, D. (2016a). The integrin-collagen connection - a glue for tissue repair? *J Cell Sci*,  
752 129(6), 1284.

753 Zeltz, C., & Gullberg, D. (2016b). The integrin-collagen connection - a glue for tissue repair? *Journal of*  
754 *Cell Science*, 129(4), 653-664.

755

756

Table 1

Composite Hydrogels - [H <sub>2</sub> O <sub>2</sub> ] = 0.6 mM					
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	[H <sub>2</sub> O <sub>2</sub> ] (mM)	[HRP] (U.mL <sup>-1</sup> )	G' (Pa)
Pure Col	0.4%	0%	0	0	252 ± 25
			0.6	0.05	189 ± 22
				0.1	209 ± 15
				0.5	196 ± 32
8:1	0.4%	0.05%	0.6	0.05	132 ± 14
				0.1	109 ± 20
				0.5	110 ± 9
4:1	0.4%	0.1%	0.6	0.05	124 ± 4
				0.1	119 ± 13
				0.5	140 ± 11
2:1	0.4%	0.2%	0.6	0.05	124 ± 17
				0.1	121 ± 10
				0.5	194 ± 19
1:1	0.4%	0.4%	0.6	0.05	131 ± 48
				0.1	195 ± 79
				0.5	369 ± 149
1:2	0.4%	0.8%	0.6	0.05	45 ± 10
				0.1	117 ± 66
				0.5	593 ± 129 *
1:5	0.4%	2%	0.6	0.05	506 ± 230 *
				0.1	844 ± 171 *
				0.5	979 ± 254 *

Table 1

Table 2

Composite Hydrogels - [HRP] = 0.5 U.mL <sup>-1</sup>					
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	[H <sub>2</sub> O <sub>2</sub> ] (mM)	G' (Pa)	G'' (Pa)
Pure Col	0.4%	0%	0.6	196 ± 32	25 ± 3
			1.1	182 ± 44	22 ± 6
8:1	0.4%	0.05%	0.6	110 ± 9	14 ± 1
			1.1	103 ± 3	15 ± 1
4:1	0.4%	0.1%	0.6	140 ± 11	15 ± 3
			1.1	107 ± 7	13 ± 1
2:1	0.4%	0.2%	0.6	194 ± 19	13 ± 1
			1.1	183 ± 22	17 ± 3
1:1	0.4%	0.4%	0.6	369 ± 149	12 ± 5
			1.1	334 ± 30	14 ± 2
1:2	0.4%	0.8%	0.6	593 ± 129	8 ± 2
			1.1	405 ± 37	9 ± 1
1:5	0.4%	2%	0.6	979 ± 254	12 ± 2
			1.1	1540 ± 189 *	17 ± 4

Figure 1

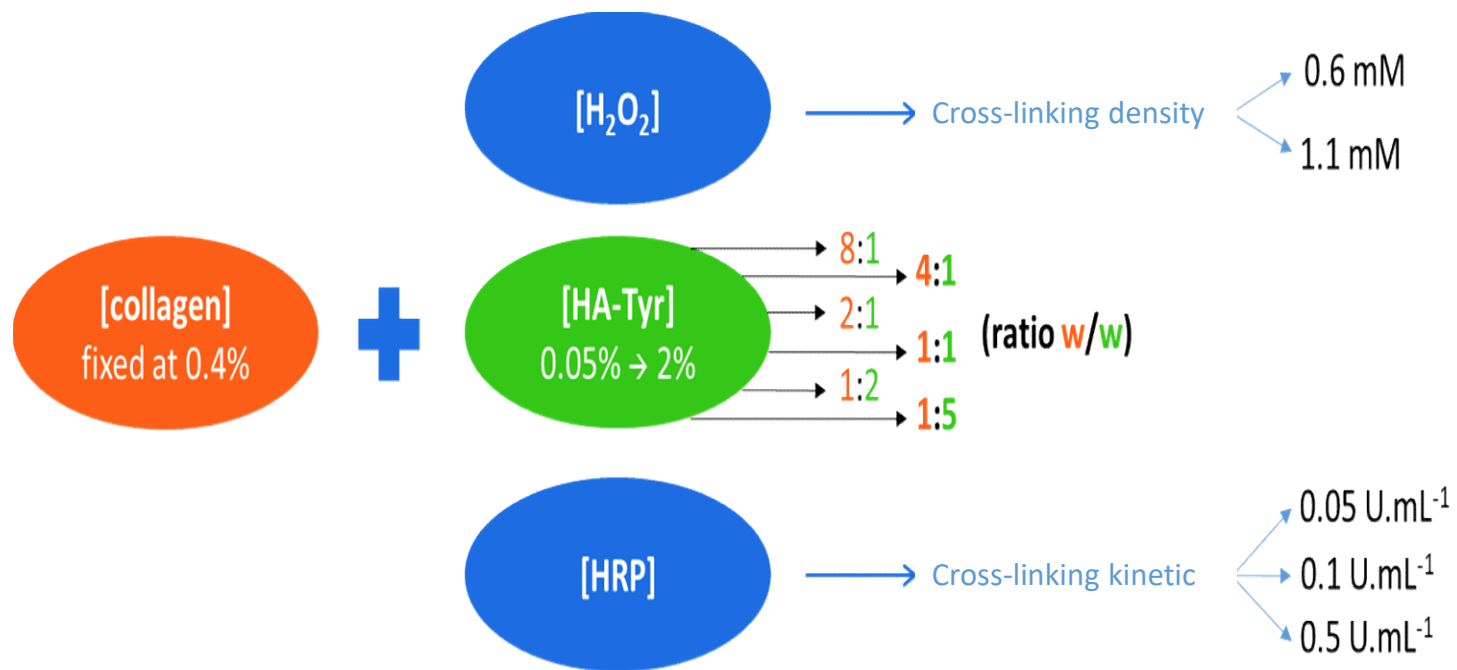


Figure 2

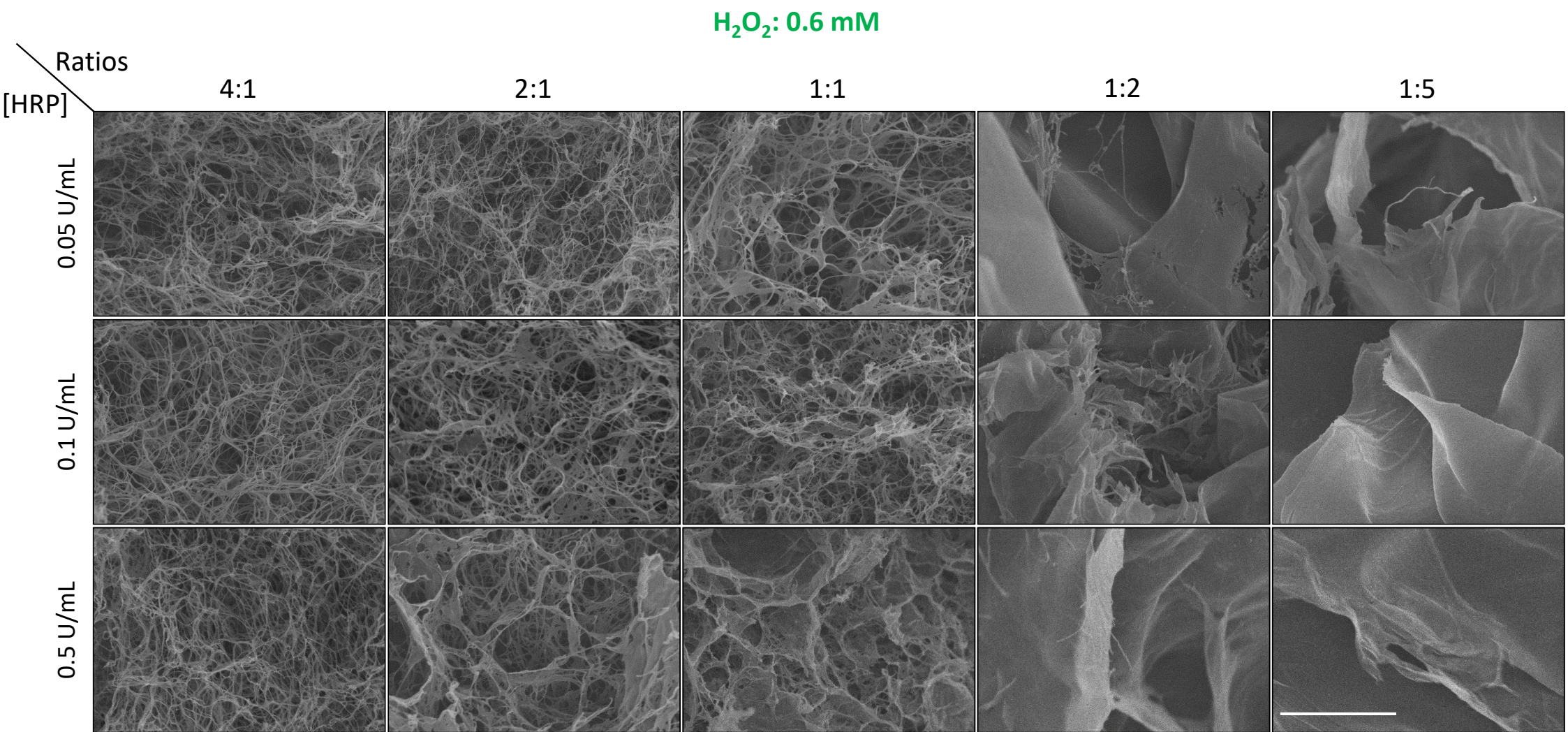




Figure 3

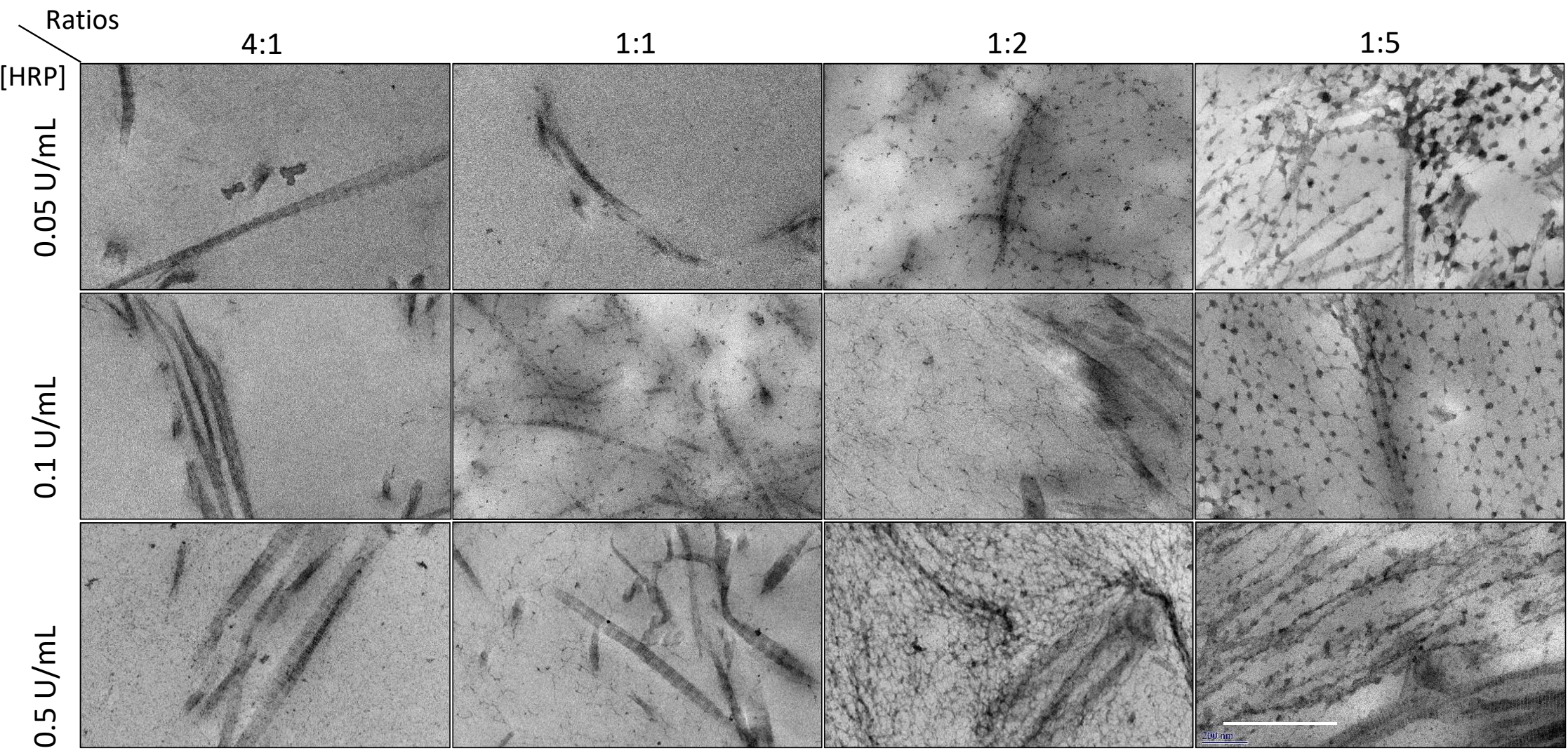




Figure 4

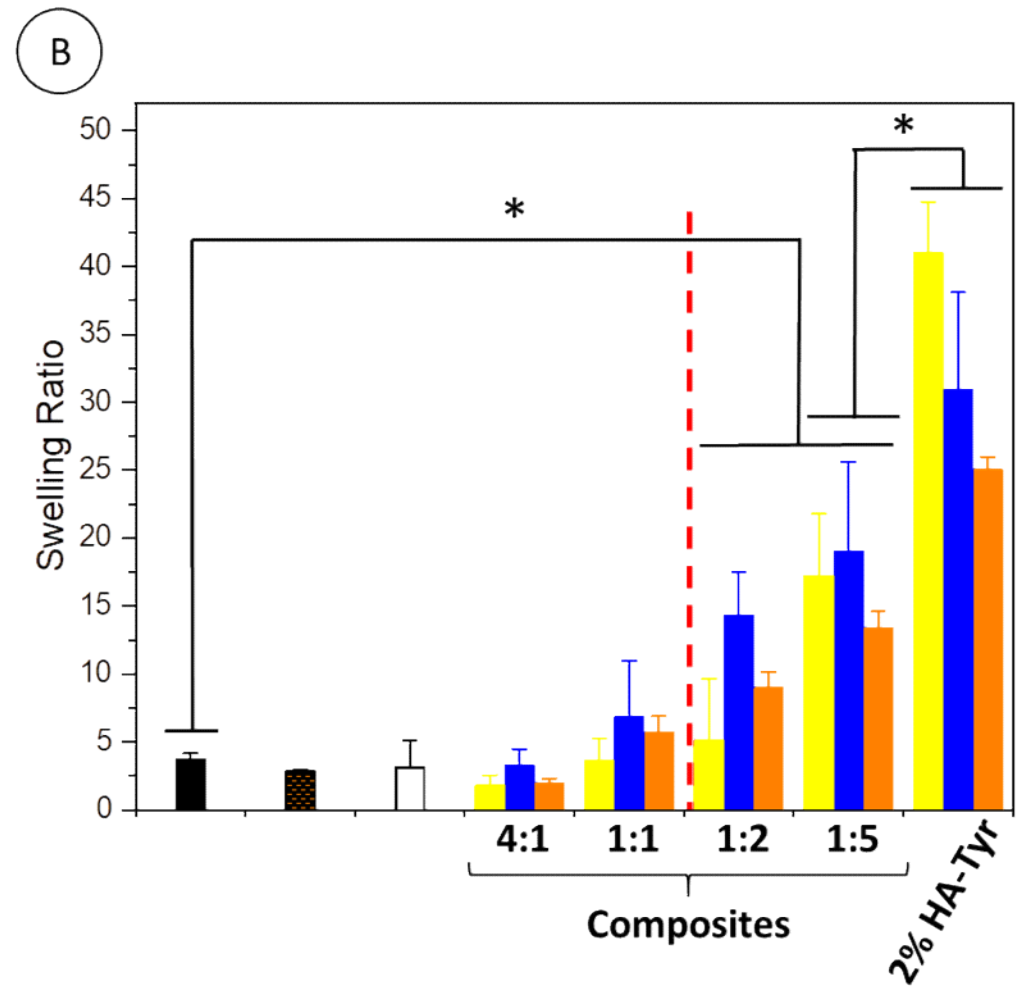
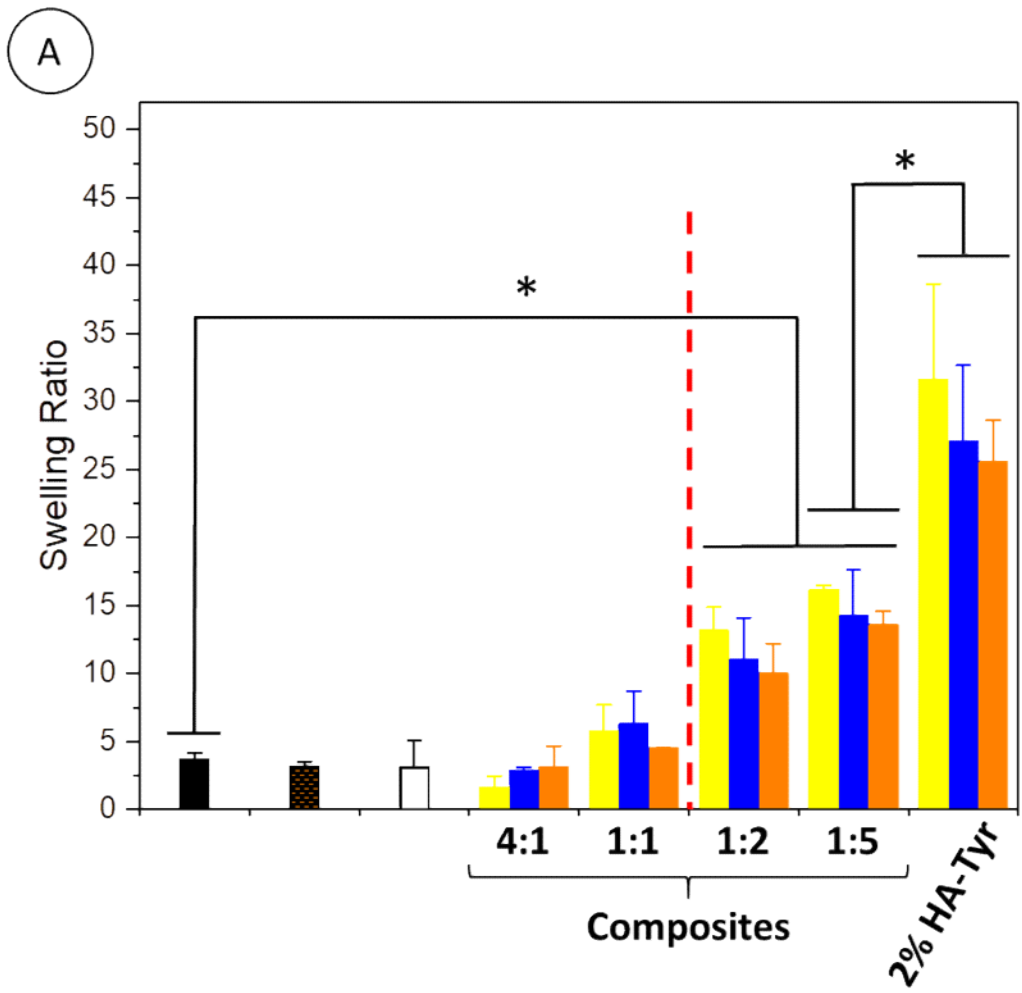


Figure 5

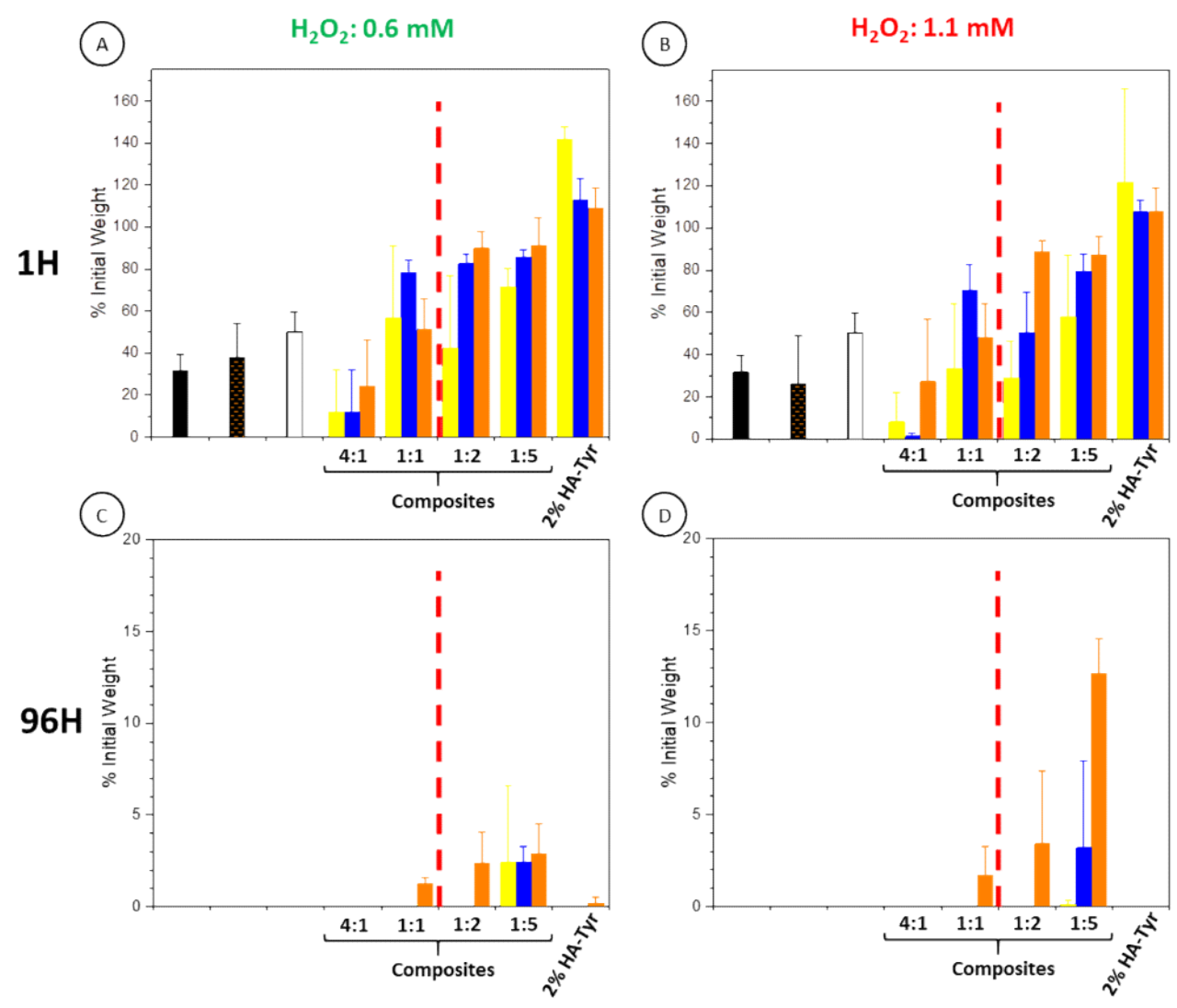


Figure 6

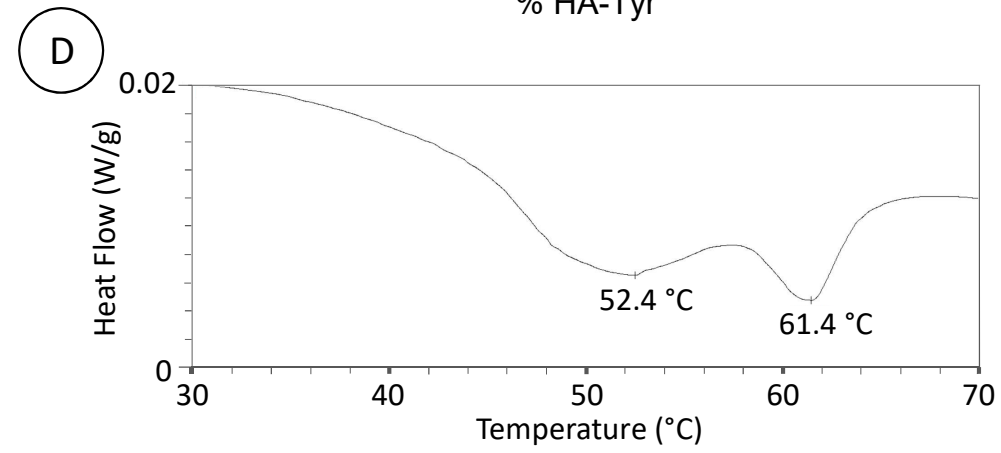
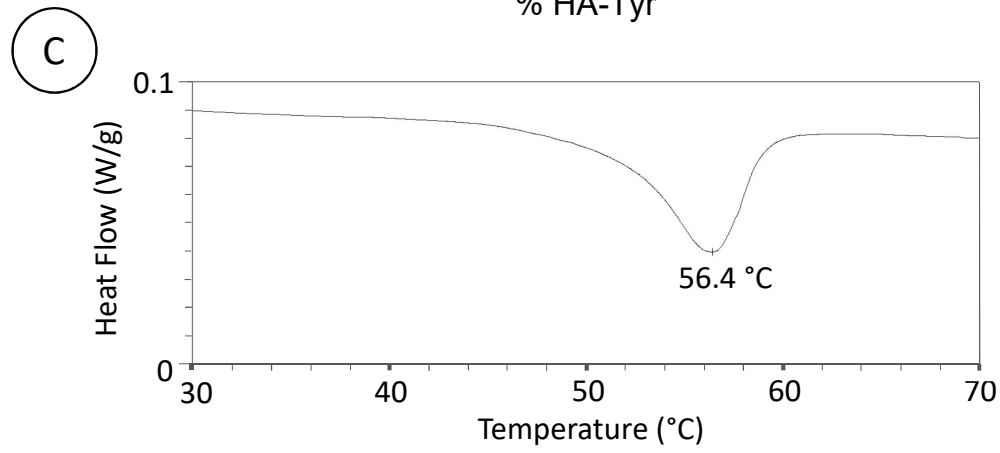
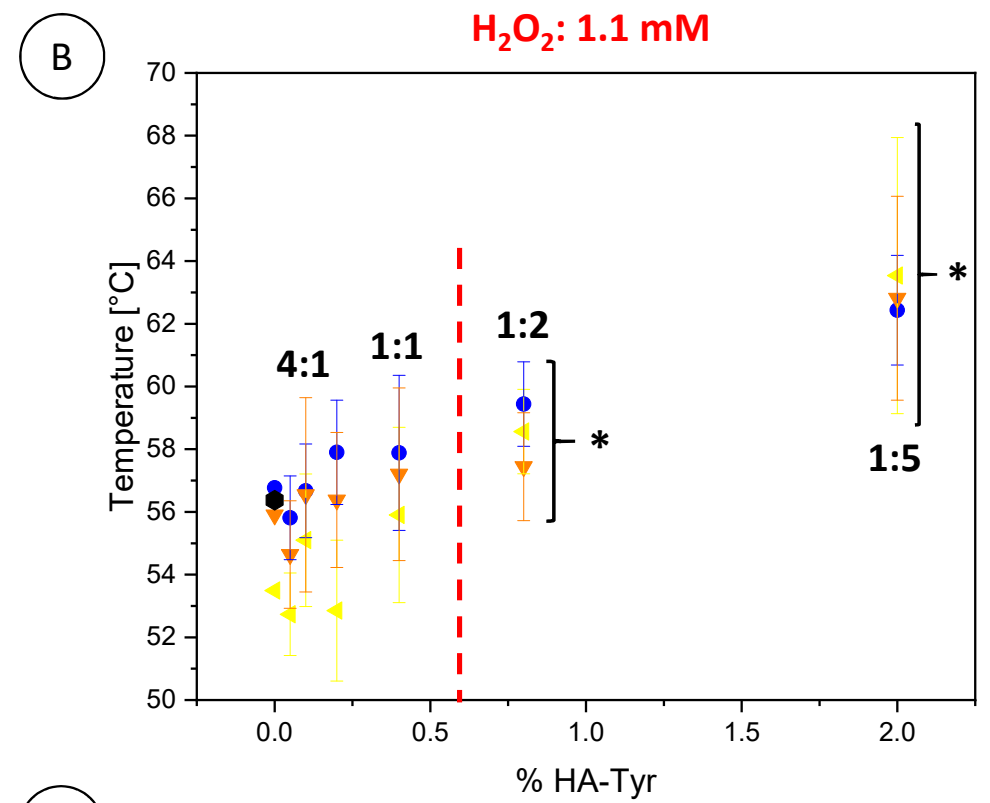
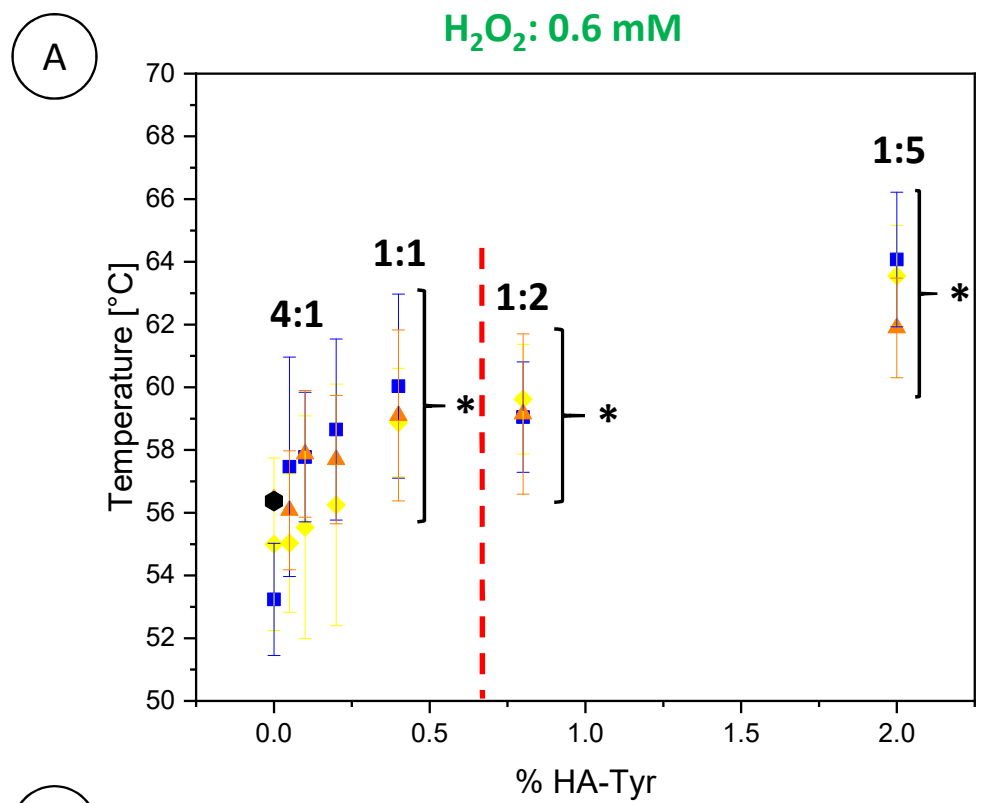


Figure 7

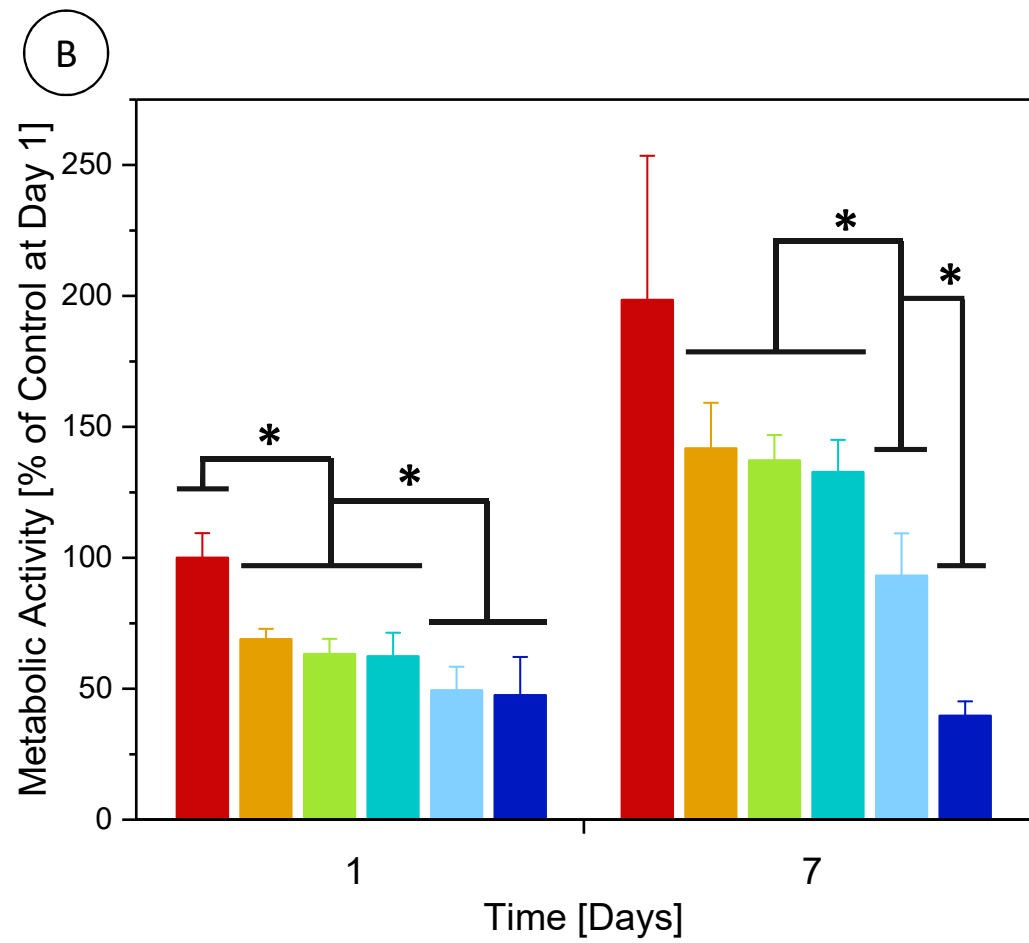
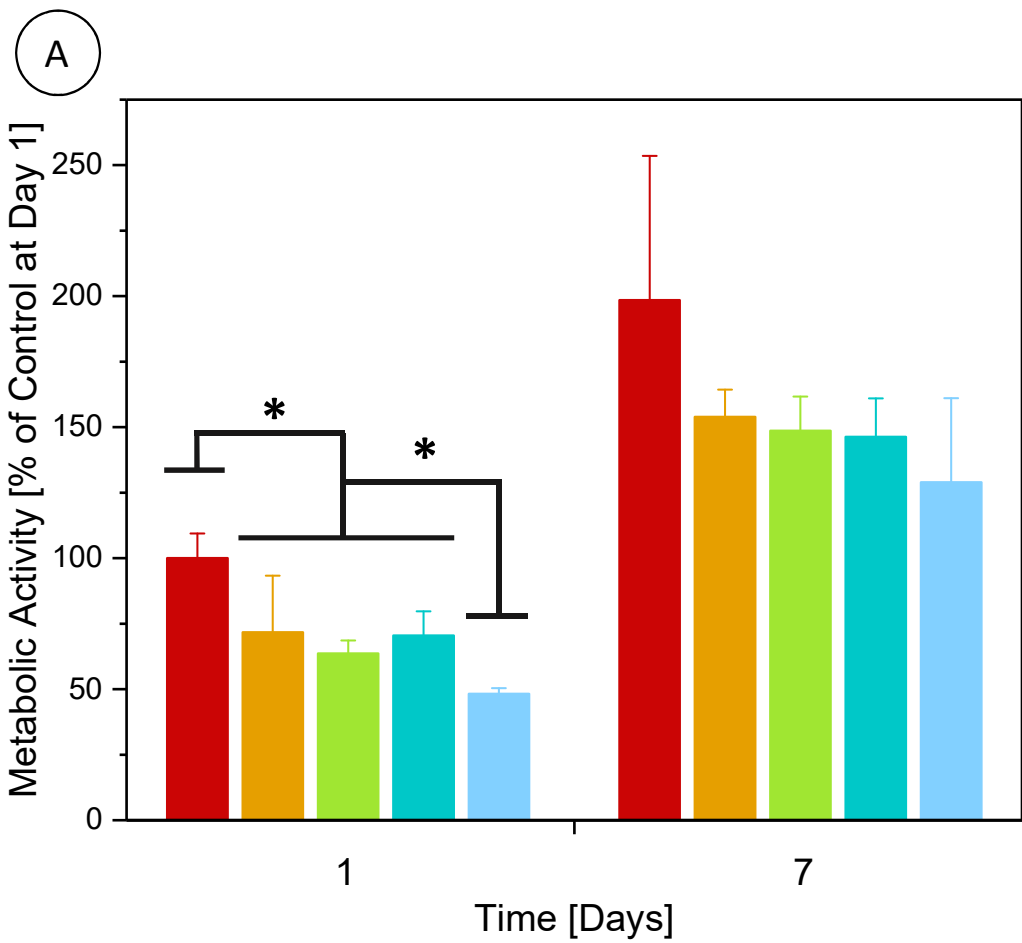
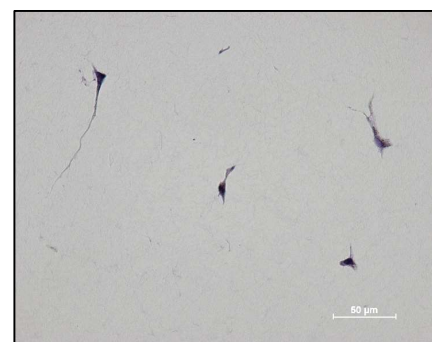
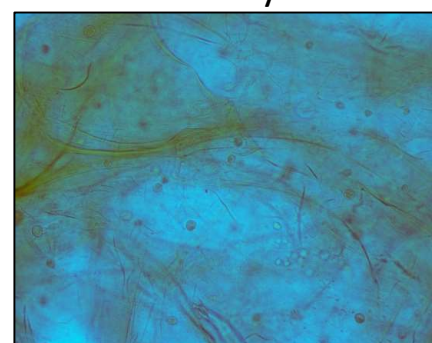


Figure 8

Collagen



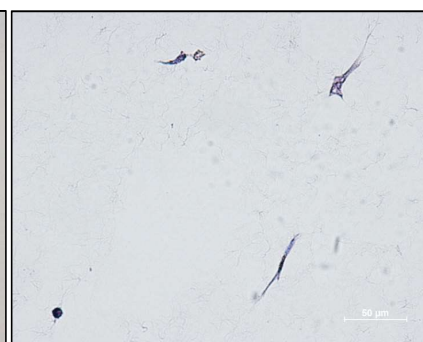
HA-Tyr



Collagen



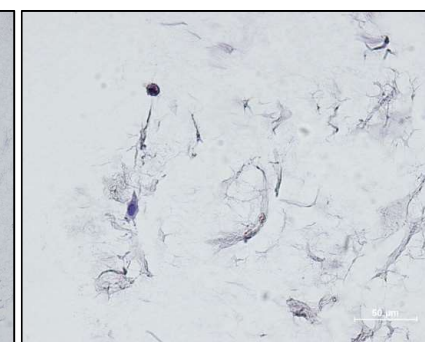
4:1



1:1

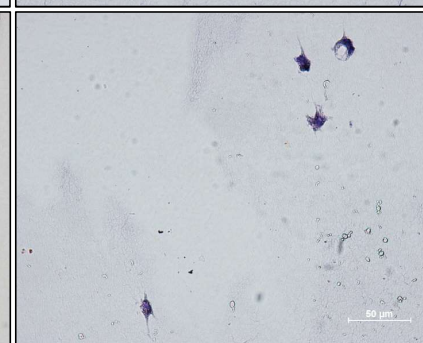
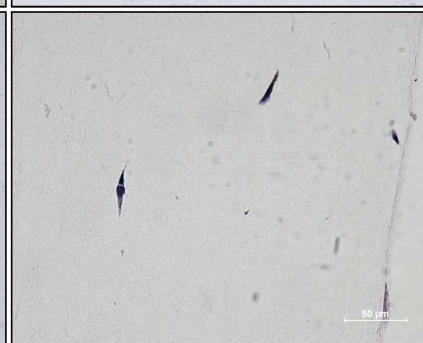
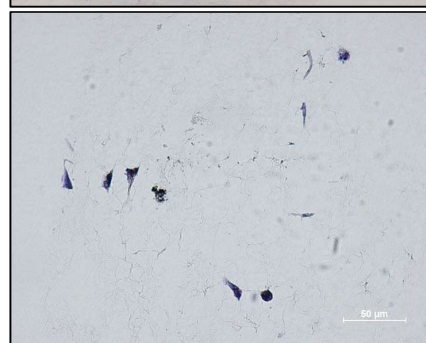


1:5



$[HRP] = 0.05 \text{ U/mL}$

$[HRP] = 0.5 \text{ U/mL}$



**Supplementary data**

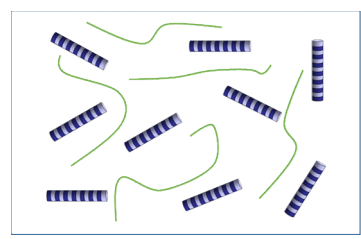
[Click here to download Supplementary data: Supporting Data Article Antoine 08-01-20.pdf](#)

### Col/HA-Tyr hydrogels



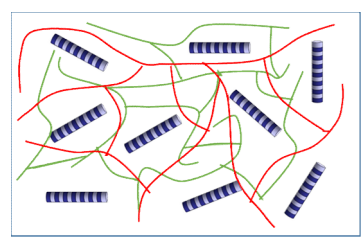
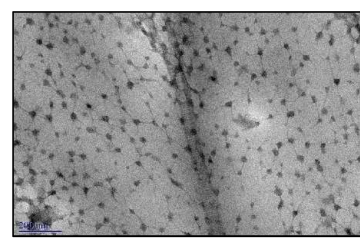
Col/HA-Tyr hydrogels

#### Low HA-Tyr Content



- Structure similar to collagen hydrogels
- Hydrogel destabilized by HA-Tyr

#### High HA-Tyr Content



- Synergistic effect
- Improved mechanical properties
- High resistance against enzymatic degradation
- Fibrillogenesis not inhibited

— Microfibrillar network  
— HA Network  
▬ Collagen fibrils