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Dr. William Wagner
McGowan Institute for Regenerative Medicine
University of Pittsburg

Trieste, 19th July 2016

Dear Dr. William Wagner,

please, find herewith enclosed the manuscript "*Enhanced bioadhesivity of dopamine-functionalized polysaccharidic membranes for general surgery applications*" by Scognamiglio F, Travan A, Borgogna M, Donati I, Marsich E, Bosmans J, Perge L, Foulc MP, Bouvy ND, and Paoletti S. that has been revised according to reviewers' comments and suggestions.

All reviewers' comments have been addressed. As indicated in the instructions that you sent to me by e-mail, the changes that were made with respect to the previous version of the main manuscript are highlighted in yellow.

Looking forward for a kind reply.

Yours sincerely,

Francesca Scognamiglio

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STATEMENT OF SIGNIFICANCE

This article describes the development of a mussels-inspired strategy for the development of an adhesive polysaccharide-based membrane for wound healing applications.
Bioadhesion was achieved by grafting dopamine moieties on the structural component on the membrane (alginate): this novel biomaterial showed improved adhesiveness to the intestinal tissue, which was demonstrated by both in vitro and in vivo studies.
Overall, this study points out how this nature-inspired strategy may be successfully exploited for the development of novel engineered biomaterials with enhanced bioadhesion, thus opening for novel applications in the field of general surgery.

RESPONSE TO REVIEWERS

- Please remove the automatically generated SEM bars below the images in Figures 4B and 4C and replace with a clear scale bar either directly on or below the image. Thank you.

  Automatically generated SEM bars have now been removed in figures 4B and 4C; new bars have been added on the images (line 371).

"Enhanced bioadhesivity of dopamine-functionalized polysaccharidic membranes for general surgery applications"

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Abstract

An emerging strategy to improve adhesiveness of biomaterials in wet conditions takes inspiration from the adhesive features of marine mussel, which reside in the chemical reactivity of catechols. In this work, a catechol-bearing molecule (dopamine) was chemically grafted onto alginate to develop a polysaccharide-based membrane with improved adhesive properties. The dopamine-modified alginates were characterized by NMR, UV spectroscopy and *in vitro* biocompatibility. Mechanical tests and *in vitro* adhesion studies pointed out the effects of the grafted dopamine within the membranes. The release of HA from these resorbable membranes was shown to stimulate fibroblasts activities (*in vitro*). Finally, a preliminary *in vivo* test was performed to evaluate the adhesiveness of the membrane on porcine intestine (serosa). Overall, this functionalized membrane was shown to be biocompatible and to possess considerable adhesive properties owing to the presence of dopamine residues grafted on the alginate backbone.

Keywords:

Dopamine, bioadhesion, membranes, alginate, functionalized polysaccharides.

1. Introduction

When designing novel biomaterials, the substrates employed can be chemically tailored by introducing organic/inorganic molecules; the latter should elicit specific biological responses or should endow the constructs with desired physical/mechanical features. For instance, in bone tissue engineering, membranes based on proteins were modified by including a bioactive epitope in the final construct, in order to promote osteogenesis and bone mineralization [1]. In other recent studies, peptide-functionalized membranes were proved to stimulate angiogenesis [2] or to prevent bacterial growth [3], thus opening for novel approaches in the development of functional biomaterials.

Bioactive and bioadhesive materials can properly fulfill their functions when an intimate contact with the target tissue is established, *i.e.* when the material is able to adhere closely to the body site; thus, bioadhesion represents a key feature of such implantable devices.

In general surgery, the ability to perform adhesion in the moist environment of the human body is a challenge and several approaches are being investigated to overcome this problem [4]. Besides the use of natural and synthetic polymeric glues, novel adhesive strategies that take inspiration from mussel's glue have been proposed in order to enable an effective adhesion of biomaterials under wet conditions [5-7]. Indeed, mussels produce and secrete protein-based adhesives that enable them to stick in marine environment [8;9]. Their adhesion ability is related to the presence of the catechol molecule L-3,4-dihydroxyphenylalanine (L-DOPA) located within those proteins [10;11]. In particular, hydroxyl groups in the catechol rings of L-DOPA residues can interact with hydrophilic surfaces through hydrogen bond formation. Moreover, under oxidizing or alkaline conditions, they are converted into ortho-quinone (o-quinone) moieties that can react via Michael type addition or Schiff base reaction with nucleophile groups (NH_2, SH, OH, COOH) exposed on different types of surfaces, thus leading to the formation of covalent bonds [12]. Based on this strategy, the chemical coupling of the dopamine moieties with different polymers such as alginate [13], hyaluronic acid [14;15], and poly(ethylene glycol) (PEG) [16;17] has been described for the development of biomaterials for medical applications. For instance, in the field of adhesive biomaterials, *Kastrup et*

al. [18] synthesized a catechol-based drug device designed to adhere to the internal walls of blood vessels for the treatment of vascular diseases. In another recent work, a catechol-based hydrogel showed improved *in vitro* adhesiveness to mouse subcutaneous tissue, which support the functionalization of polymers with catechols as a promising strategy for the development of adhesive systems for biomedical applications [19].

Recently, *Travan et al.* [20] developed a biodegradable membrane based on alginate and hyaluronic acid; the presence of hyaluronic acid (HA) within these membranes was shown to promote fibroblasts proliferation and migration, thereby accelerating the wound healing process. A potential application of this membrane can be represented by the need of promoting a faster closure of intestinal wounds following an anastomotic procedure. In fact, anastomotic leakage after the surgical treatment of intestinal cancers remains a major clinical concern and effective solutions are yet to be found [21;22]. In this perspective, such a polysaccharide-based membrane could be wrapped around the sutured part of the intestine (anastomosis) in order to enable the *in situ* release of the bioactive component (HA), thus promoting a faster wound closure. The local administration of HA would be maximized if the membrane could adhere closely to the intestinal serosa; thus, a long-term adhesiveness at the wet state represents a key requirement for the HA-releasing membrane. Recently, highly adhesive microgel films containing hyaluronan have been proposed for drug delivery applications [23], while biomaterials containing dopamine-modified hyaluronic acid manufactured in the form of hydrogels and films displayed enhanced *in vitro* and *in vivo* adhesion properties [15;24].

Given these premises, in the present work, the structural component (alginate) of this polysaccharide-based membrane has been functionalized by grafting dopamine moieties, in order to enhance its adhesion to the intestinal serosa. This novel engineered biomaterial was characterized with specific focus on its adhesive properties, both *in vitro* and *in vivo*.

2. Materials and methods

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) was kindly provided by Novamatrix FMC Biopolymer (Sandvika, Norway). Sodium hyaluronate (hyaluronan) Pharma grade HA240 (MW ~ 240000) was kindly provided by SIGEA (Area Science Park, Italy); Hepes, HBSS (Hank's Balanced Salt Solution), sodium chloride (NaCl), calcium carbonate ($CaCO_3$), D-Gluconic acid δ-lactone (GDL) and glycerol were obtained from Sigma-Aldrich Chemical Co. U.S.A. 2-Morpholinoethanesulfonic acid (MES), sodium chloride (NaCl), N-hydroxysuccinimide (NHS), N-(3-N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and dopamine hydrochloride (DOPA-HCl), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and heat-inactivated fetal bovine serum were supplied either by Sigma-Aldrich Chemical Co. U.S.A., Acros or Alfa-Aesar. Primary human dermal fibroblasts (HDFa) were purchased from Invitrogen™ Life Technologies; Medium 106 and Low Serum Growth Supplement (LSGS) from Gibco™. Mouse fibroblast-like (NIH-3T3) cell line (ATCC CRL1658), Dulbecco's Modified Eagle's Medium high glucose (DMEM) and Fetal Bovin Serum (FBS) were purchased from EuroClone (Italy).

2.1 Synthesis of dopamine-modified alginates

The synthesis of dopamine-modified alginate (D-Alg) was based on previously published articles [13;18;25]. The syntheses were carried out under nitrogen flushing to avoid oxidation of dopamine. Sodium alginate (final concentration 1% w/V) was dissolved in 100 mM MES buffer pH 6.2 and 0.5 M NaCl. NHS and EDC were added to the solution at the same concentration of DOPA-HCl and stirred for 30 min. DOPA-HCl was added at different final concentration (12.5 mM, 25 mM, 50 mM and 75 mM) to the solution in order to enable the synthesis of D-Alg with different substitution degrees, and stirred for 1 hour. The solution was precipitated in ethanol (10X the volume of the

alginate solution) and the precipitate was thoroughly washed several times with ethanol (2X the volume of the initial alginate solution) to eliminate unreacted molecules. The precipitate was then dried.

2.2 Membrane manufacturing

The preparation of the membranes was carried out following the procedure previously described by *Travan et al.* [20]. Briefly, a mixture of hyaluronan (final concentration 15 g/L), LVG alginate or dopamine-modified alginate (final concentration 15 g/L) and glycerol (used as plasticizer at final concentration of 5% V/V) was dissolved in deionised water at room temperature. Then, $CaCO_3$ (final concentration 0.2% w/V, corresponding to free Ca^{2+} 20 mM) and GDL (slowly hydrolysing agent that releases H^+ and thus enables the displacement of Ca^{2+} ions from $CaCO_3$; final concentration = 40 mM) were added; the solution was poured on a mould for 16 hours to enable the gel formation and then freeze-dried. When dopamine-modified alginates were used, the hydrogels were prepared under nitrogen flow and $CaCO_3$ was re-suspended in buffer solution (Hepes 100 mM) prior to add it to the polysaccharide mixture. After freeze-drying, the dopamine-containing membranes (D-AlgM) were stored in oxygen-free pouches to avoid oxidation.

2.3 Uniaxial tensile test

Tensile test were performed using a Universal Testing Machine (Mecmesin MultiTest 2.5-i) equipped with a 100 N cell load on samples shaped according to ASTM D638-10 standards (type 1 samples). The membranes were cut in dog-bone shape and the ends were gripped with metallic clamps. The specimens were loaded to failure at 5 mm/min. 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 was calculated as the slope of the linear portion in the stress-strain curve, considering the deformation range of 1%-3%. For each membrane formulation, five replicates were used and data were averaged.

2.4 *In vitro* adhesion studies

Adhesion studies were performed by employing an experimental setup adapted from Bernkop-Schnürch and colleagues [26]. Briefly, membranes were cut (1 cm X 1 cm), and attached to the external part of freshly harvested pig intestine that was kept moist with HBSS solution at pH 7.5. The tissue was fixed on a plastic cylinder (diameter 2.5 cm; height 11 cm) and incubated at 4°C for 16 hours. The cylinder was then immersed into a beaker containing 500 mL of deionized water at room temperature and gently shaked to mimic the action of body fluids. For each series, ten specimens were tested and the number of detached samples was recorded every 30 minutes. Three independent experiments were performed, data were averaged and the standard deviations calculated.

2.5 ^{1}H-NMR studies

Samples were prepared as described by *Grasdalen et al.* [27]. The ^{1}H-NMR spectra were recorded at 90°C with a JEOL 270 NMR (6.34 T). The chemical shifts are expressed in ppm downfield from the signal for 3-(trimethylsilyl)-1-propanesulfonate.

2.6 UV spectroscopy studies

The degree of substitution of D-Alg was determined from the molar extinction coefficient of dopamine and the absorbance of the sample. The D-Alg solution (1 g/L in citric acid/phosphate buffer pH 5.5) was analyzed by UV-spectroscopy (JASCO UV/Visible Spectrometer V6530) at λ = 280 nm. The molar extinction coefficient of dopamine in citric acid/phosphate buffer (pH 5.5) at 280 nm determined from a standard calibration curve was equal to: $\varepsilon_{280\,nm} = 0.0128$ L mol^{-1} cm^{-1}. The determination of degree of substitution was performed in triplicate.

2.7 *In vitro* biocompatibility

The biocompatibility of the compounds was evaluated through a quantitative and a qualitative analysis, both according to the ISO 10993-5:2009 International Standard. In the first case, the MTT assay was performed, while in the second case, an optical analysis of cell morphology was employed. Primary human dermal fibroblasts (HDFa) and a mouse embryonic cell line (NIH-3T3) were cultured in Medium106 and DMEM respectively, at 37°C and 5% pCO_2. Medium106 and DMEM were supplemented with 0.5% LSGS and 10% FBS respectively, both with the addition of 0.25% penicillin/streptomycin. Cells were plated on a 96-well sterile plate at final concentration of 5000 cells in each well. The dopamine-modified alginate was dissolved in cell medium at different concentrations (0.2 %, 0.1 %, 0.5 %, 0.02 % w/V) and 100 µL of sample were added to the wells. As a positive control of cell viability, cells treated with Triton X-100 (final concentration 0.01 % V/V) were considered. Cells growth in plain medium were used as negative control. The MTT assay was performed 24, 48 and 72 hours after treatment: 100 µL of MTT solution (0.5 mg/mL) were added to each well and incubation was allowed for 4 hours at 37 °C in dark. After the incubation, the MTT solution was removed and 50 µL of DMSO were added to each well for the dissolution of the formazan crystals. The absorbance of each well was read at 570 nm with a spectrophotometer (Infinite M200 PRO NanoQuant, Tecan). The percentage of viability of the negative control was set at 100 % and relative viability was calculated for all samples. For each series, eight replicates were tested and averaged.

2.8 *In vivo* adhesion studies

In vivo tests were carried out in pigs devoted to laparoscopic skill-training for surgical residents. The experimental protocol was complied with the Dutch Animal Experimental Act and approved by the Animal Experimental Committee of Maastricht University Medical Center. After laparotomy, two different membranes (3 cm X 6 cm) were placed around the intestine which was then repositioned in the abdominal cavity and the abdomen was closed in two layers. After 7 hours, the

animal was sacrificed, the treated intestine was macroscopically evaluated and the part of the intestine in direct contact with the membrane was harvested for histological analysis.

2.9 Histological analysis

Tissue samples were fixed in formalin 4% V/V for 24 hours and then embedded in liquid paraffin. Sections of 4 μm were cut, de-paraffinized in xylene and rehydrated in graded ethanol to distilled water, followed by hematoxylin-eosin staining.

2.10 Swelling tests

The swelling behavior of the membranes containing dopamine-modified alginate (D-Alg2M) was evaluated in Hank's Balanced Salt Solution (HBSS). The specimens were cut in a circular shape (Ø = 20 mm), weighted at the dry state and immersed in HBSS (5 mL). The weight of the membranes was measured over time, after removing excess solution (*i.e.* the liquid not absorbed from the membrane) by placing the sample on a filter paper for 30 seconds. The swelling ratio (s.r) was calculated using the eq.1:

$$s.r.\ (\%) = \frac{(Ws - Wd)}{Wd} \cdot 100 \qquad \text{eq. 1}$$

where W_s and W_d are the weights of the samples in the swollen and dry state, respectively. Three parallel replicates were considered.

2.11 Degradation studies

The structural stability of the membranes containing dopamine-modified alginates (D-Alg2M) was evaluated in Hank's Balanced Salt Solution (HBSS) over time. The samples (Ø = 20 mm) were immersed in 5 mL of HBSS, incubated at 37 °C and, once a day, the weight of the membranes was measured after removing excess solution (*i.e.* the liquid not absorbed from the membrane) by placing the samples on a filter paper for 30 seconds. The HBSS solution was replaced after daily

measurement of the membrane weight. As a reference, the 100% of the membrane weight was considered as the weight of the samples after 4 hours of immersion in 5 mL of HBSS. Four replicates were used and the values were averaged and standard deviations calculated.

2.12 Fourier-transformed infrared (FTIR) analyses

Fourier-transformed infrared spectra of the membranes were acquired by using Spectrometer Nicolet 6700 (Thermo Electron Corporation, Madison WI, U.S.A.) with DTGS KBr detector. The following setup was used: number of sample scans 32, resolution 6 cm^{-1} from 500 to 4000 cm^{-1}.

2.13 Scanning Electron Microscopy (SEM)

The membrane structure was investigated by using a Leica-Stereoscan 430i Scanning Electron microscope. The membranes containing dopamine-modified alginate (D-Alg2M) were analyzed after sputter-coating the samples with a layer of gold.

2.14 Wound healing assay (*in vitro*)

To evaluate the ability of the HA released from the membranes (D-Alg2M) to stimulate *in vitro* the closure of a scratch made on a confluent cell plate, the wound healing assay was carried out. For this test, D-Alg2M membranes were employed; as a control, membranes with the same composition devoid of HA (D-Alg2M-no HA) were used. The membranes were UV-sterilized and immersed in complete cell medium (DMEM) for 72 hours at 37 °C. The ratio between the weight of the membrane and the volume of the medium was maintained constant to achieve a polymer concentration of 0.5 % w/V. In order to avoid biased results due to excessive viscosity of the extraction medium, both D-Alg2M and D-Alg2M-no HA employed for the scratch test were manufactured without plasticizer (glycerol), in line with the procedure reported in *Travan et al.* [20] for the *in vitro* characterization.

NIH-3T3 (murine fibroblasts) were seeded in 6-well plates (400 000 cells per well) and incubated at 37 °C for 16 hours; after 24 hours, cells were treated with the liquids extract of the membranes (2 mL). The day after treatment, a scratch was performed in each well using a sterile 200 μL plastic tip and the scratch closure was evaluated by an optical microscope (Optech IB3 ICS) equipped with a Pentax K100D camera. The images of the scratch were acquired over time and analyzed with a software for image analyses (Image J); the cell-free area was outlined for each scratch and the percentage of closure was plotted as a function of time. The results were reported as percentage of gap closure at each time, with respect to time 0. For each sample, data were expressed as mean ± standard deviation. In order to evaluate the contribution of cell migration to the scratch closure, cell proliferation was inhibited by treating cells with mitomycin C at a non-toxic concentration (1 μg/mL).

2.15 Statistical analyses

Unpaired Student's t test were used to determine statistically significant differences.

3. Results

3.1 Synthesis of dopamine-modified alginates

Dopamine moieties were grafted on alginate in order to endow the structural component of the membrane with adhesive properties. EDC and NHS were added to the solution to activate the carboxylic groups of alginate, thereby enabling the coupling of alginate with the amino group of dopamine moieties. The reaction is shown in figure 1a.

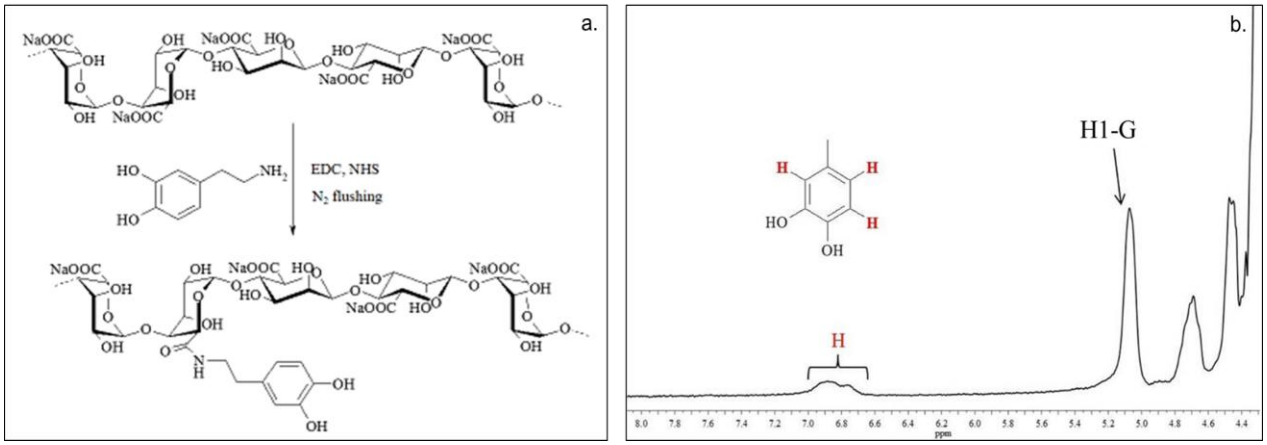

Figure 1. Grafting of dopamine on alginate backbone after activation of the carboxyl group of alginate with EDC, NHS (a); ^{1}H-NMR spectrum of dopamine-modified alginate (b).

UV spectroscopy and ^{1}H-NMR were employed for the evaluation of the degree of substitution of the functionalized polysaccharides: these analyses showed that the grafted dopamine increased by increasing the initial concentration of the compound (table 1).

Alginate formulation	**Dopamine concentration (mM)**	**Degree of substitution (%) UV-visible spectroscopy**	**Degree of substitution (%) ^{1}H-NMR spectroscopy**
D-Alg1	12.5	0.6 ± 0.1	< 1
D-Alg2	25	1.7 ± 0.2	1.2
D-Alg3	50	2.5 ± 0.1	1.8
D-Alg4	75	3.4 ± 0.4	2.8

Table 1. Dopamine-modified alginates (D-Alg). For each formulation, the initial dopamine concentration (in solution) and the degree of substitution measured by UV-visible and ^{1}H-NMR spectroscopy are reported.

^{1}H-NMR analyses confirmed this result and pointed out the successful grafting of dopamine on the alginate backbone (figure 1b). ^{1}H-NMR has been largely used to determine the degree of substitution of modified alginates [28;29]. The spectrum in figure 1b clearly shows the signal arising from three aromatic protons of the dopamine-moiety between 6.6 and 7.0 ppm. The degree of substitution can be easily calculated considering the area of the latter signal and the area of the anomeric protons of the G residues (H1-G) which, according to the composition of the starting material (as reported in the materials and methods section), accounts for 69% of the overall

anomeric protons of the polymer chain. The degree of substitution (DS) can thus be calculated according to equation 2.

$$DS\ (\%) = \frac{(Aromatic\ protons,H)/3}{(H1-G)/0.69} \cdot 100 \qquad \text{eq. 2}$$

The ^{1}H-NMR results on the degree of substitution compare rather well with the results obtained with UV spectrophotometry (table 1). The systematic lower value obtained with the former method could be due to a limited reticulation of the dopamine moieties occurred during sample preparation. These dopamine-modified alginates were employed for the preparation of calcium-reticulated hydrogels, which were finally freeze-dried to obtain the polysaccharide-based membranes, in line with the manufacturing technique described by *Travan et al.* [20]. This strategy aimed at designing a bioactive membrane for surgical applications in which the structural component is represented by alginate (modified with dopamine) while the bioactive role is ascribed to hyaluronan, whose ability to stimulate wound healing has been documented in the literature [30-33].

3.2 *In vitro* adhesion studies of membranes

The adhesive properties of the dopamine-modified membranes were evaluated *in vitro* by putting the material in direct contact with the target tissue, in line with the procedure described by Bernkop-Schnürch and colleagues [26]. In the experimental set-up, fresh porcine intestine was harvested and wrapped around a plastic cylinder in order to put the mucosa in contact with the support and to expose the external part (serosa) and the membranes were applied on it, at the serosa side. The membrane-tissue system was completely immersed in deionized water solution under gentle shaking to mimic the action of body fluids within the abdominal cavity. The time required for the detachment of the membranes from the intestinal tissue was recorded, as shown in figure 2.

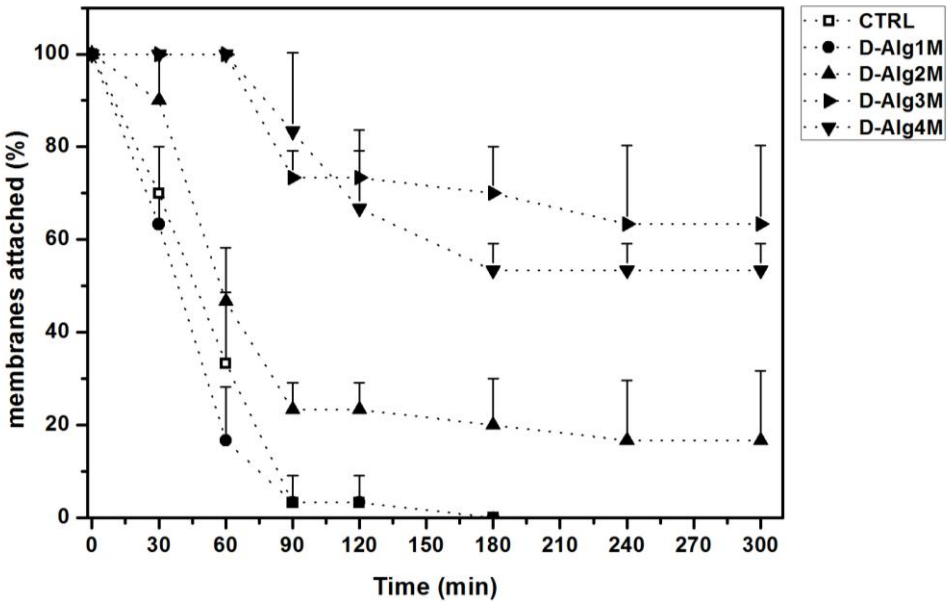

Figure 2. *In vitro* adhesion behaviour of membranes attached on explanted pig intestine and immersed in deionized water: the chart describes the detachment kinetics of the dopamine-modified membranes (D-AlgM) with respect to the control material (membrane without dopamine).

The test shows that the detachment kinetics of the dopamine-containing membranes depends on the degree of substitution (DS): high DS (D-Alg3M, D-Alg4M) correspond to high percentages of membranes attached to the intestine tissue even after 300 minutes of complete immersion. Both D-Alg3M and D-Alg4M show improved long-term adhesiveness with respect to D-Alg1M and D-Alg2M, which stems from the higher dopamine content in the modified membranes. No significant differences were observed between D-Alg3M and D-Alg4M (p-value > 0.05 considering the final time point); this data suggests that DS 2.5 ± 0.1 % (determined by UV spectroscopy) represents the maximum value for the achievement of an effective adhesion with the intestinal tissue.

The behaviour of the membranes with the lowest dopamine amount (D-Alg1M) was comparable with that of the control membrane (devoid of dopamine); at variance, when higher dopamine contents are used, the detachment profiles of the membranes (D-Alg2M, D-Alg3M and D-Alg4M) show enhanced adhesiveness. Hence, the D-Alg1M membrane was not considered for further investigations within this work.

3.3 Mechanical characterization of membranes

Starting from aqueous polysaccharide-based hydrogels, freeze-dried membranes were manufactured. The mechanical properties of the D-AlgM were tested at the dry state in a uniaxial configuration, in order to evaluate the effect of different substitution degrees on the mechanical performances of the constructs. Figure 3 shows the values of stress and strain at break and Young's Modulus of the membranes.

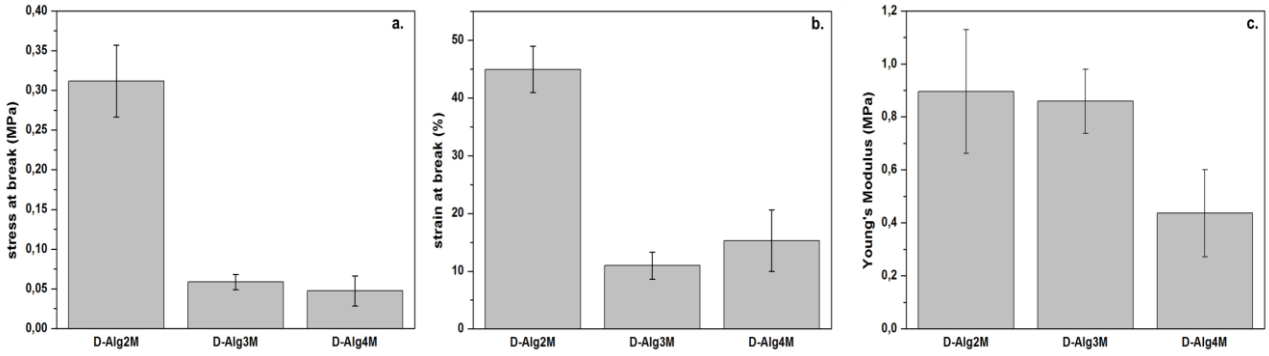

Figure 3. Mechanical properties of membranes based on dopamine-modified alginates (D-AlgM): a) stress at break, b) strain at break, c) Young's Modulus.

The results point out that the amount of grafted dopamine affects the tensile properties of the D-AlgM in terms of mechanical resistance and pliability (figure 3a and 3b). A slight reduction of the Young's modulus of the membranes was observed in the case of D-Alg4M (figure 3c).

The mechanical characterization of the modified membranes points out that high contents of dopamine (D-Alg3M, D-Alg4M) determine low material strength (0.05 – 0.06 MPa) and deformation at break (11-15%). Since a previous work of some of the authors showed that when non-modified alginate was used the tensile strength of the membrane was higher (1.31 ± 0.19 MPa), the present results suggest that the chemical modification of alginate can affect its structural properties. It should be noticed that the mechanical characterization was carried out on dried membranes because the highest stress the material should withstand is during surgical handling and positioning, *i.e.* when the material is still at the dry state [20].

Considering the results from the *in vitro* adhesion test and the mechanical characterization, the D-Alg2M membranes were considered as the best performing ones as they combine good mechanical resistance and improved adhesiveness. Thus, this membrane formulation was selected for the *in vitro* biocompatibility studies and for the evaluation of the *in vivo* adhesiveness.

3.4 Morphological evaluation of the dopamine-modified membranes

Freeze-drying of the dopamine-containing hydrogels enabled to obtain pliable membranes with homogeneous texture (figure 4a). SEM images of D-Alg2M showed that the membranes display a uniform mesh, devoid of open pores (figure 4b and 4c). The cross section micrograph highlights the compact structure of the membrane, whose thickness is in the range of 200-300 μm (figure 4c).

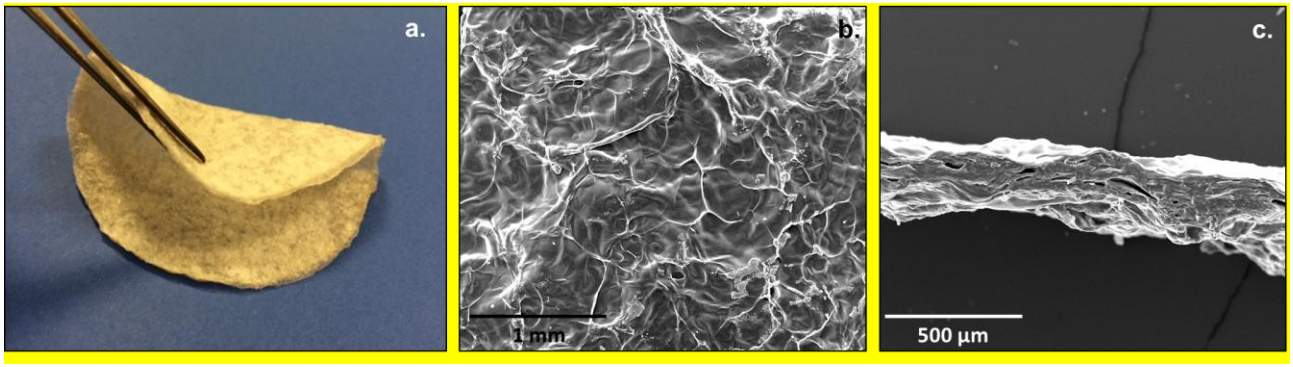

Figure 4. Images of freeze-dried D-Alg2M membrane (a), top view (b) and cross section (c) at SEM.

3.5 Rehydration and degradation studies

Rehydration and degradation studies were performed in HBSS solution in order to collect information on the *in vivo* behaviour of the membranes containing dopamine-modified alginate (D-Alg2M). The swelling test shows that the D-Alg2M membranes were able to swell considerably after immersion in HBSS solution (figure 5a). Indeed, the membranes can rapidly absorb the surrounding fluid during the first 60 minutes of incubation, reaching a swelling ratio of approximately 900% (w/w); after this point, an equilibrium is achieved.

Degradation studies were performed in similar conditions. For this analysis, the weight of each specimen was measured as a function of time and normalized by the weight of the swollen membrane (*i.e.* the weight of the membrane after 4 hours of immersion in HBSS solution).

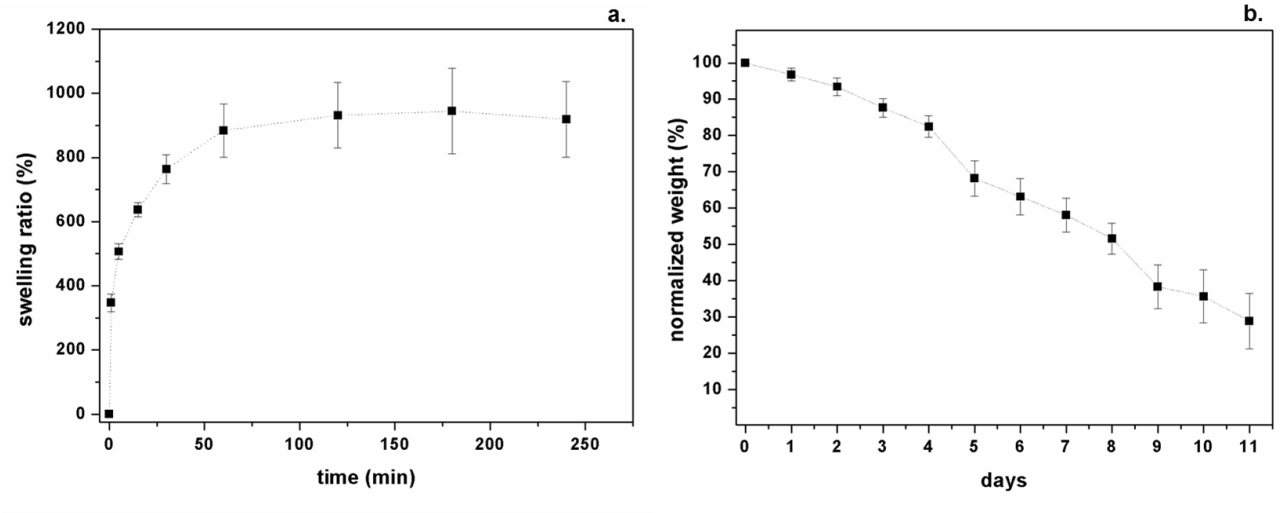

Figure 5. Swelling behaviour (a) and degradation profile (b) of D-Alg2M in HBSS solution.

The results show that, after immersion in HBSS and during the first 11 days of incubation, the D-Alg2M membranes underwent a gradual decrease of weight (figure 5b). After 11 days of incubation, only small fragments of membranes could be found in solution.

3.6 FTIR on membranes

FTIR analyses on D-Alg2M were performed in order to characterize the dopamine-modified membranes. This analysis pointed out that the spectrum of D-Alg2M was similar to that of unmodified membranes (*i.e.* devoid of dopamine), although some differences could be noticed (figure 6).

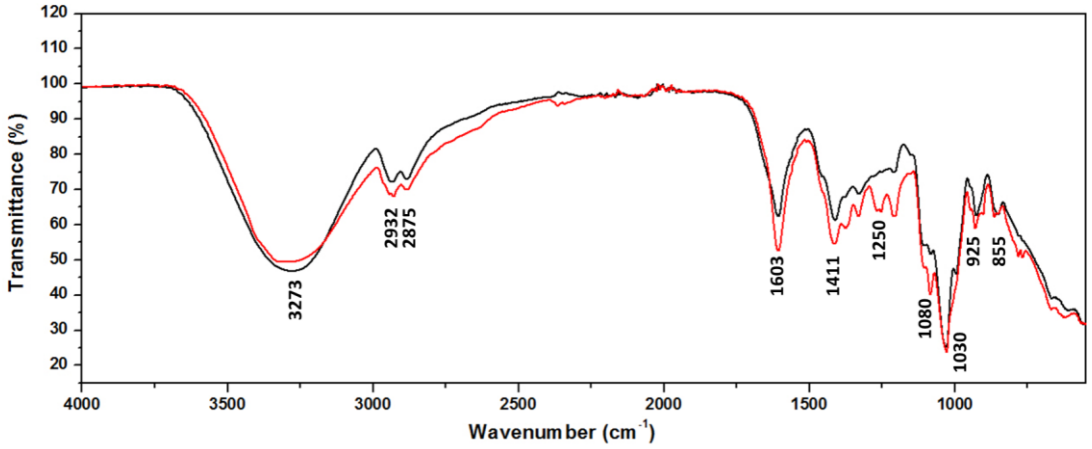

Figure 6. FTIR spectra of alginate-HA membrane (black line) and D-Alg2M membrane (red line).

Indeed, both spectra show a broad peak at 3273 cm^{-1} that corresponds to the stretching of –OH and –NH groups [34]; the peaks of moderate intensity at 2932 cm^{-1} and 2875 cm^{-1} were ascribed to C-H stretching vibrations [35]. The peaks at 1603 cm^{-1} and 1411 cm^{-1} were attributed to the C-O asymmetric and symmetric stretching of carboxyl groups of polysaccharides [35]. The D-Alg2M spectrum displays a characteristic peak at 1250 cm^{-1} that was associated to the C-O stretching of phenolic moieties [36]; this peak is not present in the spectrum of the unmodified membrane, which might confirm that dopamine residues were successfully grafted on alginate.

The peak at 1080 cm^{-1} was attributed to the C-N stretching vibrations of both HA and dopamine [34]; the peaks at 1030 cm^{-1}, 925 cm^{-1} and 855 cm^{-1} were attributed to the C-O-C stretching vibration [37] and to the G and M residues of alginate respectively [34]. Overall, the FTIR characterization of the membrane highlights the presence of grafted dopamine moieties, in line with NMR and UV-visible analyses.

3.7 *In vitro* biocompatibility

To investigate the influence of the dopamine-modified alginate on cell viability, a colorimetric assay (MTT) was carried out on primary fibroblasts (HDFa) and on a fibroblast cell-line (NIH-3T3). The modified alginate (D-Alg2) was dissolved in cell medium at various concentrations and the cell

viability was evaluated at 24, 48, and 72 hours after the treatment. As a positive control of cell viability, cells treated with a detergent that induces cell lysis (Triton X-100) were used. The results are reported in figure 7.

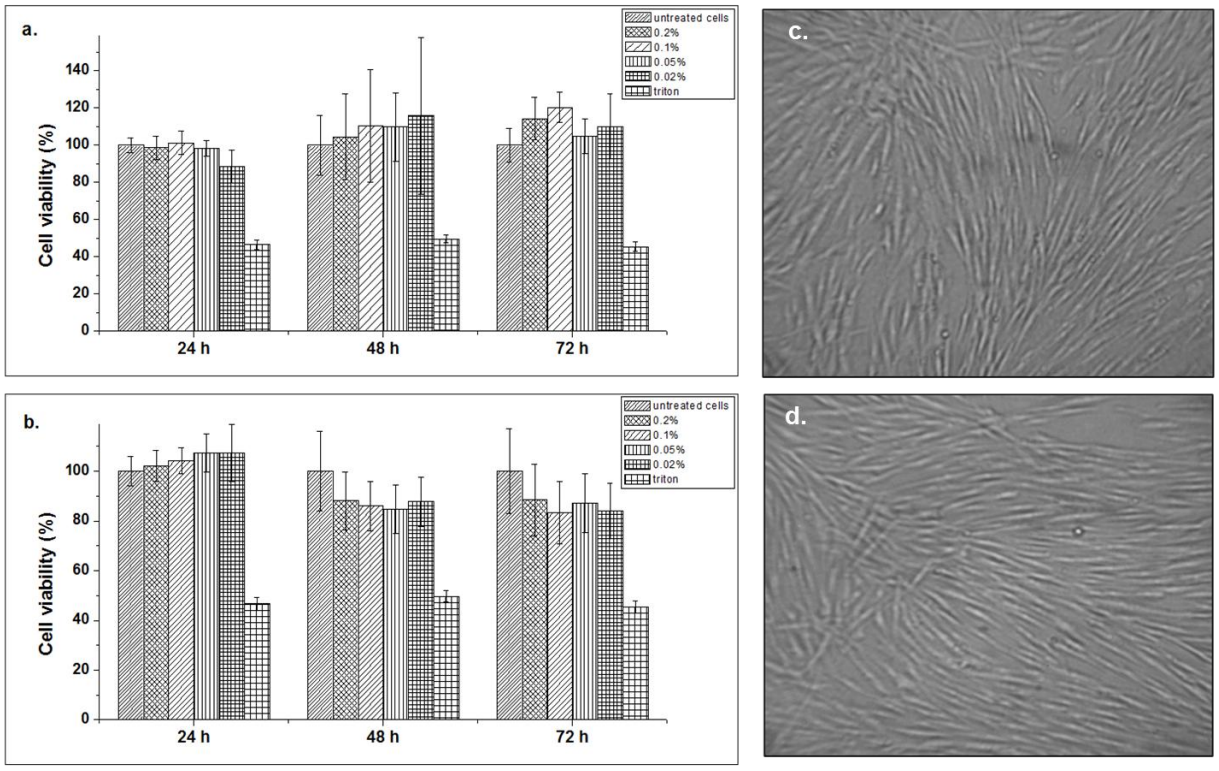

Figure 7. Cell viability (MTT test) of NIH-3T3 (a) and HDFa cells (b) treated with dopamine-modified alginate (D-Alg2) at various concentrations (0.2 %, 0.1 %, 0.05 % and 0.02 %). Optical images of untreated HDFa cells after 72 hours of culture (c) and HDFa cells treated with a dopamine-modified alginate at 0.2% (d). Student t-test was applied to evaluate significant differences between untreated and treated cells.

In the case of NIH-3T3 (figure 7a), there is no significant reduction of cell viability comparing treated cells to control cells (p-value > 0.05), which indicates the non-cytotoxicity of the tested compound at each considered time interval; the same results were obtained for HDFa primary fibroblasts at 24 hours. However, at 48 and 72 hours after treatment a slight reduction (12-16%) of the viability of treated cells could be observed at all concentrations compared to untreated cells (p-value < 0.01) (figure 7b). As a mean of comparison, the viability of cells treated with the positive control (Triton) was reduced of more than 50% for both NIH-3T3 and HDFa cells. A qualitative

evaluation of cell viability was performed by a visual analysis of the cell cultures through a microscope, in order to provide additional information on the potential cytotoxic effect of the modified alginates (figure 7c and 7d). Despite the slight reduction of the cell viability measured by the MTT assay, the optical images of both HDFa cells treated with dopamine-modified alginate after 72 hours (figure 7d) and untreated cells (figure 7c) do not point out any visible sign of cell suffering (*i.e.* change of cell morphology, cell detachment, chromatin aggregates and apoptotic bodies). It should be noticed that all the other components of the membrane were proved to be non-cytotoxic in a previous work of some of the authors [20].

3.8 *In vitro* wound healing

The ability of D-Alg2M of stimulating the wound healing was investigated *in vitro* by the scratch assay; this test enables to evaluate the effect of a compound to affect the migration and proliferation of cells, after a scratch has been performed on a confluent cell layer. The cellular response in terms of cell migration and proliferation was evaluated over time, after the treatment of fibroblasts (NIH-3T3) with the liquid extracts from D-Alg2M and from the same membrane formulation devoid of HA (D-Alg2M-no HA). Cells treated with plain medium were considered as a control. The result is reported in figures 8 A and B, which describe the percentage of gap closure as a function of time for each treatment. Optical images of the cells employed for the scratch tests are reported in figure 8 C–G.

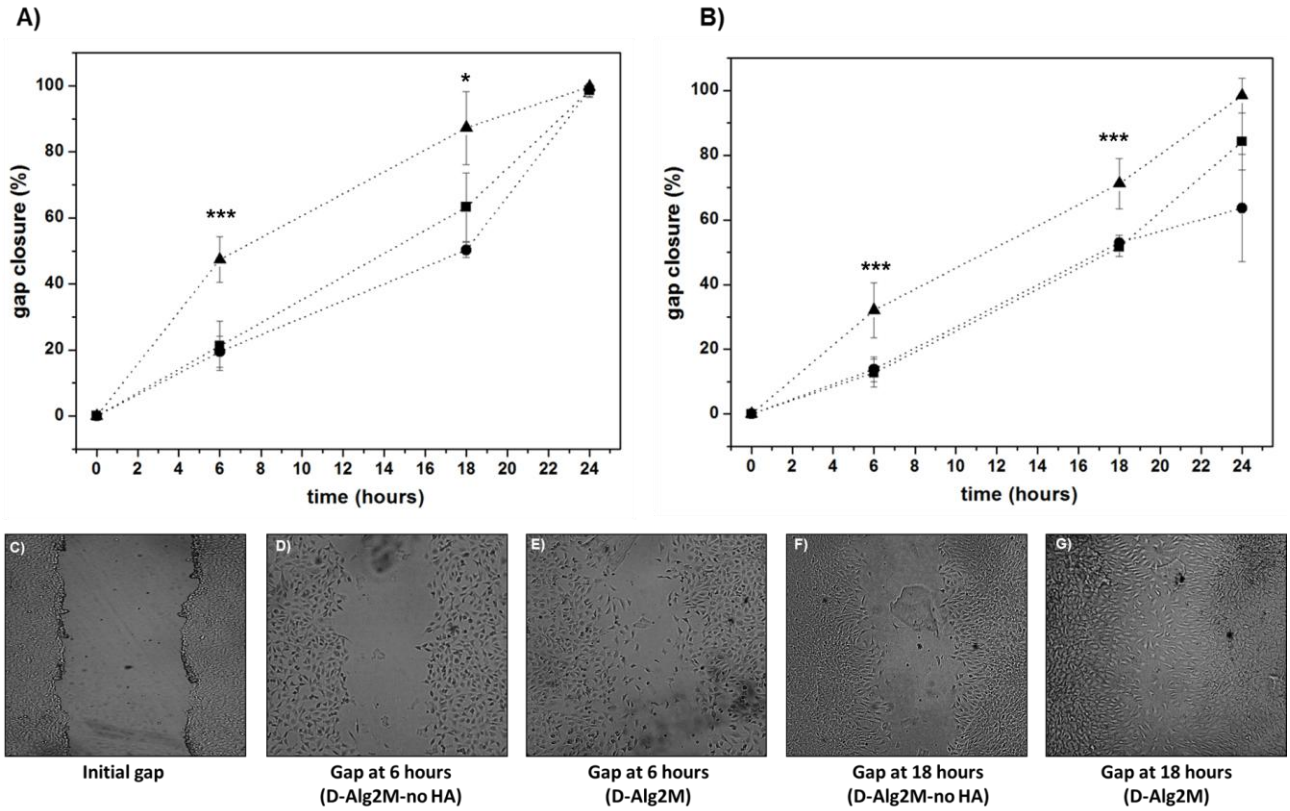

Figure 8. Effect of the liquid extracts from D-Alg2M-no HA and D-Alg2M on the gap closure of NIH-3T3 cells cultured in the absence (A) or presence (B) of mitomycin C (triangles: D-Alg2M membrane; circles: D-Alg2M-no HA membrane; squares: untreated cells). (*: p-value < 0.05; ***: p-value < 0.001).

Optical images of the cell gap at time zero (C), after 6 hours in the presence of the liquid extracted from the D-Alg2M-no HA (D) or the D-Alg2 membranes (E), after 18 hours in the presence of the liquids extracted from the D-Alg2M-no HA (F) or the D-Alg2 membranes (G).

Figure 8 A points out that both untreated cells and cells treated with the liquid extract from membranes devoid of HA (D-Alg2M-no HA) display similar kinetics of gap closure, since no significant differences were observed at each time points considered for the analysis; conversely, in the case of cells treated with the liquid extract from the HA-containing membranes (D-Alg2M), the kinetics of gap closure is accelerated. Indeed, after 6 hours and 18 hours of incubation, the percentage of gap closure reaches 47% ± 7% (p-value < 0.001) and 87% ± 11% (p-value < 0.05), respectively. In all cases, a complete scratch closure was observed after 24 hours. In parallel, the test was performed by treating cells with mitomycin C, a compound that can block cell

proliferation; thus, cell migration solely was investigated (figure 8 B). In this case, the percentage of gap closure for cells treated with the liquid extract from the D-Alg2M membranes was 32% ± 8% after 6 hours and 71% ± 7% after 18 hours, indicating that also in this case the kinetics of scratch closure is accelerated with respect to untreated cells (p-value < 0.001). This result is in line with that obtained in the absence of mitomycin C (figure 8 A), which suggests that the main phenomenon that contributes to the closure of the scratch is cell migration.

3.9 *In vivo* adhesion

The adhesiveness of the dopamine-modified membranes (D-Alg2M) was evaluated *in vivo* in a pig model. As a control, membranes prepared with non-modified alginate were used. The materials were wrapped around the pig intestine and kept in place for 7 hours after the operation. After 7 hours, the pig's abdomen was re-opened and the dopamine-based membrane was still found in place, appearing as a flexible and soft layer surrounding the intestinal walls. Moreover, the material could not be manually detached and a slight brownish colour was observed, indicating that a possible oxidation of the modified-polysaccharide occurred within the body (figure 9a). At variance, the control membrane was not found in place anymore when the abdomen was re-opened, pointing out an insufficient long-term adhesiveness of the material in such conditions.

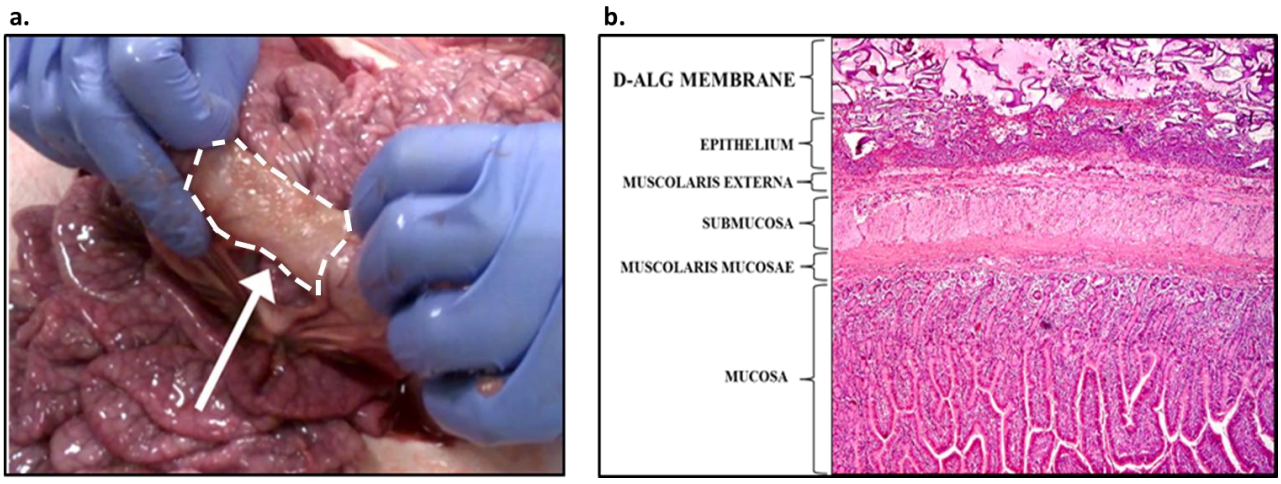

Figure 9. *In vivo* adhesion test performed by wrapping the dopamine-modified membrane (D-Alg2M) around pig intestine. a) picture taken during the explant of the material (7 hours after implantation) showing the membrane firmly wrapped around the intestine (white arrow and dotted line); b) Histological analysis of the intestine-membrane interface.

After the animal sacrifice, the tract of the intestine that was wrapped with the dopamine-containing membrane was harvested and stained with hematoxylin and eosin for the histological analysis (figure 9b). Hematoxylin and eosin are respectively basic and acid dyes able to stain the cellular structures through charge interactions in a non-specific manner, so that these compounds were employed to stain the negatively charged polysaccharide structure of the membrane in contact with the tissue. In figure 9b, the dopamine-containing membrane appears as a purple layer grafted on the intestinal epithelium (serosa), with no signs of adverse tissue reactions.

4. Discussion

The aim of this work was to evaluate if the presence of dopamine residues grafted on polysaccharide-based membranes endows the biomaterial with enhanced adhesive properties in the presence of body fluids (wet conditions). This membrane is based on the two polysaccharides alginate and HA, representing the physical matrix and the bioactive component of the system, respectively. In this perspective, the delivery of HA to the intestinal epithelium can accelerate the closure of the surgical wound, since previous studies showed that HA released from these membrane stimulates both cell migration and proliferation *in vitro* [20]. In order to guarantee an efficient release of HA at the site of intestinal anastomosis, the bioadhesion of the membrane to the intestinal walls represents a fundamental requirement for such a biomaterial, so that adhesive strategies tailored to the final medical application can be designed.

In this work, a strategy based on the functionalization of the polysaccharide alginate with dopamine was adopted because this molecule is considered the key molecular compound responsible for the adhesion mechanism of marine mussels, which occurs in water-based conditions [9;10;38]. Such

511 strong adhesion was related to the dopamine catechol rings, which can be turned into o-quinone
512 moieties in oxidizing environment; these reactive moieties are able to establish covalent bonds with
513 nucleophile groups such as amino, thiol or hydroxyl groups, which are largely present in tissue
514 proteins [39]. This mechanism appears particularly suited for the development of novel biomaterials
515 that need to be implanted and firmly adhere on internal organs, thus in the presence of abundant
516 body fluids.
517 In this work, four different dopamine-modified alginates (D-Alg) were prepared with increasing
518 dopamine content. UV-visible spectroscopy and ^{1}H-NMR pointed out a linear correlation between
519 the initial concentration of dopamine and the degree of substitution of the modified polymers. These
520 modified alginates were successfully employed for the preparation of calcium-reticulated hydrogels,
521 which were turned into pliable membranes by means of a freeze-drying procedure, according to the
522 protocol previously optimized by some of the authors of this paper [20].
523 The mechanical, chemical, adhesive and biological properties of these novel membranes were
524 investigated with particular focus on the influence of dopamine on the bioadhesion mechanism.
525 FTIR analyses performed on the modified membranes pointed out the successful grafting of
526 dopamine moieties, confirming the data obtained by UV and ^{1}H-NMR spectroscopy. The FTIR
527 spectrum of the dopamine-containing membranes shows characteristic peaks that could be ascribed
528 to the presence of phenolic moieties, in line with the findings of *Wang et al*. [34].
529 The mechanical characterization of the membranes pointed out that the higher is the substitution
530 degree of the modified alginate, the lower is the mechanical resistance (ultimate tensile strength) of
531 the dopamine-containing membranes. This behaviour can be related to the fact that chemical
532 modification of alginate is known to influence its Ca^{2+}-coordination ability, which affects the
533 reticulation process [40;41]. Indeed, it is likely that the presence of dopamine moieties might
534 interfere with the formation of the egg-box structure of the alginate chains during the gelation phase,
535 thus lowering the mechanical resistance of the freeze-dried constructs. Given the key role played by
536 guluronic acid sequences in determining such egg-box structures, it is reasonable to expect that the

distribution of the dopamine residues on the alginate chains may affect a non-negligible number of such residues, thus preventing them from participating to the calcium-mediated interchain cross-links. In order to limit such effect, a possible alternative strategy could be the derivatization with dopamine of poly(mannuronic) chains, followed by chemo-enzymatic selective epimerization to produce dopamine-derivatized alginate chains retaining the superior gel-forming ability, as suggested in a previous work by some of the authors [40].

The *in vitro* biocompatibility of dopamine-modified alginate (D-Alg2) was investigated on primary fibroblasts (HDFa) and on a fibroblast cell line (NIH-3T3): despite a slight decrease in the viability of HDFa cells was observed after 48 and 72 hours, it should be considered that, according to the ISO 10993-5:2009 method for the evaluation of the cytotoxicity of a compound, only reductions of cell viability by more than 30 % are considered as a cytotoxic effect. Furthermore, the qualitative investigation by optical microscopy confirmed the good viability of cells treated with the modified alginates even at the longest evaluation time (72 hours). These results are in line with literature data showing that catechol-containing polymers used as tissue adhesive or for the functionalization of surfaces do not exert any toxic response both *in vitro* and *in vivo* [42;43].

The bioactive role played by these alginate-HA based membranes was previously demonstrated *in vitro* by some of the authors of this paper, employing the wound healing assay [20]. This test was performed on fibroblast cells to evaluate whether the presence of the grafted dopamine could interfere with the healing process. The test proved that the bioactive role exerted by these membranes was maintained when dopamine residues were grafted on alginate; indeed, the treatment of cells with the liquid extract from the D-Alg2M improves the kinetics of scratch closure, and this phenomenon was ascribed mainly to the contribution of cell migration. In the case of untreated cells and cells treated with the liquids extracted from the D-Alg2M devoid of HA, the kinetics of cell gap closure are similar, indicating that the grafted dopamine residues do not affect neither cell migration nor cell proliferation.

562 The swelling behavior of the D-Alg2M was evaluated in HBSS solution, at 37 °C to mimic the
563 physiological conditions. This study pointed out that the modified-membranes are able to swell after
564 immersion in HBSS, which represents a positive aspect since this phenomenon favors the initial
565 adhesion of the membrane to the intestinal serosa.
566 Degradation studies on dopamine-containing membranes (D-Alg2M) provide an indication
567 regarding the behavior of the membranes *in vivo*. The test showed that D-Alg2M are gradually
568 degraded during 11 days of incubation in HBSS, which represents a desired feature for this
569 biomaterial, since HA contained within the membrane should be provided at the wound site right
570 after implantation, in order to achieve an efficient healing. After having exerted this function, the
571 membrane are designed to progressively degrade within the human body.
572 The morphological analyses performed by SEM pointed out the uniform texture and the
573 homogeneous mesh of the membrane (D-Alg2M). In a previous work of some of the authors, the
574 characterization by SEM imaging pointed out the similar morphology of the membranes prepared
575 with unmodified alginate [20], indicating that the grafting of dopamine does not affect the
576 morphological features of the membrane. Moreover, the pliability and the limited thickness (200-
577 300 μm) of the dopamine-containing membranes make them suitable for the wrapping around the
578 anastomosis.
579 Swelling tests pointed out the ability of the dopamine-containing membrane (D-Alg2M) to absorb
580 the surrounding liquids. This is a desirable feature, since it indicates that in *in vivo* conditions, the
581 membrane should be able to absorb fluids from the moist serosa, thus favouring its initial tackiness
582 to tissue.
583 *In vitro* adhesion studies were carried out to evaluate the adhesiveness of the membranes in
584 simulated physiological conditions. These tests pointed out that the adhesivity of the membranes to
585 the target tissue (intestine serosa) is enhanced when dopamine-grafted alginates are used; in
586 particular, in the case of high degrees of substitution (D-Alg3 and D-Alg4) over 50% of membranes
587 were still attached to the intestine tissue even after 300 minutes of complete immersion in deionized

588 water (a particularly demanding scenario, since in the abdominal cavity liquids would accumulate
589 only occasionally). Moreover, since D-Alg3M and D-Alg4M display a similar detachment profile, it
590 can be evinced that DS 2.5 ± 0.1 % represents the limit value for the achievement of an effective
591 adhesion to the intestinal tissue. These data appear to be in line with the finding of *Yang et al.* [44],
592 who pointed out that, over a limit value of dopamine grafted on methacrylamide-based polymers, no
593 further enhancement of the adhesion strength was observed.
594 This result proves the rationale of this paper, *i.e.* the capability of dopamine-based compounds to
595 establish adhesive bonds with proteinaceous tissues. Prompted by this result, it can be inferred that,
596 in the case of dopamine-modified membranes, bioadhesion occurs by exploiting two mechanisms: *i*)
597 an initial adhesion driven by the hydrophilic feature of the membrane itself that tends to stick to the
598 moist serosa by absorbing tissue fluids; *ii*) the establishment of chemical bonds between the
599 exposed dopamine moieties and nucleophile groups of tissue proteins (*i.e.* amino groups), which
600 favours a prolonged bioadhesion in the presence of biological fluids.
601 Moreover, considering the experimental conditions of this *in vitro* set-up, it is reasonable to assume
602 that this adhesion process could be further enhanced *in vivo*, since the oxidizing environment within
603 the human body might accelerate the oxidation of the hydroxyl groups of the catechol rings of
604 dopamine, thus boosting the bioadhesion process.
605 In agreement with the *in vitro* indications, *in vivo* adhesion studies on non-dedicated pigs pointed
606 out that both the control and the dopamine-modified membrane (D-Alg2M) displayed a good initial
607 adhesion when in contact with the moist tissue, as well as the capability to adapt to the anatomy of
608 the intestinal walls, as neither alteration of the intestinal motility or stenosis of the treated tract were
609 observed. However, 7 hours after implantation within the pig abdomen, only the dopamine-
610 modified membrane (D-Alg2M) was still laying on the intestinal serosa, while the control
611 membrane could not remain in situ; the tight integration between the D-Alg2M membrane and the
612 intestine suggests that the physiological oxidizing environment favours the bioadhesion mechanism
613 of the material. In line with this analysis, *Brubaker et al.* [45] synthetized a catechol derivatized

PEG adhesive to immobilize pancreatic islet beta cells to extrahepatic tissues, for the treatment of diabetes type I mellitus. The adhesive features of this system were proved *in vivo* and ascribed to the presence of o-quinones exerting reactivity toward amino residues such as those found in ECM proteins of tissues. This interaction was reported to be the basis of the continuous adhesive/tissue interface. Overall, the histological assessment pointed out the absence of early adverse tissue reactions upon the contact with the modified-membrane, highlighting the biocompatibility of the material after 7 hours of implantation and its deep compenetration between the material and the intestinal epithelium.

5. Conclusions

The adhesiveness of a biomaterial to the target body site is an important requirement for the accomplishment of its functions. In the case of polymeric membranes for intestinal applications, insufficient adhesiveness to the tissue might negatively affect the efficacy of the device. The polysaccharide-based membrane described in this paper has been successfully implemented by the chemical grafting of dopamine on alginate, which enabled to significantly improve the adhesiveness of the biomaterial to the intestinal tissue, as evaluated both *in vitro* and *in vivo* on a pig model. The release of HA from the resorbable membrane could stimulate fibroblasts activities (*in vitro*). This engineered membrane showed good biocompatibility in the time-frame considered and opens for novel perspectives for the development of adhesive biomaterials for general surgery applications.

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Table 1

Alginate formulation	Dopamine concentration (mM)	Degree of substitution (%) UV-visible spectroscopy	Degree of substitution (%) ^{1}H-NMR spectroscopy
D-Alg1	12.5	0.6 ± 0.1	< 1
D-Alg2	25	1.7 ± 0.2	1.2
D-Alg3	50	2.5 ± 0.1	1.8
D-Alg4	75	3.4 ± 0.4	2.8

Table 1. Dopamine-modified alginates (D-Alg). For each formulation, the initial dopamine concentration (in solution) and the degree of substitution measured by UV-visible and ^{1}H-NMR spectroscopy are reported.

Figure 1

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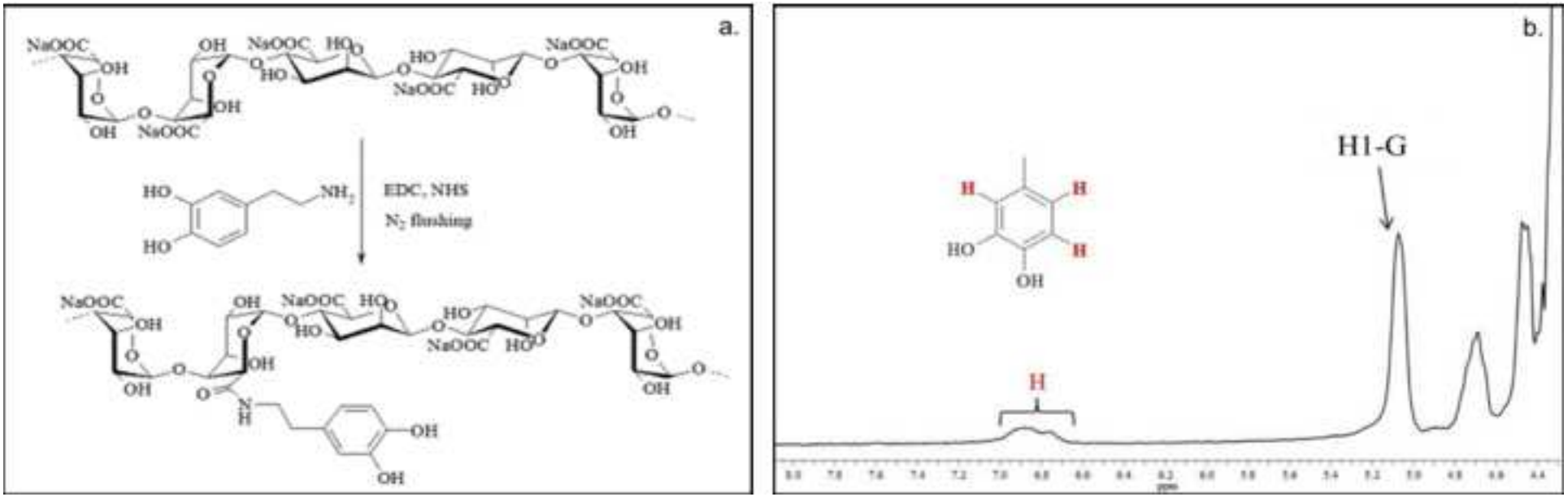

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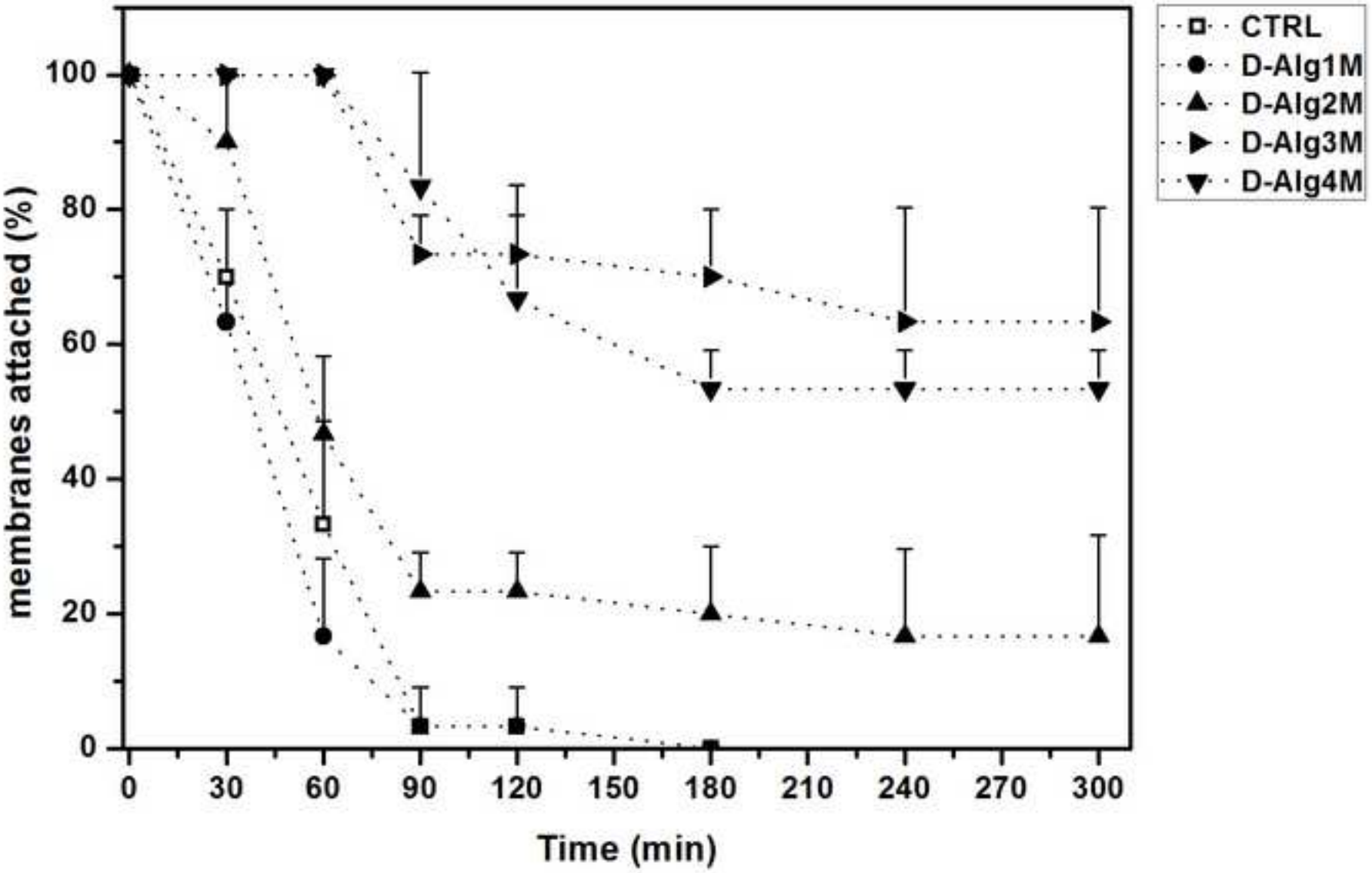

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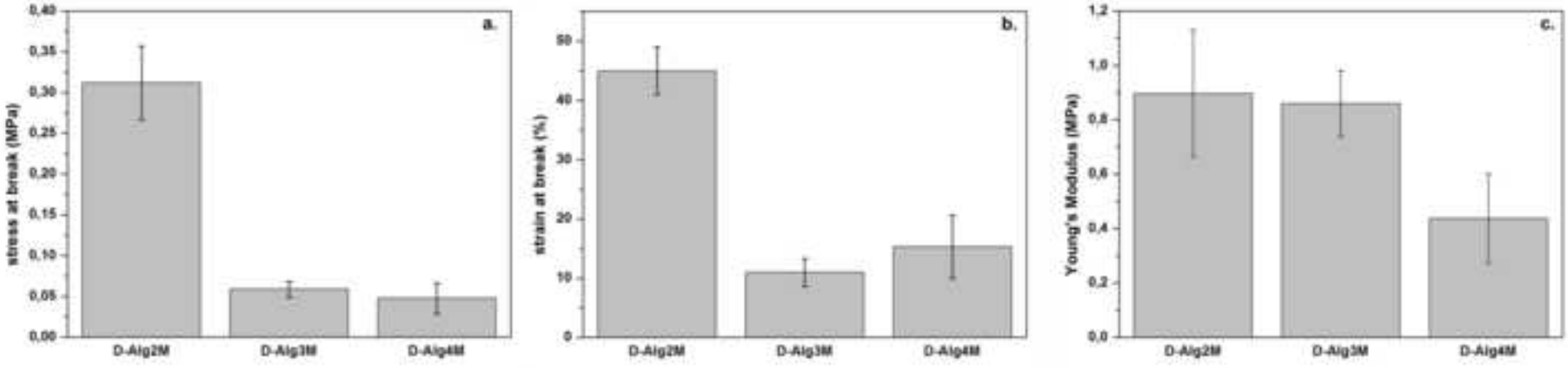

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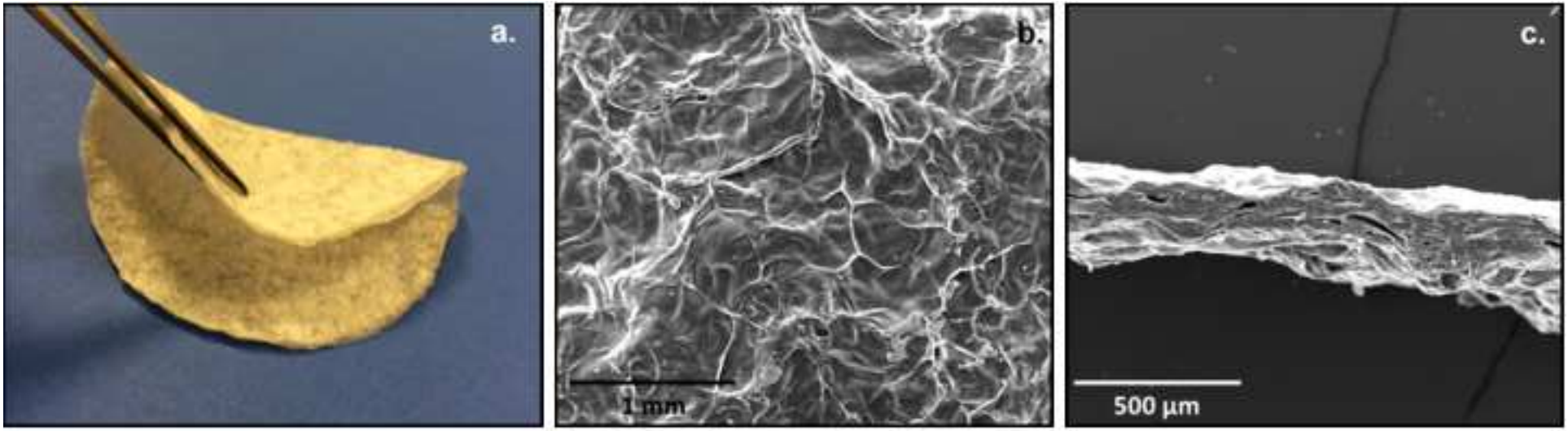

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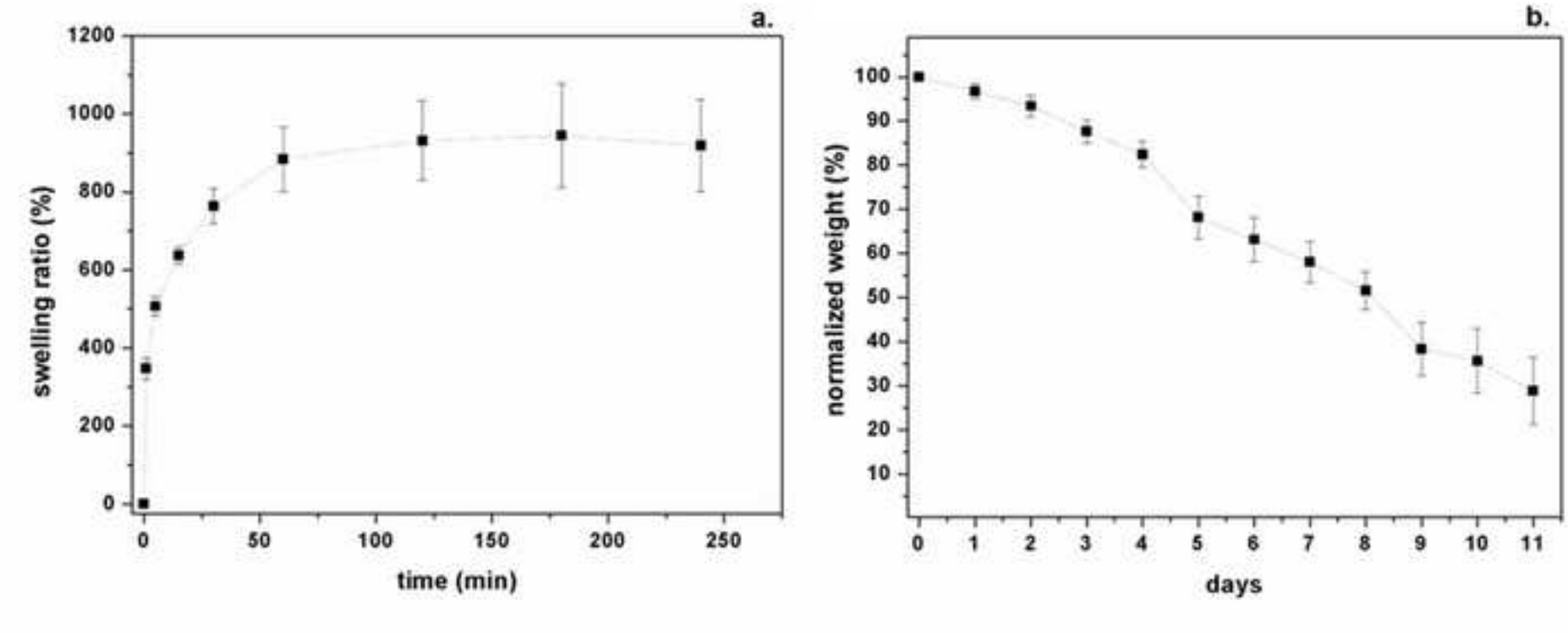

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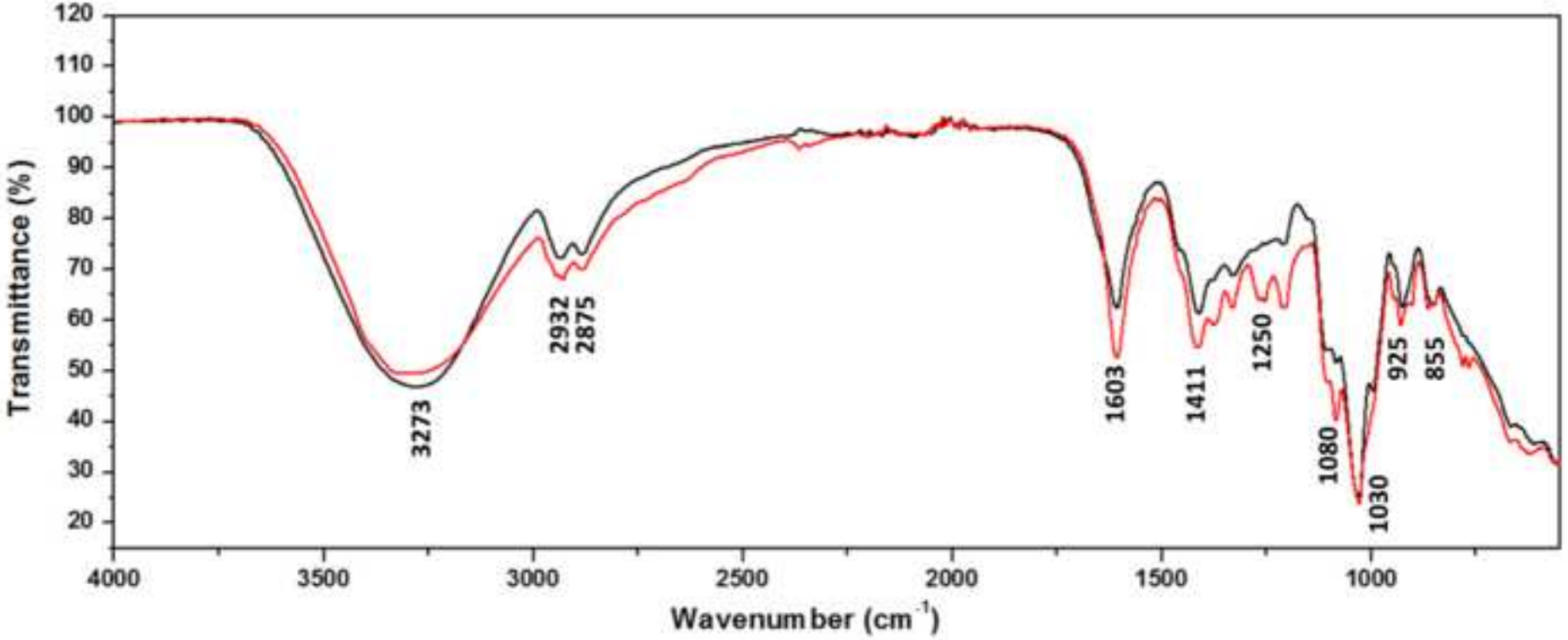

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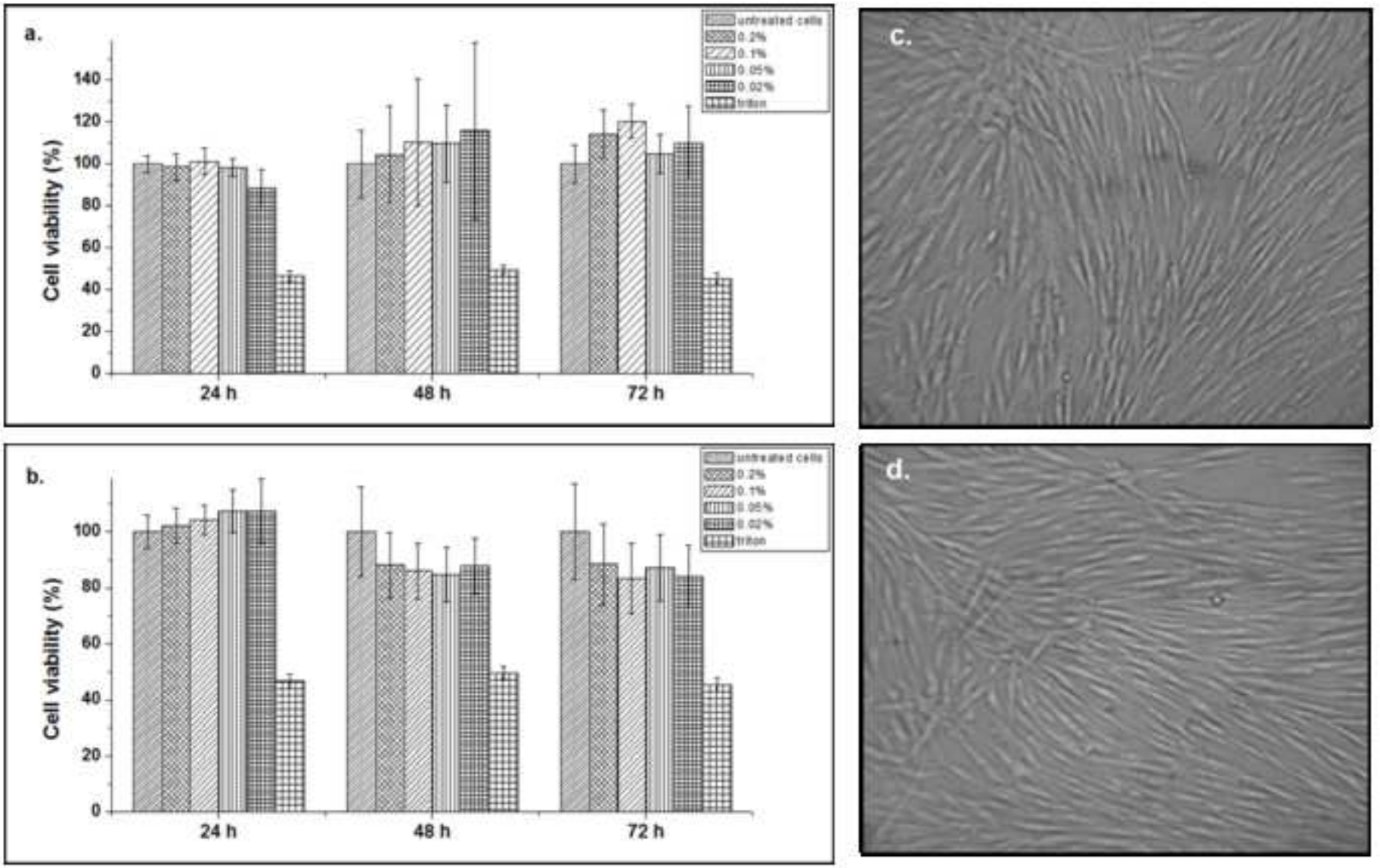

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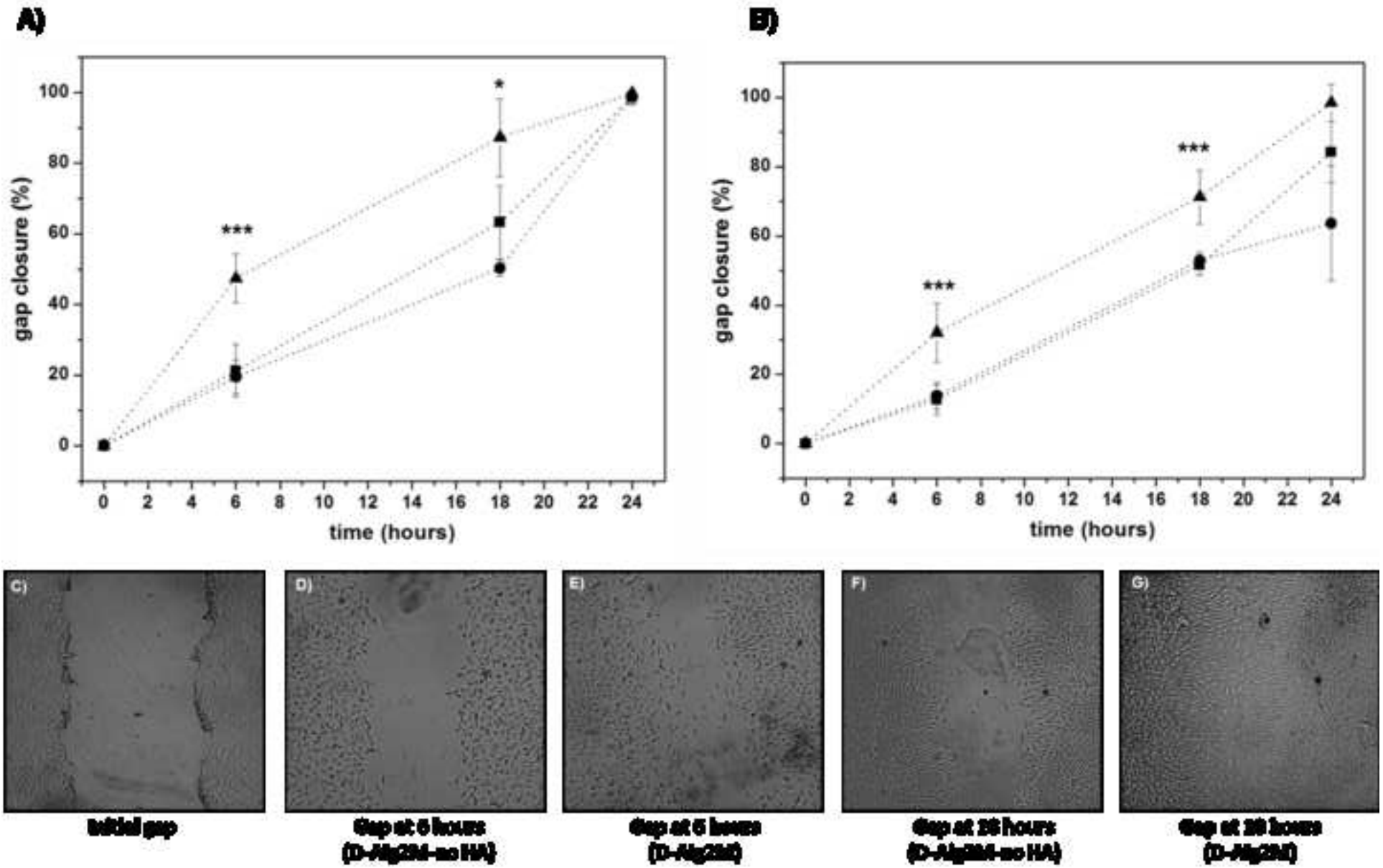

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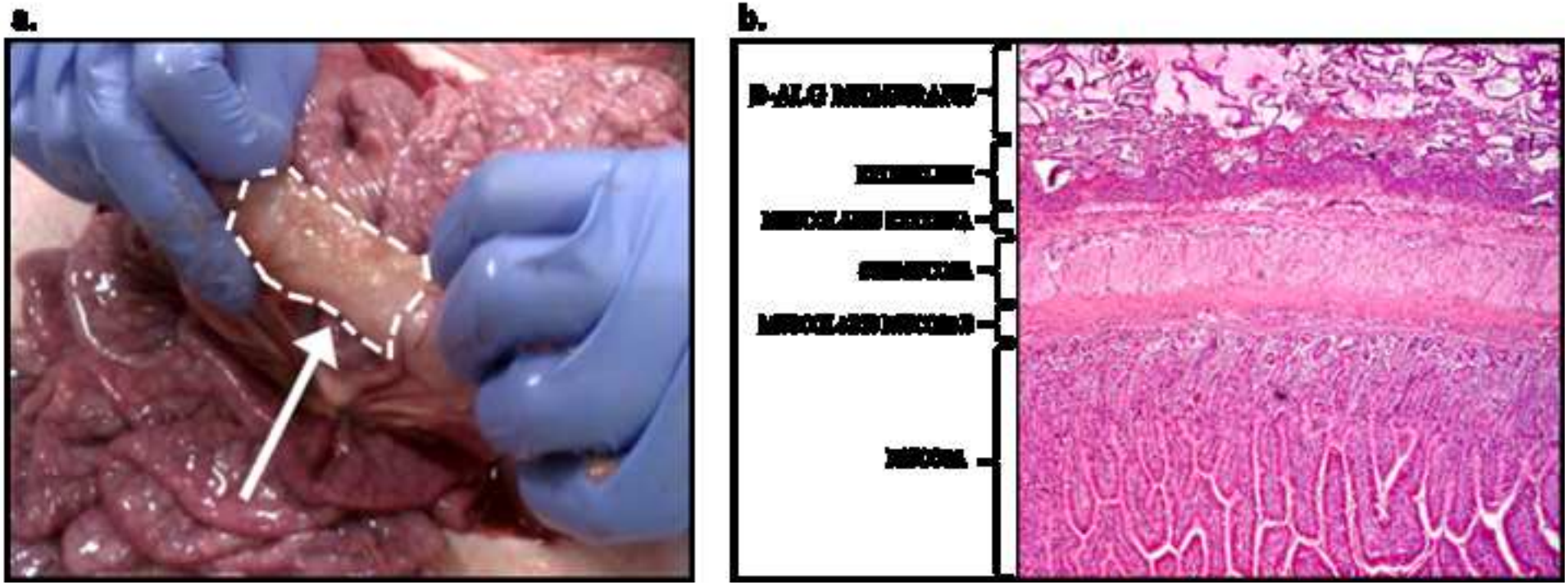

FIGURE CAPTIONS

Figure 1. Grafting of dopamine on alginate backbone after activation of the carboxyl group of alginate with EDC, NHS (a); ^{1}H-NMR spectrum of dopamine-modified alginate (b).

Table 1. Dopamine-modified alginates (D-Alg). For each formulation, the initial dopamine concentration (in solution) and the degree of substitution measured by UV-visible and ^{1}H-NMR spectroscopy are reported.

Figure 2. *In vitro* adhesion behaviour of membranes attached on explanted pig intestine and immersed in deionized water: the chart describes the detachment kinetics of the dopamine-modified membranes (D-AlgM) with respect to the control material (membrane without dopamine).

Figure 3. Mechanical properties of membranes based on dopamine-modified alginates (D-AlgM): a) stress at break, b) strain at break, c) Young's Modulus.

Figure 4. Images of freeze-dried D-Alg2M membrane (a), top view (b) and cross section (c) at SEM.

Figure 5. Swelling behaviour (a) and degradation profile (b) of D-Alg2M in HBSS solution.

Figure 6. FTIR spectra of alginate-HA membrane (black line) and D-Alg2M membrane (red line).

Figure 7. Cell viability (MTT test) of NIH-3T3 (a) and HDFa cells (b) treated with dopamine-modified alginate (D-Alg2) at various concentrations (0.2 %, 0.1 %, 0.05 % and 0.02 %). Optical images of untreated HDFa cells after 72 hours of culture (c) and HDFa cells treated with a dopamine-modified alginate at 0.2% (d). Student t-test was applied to evaluate significant differences between untreated and treated cells.

Figure 8. Effect of the liquid extracts from D-Alg2M-no HA and D-Alg2M on the gap closure of NIH-3T3 cells cultured in the absence (A) or presence (B) of mitomycin C (triangles: D-Alg2M membrane; circles: D-Alg2M-no HA membrane; squares: untreated cells). (*: p-value < 0.05; ***: p-value < 0.001).
Optical images of the cell gap at time zero (C), after 6 hours in the presence of the liquid extracted from the D-Alg2M-no HA (D) or the D-Alg2 membranes (E), after 18 hours in the presence of the liquids extracted from the D-Alg2M-no HA (F) or the D-Alg2 membranes (G).

Figure 9. *In vivo* adhesion test performed by wrapping the dopamine-modified membrane (D-Alg2M) around pig intestine. a) picture taken during the explant of the material (7 hours after implantation) showing the membrane firmly wrapped around the intestine (white arrow and dotted line); b) Histological analysis of the intestine-membrane interface.

*Graphical Abstract