Journal of Materials Science: Materials in Medicine (2019) 30:60
https://doi.org/10.1007/s10856-019-6262-6

BIOMATERIALS SYNTHESIS AND CHARACTERISATION

Original Research

Development of hyaluronan-based membranes for the healing of intestinal surgical wounds: a preliminary study

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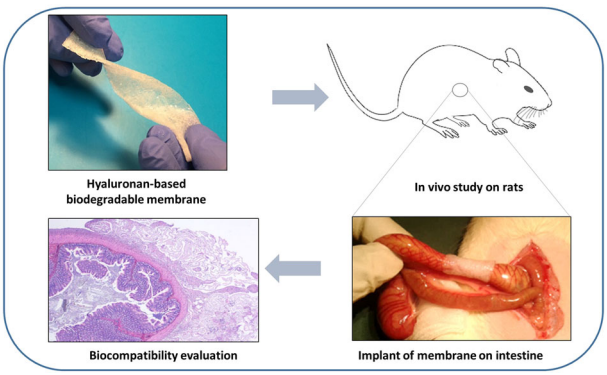
Received: 27 February 2019 / Accepted: 6 May 2019 / Published online: 24 May 2019

Abstract

Implantable membranes based on alginate and hyaluronic acid (HA) were manufactured to obtain a rapidly resorbing pliable mesh for the in situ administration of HA to intestinal tissue. Morphological analyses of this interpenetrated matrix pointed out a homogeneous polymeric texture while degradation studies demonstrated that the material is able to dissolve in physiological solutions within few days. Biological studies in vitro showed that the membrane is biocompatible towards human dermal fibroblasts and that liquid extracts from the HA-containing membrane can stimulate wound healing. A preliminary in vivo biocompatibility study on rats showed that the membranes in direct contact with the intestine did not elicit any acute adverse reaction or immune response, while only a mild inflammatory reaction was noticed at the mesenteric or serosal region. Overall, these results appear to support the application of these polysaccharide-based materials for intestinal wound healing.

Graphical Abstract



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1 Introduction

Hyaluronic acid (HA) is a linear polysaccharide widely employed in the biomedical field for its ability to regulate physiological events such as fibroblasts migration, differentiation and proliferation [1–4], making it a good candidate for the development of biomaterials that can stimulate wound healing of tissues. This property can be exploited in surgical interventions that require a fast closure of wounds, in particular in the case of internal organs in human body. Among these organs, intestine represents a challenging target, and the possibility to develop biomaterials

supporting intestinal healing has been explored in literature [5, 6]. Indeed, surgical wounds on intestinal tissues are mostly associated to the treatment of cancer, which often requires the resection of the affected tract, followed by the suture of intestinal extremities (anastomosis). A safe closure of the anastomosis occurs when a proper regeneration of the intestinal tissue takes place [7]. To prevent the occurrence of post-operative complication such as anastomotic leakage, research studies are being focused on the development of biomaterials in the form of hydrogels or membranes that could promote or assist a rapid anastomotic closure [8–11]. Some strategies employed to this aim exploit biomaterials such as suture reinforcement [11–14], glues or sealants [9, 10, 15]. Other approaches aim at the development of delivery systems of cells [16] and growth factors [8] or at the local administration of bioactive compounds to strengthen the anastomosis [17]. A promising approach to stimulate the closure of surgical wounds is based on the local delivery of HA at the wounded site through a resorbable matrix [18]. This approach has been recently proposed by Travan and colleagues who developed a biodegradable alginate-based membrane that could efficiently release HA and stimulate in vitro migration and proliferation of fibroblast cells [19]. Since fibroblast cells are abundant in the submucosa layer of intestine, it can be hypothesized that by wrapping the material around the sutured part of the intestine, the HA released from such a resorbable matrix could accelerate the wound healing process by stimulating fibroblasts. The activity of fibroblasts is required for proper tissue healing, thus limiting the risk of leakage. Although preliminary in vivo tests performed on membranes composed by alginate and hyaluronan pointed out the absence of early adverse tissue reactions when applied around pig's intestine [20, 21], dedicated in vivo studies are required to evaluate the long term biocompatibility of these polymer-based biomaterials. Given these premises, the aim of this study was to develop a biodegradable membrane for the in situ release of HA to intestine and to test its long-term biocompatibility in a preliminary in vivo study.

2 Materials and methods

2.1 Materials

Sodium alginate from *Laminaria hyperborea* (product name: 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). 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), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), mytomicin C, Hank's Balanced Salt Solution (HBSS), dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical Co. U.S.A. Primary human dermal fibroblasts (HDFa) were purchased from Invitrogen™ Life Technologies. Medium 106, 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).

2.2 Membrane manufacturing

The membranes containing alginate and hyaluronan (ALG-HA) were prepared following the procedure previously described by Travan et al. [19]. Briefly, hyaluronan (HA; final concentration 15 g/L) and LVG alginate (final concentration 7.5 g/L) were 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 (final concentration = 40 mM) were added to the mixture to enable the hydrogel formation. The solution was poured in moulds (10 cm × 10 cm) and, after 16 h, the hydrogels were freeze-dried.

2.3 Scanning electron microscopy (SEM) analyses

The membranes (ALG-HA) were gold-sputtered (Sputter Coater K550X, Emitech, Quorum Technologies Ltd, UK) and the morphological analysis was immediately performed by Scanning Electron Microscope (Quanta250 SEM, FEI, Oregon, USA) operated in secondary electron detection mode. The working distance and the accelerating voltage were adjusted in order to obtain a suitable magnification.

2.4 Mass loss evaluation

Membrane samples (ALG-HA) were cut in circular shape (Ø = 20 mm) and put in a plastic tube containing 10 mL of HBSS. All samples were loaded on an orbital shaker for the selected time points. Four hours after starting the incubation, the samples were collected, dried for 1 min on filter paper and weighed. The weight of samples at this time point was considered as a reference. Once a day the samples were collected, dried for 1 min on filter paper, weighed and then immersed in fresh HBSS. The weight of the membranes was normalized on the weight of the reference membrane

samples. Three replicates were considered at each time point, data were averaged and standard deviation calculated. Average values were reported as a function of time.

2.5 Cell cultures

Mouse fibroblast-like cells (NIH-3T3) were cultured in Dulbecco's Modified Eagle's Medium high glucose (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin. Primary human dermal fibroblasts isolated from adult skin (HDFa) were cultured in Medium106 supplemented with 0.5% Low Serum Growth Supplement (LSGS)—both provided by Gibco™—and 0.25% penicillin/streptomycin. All cells were maintained in a humidified atmosphere (5% CO_2) at 37 °C.

2.6 Preparation of membranes for in vitro studies (cell biocompatibility and scratch test)

The membranes (ALG and ALG-HA) were UV sterilized and incubated in DMEM to get membrane release at 37 °C for 72 h. The ratio between weight of the membrane and volume of the medium was calculated in order to reach a maximum ALG and HA concentration of 0.25% w/v and 0.5% w/v. In this case, 150 mg of ALG-HA membranes were soaked in 20 mL of DMEM. This approach prevents an excessive viscosity of the extraction medium, which could cause biased results. In the case of ALG membrane, 50 mg of membrane were soaked in 20 mL of DMEM, so that the theoretical concentration of alginate in solution was the same of that achieved in the case of ALG-HA. All samples were incubated at 37 °C for 72 h. After incubation, the membranes were discarded and the liquid extracted from the membranes was employed as such for the in vitro studies.

2.7 In vitro biocompatibility (MTT assay)

The biocompatibility of the ALG-HA membranes was evaluated by the MTT assay. NIH-3T3 cells were plated on 96-well sterile plates at final concentration of 5000 cells in each well and 100 µL of the liquid extracted from the membranes were added to the wells. As a negative control of cell viability, cells growth in plain medium (i.e. cell medium not conditioned with any treatment) were considered. The MTT assay was performed 24 and 48 h after treatment. The cells were incubated with MTT (100 µL of MTT solution 0.5 mg/mL in PBS 1×) for 4 h at 37 °C in the dark. After incubation, the MTT solution was removed and the dissolution of the formazan crystals was achieved by the addition of 50 µL of DMSO. 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 the results averaged.

2.8 Scratch test on fibroblasts

The scratch assay was performed on human dermal fibroblasts from dermal skin (HDFa cells) to evaluate the ability of the HA released from the membrane (ALG-HA) to stimulate their migration in vitro. HDFa cells were plated in a 6-well plate (250,000 cells in each well) and 3 mL of the liquid extract of the membranes or plain medium (negative control) were added to the wells. Incubation was allowed for 24 h at 37 °C, 5% CO_2. A scratch was performed in each well using a sterile 200 µL plastic tip after incubation. To monitor the scratch closure over time, an optical microscope (Optech IB3 ICS) equipped with a Pentax K100D camera was used. For the analysis of the kinetic of gap closure, the cell-free zone was outlined per each scratch and the percentage of closure over time was plotted. The analyses of the images were performed with a software for image analysis (Image J). The results are reported as percentage of closure of the gap area between day n and day 0. At each time point, six replicates were considered, data were averaged and expressed as mean ± standard deviation. In order to evaluate the contribution of cell migration only to the scratch closure, cell proliferation was blocked by treating cells with mitomycin C in a non-toxic concentration (1 µg/mL) for 24 h and the analysis of gap closure was performed as previously described.

2.9 Animal studies and surgical procedure

The evaluation of membrane biocompatibility in vivo was performed on Wistar rats with an average body weight of 250–300 g. Animals were housed at the Central Animal Facilities of the Maastricht University. All animals were provided *ad libitum* access to food and water and were cared for according to local standards. Postoperatively, welfare assessment was performed twice daily using a standardized method and animals were given pain medication in case of discomfort. The experimental protocol complied with the Dutch Animal Experimental Act was approved by the Animal Experimental Committee of Maastricht University Medical Center. For the surgical procedure, all rats received 0.05 mg/kg buprenorphine as analgesic and were anesthetized using isoflurane. To acquire access to the abdominal cavity, a 5 cm craniocaudal midline incision of the skin and abdominal musculature was made. The cecum was then identified and moved outside of

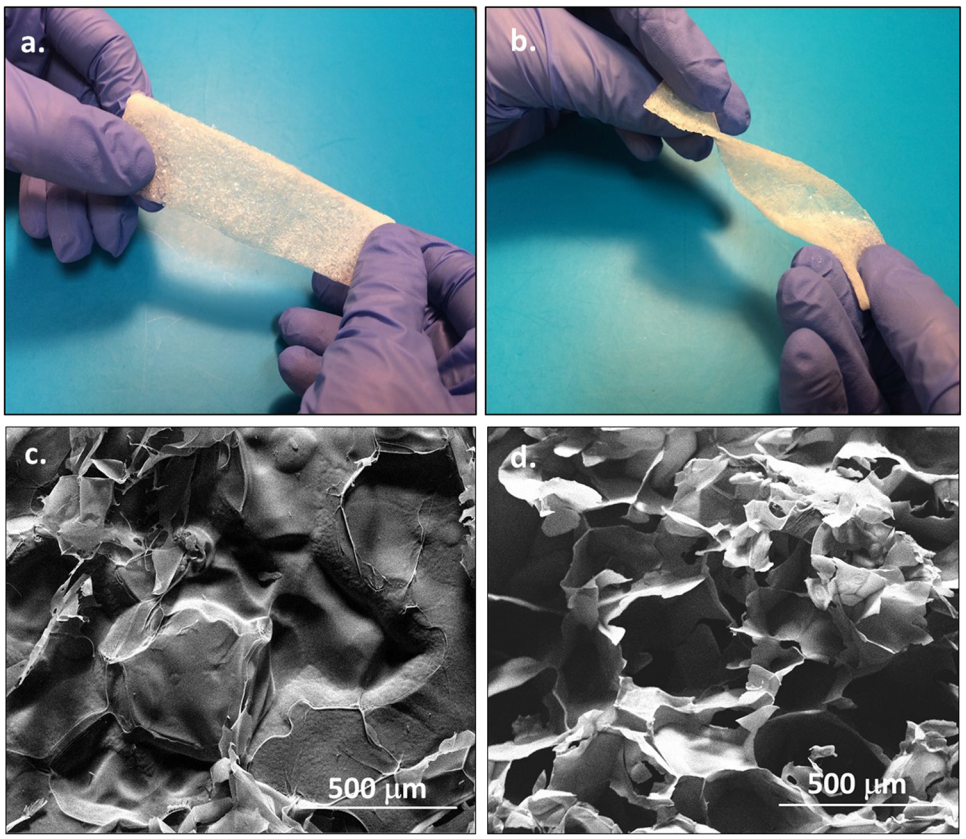

Fig. 1 ALG-HA membranes before **a** and after rehydration **b**. SEM images of membranes: top view **c** and cross-section **d**

the peritoneal cavity and onto sterile gauzes that were hydrated with sterile saline solution to prevent dehydration. For membrane positioning, the samples (ALG-HA membranes) were rehydrated with saline solution for 1 min and placed at two centimeters distal from the cecum without constructing an anastomosis. Marker sutures were applied at the edges between the membranes and tissue to identify the site at which the membranes were applied. For this study, six rats were employed with 7 days follow-up after operation.

2.10 Macroscopic tissue evaluation and histological analyses

At the animal sacrifice (7 days after surgery), rats were anesthetized and the intestinal site in contact with the membrane was dissected. Prior to fixation in formalin, the colon was cut in longitudinal direction and tissue was stretched and pinned onto a cork layer in order improve quality of histological assessment. Sections were then deparaffinized in xylene and rehydrated in graded ethanol to distilled water for histological assessment with standard hematoxylin-eosin staining. To differentiate between neutral and acid substances, tissue samples were stained with the Alcian Blue PAS staining.

2.11 Statistical analyses

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

3 Results

3.1 Membrane preparation, release and degradation studies

Polymeric membranes based on the polysaccharides alginate and HA were devised for the specific target of intestinal tissue. These membranes were prepared starting from a mixed hydrogel containing a low amount of the structural component (alginate) reticulated by Ca^{2+} ions and a high amount of the bioactive component (HA), followed by a freeze-drying step to obtain an semi-interpenetrating polymeric membrane (Fig. 1a). Given the hydrophilic nature of the polysaccharides employed, when rehydrated with aqueous media this membrane immediately becomes soft and pliable (although no plasticizers were used) as outlined in Fig. 1b. The morphological characterization of this ALG-HA membrane was carried out by SEM analyses, which pointed out a homogeneous polymeric texture with a

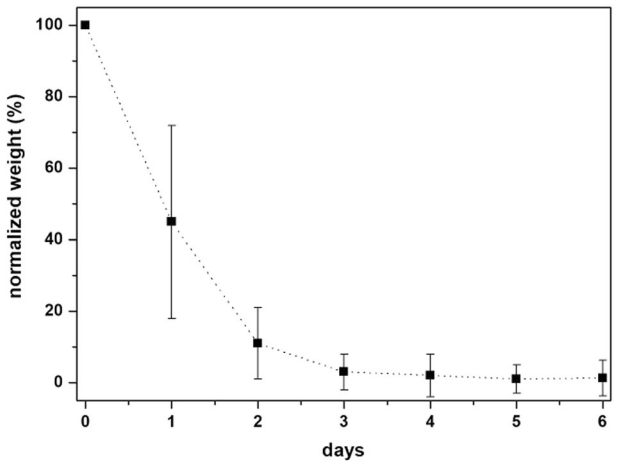

Fig. 2 Mass loss profile of ALG-HA membranes in HBSS solution

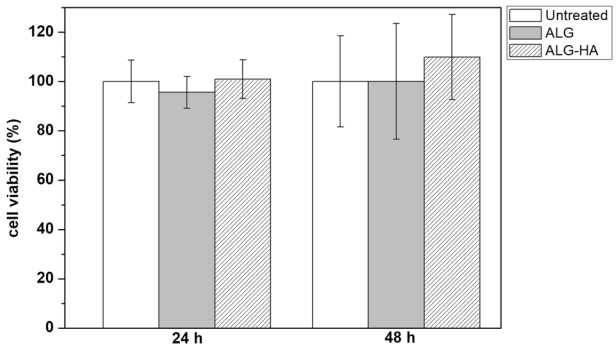

Fig. 3 Cell viability of NIH-3T3 cells treated with plain medium and with the extracts from ALG membranes and ALG-HA membranes, 24 and 48 h after incubation (white bars: untreated cells; grey bars: cells treated with liquid extract from ALG membranes; patterned bars: cells treated with the liquids extract from ALG-HA membranes)

smooth surface (top view, Fig. 1c) and a porous bulk (cross section, Fig. 1d).

The mass loss of the membrane in liquid environment was evaluated by soaking the material in HBSS and monitoring the weight loss as a function of time. Previous studies by some of the authors on the mass loss of similar alginate-HA membranes in HBSS had pointed out that HA tends to be released from the alginate matrix during the first hours of incubation in physiological medium, while at longer times mass loss is mainly associated with the physical degradation of the alginate matrix [19]. In this paper, the mass loss profile of the material is reported in Fig. 2, which points out that the largest part of the membrane components are dissolved within 2 days after incubation, while complete macroscopic dissolution occurs within 6 days.

3.2 In vitro biocompatibility

The in vitro biocompatibility of the ALG-HA membrane was investigated on fibroblast cells (NIH-3T3) by means of the MTT assay, 24 and 48 h after treatment. As controls, fibroblasts treated with the liquid extracts from alginate membranes devoid of HA (ALG) or with plain medium were used (Fig. 3).

This test pointed out that the membranes did not affect cell viability both 24 and 48 h after treatment, since no significant differences in cell viability were observed for treated cells with respect to the negative control (untreated cells).

3.3 In vitro wound healing

In order to evaluate the capability of the HA released from the ALG-HA membrane to promote the healing process, a wound healing test in vitro test was carried out on primary fibroblasts (HDFa cells). The scratches were performed on human dermal fibroblasts (HDFa) seeded on culture plates and treated with the liquid extracts from the membranes.

The results are reported in Fig. 4a, which shows the percentage of scratch closure as a function of incubation time. Representative optical images employed to quantify the gap closure are reported in Fig. 4c–g. This analysis points out that the treatment of cells with the liquid extract of ALG-HA membranes led to a complete gap closure after 32 h, while at the same time point the percentage of scratch closure of cells treated with the liquid extract from the ALG membranes (devoid of HA) was only 73%.

In order to discriminate between the contribution of cell proliferation and that of cell migration on the acceleration of gap closure, the analysis was performed also in the presence of mitomycin C, a compound that blocks cell proliferation (Fig. 4b). After treatment with mitomycin C, in the case of cells treated with ALG-HA membranes the gap closure was 80% after 72 h, while in the absence of HA (ALG membranes) the scratch closure was 40%. Overall, these in vitro studies proved that HA can be efficiently released from these membranes and stimulate a biological response.

3.4 In vivo biocompatibility of membranes

Once verified the biocompatibility of the ALG-HA membranes in vitro, the ultimate goal of this work was to evaluate in vivo the behavior of the membrane in direct contact with the intestine until material resorption, thus tentatively 1 week after implantation. For these analyses, Wistar rats were selected as animal model and the ALG-HA membranes were implanted by wrapping the material around the intact intestine (Fig. 5a). Before implantation and during the surgical procedure, ALG-HA samples were rehydrated with saline solution to obtain pliable membranes that could be

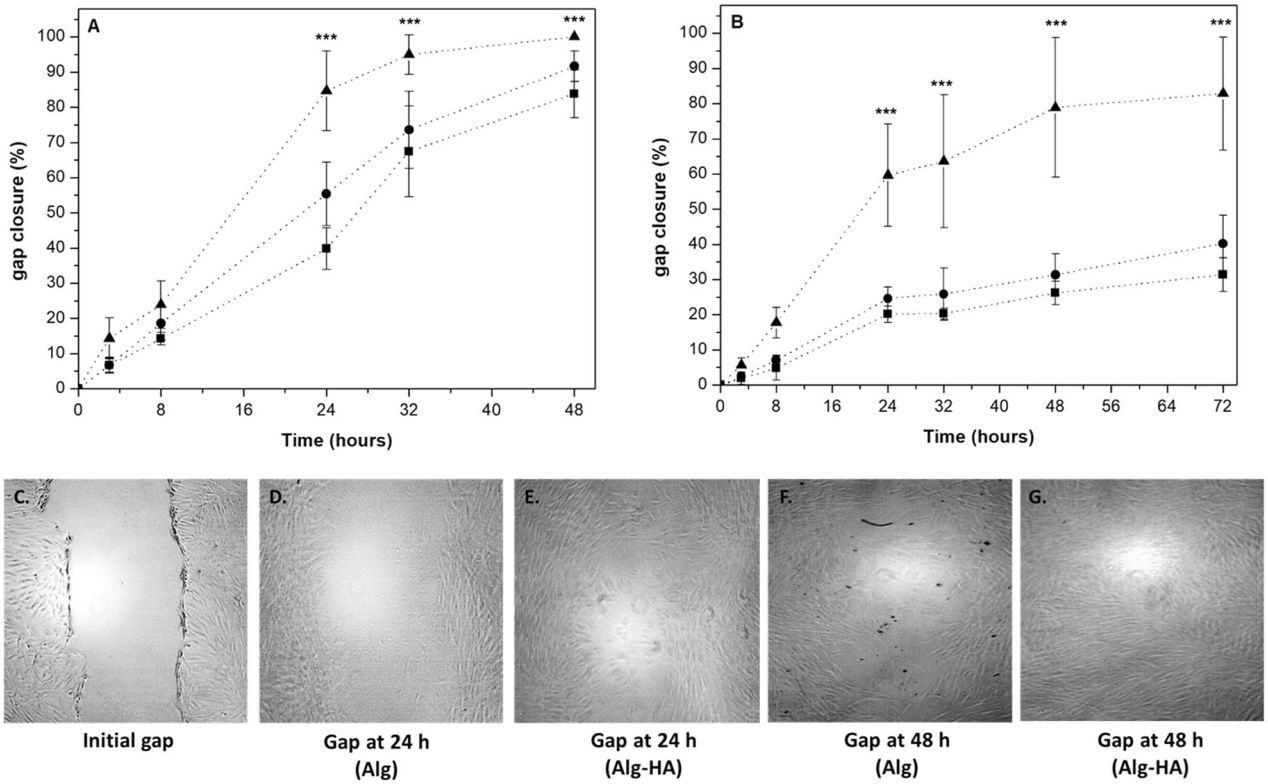

Fig. 4 Upper panel: kinetics of gap closure of HDFa cells treated with the liquid extracts of ALG membranes (rounds), ALG-HA membranes (triangles) and control cells (squares). The analysis was performed in the absence **a** or in the presence **b** of mytomicin C (***p-value < 0.001). Lower panel: optical images of the cell gap at time zero **c**, after 24 h in the presence of the liquid extracted from the ALG membranes **d** or ALG-HA membranes **e**, after 48 h in the presence of the liquid extracted from the ALG membranes **f** or ALG-HA membranes **g**

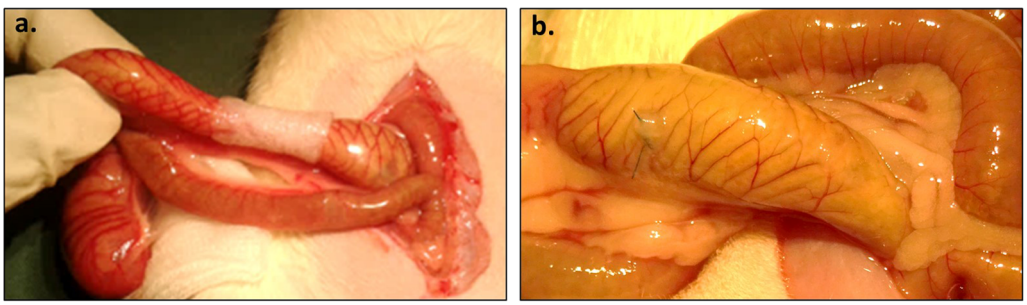

Fig. 5 Positioning of ALG-HA membrane around rat's intestine **a**. Macroscopic aspect of treated intestinal tract after 7 days incubation at animal sacrifice **b**

conveniently adapted to the shape of the bowel. After positioning the membranes around the intestine, marker sutures were applied at the edges between the membranes and tissue in order to identify the site at which the material was positioned. Based on degradation studies, it was expected that the membranes could be completely resorbed within the body during a few days of incubation. At animal sacrifice (1 week after implantation), the rat abdomen was reopened and a macroscopic qualitative evaluation of the specific intestinal site was performed (Fig. 5b). Macroscopically, no adverse tissue reactions or altered tissue morphology were observed. As expected, no membrane residues were found in correspondence of the marker suture, indicating that membrane degradation occurred within the time frame of the study.

3.5 Histological assessment

The intestinal tissues in direct contact with the implanted membranes were sectioned and stained (hematoxylin-eosin staining) for histological analyses (Fig. 6a–b, e–f). Alcian Blue PAS staining was performed to highlight the presence

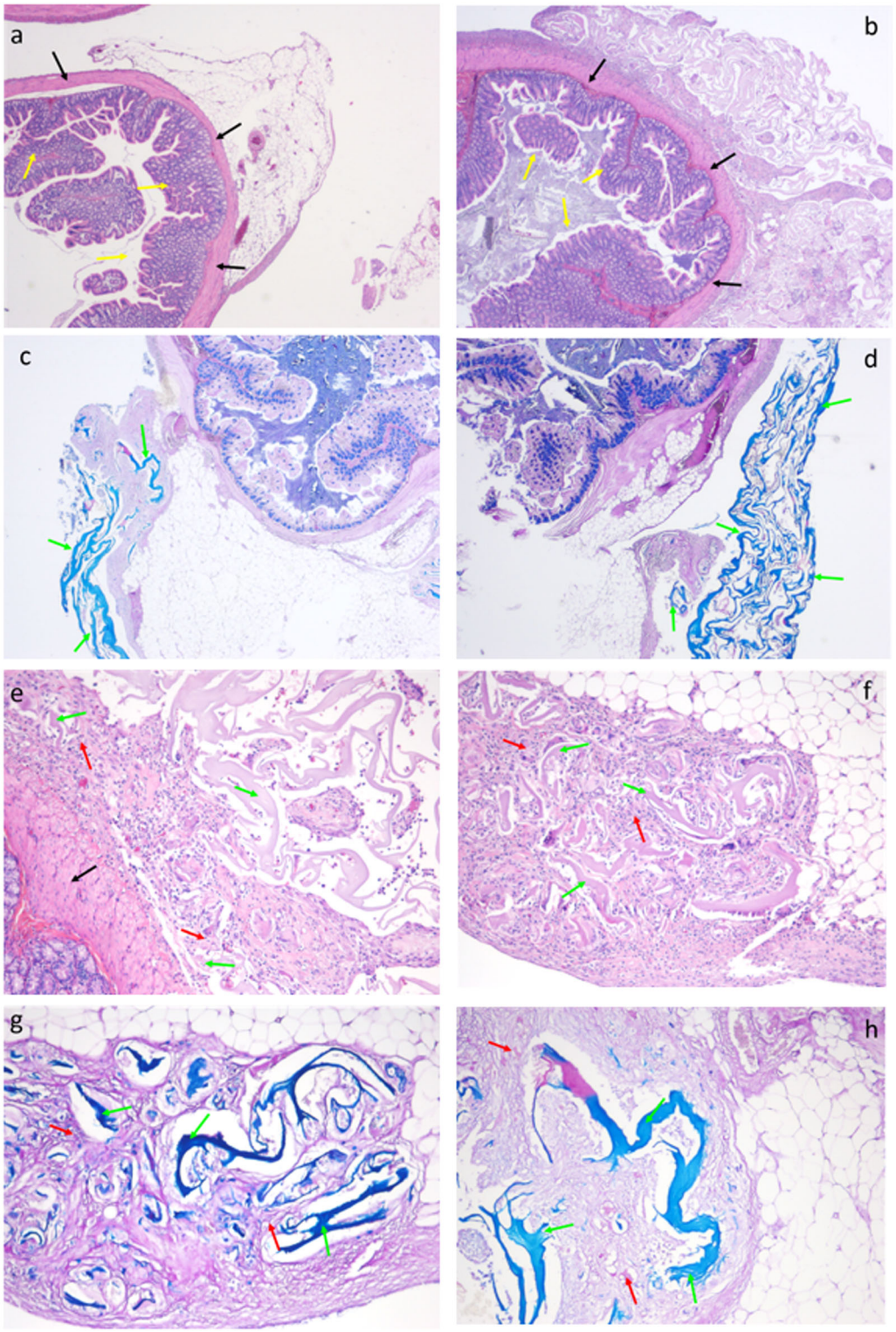

Fig. 6 Histological staining of intestinal tissue in contact with ALG-HA membrane. Hematoxylin-eosin (H.E.) staining at 2.5× (**a**, **b**) and 10× (**e**, **f**); Alcian Blue PAS (AB PAS) staining photographs at 2.5× (**c**, **d**) and 10× (**g**, **h**). Arrows were added to highlight the intestinal wall (black arrows), the mucosa (yellow arrows) and the remnants of the polysaccharide-based material (green arrows); a minor inflammatory reaction can be observed in few spots of the mesenteric/serosal region (red arrows)

of residual membrane fragments, which might not be completely resorbed (Fig. 6c–d, g–h).

The histological analyses pointed out the absence of structural damages throughout the intestinal walls. In fact, the images of Fig. 6 pointed out that the membrane left both the intestine wall and the mucosa unaltered and that the polysaccharide-based material underwent a progressive bioerosion process; a minor inflammatory reaction could be observed only in few spots of the mesenteric/serosal region. The Alcian Blue PAS staining showed the presence of microscopic membranes fragments surrounding the intestinal wall, which might have triggered a mild foreign body reaction with recruitment of macrophages and sustained by macro-histiocytes. Such mild inflammatory reaction occurred in five over six rats, while in the sixth rat no inflammation was observed.

4 Discussion

The possibility to manufacture tunable alginate-based membranes capable of ensuring an efficient release of hyaluronic acid (HA) was recently demonstrated by some of the authors [19]. Starting from these encouraging results, in this work novel polysaccharide-based membranes were devised for the specific target of intestinal tissue. In fact, considering the sensitivity of intestine to the contact with exogenous constructs, the rationale was to create a soft mesh that could rapidly resorb after the release of HA (which was previously shown to leak out of alginate matrix within few hours in physiological solution) [19]. Due to the absence of plasticizers, a rehydration step was required to obtain a pliable material that could be wrapped around intestine without breaking. The evaluation of mass loss of membranes shows that the membrane underwent dissolution within 1 week. This process can be ascribed to two phenomena: the leaking of HA out of the membrane (induced by both polymer demixing and membrane degradation) [19] and the dissolution of the Ca^{2+}-reticulated alginate matrix that in physiological solution occurs due to the competition of dissolved ions (e.g. sodium, potassium). The mass loss profile shown in Fig. 2 appears compatible with an in vivo use since the rapid dissolution minimizes the contact between biomaterial and intestine, thus limiting the risk of inflammation. After proving the biocompatibility of such biomaterial, the rationale behind the development of the ALG-HA membranes was to devise a resorbable matrix for the in situ release of HA on the intestinal tissue, which could be beneficial in the presence of bowel wounds/damages. In fact, HA is known to be effective in terms of supporting the growth of intestinal cells [5] and stimulating the wound healing process on both in vitro [22, 23] and in vivo models [24, 25]. This test was based on the simulation of a wound (scratch) on a layer of cultured (confluent) cells, followed by the monitoring of the cellular response over time; this approach was employed because it mimics what happens in the human body when wound closure occurs due to a combination of cell proliferation and cell migration phenomena. The results reported in Fig. 4a, b show that the profiles of scratch closure are similar both in the presence and in the absence of mytomicin C, indicating that the contribution of HA occurs mainly through the stimulation of cell migration, although a minor contribution of HA to cell proliferation cannot be ruled out. Once verified in vitro the proof of concept of this study (i.e. the stimulation of wound closure by a bioactive polysaccharide released from a biodegradable matrix), in vivo tests on rats were performed to evaluate the material biocompatibility 1 week after being implanted. Indeed, adverse events such as extensive inflammation, stenosis of the intestine, encapsulation of the membrane or bowel damages causing serosal injuries may not be ruled out without a specific study in an in vivo model. When the hydrated membranes were placed in direct contact with the serosa, the material displayed a considerable adhesiveness to the intestinal tissue, which could be ascribed to the hydrophilic nature of the polysaccharides employed. At the animal sacrifice, the membrane was resorbed and no adverse tissue reactions nor altered tissue morphology were observed. The histological assessment pointed out that a mild inflammatory response occurred in five over six rats. The inflammation involved the mesenteric intestinal region with the recruitment of plasma-cells, lymphocytes and granulocytes, while at the serosal side no signs of inflammation were noticed. Moreover, where present, this biological response remained localized at the mesenteric or serosal region, without damaging the intestinal wall, thus suggesting an acceptable material biocompatibility with this specific target tissue in the selected animal model.

5 Conclusions

The possibility to provide an in situ administration of HA from a resorbable device represents a promising solution for several medical needs, in particular for the treatment of wounded internal organs. This study described the development of a resorbable implantable membrane for the local release of HA to the intestinal tissue. The proposed approach enabled to obtain a rapidly hydratable and pliable membrane that could dissolve within one week in physiological solution. In vitro biological tests showed the biocompatibility of the membrane on fibroblast cells, while in vitro wound healing assays demonstrated the capability of the HA released from the membrane to support the physiological healing process. Finally, a preliminary in vivo

biocompatibility study on rats showed that the membranes wrapped around the intestine elicited only a mild inflammatory reaction at the mesenteric region, without affecting the structural integrity of the intestinal wall. A mild inflammatory response is necessary in normal intestinal wound healing as it is considered the first stage of the healing process [26]. These results support the use of the alginate-hyaluronan bioactive membranes for wound healing applications and suggest further in vivo investigations on the biological behavior of such materials on wounded intestinal tissues.

Acknowledgements The authors would like to acknowledge Dr. Gianluca Turco for providing assistance in SEM analyses. This study was supported by the EU-FP7 Project "AnastomoSEAL" (Contract Number 280929).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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