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

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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.

*Response to Reviewers

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 Hélary

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_2O_2 (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_2O_2 0.6mM	0,77	0,76	**-0,01**
Col/HA-Tyr	ratio 1:1, HRP 0.1, H_2O_2 0.6mM	1,18	1,28	**0,1**
Col/HA-Tyr	ratio 1:1, HRP 0.05, H_2O_2 1.1mM	0,72	0,85	**0,13**
Col/HA-Tyr	ratio 1:2, HRP 0.5, H_2O_2 1.1mM	1,97	2,19	**0,22**
Col/HA-Tyr	ratio 1:2, HRP 0.5, H_2O_2 0.6mM	3,42	3,93	**0,51**
Col/HA-Tyr	ratio 1:5, HRP 0.1, H_2O_2 0.6mM	2,23	2,56	**0,33**
Col/HA-Tyr	ratio 1:5, HRP 0.1, H_2O_2 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_2O_2 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_2 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).

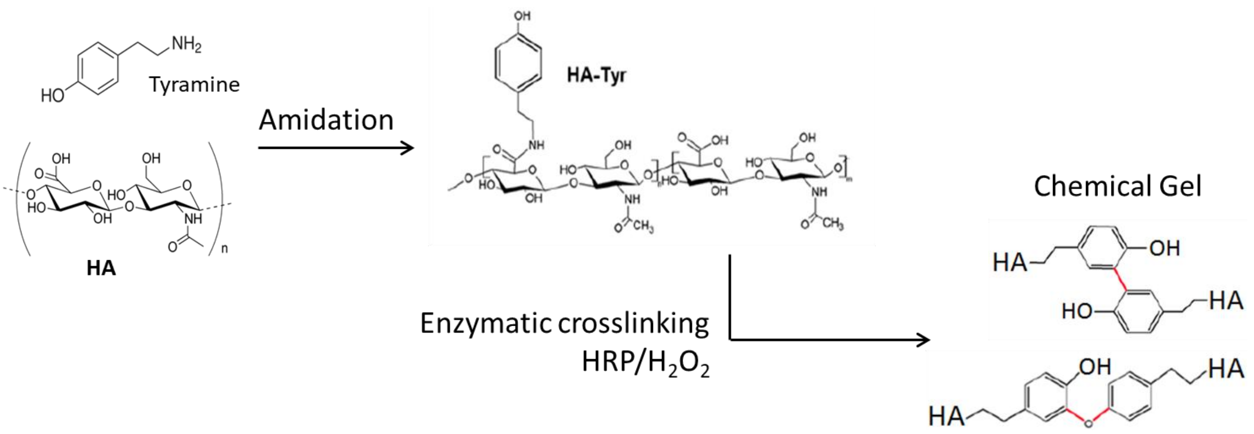
* In the text it was said that the Col concentration was 4 mg/ml, however in Fig. 1 it was expressed as %.

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

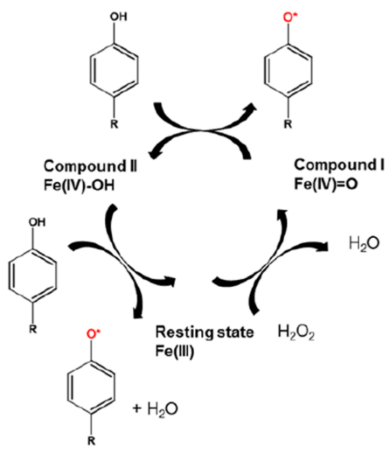
* Line 172-173: "to explore the effect of HA-Tyr cross-linking densities (dityramine bond formation)". The dityramine bond formation is the type of hydrogen bonding not the cross-linking densities and how possibly dityramine bond can from network structure.

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_2O_2 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).

(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-1 425 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^{-1} HRP are not significantly different. It is written line 426: "**H_2O_2 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 Tg (glass transition) and melting temperature when semi-crystalline synthetic polymers are studied. In this study, we are in presence of HA and Coll. No Tg 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.

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

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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 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.

1. Introduction

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

Hyaluronic acid (HA) is a natural polysaccharide and a non-sulphated glycosaminoglycan fundamental for hydration and structure of biological tissues; additionally, it plays a fundamental role in tissue regeneration and other biological processes (Garg, 2004). HA has a long track record of clinical use as dermal filler (Yeom et al., 2010), viscosupplement in osteoarthritis (Strauss, Hart, Miller, Altman, & Rosen, 2009), biomaterial to promote wound healing and in ophthalmic treatments (H. Kim et al., 2017). To form a matrix with viscoelasticity similar to ECM, this polysaccharide has to be functionalized and cross-linked, for example through covalent bonds by employing chemical, enzymatic or photochemical mechanisms (Lopez-Ruiz et al., 2019). Most often chemical cross-linking uses toxic reagents and harsh conditions not suitable for cell encapsulation (Lopez-Ruiz et al., 2019). Photo-induced cross-linking requires the utilization of UV light which triggers the formation of free radicals, toxic for cells (Loebel et al., 2017). In contrast, enzymatic cross-linking usually occurs under conditions compatible with cell encapsulation (Khunmanee, Jeong, & Park, 2017). The Tyramine derivative of HA (HA-Tyr) can be cross-linked by Horse Radish Peroxidase (HRP) and hydrogen peroxide (H_2O_2). HA-Tyr physical properties can be tuned through the modulation of the degree of substitution, the degree of cross-linking and the HA gelling kinetic mediated by the H_2O_2 and HRP concentrations (Bell et al., 1979; Loebel, D'Este, Alini, Zenobi-Wong, & Eglin, 2015; Loebel et al., 2017). Because of its scarce ability to promote cell adhesion, HA-Tyr hydrogels have been functionalized with RGD moieties or gelatin (Petta, Grijpnia, Alini, Eglin, & D'Este, 2018).

Col/HA hybrid hydrogels have been developed to take advantage of the properties of both polymers and circumvent their respective drawbacks (Raia et al., 2017). Preparation of hybrid hydrogels require the functionalization of Col and/or HA without negatively affecting cell encapsulation. Col-hydroxy benzoic acid and HA-Tyr were used to form a hybrid hydrogel by coupling their phenol moieties (Ying et al., 2019). Here, mechanical and swelling properties were impaired by the formation of the hybrid network (Ying et al., 2019). A maleilated Col in combination with thiol-modified HA coupled through Michael addition was also described (Li et al., 2017). Resulting hydrogels exhibited high mechanical properties but the process of fabrication inhibited fibrillogenesis (Davidenko, Campbell, Thian, Watson, & Cameron, 2010; Ying et al., 2019). Hence, they do not mimic the morphology of native ECMs. Cross-linked biomaterials obtained from blended HA and Col were developed, but led to heterogeneous materials (Z. H. Kim et al., 2015). Composite hydrogels exhibiting an interpenetrating network of Col and HA were reported. However, when EDC cross-linker was used to form collagen hydrogels, fibrillogenesis was also inhibited (Collin et al., 2011). Without cross-linkers, it was possible to incorporate a Col fibrillar network within a non-fibrillar network made of HA-PEG diacrylate. By driving the Col fibrillogenesis faster, the mechanical properties were increased by Col, but the presence of chemicals to trigger gelling could be harmful for cells (Walimbe, Calve, Panitch, & Sivasankar, 2019).

To date, a one-pot fabrication of Col/HA hydrogels preserving collagen fibrillogenesis and the capability of encapsulating cells has not been described. In addition, no systematic study has been performed to understand the impact of one biopolymer (type and quantity) on the other during the gelling process to find the best conditions for an optimal composite hydrogel.

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

2. Materials and methods

2.1 Materials.

Hyaluronic acid sodium salt from Streptococcus equi with low weight-average molecular weight Mw = 290 kDa and dispersity index Đ = Mw/Mn = 1.86, where Mn is the number-average molecular weight was purchased from Contipro Biotech s.r.o. (Czech Republic). 4-

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

2.2 Collagen preparation

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

2.3 Hyaluronan conjugation with tyramine

Conjugation of Hyaluronan (HA) with tyramine was performed by amidation reaction between HA carboxylic groups and the amine group of Tyramine Hydrochloride (Tyr). Hyaluronic acid sodium salt (2 g, 5 mmol carboxylic groups) was dissolved overnight in 200 mL of ultrapure water at a final concentration of 1% (w/v). The following day, the HA solution was warmed up to 37 °C using a thermostatic oil bath. Five mmol of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) were added to the HA solution. Subsequently, 5 mmol of Tyr were dissolved in *ca.* 5 mL dH_2O and added dropwise. The whole mixture was stirred at 37 °C for 24 h. Following the addition of 32 mL of a NaCl saturated solution and a 30 min stirring, the newly formed HA-Tyr was precipitated by adding dropwise 96% alcohol. After several washes, the precipitate was collected by filtration under vacuum with a Gooch filter P3 and dried at 40 °C for 48h. To detect salt residues 0.1 M silver nitrate was used. Synthesized HA-Tyr conjugates were characterized using UV-vis spectroscopy as reported previously (Loebel et al., 2015; Loebel et al., 2017). The molar degree of substitution (DS_{mol}, %) was 6%, calculated by measuring the absorbance at 275 nm of a 0.1% (w/v) HA-Tyr solution in ultrapure water using a Cary 5000 UV-Vis-NIR Spectrophotometer (Agilent Technologies).

2.4 Col/HA-Tyr hydrogels synthesis

Several combinations of Col and HA-Tyr were prepared as follows. Stock HA-Tyr solutions concentrated at 3% and 6% and Col solutions concentrated at 0.6 and 0.875% were used. For all hydrogels, the final Col concentration was kept constant at 0.4%. HA-Tyr was mixed with the Col solution to generate hydrogels with Col/HA-Tyr weight ratio of 8:1, 4:1, 2:1, 1:1, 1:2 and 1: 5 respectively (Figure 1 and S.I 1). For a 1 mL hydrogel, Col gelling was triggered by pH increase up to 7 using 100 μL of 10X-PBS and 40 μL of 0.1 M NaOH. The gelling kinetic of HA-Tyr was tuned using 3 different HRP concentrations: 0.05, 0.1 and 0.5 U mL^{-1}. H_2O_2 at a final concentration of 0.6 or 1.1 mM was added to explore the effect of HA-Tyr cross-linking densities (di-tyramine bond formation). Typically, the formation of the Col/HA-Tyr hydrogels was performed by adding the HA-Tyr with HRP, 10X Phosphate Buffered Saline (10x PBS) and 0.1 M Sodium hydroxide (NaOH). After an overnight incubation at 4 °C, Col was added to the mixture and HA-Tyr gelling triggered by H_2O_2 addition. Last, the plates were incubated at 20 °C.

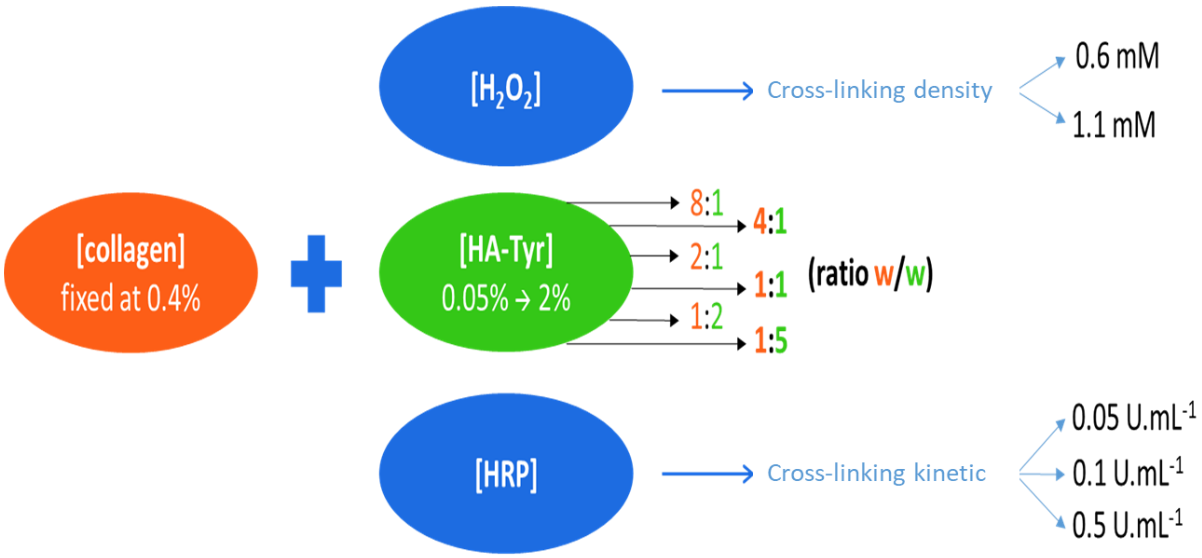

Figure 1: Formulations to synthesize Col/HA-Tyr hydrogels.

2.5 Rheological measurements

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

2.6 Scanning electron microscopy (SEM) analysis

Hydrogels were fixed in 4% paraformaldehyde in PBS (w/v) for 24 h, then washed three times with ultrapure water (10 min each) and freeze-dried overnight. Each sample was torn prior to SEM observation to see the inner structure of composite hydrogels. Samples were coated with a 15 nm-gold-layer and imaged using a Hitachi S-3400N scanning electron microscope operating at 10 kV. For each sample, pictures were acquired at magnification X10000.

2.7 Transmission electron microscopy (TEM)

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

2.8 Differential scanning calorimetry (DSC)

10-30 mg of freeze-dried hydrogels were rehydrated with 15 μL of PBS and they were analyzed using a modulated DSC TA Q20. Standby temperature was set at 20 °C. Temperature was increased from 10 °C to 80 °C at a rate of 5 °C min^{-1}. Data were analyzed using TA Universal Analysis software (n=4).

2.9 Swelling properties

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

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

2.10. *In vitro* accelerated degradation.

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

2.11 Fibroblast cell culture

Normal Human Dermal Fibroblasts (NHDF) were cultured in complete cell culture medium (DMEM supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} Fungizone) and kept at 37 °C in a 95% air - 5% CO_2 atmosphere. Cells with 80% confluency were detached with 0.1% trypsin and 0.02% EDTA and counted using a Malassez Cell.

2.12 Cell metabolic activity

NHDF were encapsulated within each hydrogel at a final cell density of 200.000 cells/hydrogel. Cells were added to the hydrogel mixture prior to H_2O_2 addition at 4 °C. After 15 min, 2 mL of complete medium was added and the newly formed hydrogels were then incubated at 37 °C in a 95% air - 5% CO_2 atmosphere. The 3D cell culture was performed over 7 days using the Coll/HA-Tyr ratios 4:1, 1:1 and 1:5. Both cellularized pure Col and HA-Tyr hydrogels were used as controls.

Cell metabolic activity was monitored after 1 and 7 days in culture using Alamar Blue assay. 3D-cellularized Col/HA-Tyr hydrogels were incubated with 300 μL of a resazurin solution at 0.1% (w/v) for 6 hours. The supernatant in each well was then collected, diluted with 700 μL of fresh medium, and the absorbance measured at λ = 570 nm and λ = 600 nm. The percentage of resazurin reduction was calculated following the formula provided by the supplier. Cell metabolic activity of hydrogels was compared to control samples, i.e. cells cultivated in pure Col hydrogel without HRP and H_2O_2. The arbitrary value 100% was given to control samples.

2.13 Cell morphology

Morphology of encapsulated cells was observed on histological sections. Hydrogels were fixed with a PFA 4% (w/v), then dehydrated with ethanol and butanol and embedded in paraffin. Five micrometer transverse sections were performed using a manual microtome (Stiassnie, France). Sections were rehydrated and stained with Hemalum for 7 min. Then, samples were rinsed with dH_2O and dehydrated again using ethanol and toluene. Last, sections were mounted between glass and coverslip using an Eukitt mounting medium. Samples were observed at X400 magnification with a Nikon Eclipse E600 POL equipped with a Nikon DS-Ri1 camera.

2.14 Statistical analysis

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

3. Results and discussion

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

The goal of this work was to obtain Col/HA-Tyr hydrogels with synergistic properties and mimicking the fibrillar structure of native connective tissues ECM. Earlier studies using covalently-modified Col and/or chemical cross-linking have highlighted that the interplay between HA-Tyr gel formation and Col fibrillogenesis, under the influence of HA-Tyr/Col interactions, was a major factor determining the properties of the composite hydrogels.

In this work, unmodified Col was used, its concentration was fixed at 0.4% (w/v) and its gelation was induced by neutralization of an acidic solution. HA-Tyr was added in various amounts and the degree of cross-linking and gelling kinetics, were tuned by the H_2O_2 and HRP concentrations, respectively (Loebel et al., 2015).

In a first step, the morphology of the hydrogels was examined using scanning electron microscopy (SEM). Pure Col hydrogels (0.4%) exhibited a typical fibrillar network (S.I. 2A). When 0.6 mM H_2O_2 was used, addition of HA-Tyr up to a Col/HA-Tyr 1:1 ratio preserved this ultrastructure for the lowest HRP concentrations (Figure 2). At higher HA-Tyr content, sheets-like morphological features reminiscent of the pure HA-Tyr hydrogels were observed (S.I. 2B). Noticeably, some fibers were still visible at the 1:2 ratio, whereas they were not visible for higher HA-Tyr content, except for lower HRP concentration, 0.05 $U.mL^{-1}$. For the highest enzyme concentration, sheet-like structures can be observed from the 1:1 ratio and remaining fibers were hardly distinguishable at 1:2. Hydrogels formed with 1.1 mM H_2O_2 exhibited similar structures and morphological evolution (S.I. 3). Mixtures of Col and HA-Tyr in the absence of HRP/H_2O_2 did not exhibit such a fibril-to-sheet transition (S.I. 4). Altogether, the hydrogel morphology as determined via SEM was driven by Col fibrillogenesis at low ratio and by HA-Tyr gelation for higher polysaccharide content. However, the observation of some Col fibrils in the latter conditions is indicative of partial Col fibrillogenesis preservation (Kreger et al., 2010). Moreover, the transition from one morphology to the other shows a strong dependence on the HRP concentration, *i.e*. on the kinetics of the HA-Tyr cross-linking reaction, and, to a lower extent, on H_2O_2 concentration, i.e. on the cross-linking density (S.I. 3).

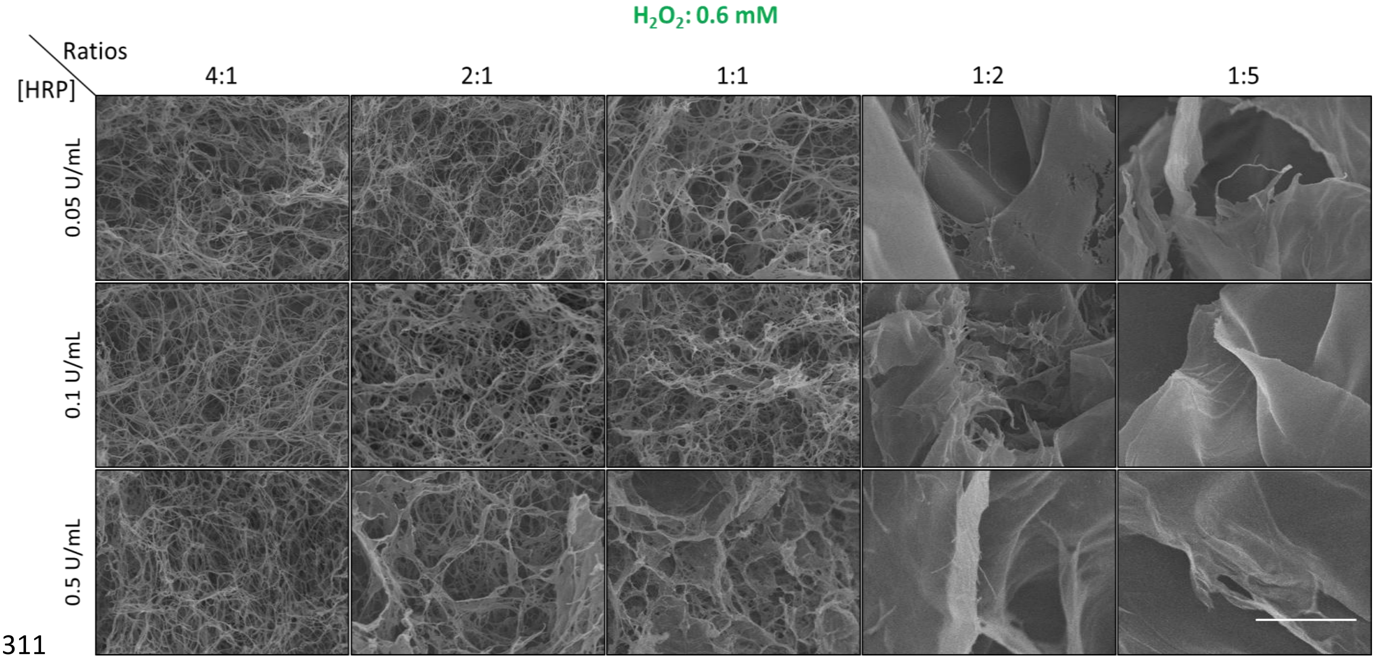

Figure 2. Ultrastructure of Col/HA-Tyr hydrogels observed by scanning electron microscopy. Cross-linking performed with 0.6 mM H_2O_2. Bar: 5 μm.

TEM imaging of hydrogels obtained at 0.6 mM H_2O_2 confirmed the prevalence of fibrillar structure of the Col/HA-Tyr hydrogels until the 1:1 ratio, irrespective of the HRP concentration. Col/HA-Tyr hydrogels exhibited a population of banded fibrils (67 nm) similar to that of pure Col (Figure 3 and S.I. 2C). From the 1:1 ratio, a microfibrillar network appeared in hydrogels prepared with HRP 0.1 and 0.5 $U.mL^{-1}$. Above this ratio, microfibrils were visible in all types of hydrogels and their number increased with HA-Tyr content and HRP concentration (Figure 3). Despite the presence of the microfibrillar network, typical collagen banded fibrils of 50 nm in diameter could be observed in all samples, but their number decreased as the microfibrillar network extended (Figure 3). These microfibrils were not visible in pure HA-Tyr and Col hydrogels, nor in uncross-linked mixtures, in agreement with the SEM data (S.I. 2C and 2D). This suggests that the microfibrils originated from the enzymatic cross-linking reaction. At this stage, two mechanisms can be considered in the network formation. First, the cross-linking between HA-Tyr chains could create a network where Col molecules are confined and fibrillogenesis is limited, leading to smaller fibrils. Second, the tyrosine residues in Col could form a covalent bond with tyramine in the presence of the HRP/H_2O_2 catalytic system, thanks to the similarity of the chemical structure of the two molecules. The tyramine-tyrosine bonds may hinder fibrillogenesis and again lead to microfibrils instead of large banded fibrils (Mazzocchi, Devarasetty, Huntwork, Soker, & Skardal, 2018). However, it can be expected that the first mechanism would lead to an increase in microfibrils number and a decrease of microfibril size with the HA-Tyr content as the network would grow in density. In contrast, in the second mechanism, the number and the size of microfibrils are expected to grow at the expense of fibrils, as more HA-Tyr is present and more HA-Tyr/Col cross-links can be formed. Eventually, some Col molecules may not react with HA-Tyr and could still self-assemble to form fibrils. To further characterize the composite, the rheological properties of the hydrogels were studied.

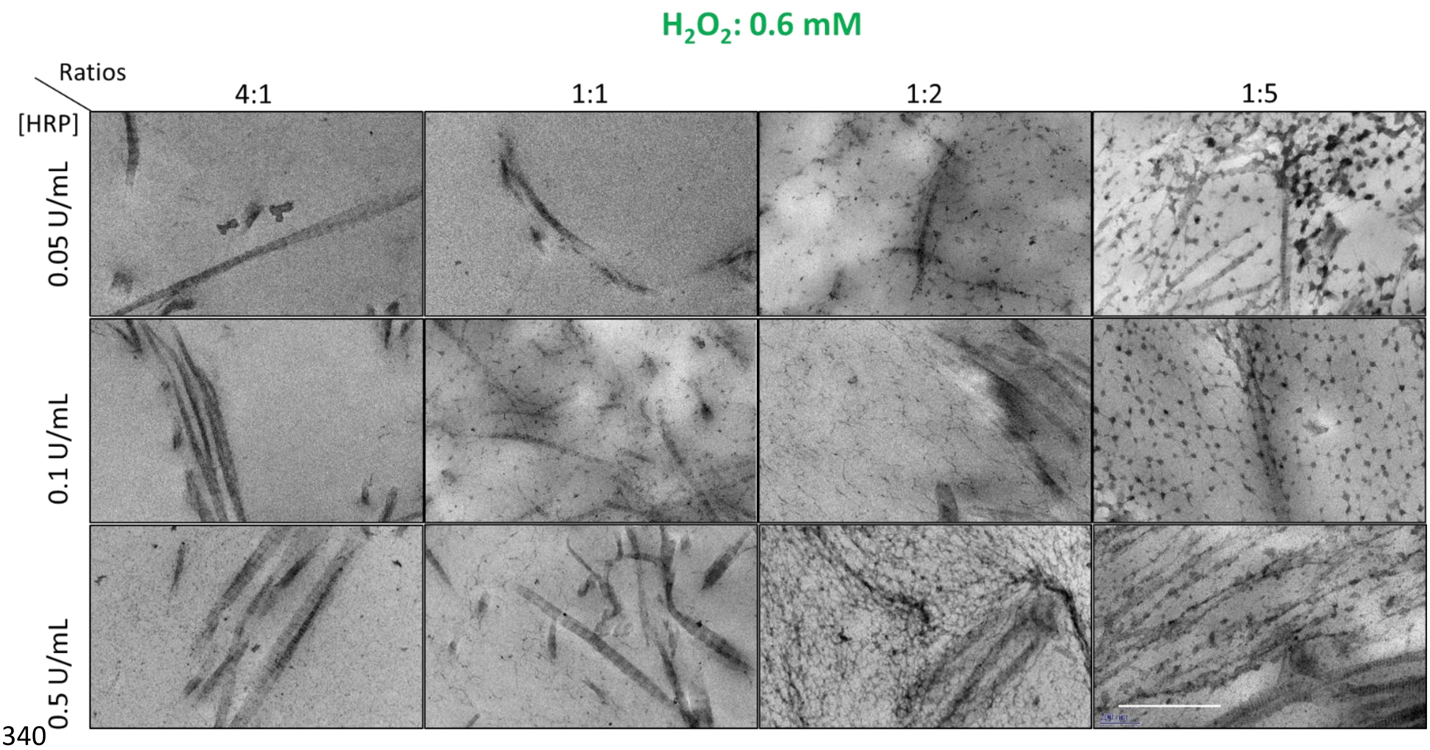

Figure 3. Ultrastructure of Col/HA-Tyr hydrogels observed by transmission electron microscopy. Cross-linking performed with 0.6 mM H_2O_2. Bar: 500 nm.

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

The influence of increasing HA-Tyr amount at fixed H_2O_2 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 in all Col/HA-Tyr composites (Table 1 and 2, S.I. 5).

Composite Hydrogels - $[H_2O_2]$ = 0.6 mM						
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	$[H_2O_2]$ (mM)	[HRP] ($U.mL^{-1}$)	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

Table 1: Effect of [HRP] on mechanical properties of Col/HA-Tyr composite hydrogels formed with $[H_2O_2]$ at 0.6 mM. (*: $p< 0.05$). Comparison between composite hydrogels and pure collagen hydrogels.

Low HA-Tyr content

For concentrations below 0.8% (w/v), pure HA-Tyr did not form a stable cross-linked network regardless of the HRP and H_2O_2 concentration used (S.I. 5). The storage moduli measured for Col/HA-Tyr hydrogels formed with a weight ratio below 1:1 were statistically smaller than those measured for pure Col hydrogels, indicating a slight destabilization of Col hydrogels by the addition of HA-Tyr, HRP and H_2O_2 as previously indicated (Docherty, Forrester, Lackie, & Gregory, 1989).

This effect was dependent on HRP concentration and HA-Tyr content (Table 1). For example, using 0.6 mM H_2O_2 and 0.5 $U.mL^{-1}$ HRP, the elastic modulus decreased to *ca.* 100 Pa with the 8:1 ratio. The storage modulus increased to reach that of pure Col hydrogels (200 Pa) with the 2:1 ratio and almost doubled to reach *ca.* 400 Pa with the 1:1 ratio. At this ratio, the storage modulus of composites formed with 0.05 $U.mL^{-1}$ HRP was still below that of Col. Additionally, when Col/HA-Tyr mixtures were used without HRP/H_2O_2, the generated hydrogels exhibited rheological properties and an ultrastructure similar to those of pure Col, thereby evidencing that fibrillogenesis and gel formation occurred (S.I. 4 and S.I. 6).

Kuznetsova et al (Kuznetsova, Chi, & Leikin, 1998) showed that polyols can inhibit Col fibril formation decreasing the mechanical properties of the resulting hydrogels (Christiansen, Huang, & Silver, 2000; Fratzl et al., 1998). Similar interactions may occur between Col and HA-Tyr, which is rich in hydroxy groups (Christiansen et al., 2000; Fratzl et al., 1998). However, since the destabilization is affected by the enzymatic cross-linking, tyramine-tyrosine covalent bonding between HA-Tyr chains and Col triple helices and fibrils cannot be ruled out (Eastoe, 1955; Gullekson, Lucas, Hewitt, & Kreplak, 2011; Loebel et al., 2015). This could impair interactions between the fibrils and hinder Col hydrogel formation. This inhibition of Col gel formation diminishes at higher HRP concentration, which can be attributed to the increase of the HA-Tyr cross-linking kinetics which occurs before collagen fibrillogenesis.

High HA-Tyr content

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

This synergistic effect has not been reported for hybrid gelatin or Col/GAG hydrogels in which mechanical behavior is driven by the HA hydrogel formation (Lou, Stowers, Nam, Xia, & Chaudhuri, 2018; Moulisova et al., 2017). The addition of HA was observed to destabilize the hybrid structure and lower its mechanical properties (Ying et al., 2019). The synergistic improvement of mechanical properties was not observed in composite HA/Col hydrogels either. Walimbe *et al*., showed that a faster Col gelling prior to the HA gel formation was required to reach high mechanical properties (Walimbe et al., 2019). Here, a slow HA gelling associated with a faster collagen gelling negatively impacted mechanical properties. A positive effect of HA is observed only when the HA gelling is fast and is probably due to the presence of the microfibrillar network.

Composite Hydrogels - [HRP] = 0.5 U.mL^{-1}					
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	[H_2O_2] (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

Table 2: Effect of [H_2O_2] on mechanical properties of Col/HA-Tyr Composite Hydrogels formed with [HRP] at 0.5 U.mL^{-1}. (*: $p< 0.05$). Comparison between composite hydrogels formed with 0.6 mM and those formed with 1.1 mM.

When the degree of cross-linking was increased by addition of 1.1 mM H_2O_2 at 0.5 U.mL^{-1} HRP, G' and G" of the hydrogels did not increased until Col/HA-Tyr 1:2 ratio, thereby suggesting that H_2O_2 was in stoichiometric excess (Table 2 and S.I. 5). In contrast, the storage modulus measured in hydrogels with the 1:5 ratio reached 1500 Pa, *i.e* 1.5 times higher than that measured in hydrogels formed with 0.6 mM H_2O_2 (Table 2). This shows that the H_2O_2 concentration has an impact on the cross-linking degree and mechanical properties only when the highest HA-Tyr content is used (Loebel et al., 2015).

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

3.3.1 Swelling properties

The influence of the Col/HA-Tyr ratio on the swelling properties was assessed after 24 hours of incubation in PBS. The swelling ratio was around 5 for pure Col and 30 for pure HA-Tyr at 2% (w/v), and all composites exhibited values in between. The measured values were in agreement with previous reports (Helary et al., 2015; Petta, Armiento, et al., 2018; Petta, Grijpnia, et al., 2018). The higher swelling of HA-Tyr can be attributed to its chemical cross-linking and the highly hydrophilic nature of HA. H_2O_2 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 swelling ratio measured in the Col/HA-Tyr hydrogels was similar to that of pure Col until Col/HA-Tyr 1:1 ratio regardless of the H_2O_2 concentration and the HRP concentration used (Figure 4A and 4B). HA-Tyr did not form a gel below the 1:2 ratio, showing that cross-linking is essential to retain water and increase the swelling properties of hydrogels. These results were confirmed by the Col/HA-Tyr hydrogels not cross-linked by H_2O_2 and HRP, which exhibited swelling properties similar to pure Col. The same swelling behavior was observed after 2 weeks with slightly increased values (S.I. 7). Thus, surprisingly, swelling and mechanical properties were scarcely correlated.

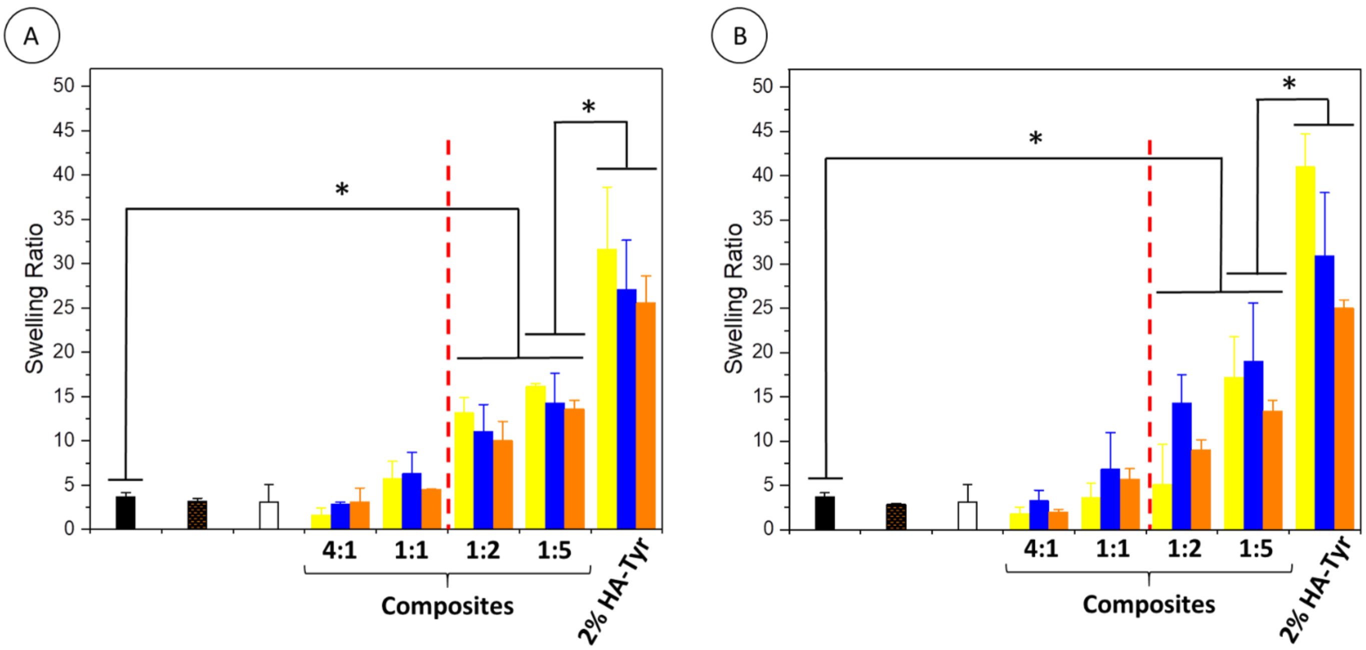

Figure 4. Swelling properties of Collagen/HA-Tyr hydrogels after one day. HA-Tyr cross-linking performed with 0.6 mM H_2O_2 (A) and 1.1 mM H_2O_2 (B). ■ : 0.4 % Col; ■ : 0.4 % Col with 0.5 U mL^{-1} HRP activity (A and B); □ : 0.4 % Col with 2 % HA-Tyr without any cross-linking agents (H_2O_2 and HRP); ■ , ■ and ■ : Col/HA-Tyr hydrogels cross-linked with 0.05, 0.1 and 0.5 $U.mL^{-1}$ HRP, respectively

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

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

From the 1:2 ratio, the swelling properties significantly increased to reach *ca*. 15 for the Col/HA-Tyr 1:5 ($p< 0.05$). This was due to the addition of large quantities of HA-Tyr and its ability to form hydrogels at these concentrations (Figure 5A and 5B). Nevertheless, Col/HA-Tyr hydrogels formed with the 1:5 ratio exhibit swelling abilities lower than 2% HA-Tyr ($p< 0.05$), despite a similar HA-Tyr content. Hence, the presence of Col decreased hydrogel swelling. The microfibrillar network observed in these hydrogels exhibited numerous cross-linking points which could be responsible for the lower swelling properties compared to pure HA. However, these hydrogels are composed of more than 90% water. In paragraph 3.2 we have shown how the [H_2O_2] had no impact on the mechanical properties of hydrogels until the 1:2 ratio, this explains the similar abilities of swelling. Mechanical properties increased with the 1:5 ratio when a higher [H_2O_2] was used (1.1 mM) but did not impact the swelling properties. This could be due to the different structure of hydrogels characterized by the presence of numerous microfibrils in hydrogels formed with 1.1 mM H_2O_2. Taken together, the results show how it is possible to modulate the swelling properties of hydrogels by tuning the HA-Tyr content (Toh, Lim, Kurisawa, & Spector, 2012).

3.3.2 Enzymatic degradation rate

The degradation by collagenase and hyaluronidase did not reveal any differences between HRP-cross-linked and not cross-linked pure Col hydrogels regardless of the quantity of H_2O_2 (Figure 5A and 5B). After 1 hour of digestion, the remaining mass was around 30%, confirming once again the absent or minor cross-linking between fibrils via di-tyrosine bonds. Col/HA-Tyr hydrogels with the 4:1 ratio exhibited a faster degradation than pure Col except for the 0.5 $U.mL^{-1}$ HRP concentration. Hence, hydrogels characterized by weak mechanical properties were more prone to degradation, as expected. Regarding the other Col/HA-Tyr hydrogels, the degradation rate was proportional to the HA-Tyr content. In these cases, the rate was also correlated with the mechanical properties of hydrogels (Table 1 and 3). After six hours of digestion, pure Col hydrogels and not cross-linked Col/HA-Tyr were completely degraded, which is in agreement with previous studies (Helary et al., 2015). Hydrogels with the 4:1 ratio were also digested and only hydrogels formed with 0.5 $U.mL^{-1}$ HRP had a residual mass. Hydrogels with the 1:5 ratio (w/w) were more degraded than 2% HA-Tyr ones despite their identical HA-Tyr content. The Col fibrils could be more prone to degradation whereas the HA-Tyr network and microfibrils could be more resistant owing to cross-linking (Figure 3).

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

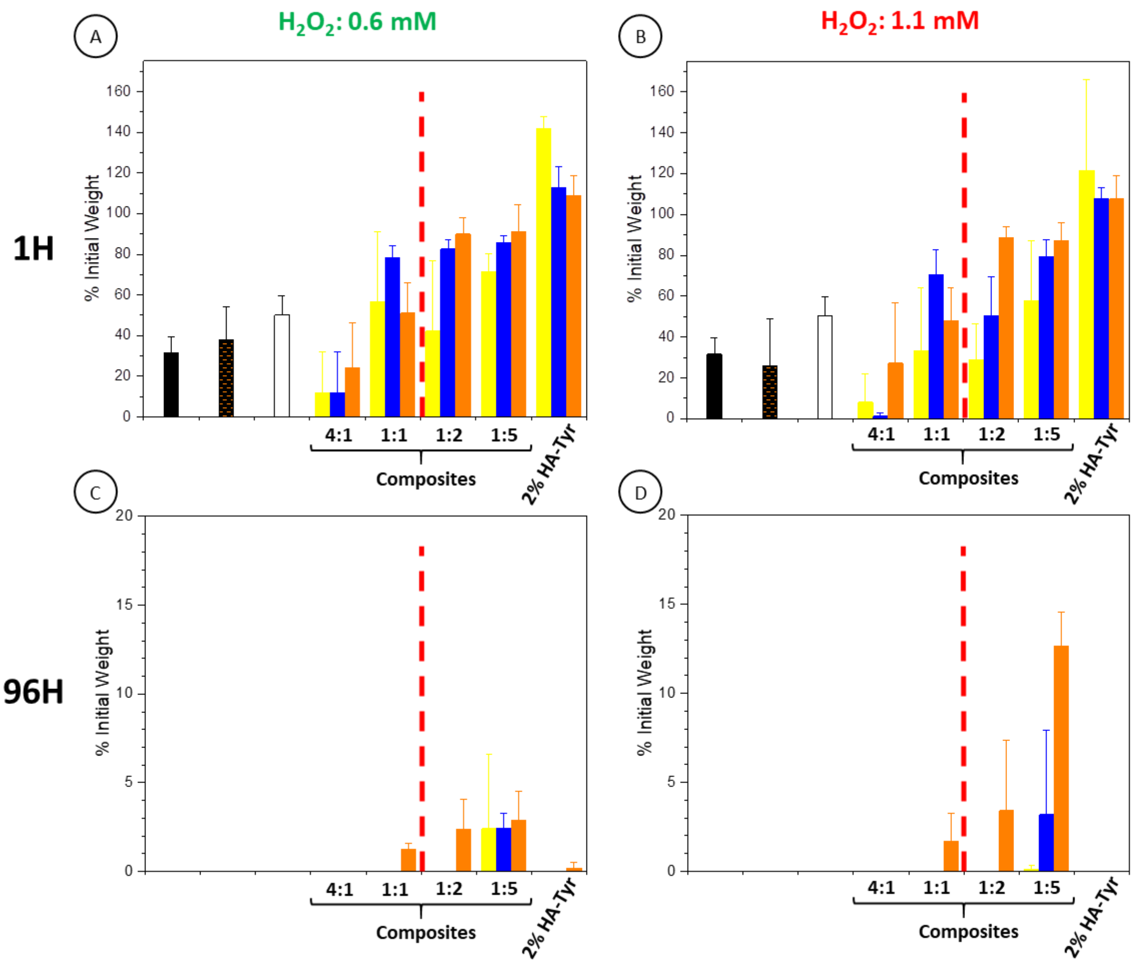

Figure 5. Accelerated enzymatic degradation of Col/HA-Tyr hydrogels. Remaining mass measured after 1 hour (A and B) and 96 hours (C and D). ■ : 0.4 % Col; ■ : 0.4 % Col with 0.5 U mL^{-1} HRP activity; □ : 0.4 % Col with 2 % HA-Tyr without any cross-linking agents; ■ , ■ and ■ : Col/HA-Tyr hydrogels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

3.3.3 Differential Scanning Calorimetry

The thermal stability of collagen fibrils within composite hydrogels was investigated via DSC. In agreement with literature data, the fibril denaturation in pure Col hydrogels was measured at 55-56 °C (Walton, Brand, & Czernuszka, 2010)(Figure 6C). Until the 2:1 ratio, the Col denaturation peak of the composites was similar to that of pure Col regardless of H_2O_2 and HRP concentrations used for HA-Tyr cross-linking (Figure 6A and 6B). For the composites, a shoulder appeared between 45 °C and 52 °C (S.I. 9A) which did not correspond to the HRP denaturation temperature (S.I. 9B). As expected, HA-Tyr did not show any denaturation/degradation temperature in this range (S.I. 9C). Rat tail triple helices denature below 35 °C and chemical cross-linking usually leads to the increase of their denaturation temperature (Leikina, Mertts, Kuznetsova, & Leikin, 2002). Hence, this shoulder could be correlated with the stabilization of Col triple helices by cross-linking *via* tyrosine-tyrosine or tyrosine/tyramine bonds triggered by HRP and H_2O_2. The shape of the shoulder suggests the presence of a heterogeneous population of cross-linked molecules (in number and degrees of interaction). As the denaturation temperature is below 55 °C, the cross-linking of Col fibrils cannot be considered. However, the Col cross-linking did not impact the structure, mechanical and physical properties of these hydrogels. From the 1:2 ratio, two denaturation peaks appeared: one around 53 °C and another one statistically higher ($p < 0.05$) around 61 °C (Figure 7D).

In addition, the thermogram profile did not depend on the H_2O_2 concentration and the HRP concentration employed in the enzymatic cross-linking (Figure 7A and 7B). As said above, the first peak at 52 °C is characteristic of native Col fibril denaturation, thereby evidencing the absence of cross-linking between fibrils. These results are in agreement with previous studies which showed that cross-linking of Col fibrils led to their aggregation and a drastic increase of thermal denaturation, which is not observable in this study (Tian, Liu, & Li, 2016). The second denaturation temperature can be tentatively associated with the microfibrillar network. Col triple helices have been stabilized by numerous Col/HA-Tyr covalent bonds, thereby increasing their denaturation temperature. This temperature depends on the cross-linking degree of Col (Calderon et al., 2010; Lin & Liu, 2007). Taken together, the results are suggestive of a microfibrillar network consisting of a hybrid Col/HA-Tyr as this structure was not visible in pure Col and HA-Tyr hydrogels (S.I. 2B and 2D). In addition, no peak at *ca.* 60-64 °C was observed in these hydrogels (S.I. 9A and 9C).

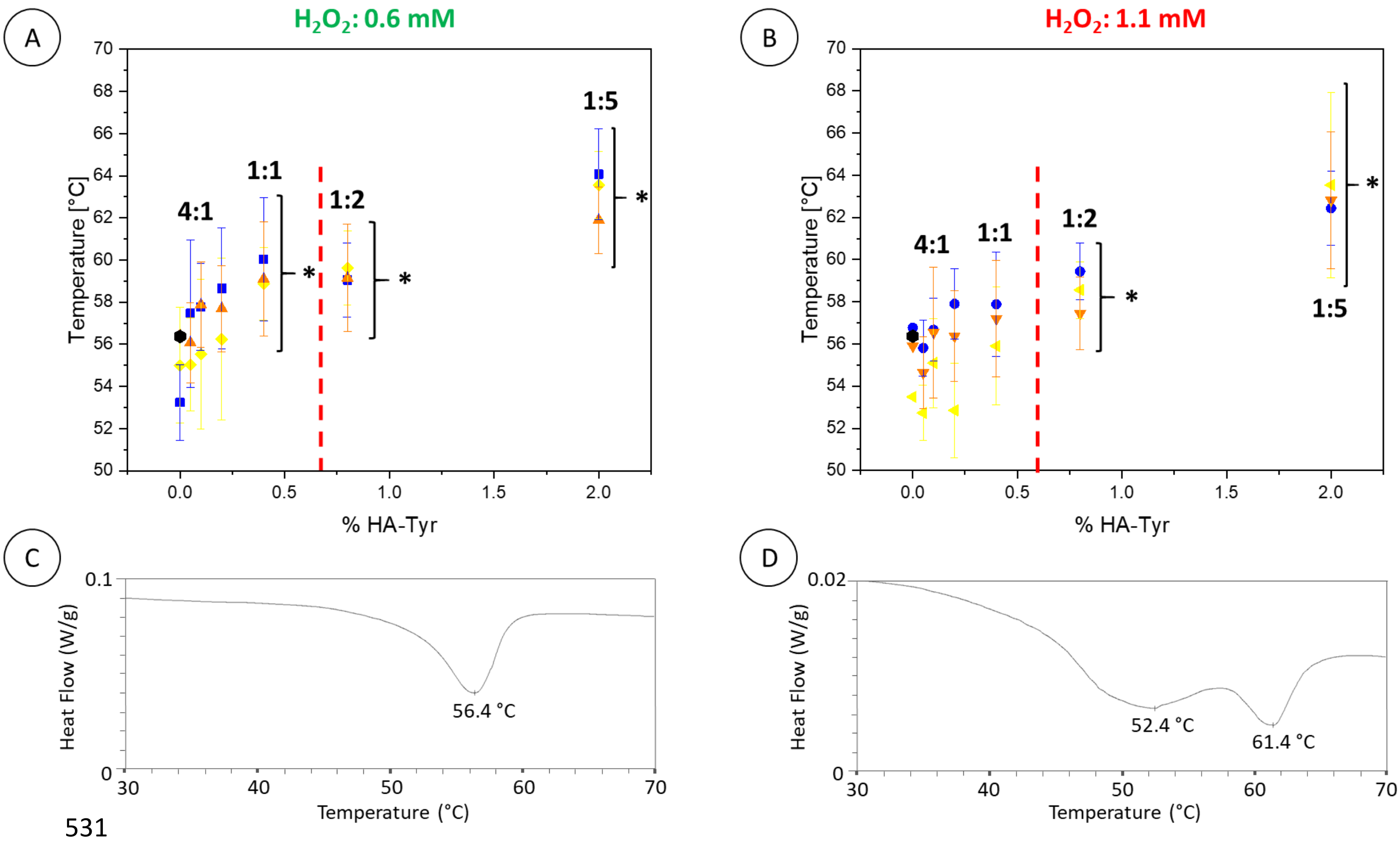

Figure 6. Denaturation temperature of collagen within Col/HA-Tyr hydrogels assessed by differential scanning calorimetry. Peak of denaturation temperature expressed as a function of the HA-Tyr content (A and B). Pure 0.4% Col thermogram (C) and thermogram of Col/HA-Tyr hydrogels with the 1:5 ratio (w/w) cross-linked with HRP at 0.5 U mL^{-1} and 0.6 mM H_2O_2 (D).

⬢ : 0.4 % Col. ◆, ■ and ▲: HA-Tyr cross-linked with 0.6 mM H_2O_2 and 0.05, 0.1 and 0.5 $U.mL^{-1}$ HRP, respectively. N = 6. *: $p < 0.05$ with a Mann-Whitney statistical test. Comparison between pure Col and Col/HA-Tyr Hydrogels.

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

3.4.1 Cell viability

Fibroblasts encapsulated within Col hydrogels formed in presence of 0.05 $U.mL^{-1}$ HRP and 0.6 mM H_2O_2 displayed 75% of the metabolic activity found in pure Col hydrogels without cross-linker after 24 h in culture (Figure 7A).

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

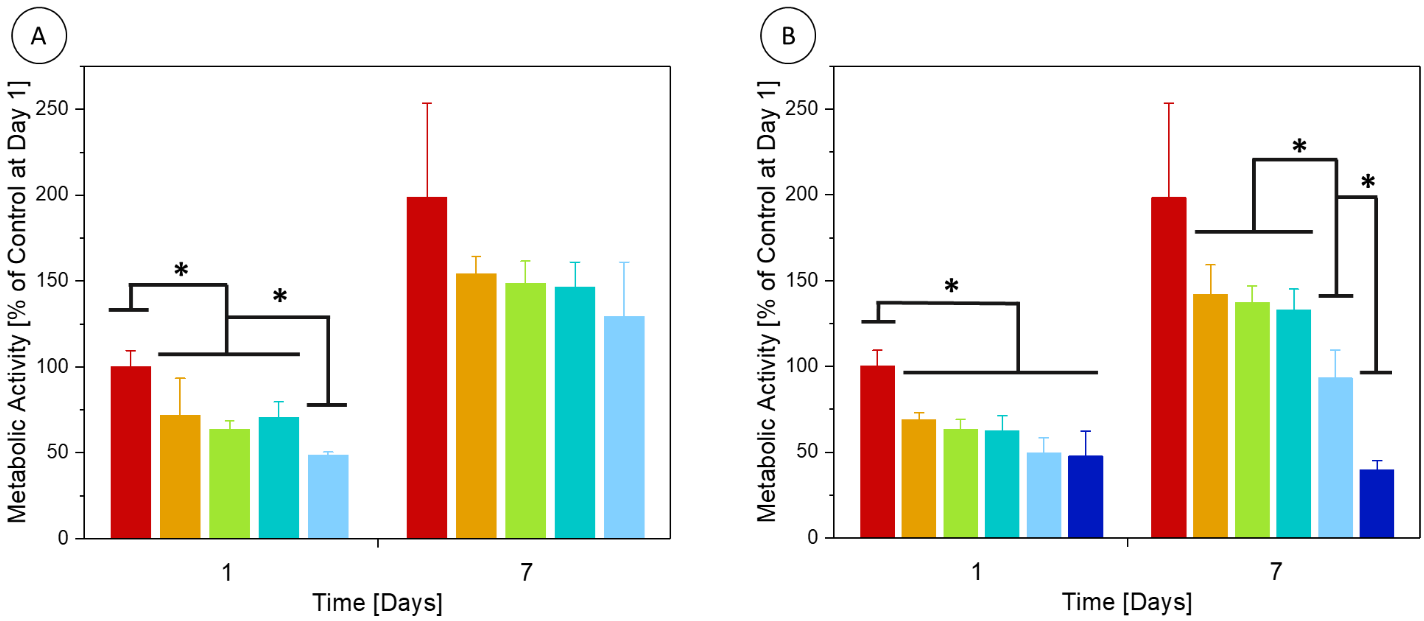

Figure 7. Metabolic activity of Normal Human Dermal Fibroblasts within Col/HA-Tyr hydrogels measured over 7 days. HA-Tyr cross-linking was performed with 0.6 mM H_2O_2 using a HRP activity at 0.05 U mL^{-1} (A) or 0.5 U.mL^{-1} (B). : 0.4 % Col; : 0.4 % Col with 0.5 U.mL^{-1} HRP and H_2O_2 : Col/HA-Tyr 4:1 ratio; : Col/HA-Tyr 1:1 ratio; : Col/HA-Tyr 1:5 ratio (w/w); : 2 % HA-Tyr. N = 4. *: $p < 0.05$: Mann-Whitney test.

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. It was previously shown that fibroblasts encapsulated within concentrated Col hydrogels

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

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

3.4.2 Fibroblast morphology

Until the 1:1 ratio, fibroblasts within hydrogels exhibited a spindle shape morphology after one and seven days in culture, characteristic of cells encapsulated within concentrated Col hydrogels (Figure 8 and S.I. 10) (Helary et al., 2010; Helary et al., 2012). The presence of HA-Tyr did not impact cell morphology except for the 1:1 ratio using 0.5 U.mL^{-1} HRP in which cells were more rounded (Figure 8). The spindle shape morphology evidenced a strong adhesion to the Col network, allowing for cell spreading. Cells encapsulated in pure HA-Tyr and Col/HA-Tyr with the 1:5 ratio did not spread and kept a round shape. This confirms the weak adhesion to the HA-Tyr network (Figure 8). These results are in agreement with the study of Doyle et al., showing that fibroblast spreading is biopolymer dependent (Doyle & Yamada, 2016). In addition, other types of cells such as mesenchymal stem cells do not spread in pure HA-Tyr hydrogels (Toh et al., 2012). HA needs to be functionalized with RGD peptides or associated with gelatin to allow for cell spreading, proliferation and differentiation (Moulisova et al., 2017; Petta, Grijpnia, et al., 2018). However, a strong adhesion is not required for stem cells, chondrocytes or NP cells. This is evidenced by their rounded shape (Collin et al., 2011). Hence a Col/HA-Tyr 1:5 ratio could be adequate for this kind of cells. For fibroblasts, a lower HA content (below 1:1 ratio) is required to get an appropriate adhesion and spreading.

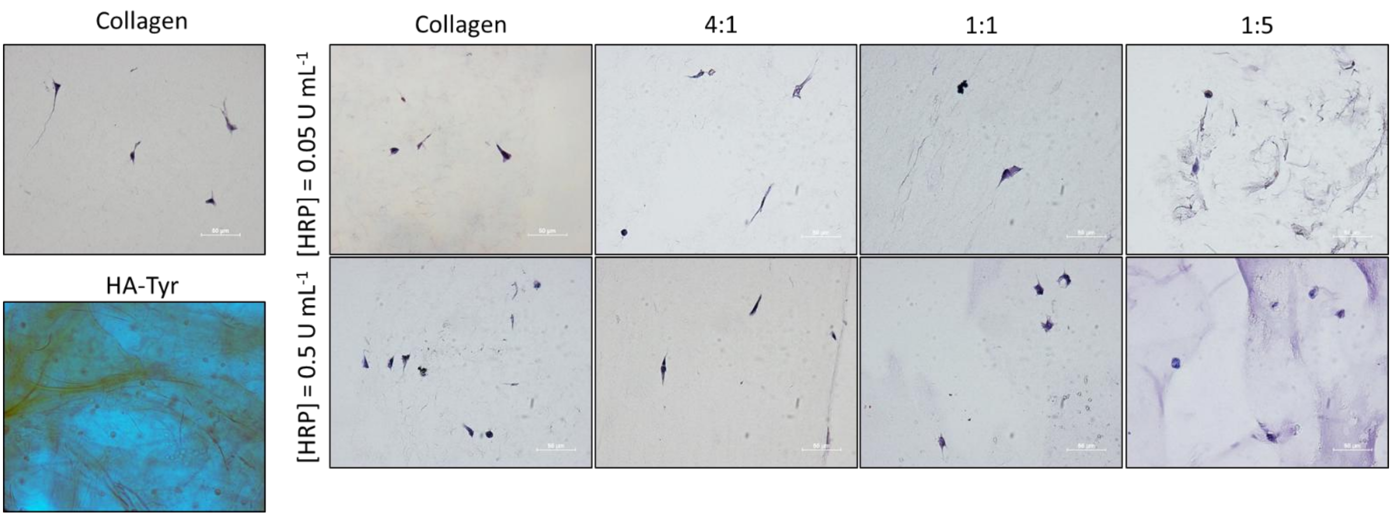

Figure 8. Morphology of normal human dermal fibroblasts encapsulated within Collagen/HA-Tyr hydrogels after one day. HA-Tyr cross-linking performed using 0.6 mM H_2O_2 and HRP concentration at 0.05 and 0.5 U.mL^{-1}. Cells nuclei were stained with Hemalun. Bar: 50 μm.

4. Conclusions

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

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Table 1

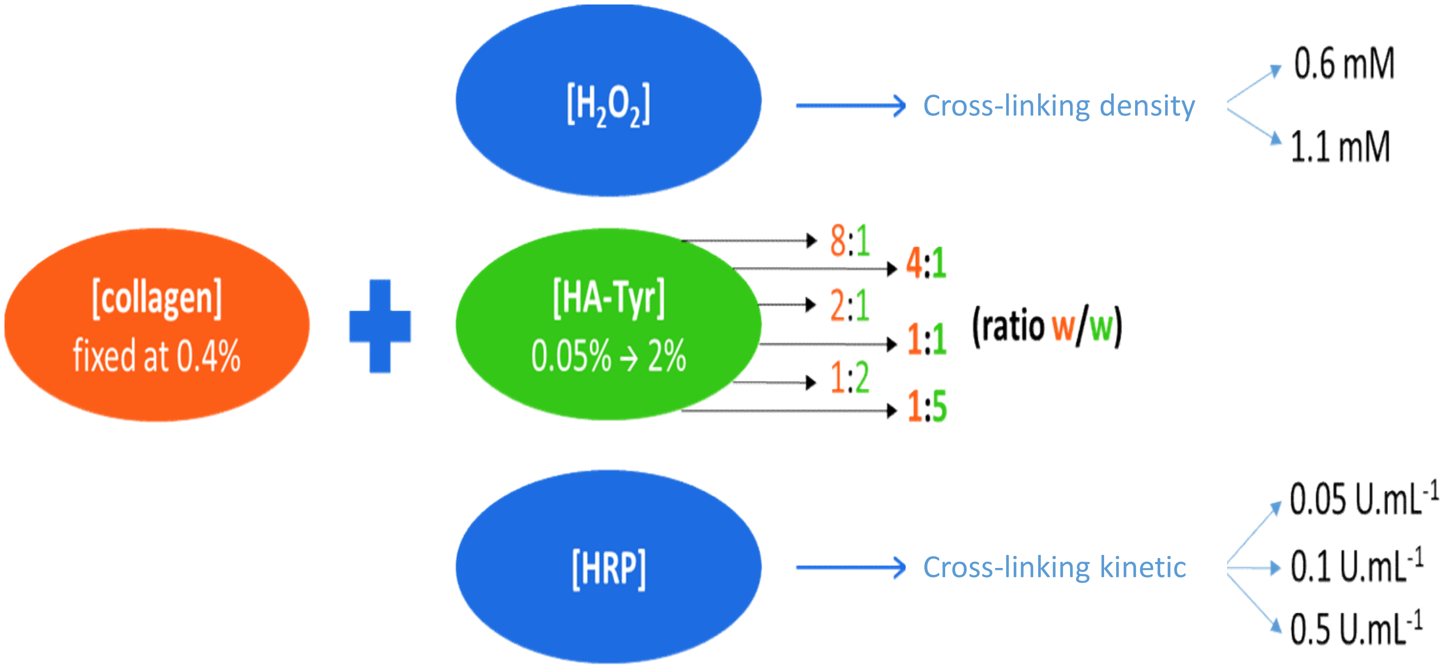
Composite Hydrogels - $[H_2O_2]$ = 0.6 mM					
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	$[H_2O_2]$ (mM)	[HRP] ($U.mL^{-1}$)	G' (Pa)
Pure Col	0.4%	0%	0	0	252 ± 25
			0.6	0.05	189 ± 22
				0.1	209 ± 15
				0.5	196 ± 32
8:1	0.4%	0.05%	0.6	0.05	132 ± 14
				0.1	109 ± 20
				0.5	110 ± 9
4:1	0.4%	0.1%	0.6	0.05	124 ± 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^{-1}					
Col/HA-Tyr Ratio	[Col]	[HA-Tyr]	[H_2O_2] (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



[H_2O_2]
Cross-linking density
0.6 mM
1.1 mM
[collagen]
fixed at 0.4%
[HA-Tyr]
0.05% → 2%
8:1
4:1
2:1
1:1
1:2
1:5
(ratio w/w)
[HRP]
Cross-linking kinetic
0.05 U.mL-1
0.1 U.mL-1
0.5 U.mL-1

Figure 2

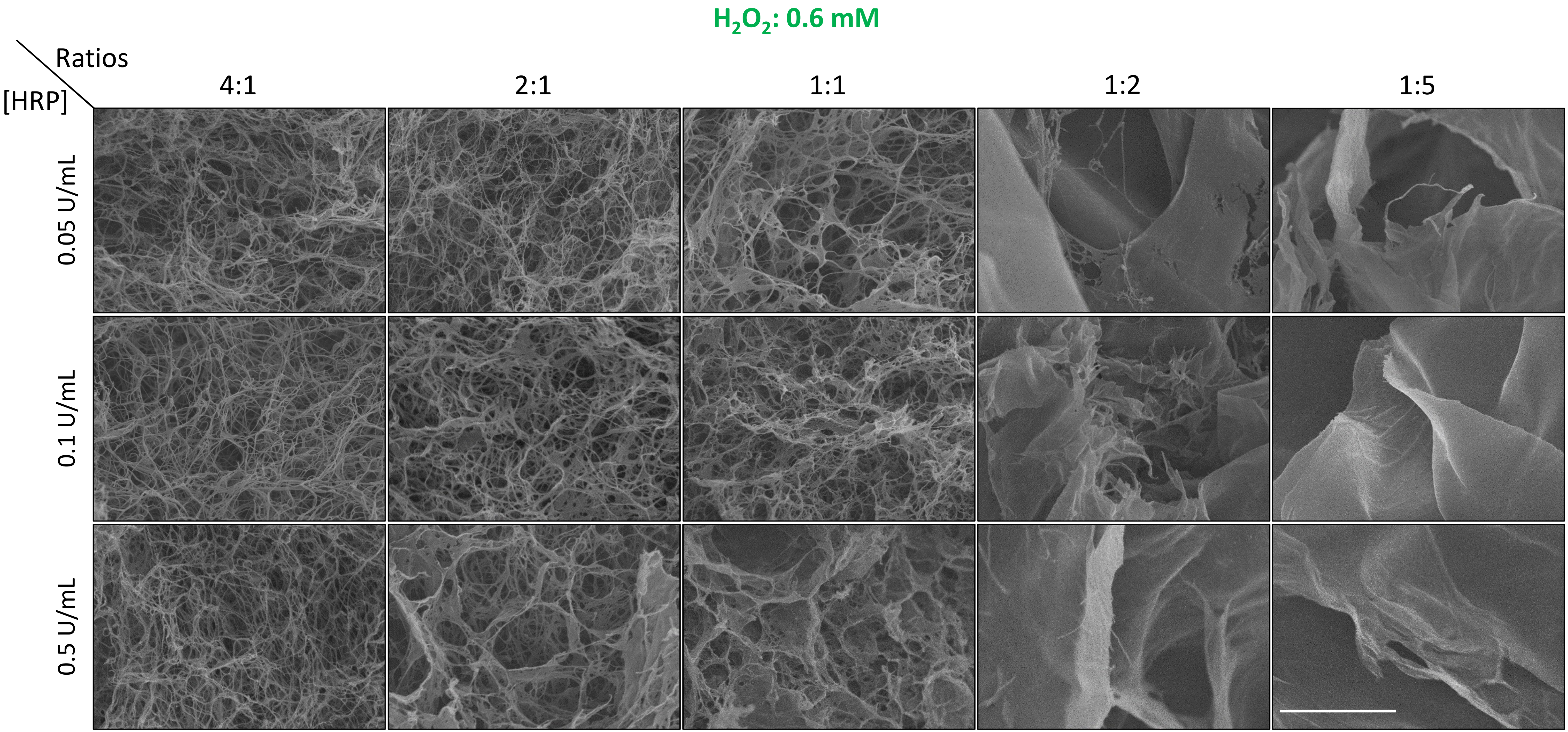


Figure 3

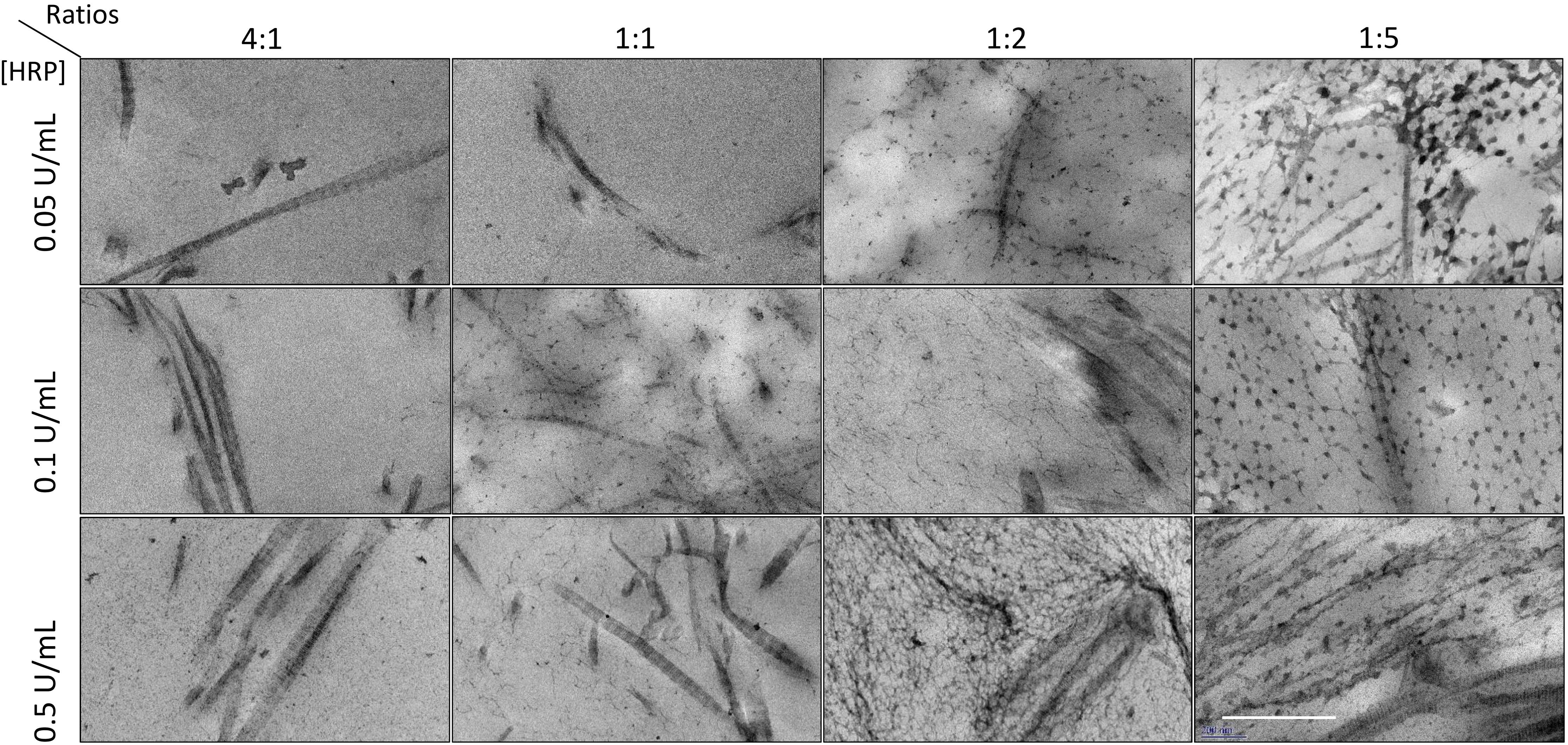
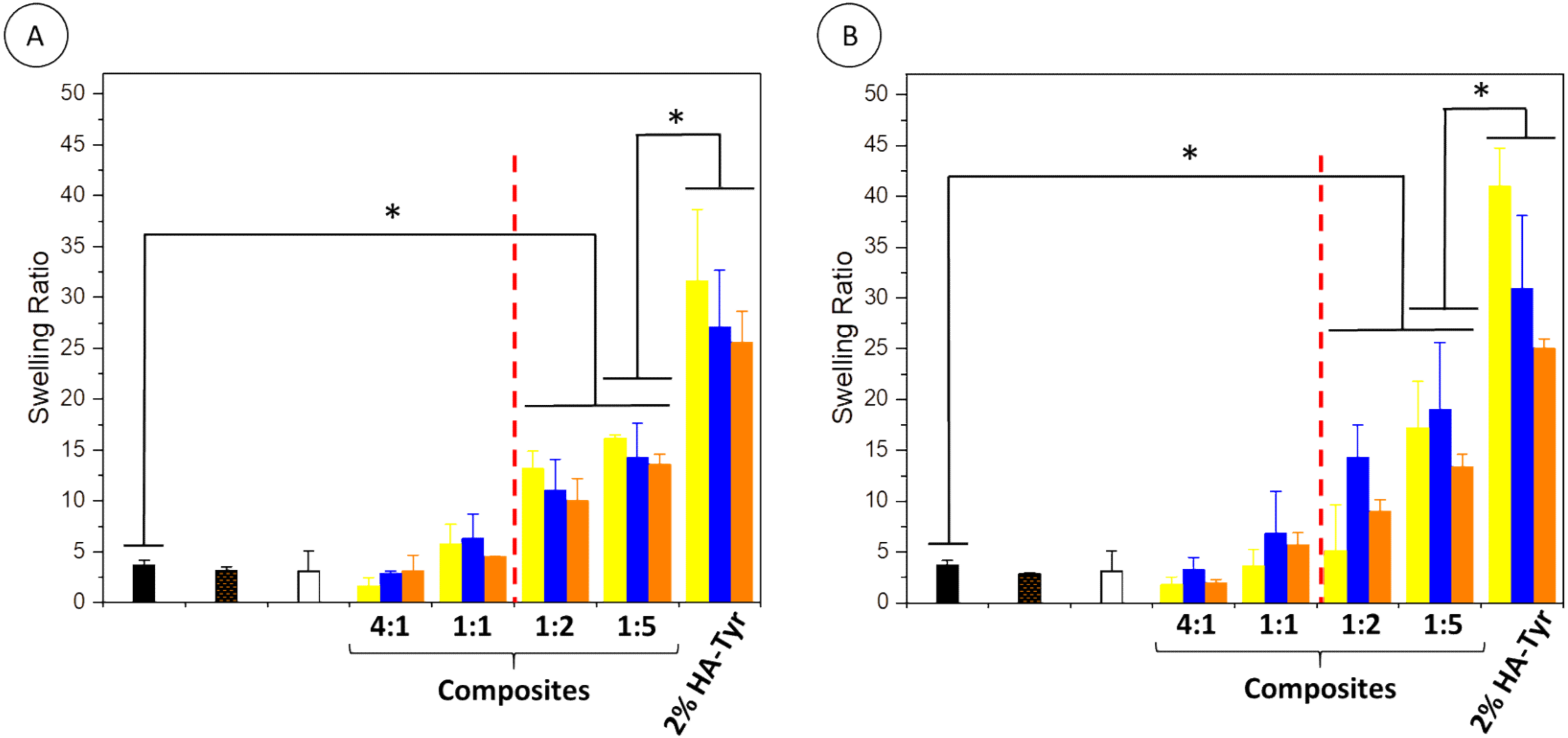


Figure 4



A
B
Swelling Ratio
0
5
10
15
20
25
30
35
40
45
50
*
4:1
1:1
1:2
1:5
Composites
2% HA-Tyr

Figure 5

Figure 5

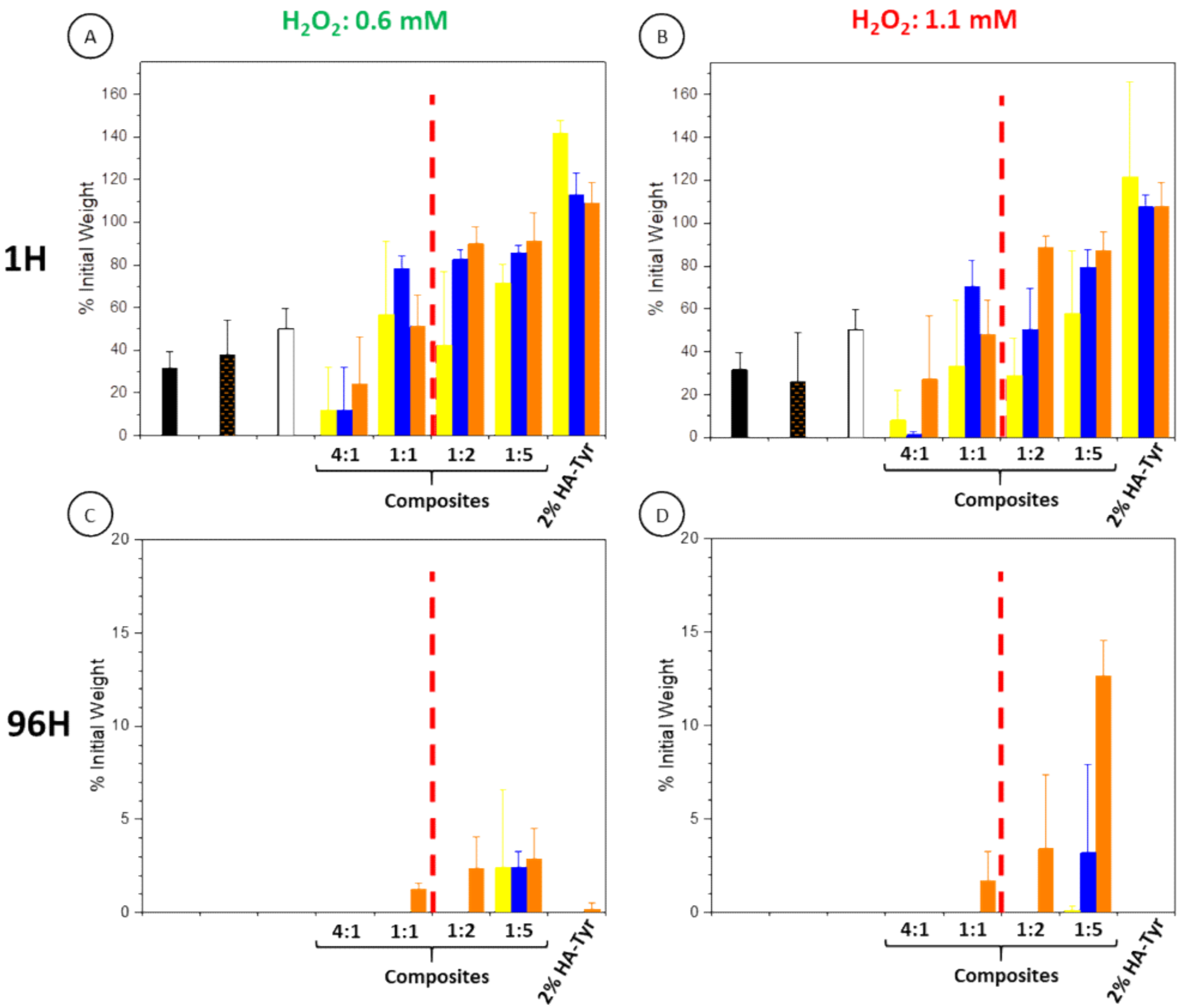


Figure 6

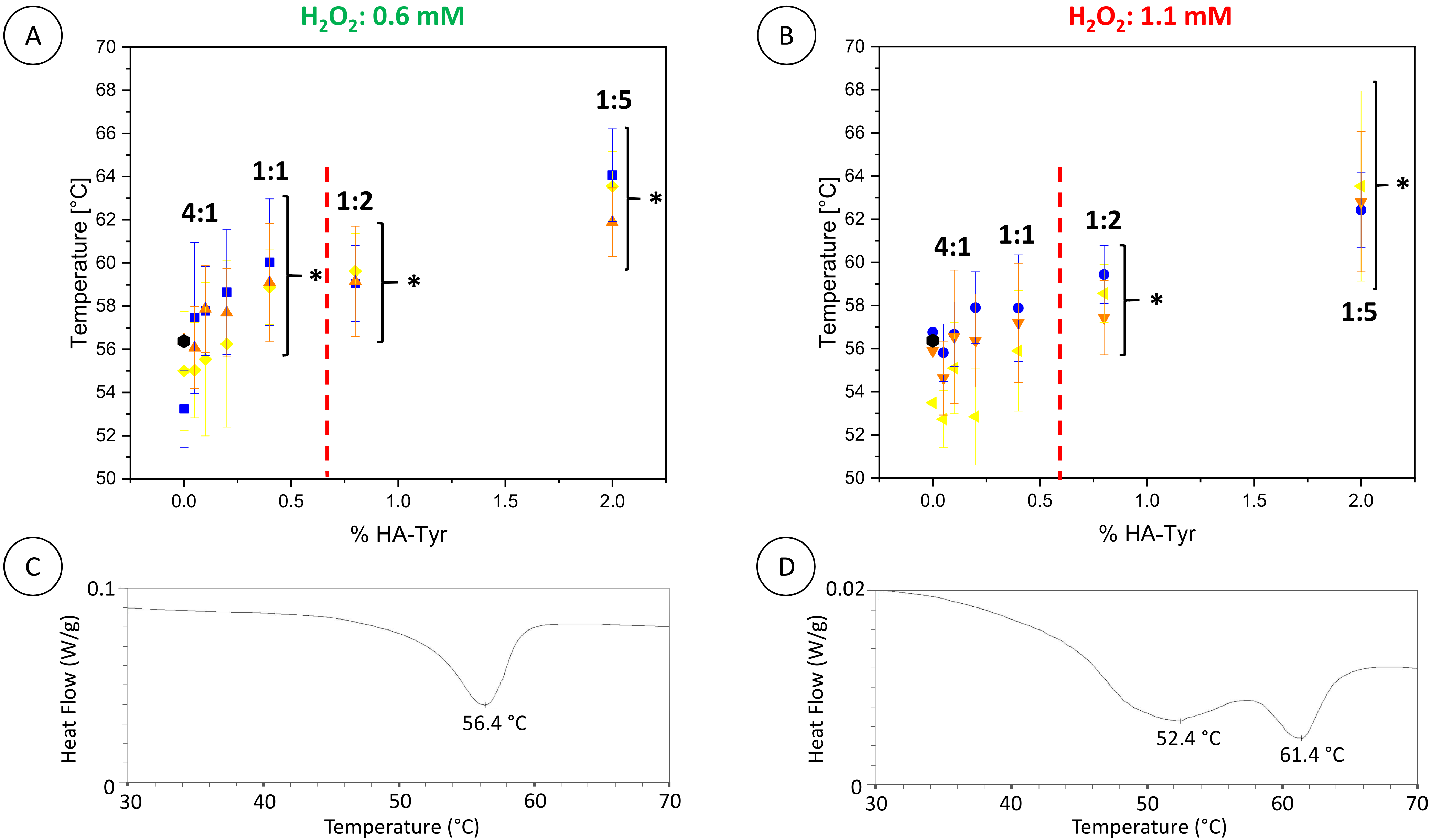
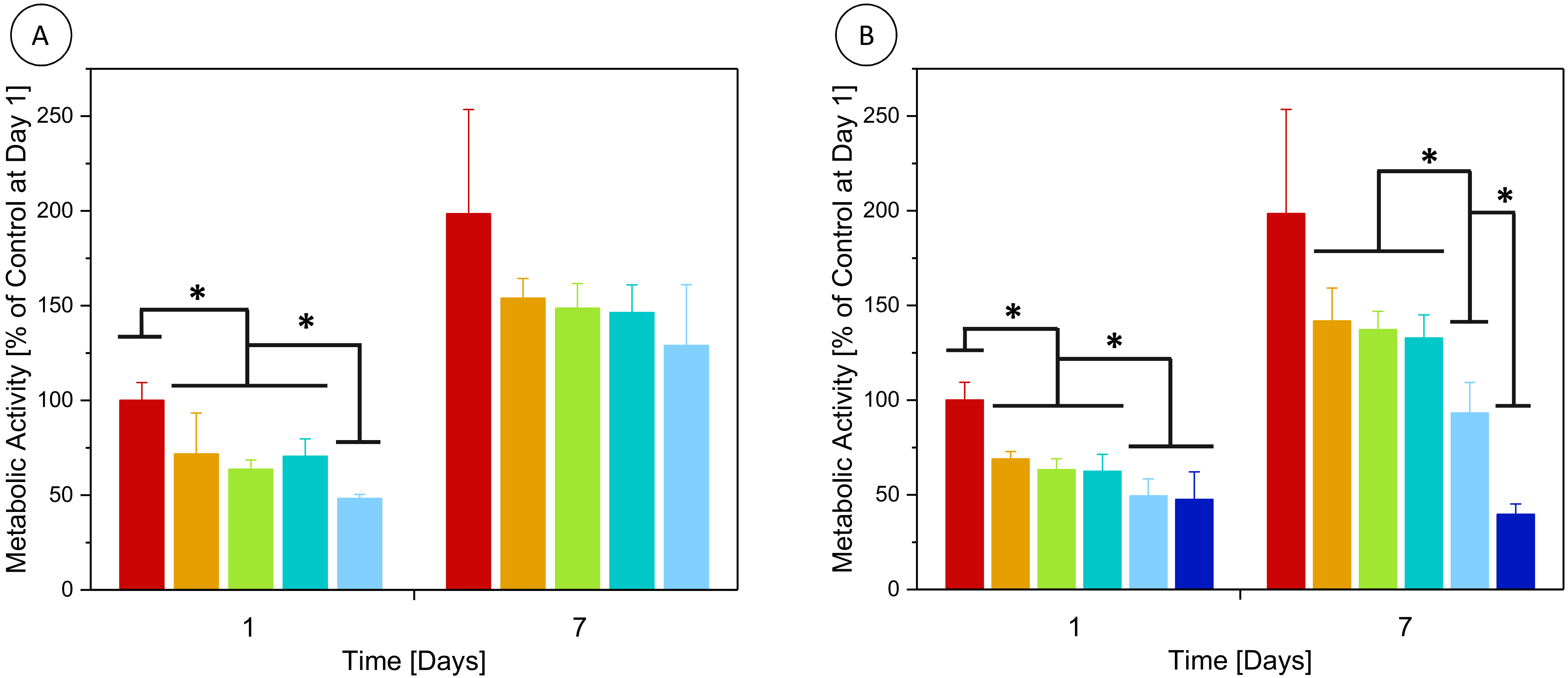
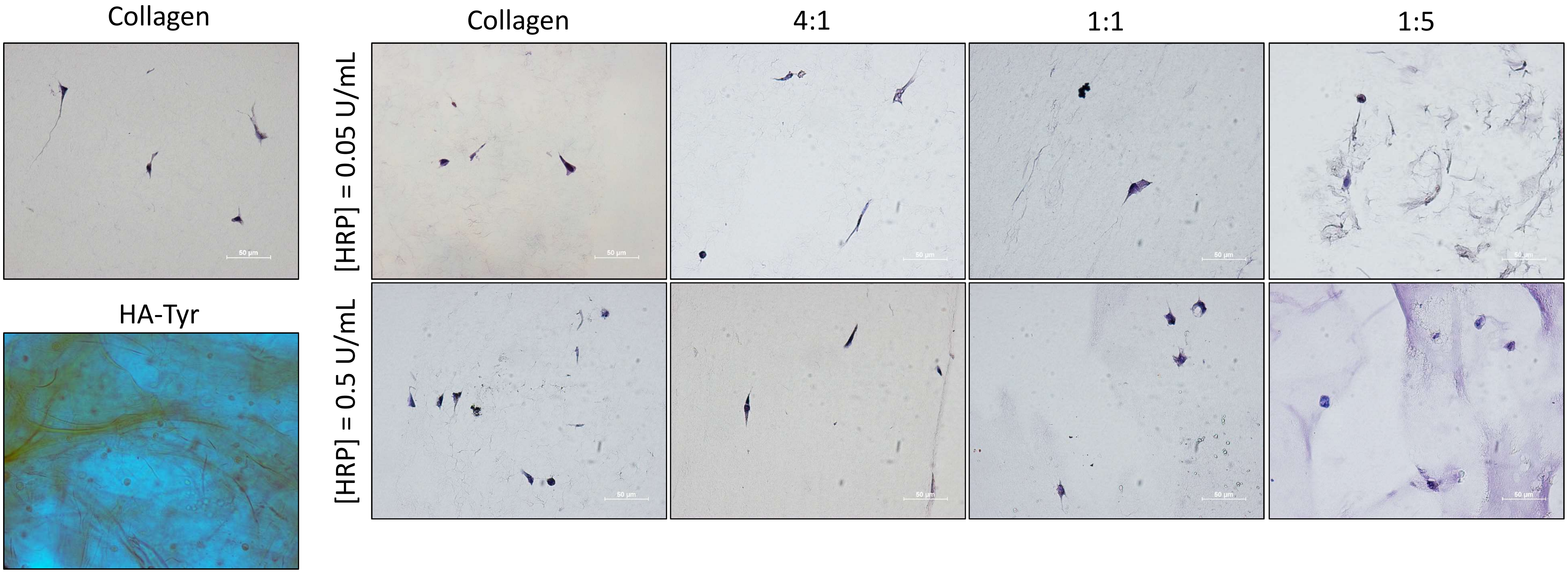

A
H_2O_2: 0.6 mM
Temperature [°C]
% HA-Tyr
4:1
1:1
1:2
1:5
*
B
H_2O_2: 1.1 mM
Temperature [°C]
% HA-Tyr
4:1
1:1
1:2
1:5
*
C
Heat Flow (W/g)
Temperature (°C)
56.4 °C
D
Heat Flow (W/g)
Temperature (°C)
52.4 °C
61.4 °C

Figure 7



A
Metabolic Activity [% of Control at Day 1]
0
50
100
150
200
250
*
*
1
7
Time [Days]
B
Metabolic Activity [% of Control at Day 1]
0
50
100
150
200
250
*
*
*
*
1
7
Time [Days]

Figure 8



Supplementary data
Click here to download Supplementary data: Supporting Data Article Antoine 08-01-20.pdf

Graphical Abstract