International Journal of Pharmaceutics 378 (2009) 108–115

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Collagen-based modified membranes for tissue engineering: Influence of type and molecular weight of GAGs on cell proliferation

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ARTICLE INFO

Article history:
Received 30 March 2009
Received in revised form 19 May 2009
Accepted 25 May 2009
Available online 6 June 2009

Keywords:
Collagen membranes
Glycosaminoglycans
Tissue engineering
Physico-chemical characterization
Cell adhesion
Cell proliferation

ABSTRACT

This study aims to evaluate the effects of the two most widely used glycosaminoglycans (dermatan sulphate and heparin) on both the structural and biological properties of collagen-based modified membranes (COL/GAGs membranes) designed for tissue engineering. The molecular weight of dermatan sulphate and heparins was correlated with the membrane feasibility and the cell (fibroblasts and keratinocytes) ability to adhere and proliferate on the COL/GAG membranes.

Microstructure and physico-chemical properties of COL/GAGs membranes were examined using scanning electron microscopy and differential scanning calorimetry; the free amino group content and the swelling properties were also detected. The morphology, proliferation and growth behaviour of keratinocytes and fibroblasts were investigated using microscopical approach and *in vitro* colorimetric assay. Both fibroblasts and keratinocytes are able to grow and proliferate on COL/dermatan sulphate membranes. Fibroblasts revealed significantly higher proliferation on the membranes prepared with heparin if compared to the proliferation on the membrane without heparin (COL membrane). Particularly, a combination of the membranes formulated adding high molecular weight dermatan sulphate and high molecular weight heparin could be suitable to be used as biomaterials for epidermal substitute.

1. Introduction

Tissue engineering is a young diagnostic and therapeutic discipline compelling the fundamental principles of materials engineering and molecular biology with the aim of tissues and organs regeneration (Kim et al., 2008).

Collagen (COL) is widely applied in the biomedical field and in tissue engineering owing to the low immunogenicity and toxicity and to its unique special properties (Elsdale and Bard, 1972; Sheridan and Tompkins, 1999; Auger et al., 1998). In fact, besides outstanding mechanical, haemostatic and cell-binding properties, collagen exhibits an excellent biocompatibility profile and predictable biodegradability kinetics capable to overcome the need for any surgery for its removal. Due to its fibrous structure, collagen confers a strength and stability to bodily tissue, such as tendons, ligaments and skin; as a consequence, collagen plays an integral role in providing a stabilizing membrane or matrix for cell seeding (Lu et al., 2007a; Qiang et al., 2005; Lee et al., 2001b). Particularly, type I collagen represents an ideal substrate for the histo-reparation processes. Several studies demonstrated that different types of cells (epithelial, endothelial, keratinocytes, fibroblasts, osteoblasts and chondrocytes responsible for the synthesis of the tissue structures) are able to adhere and proliferate to the fibers of type I collagen, and thus to be orientated in order to reshape the damaged tissue (Schor, 1980; Pieper et al., 2002; Gruber et al., 2006).

Glycosaminoglycans (GAGs) were added to type I collagen to obtain scaffolds widely utilized as biological support analogs for skin regeneration (Lee et al., 2001a; Zhong et al., 2007). GAGs (chondroitin-4-sulphate, chondroitin-6-sulphate, keratin sulphate, dermatan sulphate, hyaluronan, heparin sulphate and heparin) are negatively charged polysaccharides capable to maintain the structural integrity of the cartilage. Therefore, the rationale of their addition in the scaffold preparation is based on the favorable effect of the GAGs on cell adhesion, proliferation and on differentiation processes, exercised by means of binding molecules such as growth factors and cytokines (Jackson et al., 1991; Chen et al., 2007).

GAGs, such as heparin and dermatan sulphate, play a variety of roles; for example, as modifiers of key biological response, they can act (1) as stabilizers, cofactors and co-receptors for growth factors, cytokines and chemokines, (2) as regulators of enzyme activity and (3) as signaling molecules in response to cellular damage (Trowbridge and Gallo, 2002). Since, dermatan sulphate is predom-

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doi:10.1016/j.ijpharm.2009.05.049

inantly expressed in the skin, several experiments demonstrated that it could be considered as a multifunctional facilitator of the wound repair process (Penc et al., 1998; Couchman and Hook, 1988; Kirker et al., 2002; Van Susante et al., 2001). The presence of heparin into the collagen-based scaffolds promotes the attachment of a variety of growth factors and proteins producing a good environment for cell growth; thus the tissue development is facilitated (Lindahl et al., 1994; Casu and Lindahl, 2001; Lu et al., 2007b).

The aim of this study is to investigate the effects of dermatan sulphate and heparin on both the structural and biological properties of collagen-based modified membranes (COL/GAGs membranes). Particularly, to prepare COL/GAGs membranes as support for skin regeneration, dermatan sulphate and heparin with different molecular weights were added to equine type I collagen, notwithstanding usually papers dealt with the application of bovine collagen in scaffolds for tissue regeneration (Mizuno and Glowacki, 1996; Rodrigues et al., 2003; Doillon et al., 2003). Moreover, the choice of using equine collagen can avoid the possible bovine spongiform encephalopathy (BSE) transmission (Ruozi et al., 2007). To evaluate both cell attachment and proliferation to these scaffolds, experiments were carried out using fibroblast and keratinocyte cell cultures.

Fibroblasts are a group of cells involved in the deposition, maintenance and re-modulation of the extracellular matrix. Keratinocytes represent one of the major classes of constituents of the epidermal cells (Radhika et al., 1999). Skin is identified by two components, i.e. the dermal equivalent consisting of collagen gel populated with fibroblasts and the epidermal matrix formed by keratinocytes on the top of the dermal base (Bell et al., 1979).

2. Material and methods

2.1. Substrate materials

2.1.1. Type I collagen separation

Type I collagen was isolated from equine Achilles' tendons using the standardized industrial protocol of OPOCRIN Spa (Corlo, Formigine, Italy). The inner part of tendons of horses, collected in a certificated slaughterhouse under strict control of the veterinary authorities, was dissected ensuring the complete removal of the surrounding membrane. An exact amount of tendon inner part was then minced and suspended in acidic solution pH of 2.5. To prepare the telopeptide-free collagen, a specific enzymatic treatment was carried out on the suspension, adding 1% (w/w) of pepsin (with respect of the tendon weight) overnight removing the non-helical regions of the molecules (Miller and Rhodes, 1982; Bashley et al., 1978). In order to obtain non-immunogenic collagen (as atelocollagen), telopeptides, constituting the terminal part of the collagen fibrilla, are removed (Bianchini and Parma, 2001). The temperature was maintained at 25 ± 0.2 °C throughout the enzymatic procedure. After the enzymatic treatment, the telopeptide-free collagen fibers were precipitated by adding 1N NaOH until the solution reached a pH value of 5.6. After the aqueous phase removal, the telopeptide-free collagen fibers were washed several times with purified water.

In order to remove the glycosidic portions and to inactivate potential virus, the fibers were suspended in 1 N NaOH solution for 1 h. After this period, collagen fibers were precipitated again and repeatedly washed with purified water to remove impurities and salts. Finally, the collagen fibers were acidified up to pH of 3.5 using 0.3% (v/v) acetic acid and vigorously stirred with Sterilmixer (11,000–13,000 rpm) (PBI International, Milan, Italy) until homogeneous suspensions were obtained.

2.1.2. Glycosaminoglycans (GAGs)

Dermatan sulphate (DS; also known as chondroitin sulphate B; *lot* 90303, Mw 25.1 kDa, SO_3/COO^- 1.16, AXaU/mg 1.19), low-molecular-weight dermatan sulphate (LMWDS; *lot* 3*L*, Mw 6.962 kDa, SO_3/COO^- 0.97, AXaU/mg 1.02), un-fractioned heparin (UFH; *lot* 15839, Mw 14.7 kDa, SO_3/COO^- ND, AXaU/mg 182.55), low-molecular-weight heparin (LMWH; Pernaparin, *lot* 84631, Mw 5.215 kDa, SO_3/COO^- 2.21, AXaU/mg 92.69), very-low-molecular-weight heparin (OLIGOH; Deligoparin, *lot* 84390, Mw 3.1 kDa, SO_3/COO^- 2.30, AXaU/mg 71.64) were purchased from OPOCRIN Spa.

2.2. Membranes preparation

To prepare COL/GAGs membranes, GAG solutions were added at a rate of 2–6 mL/min to type I equine telopeptides-free collagen suspension (1%, w/w) under vigorous stirring with Sterilmixer (11,000 rpm). The mixtures were homogenized for an additional 15 min and then stirred under vacuum (10 mmHg) for 12 h to be degassed. Several concentrations of GAGs were tested (0.25–0.5–1.0–2.0% of DS and LMWDS; 0.1–0.2–0.4–0.8–1.0% of UFH; 0.1–0.25–0.4–0.8–1.0% of LMWH and OLIGOH), but only few preparations demonstrated appreciable stability conditions (absence of precipitate, miscibility, density, homogeneity and alteration of pH) suitable for membrane preparation (Table 1). The stable gels were used for the production of membranes according to OPOCRIN Patent EP1307247 (Parma, 2003). Briefly, the collagen gel is poured into eletrostatically charged tray and subsequently slowly dried at 26 °C in a turbulence free ventilated incubator, until obtaining membranes with a water content not exceeding 15% (w/w).

The membranes were packed in blister and sterilized with gamma rays (25 kGy) by GAMMARAD Italia Spa (Minerbio, Italy).

The collagen membranes contained 9 mg collagen/cm^2 and their thickness was approximately 0.150 mm ± 0.03. Macroscopically, they showed two surfaces: one "opaque" and one "bright". The opaque surface was the free surface, i.e. the surface into contact

Table 1
Physico-chemical parameters of membranes.

Membranes	Composition[a]	W[b] (%)	T_{peak}[c] (°C)	T_{onset}[d] (°C)	AH[e] (J g^{-1})	Amino group contents[f]
COL	COL	174 ± 3	46.0 ± 2.0	42.1 ± 1.1	29.7 ± 4.0	20.1 ± 2.0
COL/DS	COL + 1% DS	181 ± 13	48.7 ± 0.5	44.6 ± 0.6	43.2 ± 2.6	23.2 ± 1.8
COL/LMWDS	COL + 1% LMWDS	166 ± 22	41.4 ± 6.0	38.4 ± 2.9	74.5 ± 6.0	20.2 ± 1.3
COL/UFH	COL + 0.4% UFH	150 ± 11	49.5 ± 3.0	47.3 ± 3.7	68.5 ± 8.0	19.8 ± 0.7
COL/LMWH	COL + 0.25% LMWH	196 ± 23	48.7 ± 1.5	43.5 ± 1.0	41.2 ± 6.4	17.9 ± 1.6
COL/OLIGOH	COL + 0.25% OLIGOH	160 ± 8	47.6 ± 5.1	42.5 ± 2.1	34.3 ± 6.8	19.9 ± 1.4

[a] The percentage of GAGs are expressed as percent (w/w) respect to collagen.
[b] Water binding capacity. Values are mean ± SD (n = 6).
[c] T_{peak} was determined as the peak value of the corresponding endothermic phenomena. Values are mean ± SD (n = 3).
[d] T_{onset} is chosen as denaturation temperature. Values are mean ± SD (n = 3).
[e] Denaturation enthalpy represent the total area of peak between T_i (initial temperature) and T_f (final temperature). Values are mean ± SD (n = 3).
[f] Expressed as moles of free amine groups/g of collagen × 10^5. Values are mean ± SD (n = 5).

with the air, whereas the bright surface was the membrane surface into contact with the template bottom.

2.3. Characterization of membranes

2.3.1. Amino group content

The free amino group content in the collagen was evaluated by reaction with 2,4,6-trinitrobenzenesulphonic acid (TNBS) according to Ofner and Bubnis (1996). Briefly, an exactly weighed amount of collagen membranes (about 11 mg) was treated for 4 h at 40 °C with $NaHCO_3$ (4% (w/v) water solution; 1 mL) (Carlo Erba, Milan, Italy) and trinitrobenzensulphonic acid solution (TNBS; 1 mL) (Fluka Chemie, Buchs, Switzerland). Then, 6N HCl (3 mL) was added and the mixture autoclaved (model NF 732 FV/2, Fedegari Autoclavi, Albuzzano, Italy) for 1 h at 120 °C and 15–17 psi. The hydrolysed product was diluted with water (5 mL) and extracted with ethyl ether (5 mL). Then an aliquot of the aqueous phase (5 mL) was withdrawn and warmed at 37 °C for 15 min, cooled to room temperature and diluted again with water (15 mL). The moles of the free amino group/g of collagen were determined by spectrophotometrical determination (V-530 Jasco, Jasco Europe, Carpi, Italy) at the wavelength of 346 nm against a blank. The reference solution (blank) was prepared as above described, but adding 6N HCl before TNBS in order to block TNBS reaction with the amino groups of collagen. All the data expressed were the mean of at least five determinations.

2.3.2. Swelling ratio of collagen membranes

The water absorption test was carried out by measuring the amount of water absorbed by collagen membranes (1 cm × 1 cm; mean dry weight 12 mg) according to the method previously proposed by Nogami et al. (1969). Briefly, the membrane was placed on the glass filter of the Enslin apparatus and the volume of purified water absorbed at room temperature (25 ± 1 °C) was observed during 24 h of the experiment. After 24 h, to evaluate the behaviour of membrane (normalized as regards the weight) the water binding capacity was calculated according to Pieper et al. (1999), using the following equation:

$$W\,(\%) = \frac{W_w - W_d}{W_d} \times 100$$

where W_w and W_d are the weights of wet and dried samples, respectively.

2.3.3. Calorimetric measurements

DSC curves were recorded on a Netzsch DSC 200PC "Phox®" differential scanning calorimeter equipped with a computerized data station (Netzsch GmbH, Selb, Germany). Indium (99.99%; m.p. 156.6 °C; $AH_f = 28.45\ J\ g^{-1}$) was used to check the instrument. Briefly, about 3.5 mg of membranes was introduced in an aluminium pans and hydrated with deionised water (20 μL). The pans were hermetically sealed and kept at 4 °C for about 15 h. After this time period, the samples were equilibrated for 2 min at 10 °C, before to be heated to 100 °C at a scanning rate of $2\ °C\ min^{-1}$ using dry nitrogen flow ($20\ mL\ min^{-1}$) versus a control sealed pan containing only the same amount of deionised water (20 μL).

2.4. Preparation of membranes for cellular studies

Preliminarily, the COL/GAGs membranes were conditioned as described by Croce et al. (2004). Then, under sterile conditions, the membranes were washed twice for 30 min and soaked overnight in distilled water. In the morning, the pH of water was checked and the membranes were washed with PBS three times for 15 min, conditioned in growth culture medium, cut into small pieces of 19 mm × 20 mm and inserted in the wells.

2.5. Cell cultures

Two cell lines were used to evaluate the ability of the COL/GAGs membranes to promote the cellular growth and proliferation. The 3T6 cell line (standard fibroblast cell line) was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (Brescia, Italy). The immortalized keratinocyte cell line HaCaT (Boukamp et al., 1988) was kindly provided by Professor N.E. Fusenig (German Cancer Research Center, Heidelberg, Germany). Both the 3T6 cell line and the HaCaT cell line were cultured in Dulbecco's modified Eagle essential medium (DMEM; Euroclone Celbio, Milan, Italy) supplemented with 10% (v/v) foetal bovine serum (FBS; Euroclone Celbio, Milan, Italy), 2 mM L-glutamine, 50 UI/mL penicillin and 50 μg/mL streptomycin, and passaged every 3 days. For all experiments, $(20–25) \times 10^4$ cells were seeded in a "Chamber Slide" (Lab-Tek, Nunc, Roskilde, Denmark, 2 wells, surface area 4 cm^2) coated with COL/GAGs membranes in a total volume of 2 mL of complete culture medium. The cells were seeded on the "opaque" surface of the membranes. Cells were cultivated at 37 °C in humidified atmosphere (95% air and 5% CO_2) for 3, 6, 9 and 12 days. The culture medium was renewed every 2 days.

2.6. Cellular adhesion and morphology

Scanning electron microscopy (SEM) was used to analyse (1) the morphology of the membranes and (2) the cell adhesion and density following seeding of the cells.

Both cellular adhesion and morphology were evaluated at culture day 3. COL/GAGs membranes with and without cells were washed with PBS and fixed in 2.5% (w/v) glutaraldehyde in 0.1 M Tyrode's solution, pH 7.3. After 24 h, both samples were washed in PBS and dehydrated in ethanol (from 30 to 100% (v/v) ethanol in aqueous solution). Then, the membranes were "critical point" dried, mounted on aluminium stubs (TAAB Laboratories Equipment, Berks, UK) using a double side sticky tab (TAAB Laboratories Equipment), vacuum coated with a 10 nm gold palladium in an argon atmosphere (Emitech-K550 Sputter Coater, Houston, TX, USA) and observed in a SEM (XL-30, FEI Company, Oxford Instruments).

2.7. Cellular viability assay

Cell proliferation was evaluated using the colorimetric assay (MTT; 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma Chemical, St. Lewis, MO, USA) that is based on evaluation of mitochondrial (metabolic) activity in the cell culture; the MTT tetrazolium dye is reduced by live cells to a purple formazan product insoluble in aqueous solution (Mosmann, 1983; Ciapetti et al., 1993). The amount of generated formazan is directly proportional to the number of viable cells. Briefly, at 3, 6, 9 and 12 days of culture, the medium was removed and the cells growth with and without COL/GAGs membranes, were washed in PBS; 2 mL of medium containing 200 μL MTT reagent (5 mg/mL in PBS) was added to the wells. After 6 h in the dark (at 37 °C, 5% CO_2 and 95% relative atmospheric humidity) the membranes were transferred into a clean cell-culture plates and 2 mL of dimethyl sulphoxide (DMSO) were added to each well to dissolve purple crystals of formazan; the spectrophotometrical absorbance of the solutions was measured at a wavelength of 540 nm. The results were expressed as optical density (OD) after blank (free COL/GAGs membranes) subtraction. All the experiments were repeated at least thrice and the values were expressed in the form of mean ± SD. Statistical analysis was performed using *t*-test, with the significant level set as $p < 0.05$. Statistical significance was discussed only regarding the results obtained versus COL membrane.

3. Results and discussion

3.1. Physico-chemical characteristics of COL/GAGs membranes

Physico-chemical characteristics of COL/GAGs membranes are shown in Table 1.

In order to evaluate the membrane properties for tissue engineering, the ability of a membrane to preserve water is an important aspect. All samples rapidly absorbed great amounts of water and no significant differences in water binding capacity were observed between COL/GAGs and COL membranes.

The behaviour of the membrane as function of temperature was evaluated by DSC. As described by Mentink et al. (2002), when collagen in hydrated state is heated, the crystalline triple helix of the collagen is transformed into amorphous random coil, resulting in shrinkage of the collagen. Typical parameters derived from DSC thermogram describing the phase transition, are reported in Table 1. Particularly, T_{onset} (the intersection point between the base line and the linear section of the ascending endothermic curve) was chosen to be representative for the denaturation temperature. In fact T_{onset} is less influenced by changes in methods parameter like scan rate than T_{peak} (maximum temperature of denaturation). COL membrane had a denaturation temperature close to 42 °C. Thermal behaviour of COL/GAGs membranes vary in function of the type of GAGs used in the preparation. In fact, COL/DS membrane showed a slightly increase of the collagen denaturation temperature (about 44 °C) while the COL/LMWDS membrane showed a more weakly degree of the collagen denaturation temperature (about 38 °C). The presence of UFH markedly increased the collagen denaturation membrane over 48 °C, while both the LMWH and the OLIGOH do not remarkably affect the collagen transition. Besides, the presence of GAGs influences the DSC curves (Fig. 1). Both the peak shape and width of all COL/GAGs samples showed differences, respect to the COL membrane. From Fig. 1, the broadening and the distortion of the peaks especially in the COL/UFH and COL/LMWDS curves can be observed. Then, the transition enthalpies increased in comparison of COL membrane in all the samples, chiefly in COL/LMWDS (Table 1).

No remarkable differences in the free amino group content were observed between the different membranes (Table 1).

3.2. Morphological analysis of collagen-based modified membranes

As previously reported, the superior and the lowest surface of the collagen membranes showed a different organization. Bright surface (in contact of the support during preparation) is smooth and characterized by fibers arranged in parallel way; the opaque (in contact with air during preparation) is roughly owing to disorganized and random disposed fibers (Croce et al., 2004). Owing to the less organization, the cells have been seeded on the "opaque" surface of the membranes. Analysing this surface, the collagen-based modified membranes vary in superficial characteristics in function both of type and concentration of GAGs added during preparation. The opaque surface of COL/DS membrane appeared homogeneous (Fig. 2d). On the contrary, the surface of COL/LMWDS membrane varied significantly if compared to COL membrane (Fig. 2a); as shown in Fig. 2g the surface was extremely disorganised and characterized by a tangle of thin fibers.

As well described by the magnifications reported in Fig. 3, the "opaque" surface of COL/Heparin membranes (particularly COL/LMWH and COL/OLIGOH) was characterized by having a roughness morphology with irregular texture. The fibers cannot be clearly pointed out, but we can recognize the presence of cluster emerging from an indistinct structure.

3.3. Cell cultures on membranes

3T6 (fibroblast) and HaCaT (keratinocyte) cells seeded on COL/GAGs membranes were observed by SEM and the proliferation compared with the control group (cells grown on COL membrane) during culture day 3. The density of cells on the samples was used as an indirect assessment of cell adhesion/proliferation. The images demonstrated that both 3T6 and HaCaT cells were adherent and still isolated to the COL membrane; 3T6 cells were elongated (Fig. 2b) and HaCaT cells characterized by a spindle-shaped structure (Fig. 2c). At the day 3 of culture both 3T6 and HaCaT cells showed better growth on COL/DS than that obtained on COL membrane. 3T6 cells formed a "textured" surface on COL/DS membrane and near a filamentous structure we observed round cells (Fig. 2e). Notwithstanding, HaCaT cells tended to form a multilayered and

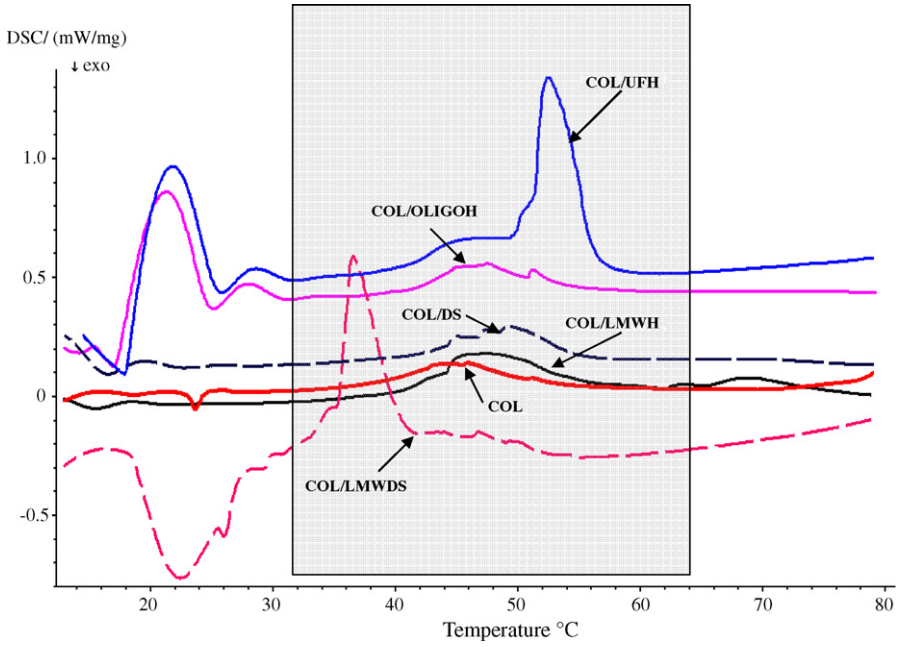

Fig. 1. DSC thermograms of COL and COL/GAGs membranes (COL/DS, COL/LMWDS, COL/UFH, COL/LMWH, COL/OLIGOH).

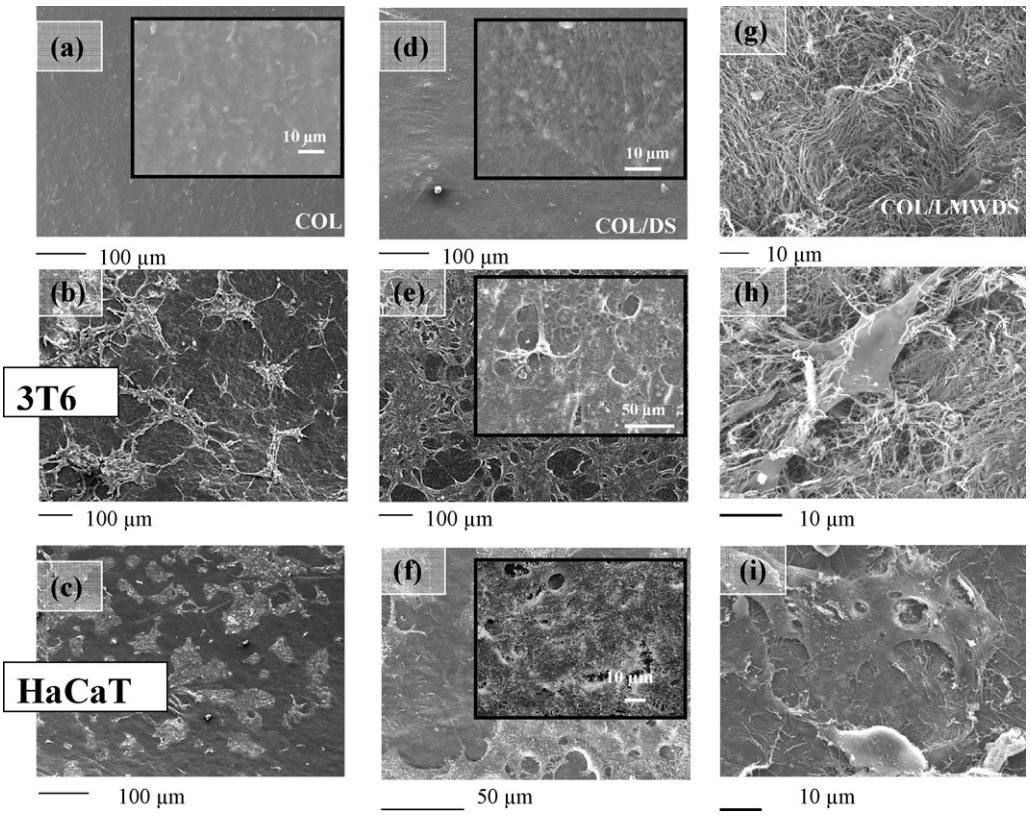

Fig. 2. Scanning electron micrographs of COL and COL/dermatan sulphate membranes [first line: (a) COL, (d) COL/DS, (g) COL/LMWDS] and 3 days after having seeded the cells [second line, 3T6 cells: (b) COL, (e) COL/DS, (h) COL/LMWDS and third line, HaCaT cells: (c) COL, (f) COL/DS, (i) COL/LMWDS]. In the panel the magnification (1500–2000×) of a scanning area.

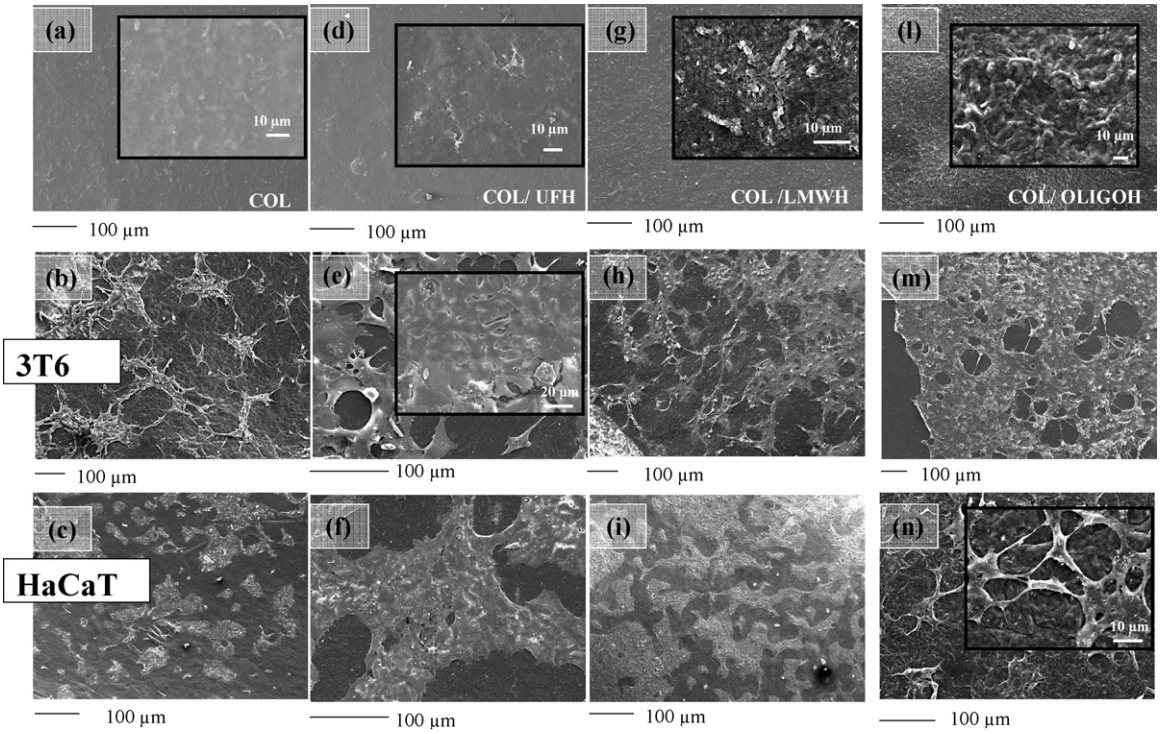

Fig. 3. Scanning electron micrographs of COL and COL/Heparin membranes [first line: (a) COL, (d) COL/UFH, (g) COL/LMWH, (l) COL/OLIGOH] and 3 days after having seeded the cells [second line, 3T6 cells: (b) COL, (e) COL/UFH, (h) COL/LMWH, (m) COL/OLIGOH, and third line, HaCaT cells: (c) COL, (f) COL/UFH, (i) COL/LMWH, (n) COL/OLIGOH. In the panel the magnification (1500–2000×) of a scanning area.

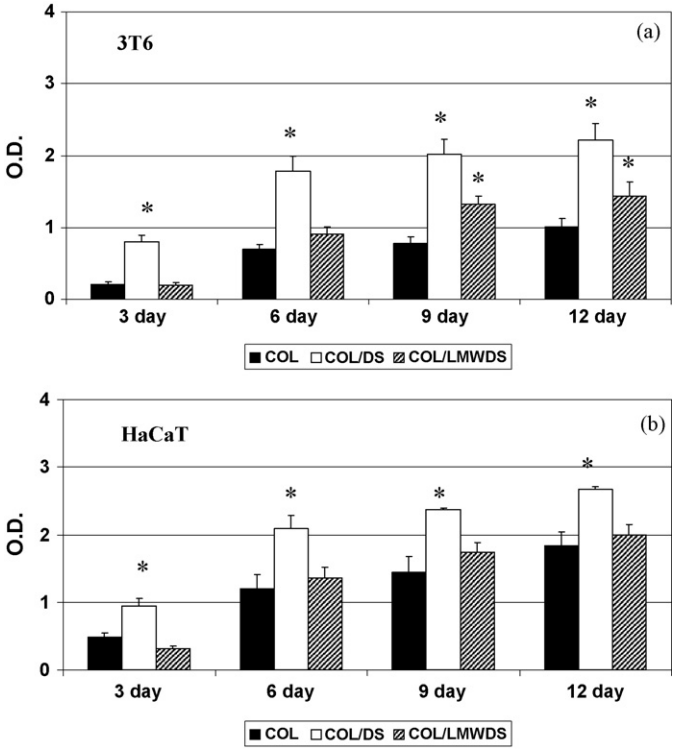

Fig. 4. Cell viability (MTT assay) of (a) 3T6 and (b) HaCaT cells cultured for 3, 6, 9 and 12 days on COL, COL/DS and COL/LMWDS membranes (*$p < 0.05$) [MTT = 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide].

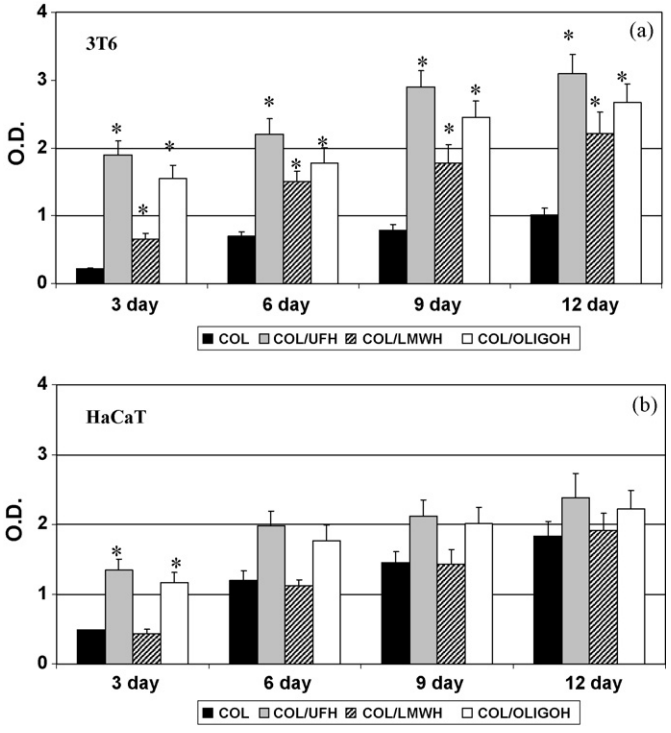

Fig. 5. Cell viability (MTT assay) of (a) 3T6 and (b) HaCaT cells cultured for 3, 6, 9 and 12 days on COL, COL/UFH, COL/LMWH, COL/OLIGOH membranes (*$p < 0.05$) [MTT = 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide].

compact surface. The number of cells was higher than the COL membrane (90% cellular confluence) and the evaluation of the morphology was difficult (Fig. 2f). Both 3T6 and HaCaT cells grown on low molecular weight dermatan sulphate collagen membrane (COL/LMWDS) were found to have lower densities if compared with the collagen membrane (COL). Notwithstanding, both the 3T6 and the HaCaT cells tended to penetrate the confused fibrotic surface of the COL/LMWDS membrane, the physico-chemical characteristics of this support do not provide a microenvironment able to promote the cell proliferation (Fig. 2h and i).

Cell proliferation on COL/Heparin membranes is reported in Fig. 3. Density of 3T6 cells on all COL/Heparin membranes (Fig. 3e, h and m**)** was sharply increased and significantly higher than that recorded on collagen membrane (COL) (Fig. 3b); moreover a compact cell-to-cell packing was observed (cellular confluence near 90%). Also HaCaT cells tended to form a compact monolayer on COL/Heparin membranes (Fig. 3f, i and n) in which the cells showed several contacts among both themselves and with the substrate.

3.4. Fibroblasts and keratinocytes proliferation

Both the cell growth and viability were analyzed by the MTT method. The cell proliferation evaluated by MTT test at 3, 6, 9 and 12 days after the seeding of cells on COL/DS and COL/LMWDS membranes is reported in Fig. 4. The proliferation curves, related to the experiments carried out on COL/LMWDS, were similar with both 3T6 and HaCaT (Fig. 4a and b) and no significant differences were observed between cells cultured on collagen membrane (COL). On the contrary, using 1% of DS on membrane preparation (COL/DS), the growth of both 3T6 and HaCaT cells steadily increased from day 3 after cell seeding to culture day 12. In fact, the cell proliferation was found to be significantly higher when compared to the control (COL membrane).

Fig. 5 shows the cell proliferation on COL/Heparin membranes. Heparins tend to promote proliferation of 3T6 cells to a greater level on all tested membranes, when compared to the control (COL membrane) (Fig. 5a). Particularly, the growth of these cells on membranes, prepared using UFH and OLIGOH as additives, was sharply increased already after culture day 3; a lower and slower 3T6 cells growth was supported incorporating the LMWH into collagen membrane (COL/LMWH).

On the contrary, the HaCaT proliferation seeded on the different COL/Heparin membranes showed low differences, when compared to the control (COL) (Fig. 5b). As we observed an initial increase of HaCaT proliferation both on COL/UFH and COL/OLIGOH membranes, the trend of cell proliferation on COL/LMWH membrane was similar and, considering the standard deviation, comparable with the control's one.

4. Discussion

Collagen is the major component of the extracellular matrix and is well recognised as an ideal material for tissue engineering and wound dressing application (Nair and Laurencin, 2007). Many studies have highlighted the importance of scaffolds prepared conjugating collagen and GAGs in order to optimize the tissue engineering applications. Particularly, the association of heparin and dermatan sulphate with the collagen plays a key role in binding the growth factor and cell surface ligands (Tierney et al., 2009). This study demonstrated that fibroblasts and keratinocytes cultured on COL/GAGs membrane showed several differences in term of rate and density of cell proliferation. Besides, in our experiments we correlated the molecular weight of dermatan sulphate and heparins with the cell ability to adhere and proliferate on the collagen modified membranes. The type of cells used represents a realistic model to evaluate the suitability of the biomaterials as tissue substitute for the *in vivo* applications (Johnen et al., 2008).

Adhesion, cell morphology, density and proliferation of cells seeded into COL/GAGs membranes were compared with the control represented by the culture on COL membrane.

The COL/GAGs membranes were fabricated using only a selected stabilized COL/GAGs suspension. Parameters such as morphology, water binding capacity, denaturation temperature and amino group content were assayed in order to characterize the new membranes and to define them as new materials for tissue engineering.

SEM culture images at day 3, after the addition of cells, suggested a different cell density on collagen membrane prepared using dermatan sulphate. Using SEM observation, it has been elucidated that both the fibroblasts and the keratinocytes cultured on COL/DS membrane showed higher proliferation, if compared with the COL/LMWDS membrane. The thermal behaviour of COL/LMWDS membrane confirmed that a different molecular arrangement and stabilization occurred in this sample when compared both to COL and COL/DS membranes. In fact, the presence of this GAG into collagen membrane changed the typical DSC thermogram of collagen; there was a significant effect both on peak (shape and width) and ΔH, indicating a different molecular organization of collagen chains. Besides, as demonstrated for chitosan/collagen sponges (Arpornmaeklong et al., 2007), the microstructure of membrane surface influences both the attachment and the growth of cells. Presumably, the irregular structure of COL/LMWDS membrane hindered cell binding. On the contrary, the structural homogeneity was maintained when high molecular weight dermatan sulphate (DS) was added to collagen membrane and in parallel the density of cells tended to increase. The cells were able to adhere and proliferate on COL/DS membrane with the best viability observed; SEM images revealed that close to the elongated structure, fibroblasts seeded on COL/DS membrane exhibited the round structure that can be ascribed to recently divided cells by mitosis (Silvério et al., 2007) suggesting a relevant mitogenic activity. These data were confirmed by the MTT assay; whether fibroblasts or keratinocytes cultured on COL/DS membrane exhibited higher absorbance compared with the control (COL membrane).

COL/Heparin membranes exhibited similar physico-chemical parameters and a regular and well-organized structure allowing an effective cell seeding and proliferation. Density and proliferation of fibroblasts on COL/Heparin membranes were strongly enhanced when compared both to COL and to COL/DS membranes. Both SEM analysis and MTT assay revealed that COL/UFH membrane seemed particularly suitable for 3T6 adhesion and proliferation. On the contrary, the proliferation of keratinocytes on COL/Heparin membranes was slightly affected by the molecular weight of GAG used and not superior to that recorded on COL membrane. Certainly, low molecular weight heparins (LMWH, ≈5 kDa, and OLIGOH, ≈3 kDa) showed different behavior in MTT assay. Along with the desirable pharmacokinetics and pharmacological properties as antithrombotic agent (Bianchini et al., 1995), OLIGOH seemed to provide for a better ability in promoting both the cell interaction and the proliferation on COL membranes than the LMWH.

Therefore, these *in vitro* experiments suggested that is important to evaluate the molecular weight of GAGs. It can be concluded that the use of COL/DS membrane supported proliferation both of fibroblasts and keratinocytes whereas COL/Heparin membranes created a good microenvironment allowing an effective cell seeding and proliferation only of fibroblasts if compared with COL membrane. Particularly, a combination of membrane prepared adding high molecular weight dermatan sulphate (DS) and high molecular weight heparin (UFH) should create an appropriate environment for the growth of fibroblasts and keratinocytes, providing an epidermal substitute.

Acknowledgements

The authors wish to thank Prof. R. Tiozzo and Prof. R. Battini (Department of Biomedical Sciences) for their advice on cellular experiments.

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