Characterization of Composite Networks Made of Type I Collagen, Hyaluronic Acid and Decorin

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Summary: To ultimately assess the ability of macromolecular medicines e.g. liposomes, non viral gene delivery systems, to penetrate one of the least studied physiological barriers, the extracellular matrix (ECM), composite networks made of different ECM components i.e. type I collagen, hyaluronic acid and a proteoglycan, decorin, were prepared. These composite networks were characterized by rheology, Confocal Reflection Microscopy, Fluorescence Recovery After Photobleaching and Transmission Electron Microscopy. While being at the low end of its physiological concentration, collagen appears to be the backbone of the composite networks as it provides the elastic modulus. On the other hand, 15 kDa and 1.1 MDa hyaluronic acid, when present at physiological levels interpenetrate the collagen network. When approaching their overlap concentration, hyaluronic acid chains lead to an increase of the population of collagen fibrils. Finally, while decorin increased the population of fibrils in pure collagen networks, its role in presence of hyaluronic acid remains unclear as it does not alter the diameter of fibrils nor their population.

Keywords: collagen; decorin; extracellular matrix; hyaluronic acid; network

Introduction

As the interstitium and more precisely the extracellular matrix (ECM) have been recognized as one of the physiological barriers macromolecular drugs (liposomes, non viral gene delivery systems) have to overcome,[1] devising composite networks mimicking the ECM is relevant in order to evaluate the respective influence of each the components on the architecture of the ECM and ultimately their influence on novel macromolecular drugs penetration e.g. friction, density.

The ECM, a Composite Network, Hindering Macromolecular Drugs Transport

For a period of time, the ECM can be considered as a fixed composite network made of glycoaminoglycans (GAG) especially hyaluronic acid (HA), proteoglycans *e.g.* decorin and proteins such as type I collagen. Consequently, the organisation of biomacromolecules and their concentrations are expected to affect macromolecular drugs penetration. Hyaluronic acid, a glycosaminoglycan made of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine disaccharide able to have up to few MDa,[2] is likely to form entanglements. In addition to this property, its ability to bind to water and swell helped it to be considered as the main ECM component with regards to transport hindrance.[3,4] Interestingly, this concept has been challenged in the late 1990 s by Jain and co-workers[5,6] who have reported that *in vivo* tumours with a high fibrillar collagen content resulted in a high hindrance to macromolecular transport. These findings were later supported by Ramanujan et al. who correlated hindrance in collagen networks to tumours hindrance after correcting for geometric tortuosity.[7] Though these results confirm the importance of

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fibrillar collagen, it is unlikely that transport hindrance may be attributed to a single component of the ECM. Consequently, Clague et al.[8] proposed in a model that hindrance to transport is not reduced to one component but to the synergistic effect of all components *e.g.* collagen and hyaluronic acid. Though this concept is interesting, little is known on the interaction of these macromolecules and their influence on the resulting network and the resulting hindrance to macromolecular transport. Consequently, studying the formation and characterizing composite networks made of type I collagen, hyaluronic acid and, additionally decorin, a proteoglycan, without artificial cross-linking is of interest as it may lead ultimately to a better understanding of transport and drug delivery.

Previous Experimental Studies on Collagen Hyaluronic Acid Networks

Overall, to investigate the influence of ECM components, studies often relied on enzyme treatment to support their conclusions[5,6] which may affect cells and interstitial space and, consequently, limit the understanding of each ECM components influence on transport. Experimental studies have already involved collagen and HA networks: Xin et al.[9] focused on rheology and used two HA molecular weight and a low collagen concentration (2.8 mg/ml) similar to us suggested that their low molecular weight (155 kDa) interacted with collagen fibres while high molecular weight (1.2 MDa) was thought to be too slow which prevented it to mix with collagen. Recently, Salchert et al.[10] have investigated the influence of HA and heparin on the formation of fibrils and the organisation of surface bound fibrillar collagen by different microscopies. They concluded that the shape of fibrils depended on the presence of GAGs and, heparin at high concentration resulted in formation of larger fibrils.

Biopharmaceutical Relevance of the Present Work

In the present study, we chose to design composite matrices with content as close as possible to physiological properties of tumour and/or normal ECM. As we are ultimately willing to understand how biomacromolecules transport in tissue and, to address the penetration problems of novel medicines, which, to a great extent, limit the interest of the pharmaceutical industry. As a result, the present study on composite matrices not only considers collagen or hyaluronic acid contents but also the importance of HA chain length (or molecular weight) as entanglements are related to chain length[11] and tumours may present shorter HA chains than normal tissues. In fact, HA molecular weight has been correlated with the invasiveness of tumours:[12] HA chain length may vary from 20,000 Da to few MDa depending on the presence or the absence of the disease.[13–15] In addition, small HA has been observed in saliva of patients with head and neck squamous carcinomas.[16] Consequently it is likely that the presence of short HA chains will not result in the same network i.e. reduced probability to form entanglements, electrostatic interactions different mobilities of polymer chains, which, ultimately, should lead to a different hindrance to macromolecular transport. Finally, HA chains in the present study are not artificially cross-linked to collagen fibres, thus providing a more realistic approach as it also has been noted that the mobile pool of tissues is made of small molecular weight HA.[14]

Decorin, a proteoglycan thought to bind to collagen and increase the formation of bundles,[17–19] was added at a concentration (1:25) chosen on a study on the alignment and mechanical stress of collagen/decorin fibres by Pins et al.[19] Decorin was added to hopefully form large collagen fibres though reports in the literature may suggest that it may also form complexes with HA.[18]

By taking advantage of different confocal microscopy techniques such as Fluorescence Recovery After Photobleaching (FRAP) and Confocal Reflection Microscopy (CRM), Transmission Electron Microscopy (TEM) and rheology, composite networks were characterized. FRAP

has already been used to measure diffusion in HA solutions.[20] CRM which allows the observation of proteins (collagen) has been in different recent studies to observe collagen scaffold of pure collagen or in presence of HA and decorin.[21,22] Electron microscopy has been widely used to determine fibres diameter and the presence of molecules[6,9,18] while finally rheology is one of the classic tools used in polymer studies to determine gel formation or sol/gel transitions and the characterisation of these systems e.g. collagen networks.[5,23]

The aim of the present study was to prepare these composite and characterise their organisation and mutual influence. While rheology indicates that the mechanical properties are largely influenced by collagen, CRM and TEM studies point out at the influence of HA size and concentration in composite networks.

Materials and Methods

Preparation of Hyaluronic Acid Solutions

Lyophilised hyaluronic acid sodium salts (HA) 1.1 MDa (high molecular weight, HMW) and 15 kDa (low molecular weight, LMW) were purchased from Medipol SA (Switzerland) and dissolved by addition of PBS (pH = 7.4) at 4 °C overnight for a concentration range from 1 to 10 mg/mL. After being poured in a cavity slide, HA samples were incubated at 37 °C.

Preparation of Collagen Networks

Vitrogen 100 purified peptide solubilised type I bovine collagen was purchased from Cohesion Technologies (Palo Alto, CA). pH was adjusted between 7.4 ± 0.2 by addition of 0.1 M NaOH before dilution and 0.2 M after centrifugation, and of 10X phosphate buffered saline (PBS, Gibco, Invitrogen Corporation, Paisley, UK). Concentration of the solution (3–15 mg/mL) was performed by centrifugation (Labofuge 400R, Heraeus, Germany) at 25 °C at 4500 rpm in presence of 0.5 M NaCl. Following concentration of the solutions, supernatant was extracted and pellets were stored at 4 °C. UV spectroscopy (207 nm on a PerkinElmer Lambda 25 UV/VIS spectrometer) was used to calculate the collagen concentration in the supernatant and therefore in the pellets. The collagen concentration of the pellets obtained by centrifugation was adjusted by addition of PBS.

Preparation of Composite Networks

For collagen/hyaluronic acid composite networks, collagen solution were first concentrated then HA was added and the resulting solution was stirred for 15 to 30 minutes before incubation at 37 °C. For composite networks containing the proteoglycan, decorin from bovine articular cartilage was purchased from Sigma Steinheim, Germany). 0.5 mg of lyophilized decorin was dissolved with 250 μL of PBS (final decorin:collagen ratio 1:25). When decorin was added to samples based on centrifugated collagen the solution was magnetically stirred for 15 minutes at room temperature.

Measurement of the Viscoelastic Properties of the Systems

Elastic modulus G' and phase angle δ were measured by rheology using a Bohlin CVO120 rheometer (Malvern Instruments Ltd, UK). Experiments were stress-controlled and carried out at 37 °C using plate-plate geometry and a gap of 100 μm between the plates. 50 μL dodecan 99% (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany) was added around the plates to avoid desiccation of the sample during incubation. Amplitude tests were carried out for shear stress σ ranging from 0.23 to 100 Pa. Frequency sweeps were performed between 0.05 and 10 Hz at $\sigma = 12$ Pa. The applied strains varied between 0.0006 and 1. A loss angle value lower than 1 will be used to consider a network has been formed.[24]

Measurement of Diffusion Coefficients

Fluorescence Recovery After Photobleaching (FRAP) was used to measure the diffusion coefficients of Bodipy-Fl HA (200 kDa) purchased from Invitrogen (Paisley, UK). Solutions containing 0.01 mg per ml of

Bodipy-Fl HA in phosphate buffered saline (PBS) and were either directly used after stirring of the solution to measure diffusion in solution or let infuse in networks before measuring diffusion. Samples were contained in microscope cavity slides and incubated for 30 min. FRAP was carried out using a Zeiss Combi LSM 510 Meta Confocor II (Zeiss, Jena, Germany). The 40X1.3NA objective and the 488 nm wavelength of Argon laser were used for bleaching and excitation. Measurements of the diffusion coefficient were carried out using a region of interest (ROI) of a diameter of 18 μm. Fast Fourier Transform analysis results in exponentional decay. The diffusion time was extracted by fitting with monoexponential decay and the diffusion coefficient, D, obtained with $D = R^2/(4 \cdot \tau_D)$ where R is the radius of the ROI and τ_D the diffusion time. 7–12 FRAP experiments are performed on each sample.

Visualisation by Confocal Microscopy: Fluorescence Scanning Microscopy or Confocal Reflection Microscopy

Confocal Reflection Microscopy (CRM) is performed using the Meta detector of the Zeiss Combi LSM 510 Meta Confocor II (Zeiss, Jena, Germany). The sample is excited with a 633 nm wavelength of a He-Ne laser and the reflected light is collected between 628 and 638 nm. A 40X1.3NA oil objective is used. When the hyaluronic acid solution was observed, BODIPY-FL Hyaluronic acid (Molecular Probes, Eugene, Oregon) was introduced in hyaluronic acid solutions at a concentration of 0.01 mg/ml. Then the HA network was visualized by scanning laser microscopy (excitation 488/ emission 520 m).

Preparation of Samples and Observation of the Networks by Transmission Electron Microscopy

Samples prepared for the TEM were fixed after 0.5–2 h of incubation at 37 °C by addition of gluteraldehyde 1% in cacodylate buffer (pH = 7.2). Freshly cut pieces of the fixed samples were then washed in this buffer before further fixation in osmium tetroxide 1% in cacodylate buffer. After 1 h the fixative was removed and the sample was washed 3 times in the buffer and once with deionised water. After staining in diluted uranyl acetate overnight the samples were washed with deionised water and dehydrated in 50, 75, 90 and 100% acetone (15 minutes in each mixture). Diluted epoxy resin was then added to the sample before final embedding in the pure resin, hardening of the embedded sample in a 80 °C oven and cutting with and ultramicrotome (Reichert-Jung Ultracut E). 1% uranyl acetate in water was added on the grids for 10 min, followed by 0.3% lead citrate in water. TEM pictures were obtained with a Philips/FEI Tecnai 12 Biotwin Transmission Electron Microscope.

Results and Discussion

As we ultimately wish to understand macromolecular drugs penetration in tumours, we aimed at preparing composite networks with concentrations similar to what encountered in tumours. Therefore, the choice of the concentrations for the networks studied in this section was influenced by two parameters: (1) the tumour ECM content determined by Netti et al.[5] and Ramanujan et al.[7] – for LS174T and MCaIV tumours, interstitial collagen concentration of approximately 9.0 mg/ml and a HA content ranging from 0.5 to 1.0 mg/ml - and (2) two different molecular weights of HA (15 kDa and 1.1 MDa) as hyaluronic acid chains tend to be smaller in tumour tissue especially invasive tumours comparatively to normal tissue.[2] Based on these facts, different composite networks were prepared with collagen concentrations of 1.5 mg/ml and 10 mg/ml and HA concentrations of 1 mg/ml and 10 mg/ml. It is important to note that only 10 mg/ml collagen network concentration has any physiological relevance as Netti et al. noted that low collagen content tumours had approximately 10 mg/ml (MCaIV,

LS174T cell lines).[5] While the collagen concentrations are at best in the low collagen concentrations observed in tumours, it should be noted that HA concentrations here are higher that what was observed in tumours (up to 10 mg/ml in this study). The HA range approaches concentrations in tumours and normal tissues[2] as we aimed to find out whether the dispute on the respective influence of collagen and hyaluronic acid was related to their concentrations.

Determination of the Incubation Time

To determine the minimum incubation time necessary to form a gel, the elastic modulus of the lowest collagen concentration used in the present study (1.5 mg/ml) was determined at different time (0 min to 90 min) as previous studies have shown that the lowest concentration need more time to form the network.[23] Figure 1 presents the variation of the elastic modulus and the phase angle as a function of time. As the elastic modulus is stable and the phase angle is less than 45 ° after 30 minutes of incubation, this incubation time will be then used for the rest of the study unless specified. It should be noted that this value is in good agreement with other data from literature.[9,23]

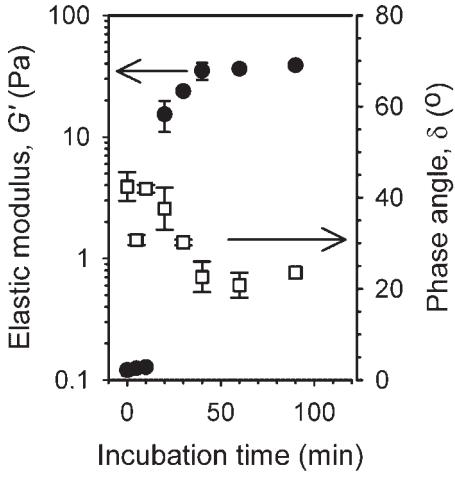

Figure 1.
Determination incubation time of low collagen network (1.5 mg/ml). Variation of the elastic modulus (closed symbols – left ordinate) and phase angle (open symbols – right ordinate) as a function of time. Incubation time varied between 0 and 2 hrs. Minimum incubation time taken as beginning of plateau value. Note data points between 0 and 20 min (included) are not reliable due to inertia of rheometer. Incubation at T = 37 °C and experiments at T = 37 °C and $\omega = 1$ Hz, $\sigma = 12$ Pa.

Rheological Behaviour of Composite Matrices

Though proteins and polysaccharides may result in phase separation and result in complex behaviour in rheology, a simple approach has been taken for these studies as (i) Forgacs, Newman and coworkers[25–27] have shown that the presence of phase separation between collagen and polystyrene beads depend on the polymer used to coat beads and (ii) rheological measurements were done at the equivalent time after preparation to compare a network at a given time. Figure 2(a) presents the influence of HMW HA on the mechanical properties of the network.

The elastic modulii, G', of a 10 mg/ml pure collagen network and a composite network containing 15 mg/ml collagen and 10 mg/ml HA 1.1 MDa do not appear to be significantly different which suggests that collagen mainly dictates the elastic modulus of the composite system (as indicated when comparing with the pure HA solution). Figure 2(b) which compares the elastic modulus of 1.1 MDa HA composite networks and pure collagen networks at different collagen concentrations, confirms that if, an effect of long HA chains on the elastic modulus of composite networks exists, it is limited and, overall, the elastic modulus of all networks, G', seems to vary with the square of the collagen concentration. Altogether these results tend to support the explanation proposed by Xin et al.[9] who suggested an absence of influence of long HA chains on elastic modulus possibly due to their lower mobility or inability to penetrate the collagen network during network formation. Consequently it is important to find out whether HA penetration is chain size dependent and whether HA and/or decorin

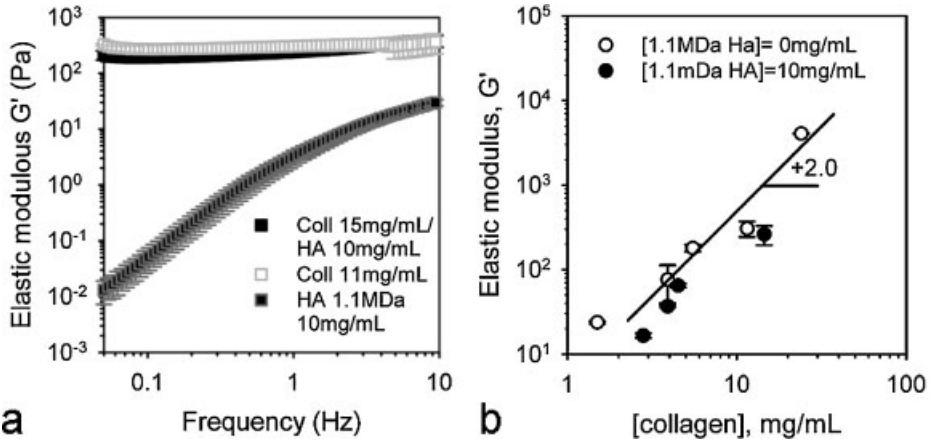

Figure 2.
rheology of mixed networks. (a) Variation of the elastic modulus of 3 different preparations is provided (collagen 15 mg/ml and HA 10 mg/ml, collagen 11 mg/ml and, HA 1.1 MDa 10 mg/ml) as a function of the sweep frequency (Hz). G' is higher and continuous only for collagen containing systems – Experiments done at $T = 37\,^{\circ}C$ and $\sigma = 12$ Pa. (b) Variation of the elastic modulus, G', at 1 Hz $\sigma = 12$ Pa as a function of the collagen concentration in presence (filled symbols) and absence (open symbols) of 10 mg/ml 1.1 M Da HA. The lines is indicative and not the result of a fit – Experiments done at $T = 37\,^{\circ}C$.

affect to collagen fibrils formation and possibly bind to the collagen as suggested by Salchert et al. and Turley et al.[10,18]

Hyaluronic Acid Penetrates Collagen Network

Whereas De Rosa et al. and Xin et al.[9,28] suggest their 1.2 MDa HA chains do not penetrate networks but barely binds to collagen (while the 150 kDa HA chains do), Figure 3(a) and 3(b) present the visualisation of a 1.5 mg/ml collagen/10 mg/ml 15 kDa HA composite network including of a fluorescently-labelled 200 kDa HA chains (Bodipy-HA) added at a 0.01 mg/ml concentration. While Figure 3(b) present the network of collagen fibrils observed by CRM, Figure 3(a) shows the distribution of Bodipy-HA (light grey values) in the same network by Confocal Scanning Laser microscopy. The apparent heterogeneous distribution of collagen fibres observed Figure 3(b) may be either due to phase separation or to the network/glass coverslip interface. Arrows in Figure 3(a) indicate where darker grey lines may reflect the absence of fluorescence from Bodipy-HA due to the presence of collagen fibrils. This indicates that up to at least 200 kDa, HA chains are able to penetrate the collagen network and the majority of fluorescently labelled polysaccharide seems to remain in the bulk. As Fig. 3(a)/(b) does not provide a direct answer for 1.1 MDa penetration ability, we chose to determine whether Bodipy-HA diffusion was more hindered in composite networks containing 1.1MDaHA than in 15 kDa HA composite networks or in pure collagen networks.

As transport hindrance is reflected by the ratio of diffusivities i.e. the ratio of the diffusion in constrained environment over the diffusion in free solution (D/D_0), Bodipy-HA diffusion was measured by FRAP in these networks and the results presented in Figure 3(c) point out to a different element of HA chains interpenetration: overall, the hindrance of Bodipy-HA increases with collagen concentration as expected. In low collagen concentration composite networks, Bodipy-HA transport is significantly affected in presence of 1.1 MDa HA while 15 kDa HA composite networks do not offer a hindrance significantly different from pure collagen networks. At high collagen concentration

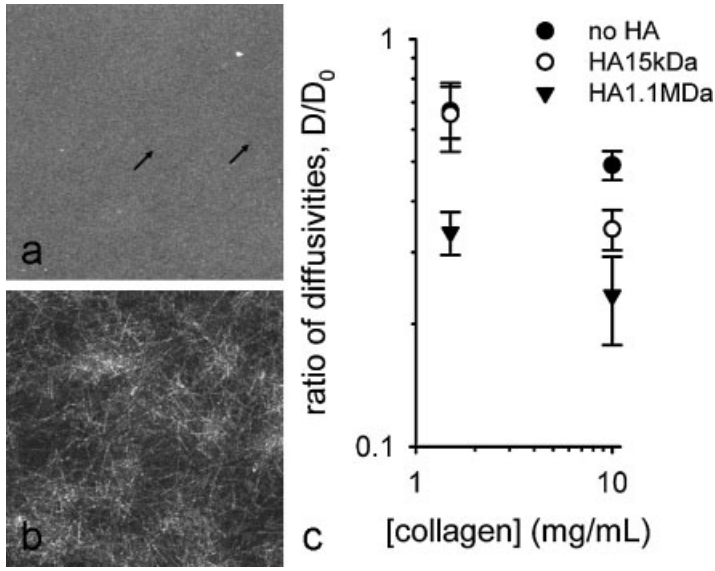

Figure 3.
Interpenetration of HA in the collagen network. (a) distribution of Bodipy 200 kDa HA chains in a composite network. Fluorescent image of Bodipy 200 kDa HA chains at a 0.01 mg/ml concentration (light grey colour viewed by confocal microscopy) in a 1.5 mg/ml collagen and 10 mg/ml 15 kDa HA composite network. Arrows point at lines (darker grey values) indicating the presence of collagen fibrils. The size of the image is $115.2 \times 115.2\ \mu m^2$. (b) Visualization collagen fibrils by Confocal Reflection Microscopy. Collagen fibrils of a 1.5 mg/ml collagen and 10 mg/ml 15 kDa HA composite network in white over dark background. The size of the image is $115.2 \times 115.2\ \mu m^2$. c. Ratio of diffusivities of Bodipy 200 kDa HA (D/D_0 *i.e.* the ratio of the diffusion coefficient in networks, D, over the diffusion coefficient in buffer, D_0) as a function of collagen concentration in absence (closed circles) or presence of HA (HA total concentration is constant and equal to 10 mg/ml) for each of the HA molecular weight i.e. 15 kDa (open circles) and 1.1 MDa (closed inverted triangles). Each point is the result of 7–12 measurements at room temperature.

while both HA chain lengths contribute to an increased hindrance to Bodipy-HA diffusion, the difference of the ratio of diffusivities between 15 kDa HA composite networks and the 1.1 MDa HA composite networks is significantly reduced. This indicates that (i) Bodipy-HA hindrance is not only due to the geometry/viscosity of collagen fibrils and, (ii) not only a HA network fills the gaps between the collagen fibres as suggested by Figure 3(b), but its effect varies with the HA concentration, its molecular weight and, possibly, the overlap concentration of HA chains.

This hypothesis is supported as data from the literature[11,29,30] suggest overlap concentrations of approximately 1 mg/ml and 10 mg/ml for 1.1 MDa HA and 15 kDa HA respectively. Therefore at 10 mg/ml collagen concentration 15 kDa HA chains are close enough to approach their overlap concentration and to affect significantly Bodipy-HA diffusion (Figure 3(c)) while this is observed at 1 mg/ml in 1.1 MDa HA composite networks. Finally at high collagen concentration, 1.1 MDa HA has a relatively reduced effect on hindrance which may suggest that, as the preparation of composite networks rests on HA chains to diffuse in the collagen network, the maximum amount of 1.1 MDa HA chains able to penetrate has already been reached at a lower concentration and the remaining chains are excluded from the collagen network or possibly phase separation. The possibility of a change of regime in the diffusion of Bodipy-HA was ruled out as the gyration radius of Bodipy-HA can be estimated at 55 nm[31] and, on the other hand, previous studies on hyaluronic

acid[20] suggest that the mesh size of a 10 mg/ml 950 kDa HA solution is about 25 nm suggesting that at both concentrations Bodipy-HA is likely to diffuse by reptation.

The Presence of HA Affects the Collagen Interfibrillar Spacing

The previous section indicated both HA chain lengths were able to penetrate the network and Figure 2 suggested little effect on the mechanical properties of the networks. Consequently, finding whether HA chains affect collagen interfibrillar spacing is important as it is unclear whether direct interactions exist between HA and collagen. Collagen fibrils spacing hypothesis was assessed using Confocal Reflection Microscopy (CRM).

Figure 4 presents an example of the organisation of collagen fibrils as observed by CRM in different conditions (1.5 and 10 mg/ml collagen, 10 mg/ml 15 kDa and with and without decorin). Similarly to Ramanujan et al.[7] in collagen networks, the collagen network appears without preferential orientation of the fibrils but dense areas of short fibrils can be noticed (see Figure 4).

At first sight, in presence of 15 kDa HA, the increase of collagen concentration seems to lead to a fuzzier image, but more interestingly the addition of the proteoglycan decorin in the composite network, seem to lead to longer, thinner collagen fibrils. Table 1 provides an estimation of the space between the fibrils based on CRM images.

Table 1 lists the interfibrillar spaces determined as an area between fibrils and it is assumed that (i) interfibrillar spacing determined in a 2 dimensional analysis is valid for a 3 dimensions network and (ii) CRM images will only show fibrils large enough to be detected i.e. microfibrils are unlikely to provide a strong signal/noise ratio. As expected the average space between fibrils decreases with increasing collagen content (non italicized data/upper part of Table 1).

The presence of (LMW or HMW) HA leads to a decrease of the average space between fibrils. In low collagen content networks this decrease is limited but the heterogeneity of networks (coefficient of variation) is increased while for high collagen content networks the decrease of the fibrils spacing is more pronounced

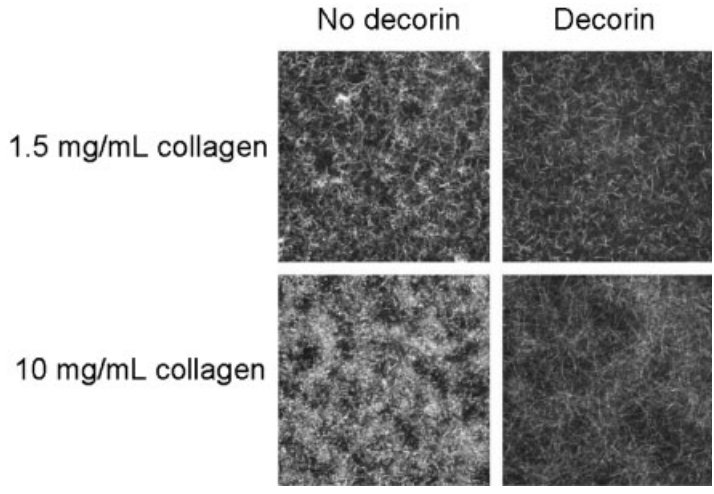

Figure 4.
Examples of visualisation of composite networks by Confocal Reflection Microscopy indicating the influence of LMW HA and decorin on fibrils formation. Left column shows the influence of the variation of the collagen content in 10 mg/mL 1.5 kDa HA composite networks. Right column presents the effect of collagen content on 10 mg/mL 1.5 kDa HA composite networks in presence of decorin at a decorin:collagen ratio 1:25. Fibrils appear light grey over background. Images size is $230 \times 230\ \mu m^2$.

Table 1.
Average area between the collagen fibrils depending on the collagen concentration and the addition of size and concentration of HA and/or decorin (*italicised*). The interfibrillar space was done using the image software analysis ImageJ (NIH) and images obtained by Confocal Reflection Microscopy. The average area between fibrils is in bold while the other data correspond to one standard deviation. The last column corresponds to the coefficient of variation, CV. Data are the result of the fit using a skewed distribution on more than 100 measurements for each network type.

[Collagen] (mg/ml)	HA type	[HA] (mg/ml)	Average area between the fibrils (μm^2)	C.V.
1.5	–	–	$4.6 < \mathbf{12.0} < 30.8$	1.6
10	–	–	$3.5 < \mathbf{7.5} < 16.2$	1.2
1.5	15 kDa	10	$2.0 < \mathbf{10.7} < 57.5$	4.4
10	15 kDa	10	$1.4 < \mathbf{5.3} < 19.7$	2.7
1.5	1.1 MDa	10	$1.8 < \mathbf{9.1} < 45.8$	4.0
10	1.1 MDa	10	$0 < \mathbf{2.2} < 6.1$	1.8
8.3	–	–	$0 < 2.1 < 6.4$	*2.0*
1.5	*15 kDa*	*10*	$1.6 < 6.9 < 29.1$	*3.2*
10	*15 kDa*	*10*	$1.9 < 5.7 < 17.2$	*2.0*
1.5	*1.1 MDa*	*10*	$1.8 < 4.8 < 12.8$	*1.7*
10	*1.1 MDa*	*10*	$2.2 < 6.1 < 17.0$	*1.3*

without resulting in an increased heterogeneity.

The second part of Table 1 (italicized data) provides the interfibrillar spacing of pure collagen and composite networks in presence of decorin. The apparent effect of decorin on the area between fibrils appears complex. Interestingly the main effect of decorin can be observed in collagen networks as the average space between fibrils varies from about 10 μm^2 in absence of decorin to about 2 μm^2 which suggests decorin participate to fibrillogenesis. The effect of decorin on collagen/HA networks is less clear: a comparison of the same collagen/hyaluronic acid network with and without decorin indicates that (i) low collagen content networks see their average space between fibrils decreased (as well as their coefficient of variation) and (ii) in high collagen content composite networks present apparently opposite results: while 15 kDa HA composite networks are not affected by the presence of decorin, 1.1 MDa Ha composite networks have larger interfibrillar spaces.

As FRAP confirmed the ability of HA to penetrate the space between collagen fibrils and CRM images (Figure 3) suggested an influence of HA on collagen fibrils, TEM (Figure 5(a)) was involved to determine whether HA binds or influence the formation of collagen fibrils. While fibrils can be easily seen, a second population of smaller fibrils is observed (as indicated by arrow). The diameter of collagen fibrils was determined from TEM micrographs. Figure 5(b) shows the typical distribution of fibrils diameters determined for a 10 mg/ml collagen network seems to indicate the presence of two populations of collagen fibrils.

Consequently, the smallest fibrils population will be referred as microfibrils whereas the largest fibrils population will be called fibrils.

Finding out whether these populations and fibrils sizes were affected by parameters such as the incubation time, the presence of LMW or HMW HA appeared important. Table 2–4 present the variation of the collagen fibrils distributions and the percentage of fibrils in each of the population as a function of incubation time and in presence of different amount of 15 kDa HA and 1.1 MDa.

Table 2 indicates that increasing the incubation time does not change significantly the populations and the diameter of the fibrils in pure collagen networks confirming that a 30 minutes incubation time is enough which supports results from Figure 1. Conversely, the networks preparation method only allow to synthesize at best fibrils as the average diameter of the second peak is approx. 25 nm which is close

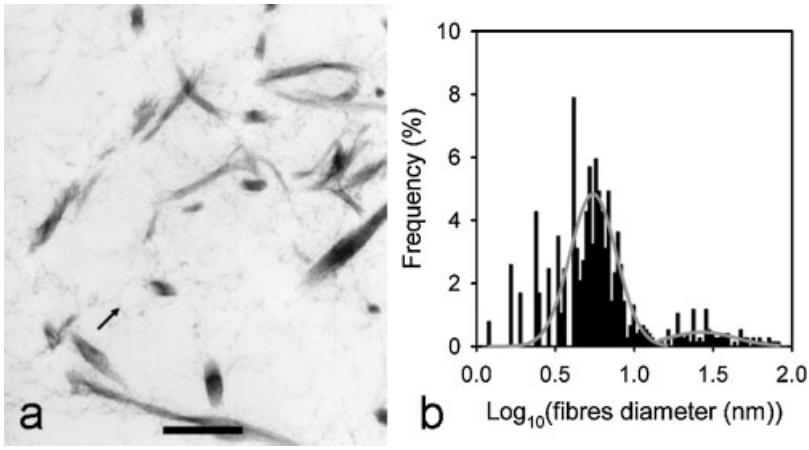

Figure 5.
(a) TEM micrograph showing collagen fibrils (bar represents 300 nm). The diameter of these fibrils was determined using such these micrographs to find out the influence HA and decorin on fibrils formation. Collagen concentration was 10 mg/ml. (b) Example of distribution of collagen fibrils diameters observed in a 10 mg/ml collagen network. Distribution is expressed as a percentage of the total number of fibrils observed for each concentration as a function of the logarithm of the diameters (observed skewed distribution). The grey line represents the result of the fits of skewed distributions considering two populations of fibrils.

to literature data on the average size of fibrils (considering a diameter of approximately 20 nm for fibrils[6,19,32]) and microfibrils.[33]

Table 3 and 4 point out to different influences of 15 kDa HA and 1.1 MDa HA as a function of their concentration in the composite networks.

Table 2.
Distribution of the average diameter of the collagen fibrils depending on the incubation time at 37 °C in a 10 mg/ml collagen network. The collagen fibrils diameter is in bold while the other data correspond to one standard deviation. The frequency (%) corresponds to the percentage of diameters under one peak (skewed distributions see Figure 5b). Data are the result of fits using skewed distributions on more than 100 measurements for each incubation time.

Incubation time (min)	Bimodal distribution			
	Average diameter (nm)		Frequency (%)	
	1st peak	2nd peak	1st peak	2nd peak
30	3.9 < **5.5** < 7.7	16.6 < **26.9** < 43.5	89	11
120	5.8 < **8.3** < 11.8	12.7 < **23.3** < 42.8	93	7

Table 3.
Distribution of the average diameter of the collagen fibres depending on the HA15 kDa concentration after 30 min of incubation at 37 °C. The collagen fibrils diameter is in bold while the other data correspond to one standard deviation. The frequency (%) corresponds to the percentage of diameters under one peak (skewed distributions see Figure 5b). Data are the result of the fit using skewed distributions on more than 100 measurements for each HA concentration.

[HA] (mg/ml)	Bimodal distribution			
	Average diameter (nm)		Frequency (%)	
	1st peak	2nd peak	1st peak	2nd peak
0	3.9 < **5.5** < 7.7	16.6 < **26.9** < 43.5	89	11
1	4.5 < **6.3** < 8.8	22.8 < **32.5** < 46.4	84	16
5	4.6 < **6.3** < 8.6	21.3 < **31.9** < 47.8	82	18
10	4.2 < **6.6** < 10.1	20.8 < **34.4** < 56.9	38	62

Table 4.
Distribution of the average diameter of the collagen fibrils depending on the HA1.1 MDa concentration after 30 min of incubation at 37 °C. The collagen fibrils diameter is in bold while the other data correspond to one standard deviation. The frequency (%) corresponds to the percentage of diameters under one peak (skewed distributions see Figure 5b). Data are the result of fits using skewed distributions on more than 100 measurements for each HA concentration.

[HA] (mg/ml)	Bimodal distribution			
	Average diameter (nm)		Frequency (%)	
	1st peak	2nd peak	1st peak	2nd peak
0	3.9 < **5.5** < 7.7	16.6 < **26.9** < 43.5	89	11
1	5.0 < **7.0** < 9.6	21.3 < **27.0** < 34.2	31	69
5	4.2 < **5.5** < 7.3	20.0 < **27.4** < 37.6	57	43
10	3.5 < **5.1** < 7.4	15.6 < **28.0** < 61.3	75	25

While the average diameters of microfibrils and fibrils population do not significantly differ with concentration or with the HA chain length, the percentage of population under each peak vary in a different manner and depends on HA concentration: (i) the population of collagen fibrils seems unaffected until a high content of 15 kDa HA (62% at 10 mg/ml, approximately 6 times the amount in pure collagen networks) and (ii) 1.1 MDa HA strongly influences the population of fibrils at low concentration (69% at 1 mg/ml, approximately 7 fold the amount of fibrils in pure collagen networks) then the proportion of fibrils decreases with the theoretical amount of HA to reach only 25% at 10 mg/ml (about 2.5 more than pure collagen networks). It is worth mentioning that, at 10 mg/ml 1.1 MDa HA, fibril diameters are more heterogeneous than for lower concentrations.

Overall 15 kDa HA seems to have little effect on the collagen fibrils formation at observed tumour content while 1.1 MDa HA has. As for the FRAP study, these effects seem to be related to the overlap concentration as data published elsewhere in the literature[11,29,34] suggest that the overlap concentration for 15 kDa HA should of the order of 10 mg/ml and 1 mg/ml for 15 kDa Ha and 1.1 MDa HA respectively. But as observed for 1.1 MDa HA composite networks (see Table 4), at concentration higher than the overlap concentration, other phenomena e.g. osmotic stress, exclusion, may be involved leading to reduced formation of fibrils. Though not demonstrated in this study, binding of HA to collagen, previously described by Turley et al.[18] or more recently by Xin et al.[9] and Salchert et al.,[10] should be considered as collagen is positively charged at pH 7.3 ($pI = 9$[23]) while HA is negatively charged.[35] Overall, this concentration size dependence is likely to have implications for the estimation of macromolecular transport.

Effect of Decorin

Table 5 present the variation of the collagen fibrils diameter in presence or not of decorin for pure collagen networks at similar concentration (decorin positive fibrils diameters are italicized).

Though we did not observe an increase in the diameter of fibrils in presence of decorin, it is should be noted that this proteoglycan, at the ratio used in these experiments, has an influence on the population of the fibrils observed: the addition of decorin leads to a 6 fold increase of the population of collagen fibrils and this influence is independent of the incubation time. Actually, this increase of the population of fibrils correlates CRM based interfibrillar spacing which indicated that decorin added to pure collagen solution induced a decrease in the space between fibrils.

We then determined whether derorin had a similar influence on fibrils population in composite networks. Table 6 indicates the effect of decorin on composite networks containing 10 mg/ml HA. Interestingly our results suggest that decorin is not beneficial

Table 5.
Distribution of the average diameter of the collagen fibres depending on the collagen concentration and the addition of decorin (*italicised*) after 30 min (*), 150 min of incubation at 37 °C in an eppendorf shacked at 700 rpm, and 30 min at 37 °C in an incubator (**) or 180 min of incubation at 37 °C. The collagen fibrils diameter is in bold while the other data correspond to one standard deviation. The frequency (%) corresponds to the percentage of diameters under one peak (skewed distributions see Figure 5b). Data are the result of fits using skewed distributions on more than 100 measurements for each sample.

[Collagen] (mg/ml)	Bimodal distribution			
	Average diameter (nm)		Frequency (%)	
	1st peak	2nd peak	1st peak	2nd peak
10*	3.9 < **5.5** < 7.7	16.6 < **26.9** < 43.5	89	11
8.4	*3.2 < 4.8 < 7.2*	*14.8 < 24.5 < 40.4*	*43*	*57*
*8.3***	*3.3 < 4.8 < 7.0*	*15.6 < 25.3 < 40.9*	*39*	*61*

to fibrils formation in composite networks as, in 10 mg/ml of 15 kDa HA or 10 mg/ml 1.1 MDa HA composite networks, their population do not change significantly in presence or absence of the proteoglycan.

As observed for Table 5, these results are supported by the observation by CRM. It is possible that the absence of additive influence of decorin on the formation of fibrils in composite networks may be related to the mode of preparation of the networks. The lack of noticeable effect may be the result of a competition between the three components of the network. Though we lack of direct information on the mechanism, it should be noted that: (i) as the populations of fibrils in collagen/HA/decorin composite networks are closer to what previously determined in collagen/HA networks than in pure collagen, this may suggest decorin cannot compete against HA ability to form microfibrils, (ii) this may be explained as Turley et al.[18] noted decorin binding to collagen could be boosted by hyaluronic acid but also mentioned that complexes made of HA and decorin bind to collagen or stay between collagen fibres.

Interestingly, Brightman et al.[21] did not see any variation of the collagen fibres diameter in the influence of decorin at ratios 1:20 or 1:80 but did note that the presence of GAGs especially decorin increased the lag time i.e. slows down the fibrillogenesis. This point is interesting as it shows that decorin concentration does not seem to be the element that may have lead to a variation of the collagen fibrils diameters. Additionally, the effect of heparin on fibrils formation observed by Salchert et al.[10] is observed at much higher ratio (1.2 mg/ml collagen and between 0.4 mg/ml and 5 mg/ml heparin) and the collagen fibres preparation method is different evaporation of buffer at room temperature which increases the extracel-

Table 6.
Distribution of the average diameter of the collagen fibres depending on HA chain size and decorin presence. Composite networks presented contain 8.6 mg/ml collagen networks containing decorin, 10 mg/ml HA and in presence or absence of decorin (1:25). The collagen fibrils diameter is in bold while the other data correspond to one standard deviation. The frequency (%) corresponds to the percentage of diameters under one peak (skewed distributions see Figure 5b). Data are the result of fits using skewed distributions on more than 100 measurements for each sample.

[HA] (mg/ml)	Decorin 1:25	Bimodal distribution			
		Average diameter (nm)		Frequency (%)	
		1st peak	2nd peak	1st peak	2nd peak
15 kDa: 10	No	4.2 < **6.6** < 10.1	20.8 < **34.4** < 56.9	38	62
15 kDa: 10	*Yes*	*3.3 < 5.3 < 8.5*	*16.7 < 28.5 < 48.7*	*37*	*63*
1.1 MDa: 10	No	3.5 < **5.1** < 7.4	15.6 < **28.0** < 61.3	75	25
1.1 MDa: 10	*Yes*	*3.9 < 5.5 < 7.7*	*15.0 < 29.2 < 56.9*	*72*	*28*

lular materials concentration with time and, consequently, the probability of forming fibres.

Conclusion

Enhancing macromolecular medicines delivery requires to overcome physiological barriers such as tissue extracellular matrix. Therefore a better understanding of the ECM components organisation, interaction and their respective influence on macromolecular transport is needed to design more efficient novel medicines. We therefore aimed to prepare and characterise composite networks made of three components of the ECM namely type I collagen, hyaluronic acid and decorin. While we did not succeed to obtain large collagen fibres which limits the direct comparison to in vivo, composite networks still presented interesting features such as the role of fibrillar collagen in the mechanical properties of the composite network i.e. acting as the backbone as in tissues. While both 15 kDa and 1.1 MDa HA chains penetrate the collagen network and increase transport hindrance of the composite networks as determined by FRAP measurements, ECM components interactions lead to interesting results. Though only fibrils were formed with this method and the chosen collagen, it is noteworthy that the fibrils formation and population is influenced by HA content and molecular weight and decorin (in absence of HA). In fact the HA overlap concentration seem to play a critical role both for fibril formation and HA incorporation in the composite network which may be regarded as crowding, complexation, binding and osmosis. Studies have already dealt with similar issues for globular proteins and polyelectrolyte or polymers.[36,37] Though their work is not directly applicable to fibrils formation, it would be interesting to explore this lead.

Finally, the critical influence of the HA chain overlap concentration on the formation of collagen fibrils and transport hindrance as observed for Bodipy-HA chains is likely to result in different abilities of novel medicines to penetrate tumour relevant composite networks (0–1 mg/ml HA and 10 mg/ml collagen). Conversely, the state of the disease may results in different levels of macromolecular drug penetration as invasive tumours have much smaller HA chains (tens of kDa) than non invasive tumours or normal tissues (few MDa).

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