Hyaluronic acid/lactose-modified chitosan electrospun wound dressings – Crosslinking and stability criticalities

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ABSTRACT

Polysaccharide electrospun wound dressings should be an effective strategy in the field of wound care, as they combine an extracellular matrix-like structure with excellent biomimicry. However, their high hydrophilicity and large surface area cause a rapid dissolution in aqueous environments, compromising their clinical employment. In the present paper, electrospun membranes prepared using hyaluronic acid, a bioactive lactose-modified chitosan (CTL), and polyethylene oxide have been crosslinked using glutaraldehyde, genipin, EDC/NHS or thermal treatments, obtaining very poor results in terms of membrane stability. Therefore, carbonyldiimidazole (CDI) and methacrylic anhydride were investigated in an innovative way, where CDI proved to be the best compromise between nanofiber water resistance, architecture maintenance and degradability. Indeed, the swelling and degradation behavior as well as the water vapor permeability of these matrices were tested, revealing the effectiveness of the electrospun products in absorbing large amount of liquid while maintaining the balance between water retention and gas permeability.

1. Introduction

Non-healing or chronic wounds are characterized by a dysregulated healing path, where the normal timeline of coagulation and hemostasis, inflammation, proliferation, and remodeling stalls in the inflammation phase, causing fibrosis, tissue loss, and insurgence of infections (Andrabi et al., 2021; Hauck et al., 2021; Yang, Zhao, et al., 2021; Zhang et al., 2021). Surgical debridement and negative pressure are common strategies for the cleaning and preparation of the wound bed, but a wound dressing (passive or active) must be then applied to protect the wound site (Khandelwal et al., 2021). There is no single definition of the characteristics that an ideal wound dressing should have, but some key factors clearly influence the goodness of the medical device; these include, for example, biocompatibility, antimicrobial and anti-scarring potential, water adsorption capacity along with gas permeability, adaptability to the wound shape, mimicking of extracellular matrix (ECM) structure and mechanical properties, and cost-effectiveness (Fu et al., 2021; Kraskouski et al., 2021; Liu et al., 2011). In this context, electrospinning has received much attention as a simple and effective technique to produce biomimetic nanofibrous wound dressings, with a large surface area and a highly interconnected porous structure, that mimics ECM architecture and favors gaseous exchanges, drainage of excess fluid, and hemostasis Li, Wang, et al., 2021; Tonda-Turo et al., 2018). This moisturizing ability is then responsible for the anti-scarring potential of electrospun mats, as they accelerate wound repair and closure avoiding scar insurgence (Ekambaram & Dharmalingam, 2020).

The use of FDA-approved synthetic polymers as bulk materials for electrospun wound dressings has been largely exploited in recent years due to their good biocompatibility and mechanical strength as well as their good degradation profile or thermal stability. However, they are usually hydrophobic and lack intrinsic bioactive properties and biological cues directly recognized by cells (Kai et al., 2014; Wang, Song, et al., 2021; Zhou et al., 2021). Attention was then drawn to natural polymers, namely proteins (such as collagen, fibrinogen, fibroin) and

Abbreviations: CDI, carbonyldiimidazole; CTL, lactose-modified chitosan; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; ECM, extracellular matrix; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; HA, hyaluronic acid; NHS, N-hydroxysuccinimide; PEO, polyethylene oxide; PCL, poly(−ε-caprolactone); WVTR, water vapor transmission rate.

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polysaccharides (such as alginate, chitosan, hyaluronic acid, cellulose, dextran, to name a few). In addition to their excellent biocompatibility and ability to mimic ECM composition, which is essentially fibrous structural proteins (mainly collagen and elastin) and polysaccharides (such as hyaluronan and dermatan sulfate), natural polymers are hydrophilic and provide an optimal regenerative substrate due to their recognition signals for cells, even if they do not confer proper mechanical strength to the final product (Dodero, Schlatter, et al., 2021; Izadyari Aghmiuni et al., 2021; Memic et al., 2019). Among them, hyaluronic acid and chitosan are widely employed in the synthesis of wound dressing devices (Suo et al., 2021; Wu & Li, 2021; Xia et al., 2020; Yang, Xie, et al., 2021; Zhong et al., 2020). Hyaluronic acid is a high molecular weight non-sulfated glycosaminoglycan given by a linear repetition of (β 1 → 4)-glucuronic acid and (β 1 → 3)-*N*-acetyl-D-glucosamine. The ECM of skin contains up to 50% of the total amount of hyaluronic acid in the body, where it acts as lubricant preventing skin dehydration. Furthermore, hyaluronic acid can be recognized by cells through CD44 receptor, promoting cell adhesion, proliferation, and differentiation (Alven & Aderibigbe, 2021; Makvandi et al., 2019; Mauro et al., 2021; Tokudome et al., 2018). All these properties, together with its antioxidant and anti-inflammatory activity, make it an ideal candidate for the preparation of wound dressings (Chen et al., 2018; Corrêa et al., 2020). Chitosan, on the other hand, is a high molecular weight polysaccharide that is widely used as a biocompatible, biodegradable, hemostatic, and antimicrobial polymer. It is obtained from the deacetylation of chitin and is structured in a linear repeat of (β 1 → 4)-linked glucosamine and *N*-acetylglucosamine residues (Chen, Lin, et al., 2021). Thanks to the presence of numerous available amino groups, highly deacetylated chitosan can be exploited for the insertion of specific ligands, such as oligosaccharides (Donati, Haung, et al., 2007). Among them, CTL (1-deoxylactic-1-γ-L-chitosan also known as Chitlac) is a hydrophilic lactose-modified chitosan obtained by reductive amination with the lactose aldehydic group. This gives CTL several chemical-physical advantages over chitosan, including higher solubility at pH values closer to neutrality (Cok et al., 2018; Donati, Borgogna, et al., 2007), allowing the employment of non-toxic solvents, as water, ensuring the biocompatibility of the final product. CTL even possesses bioactive properties; for example, it induces the aggregation of articular chondrocytes by stimulating the production of glycosaminoglycans and type-II collagen and interacting with Galectin-1 (Marcon et al., 2005) or it promotes the differentiation of multipotent stem cells (namely, human dental pulp stem cells) into an osteoblast phenotype (Porrelli, Gruppuso, et al., 2021).

Despite all the advantages, electrospinning of natural polymers has some difficulties, due to the high viscosity, surface tension, and conductivity of the solutions, so that the addition of synthetic polymers and/or surfactants to the polysaccharide solutions is necessary (Bazmandeh et al., 2020; Rošic et al., 2012). This includes polyethylene oxide (PEO), a hydrophilic, water-soluble, inert synthetic polymer often used to increase polymer chain entanglement in the solution and refine polysaccharides electrospinnability (Darbasizadeh et al., 2019; Zhao et al., 2016). On the other hand, the addition of surfactants, as Tween® 20, reduces the surface tension of the solution and improves its conductivity, resulting in thinner and bead-free fibers (Kriegel et al., 2009; Liu et al., 2011).

However, the most important and critical issue related to the electrospinning of natural polymers, and especially polysaccharides, is represented by their high solubility and thus almost immediate dissolution in aqueous environment, which requires an additional and properly selected crosslinking step (Baker et al., 2016; Campiglio et al., 2019; Zheng, Yang, et al., 2021). Over the years, numerous crosslinking strategies have been employed, ranging from physical to chemical to enzymatic methods (Gruppuso et al., 2021). Physical methods include irradiation (γ-irradiation, UV irradiation, high-energy electron beam irradiation) or heat treatment. Chemical crosslinking, on the other hand, involves the formation of covalent bonds between the functional units of the polymer chains. This is the case of glutaraldehyde, genipin, or EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide), to name the most commonly used methods. The enzymatic approach exploits enzymes (as transglutaminase or oxidoreductases) to catalyze specific chemical reactions (Dodero, Scarfi, et al., 2021; Grabska-Zielińska et al., 2021; Koosha et al., 2019).

The aim of this work is to present a novel electrospun wound dressing based on hyaluronic acid and CTL, paying special attention to all critical aspects related to the chemical and structural stabilization of the final matrix. Despite the huge variety of chitosan-based wound dressing materials, to the best of the author's knowledge, this is the first time that CTL-based electrospun wound dressings are produced, with numerous possible advantages, firstly related to the use of water as solvent and to the multiplicity of bioactive properties exhibited by CTL. Moreover, various crosslinking methods are here reported, by comparing strategies documented in literature with innovative ones. The results show that all the exploited traditional approaches do not stabilize the mats or maintain their fibrous structure, mechanical strength, and integrity. Hyaluronic acid/CTL mats were also compared with electrospun matrices based on poly(−ε-caprolactone) (PCL), non-electrospun polysaccharide membranes, and the commercial product Chitoderm® to highlight the advantages of using electrospun matrices compared to non electrospun ones, where the use of bioactive polysaccharides enables to switch from biologically inert to bioactive medical devices. The hypotheses on which this work is based are: i) that it will be possible to electrospun water-soluble CTL, ii) that it will be possible, given the chemical structure of hyaluronic acid and CTL, to stabilize the electrospun membranes produced with these polymers availing of novel crosslinking methods, and iii) that the polysaccharide-based electrospun membranes here produced will present swelling and degradation behaviors, together with vapor permeability, exploitable for wound dressing applications.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA) (M_W = 40–50 kDa; Batch N# 2018082984) and CTL hydrochloride (lactose-modified chitosan; Batch N# 350118) were provided by Sigea S.R.L. (Trieste, Italy) and biopoLife S.R.L. (Trieste, Italy), respectively. CTL final composition, determined through ^{1}H NMR, was as follow: glucosamine residue 27%, *N*-acetylglucosamine 18%, and 2-(lactit-1-yl)-glucosamine 55%; the calculated relative M_W of CTL is around 1.5 × 10^3 kDa, as determined by viscometry (Porrelli, Gruppuso, et al., 2021). Polyethylene oxide (PEO) (M_W = 900 kDa), poly (ε-caprolactone) (PCL) (M_W = 80 kDa), Tween® 20, dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), sodium hydroxide, methanol, acetone, 1,1′-Carbonyldiimidazole (CDI), methacrylic anhydride, glutaraldehyde (25 wt% in water), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), and sodium chloride were purchased from Sigma-Aldrich (Chemical Co. USA). Genipin (purity 98%) was acquired from Challenge Bioproducts Co., Ltd. (Yun-Lin Hsien, Taiwan). Fortuna Optima glass syringes (an inner diameter of 9 mm) were purchased from Sigma-Aldrich (USA). The D-ES30PN-20 W potential generator was purchased from Gamma High Voltage Research Inc. The syringe pump, model KDS-100-CE, was acquired from KD Scientific (Holliston, MA, USA) (Ormond Beach, FL, USA).

2.2. Membrane preparation

2.2.1. HA/CTL/PEO based membranes

The polysaccharide solution was prepared by separately dissolving hyaluronic acid (HA), lactose-modified chitosan (CTL), and polyethylene oxide (PEO) in deionized water. After their complete dissolution overnight (o.n.), the pH of HA and CTL was adjusted to 7.5, with the aim to avoid the formation of complexes between the positively charged CTL and the negatively charged HA. CTL was then added to PEO solution

and stirred for 3 h, after which also HA was included to obtain a ternary mixture with final HA/CTL/PEO concentrations of 2% (w/v), 1% (w/v), 2% (w/v), respectively. After 30 min of equilibration 1% (w/v) Tween® 20 was added as surfactant and the mixture was stirred overnight at room temperature before electrospinning.

The nanofibrous mats were obtained with a custom-made horizontal electrospinning device after 90 min of electrospinning, performed with the following parameters: voltage, 30 kV; distance between the tip of the needle and the collector, 20 cm; flow rate, 1.2 mL/h; needle gauge, 23 G. The negative pole of the high voltage power supply was set over the collector.

The same solution used in the electrospinning procedure was cured in 6 cm (ø) Petri dishes and freeze-dried for 1 day (ALPHA 1–2 LD plus freeze-dryer, CHRIST, Osterode am Harz, Germany) to obtain non-fibrous membranes to be used for comparison.

2.2.2. PCL based membranes

PCL solution was prepared according to what reported by Porrelli, Mardirossian, et al., 2021. Briefly, 12% (w/v) PCL was dissolved in a DCM: DMF (7:3) mixture, by first dissolving PCL in DCM overnight, followed by the addition of DMF the day after.

The membranes were obtained after 1 h of electrospinning using the following parameters: voltage, 17 kV; distance between the tip of the needle and the collector, 25 cm; flow rate, 0.6 mL/h; needle gauge, 27 G. The negative pole of the high voltage power supply was set over the collector.

Some of the PCL mats were activated with air-plasma treatment, converting their basically hydrophobic behavior into a hydrophilic one. The process was carried out with a PDC 32-G Plasma Cleaner (Harrick Plasma, Ithaca, USA) used in low power mode (6.8 W) for 5 min, with a pressure of 0.1 mTorr.

Activated PCL mats were even used to produce polysaccharide-coated PCL membranes. In this case, both HA and CTL were solubilized in deionized water at the final concentration of 0.2% (*w/V*) and their pH was adjusted to 7.2–7.4. CTL was cured on PCL samples until complete adsorption and air-dried. CTL-coated membranes were then washed in deionized water and air-dried for a second time. Subsequently, HA was cured on the top of PCL-CTL samples, which were washed in deionized water and air-dried again.

2.3. Crosslinking strategies

Due to the high instability and complete dissolution of polysaccharide-based membranes in aqueous environments, HA/CTL/PEO mats have been subjected to various crosslinking treatments in the attempt to stabilize them in water and enable their application for biomedical purposes.

2.3.1. Carbonyldiimidazole (CDI) crosslinking

Fifty equivalents of CDI per mol of CTL glucosamine residues were used. CDI powder was dissolved in DMF, and the polysaccharide mats were incubated at different time points (1 h, 2 h, 3 h, 4 h, 5 h, 7 h, 8 h, overnight) to find the optimal reaction conditions and the best compromise between mat stability and fiber loss. At the end of each selected timepoint, the membranes were washed in ethanol and air-dried.

2.3.2. Methacrylic anhydride crosslinking

Pure methacrylic anhydride was cured on HA/CTL/PEO samples until complete adsorption and air-drying. Membranes were then washed with various solvents, namely deionized water, sodium hydroxide, methanol, acetone, and dimethylformamide, to determine the best washing method.

2.3.3. Glutaraldehyde vapor crosslinking

Three Petri dishes (⌀ = 6 mm) filled with 25% glutaraldehyde in water were placed on the bottom of a vacuum chamber. Membrane samples were stabilized on the grid of the sealed chamber and crosslinked under vacuum conditions for 4 h or 2 h. In the last case, the electrospun mats were heat treated for 24 h at 60 °C to stabilize the crosslinking between nanofibers.

2.3.4. EDC/NHS crosslinking

EDC and NHS were added to the polymer solution 30 min before electrospinning at a final concentration of 2% (w/v) and 1% (w/v), respectively. In one case, the powder was added directly to the final electrospinning solution; alternatively, EDC/NHS were first dissolved in deionized water and then added to the polymer solution.

2.3.5. Genipin crosslinking

Genipin crosslinking was performed according to two different procedures. In one case, genipin was directly added to the polymeric solution at a final concentration of 0.2% or 0.05% (w/v) 5 min before electrospinning. After preparation, the electrospun matrices were placed at 37 °C for 24 h to 7 days with the aim to promote genipin reaction. Otherwise, electrospun polysaccharide mats were further treated with 0.5% (w/v) genipin dissolved in ethanol for 15 min, 30 min, and 45 min, and then they were heated at 37 °C for 24 h to activate genipin reaction.

2.3.6. Heat treatment

Heat treatment was performed alone, by placing the electrospun samples in a convection oven at 80 °C for 4 h, or in conjunction with CDI crosslinking, by first crosslinking the polysaccharide matrices with 50 equivalents of CDI in DMF and then heating them for 4 h at 80 °C. Another type of thermal treatment was performed by using a vacuum oven, in which the samples were heated for 1 h (after equilibrating from 20 °C to 80 °C) or 1 h, 2 h, 3 h at 80 °C.

2.4. Scanning electron microscope (SEM) analysis

In all cases, dried membrane samples were sputter-coated with gold through a Sputter Coater K550X.

(Emitech, Quorum Technologies Ltd., UK) and placed on aluminum stubs covered with a double-sided carbon tape. The morphological analysis was then performed with a scanning electron microscope (Quanta 250 SEM, FEI, Oregon, USA) working in secondary electron detection mode. The working distance was set at 10 mm to obtain the appropriate magnifications, and the acceleration voltage was set between 20 and 30 kV. Fiber diameters were calculated by 100 measurements per sample using Fiji software.

2.5. Attenuated total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy

ATR-FTIR was performed to assess the occurred crosslinking in the presence of overnight CDI-treated samples. IR spectra were recorded in transmittance mode with a Nicolet iS50 FT-IR spectrometer (Thermo Scientific, MI, Italy), within a wavenumber range of 4000–400 cm^{-1}. HA/CTL/PEO membranes before and after CDI crosslinking were analyzed as well as HA, CTL, PEO, and CDI pure spectra as comparison. All the spectra were acquired with 32 scans and a resolution of 4 cm^{-1}.

2.6. Swelling tests

The swelling behavior was quantified after rehydration of the samples in deionized water or saline solution (NaCl 150 mM) by measuring the weight changes as a function of immersion time. Seven types of samples were compared here: CDI-crosslinked and methacrylic anhydride-crosslinked electrospun polysaccharide mats, CDI-crosslinked freeze-dried polysaccharide mats, PCL electrospun matrices (non-activated, activated, or coated with CTL/HA), and the commercial product Chitoderm® (Pietrasanta Pharma S.p.A.).

Once measured dry weights, wet weights were determined at each timepoint (15′, 30′, 45′, 1 h, 2 h, 3 h, 5 h, 7 h, 24 h) by gentle blotting with a filter paper to remove exceeding surface liquid. The swelling ratio was calculated according to Eq. (1), as proposed by Porrelli, Gruppuso, et al. (2021):

$$\text{Swelling}\ (\%) = \left(\frac{(\text{Ws} - \text{Wd})}{\text{Wd}}\right) \times 100 \quad (1)$$

where W_d and W_s are the weights of the samples in the dry and the swollen state, respectively. The results were taken as the mean values of four samples for each condition.

2.7. Degradation tests

The rate of degradation of the same samples investigated for their swelling behavior (Section 2.6) was assessed both in water and saline solution (NaCl 150 mM). Their stability was evaluated after 1, 3, 5, 7 days of immersion at 37 °C. The wet weight was measured after 10 min equilibration and related to weight variations in time, which were calculated using Eq. (2), adapting the protocol proposed by Turco et al. (2009):

$$\text{Weight variation}\ (\%) = \left[1 - \left(\frac{\text{Wtn}}{\text{W10}min}\right)\right] \quad (2)$$

where W_{tn} and W_{10min} are the wet weights of the samples at the defined time and after 10 min of rehydration, respectively. Four replicates were performed for each condition.

2.8. Water vapor transmission rate (WVTR)

The ability of membranes to transmit water vapor was assessed for CDI-crosslinked electrospun mats, PCL electrospun mats (non-activated, activated, and coated with CTL/HA), and the commercial product Chitoderm® (Pietrasanta Pharma S.p.A.).

Glass vials with a top closure of 13 mm of diameter were filled with deionized water until a 2 cm gap remained between the water and the sample, which was placed on the top of the vial and sealed on the side with Parafilm®. The vials were then weighted and incubated at 37 °C for 24 h and 48 h, by measuring water loss at each timepoint. Uncapped vials and vials capped with Parafilm® were used as free evaporation and no evaporation controls, respectively. Water vapor transmission rate was calculated using Eq. (3), as proposed by Tarusha et al. (2018):

$$\text{WVTR}\left(\frac{\text{g}}{\text{m}^2\text{h}}\right) = \left(\frac{(\text{Wtx} - \text{Wt0})}{\text{A} \times \text{h}}\right) \quad (3)$$

where, W_{tx} is the weight after 24 h or 48 h, W_{t0} is the initial weight of the vial, and A is the area of the top closure of the vial. Three replicates were analyzed per each sample.

2.9. Statistical analyses

Statistical analyses were performed with GraphPad software (Graphpad Holdings, LLC). Data not satisfying normality (Shapiro-Wilk test) assumptions were analyzed by means of Kruskal-Wallis and Mann–Whitney non-parametric tests, applying Bonferroni's correction. Data that satisfied the normality assumption were analyzed with one-way ANOVA test, applying Bonferroni's correction. Statistical significance was pre-set at $\alpha = 0.05$.

3. Results

3.1. Electrospun membranes and fiber morphology

Polysaccharide membranes based on hyaluronic acid (HA), lactose-modified chitosan (CTL), and polyethylene oxide (PEO) were prepared by dissolving the three polymers separately in deionized water and then adding CTL and HA to PEO solution, after 3 h of equilibration between the two. Tween® 20 was added as a surfactant to reduce surface tension of the solution and improve its electrospinnability (Fig. 1). The membranes obtained after 90 min of electrospinning (Fig. 2A-B) are highly reproducible and show an optimal morphology, with thin, uniform, and bead-free fibers. Fiber diameter (Fig. 2C), calculated from 100 randomly selected fibers on the sample, shows a normal distribution, with an average diameter of 442 ± 117 nm.

To analyze the differences between electrospun matrices of various composition, PCL membranes were produced as a comparison. Three types of PCL mats obtained with the same electrospinning process, were considered: (i) untreated, non-activated PCL, (ii) air-plasma activated PCL, and (iii) PCL coated with CTL/HA. In all cases, a uniform distribution of fibers was observed, with the activation process causing no significant changes in fiber morphology. On the other hand, the presence of the coating slightly alters the overall morphology and leads to a slight increase in fibers diameters due to rehydration, resulting in a statistically significant difference compared with pristine or activate PCL (Fig. 3).

3.2. Non-electrospun membranes

Freeze-dried mats were prepared with the same solution used for the synthesis of polysaccharide-based electrospun mats, to highlight the advantages underlying the employment of a nanofibrous structure over a non-fibrous one. As confirmed by SEM imaging (Fig. SI-1), the freeze-drying process yields membranes characterized by a thin amorphous layer of the polymer blend. As further non-electrospun control, a commercial product, namely Chitoderm®, was chosen for its composition and structure. It consists of an outer polyurethan layer which serves as protection against the external environment, and a lower chitosan pad with a macro-fibrous structure.

3.3. Crosslinking strategies for electrospun membranes

The high degree of hydrophilicity of the polysaccharides used, combined with the high surface-to-volume ratio characteristic of nanofiber structures, imparts considerable structural instability to electrospun membranes in aqueous environments, as might be expected. Indeed, the nanofibrous mesh collapses in seconds when exposed to water, causing the immediate dissolution of the biomaterial.

Therefore, different crosslinking strategies were tried to impart water resistance to membranes while maintaining the fiber structure (Fig. 4), with different results depending on the strategy chosen (Table 1).

3.3.1. CDI

With the aim of finding the optimal reaction time in addition to the best compromise between aqueous stability and fiber loss, crosslinking was carried out with 50 equivalents of CDI per mol of CTL glucosamine residues in DMF at different time points, namely 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, 8 h, overnight, with the proposed mechanism illustrated in Fig. SI-2. Comparing the morphology of the crosslinked mats shows that the overall nanofibrous architecture was slightly affected after the crosslinking step, with a kind of partial fiber fusion. The best results were obtained after overnight incubation, since the slight morphological alteration is balanced by the optimal water resistance. Indeed, considering the first and the last crosslinking time points (Fig. 5), the overnight crosslinked fibers are stable in water while retaining almost the same morphology observed after the crosslinking step. All the time-points tested are reported in Fig. SI-3a-i.

3.3.2. Methacrylic anhydride

Membranes crosslinked with pure methacrylic anhydride were

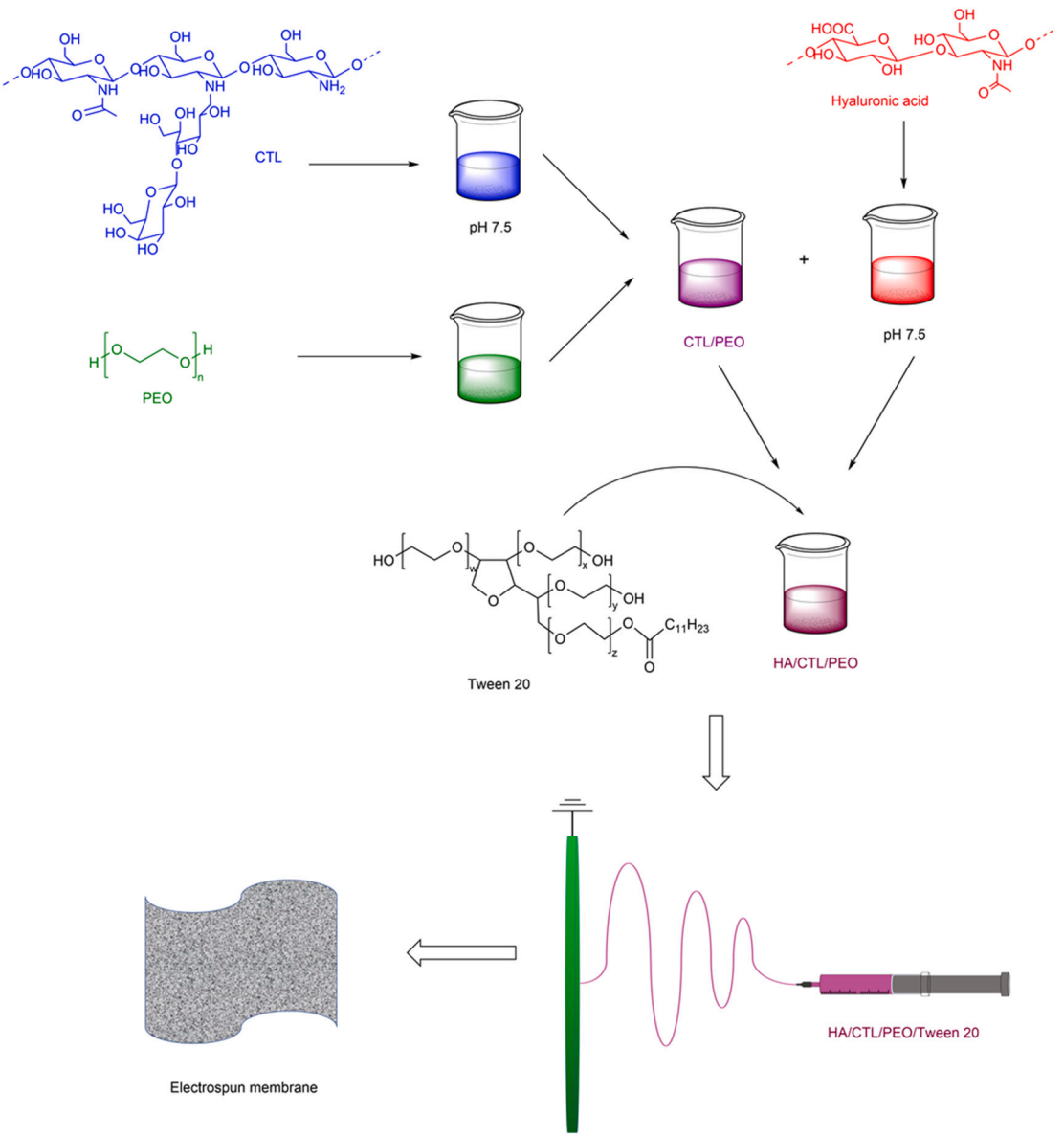

Fig. 1. Schematic representation of HA/CTL/PEO membranes preparation.

subjected to an additional washing step to remove the excess methacrylic anhydride while retaining the nanofibrous architecture. Of the different methods tested (Fig. SI-4), only washings in acetone and dimethylformamide gave satisfactory results in terms of structural integrity, but stability in water was not as high as for CDI-crosslinked mats.

3.3.3. Glutaraldehyde

Glutaraldehyde vapor was used to crosslink polysaccharide-based nanofibers using two different procedures: i) 4 h of crosslinking in a vacuum chamber and ii) 2 h of crosslinking in a vacuum chamber combined with heat treatment (Fig. SI-5). In the first case, a partial loss of the fibrous structure was observed, even though the overall architecture was still recognizable; in the second case, the nanofibrous architecture was completely lost (Fig. SI-6). Nevertheless, aqueous stability was not achieved in either case.

3.3.4. EDC/NHS

In the case of EDC/NHS, the crosslinkers were added to the polymeric solution 30 min before electrospinning as powders or after their solubilization in water (Fig. SI-7). In both cases, an almost immediate gelation occurred hindering the electrospinning process (Fig. SI-8).

3.3.5. Genipin

Genipin was electrospun in the polysaccharide solution or the membranes were post-processed with genipin dissolved in ethanol at different timepoints (15, 30, 45 min) (Fig. SI-9). In both cases, the samples were subjected to heat treatment at 37 °C to activate the genipin and promote crosslinking between fiber meshes. Nevertheless, crosslinking did not occur in any case, not even after 7 days, with the complete membrane dissolution upon contact with water (Fig. SI-10).

3.3.6. Heat

Heat treatment was performed at 80 °C for 4 h both alone and in combination with chemical crosslinking by CDI. In both cases, the nanofibrous structure was lost and the membranes showed absolute instability in water (Fig. SI-11). With the aim of improving the thermal treatment procedure, the membranes were even crosslinked in a vacuum oven at 80 °C with different time settings, but in all cases neither the fibrous architecture was maintained nor the stability in water was

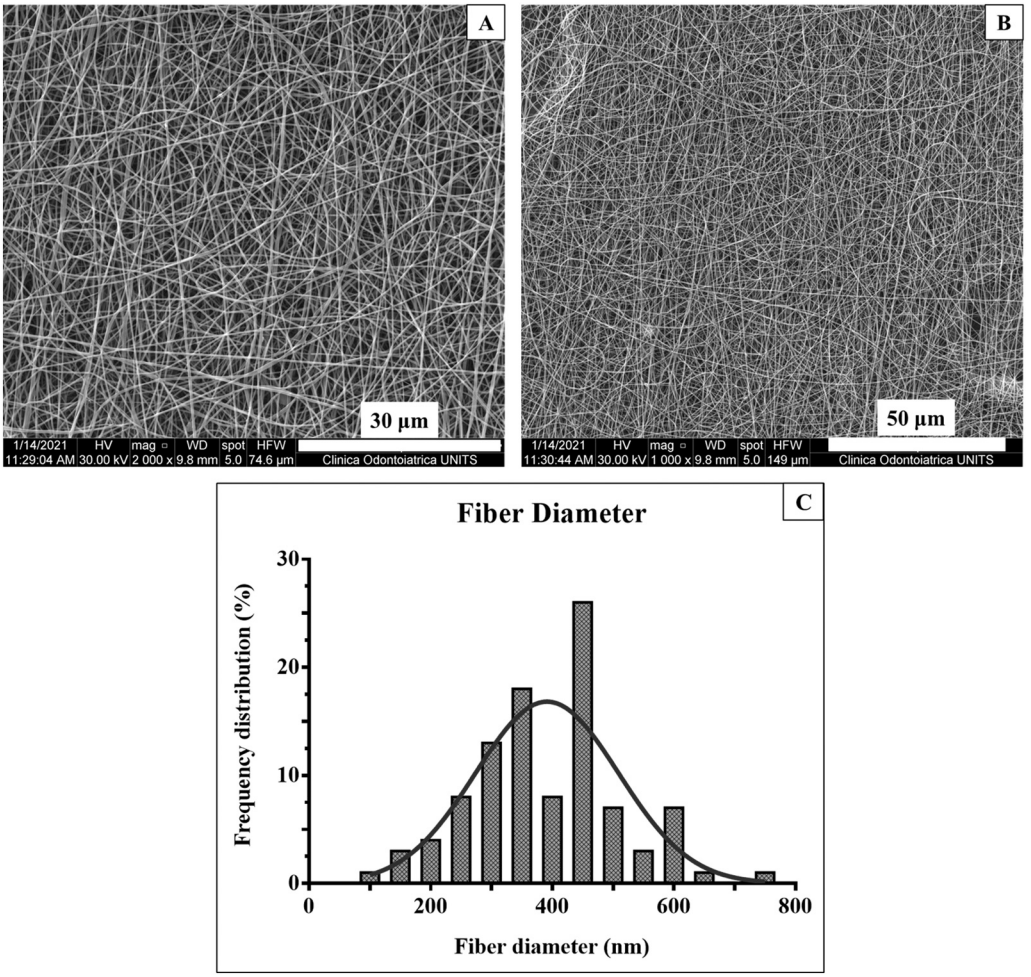

Fig. 2. Morphological characterization of HA/CTL/PEO membranes: (**A**) and (**B**) SEM micrographs at different magnifications, 2000× and 1000×, respectively; (**C**) distribution of fiber diameters throughout the membrane.

achieved (Fig. SI-12).

3.4. Membrane characterization by attenuated Total reflectance – Fourier transform infrared (ATR-FTIR) spectroscopy

Once assessed the goodness of CDI-mediated crosslinking among the other methods tested, with an adequate equilibrium between fiber loss and aqueous resistance, ATR-FTIR spectroscopy was used to analyze the occurred amide bond formation within HA/CTL/PEO nanofibrous mats after the overnight treatment with CDI, as reported in Fig. 6. The not-crosslinked and crosslinked membrane spectra were compared with the spectra of their single components (namely, hyaluronic acid, CTL, PEO) and of CDI, and their characteristics bands were distinguishable within the mat spectra. However, no CDI signals were detected in the case of CDI-crosslinked meshes, since CDI is a coupling agent which only mediates the formation of the amidic bond without being incorporated within the structure (Vaidyanathan et al., 2004). On the other hand, the signal related to the amide bond formation was superimposed on the IR signals of carbonyl (C=O) and carboxylic (-COOH) groups.

3.5. Swelling behavior

The swelling behavior of CDI- and methacrylic anhydride-crosslinked matrices was evaluated in both water and saline solution (NaCl 150 mM), chosen to partially mimic physiological conditions (Chen et al., 2020; Oliveira et al., 2014; Sharma et al., 2017). Polysaccharide membranes were compared with electrospun mats based on synthetic polymers and non-electrospun products. In the first case, non-activated, activated, or CTL/HA-coated PCL matrices were chosen. In the second case, freeze-dried polysaccharide membranes obtained from the same solution as the electrospun membranes and the commercial product Chitoderm® were selected. As can be seen in Fig. 7, the nanofibrous structure along with the high hydrophilicity of the polysaccharides in the CDI-crosslinked electrospun membranes have a strong influence on the swelling capacity of the matrix in both water and saline solution. For similar reasons, activated PCL membranes showed a remarkable increase in the swelling ratio up to 24 h compared to non-activated PCL and polysaccharide-coated mats (Fig. 7B). The lower swelling behavior in the presence of polysaccharide-coating could be explained by the rapid formation of a hydrated uniform layer which affects membrane porosity and reduces the absorption capacity of the material. CDI-crosslinked electrospun polysaccharidic membranes showed swelling behavior close to that of activated PCL ones. This indicates that these membranes retain a high swelling capacity, despite the partial loss of the fibrous structure. In contrast, the membranes cross-linked with methacrylic anhydride were not able to retain large amounts of liquid as they start dissolving within the first hour of immersion. Furthermore, despite the high stability of the commercial product Chitoderm®, its ability to retain water was about 3 times lower at 24 h than CDI-crosslinked electrospun mats (Fig. 7A). A similar trend was observed with non-electrospun membranes, thus confirming the crucial role played by the nanofibrous structure in determining the swelling capacity of the device. The high hydrophilicity of CDI-crosslinked polysaccharide membranes and of activated PCL membranes, their

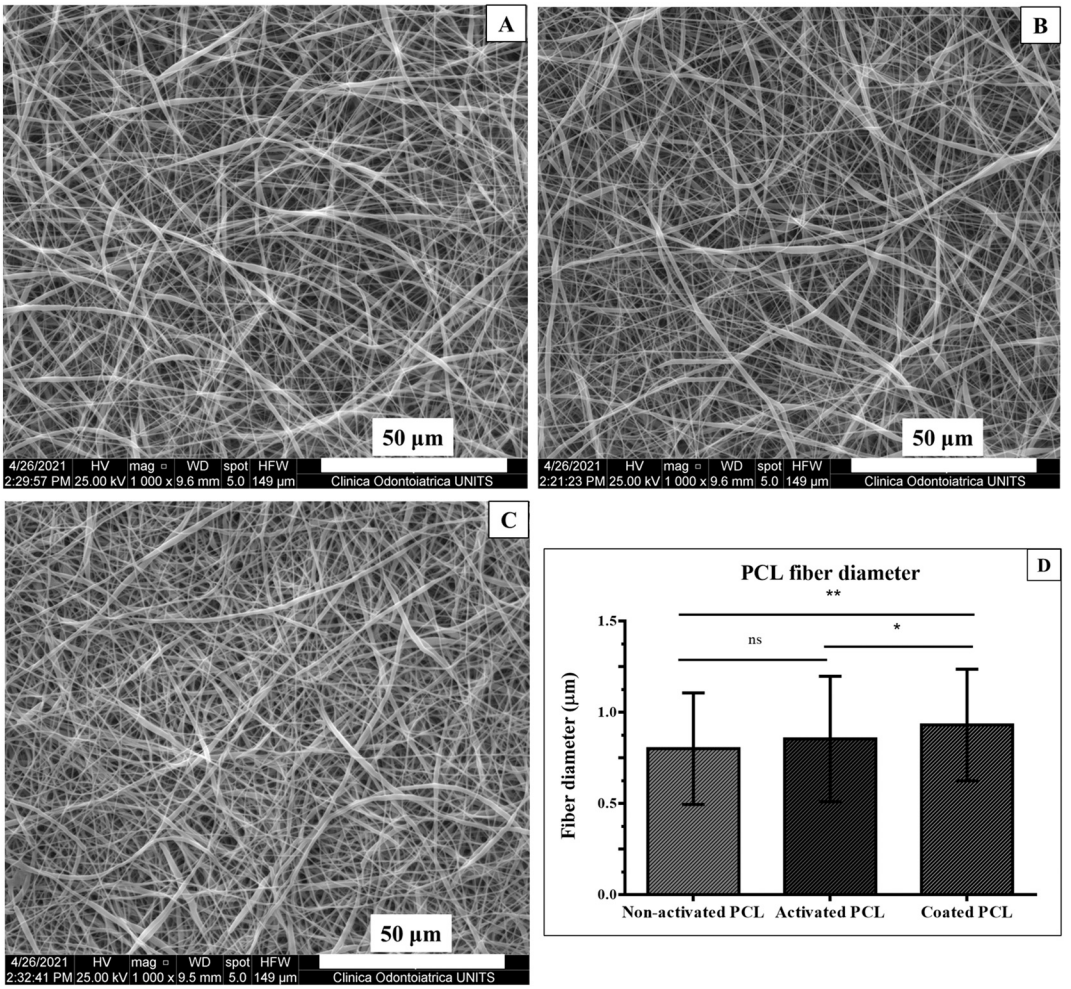

Fig. 3. SEM micrographs of PCL matrices, namely (**A**) non-activated, (**B**) activated, and (**C**) coated nanofibers. On the lower right, (**D**) a comparison of fiber diameters. Statistical analysis was performed with Kruskal-Wallis test and Mann–Whitney test for two-groups comparison, applying Bonferroni's correction. Statistically significant differences are indicated as asterisks (*).

marked swelling behavior and thus the large amount of absorbed fluids, are responsible for the higher variability of the results with respect to other materials.

3.6. Degradation tests

The stability of the electrospun membranes was evaluated for up to 7 days in both water and saline solution, by observing the change in membrane weight over time (Fig. 8). Polysaccharide-based matrices crosslinked with CDI and methacrylic anhydride were compared with PCL membranes (non-activated, activated, coated), Chitoderm®, and freeze-dried polysaccharide membranes even crosslinked with CDI. The results confirmed the overall stability of the PCL membranes, even in the presence of the coating, which showed a slight degradation behavior, maybe due to the coating desorption in the aqueous environment. On the other hand, the effectiveness of CDI crosslinking was evaluated in comparison to methacrylic anhydride, with the membranes showing progressive degradation behavior already after 1 day. In fact, CDI-crosslinked mats revealed an optimal stability in water up to day 7, while they were stable in saline solution up to 3 days. After that a slight degradation trend was noticeable net of standard deviations, possibly caused by the presence of salts destabilizing the polysaccharide structure. On the other hand, CDI-crosslinked membranes obtained by freeze-drying underwent a mild degradation between one and three days in water or saline solution, without showing any degradation in the next time points; in contrast, Chitoderm® samples were stable over time regardless of the tested fluid.

3.7. Water vapor transmission rate (WVTR)

The ability to transmit water vapor was assessed on electrospun membranes crosslinked with CDI, which were compared with PCL membranes in different states (namely, non-activated, activated, or coated with CTL/HA) and Chitoderm®. Parafilm® was used as control for non-permeability; uncapped vials were tested as control for total evaporation. The results (Fig. 9) indicate good water vapor transmission ability in the case of PCL membranes, which all showed similar results. CDI-crosslinked polysaccharide mats revealed a similar trend to PCL mats after 24 h, but the transmission rate was lower than PCL at 48 h. This could be due to the composition of the membrane, based on highly hydrophilic polymers. Indeed, owing to its polysaccharide nature, its great ability to retain water could result in water being trapped in the nanofiber structure, leading to a consequent increase of weight when measuring the amount of residual water in the system. Nevertheless, the water vapor permeability of polysaccharidic electrospun mats was still higher than that of Chitoderm®, due to the outer polyurethane layer which shields pathogen entry, while reducing water evaporation.

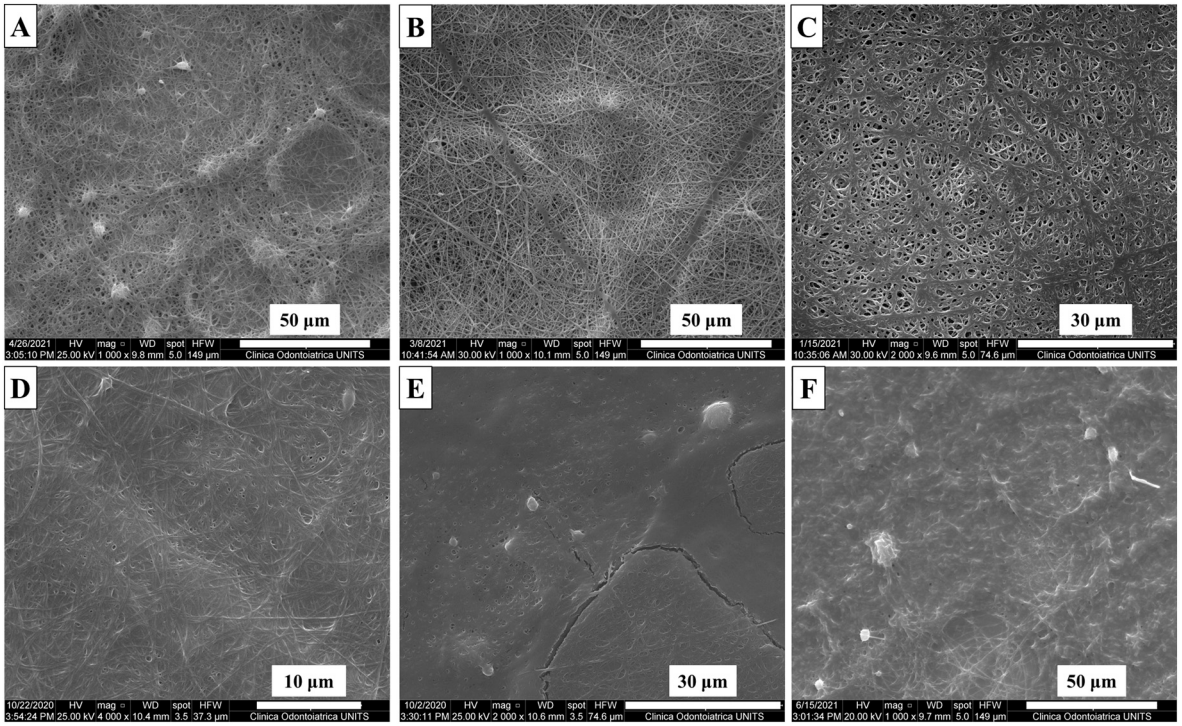

Fig. 4. SEM micrographs of HA/CTL/PEO membranes crosslinked with various strategies: (**A**) CDI overnight; (**B**) methacrylic anhydride coupled with acetone washing; (**C**) glutaraldehyde (25% in water) vapor after 4 h in a vacuum chamber; (**D**) genipin in ethanol after 45 min of immersion; (**E**) heat at 80 °C for 4 h; (**F**) heat in a vacuum oven at 80 °C for 3 h.

Table 1
Summary of the crosslinking strategies attempted on HA/CTL/PEO membranes and the effects on membrane stability, thought of as both water resistance and architecture preservation.

Crosslinking treatment	Outcome
CDI	Partial fiber fusion, but optimal stability in water
Methacrylic anhydride	Preservation of fibers after washing in dimethylformamide and acetone, despite rapid degradation compared to CDI crosslinking
Glutaraldehyde	No resistance in water. Fiber fusion or loss when coupled with thermal treatment
EDC/NHS	Immediate gelation after EDC/NHS addition to the solution
Genipin	Fiber loss and total instability in water, both when added before electrospinning or after membrane production
Heat	Fiber loss and immediate dissolution in water

4. Discussion

Considering the compelling need for wound dressings able to mimic the extracellular matrix (ECM) architecture and composition, absorb exudates, and provide a large surface area for gaseous exchanges (Ekambaram & Dharmalingam, 2020; Luan et al., 2021; Zheng, Zhang, et al., 2021), the current work presents the production of electrospun wound dressings based on two different polysaccharides, namely hyaluronic acid and a bioactive lactose derivative of chitosan (CTL). The presence of these two polysaccharides should strongly favor the wound healing process. Hyaluronic acid is indeed a natural component of the ECM and provides excellent biomimicry. On the other hand, CTL brings the enormous advantage of being water soluble at neutral pH values, combining chitosan properties with the possibility to use aqueous, non-toxic solvents. It has been studied for its bioactivity in numerous fields, such as osteochondral or neuronal regeneration (Donati et al., 2005; Medelin et al., 2018; Porrelli, Gruppuso, et al., 2021). As a derivative of chitosan, it should even exert some of the advantages given by chitosan itself, such as hemostatic or antibacterial activity, due to the presence of positive charges along the polymeric chains that bind negatively charged bacterial cell walls (Hu et al., 2021; Sharifi et al., 2021). Moreover, CTL can be easily functionalized to enhance its bioactive properties. For example, Porrelli and coworkers enriched CTL with nAg (CTL-nAg) to produce PCL-based antibacterial electrospun membranes coated with CTL-nAg, thereby increasing their antibacterial potential (Porrelli, Mardirossian, et al., 2021). Given the need to combine polysaccharides with synthetic polymers, able to improve their electrospinnability, and surfactants which can reduce the surface tension of the solution to favor the extrusion of the filament (Fahimirad et al., 2021; Kriegel et al., 2009; Liu et al., 2011; Rezaei et al., 2021), HA and CTL were used in a ternary mixture with polyethylene oxide (PEO), while Tween® 20 was used as a surfactant. This allowed to obtain for the first time a CTL-based nanofibrous mesh, with homogeneous, thin, uniform, and defect-free nanofibers, in a diameter range encountering the dimension of natural collagen nanofibers (50–500 nm) (Xue et al., 2019).

The challenge in producing polysaccharide-based electrospun matrices is not only in the electrospinning process, but also in the post-processing step. Indeed, due to their highly hydrophilic nature combined with a large surface area available, polysaccharide membranes are extremely soluble in aqueous environments, hampering their application for biomedical purposes, where the medical device is expected to interact with biological fluids (Li et al., 2005; Li et al., 2015; Mirzaei et al., 2014). Both hyaluronic acid and CTL are highly hydrophilic polymers, leading to the immediate dissolution of the synthetized membranes upon contact with water. For this reason, based on the numerous literature example (Dodero, Scarfi, et al., 2021; Gruppuso et al., 2021; Lin et al., 2020; Mak & Leung, 2019), different crosslinking strategies have been tested in this work with the aim of stabilizing HA/CTL/PEO membranes, namely physical (heat treatment) and chemical (glutaraldehyde, genipin, EDC/NHS, methacrylic anhydride, CDI) methods.

The use of carbodiimide-based coupling agents, such as EDC/NHS, is

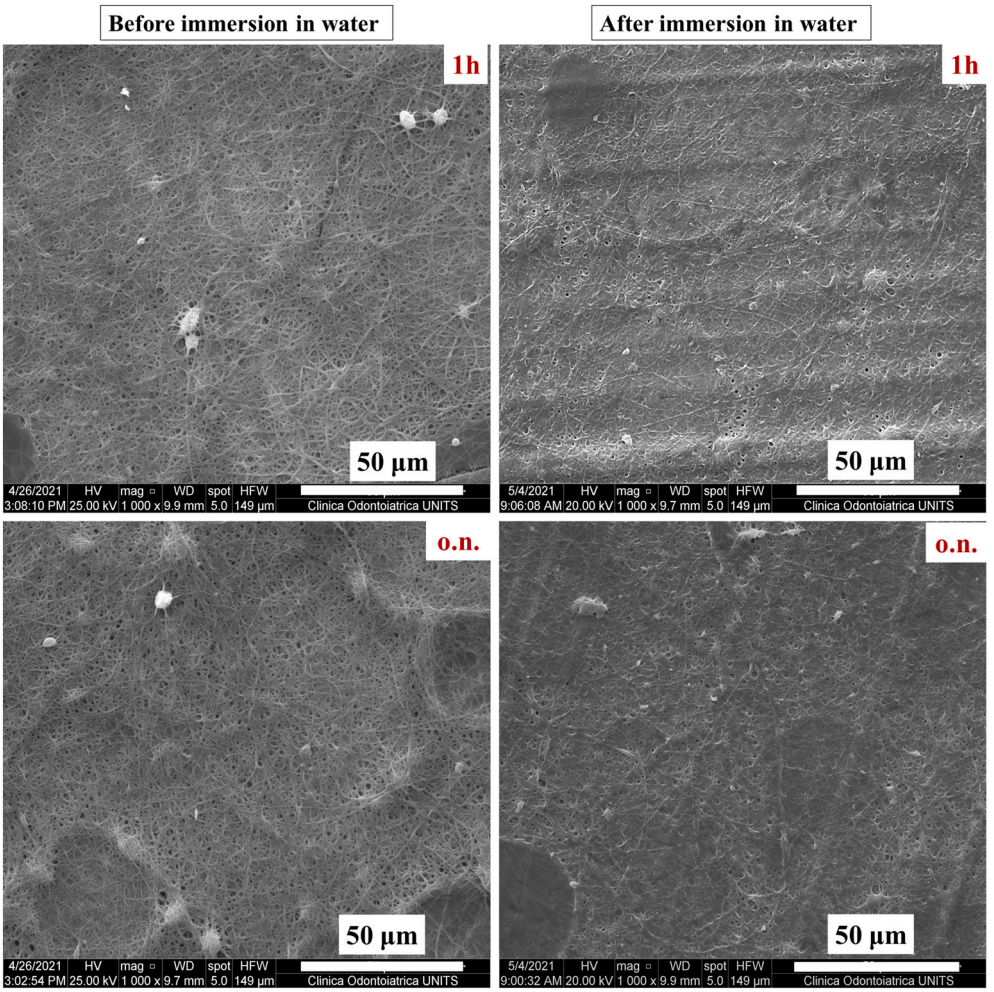

Fig. 5. HA/CTL/PEO membranes crosslinked with CDI for 1 h and overnight (o.n.) before and after their immersion in water. After 1 h of crosslinking, the nanofibrous mesh is thinner and unstable in water, while the overnight-crosslinked nanofibers retain their morphology and resist longer in water.

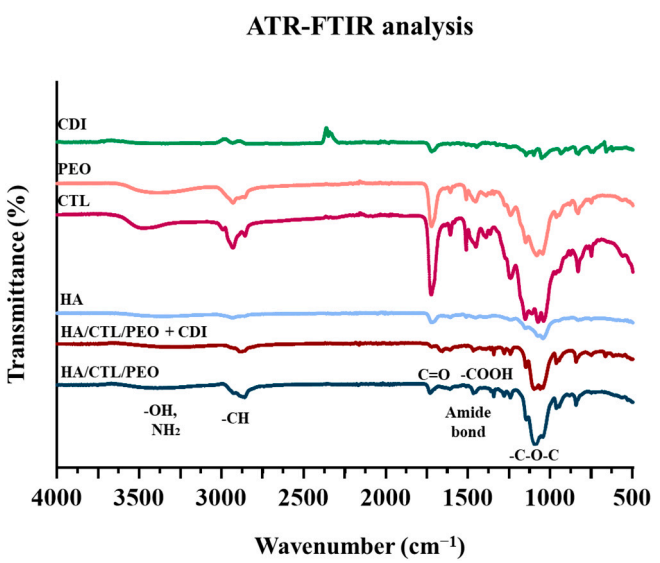

Fig. 6. ATR-FTIR spectra of CDI-crosslinked and un-crosslinked HA/CTL/PEO mats, compared with the spectra of the single membrane constituents (that are hyaluronic acid, CTL, PEO) and of CDI (in the case of crosslinked mats).

well documented in the literature (Castro et al., 2021; Federico et al., 2021; Keshvardoostchokami et al., 2021), as they promote the formation of an amide bond by the activation of carboxylic groups, that react with the crosslinking agents. Subsequently, in the presence of amino- or alcohol groups, a nucleophilic attack on the activated species unseat the crosslinker, leading to the formation of the amide bond without incorporating the reactive adduct (Khunmanee et al., 2017). In this context, Séon-Lutz and coworkers synthetized hyaluronic acid/polyvinyl alcohol nanofibrous dressing by electrospinning in water and crosslinking with EDC/NHS and obtained stable and only partially fused nanofibers at different relative humidity levels (up to 90%) (Séon-Lutz et al., 2019); moreover they did not observe any cytotoxic effect of EDC/NHS. Following these observations, HA/CTL/PEO nanofibers were crosslinked by direct addition of EDC/NHS to the electrospinning solution, both in powder and previously dissolved in water. Unfortunately, immediate gelation occurred, impeding the electrospinning of the solution. This could be due to the high reactivity of both HA and CTL that together hinders the maintenance of the solution state after crosslinker addition.

In addition to EDC/NHS, glutaraldehyde stands out as an efficient aldehydic chemical crosslinker (Ardekani et al., 2019; Chen, Meng, et al., 2021b; Darbasizadeh et al., 2019; Mistry et al., 2021). Glutaraldehyde mediates the formation of imminic bonds (C≡N) through the Shiff's base reaction between amino groups and glutaraldehyde aldehydic groups (Vondran et al., 2008), allowing it to potentially interact with CTL chains, thus creating a mesh able to entangle hyaluronic acid and PEO chains. The glutaraldehyde treatment has been reported in

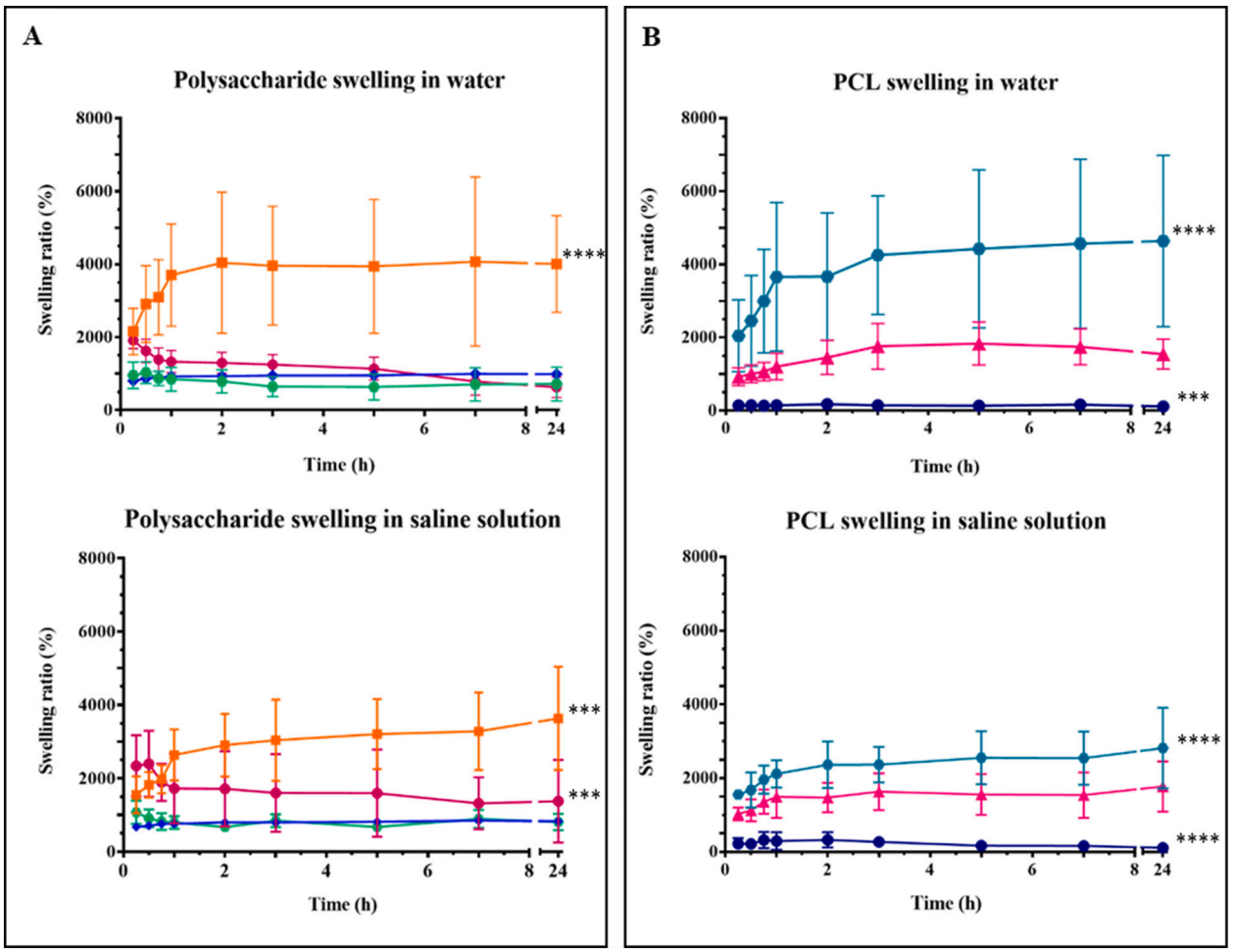

Fig. 7. Swelling capacity of electrospun and non-electrospun membranes expressed as swelling ratio (%) as a function of the immersion time. *Panel A*: swelling behavior of (■) polysaccharide-based electrospun membranes crosslinked with CDI or (●) methacrylic anhydride, (⬢) polysaccharide-based freeze-dried membranes and (◆) Chitoderm®. *Panel B*: swelling behavior of (●) non-activated, (⬢) activated, and (▲) polysaccharide-coated PCL membranes. In the case of polysaccharides in water, statistical analysis was performed with Kruskal-Wallis test and Mann-Whitney test for two-groups comparison, applying Bonferroni's correction. In the case of polysaccharides in saline solution, PCL in water, and PCL in saline solution, statistical analysis was performed with one-way ANOVA, applying Bonferroni's correction.
Statistically significant differences are indicated with asterisks (*). *** $= p < 0.001$.

literature following the most varied timepoints, from 4 h to 12–16 h to 3 days (Akbari et al., 2022; Ali et al., 2022; Qian et al., 2011). Two different strategies were here attempted by using glutaraldehyde vapor at different times (namely, 4 h and 2 h), in combination with heat treatment in the case of 2 h crosslinking for further stabilization. Prolonged reaction times were not possible, since partial fiber fusion was observed when membranes were crosslinked with glutaraldehyde only and the structure was completely lost after heat treatment. According to Ahmadi and co-workers, this could be due to the release of water as a by-product of the reaction, which could be entrapped into polymer chains causing fiber swelling and the alteration of the morphology (Ahmadi et al., 2021). This is particularly evident in the case of HA/CTL/PEO mats, where the presence of highly hydrophilic hyaluronic acid and CTL increases this swelling phenomenon, hampering membrane exposure to a wet environment for extended times. On the other hand, the heat treatment would be too strong, which would irreparably damage polysaccharide mats. In both cases, the membranes were completely unstable in water, undergoing immediate dissolution after a few seconds, showing the ineffectiveness of glutaraldehyde crosslinking in the case of HA/CTL/PEO membranes.

Considering the potentially toxic effects of glutaraldehyde, the use of other chemical crosslinkers, such as genipin, has been investigated over the years (Lau et al., 2018; Mirzaei et al., 2014); indeed, it was proved that it is possible to substitute glutaraldehyde with genipin for the preparation of biocompatible chitosan-based biomaterials (Lai, 2012). Genipin crosslinking reaction occurs through the nucleophilic substitution of the genipin ester function by primary amines to form secondary amides or through the nucleophilic attack on genipin dihydropyran ring, creating a six membered nitrogen heterocycle (Aubert-Viard et al., 2019). In the specific case of nanofibrous devices, genipin can be electrospun by direct addition to the polymeric solution or it can be used as post-electrospinning crosslinker by solubilization in ethanol or PBS and immersion of the electrospun matrices in the crosslinking medium (Li et al., 2015; Mak & Leung, 2019; Sergi et al., 2020). For example, Panzavolta and coworkers synthetized gelatin-based nanofiber mats crosslinking them by direct genipin addition in the solution 30 min before electrospinning and post-treating the membranes by immersion in ethanol-solubilized genipin up to 7 days (Panzavolta et al., 2011). Both approaches have been tried in this case, where, as for glutaraldehyde, genipin could react with CTL aminic moieties producing an entangled nanofibrous mesh, in which hyaluronic acid and PEO chains are entrapped. Electrospinning of genipin did not alter polysaccharide solution properties, thus allowing the production of optimal nanofibers, with a uniform and defect-free morphology. The matrices obtained were

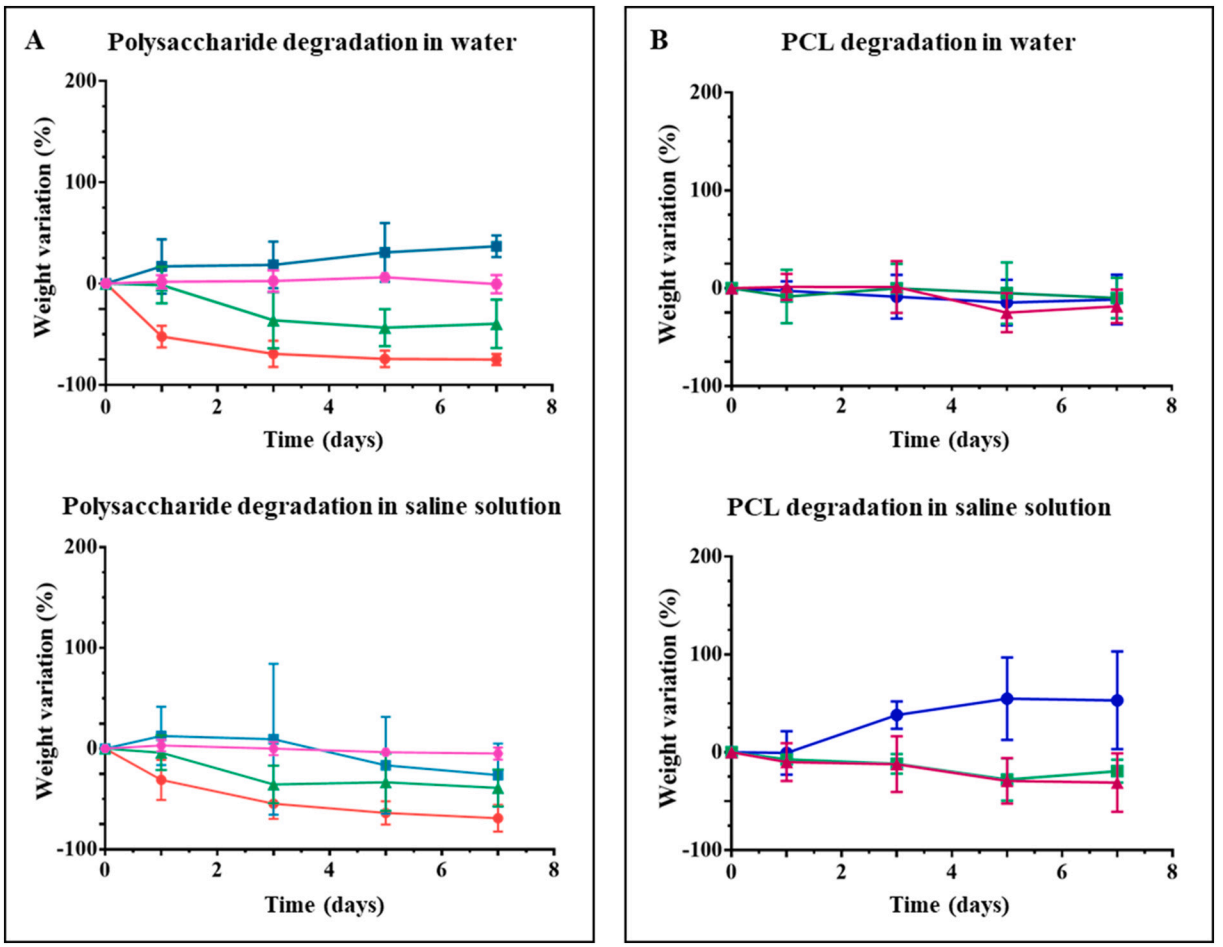

Fig. 8. Degradation behavior of electrospun and non-electrospun membranes expressed as weight variation (%) in time. *Panel A*: degradation of (■) polysaccharide-based electrospun membranes crosslinked with CDI or (●) methacrylic anhydride, (▲) polysaccharide-based freeze-dried membranes and (⬣) Chitoderm®. *Panel B*: degradation of (●) non-activated, (■) activated, and (▲) coated with polysaccharides PCL membranes. In all cases, statistical analysis was performed with Kruskal-Wallis test and Mann-Whitney test for two-groups comparison, applying Bonferroni's correction; no statistical differences were found.

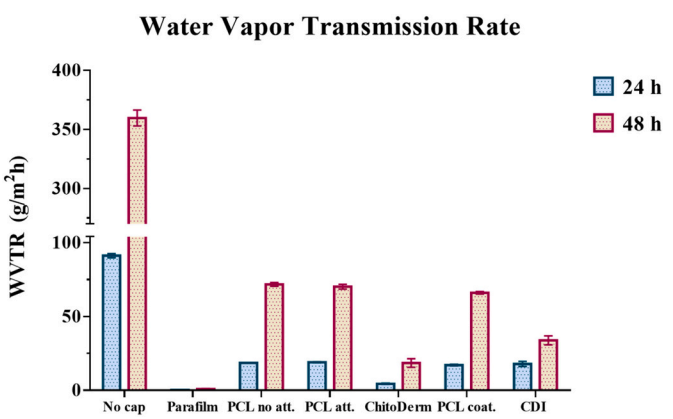

Fig. 9. Water vapor transmission ability of PCL (non-activated, activated, coated) mats, CDI-crosslinked polysaccharidic membranes, and Chitoderm® after 24 h and 48 h.

then heated at 37 °C for 24 h to 7 days, with the aim to activate genipin reaction and facilitate the crosslinking between the polymer meshes. However, the reaction did not take place and even after 7 days of incubation membranes were completely unstable in an aqueous environment. This could be due to the dry environment, which is not a suitable condition for genipin activity. Indeed, during the electrospinning process the solvent evaporates and the fibers are deposited on the collector as ideally solvent-free fibers. On the other hand, the post-electrospinning crosslinking has been performed by dissolving genipin in ethanol and embedding the polysaccharidic membranes at different timepoints. The membranes were then heated to favor genipin reactivity, but also in this case the crosslinking reaction did not occur, besides leading to the loss of the fibrous structure. In fact, the extended immersion in ethanol disrupts the nanofibrous morphology and heat treatment seemed to further worsen the already altered architecture.

With the objective to assess the effects of thermal treatment alone on nanofibrous meshes, heat was used as possible crosslinking strategy. Thermal crosslinking is a physical method to induce crystallization of the electrospun polymers and stabilize the resultant structure (Sarhan & Azzazy, 2015). Two different methods were adopted: membranes were heated at 80 °C in a convection oven or in a vacuum oven. Despite the higher temperatures reported in the literature (Esparza et al., 2017; Sandri et al., 2019; Sarhan & Azzazy, 2015), burning of membranes was observed above the 80 °C limit. On the other hand, the resulting membranes were completely unstable in water apart from losing their nanostructure, thus demonstrating the ineffectiveness of thermal treatment to crosslink HA/CTL/PEO membranes. For this reason, the thermal treatment was combined with chemical treatment involving carbonyldiimidazole (CDI), a carbodiimide coupling agent that mediates the formation of amide bonds between carboxyl- or hydroxyl-groups and aminic moieties without being incorporated (Woodman et al., 2009), as revealed by ATR-FTIR analysis. Indeed, the presence of CDI was not

detectable in the spectrum of CDI-crosslinked mats, which was comparable to that of not crosslinked ones. Moreover, in the presence of the characteristic carbonyl (C=O) and carboxyl (-COOH) bands, the amide bond IR spectrum was not clearly distinguishable, thus requiring further characterization of the reaction mechanism by ^{1}H NMR. CDI has been studied as covalent crosslinker to immobilize specific molecules on the surface of electrospun membranes, as reported by Baştürk and coworkers, which used CDI to covalently bind α-amylase on the surface of poly(vinyl alcohol)/poly(acrylic acid) (PVA/PAA) nanofibers (Baştürk et al., 2013). It has also been employed as crosslinker in pullulan/gelatin hydrogels, with a "one-step" method enrolling aqueous DMSO as reaction solvent (Han & Lv, 2019). However, CDI use as coupling agent for electrospun nanofibers, where it can induce amide bond formation between polymeric chains, is not documented to the best of the author's knowledge. Nonetheless, CDI provided the best crosslinking results, as it represents a promising compromise between mat stability and nanofiber morphology. Indeed, as shown by the morphological characterization, CDI-crosslinked matrices did not completely lose their nanofibrous architecture despite the partial fiber fusion. Moreover, they were particularly stable in aqueous environment, maintaining their macroscopic integrity. This was confirmed by swelling and degradation studies, which highlighted the high absorption potential of CDI-crosslinked membranes as well as their adequate stability in water and saline solution. Indeed, the ability to absorb and retain exudates is of remarkable importance when considering the effectiveness of wound dressings and their potential use in application for mild-to-highly exudating wounds (Tamer et al., 2021; Wang, Song, et al., 2021b). A comparison with polycaprolactone (PCL) electrospun membranes was useful to assess the efficacy of electrospun products. Indeed, PCL is a widely exploited polymer in numerous fields, including wound management; several PCL wound dressing have been produced over the years, with *in vitro* and *in vivo* studies supporting the usefulness of employing this polymer for wound healing purposes (Ehterami et al., 2018; Karizmeh et al., 2022; Thomas et al., 2015; Zhou et al., 2022). Nonetheless, one of the major drawbacks of PCL lies in its hydrophobicity, thus requiring the association with hydrophilic polymers or surface modifications allowing the interaction with biological environments (Asghari et al., 2022; Jacobs et al., 2013; Patel et al., 2021). In this direction, Porrelli and coworkers synthetized PCL-based nanofibrous scaffolds modifying their surface with air-plasma treatment to confer hydrophilicity to the final structure, thus increasing membrane wettability and allowing the interaction with cells (Porrelli, Mardirossian, et al., 2021). In this study, PCL membranes were employed as pristine (non-activated membranes), air-plasma activated membranes, and coated with a polysaccharide layer of CTL/HA (to compare the presence of a polysaccharide coating with the use of polysaccharides as components of the nanofiber matrix itself). In terms of swelling capability, activated PCL membranes showed similar results to the CDI-crosslinked polysaccharide mats, indicating that a good swelling capacity can be achieved by using large-area materials as well as by using strongly hydrophilic surfaces. On the other hand, the polysaccharide coating of the PCL membranes slightly affected their ability to absorb fluids, perhaps because the swelling of the polysaccharide layer drowns the porosity of the electrospun meshes, thereby hindering the water uptake capacity of these membranes. The advantage of using electrospun wound dressings was further demonstrated by the comparison with a commercial product, namely Chitoderm®, and CDI-crosslinked membranes produced by freeze-drying. In both cases, the swelling capacity was significantly lower than that of electrospun membranes, despite their stability in both water and saline solution. Overall, the swelling capacity was slightly lower in saline solution compared to water. This could be due to the osmotic pressure established by the mobile ions between the saline solvent and the nanofibrous network. According to Donnan's equilibrium theory, the ionic forces are determined by the counterions present in the solvent and the fixed ionizable groups on the material. In the presence of free sodium cations, a charge-screening effect could occur, thus reducing the osmotic pressure and moving the absorbed solution back towards the medium (Ferfera-Harrar et al., 2016; Ricka & Tanaka, 1984).

The stability of CDI crosslinking became even more evident when compared to methacrylic anhydride crosslinking. Methacrylic anhydride is a methacrylating agent often used to chemically modify polymer chains through the introduction of methacryloyl moieties, allowing thereafter the photo-crosslinking of the methacrylated polymers, without affecting the biocompatibility of the final product (Joshi et al., 2021; Nazir et al., 2021; Samani et al., 2021; Seo et al., 2021; Skardal et al., 2010; Zhang et al., 2020; Zhu & Bratlie, 2018). Here, pure methacrylic anhydride was used as post-electrospinning treatment on HA/CTL/PEO membranes. However, despite the good morphology displayed by treated membranes washed in acetone or dimethylformamide, the use of methacrylic anhydride alone was not sufficient to achieve long-term polysaccharidic membranes stabilization, resulting in their degradation from a few hours to 7 days. Probably, the interaction between the methacrylate moieties introduced after the electrospinning process determined a weak and temporary stabilization of the nanofibrous mesh, subsequently leading to its rapid degradation. On the contrary, CDI-crosslinked mats were stable up to 7 days in water, while in saline a mild degradation started after 3 days. Indeed, as described above, there should be a change in osmotic pressure, causing membrane shrinkage in the presence of salts, a phenomenon that varies depending on the composition of the material considered. Nevertheless, the frequency of dressings changes must be taken into account, which is approximately 2–3 times per week, depending on the type of wound considered (Akita et al., 2006; Lindholm & Searle, 2016; Resch et al., 2021). On the other hand, the advantages of a local release of the polysaccharides employed to favor skin regeneration should not be neglected (Hauck et al., 2021; Li et al., 2022; Liang et al., 2020; Wang, Feng, et al., 2021; Yao et al., 2022). Hence, the degradation behavior exhibited by CDI-crosslinked membranes should not affect the quality of the final product but should be an added value in promoting skin regeneration.

Another important parameter to consider in the synthesis of wound dressings is their ability to transmit water vapor and favor gaseous exchanges (Catanzano et al., 2021; Chen, Pan, et al., 2021; Salami et al., 2021). In fact, in 1962 Winter demonstrated how in the presence of a dry environment, with the wound covered by superficial scab, the regeneration process is delayed compared to a wound maintained in a moist environment, preventing the formation of the scab (Winter, 1962). Consequently, an ideal wound dressing should ensure the proper equilibrium between a high evaporation rate, which would hamper the maintenance of a moist environment, and an occlusive behavior, which would not allow a correct drainage of fluids, leading to skin maceration and paving the way for infections (Du et al., 2021; Zoghi et al., 2021). After having assessed the goodness of CDI crosslinking among the different strategies tried, the ability to transmit water vapor was tested by a comparison with PCL electrospun membranes and Chitoderm®. The water vapor transmission rate of electrospun products was higher than that of the commercial product, due to Chitoderm® external polyurethan layer, which acts as a barrier against microorganisms as well as being occlusive to water. On the other hand, the evaporation rate of CDI-crosslinked membranes was lower than that of the electrospun PCL products after 48 h, possibly due to their high efficiency in entrapping water molecules. In fact, the water vapor transmission ability also depends on the diffusivity and solubility of the water molecules in the polymer meshes (Li, Ma, et al., 2021b); consequently, the presence of hyaluronic acid and CTL, with their high hydrophilicity, should cause a slight water retention on the nanofibrous mat after 48 h. Nevertheless, the ability of electrospun devices to transmit water vapor facilitating gaseous exchanges is well halfway between the complete evaporation and total impermeability, as expected from an ideal wound dressing.

Based on these considerations, the present polysaccharide-based electrospun matrices should be a good starting point for the production of polysaccharide-based wound dressings, despite all the critical

aspects related to the crosslinking with the numerous crosslinkers currently available. The mechanical stability of such products could be a critical issue too; therefore, it should make sense to combine them with a supporting synthetic matrix, resulting in a two-phase system with a protective outer synthetic layer and a bioactive inner layer. It will be also important to thoroughly characterize the membranes cytotoxicity and biocompatibility especially after crosslinking, as well as their mechanical properties in order to optimize their stability, their strength, their handling and their adaptability to the wound site. Although CDI has been already used for the production of biocompatible materials (Olsson et al., 2014), and FTIR analysis did not show the presence of residual CDI biproducts in the membranes here produced, cytotoxic assays are needed to confirm the biocompatibility of crosslinked membranes. The as-obtained matrix could be even functionalized with antibacterial agents, such as silver nanoparticles, with the aim of preventing undesired bacterial infection at the wound site (El-Aassar et al., 2020; 2021). As a matter of facts, CTL-nAg has already been produced and characterized as coating for electrospun membranes (Porrelli, Mardirossian, et al., 2021), thus encouraging its electrospinning for wound dressing production. Once assessed the stability of such devices, even in terms of long-time storage, the way for the biological characterization could be taken to translate *in vitro* research into clinical application.

5. Conclusions

Polysaccharide-based electrospun wound dressings were synthetized, using hyaluronic acid, a lactose-modified chitosan (CTL), and polyethylene oxide as the main components. The major problem with these nanofibrous matrices is their instability in aqueous environment, requiring a further crosslinking step. Based on the numerous examples in the literature, different strategies, such as glutaraldehyde vapor, genipin, EDC/NHS, or heat treatment were explored, but did not give satisfactory results. Therefore, two new crosslinking methods were tested for their ability to stabilize the nanofibrous structure, namely methacrylic anhydride and carbonyldiimidazole, the latter being the most effective combination of structure preservation and water resistance. The membranes thus produced were tested for their ability to absorb exudate and retain their structure apart from their capability to transmit water vapor and favor gas permeation, revealing promising results. Undoubtedly, the nanofibrous morphology represents a major advantage in terms of swelling capacity and water vapor permeability, as demonstrated by a comparison with synthetic polymer-based electrospun products. The presence of polysaccharides should bring further advantages in terms of dressing bioactivity and interaction with the surrounding biological environment and the system should be even implemented with bioactive moieties, as antibacterial ones. For this reason, further efforts are being made to achieve robust mechanical stabilization of the polysaccharidic dressings, which will be characterized in terms of mechanical properties, cytotoxicity and biological properties, with the aim to produce a long-term stable and useful wound dressing.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors Gianluca Turco and Eleonora Marsich declare to be shares holders of the company BiopoLife.

Appendix A. Supplementary data

Morphological characterization of HA/CTL/PEO freeze-dried membranes along with polysaccharidic membranes after different crosslinking treatments, reaction mechanism schemes. Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.119375.

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