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Effects of fibronectin and laminin on structural, mechanical and transport properties of 3D collageneous network

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Abstract Recent studies, on cells cultured in 3D collagen gels, have shown that, beside from their well known biochemical role, fibronectin (FN) and laminin (LM) affect cell functions via a modification of mechanical and structural properties of matrix due to interaction with collagen molecules. Though biochemical properties of FN and LM have been widely studied, little is known about their role in collagen matrix assembly. The aim of this work was to characterize FN- and LM-based collagen semi-interpenetrating polymer networks (semi-IPNs), in order to understand how these biomacromolecular species can affect collagen network assembly and properties. Morphology, viscoelasticity and diffusivity of collagen gels and FN- and LM-based collagen semi-IPNs were analysed by Confocal Laser Scanning microscopy (CLSM), Environmental Scanning Electron microscopy (ESEM), Transmission Electron microscopy (TEM), Rheometry and Fluorescence Recovery After Photobleaching (FRAP) techniques. It was found that FN and LM were organized in aggregates, interspersed in collagen gel, and in thin fibrils, distributed along collagen fibres. In addition, high FN and LM concentrations affected collagen fibre assembly and structure and induced drastic effects on rheological and transport properties.

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Introduction

Recently, some studies have focused their attention to elucidate the role of fibronectin (FN) and laminin (LM), two extracellular matrix (ECM) components, in affecting cell behaviour in 3D matrices both *in vivo* and *in vitro* [1–5]. These results indicate that FN and LM are involved in many cellular processes. In particular, FN sometimes acts as a general cell adhesion molecule by anchoring cells to ECM molecules like collagen or proteoglycans. It also can serve to organize cellular interaction with the ECM by binding to different components of the extracellular matrix and to membrane-bound FN receptors on cell surfaces [6]. Furthermore the role of FN in cell migration events is fundamental during embryogenesis [1]. On the other hand, also laminin exhibits a variety of biological activities, including stimulation of growth and differentiation, neurite outgrowth promotion, and mediation of cell communication. Moreover, laminin is the first ECM protein detected during embryogenesis; it is present at the two-cell stage in the mouse embryo. In later development and in mature tissues it serves as an ubiquitous and major non-collagenous component of basement membranes [7–9]. It participates in the assembly of this specialized form of the ECM and mediates cell attachment and maintenance of the differentiated state of epithelial and endothelial cell layers that are intimately associated with their basement membranes [10]. Furthermore, FN and LM can interact with other ECM constituents, thanks to their complex molecular structures [11].

Recent studies have hypothesized that the effects of FN and LM on cell functions derives, not only from their well-known biochemical properties, but also from a modification of mechanical and structural properties of matrix due to FN and LM interaction with collagen molecules [4, 5]. Collagen is the most abundant protein in the mammalian organisms

and it is the major fibrous component of the ECM of the connective tissue providing tissue strength. Moreover, collagen is able to interact with nearly 50 different molecules [12] and to *in vitro* self-assemble.

Mechanistically, collagen fibrillogenesis is described as a multi-step process. The first step is the assembly of short, small diameter fibril intermediates or segments [13, 14], that are formed by assembly of procollagen/collagen molecules. The subsequent collagen fibre assembly, starting from these immature fibril intermediates, has been reported to occur following two models [15]. According to the first one, fibril segments can laterally associate and fuse together [16–18]. Conversely, the alternative model of fibril growth is an accretion model in which collagen fibril segments linearly grow as monomeric collagen is added [19–21]. The fibril growth step involves a regulated balance between linear and lateral growth both *in vivo* and *in vitro*. During this phase, the interaction of immature fibrils with specific macromolecules, associated with the fibril surface, can control fibril growth. These molecules include the small leucine-rich repeat proteoglycans, known to associate with collagen fibrils [22–24] and another class of fibril-associated molecules, the FACIT collagens [25–30].

Nevertheless it is well-known that FN and LM can interact with collagen molecules, a full structural characterization of collagen scaffolds, in presence of FN and LM, has not been performed. Consequently, the way by which FN and LM can influence collagen assembly has not yet been understood.

The aim of this work is to characterize collagen gels and collagen FN- and LM-based semi-IPNs, in order to understand how these biomacromolecular species can affect collagen network assembly and properties. Morphological, viscoelastic and diffusion properties of collagen gels and FN- and LM-based semi-IPNs were respectively analysed by Confocal Laser Scanning microscopy (CLSM), Environmental Scanning Electron microscopy (ESEM), Transmission Electron microscopy (TEM), Rheometric techniques and Fluorescence Recovery After Photobleaching (FRAP) techniques.

Materials and methods

Materials and reagents

Pepsin-solubilized bovine dermal type I Collagen (Vitrogen, Angiotech Biomaterials, Corp., Palo Alto, CA) was prepared following manufacturer's procedures. Briefly, 1 ml of 10X DMEM (Gibco, Life Technologies) was added to 8 ml collagen (stock solution: 3 mg/ml). Next, 1 ml of 0.1 N NaOH was added and pH 7.2 was reached by adding 0.1 N HCl. The solution was incubated at 37°C for 45 min to allow collagen fibrillogenesis.

Fibronectin and laminin-collagen semi-interpenetrating gels (networks)

For laminin (LM) and fibronectin (FN)-based semi-interpenetrating polymer networks (semi-IPNs), fibronectin and laminin-1 (Sigma) were added to collagen solution (1.2 mg/ml) before fibrillogenesis was initiated. The final concentrations of fibronectin and laminin were 10, 50, 100 μg/ml. The solutions were incubated at 37°C for 45 min to allow collagen fibrillogenesis. After the incubation, the semi-IPNs appeared as a hydrated gel.

Diffusing probe

Fluorescently labelled dextrans of 500 kg/mol (Molecular Probe Inc. - Eugene, OR) were used as diffusing probes. The collageneous samples were soaked overnight in a PBS phosphate buffer solution (pH 7.4) containing the fluorescent probe (0.1 mg/ml).

Indirect immunofluorescence

Samples were prepared in chamber slides, fixed with 4% paraphormaldeyde for 20 min at RT, rinsed twice with PBS buffer and incubated with PBS-BSA 0.5% to block unspecific binding. The primary antibodies, mouse anti-collagen type I (Chemicon), mouse anti-FN (Sigma) and rabbit anti-LM (Sigma), diluted in PBS-BSA 0.5%, were incubated for 1h at RT. Samples were then rinsed 3 times with PBS-BSA 0.5% and incubated with secondary anti-mouse fluorescein-isothiocyanate (FITC) and anti-rabbit tetramethylrhodamine isothiocyanate (TRITC) antibodies (Chemicon), respectively. Finally, samples were rinsed with PBS.

Fluorescence analyses were performed by using a confocal microscope Zeiss LSM 510, equipped with an argon laser, at a wavelength of 488 nm, and a He-Ne laser, at a wavelength of 543 nm, and objectives 10 and 20X. Images were acquired with a resolution of 512 × 512 or 1024 × 1024 pixels. The emitted fluorescence was detected using filters LP 505 and HFT 488 for FITC and BP 560–600 and HFT 488/543 for TRITC.

ESEM analyses

For environmental scanning electron microscopy (ESEM), collagen matrices were fixed with 2.5% glutaraldehyde and dehydrated in increasing ethanol series (70%, 80%, 95% and 100%). Samples were then analyzed by ESEM Quanta

200 (FEI Company), using low-vacuum mode (0.75 Torr), at 10 kV and 7.7 mm working distance.

TEM

For structural analysis, small blocks of the gels were fixed for 60 min in 3% glutaraldehyde containing 0.05% tannic acid buffered with 0.1 M cacodylate buffer, pH 7.4. Samples were then washed in 0.1 M cacodylate buffer, post fixed for 60 min in 0.1 M cacodylate buffered OsO_4, rinsed again in buffer, and then dehydrated in a graded series of ethanol to 100%. The gels were washed in 100% propylene oxide then infiltrated and embedded in Spurrs epoxy.

For immuno-electron microscopy, small blocks of the gels were fixed for 60 min in 0.1 M cacodylate buffered 0.1% glutaraldehyde then rinsed extensively in buffer followed by a rinse in Dulbecco's Modification of Eagle's Medium (DMEM). The gels were then incubated in either Rabbit anti-laminin (Sigma L9393) or Mouse anti-fibronectin (Sigma F0791) antibody diluted 1:5 in DMEM overnight at 4°C, rinsed extensively in DMEM then immersed in appropriate Goat anti-mouse or anti-rabbit one nm gold secondary conjugate (Amersham Biosciences) overnight at 4°C, rinsed in PBS several hours, then gold enhanced using a Nanoprobe gold enhancement kit. Samples were rinsed again in SFM, fixed in 1.5% glutataldehyde/1.5% paraformaldehyde containing 0.05% tannic acid, then post fixed in OsO_4 and finally dehydrated and embedded as described above. For all samples, 60–80 nm thick ultrathin sections were contrasted in Uranyl Acetate and Reynold's lead citrate and observed using a Philips EM 410LS transmission electron microscope. Collagen fibre analyses were carried out on at least 50 images of every sample.

Rheological measurements

The collagen gel and FN- and LM-based collagen semi-IPNs rheological properties were evaluated by small amplitude oscillatory shear tests using a stress controlled rotational rheometer (GEMINI Bohlin instruments, Malvern, UK), in a parallel plate geometry (15 mm of diameter). The instruments was preheated to 37 ± 0.01°C and maintained at constant temperature throughout the tests. The gels were prepared as described above and placed on the plate filling about 1 mm gap. A humid environment was used to prevent evaporation during the measurements. G' and G'' moduli were evaluated as function of frequency.

Preliminary strain sweep tests at a fixed oscillation frequency (consisting in monitoring the properties while logarithmically varying the strain amplitude) were performed on the materials to determine the strain amplitude range at which linear viscoelasticity holds [31]. The oscillation frequency has been varied from 0.1 to 1 Hz. The tests were repeated at least four times.

Diffusion coefficient measurements

The diffusion coefficients of the probes were measured by Fluorescence Recovery After Photobleaching (FRAP) technique [32, 33]. The home-made apparatus was composed of a direct microscope (AX60 Olympus), a mercury lamp (100 W; USH-02D Ushio), a monochromatic argon laser (488 nm; Innova 90-2) supplied of shutters and spatial filter (100 μm; M900 Newport) to bleach the fluorescence, a CCD camera (PENTAMAX, Princeton Instruments) controlled by a computer. The diffusion coefficient was evaluated by a Spatial Frequency Analysis (SFA) [34–37]. The experiments were performed by using Metamorph software (Universal Imaging Corp.) and the data were analysed through a Matlab (MathWorks, Inc.) program specifically developed.

Results

Distribution of FN and LM in collagen gels and in FN- or LM-based semi-interpenetrating networks (semi-IPNs)

In order to investigate the microscopic structure of gels and semi-IPNs, we assessed spatial distribution of collagen, FN and LM in the gels, by immunofluorescence analyses. While collagen was homogeneously distributed either in collagen gels and in FN-and LM-based semi-IPNs (Fig. 1 panel A–D and I–L), FN and LM resulted localized in discrete spots (Fig. 1 panel F–H and N–P). The spots appeared heterogeneous in shape and size and their number increased with increasing FN or LM concentrations. In addition, in presence of LM, some thin fibrillar structures were detected (Fig. 1 panel N-P). Control panels are reported (Fig. 1 panel E and M), where no signal was observed when anti-FN and anti-LM antibodies were used on collagen gels. These results suggest that LM and FN proteins are not uniformly distributed throughout the gel and tent to aggregate in macromolecular structures.

Morphological analyses of collagen gels and FN- or LM-based semi-IPNs

ESEM

To better analyse the network structure formed in collagen gels and semi-IPNs, we performed ESEM analyses on collagen gels and LM- or FN-based semi-IPNs with LM and FN ranging from 10 to 100 μg/ml. Collagen matrices had loose network areas (Fig. 2(A)) and very homogeneous fibres diameters, approximately 100 nm (Fig. 3). FN concentrations

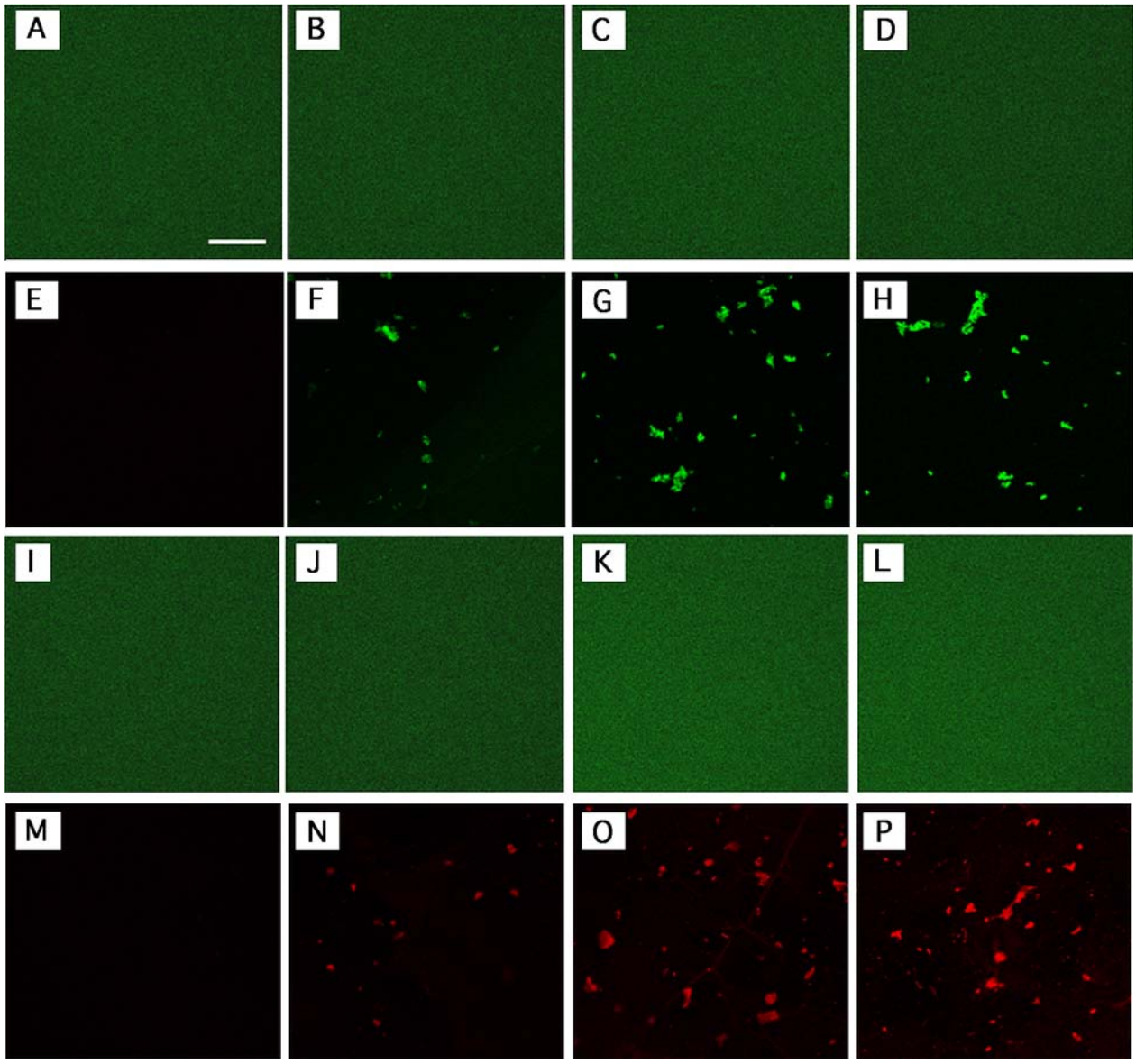

Fig. 1 Indirect immunofluorescence of collagen gels and FN- and LM-based semi-IPNs. A, E, I and M. 1.2 mg/ml collagen gel; B and F. 1.2 mg/ml collagen gel + 10 μg/ml of FN; C and G. 1.2 mg/ml collagen gel + 50 μg/ml of FN; D and H. 1.2 mg/ml collagen gel + 100 μg/ml of FN; J and N. 1.2 mg/ml collagen gel + 10 μg/ml of LM; K and O. 1.2 mg/ml collagen gel + 50 μg/ml of LM; L and P. 1.2 mg/ml collagen gel + 100 μg/ml of LM. A, B, C, D, I, J, K, L were treated with anti-collagen antibody. E, F, G, H were treated with anti-FN antibody. M, N, O, P were treated with anti-LM antibody. (Bar 100 μm)

of 10 and 50 μg/ml did not substantially alter the network structure of collagen gels (Fig. 2(B) and (C)). Conversely, at FN concentrations of 100 μg/ml, the structure of the gels appeared more heterogeneous, with some amorphous regions and less defined fibre boundaries (Fig. 2(D)). In addition, there were some regions where areas of amorphous material were interspersed in collagen fibres. Similar results were obtained for LM-based semi-IPNs (Fig. 2(E)–(G)), also for these semi-IPNs, only higher LM concentrations led to a modification of the network structure.

TEM

To gain more information on single fibre structure, we performed TEM analyses. In collagen gels and in semi-IPNs at low FN and LM concentrations, fibril cross-sections were irregular and appeared to be fusion products of smaller fibrils (Fig. 4(A) and (C)). Conversely, the addition of increasing amount of FN and LM induced modification in fibrils structure, leading to linear fibrils with a regular circular cross-section profile (Fig. 4(B), (D) and (E)). Moreover, in presence

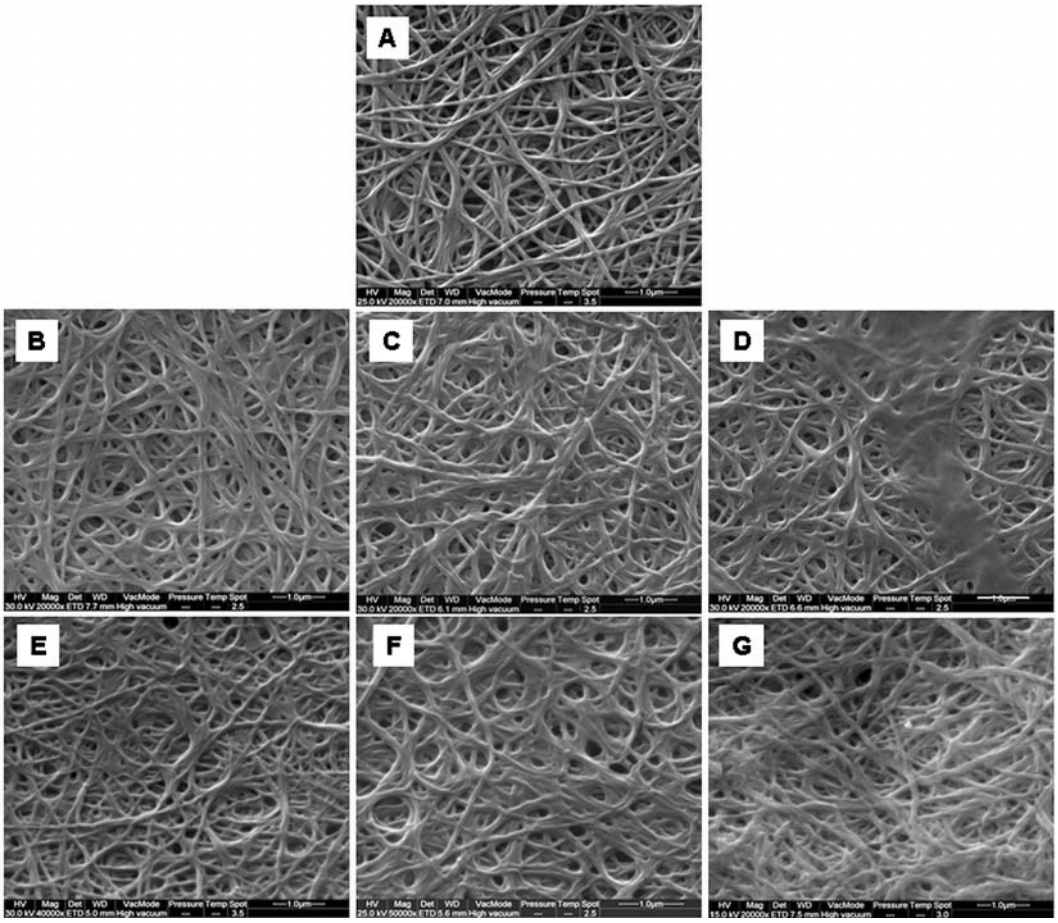

Fig. 2 ESEM micrographs of collagen gels, FN-based semi-IPNs and LM-based semi-IPNs. A. 1.2 mg/ml collagen gel; B. 1.2 mg/ml collagen gel + 10 μg/ml of FN; C. 1.2 mg/ml collagen gel + 50 μg/ml of FN; D. 1.2 mg/ml collagen gel + 100 μg/ml of FN; E. 1.2 mg/ml collagen gel + 10 μg/ml of LM; F. 1.2 mg/ml collagen gel + 50 μg/ml of LM; G. 1.2 mg/ml collagen gel + 100 μg/ml of LM. (Magnification ×20,000)

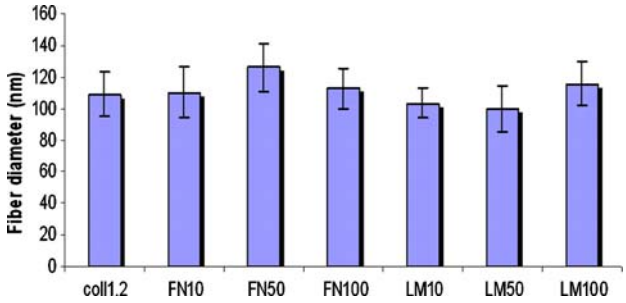

Fig. 3 Diagrams of collagen fibres diameters in collagen gels and in FN- or LM-based semi-IPNs

of FN or LM, thin fibrils were detected along collagen fibres (Fig. 4(B) arrows).

In order to investigate FN and LM distribution and organization in collagen gels, we performed immunogold labelling of TEM sections with anti-FN and anti-LM antibodies (Fig. 5). In LM-based semi-IPNs, LM formed thin fibrils along the collagen fibrils (Fig. 5(C)–(E)). In addition, in agreement with immunofluorescence results, anti-LM antibodies reactivity was localized around amorphous structures, indicating thus the existence of discrete aggregates of laminin interspersing in 3D collagen structure. Conversely, no signal was detected in the control collagen gels and FN-based semi-IPNs treated with anti-LM antibodies (Fig. 5(A) and (B)). FN was similarly distributed, being localized along collagen fibrils as thin fibrils (not shown).

Rheological analyses of collagen gels and FN- or LM-based semi-IPNs

The structural results obtained indicated that high FN and LM concentrations affected collagen structure. In order to investigate if the structural modifications altered the mechanical properties of the semi-IPNs, we performed rheological analyses. In Fig. 6 it is shown the mechanical spectra of the collagen/LM and collagen/FN semi-IPNs (50 μg/ml of FN or LM) while in Table 1 the value of the G′ at (1 Hz) for the different gels are reported. The rheological behaviour was typical for weak gel material, with G′ one order of magnitude higher than G″ and both moduli almost frequency independent. With the addition of laminin or fibronectin, the

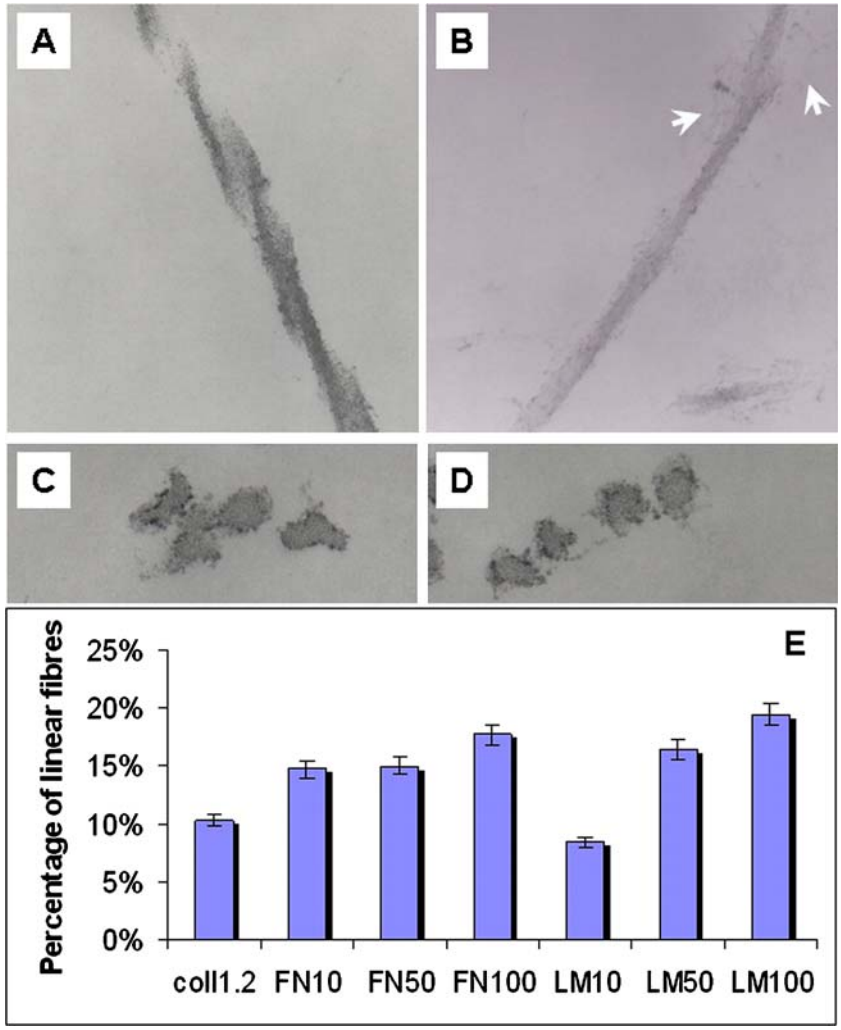

Fig. 4 TEM micrographs of collagen gels, FN-based semi-IPNs and LM-based semi-IPNs. A. 1.2 mg/ml collagen gel; B. 1.2 mg/ml collagen gel + 100 μg/ml of LM; C. 1.2 mg/ml collagen gel (fibril cross sections); D. 1.2 mg/ml collagen gel + 100 μg/ml of LM (fibril cross sections); E. Dependence of collagen fibres shape on collagen gel composition. (Bar 500 nm)

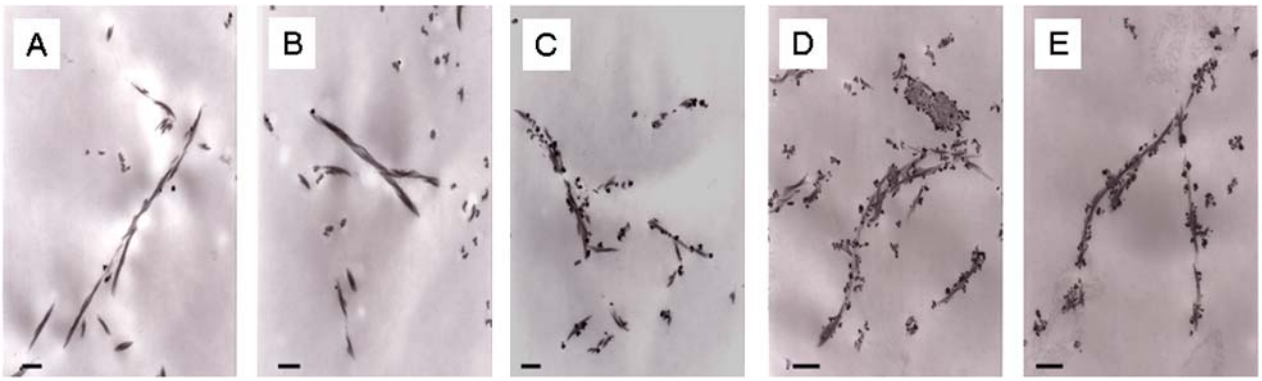

Fig. 5 Immunogold labelling of FN and LM in collagen gels, FN-based semi-IPNs and LM-based semi-IPNs. A. 1.2 mg/ml collagen gel; B. 1.2 mg/ml collagen gel + 50 μg/ml of FN; C. 1.2 mg/ml collagen gel + 10 μg/ml of LM; D. 1.2 mg/ml collagen gel + 50 μg/ml of LM; E. 1.2 mg/ml collagen gel + 100 μg/ml of LM. Samples treated with anti-FN antibody. (Bar 500 nm)

collagen gels still behaved as "weak gel" (Fig. 6), while the elastic modulus values changed. Moreover LM and FN-based semi-IPNs produced similar response to shear: the addition of small amounts of laminin or fibronectin (<=50 μg/ml) caused no significant changes in both moduli, whereas larger amounts (>=100 μg/ml) resulted in a substantial moduli decrease over the plain collagen gels. Indeed, G′ was about 16 ± 1.12 Pa for the gel at 10 μg/ml of FN and LM as the

Table 1 Storage moduli at 1 hz for 1.2 mg/ml collagen gel with varying concentration of fibronectin and laminin

Amount of FN or LM (μg/ml)	G′ gel with LM (Pa)	G″ gel with LM (Pa)
0	16	16
10	16	14.7
50	12.9	12.1
100	5.4	5

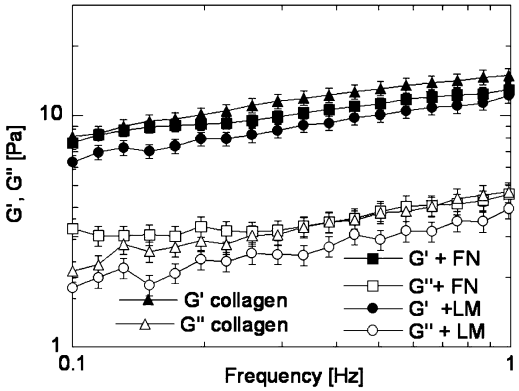

Fig. 6 Storage and loss moduli as function of frequency for collagen gel (1.2 mg/ml) added with 50 μg/ml of fibronectin or laminin ($T = 37\ ^{\circ}$C)

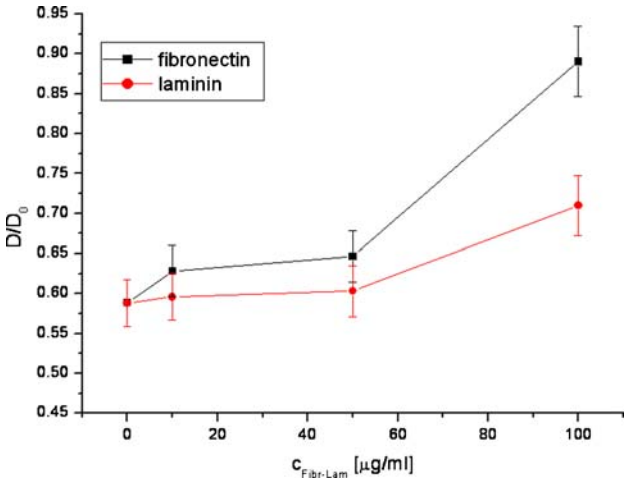

Fig. 7 Diffusion coefficient of dextran (500 kg/mol) in fibronectin (square)—and laminin (circle)—based semi-IPN, normalized respect its value in water, versus additive concentration

control, while it decreased to about 5 ± 0.35 Pa for the gel at 100 μg/ml of FN and LM (Table 1).

FRAP analyses of collagen gels and FN- and LM-based semi-IPNs

The transport properties of the semi-IPNs were investigated by means the diffusion coefficient of the dextran (500 kg/mol), within the different matrices (Fig. 7). The collected data indicated that the molecular diffusivity was not affected by low LM and FN concentrations. Conversely, at higher concentration (100 μg/ml), the diffusivity increased. The effect was more evident for FN. The dextran diffusion coefficient, indeed, increased from $9.65 \pm 0.13 \cdot 10^{-7}$ cm^2/s in pure collagen to $1.16 \pm 0.25 \cdot 10^{-7}$cm^2/s at high LM concentration and to $1.46 \pm 0.14 \cdot 10^{-7}$ cm^2/s at high FN concentration.

Discussion

Immunofluorescence results showed that FN and LM, two fibrillar proteins, were not homogeneously distributed in collagen gel, but formed discrete amorphous aggregates interspersed in the network (Fig. 1). These aggregates increased in number and dimension at increasing of FN and LM concentrations. The presence of FN and LM aggregates was demonstrated also by ESEM analysis that revealed no morphological change of collagen gel structure in presence of lower FN and LM concentrations (10 and 50 μg/ml) while, at higher concentrations of FN and LM (100 μg/ml), collagen network appeared more dense and collagen fibre boundaries less defined because of amorphous material interspersed in them (Fig. 2). However, collagen fibres diameters were not particularly affected by both low and high FN and LM concentrations (Fig. 3).

Looking in deeper details at the structure of semi-IPNs, by using the TEM and immunogold analyses, it was possible to observe that FN and LM were organized, not only, in aggregates, but also, in very thin fibrils distributed along collagen fibres and closely associated to them (Fig. 5). These data suggested an effective interaction between FN and LM with collagen, likely due to specific domains present in protein molecules. Further analyses of TEM sections showed that most of collagen fibres appeared as small segments laterally apposed and fused together (Fig. 4(A)). The presence of high concentrations of FN and LM (100 μg/ml) reduced the tendency of fibrils to fuse together, promoting the formation of linear collagen fibres rather than lateral apposition of intermediate fibrils (Fig. 4). These results were in agreement with previous data described in literature. Indeed, it has been suggested that the constraints involved in incorporating a molecule that would make packing less regular, e.g. a longer type V molecule with type I collagen, would limit lateral filamentous growth [38–40]. These structural results were confirmed by the rheological and transport properties of semi-IPNs. From a mechanical point of view, indeed, the collagen gel had a "weak gel behaviour", that is G′ one order of magnitude higher than G″ and both moduli almost frequency independent and parallel to each other, typical of the physically cross-linked network (Fig. 6). The nature of crosslinks, in the case of collagen could involve disparate forces such as electrostatic, hydrophobic and hydrogen bonding

interaction arising during the fibrillogenesis [41]. The addition of fibronectin and laminin to collagen did not qualitatively alter the rheological behaviour, that was still typical of a weak gel (Fig. 6), and modify the network structure. Conversely, their addition led to a quantitative variation of the dynamic moduli (Table 1). In particular small amounts of laminin and fibronectin (10, 50 μg/ml) did not cause a significant difference in the moduli whereas larger amount (100 μg/ml) caused a progressive reduction in both dynamic moduli. These results were in agreement with the structural findings indicating that the presence of FN and LM prevented the collagen fibril intermediate lateral apposition, enhanced linear growth and, thus, led to a weaker and looser network structure. The decrease of the viscoelastic moduli were associated with an increase of diffusivity coefficient at higher FN and LM concentration (100 μg/ml). Indeed, the presence of high FN and LM concentration, inducing the formation of a looser collagen structure, resulted in a hindrance reduction.

Conclusions

In this work, the structural and mechanical characterization of FN- and LM-based collagen semi-interpenetrating polymer networks (semi-IPNs), compared to pure collagen gels, was performed in order to understand how these biomacromolecular species can affect collagen network assembly and properties. Morphological analyses suggested that FN and LM were both organized in thin fibrils, distributed along collagen fibres, and in spot like structures. The presence of FN and LM affected collagen fibril assembly and final structure, promoting linear fibril growth rather than lateral one. Moreover, at higher concentrations, FN and LM induced net effects on rheological and transport properties, decreasing the elastic modulus and increasing diffusion coefficient.

In conclusion, we demonstrated that the active interplay, between FN and LM molecules with collagen fibrils, affected structural, mechanical and transport properties of collagen gels.

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