Elsevier Editorial System(tm) for Carbohydrate Polymers

Manuscript Draft

Manuscript Number: CARBPOL-D-16-00212R2

Title: Hyaluronan delivery by polymer demixing in polysaccharide-based hydrogels and membranes for biomedical applications

Article Type: Research Paper

Keywords: Polysaccharides, hydrogels, polymer demixing, membranes, wound healing activity.

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Abstract: Alginate-based membranes containing hyaluronic acid (HA) were manufactured by freeze-drying calcium-reticulated hydrogels. The study of the distribution of the two macromolecules within the hydrogel enabled to highlight a polymer demixing mechanism that tends to segregate HA in the external parts of the constructs. Resistance and pliability of the membranes were tuned, while release and degradation studies enabled to quantify the diffusion of both polysaccharides in physiological solution and to measure the viable lifetime of the membranes. Biological studies in vitro proved that the liquid extracts from the HA-containing membranes stimulate wound healing and that fibroblasts are able to colonize the membranes. Overall, such novel alginate-HA membranes represent a promising solution for several medical needs, in particular for wound treatment, giving the possibility to provide an in situ administration of HA from a resorbable device.

Dr. Manuel A. Coimbra

14th March 2016

Manuscript Ref. No: **CARBPOL-D-16-00212**

Dear Dr. Coimbra,

please, find herewith enclosed the revised version of the manuscript "*Hyaluronan delivery by polymer demixing in polysaccharide–based hydrogels and membranes for biomedical applications*" by Travan A., Scognamiglio F., Borgogna M., Marsich E., Donati I., Tarusha L., Grassi M., and Paoletti S.

All the revisions suggested by the reviewers and the editor have been followed and all questions have been answered.

Yours sincerely,

Andrea Travan

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Authors declarations:

The authors confirm that the manuscript, or its contents in some other form, has not been published previously by any of the authors and/or is not under consideration for publication in another journal at the time of submission. All authors have seen and approved the submission of the manuscript.

Highlights

- Alginate-hyaluronan hydrogels were obtained exploiting a polymer demixing process.
- Freeze-dried membranes with tunable mechanical properties were manufactured.
- Hyaluronan released from membranes stimulates fibroblasts migration/proliferation.
- The molecular arrangement of hyaluronan favors its in situ release.

*Response to Reviewers

Hyaluronan delivery by polymer demixing in polysaccharide–based hydrogels and membranes for biomedical applications

Andrea Travan[†,*], *Francesca Scognamiglio*[†], *Massimiliano Borgogna*[†], *Eleonora Marsich*[‡], *Ivan Donati*[†], *Lorena Tarusha*[†], *Mario Grassi*[‖] *and Sergio Paoletti*[†]

Reviewer #1: The work of Andrea Travan et al. presented in this article is of interest to the readers of Carbohydrate Polymers. It represents a relatively interesting study of Hyaluronan delivery by polymer demixing in polysaccharide-based hydrogels and membranes for biomedical applications. However, I have a number of recommendations for the authors.

Q1 There are still some spaces which need be improved in terms of the journal quality. The abstract are written too broadly thus lack attraction of readers, especially, It appears to be chaotic and disorderly.

The abstract has now been shortened and clarified.

Q2, What is the mechanism of Freeze-drying the hydrogels? There are much paper reported about freeze-drying the fibers , hydrogel, membrane, and particale..such as Carbohydrate Polymers, Volume 88, Issue 2, 2 April 2012, Pages 734-742.

As described in the Materials and Methods section, the mechanism of freeze-drying the hydrogels is based on two steps: 1) cooling the hydrogels to -20°C; 2) putting the frozen hydrogels under vacuum using a Single-Chamber Freeze-Dryer until all water sublimates from the hydrogels.

Q3, The authors say in the page of 17, To the best of our knowledge, our work is the first reported case of a demixing process between alginate and hyaluronan clearly identified as such. Such a sentence, sounds arbitrary and makes you think that you are the first to reported such a material?

Yes, indeed to the best of our knowledge, this is the first documented case that specifically highlights this demixing mechanism between alginate and hyaluronan in this type of hydrogels.

Q4, How were the release and measured? Give the details.

More details have been added in chapter 2.8 of the Materials and methods section.

Q5, SEM image should be shown from different image from sample in order to observe.

An additional SEM image has been added in Figure 7 to highlight the presence of cells on the membrane.

Q6, r365 p15 a reference should be reported, at least.

This sentence is a comment to our results and not a reference to literature data. In order to make it clearer, this aspect has been stressed by adding the words "The mechanical spectra showed that…" in section 3.2.

Q7, There are many places need to be unified in writing, such as MW on page of 8, 20mm on page of 10, and 10ml on page of 11.

These corrections have been done throughout the manuscript.

Q8, The reference of such as in the paper should be unified (Zhang Y, Zhang X, Shi B, & Miron RJ, 2013),

The style of this reference has now been unified with the rest of the manuscript.

Reviewer #2: Overall I thought the manuscript was generally well written and demonstrated a comprehensive and logical experimental approach. There are however a number of small revisions that I believe would improve the paper.

1. The introduction could be shortened. The first paragraph is not needed (start the introduction from line 53-54)

As suggested by Reviewer 2, the whole paragraph indicated has been deleted. The introduction now starts from the indicated line.

2. Line 169 use 'roughened' rather than 'shagreened'

The word has now been changed in the text.

3. Line 179 what is solicitation frequency? Do you mean oscillation frequency?

Yes. The word has now been changed in the text.

4. Section 2.7 give details of the excitation and emission wavelengths

The details have been inserted in the text.

5. Line 216 composed from alginate not composed by alginate

The word has been corrected.

6. Section 2.10 use the past tense throughout see line 235

The past tense has been used.

7. Line 237 w/v not w/V

The change has been done in the text.

8. Line 260 the kinetic of the closure?

The word "kinetic" has been changed with "time required for".

9. Line 304 a reference would be useful here to describe the homogenous gelation using CaCO3 perhaps Draget et al 1990 Carbohydrate Polymers 14 159-178; Moe et al 1992 Carbohydrate Polymers 19 279-284; Draget et al 1993 Hydrobiologica 260 563-569

References have been inserted.

10. Line 308 references would be useful here to show alginate degrades in physiological conditions perhaps Rowley et al 1999 Biomaterials 20 (1), 45-53; Hunt et al 2010 Acta Biomaterialia 6(9)3649-56; Jahromi et al 2011 J Mech Behav Biomed Mater. 2011 4(7):1157-66

References have been inserted.

11. Clearer scale bars should be added to micrographs

The SEM images have now been up-loaded at higher definition, so that the scale bars are more clear.

12. Fig 5B should the x axis be days? I think that would be clearer than shifts

The x axis has been modified according to the reviewer's suggestion.

13. Line 493 from the micrographs in Fig 7 I think that the cells look more rounded (as would be expected with a non cell-adhesive material such as alginate) than flattened and strongly attached. There may be some attachment to the hyaluronan segments at the surface but these are not elongated attached cells "suggesting strong biological interactions" I would tone down this claim to something like "there appears to be some evidence of cell attachment which is likely to be due to the presence of regions of hyaluronan on the surface"

The sentence has been rephrased according to the reviewer suggestion.

14. Figure 8 Add the micrograph for the Alg alone at 48h for comparison with Alg-HA also there is no mention of figures 8C-F in the text

The micrograph for the Alginate alone at 48 hours has been added to figure 8 (8F), as suggested by the reviewer (figure caption has been updated accordingly). The optical images of the cultured cells are now mentioned in the text.

15. Conclusion needs shortening, start at line 533. Also line 536 says that "the process can be easily scaled up" where any studies on scale up done? If not remove it from the conclusion

According to the reviewer's suggestion, conclusions have been shortened and the reference to the scale-up has been removed.

Editor: Highlights should not have more than 85 characters each, including spaces.

Highlights have been shortened as indicated by the Editor.

Hyaluronan delivery by polymer demixing in polysaccharide–based hydrogels and membranes for biomedical applications

Andrea Travan[†,*], *Francesca Scognamiglio*[†], *Massimiliano Borgogna*[†], *Eleonora Marsich*[‡], *Ivan Donati*[†], *Lorena Tarusha*[†], *Mario Grassi*[‖] *and Sergio Paoletti*[†]

Itemized list of changes which have been made in response to the reviewer's comments/suggestions:

Abstract

Original abstract:

Alginate-based membranes containing hyaluronic acid (HA) were manufactured by freeze-drying calcium-reticulated hydrogels. The study of the distribution of the two macromolecules within the hydrogel enabled to highlight a polymer demixing mechanism that tends to segregate HA in the external parts of the constructs. Resistance and pliability of the membranes were tuned based on mechanical characterization. Release and degradation studies enabled to quantify the diffusion of both polysaccharides in physiological solution and to measure the viable lifetime of the membranes. The liquid extracts from the membranes were proved to be biocompatible and to stimulate wound healing *in vitro*. SEM analyses showed that fibroblasts were able to colonize the membranes, pointing to their possible use for tissue engineering applications. Overall, such novel alginate-HA membranes represent a promising solution for several medical needs, in particular for wound treatment, giving the possibility to provide an in situ administration of HA from a resorbable device.

Modified abstract:

Alginate-based membranes containing hyaluronic acid (HA) were manufactured by freeze-drying calcium-reticulated hydrogels. The study of the distribution of the two macromolecules within the hydrogel enabled to highlight a polymer demixing mechanism that tends to segregate HA in the external parts of the constructs. Resistance and pliability of the membranes were tuned, while release and degradation studies enabled to quantify the diffusion of both polysaccharides in physiological solution and to measure the viable lifetime of the membranes. Biological studies *in vitro* proved that the liquid extracts from the HA-containing membranes stimulate wound healing and that fibroblasts are able to colonize the membranes. Overall, such novel alginate-HA membranes represent a promising solution for several medical needs, in particular for wound treatment, giving the possibility to provide an *in situ* administration of HA from a resorbable device.

Introduction

The following paragraph has been deleted:

"The cross fertilization between biological sciences and polymer science (and technology) has been steadily increasing from Staudinger's groundbreaking elucidation of the nature of the high-molecular weight compounds. Apart from the vital tribute that proteins and nucleic acids have to pay to the concept of macromolecule, theoretical approaches, models, theories from polymer science but also synthetic routes and design of polymer-based supramolecular architectures have produced an enormous, positive impact on biology, biotechnology, pharmaceutical sciences, medical devices. To name one, the role of polymers in the development of effective drug-delivery systems is of primary importance (Liechty, Kryscio, Slaughter, & Peppas, 2010)".

Section "2.4. Mechanical spectroscopy"

- *The word "'shagreened" has been changed with the word "roughened".*
- *The words "solicitation frequency" have been changed with "oscillation frequency".*

Section "2.5. Degradation studies"

The style of the following figures has been unified: 20 mm; 10 mL.

Section "2.7. Fluorescent microcopy"

Details related to excitation and emission wavelengths have been inserted in this section: (excitation wavelength: 460 nm; emission wavelength: 515 nm).

Section "2.8. Release studies"

- *The sentence "Membranes composed by alginate…" has been changed in "Membranes composed from alginate…" according to reviewer's comment.*
- *The style of the following figure has been unified: 10 mL.*
- *The following paragraph has been added: "Briefly, ^{1}H NMR spectra of supernatants were recorded at 60 °C with a JEOL 270 NMR spectrometer (6.34 T) operating at 270 MHz for proton. Samples were dissolved in D_2O and 40 μL of NaOD 20% in D2O (final NaOD concentration 0.3 M) were added prior to the analysis. The chemical shifts are expressed in ppm downfield from the signal for 3-(trimethylsilyl)propanesulfonate. The composition of the supernatants was determined from the ratio between the area of the anomeric proton of guluronic residues of alginate and the area of the methyl signal on the acetyl group of hyaluronan. Binary mixtures of alginate and hyaluronan at known composition were used for the calibration curve".*

Section "2.10. In vitro biocompatibility (LDH assay)"

- *The present perfect tense of the sentence "UV-sterilized specimens of membranes have been soaked" has been changed with the past tense form "UV-sterilized specimens of membranes were soaked".*
- *"w/V" has been changed in "w/v"*

Section "2.11. In vitro wound healing (scratch assay)"

The sentence "the kinetic of the closure" has been modified in "the time required for the closure the closure of the cell gap".

Section "3.1. Hydrogel preparation and confocal analysis"

The following references have been inserted in the text:

"Draget, Siemensen, Onsøyen, & Smidsrød, 1993; Moe, Draget, Skåk-Bræk, & Simdød, 1992";

"Markovsky et al., 2012; Shoichet, Li, White, & Winn, 1996".

Section "3.2. Rheological characterization of hydrogels"

The following sentence has been inserted in the text: "The mechanical spectra showed that".

Section: "3.5. Polysaccharide release and membrane degradation"

The x axis of figure 5b has been changed (the word "shifts" has been changed in "days").

Section "3.6. In vitro biocompatibility"

- *The following sentence has been inserted in the text: "there appears to be some evidence of cell attachment which is likely due to the presence of regions of hyaluronan on the surface".*
- *SEM images have been loaded at higher resolution.*
- *An additional SEM image has been added to figure 7.*

Section: "3.7. In vitro wound healing (scratch assay)"

- *The sentence "Representative optical images of cultured cells used for the scratch tests are reported in Figures 8C-G" has been inserted.*
- *An additional micrograph has been added to figure 8.*

Section: "4. Conclusions"

- *The following sentence "Bioactive membranes designed for the acceleration of the wound healing process…" has been changed in "A promising approach for the preparation of bioactive membranes for wound healing applications is…"*
- *The sentence "the process can be easily scaled up" has been removed.*

Section: "Reference list"

The style of the following reference has been unified:

"Zhang, Y., Zhang, X., Shi, B., & Miron, R. J. (2013). Membranes for guided tissue and bone regeneration. Annals of Oral & Maxillofacial Surgery, 1(1):10."

Section: "Figure captions"

The caption of figure 8 has been modified as reported hereafter:

Figure 8: Upper part: effect of the membranes on the closure of gaps within HDFa cells cultured for the scratch tests in the absence (A) or presence (B) of mitomycin C. Triangles: alginate-HA membrane (Formulation D); circles: alginate membrane (Formulation A); squares: untreated cells (control). (: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001). Lower part: optical images of the cell gap at time zero (C), after 24 hours in the presence of the liquid extracted from the alginate membranes (D) or the alginate-HA membranes (E), after 48 hours in the presence of the liquid extracted from the alginate membranes (F) or the alginate-HA membranes (G).*

Highlights

Highlights have been shortened as indicated by the Editor.

Hyaluronan delivery by polymer demixing in polysaccharide–based hydrogels and membranes for biomedical applications

Andrea Travan[†,*], *Francesca Scognamiglio*[†], *Massimiliano Borgogna*[†], *Eleonora Marsich*[‡], *Ivan Donati*[†], *Lorena Tarusha*[†], *Mario Grassi*[‖] *and Sergio Paoletti*[†]

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Abstract

Alginate-based membranes containing hyaluronic acid (HA) were manufactured by freeze-drying calcium-reticulated hydrogels. The study of the distribution of the two macromolecules within the hydrogel enabled to highlight a polymer demixing mechanism that tends to segregate HA in the external parts of the constructs. Resistance and pliability of the membranes were tuned, while release and degradation studies enabled to quantify the diffusion of both polysaccharides in physiological solution and to measure the viable lifetime of the membranes. Biological studies *in vitro* proved that the liquid extracts from the HA-containing membranes stimulate wound healing and that fibroblasts are able to colonize the membranes. Overall, such novel alginate-HA membranes represent a promising solution for several medical needs, in particular for wound treatment, giving the possibility to provide an *in situ* administration of HA from a resorbable device.

Keywords:

Polysaccharides, hydrogels, polymer demixing, membranes, wound healing activity.

1. Introduction

Novel polymer-based biomaterials in the form of surgical membranes, meshes and dressings are being developed for biomedical use in tissue engineering (Zhang, Zhang, Shi, & Miron, 2013), for the treatment of topical wounds and burns (Okamura et al., 2013; Plettig et al., 2014; Vasile, Pieptu, Dumitriu, Panzariu, & Profire, 2013; Xu et al., 2015), for the repair of hernias (Beale, Hoxworth, Livingston, & Trussler, 2012; Novitsky, 2013) and for the development of haemostatic devices (Granville-Chapman, Jacobs, & Midwinter, 2011; Smith, Laird, Porter, & Bloch, 2013; Gaharwar et al., 2014; Ohta et al., 2014). In the field of wound treatment, membranes based on both natural and synthetic polymers have been reported (Khil, Cha, Kim, Kim, & Bhattarai, 2003; Sionkowska, 2011). The composition of wound dressings can be optimized in order to tailor the physical-chemical properties of the biomaterial and to meet the needs of each wound stage (Fonder et al., 2008). For instance, Fleck and colleagues reported the possibility to modify the composition of membranes to tune their bioactive properties in terms of stimulating or accelerating the healing of the damaged tissue (Fleck & Chakravarthy, 2007). Several biopolymers from natural sources have been proposed as candidates to manufacture surgical membranes; the advantage of these materials is that their chemical structure can mimic the macromolecular environment of the extracellular matrix (ECM). Recently, membranes prepared from biopolymers like polysaccharides and collagen have been proposed for biomedical use (Fleck & Simman, 2010; Francesko & Tzanov, 2011; Cardoso, hado-Silva, Sabino, Santos, Jr., & Zavaglia, 2014; Lopes, Riegel-Vidotti, Grein, Tischer, & Faria-Tischer, 2014; Majumdar et al., 2015; Pandis et al., 2014; Zheng et al., 2014). Among natural polymers, polysaccharides like alginate, chitosan and hyaluronic acid (HA) offer several advantages in terms of biocompatibility, hydrophilicity and bioactive properties (Maggiori et al., 2010; Boateng, Matthews, Stevens, & Eccleston, 2008; Jayakumar, Prabaharan, Sudheesh Kumar, Nair, & Tamura, 2011; Powers, Morton, & Phillips, 2013). The use of 3D matrices based on the combination of several polysaccharides has been previously reported by some of the authors of this

paper (Marsich et al., 2008; Marsich et al., 2013; Travan et al., 2009). HA and alginate are particularly appealing for the preparation of bioactive and biodegradable membranes for wound healing applications, as the former displays wound healing bioactivity (Dicker et al., 2014) while the latter is a biocompatible polysaccharide with considerable structural and mechanical properties (Lee & Mooney, 2012).

HA is a linear polysaccharide belonging to the family of glycosaminoglycans (GAGs), the main constituents of the ECM. At physiological pH values, it has a polyanionic structure that imparts excellent hydro-coordinating properties enabling the retention of large amounts of water. HA is involved in the regulation of several biological phenomena such as cell migration, differentiation, growth, adhesion, angiogenesis as well as the control of the immune response and the interaction with collagen fibres (Jiang, Liang, & Noble, 2007). In the human body, HA undergoes degradation through enzymatic catalysis (Markovsky et al., 2012). The wound-repairing properties of HA have been correlated to its effects on the inflammatory response, chemotaxis, cell migration, proliferation and angiogenesis (Jiang et al., 2007).

Alginate is a polysaccharide mainly isolated from brown algae. It is a linear copolymer whose comonomers are guluronic (G) and mannuronic (M) acids; it can form hydrogels in the presence of divalent cations such as calcium, which bind preferentially to the G-blocks (but to MG sequences as well, albeit to a lesser extent) in a highly cooperative manner. Alginates have long been known to possess haemostatic properties (Blair, Jarvis, Salmon, & McCollum, 1990; Sayag, Meaume, & Bohbot, 1996) and recently alginate-based dressings have been shown to reduce postoperative drainage volume in patients undergoing elective rectal resection for cancer (Maggiori et al., 2010). Alginate-based biomaterials can be manufactured into films (Esser & Tessmar, 2013; Rosellini, Cristallini, Barbani, Vozzi, & Giusti, 2009), fibres (Cuadros, Skurtys, & Aguilera, 2012; Kang et al., 2012) gels (Donati et al., 2005; Turco et al., 2011) and foams (Andersen et al., 2014a; Andersen, Melvik, Gåserød, Alsberg, & Christensen, 2014b), according to their final applications. In the human body, this polysaccharide is bio-eroded, partially degraded by macrophages and excreted

through kidneys (Markovsky et al., 2012). Mixtures of alginate and HA have been previously investigated, aiming at combining the respective peculiar properties of those polysaccharides for various types of biomedical applications (Donati et al., 2011; Geremia et al., 2014; Oerther et al., 1999; Oerther et al., 1999). Alginate hydrogels containing HA have been prepared and proposed for diverse applications, like cartilage transplant (Dausse et al., 2003; Gerard et al., 2005), articular surgery (Aulin et al., 2011; Chung et al., 2014) and wound healing (Catanzano et al., 2015).

Given these premises, the aim of this work was to develop novel biodegradable dressings in the form of pliable membranes based on HA and alginate starting from aqueous hydrogels obtained by freeze-casting from the mixture of these two polysaccharides. In essence, the construct design foresees a bi-component polymeric biomaterial which should release the bioactive polysaccharide, *i.e.* HA, from the 3D solid-like architecture of the calcium alginate structure. The investigation of the macromolecular distribution of the structural and mechanical features of the hydrogels was followed by a thorough characterization of the membranes, in terms of mechanical, functional (degradation, release), and biological (biocompatibility, wound healing capability) properties. From the fundamental macromolecular physical-chemical standpoint, particular attention was given to analyse the distribution profile of the two biopolymers arising from their demixing.

2. Materials and methods

2.1. Materials. Sodium alginate from *Laminaria hyperborea* (Alginate Pronova UP LVG, molecular weight, MW, 120 000; fraction of guluronic G residues, F_G = 0.69; fraction of guluronic diads, F_{GG} = 0.59; number average of G residues in G-blocks, $N_{G>1}$ = 16.3) and sodium hyaluronate (HA) Pharma grade (MW, 800 000) were kindly provided by Novamatrix/FMC Biopolymer (Sandvika, Norway). HA (MW~240 000, Phylcare Sodium Hyaluronate extra LW) was kindly provided by Sigea S.r.l. (Trieste, Italy). Calcium carbonate ($CaCO_3$), D-Gluconic acid δ-lactone (GDL), glycerol, C1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), N-

hydroxusuccinimide (NHS), fluoresceinamine, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), LDH (lactate dehydrogenase)-based TOX-7 kit, mitomycin C, 2-(N-Morpholino)ethanesulfonic acid (MES), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), hydrochloric acid (HCl), calcium chloride ($CaCl_2$), glutaraldehyde, glucose, ethanol and Hanks' Balanced Salt solution (HBSS, product code H8264) were purchased from Sigma Aldrich.

2.2. Membrane preparation

All membranes were prepared according to the following procedure: the polysaccharides were dissolved in deionized water and glycerol was added as a plasticizer (final concentration = 5% v/v). Then, $CaCO_3$ and GDL were added and the mixture was poured into rectangular moulds for the *in situ* gelation of the solution. The ratio $CaCO_3$ / GDL was 0.5 for each formulation studied; suspensions of $CaCO_3$ corresponding to $[Ca^{2+}]$ 20 mM or 50 mM were used. Subsequently, the hydrogels were cooled by immersion in a liquid cryostat; ethylene glycol in water (3:1) was used as refrigerant fluid. Temperature was decreased stepwise from +20°C to -20°C by 5 °C steps with 30 min intervals for equilibration. Finally, the frozen hydrogels were dried under vacuum using a Single-Chamber Freeze-Dryer (Christ Alpha 1-2 LDplus). Several membrane formulations were prepared by varying the compositions: the list of formulations employed is reported hereafter:

- Formulation A: Alginate 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v;
- Formulation B: Alginate 15 g/L, HA (800 kDa) 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v;
- Formulation C: Alginate 20 g/L, HA (240 kDa) 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v;
- Formulation D: Alginate 15 g/L, HA (240 kDa) 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v;

- Formulation E: Alginate 15 g/L, HA (240 kDa) 15 g/L, $CaCO_3$ 50 mM, GDL 100 mM, glycerol 5% v/v.

2.3. Mechanical characterization

The membranes were cut in dog-bone shapes according to ASTM D638-10 standards (type 1 samples); their mechanical properties were studied using a Universal Testing Machine (Mecmesin Multitest 2.5-i) equipped with a 100 N load cell. Tensile tests were performed at a crosshead speed of 5 mm/min. The cross section of the samples was measured with a caliper. Tensile stress was calculated dividing the load by the average original cross sectional area in the gage length segment of the specimen. Young's Modulus (*E*) was calculated as the slope of the linear portion in the stress-strain curve, considering the deformation range of 1%-3%. For each formulation, 5 replicates were used and the data were averaged and standard deviations calculated.

2.4. Mechanical spectroscopy

Rheological characterization of cylindrical hydrogels was performed by means of a controlled stress rheometer Haake Rheo-Stress RS150 operating at 25 °C using, as measuring device, a roughened plate apparatus (HPP20 *profiliert*: Ø = 20 mm). To avoid water evaporation from the hydrogel, the measurements were performed in a water-saturated environment realized by using a glass bell (solvent trap) containing a wet cloth. In addition, to prevent both wall-slippage and excessive gel squeezing (Lapasin & Pricl, 1995), the gap between plates was adjusted, for each sample, by executing a series of short stress sweep tests (f = 1 Hz; stress range 1 - 5 Pa; maximum deformation < 0.1%) characterized by a reducing gap. The gap was selected in order to maximize the value of the elastic modulus G' (used gaps ranged between 2.5 and 2.0 mm). For each hydrogel, the linear viscoelastic range was determined by means of a stress sweep test consisting in measuring elastic (G') and viscous (G") moduli variation with increasing shear stress (1 Pa < τ < 100 Pa) at oscillation frequency of ν = 1 Hz ($\omega = 2\pi\nu$ = 6.28 rad s^{-1}). Mechanical spectrum of the hydrogel was

determined by measuring the dependence of the elastic (G') and viscous (G") moduli from the pulsation (ω) at constant shear stress τ = 3 Pa (well within the linear viscoelastic range that, for all samples, spans up to at least 30 Pa).

2.5. Degradation studies

Circular samples of the membrane (Ø = 20 mm) were soaked with 10 mL of Hanks' balanced salt solution (HBSS) at room temperature and once per day the samples were collected, dried for 1 minute on filter paper, weighted and then immersed in fresh HBSS. The weight variation was recorded as a function of solution shifts. As a reference, the 100% of the weight was considered as the weight of the samples after 4 hours of immersion in 10 mL HBSS. Six parallel replicates were used and the values were averaged and standard deviations calculated.

2.6. Polysaccharide labeling

Alginate and HA were labelled with fluoresceinamine according to the procedure reported by Donati et al. (Donati et al., 2011). Briefly, NHS and EDC ([EDC]/[PolymRU] = 1.5; [NHS]/[EDC] = 1; PolymRU refers to the polymer repeating units) were added to each polysaccharide solution (3 g/L) in MES buffer (50 mM, pH 5.5) containing 10 % ethanol. A solution of fluoresceinamine in ethanol was added to label 1/500 of the available carboxylic groups of alginate, and 1/250 of the available carboxylic groups of HA. The solutions were stirred overnight in the dark at room temperature, dialyzed against 0.05 M $NaHCO_3$ (2 shifts), 0.05 M NaCl (2 shifts), and then extensively against deionized water (until the conductivity at 4 °C was below 2 µS). The pH was adjusted to 7.4; the solutions were filtered through 0.45 µm Millipore filters and then freeze-dried.

2.7. Fluorescent microcopy

The distribution of the polysaccharide within the hydrogels was analyzed by confocal microscopy (Nikon Eclipse C1 confocal microscope system on a Nikon TE-2000U inverted microscope). The

compositions analyzed were Formulation D and Formulation E prepared with either fluorescently-labelled alginate or fluorescently-labelled HA, employed one at a time in different gels. The hydrogels were sectioned and the fluorescent signal was acquired on the cross section surfaces (*xy* plane) from edge to edge of the samples (excitation wavelength: 460 nm; emission wavelength: 515 nm). Image analysis was carried out with the software Image J in order to measure the intensity profiles of the fluorescent sections. For each sample, fluorescence intensity was normalized on the maximum intensity recorded, while the cross section width was normalized on the hydrogel thickness in order to qualitatively compare the different samples. Five replicates per sample were used.

2.8. Release studies

Membranes composed from alginate and HA were incubated in 10 mL of HBSS for selected time intervals. After incubation, the supernatants were collected and dialyzed for two days against 0.1 M HCl (4 shifts) and deionized water until the conductivity of the solution was below 4 µS. Then, the solution was collected and the pH set at 7.2. The supernatants were dried, weighted and analyzed by NMR according to the procedure described by Geremia et al. (Geremia et al., 2014). Briefly, ^{1}H NMR spectra of supernatants were recorded at 60 °C with a JEOL 270 NMR spectrometer (6.34 T) operating at 270 MHz for proton. Samples were dissolved in D_2O and 40 µL of NaOD 20% in D_2O (final NaOD concentration 0.3 M) were added prior to the analysis. The chemical shifts are expressed in ppm downfield from the signal for 3-(trimethylsilyl)propanesulfonate. The composition of the supernatants was determined from the ratio between the area of the anomeric proton of guluronic residues of alginate and the area of the methyl signal on the acetyl group of hyaluronan. Binary mixtures of alginate and hyaluronan at known composition were used for the calibration curve.

2.9. Cell cultures

Primary human dermal fibroblasts isolated from adult skin (HDFa) were purchased from Invitrogen™ Life Technologies. The cells were cultured in Medium 106 and supplemented with Low Serum Growth Supplement (LSGS), both provided by Invitrogen™ Life Technologies, 100 U/mL penicillin, 100 µg/mL streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 at 37 °C.

2.10. *In vitro* biocompatibility (LDH assay)

The biocompatibility of the biopolymeric membranes (formulations A and D) was evaluated through a quantitative analysis of the effect of the liquid extracts of the materials, according to the ISO 10993-5:2009 International Standard; the lactate dehydrogenase (LDH) assay was used. Since the extracting conditions should simulate or exaggerate the clinical use conditions, the following procedure has been developed. UV-sterilized specimens of membranes were soaked and incubated in the extraction culture medium for 72 hours at 37 °C. The ratio between weight of the patch and volume of the medium was maintained constant and selected in order to reach a polymer concentration (on the basis of the release studies) of 0.5 % w/v, which avoided biased results due to excessive viscosity of the extraction medium. The samples were also diluted 1:10 to reach polymer concentration of 0.05%. HDFa cells were trypsinized and seeded on a 24-well sterile plate at final concentration of 40 000 cells per well. Aliquots of 500 µL of liquid extract of the membranes were added to the wells. Untreated cells and cells treated with Triton X-100 0.1% were considered, respectively, as a negative and positive control. The LDH assay was performed 24 and 72 hours after the treatment: the level of cytotoxicity was evaluated by comparing the LDH values measured for the samples and those corresponding to the total amount of intracellular LDH calculated by inducing cellular lysis. For each series, four replicates were tested. 45 µL of both cell medium from tested samples and cellular lysates were added to LDH mix (30 µL LDH assay substrate, 30 µL LDH cofactor, 30 µL dye solution) and the incubation was allowed 30 minutes in dark. The

enzymatic reaction was stopped by adding 1/10 HCl 1N to each sample. The plate was read at 490 nm and 690 nm with a spectrophotometer (Infinite M200 PRO NanoQuant, Tecan). Evaluation of cytotoxicity was calculated according to the formula: LDH released (%)=[(A-B)/(C-B)]·100, with A: LDH activity in the culture medium of treated cells, B: LDH activity of culture medium from untreated cells and C: LDH activity after total cell lysis at 24 and 72 hours, respectively.

2.11. *In vitro* wound healing (scratch assay)

The scratch assay was performed to evaluate the ability of the HA released from the membranes to stimulate the closure of the scratch on the confluent cell plate; this assay is a well-developed method to measure cell migration *in vitro*, enabling the study of cell-matrix and cell-cell interactions also during the wound healing process (Liang et al., Park, & Guan, 2007). For this test the plasticizer (glycerol) was removed from the formulation, in order to avoid biased results due to the increased viscosity of the aliquots of liquid extract. The time required for the closure of the cell gap was monitored and measured by using a microscope equipped with a camera, and a software for image analysis. HDFa cells were seeded at a density of 250 000 cells per well in 6-well plate and incubated at 37 °C for 16 hours, in order to enable cell adhesion on the cell plate. Cells were treated with the liquid extract of the membranes (3 mL). 24 hours after the treatment a scratch was performed in each well using a sterile 200 µL plastic tip and the scratch closure was followed over time through an optical microscope (Optech IB3 ICS) equipped with a Pentax K100D camera and the images of the scratch were acquired over time to monitor the wound closure. The analysis was performed using the software Image J: the opened area, defined region of interest (ROI), was outlined per each scratch and the percentage of closure over time was plotted. The results are reported as percentage of closure of the gap area between day n and day 0. For each sample, data are expressed as mean ± standard deviation. In order to discriminate the contribution of cell migration to the scratch closure, cells were also treated for 24 hours with a non-toxic concentration

of mitomycin C (1 µg/mL), a drug that blocks the proliferation of cells at G_0 phase, to inhibit cell proliferation.

2.12. *In vitro* cell adhesion

The membranes (Formulation D) were immobilized on the bottom of the well of a 6-well sterile plate by means of a ring CellCrown (Scaffdex) to enable the complete immersion in cell medium. 230 000 cultured cells (primary fibroblasts) were re-suspended in 400 µL of medium and then seeded on each membrane specimen. After 1.5 h, 2 mL of medium were further added to the wells. After 24 h, the membranes were removed from the cell culture medium and prepared for SEM analysis according to the following steps. The membranes were rinsed twice for 30 minutes with 10 mM Hepes buffer, 0.1 M NaCl, 10 mM $CaCl_2$, 5 mM glucose, pH 7.4. Then, the samples were fixed by using 10% glutaraldehyde in 10 mM Hepes, 0.1 M NaCl, 10 mM $CaCl_2$, 5 mM glucose for 1 h and finally washed with deionized water 3 times for 10 minutes. The membranes were dehydrated by sequential immersions in ethanol 70%, 95% and 100%. Before SEM analysis, the dehydrated samples were gold-sputtered.

2.13. Scanning Electron Microscopy (SEM)

The gold-sputtered membranes (with and without cells) were analyzed using a Leica-Stereoscan 430i Scanning Electron Microscope (SEM).

2.14. Statistical analyses

Data are expressed as means and standard deviations (SD). Statistical analyses were performed using Student's t test, and a p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Hydrogel preparation and confocal analysis

For the development of membranes based on alginate and HA, the preparation of calcium alginate hydrogels has been employed in order to prepare 3D systems by avoiding the use of synthetic crosslinking agents that are potentially toxic for the organism. This approach is based on the use of Ca^{2+} ions (derived from $CaCO_3$ dissociation) to obtain an alginate cross-linked matrix (Draget, Siemensen, Onsøyen, & Smidsrød, 1993; Moe, Draget, Skåk-Bræk, & Simdød, 1992), which is meant to provide for the structural stability, while the bioactive component HA is expected to be entrapped within the reticulated matrix and to be released upon contact with the target tissue. Moreover, the crosslinking strategy based on Ca^{2+} ions aims at obtaining a structure that progressively degrades or erodes in contact with body fluids, with complete membrane disappearance within few weeks after implantation (Markovsky et al., 2012; Shoichet, Li, White, & Winn, 1996). A plasticizer already used for the preparation of medical devices (glycerol) (Kayaoglu, Ozkan, Hazinedaroglu, Ersoy, & Koseoglu, 2005; Noishiki & Shintani, 2010) was added to obtain a pliable structure (membrane) from the freeze-dried hydrogel, thus avoiding any re-hydration step. The mixture of the two anionic polysaccharides resulted in a homogeneous solution, in agreement with previous studies by some of the authors (Travan et al., 2015), while the *in situ* gelation strategy based on the gradual release of Ca^{2+} ions enabled to obtain transparent hydrogels (Figure 1C). Despite the apparent macroscopic homogeneity of the hydrogels, the molecular arrangement of the two polyanions was investigated to evaluate the distribution of the two polysaccharides after gel formation; to this end, fluorescently-labelled HA and alginate were employed for the preparation of hydrogels in order to spot their localization within the interpenetrated network. Two different concentrations of the reticulating agent Ca^{2+} (20 mM and 50 mM), were employed to modulate alginate crosslinking density, which increases with increasing Ca^{2+} concentration (Jang et al., 2014). The fluorescence profiles of the two polysaccharides recorded with the confocal microscopy are shown in Figures 1A-B. Minor variations in alginate

profile throughout the hydrogel thickness were observed, regardless of Ca^{2+} concentration (Figure 1B). The molecular arrangement of alginate is in line with the study of Kuo and Ma (Kuo & Ma, 2001), which pointed out the homogeneous distribution of this macromolecule within hydrogels prepared by *in situ* crosslinking. At variance, the analysis of a gel with $CaCO_3$ concentration of 20 mM revealed an upward-concave profile for HA concentration, with HA tending to localize preferentially on the edges of the gel: in fact, the fluorescence intensity is higher on the gel edges, while the minimum intensity corresponds to the central part of the gel section (Figure 1A). To assess the effect of an increased gel crosslinking, hydrogels with a higher concentration of $CaCO_3$ (*i.e.* 50 mM) were prepared: the result shows that when the concentration of Ca^{2+} increases, this behaviour becomes more evident. This finding suggests that the higher is the crosslinking degree of alginate, the higher is the tendency of HA to segregate into larger domains which preferentially localize at the edges of the hydrogels (as a consequence of polymer demixing), while alginate appears essentially homogeneously distributed within the gel structure. This behavior seems to conform to theoretical expectations (Wang, van Dijk, Odijk, & Smit, 2001). A graphical representation of this demixing mechanism is sketched in Figure 1D.

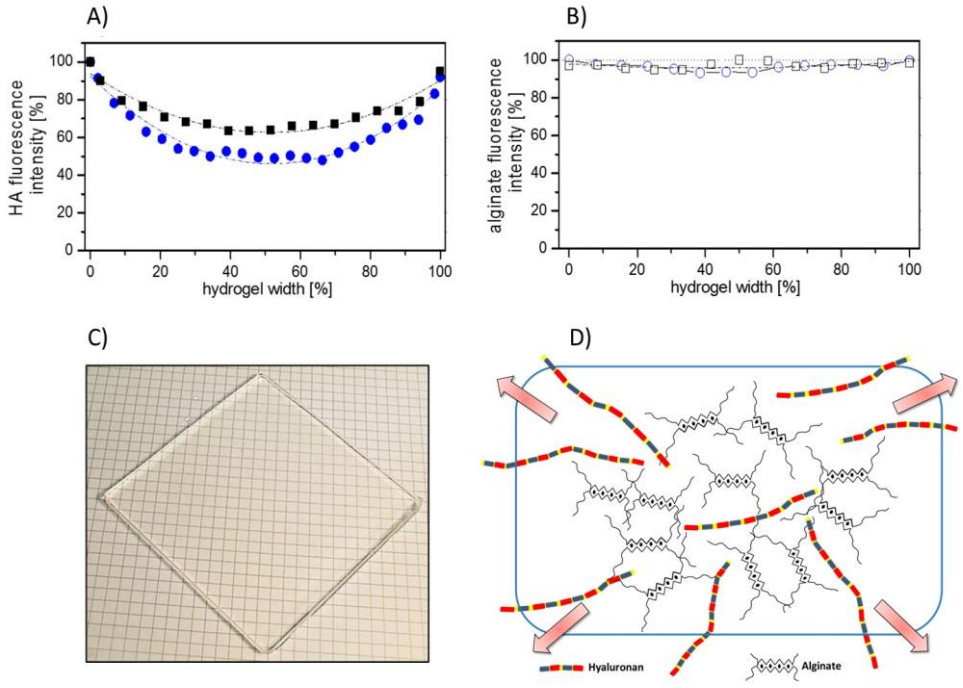

Figure 1

More or less pronounced non-linear effects in mixtures of HA and alginate had been reported over the years. Oerther et al. noticed that, although mixtures of hyaluronate-alginate exhibit physical-chemical properties slightly different from those of the single polysaccharides, existing interactions between the dissolved macromolecules are of little effect on the viscoelastic properties of the mixture (Oerther et al., 1999). According to Catanzano et al., the presence of HA can affect alginate gelation time, with limited influence on the overall properties of hydrogels (Catanzano et al., 2015). Finally, Lindenhayn et al. reported that hyaluronan is able to diffuse out of alginate beads depending on alginate concentration (Lindenhayn et al., 1999). To the best of our knowledge, our work is the first reported case of a demixing process between alginate and hyaluronan clearly identified as such.

3.2. Rheological characterization of hydrogels

The mechanical behavior of the alginate-based hydrogels was investigated by means of rheological studies to evaluate the influence of the presence of HA (240 kDa); thus, alginate gels (Formulation A) were compared to alginate-HA gels (Formulation D). The values of the elastic (G') and of the viscous (G") modulus are reported in Figure 2 as a function of the pulsation ($\omega = 2\pi\nu$).

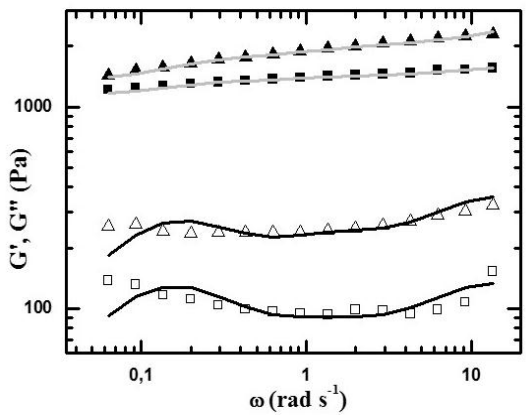

Figure 2.

Experimental data were modeled as a function of the pulsation frequency (ω) by means of a generalized Maxwell model composed of a sequence of elements in parallel (spring and dashpot) to which an additional spring has been added (Turco et al., 2011).

Figure 2 points out that, for both compositions, the elastic modulus (G') is higher than the viscous modulus (G''), which indicates that the samples are firm gels. The mechanical spectra showed that the addition of HA causes an increase of both elastic and viscous modulus leading to a dependence of G' on the pulsation, with a slight curvature at low values of pulsation; such increase can be ascribed to the HA macromolecules trapped within the alginate matrix. This behaviour was even more evident when HA having a higher molecular weight (800 kDa) was used, since a further increase of the elastic response of the hydrogels was measured (Figure S1, Supporting Information). The spring constant (G_i) and dashpot viscosity (η_i) as a function of the relaxation time (λ_i) of the Maxwell elements are reported in the Supporting Information (figure S2).

3.3. Membrane preparation and morphological analysis

After hydrogel formation, dried membranes were obtained through a procedure based on a temperature-controlled freeze-drying; this procedure enabled to obtain pliable membranes with a homogeneous mesh (Figure 3a). A morphological analysis of the membrane at the microscopic scale was carried out by SEM microscopy (Figure 3b). SEM analysis highlighted the homogeneous polymeric texture composing the membrane. Cross section micrographs display an average thickness of approximately 300 μm (Figure 3c); such limited thickness contributes to make the material suitable to be fold and wrapped by hand and stays within the thickness range of the main commercial surgical membranes for internal use (Coda, Lamberti, & Martorana, 2012).

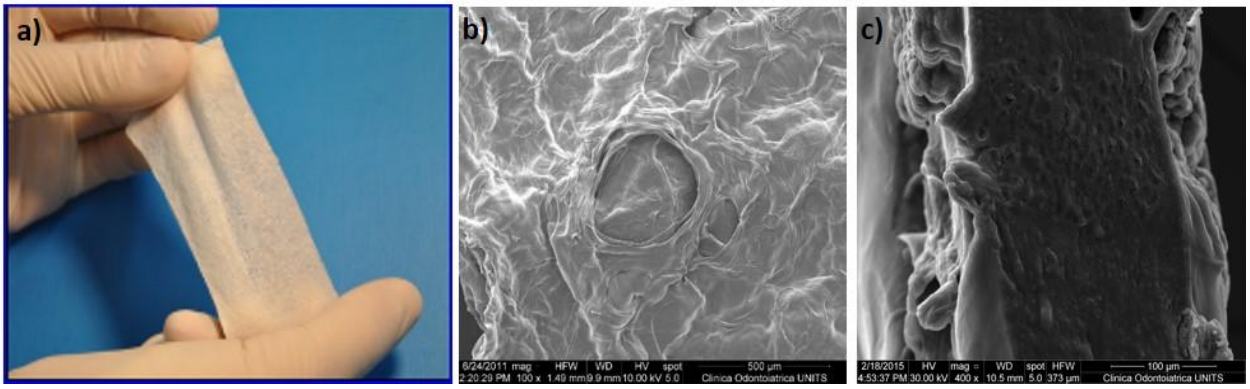

Figure 3.

3.4. Mechanical characterization of membranes

During a clinical procedure, it is important to be able to handle, position and adapt a surgical membrane to the target site without breaking or tearing the material; for this reason, the mechanical performance of the membranes were studied in terms of stiffness, resistance and pliability. To this end, tensile tests on the freeze-dried membranes were performed and Young's modulus, stress and strain at break of several membrane formulations were calculated. It should be noted that these membranes are conceived to withstand the maximum stress during the positioning of the device on the target body site, *i.e.* at the dry state; once in site, the membranes are designed to gradually rehydrate by absorbing body fluids, to release HA and to ultimately dissolve *via* adsorption and bioerosion of the components. The mechanical behavior of several membrane formulations is reported in Figure 4.

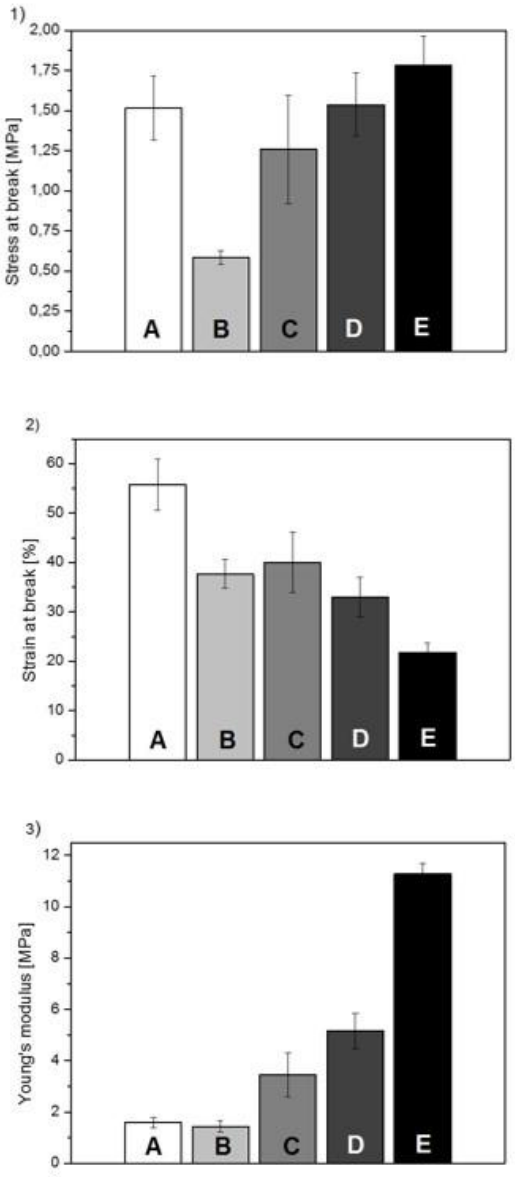

Figure 4.

All membranes displayed a tensile strength in the range of 0.5 – 1.8 MPa; despite the absence of covalent crosslinks (the structure is obtained owing the ionotropic crosslinking induced by calcium ions), this range is line with the mechanical resistance of commercial collagen-based membranes for surgical use (Birkenfeld, Behrens, Kern, Gassling, & Wiltfang, 2015). When both alginate and HA were present, the membranes were able to deform plastically at a high extent (20% - 40% of strain) before breaking, pointing out the compliant behavior of the material.

In order to optimize the membrane formulation from a mechanical point of view, the effect of polymer concentration, reticulating agent (Ca^{2+} amount) and molecular weight of HA on the

mechanical behavior of the membranes was explored. Considering membranes of alginate alone (Formulation A) as a reference, when HA with molecular weight 800 kDa was added, the stress at break decreased from 1.5 ± 0.2 MPa to 0.6 ± 0.1 MPa (Formulation B) and the strain at break decreased from 55 ± 5% to 37 ± 3%, while the Young's Modulus remained approximately unaltered. At variance, when HA with a lower molecular weight (240 kDa) was added (Formulation D), the stress at break remained around 1.5 MPa, the Young's Modulus increased from 1.5 ± 0.2 MPa to 5.2 ± 0.4 MPa and the strain at break decreased from 55 ± 5% to 33 ± 4%.

Comparing membranes with a fixed concentration of alginate (15 g/L) and HA at 240 kDa (15 g/L), when the concentrations of $CaCO_3$ (and GDL) increased by 2.5-fold (Formulation E), the Young's Modulus increased from 5.2 ± 0.7 MPa to 11.3 ± 0.4 MPa, the stress at break slightly increased from 1.5 ± 0.2 MPa to 1.8 ± 0.2 MPa, while the strain at break decreased from 33 ± 4 % to 22 ± 2 %. Considering the effect of the alginate content in membranes prepared with 15 g/L HA (240 kDa), 20 mM $CaCO_3$ and 40 mM GDL (Formulation D), an increase of alginate concentration from 15 g/L to 20 g/L (Formulation C) caused a decrease in the Young's Modulus (from 5.2 ± 0.7 MPa to 3.5 ± 0.9 MPa), a slight decrease of the stress at break and an increase in the strain at break (from 33 ± 4% to 40 ± 6%).

These results point out that, at a fixed alginate concentration, the addition of low molecular weight HA (240 kDa) leads to a stiffer membrane when compared with high molecular weight HA (800 kDa). This could be ascribed to the fact that the high molecular weight HA causes a more pronounced destabilization of the alginate matrix due to its conformation and to a much higher excluded volume effect, while the low molecular weight HA could be better integrated within the reticulated structure. Moreover, increasing the concentration of both $CaCO_3$ and GDL leads to an increase of material stiffness and strength; on the other hand, the membrane becomes less prone to deformation. This phenomenon is due to the increase of ionic crosslinking among alginate chains when increasing the concentration of $CaCO_3$; in fact, alginate chains are cross-linked with Ca^{2+}, which accounts for the mechanical resistance and stiffness of the membranes. An increase in

alginate concentration results in a decrease of membrane stiffness and in a slight increase of its maximal elongation. Given the equal concentration of Ca^{2+}, this occurs because there is a larger number of alginate chains and consequently there are less crosslinking points between the alginate chains.

Overall, the mechanical characterization of the membranes subjected to tensile stress indicated Formulation D as the best compromise among resistance (stress at break), stiffness (Young's Modulus) and compliance (strain at break); for this reason, this membrane was selected for further investigations in terms of polysaccharides release, degradation and biological behavior.

3.5. Polysaccharide release and membrane degradation

These polymeric membranes were designed to enable the *in situ* release of the bioactive component HA and to have a structure which progressively degrades within the body: in this perspective, the release profile of the two polysaccharides was studied by means of NMR analysis according to a procedure previously devised by some of the authors (Geremia et al., 2014). The kinetics of release of alginate and HA from the membrane (Formulation D) was studied by measuring the amount of the two polysaccharides released from membranes incubated in HBSS as a function of time. The values of the release of the two polysaccharides, expressed as percentage at given time with respect to the initial amount of the polymers within the membrane (time zero), are shown in Figure 5A.

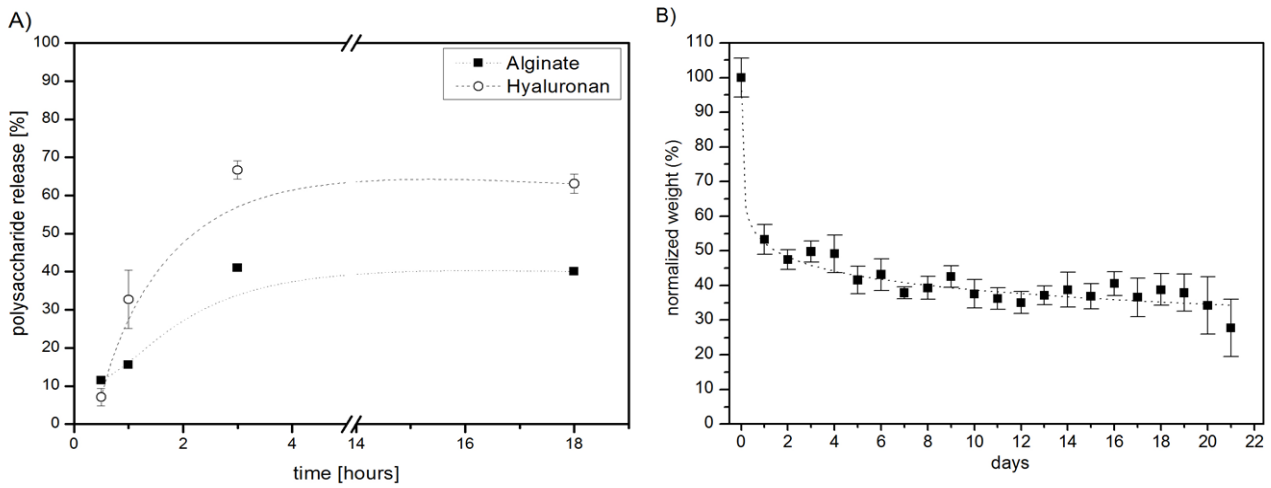

Figure 5

The results of the release kinetics show that both polysaccharides start to be gradually released during the first 3 hours of immersion, after which the release profiles reach a plateau. HA displays a fast release during the first hours (approximately two thirds of the initial content), which represents a positive feature of the membrane since this bioactive component should be effectively provided locally on the wounded site to stimulate tissue healing; this behavior appears in line with the study performed by Lindenhayn et al.(Lindenhayn et al., 1999) who showed that more than 40% of the HA entrapped in alginate beads (with a much higher degree of crosslinking) is released during the first 3 days in cell culture medium. With our membranes, alginate displays dissolution of about 35% during the same time frame, which could be ascribed to the presence of surface domains where the crosslinking of alginate was partially affected by the accumulation of HA.

The degradation profile was studied by immersing the membrane in HBSS solution at 37 °C and evaluating the mass upon daily shifts of solution. The original weight of the membranes was set as the weight of the swollen membrane after 4 hours of incubation in the solvent, since at this time point the initial weight increase due to water absorption reaches a plateau (see Supporting Information, Figure S3). Figure 5B shows an initial rapid loss of weight that is consistent with the release of HA and of a small fraction of alginate from the patch during the first hours of immersion, as observed in the release test above described. Then, the degradation rate decreases, with a gradual

weight reduction for the successive 3 weeks, after which only small fragments of the membrane could be found in solution.

3.6. *In vitro* biocompatibility

In view of a potential application of the polysaccharide membranes on dermal wounds, human dermal fibroblasts (HDFa cells) have been employed for the *in vitro* biological characterization. The biocompatibility of the alginate-HA membranes (Formulation D) and of the alginate membranes devoid of HA, taken as the reference for the contribution of the matrix (Formulation A), was evaluated by analyzing the effect of the treatment of HDFa cells with the components released from the membranes and contained in the liquid extracts (as such and diluted 1:10). The evaluation was carried out through the quantification of lactate dehydrogenase (LDH), a cytosolic enzyme that is released in culture medium upon cellular membrane permeabilization caused by the effect of non-biocompatible substances and materials. The LDH quantification data reported in Figure 6 show that there are no significant differences in the release of the LDH between untreated cells and cells treated with the samples. On the contrary, the enzyme release quantified in the medium of cells treated with Triton X-100, taken as the positive cytotoxicity control, is significantly higher.

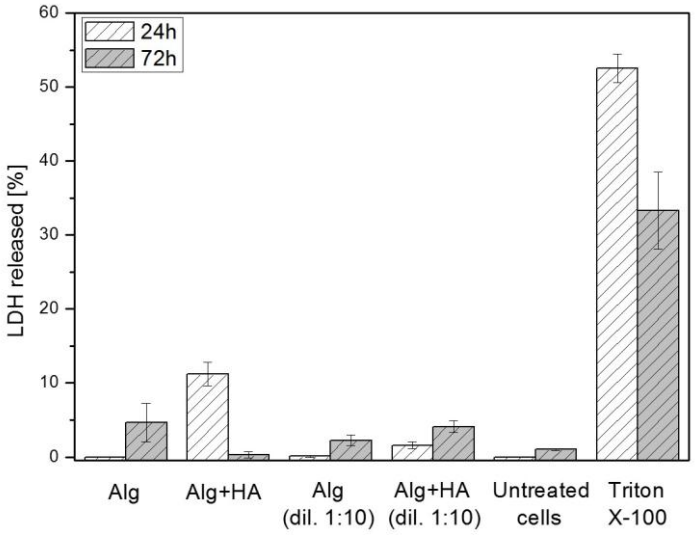

Figure 6.

These data point out the *in vitro* biocompatibility of the membranes based on the two selected polysaccharides. This feature was also confirmed by a SEM investigation of primary human dermal fibroblasts (HDFa) seeded on the alginate-HA membranes: Figure 7 shows that, 24 hours after seeding, fibroblasts were able to colonize the material and to spread firmly on it; there appears to be some evidence of cell attachment which is likely due to the presence of regions of hyaluronan on the surface. It was interesting to observe the deep physical integration of the cells with the polysaccharide matrix, suggesting the existence of strong biological interactions between cells and substrate.

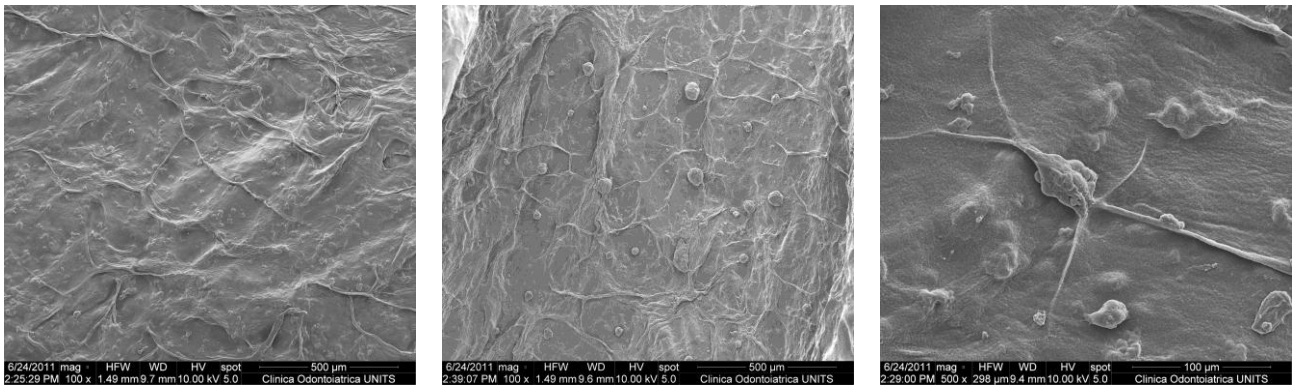

Figure 7.

These biological results suggest a possible use of this membrane also for tissue engineering applications, given its ability to support cell adhesion and proliferation.

3.7. *In vitro* wound healing (scratch assay)

The effect of such membranes on the wound healing process has been evaluated *in vitro* by use of the scratch assay, which is based on the simulation of a wound (scratch) on a layer of cultured (confluent) cells, followed by the monitoring of the cellular response; this approach aims to mimic what happens in the human body when wound closure occurs owing to a combination of cell migration and cell proliferation phenomena. For this test, the scratches were performed on fibroblasts (HDFa) seeded on culture plates and treated with the liquid extracts from the membranes.

Figures 8A-B report the percentage of closure of the scratches (gap closure) as a function of incubation time; in order to highlight the individual contribution of proliferation and migration to the scratch closure, the experiments were performed in the absence (Figure 8A) or in the presence (Figure 8B) of mitomycin C, which is able to inhibit cell mitosis. Representative optical images of cultured cells used for the scratch tests are reported in Figures 8C-G.

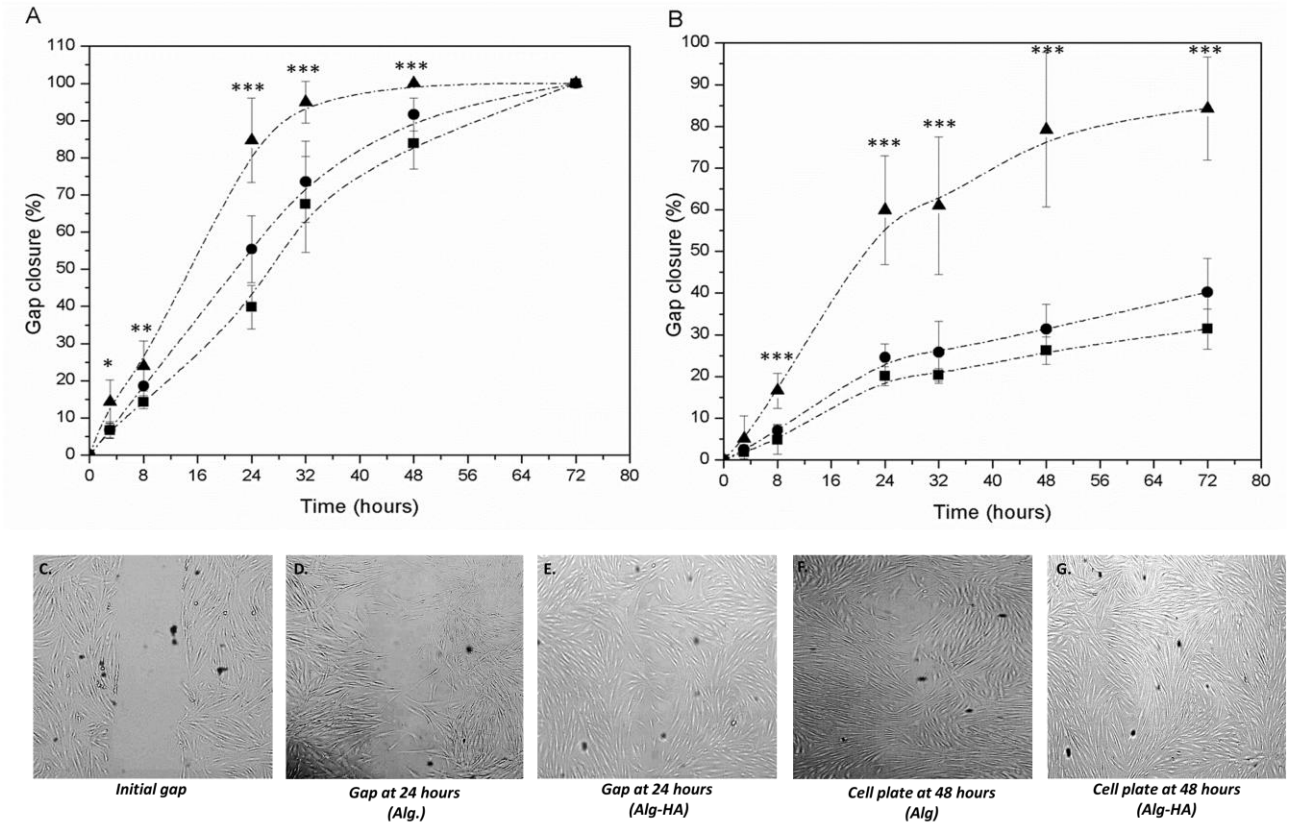

Figure 8.

Figure 8A shows that when treated with the liquid extract of the alginate-HA membranes (Formulation D) the cells showed a complete closure of the gap after 32 hours, whereas the percentage of closure at this time was around 70% for both non-treated cells and cells treated with alginate membranes (Formulation A). In these cases complete closure was achieved only after 72 hours. Figure 8B shows that, when cell proliferation was blocked by mitomycin C, in the case of cells treated with alginate-HA membranes the gap closure was 60% after 32 hours and 80% after 72 hours, while in the absence of HA the gap closure was less than 40% at 72 hours. This behavior can

thus be ascribed to the migratory ability of fibroblasts, which appears as the main mechanism involved in the closure of the gap; however, since in the presence of mitomycin C a complete gap closure was not reached, a minor contribution of HA to cell proliferation appeared to be involved. This biological study proved that HA released from the membrane provides a significant support to the physiological healing process.

4. Conclusions

A promising approach for the preparation of bioactive membranes for wound healing applications is the use of natural polysaccharides able to stimulate fibroblast activity before being degraded after contact with physiological fluids. The proposed approach enables to manufacture membranes based on HA and alginate hydrogels reticulated by calcium ions; a freeze-casting procedure was devised to obtain pliable membranes with homogeneous texture starting from the hydrogels (in the presence of glycerol as a plasticizer). The process can be easily tailored to manufacture custom-shaped membranes incorporating high amounts of HA for wound healing and surgical applications. The mechanical properties of the membranes were optimized in terms of strength, stiffness and compliance by studying the influence of polysaccharides concentration, molecular weight and amount of reticulating agent (Ca^{2+}). HA was shown to be actively depleted from the membrane reservoir *via* a polymer demixing process, which for the first time was demonstrated to take place between HA and calcium cross-linked alginate. When in contact with physiological solutions, most of HA is released during the first 3 hours of immersion, owing to both polymer demixing and membrane degradation.

This finding represents a positive feature of the membrane since this bioactive component should be efficiently provided on the wounded site to stimulate tissue healing. *In vitro* biological tests proved the biocompatibility of the membranes towards human dermal fibroblasts, which are also able to colonize the substrate and integrate within the polysaccharide matrix. Scratch assays demonstrated

the excellent capability of the HA released by the membrane to support the physiological healing process.

Overall, these novel alginate-HA membranes represent a promising solution for several medical needs, in particular when the *in situ* administration of HA from a resorbable device is required. This strategy appears well suited both for the treatment of topical wounds as well as to promote the healing of internal tissues that have undergone surgery.

Supplementary information

Rheological properties of alginate-based hydrogels in the presence or absence of HA, the spring constant and the dashpot viscosity as a function of the relaxation time of the Maxwell elements for alginate hydrogels and alginate-HA hydrogels, and data of swelling test are available as supplementary information.

Acknowledgements

The authors would like to acknowledge Dr. Gianluca Turco for providing assistance in SEM analysis and Miss Ilaria Geremia for support in hydrogels characterization. This study was supported by the EU-FP7 Project “AnastomoSEAL” (Contract Number 280929).

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FIGURE CAPTIONS

Figure 1: Alginate-HA hydrogels characterization: A) Profiles of HA localization in the cross-section (edge to edge) of hydrogels prepared with different $CaCO_3$ concentrations (black squares: 20 mM, Formulation D); blue circles (50 mM, Formulation E). The profiles reported were chosen as representative trends among 5 replicates. Dotted lines are drawn to guide the eye. B) Profiles of alginate localization in the cross-section (edge to edge) of hydrogels prepared with different $CaCO_3$ concentrations (black squares: 20 mM, Formulation D; blue circles: 50 mM, Formulation E); C) Macroscopic image of the transparent hydrogel (Formulation D); D) Graphical representation of the process of hyaluronan demixing from the structure of the alginate hydrogel.

Figure 2. Rheological properties of alginate-based hydrogels in the presence or absence of HA; elastic modulus G' (full symbols) and viscous modulus G" (empty symbols) are reported as a function of the pulsation frequency, ω. Squares: alginate hydrogels; Triangles: alginate-HA hydrogels. Crosslinking with 20 mM $CaCO_3$ and 40 mM GDL; polymer concentration 15 g/L for both HA and alginate. The lines represent the best-fit of the experimental data obtained with the Maxwell model.

Figure 3: Images of alginate-HA freeze-dried membranes (Formulation D): a) Macroscopic view; b) Top view at SEM; c) Cross section at SEM.

Figure 4: Mechanical properties of freeze-dried membranes of different compositions: 1) Stress at break, 2) Strain at break, 3) Young's Modulus. Membrane formulations: A) Alginate 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v; B) Alginate 15 g/L, HA (800 kDa) 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v C) Alginate 20 g/L, HA

(240 kDa) 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v; D) Alginate 15 g/L, HA (240 kDa) 15 g/L, $CaCO_3$ 20 mM, GDL 40 mM, glycerol 5% v/v; E) Alginate 15 g/L, HA (240 kDa) 15 g/L, $CaCO_3$ 50 mM, GDL 100 mM, glycerol 5% v/v.

Figure 5: Polysaccharide release and degradation profile in HBSS of the alginate-HA membrane (Formulation D). A) Polysaccharide release from alginate-HA membranes as a function of the immersion time. Square symbols: alginate release. Round symbols: HA release. B) Degradation profile of the membrane in HBSS upon daily solution shift. Dashed and dotted lines were drawn to guide the eye.

Figure 6. In vitro biocompatibility (LDH test) of primary human dermal fibroblasts (HDFa) treated with the polysaccharides released from the alginate (Alg, Formulation A, as matrix control) or alginate-HA (Alg+HA, Formulation D) membranes.

Figure 7: Primary human dermal fibroblasts (HDFa) grown on alginate-HA membrane (Formulation D).

Figure 8: Upper part: effect of the membranes on the closure of gaps within HDFa cells cultured for the scratch tests in the absence (A) or presence (B) of mitomycin C. Triangles: alginate-HA membrane (Formulation D); circles: alginate membrane (Formulation A); squares: untreated cells (control). (*: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001). Lower part: optical images of the cell gap at time zero (C), after 24 hours in the presence of the liquid extracted from the alginate membranes (D) or the alginate-HA membranes (E), after 48 hours in the presence of the liquid extracted from the alginate membranes (F) or the alginate-HA membranes (G).

Figure 1

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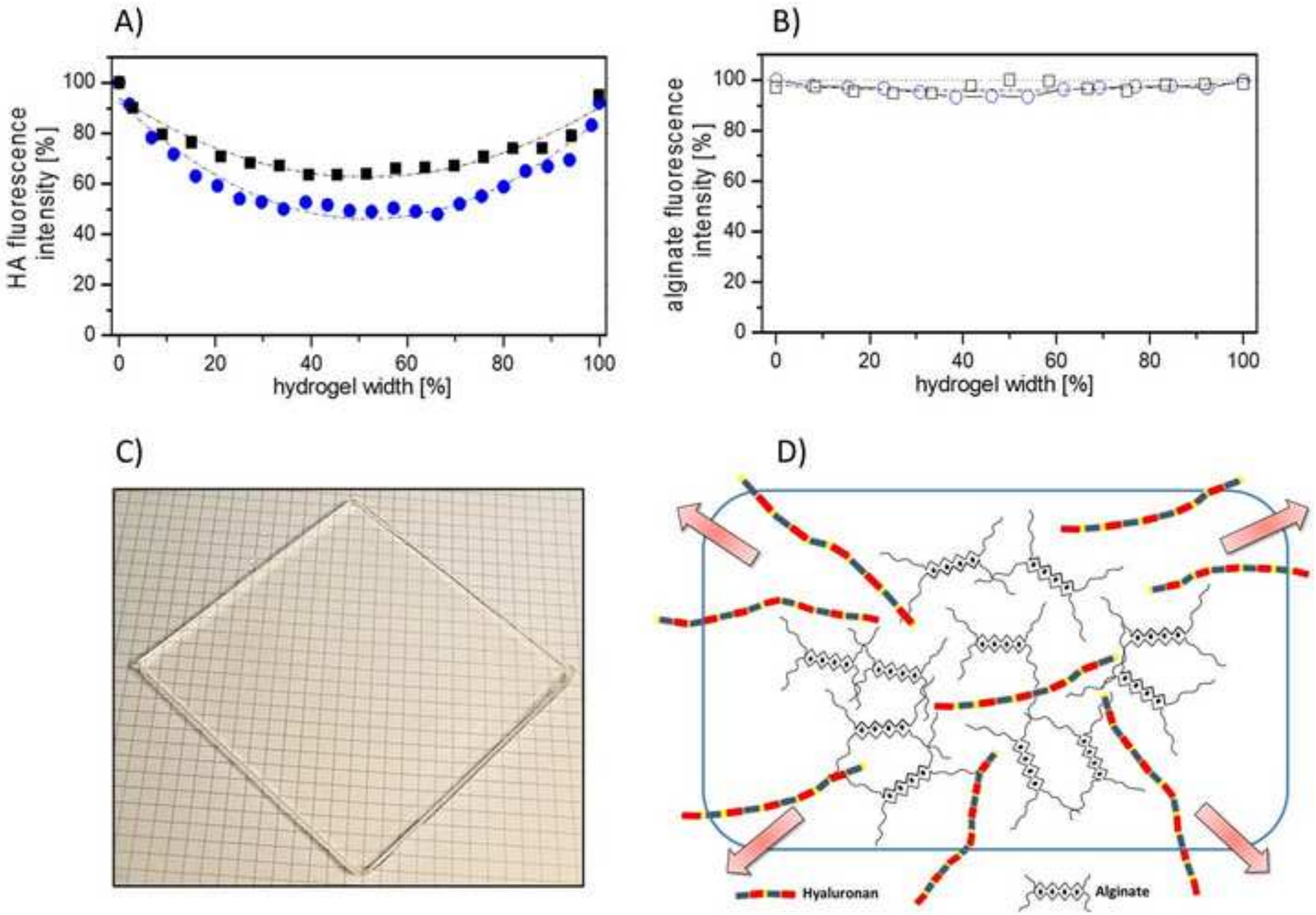

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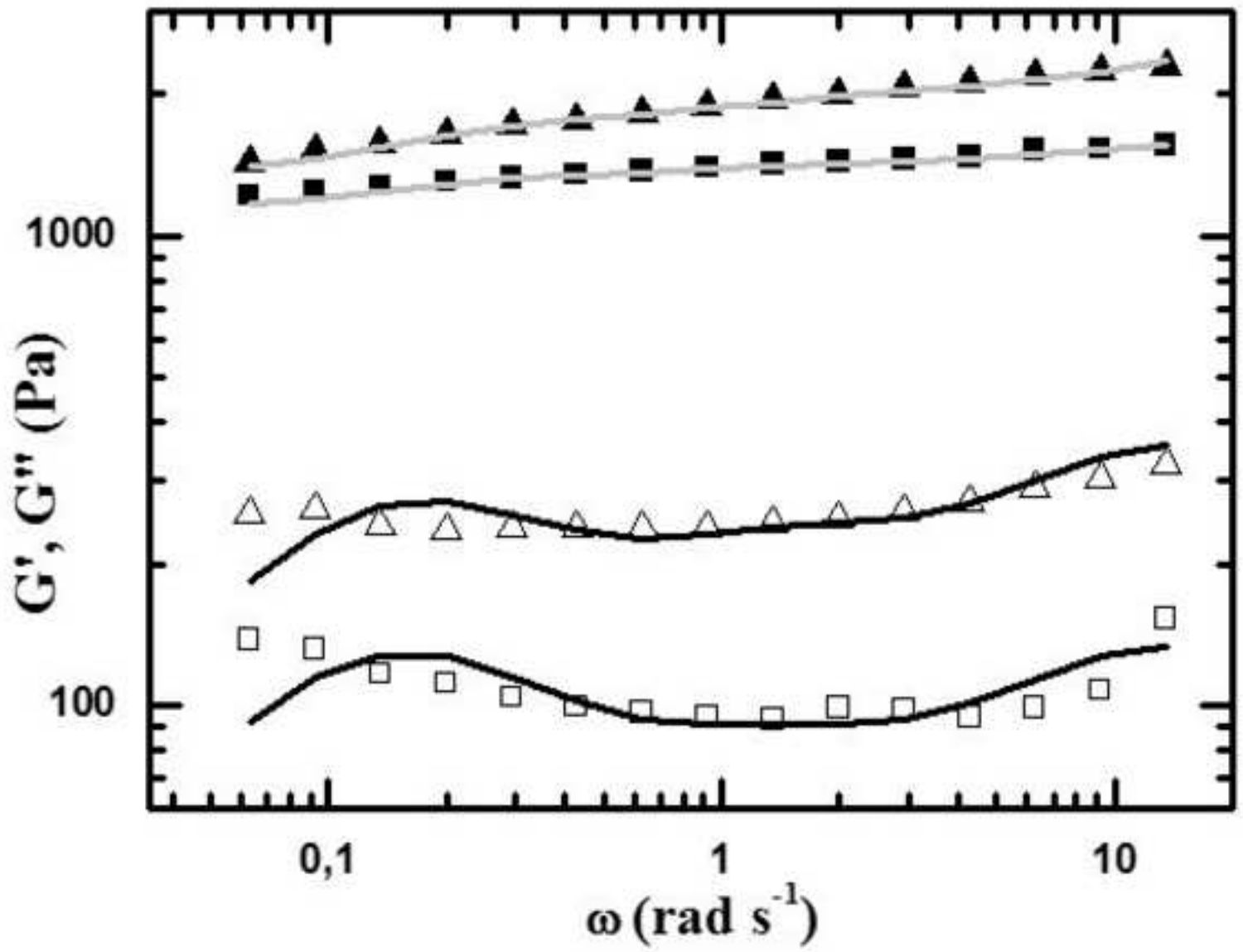

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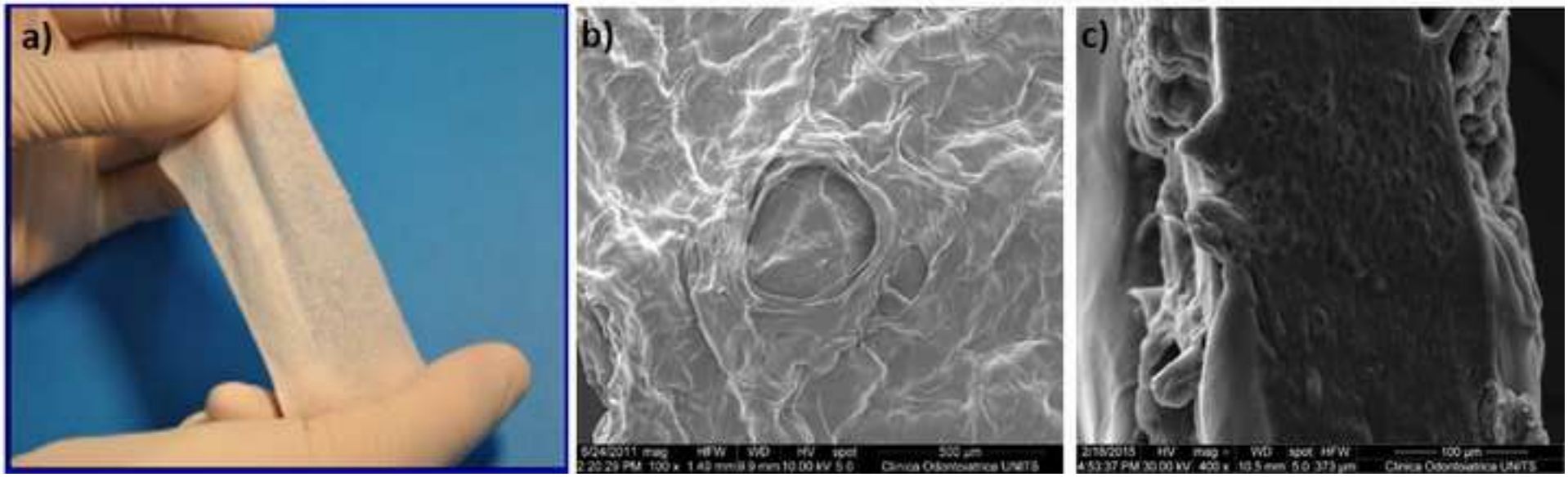

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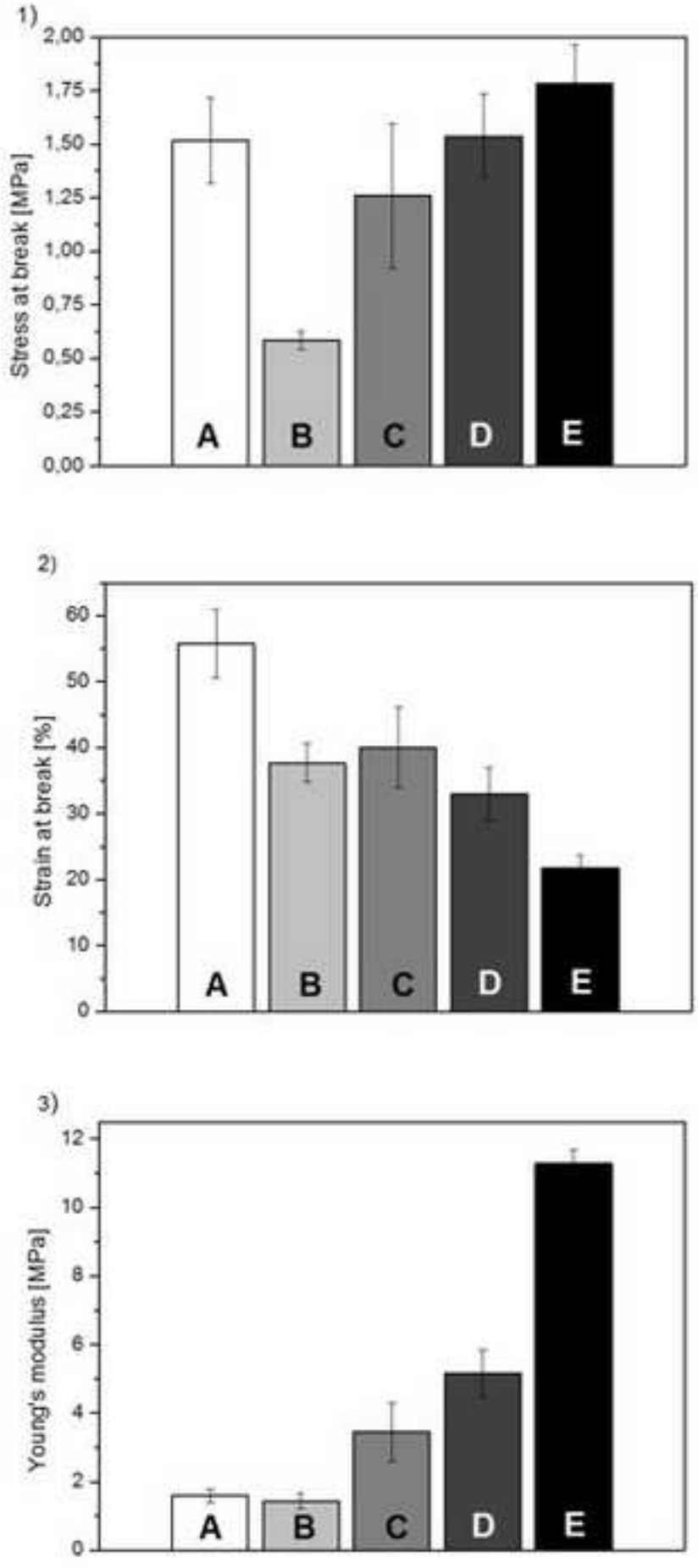

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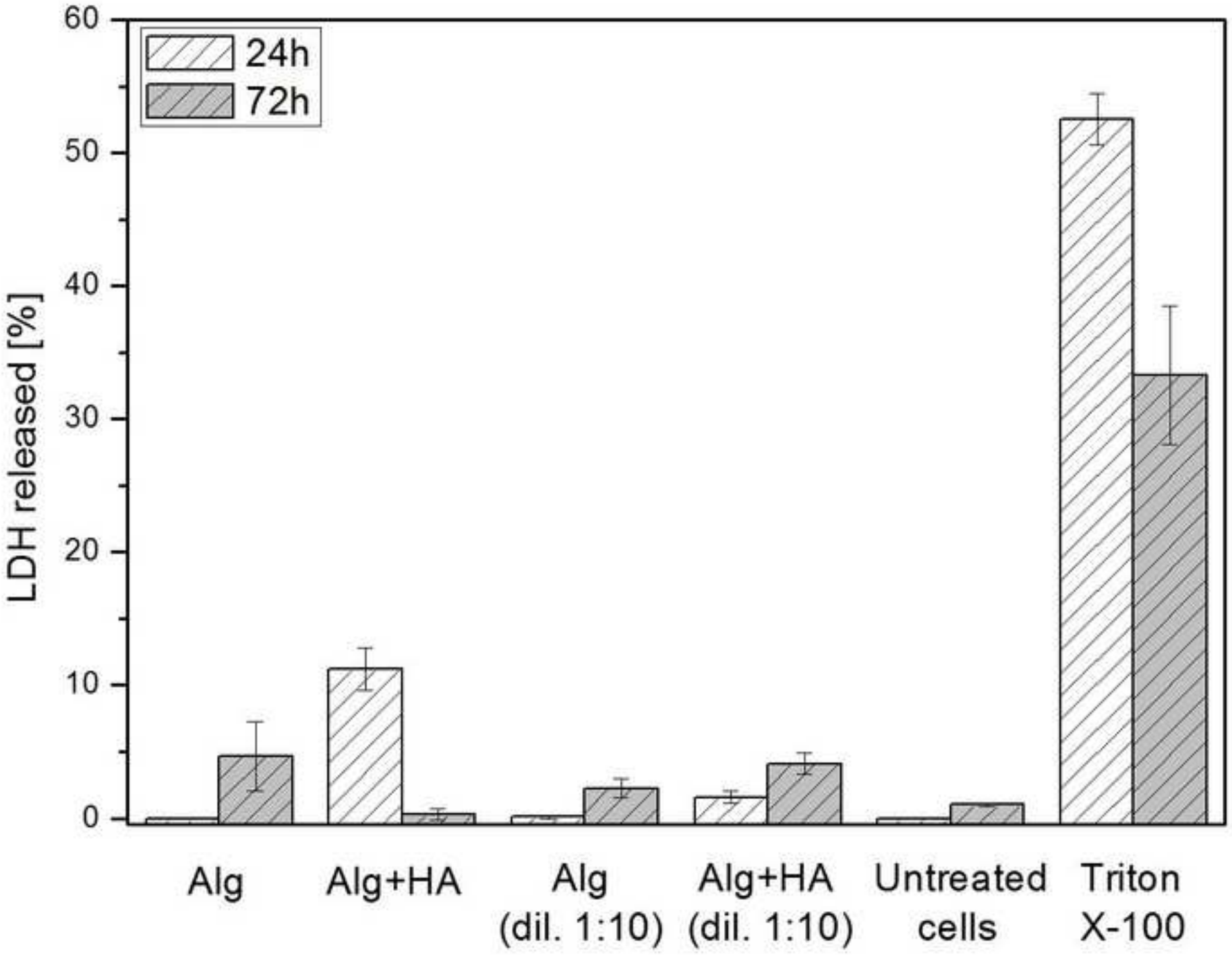

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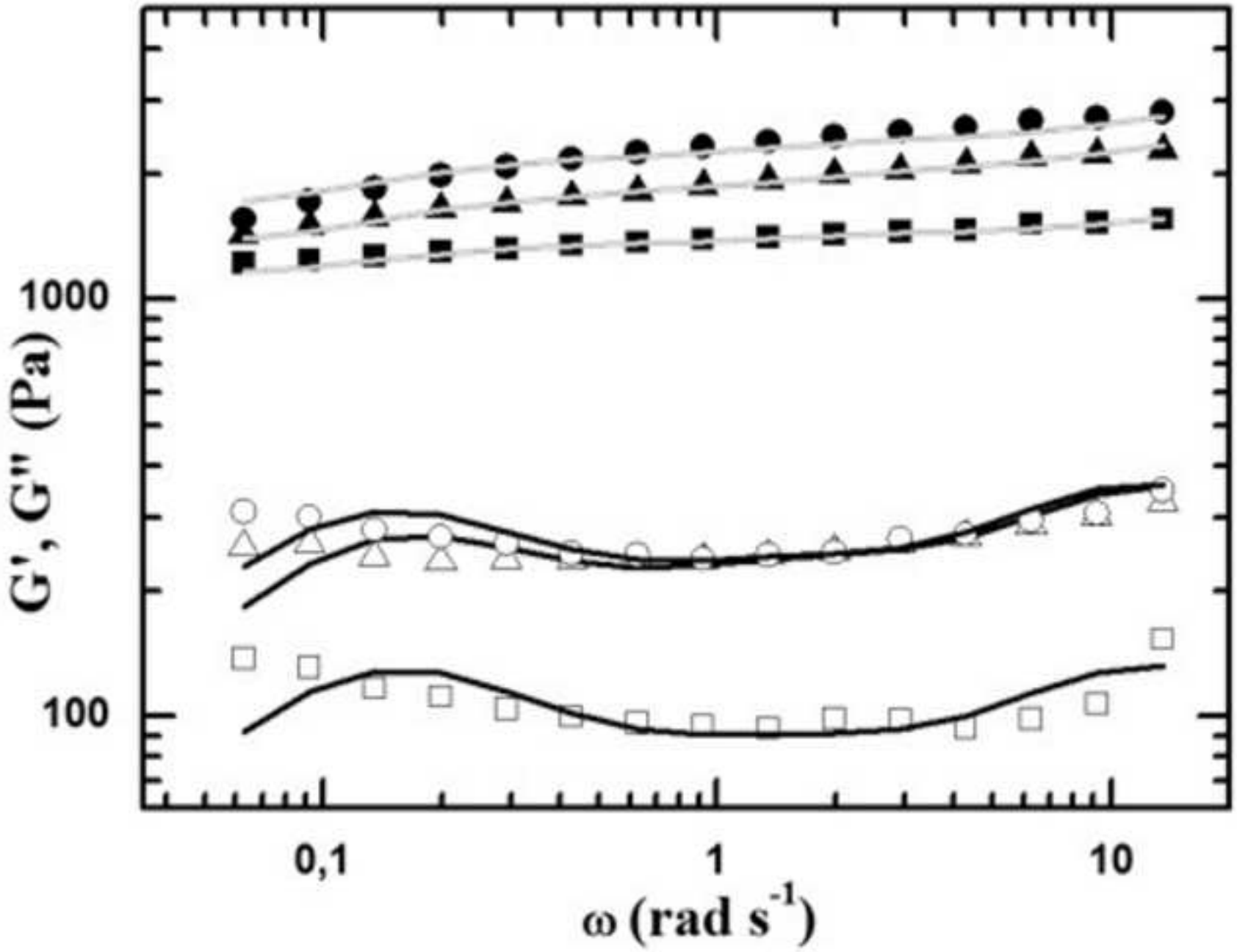

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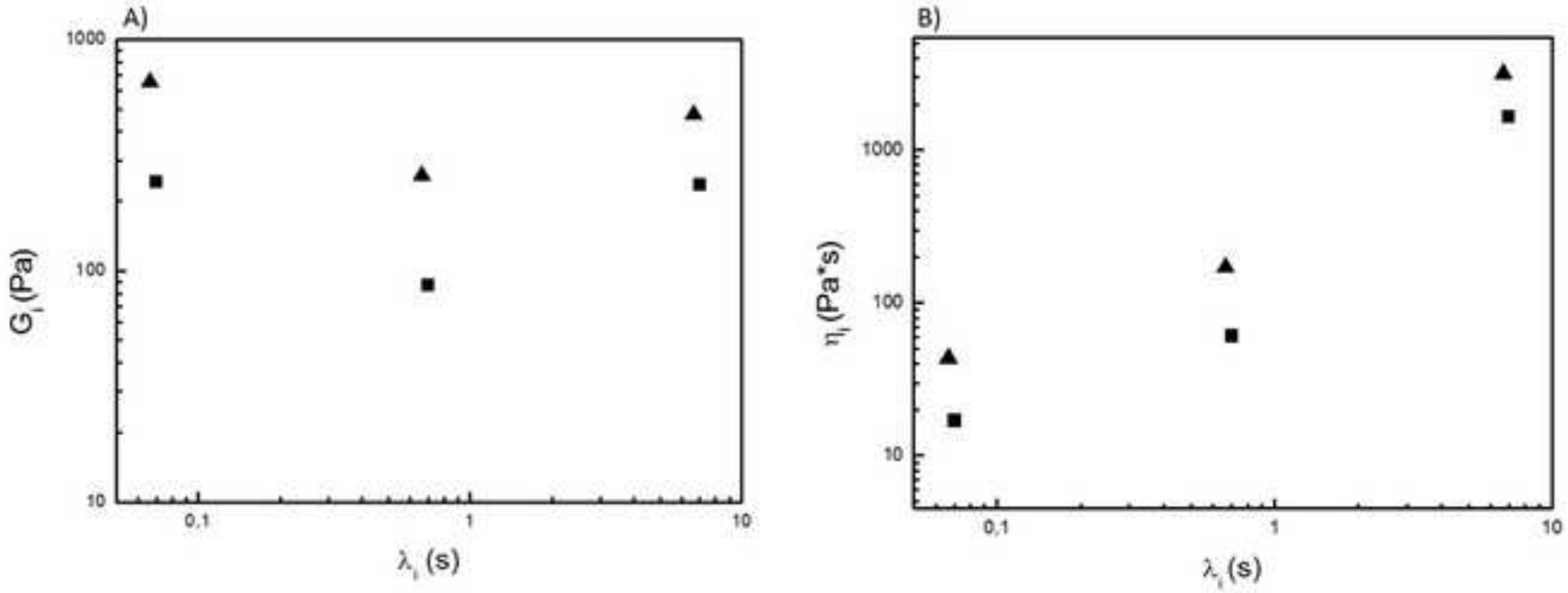

Figure S3

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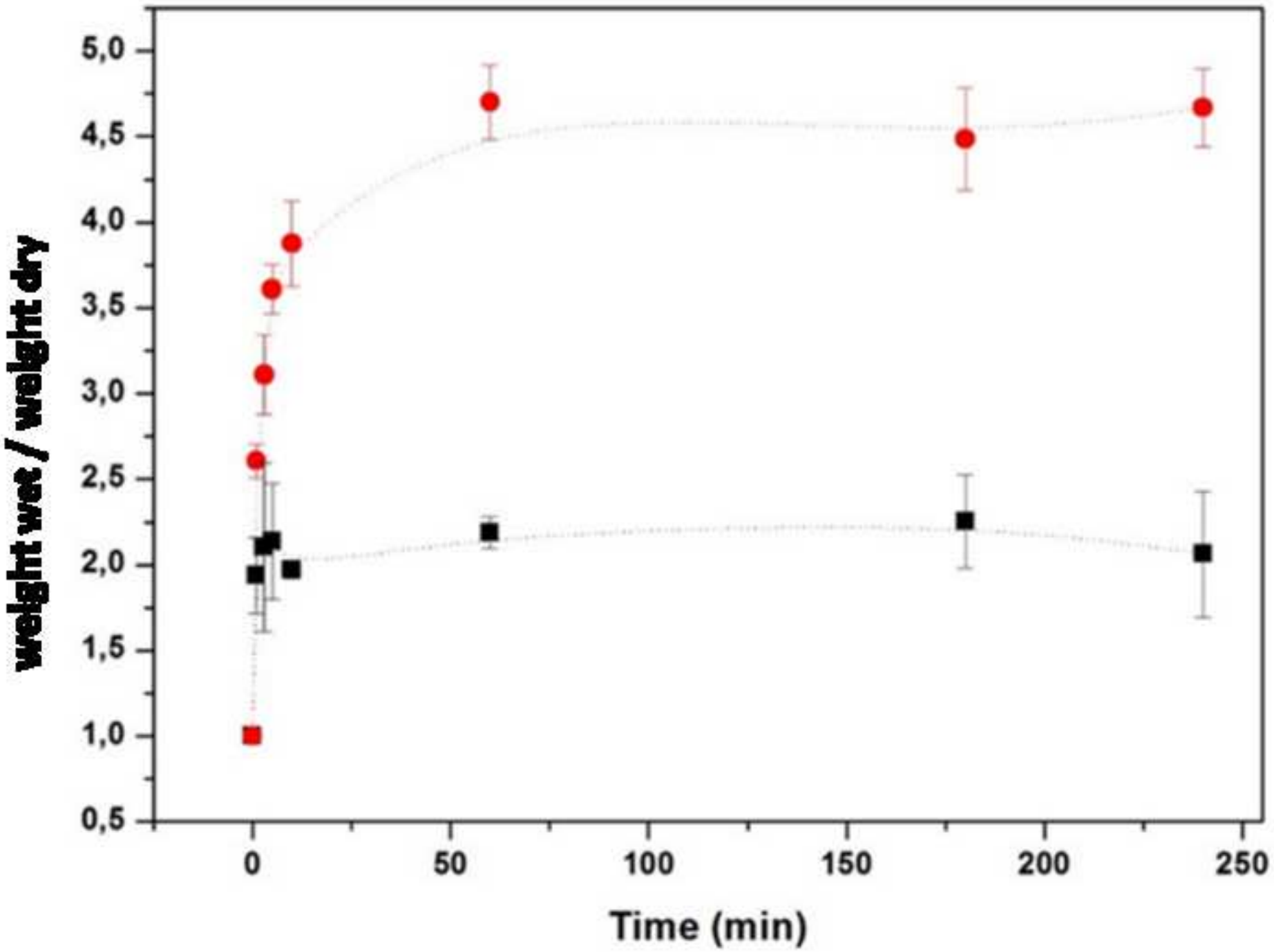

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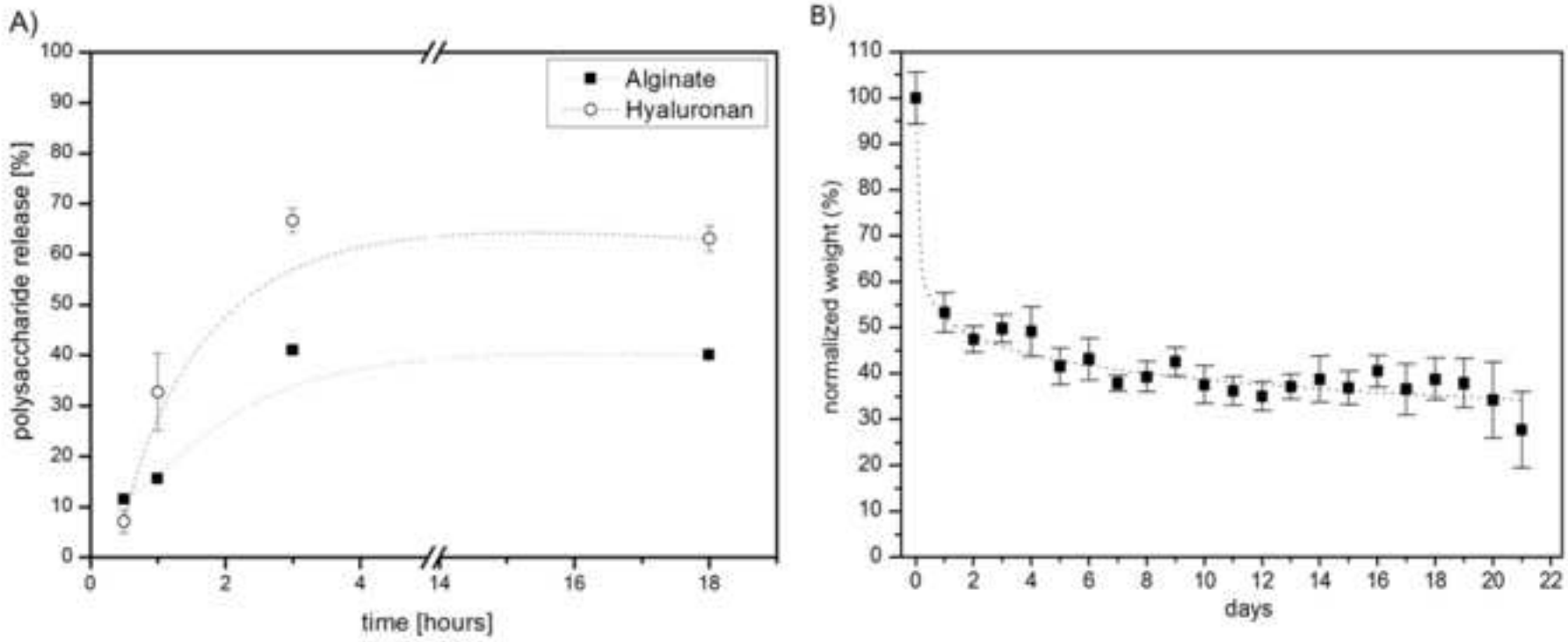

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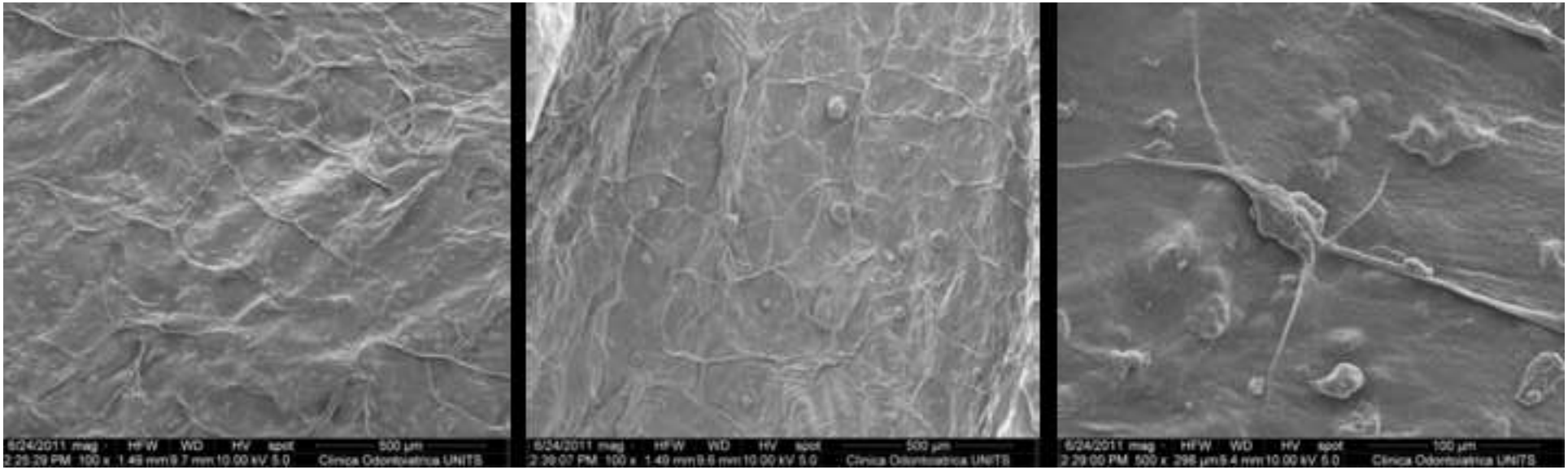

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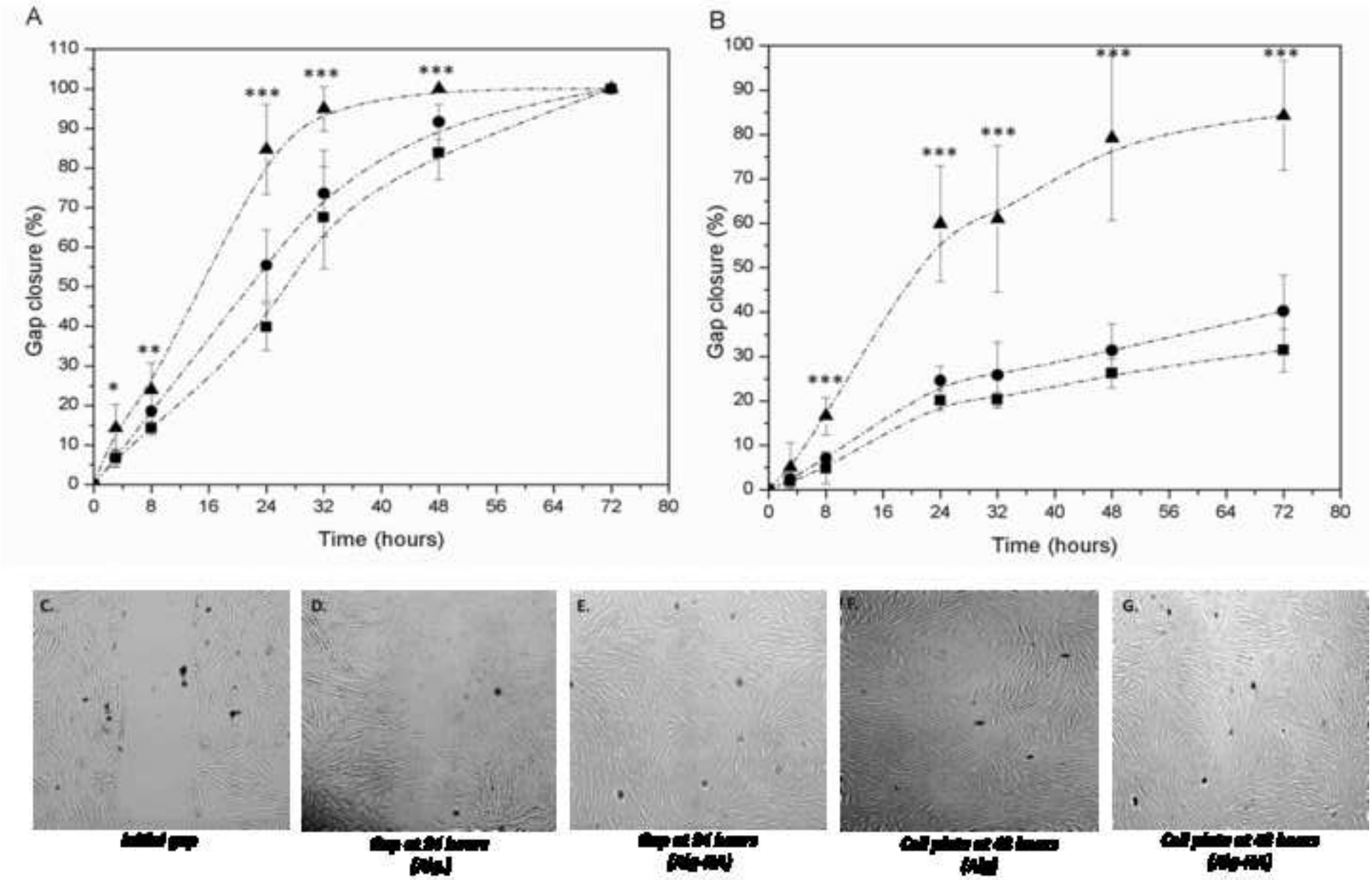

Supplementary data

Hyaluronan delivery by polymer demixing in polysaccharide–based hydrogels and membranes for biomedical applications

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SUPPORTING EXPERIMENTS

S1 and S2. Mechanical spectroscopy

Rheological characterization of cylindrical hydrogels was performed by means of a controlled stress rheometer Haake Rheo-Stress RS150 operating at 25 °C using, as measuring device, a shagreened plate apparatus (HPP20 *profiliert*: Ø = 20 mm). To avoid water evaporation from the hydrogel, the measurements were performed in a water-saturated environment realized by using a glass bell (solvent trap) containing a wet cloth. In addition, to prevent both wall-slippage and excessive gel squeezing (Lapasin & Pricl, 1995), the gap between plates was adjusted, for each sample, by executing a series of short stress sweep tests (f = 1 Hz; stress range 1 - 5 Pa; maximum deformation < 0.1%) characterized by a reducing gap. The gap was selected in order to maximize the value of the elastic modulus G' (used gaps ranged between 2.5 and 2.0 mm). For each hydrogel, the linear viscoelastic range was determined by means of a stress sweep test consisting in measuring elastic (G') and viscous (G") moduli variation with increasing shear stress (1 Pa < τ < 100 Pa) at solicitation frequency of ν = 1 Hz ($\omega = 2\pi\nu$ = 6.28 rad s^{-1}). Mechanical spectrum of the hydrogel was determined by measuring the dependence of the elastic (G') and viscous (G") moduli from the pulsation (ω) at constant shear stress τ = 3 Pa (well within the linear viscoelastic range that, for all samples, spans up to at least 30 Pa).

S3. Swelling test

Circular samples of the membrane (formulations A and D; Ø = 20mm) were weighted at the dry state. The samples were soaked with 4 mL of SBF and the weight of the hydrated membranes was measured after drying the samples on a filter paper. Data were expressed as the ratio between the weight of the wet and of the dry membranes, as a function of the time. Three parallel replicates were considered.

Results

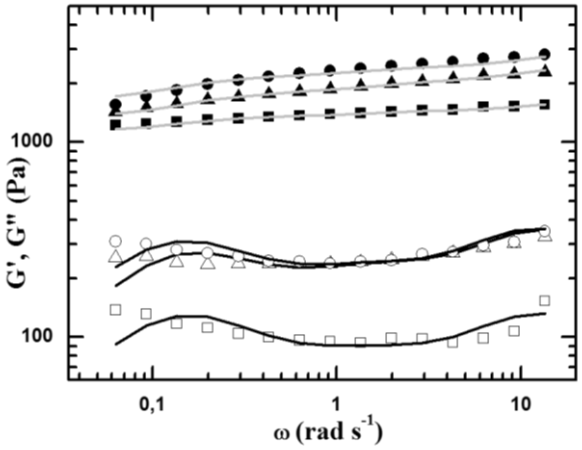

Figure S1: Rheological properties of alginate-based hydrogels in the presence or absence of HA; elastic modulus G' (full symbols) and viscous modulus G" (empty symbols) are reported as a function of the pulsation frequency, ω. Squares: alginate hydrogels; triangles: alginate-HA240 kDa hydrogels; rounds: alginate-HA800 kDa hydrogels. Crosslinking with 20 mM $CaCO_3$ and 40 mM GDL; polymer concentration for both HA and alginate corresponds to 15 g/L. The lines represent the best-fit of the experimental data obtained with the Maxwell model.

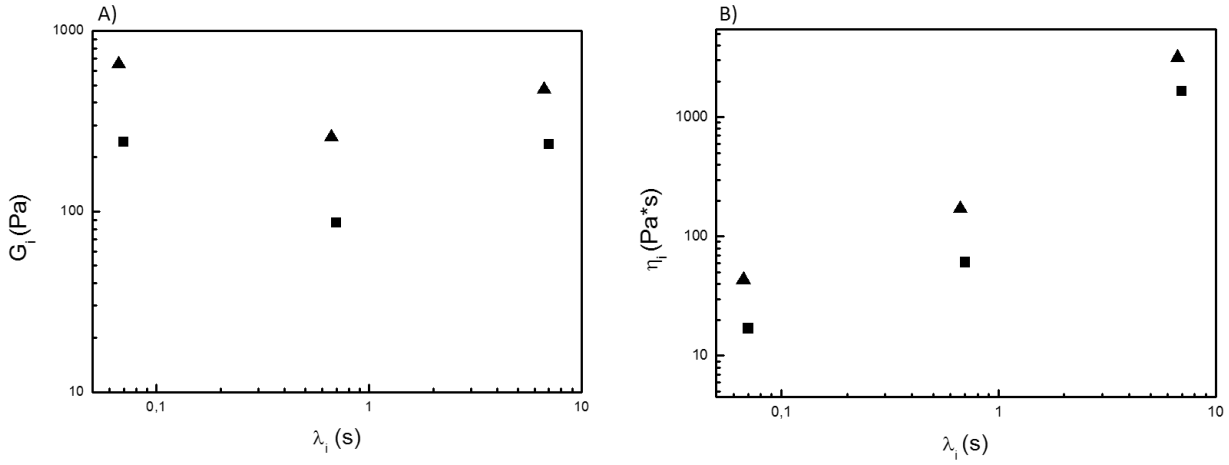

Figure S2. A) Spring constant (G_i) and B) dashpot viscosity (η_i) as a function of the relaxation time (λ_i) of the Maxwell elements for alginate hydrogels (squares) and alginate-HA hydrogels (triangles) crosslinked with 20 mM $CaCO_3$ and 40 mM GDL; polymer concentration for both HA and alginate corresponds to 15 g/L.

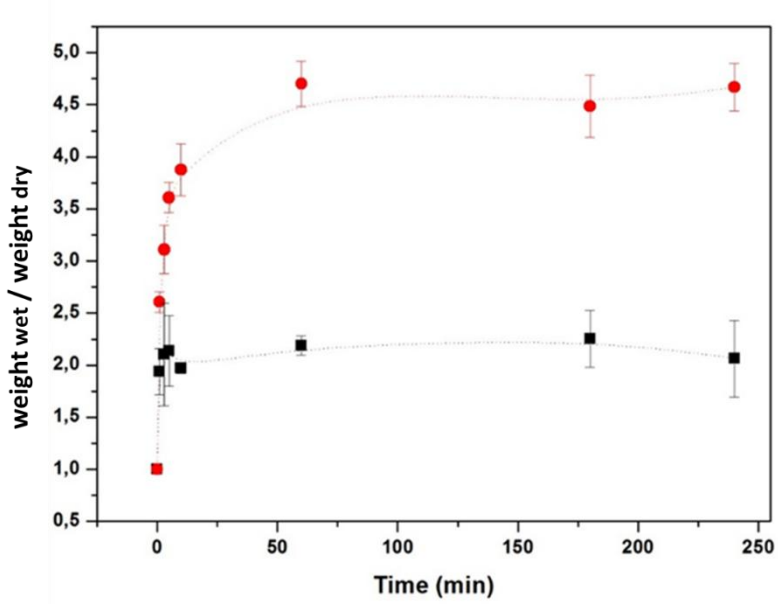

Figure S3. Rehydration kinetics of alginate (black squares) and alginate-HA membranes (red rounds) in SBF solution. Data are reported as ratio between the weight of hydrated membranes and that of dry membranes.