Gel-Like Structure of a Hexadecyl Derivative of Hyaluronic Acid for the Treatment of Osteoarthritis

Ivana Finelli, Ester Chiessi, Devis Galesso, Davide Renier, Gaio Paradossi*

Hyaluronic acid is a polysaccharide with viscoelastic and mechanical properties that are crucial for the normal functioning of osteoarticular junctions. It is demonstrated that introduction of a hexadecyl side chain into HA yields an injectable polysaccharide capable of forming physical hydrogels, which are stable at very low polymer concentrations, whereas native hyaluronic acid forms viscous solutions at concentrations that are ten times higher. Characterization of this system showed that the driving force for its gel-like behavior is the occurrence of hydrophobic interactions involving aliphatic side chains, despite the low degree of substitution, as confirmed by molecular dynamics simulations of HYADD4 and HA hydrogels.

Introduction

Hyaluronic acid, HA, is a natural polysaccharide with a disaccharide repeating unit \( \bigl[ \rightarrow 4 \bigr] \beta-D\text{-glucuronic acid–(1 \rightarrow 3)} \times 2-D\text{-deoxy-2-acetamido-}\beta-D\text{-glucose–(1 \rightarrow )} \bigl), \) capable of forming viscous solutions in water.\(^1\) The functions of this biopolymer in nature are diverse, generally involving rheological and osmotic regulatory mechanisms in mammalian extracellular matrices and in synovial fluids.\(^2\)

Currently, HA is widely used in the treatment of inflammatory and degenerative joint diseases, a group of pathologies with a high societal impact since they contribute heavily towards increasing health costs and affect quality of life. However, the rationale behind these applications is often developed on an empirical basis and it is not always clear. In this respect, medical treatments are limited to the local replacement of synovial fluids and an aqueous hyaluronic acid solution has until now been the system most frequently used as a filler in osteoarticular junctions.\(^3,4\)

Alkyl derivatives of HA provide the possibility of controlling the hydrophobically driven association of the polysaccharide chains. A study on derivatives of HA having C-8, C-10, C-12, C-14, and C-16 alkyl side chains provided evidence for the formation of hydrophobic domains and of the presence of transient network structures at concentrations above \( 1 \text{ g} \cdot \text{L}^{-1}.\)\(^5\)

Recently we have focused our attention on a slightly modified hyaluronic acid, HYADD4 (CAS RN 1022186-37-3), where the polysaccharidic backbone is derivatized with hexadecylic (C-16) side chains, through amide bonds, with a 1–3 mol-% degree of substitution of repeating units (see Scheme 1).

Despite the low degree of modification, the grafting of alkyl side chains on the backbone dramatically alters the physical properties of macromolecular dispersion, resulting in a stable hydrogel at polymer concentrations higher...
Experimental Part

Materials

HA tetrabutylammonium salt (HATBA), obtained from hyaluronic acid sodium salt from a bacterial source ($M_w = 7 \times 10^6$ Da) was provided by Fidia Farmaceutici SpA (Abano Terme, Italy) as a freeze-dried powder. Synthesis of HYADD4 was performed in Fidia Farmaceutici Chemical Research Laboratories (see Methods section). Methanesulfonic acid, 1,1'-carbonyldiimidazole, hexadecylamine, o-phthalaldehyde, fluorescein isothiocyanate (FITC)-conjugated dextran with molecular weights of 500, 250, and 20 kDa (dextran-FITC) with a label density of 0.003 to 0.02 mol FITC per mol glucose were supplied from Sigma and used without further purification. Monodispersed polystyrene latex beads (diameter 57 nm) aqueous suspension was purchased from Polyscience (USA).

Dimethyl sulfoxide (DMSO), methanol (MeOH), and ethanol (EtOH), were Carlo Erba, RPE grade products. MilliQ water was used throughout this study.

Synthesis and Characterization

Synthesis of HYADD4

HATBA (1 g) was dissolved at room temperature in 100 mL of DMSO, then 30 μL of methanesulfonic acid and 26 mg of 1,1'-carbonyldiimidazole were added slowly. This activation phase was performed at room temperature under stirring for 60 min. Subsequently, 272 mg of hexadecylamine was then added and the amidation reaction was performed at 42 °C for 16 h with stirring. Saturated aqueous NaCl solution (10 mL) was added and the product was recovered by pouring 200 mL of EtOH into the solution and filtering the precipitate. The powder was then washed several times using a mixture of EtOH and water and finally dried in an oven. High-performance liquid chromatography (HPLC) with refractive index (RI) detection was used to assess the absence of residual tetrabutylammonium ions. Following this procedure, 600 mg of HYADD4 was obtained.

The determination of the degree of amide substitution was performed by HPLC/fluorimetry analysis of total hexadecylamine (HPLC system Perkin Elmer series 200, detector Luminescent Spectr. LS30, column Versapack C18 10 μm 25 cm, mobile phase MeOH/H2O 95/5, flow rate 1.3 mL·min$^{-1}$) after alkaline hydrolysis (NaOH 2 M at 70 °C for 3 h under stirring), neutralization, extraction with MeOH, and derivatization of the amine with o-phthalaldehyde (OPA). The fluorometric detector was set at an excitation wavelength of 330 nm and emission wavelength of 440 nm.

The absence of free hexadecylamine as an indicator of impurity in the reaction was confirmed by resuspending a sample of HYADD4 in MeOH for 30 min, in order to extract nonbonded amine. The collected supernatant was then treated with OPA for amine derivatization and the hexadecylamine-OPA product was quantified using the HPLC/fluorimetry method, as described above.

than 0.3% (w/v), whereas native hyaluronic acid forms viscous solutions even at ten times higher concentrations. Autoclaving of HYADD4 dispersions, in addition to ensuring the production of a sterile material, is necessary to obtain a final product with good homogeneity.$^6$

Substitution onto HA of 1–3 mol-% does not affect other relevant features of the biopolymer, such as charge density along the chain and swelling ability in addition to its interaction behavior with metabolites and cellular material in the synovial matrix. Further evidence of the peculiar properties of this novel biomaterial can be found by examining the behavior of another HA derivative, HYCp45,$^7,8$ in which the steroidal anti-inflammatory drug 6α-methylprednisolone was grafted onto the polysaccharide through ester bonds with variable degrees of substitution. In spite of the marked derivatization of HYCp45 compared to HYADD4 and the rather high substitution, HYCp45 solubilities in water were higher than 0.3% (w/v), whereas native hyaluronic acid forms viscous solutions even at ten times higher concentrations. Autoclaving of HYADD4 dispersions, in addition to ensuring the production of a sterile material, is necessary to obtain a final product with good homogeneity.$^6$
The product synthesized for the characterization described here showed a degree of substitution of 2.4 mol-%.

The HYADD4 chemical structure was confirmed by Fourier-transform infrared (FT-IR) spectroscopy and two-dimensional (2D) NMR spectroscopy techniques (data not shown).

Preparation of HYADD4 gels

HYADD4 hydrogels were prepared by dissolving the polymer in phosphate-buffered saline (PBS) (pH = 6.9) at concentrations ranging between 0.3% and 0.8% (w/v). The polymer was dispersed in PBS by overnight stirring and the obtained milky dispersion (5 mL) was then autoclaved (Carlo Erba, 760 autoclave, Italy) for 15 min in a stoppered glass cylindrical vial. The resulting hydrogel, cooled at room temperature, was visibly clear, thus indicating the absence of inhomogeneities.

Dynamic Light Scattering

The dynamic light scattering (DLS) setup consisted of a 200-mW solid-state laser source (Photop Suevtech, Shangai, China) emitting at 532 nm, typically used at an output power of 20 mW with a vat cell mounted on a BI-200SM goniometer plate (Brookhaven Instruments, USA) and equipped with a HC120 photomultiplier (Brookhaven Instruments, USA). A correlator board (BI-9000AT) and specific software (Brookhaven Instruments, USA) were used with an array of 200 logarithmically spaced channels for processing and data storage. A refrigerated thermostat (Julabo, F30-C, France) was used to control the temperature from 25 to 60 °C.

The instrumental coherence factor β was determined by measuring the normalized autocorrelation function of the scattered light, \( g_2(q,t) \), of a standard polystyrene latex dispersion. In all cases, the value of \( \beta \) was >0.8.

Autocorrelation functions were taken at scattering angles \( \theta = 60, 90, \) and \( 120^\circ \). The correlation function build up was performed for 15 min.

Direct measurable quantities are the time-average intensity \( \langle I(q) \rangle_T \) and the ensemble-average intensity \( \langle I(q) \rangle_E \). When a gel is in a cell fixed in position, the time-average intensity is obtained. The ensemble-average intensity is measured by rotating the cell with a stepping motor at a constant angular speed of 10 rpm.

Fluorescence Recovery after Photobleaching (FRAP)

FITC-labeled dextran fractions with different molecular weights were added to HYADD4 dispersions. The fluorescent probe was added to liquid hot autoclaved HYADD4 dispersion to a final concentration of 0.08% (w/v). FRAP experiments were performed by placing approximately 50 μL of hydrogel on a microscope slide, which was then sealed under a coverslip. The HYADD4 concentration ranged from 0.3 to 0.8% (w/v).

Photobleaching experiments were performed using a confocal laser scanning microscopy [11, 12] (Nikon PCM 2000, Nikon Instruments, Japan) with a 60 × 1.4 oil immersion objective. The 488 nm line of a 100 mW argon ion laser was used for sample bleaching and fluorescence excitation. For bleaching and recovery images, a pixel size of 0.414 μm and an entire field of 512 × 512 pixels were used. A square central region of 30 × 30 pixels of the bleached spot was selected for image scans. Typically, a frame was taken every 1.2 s.

The normalized fluorescence intensity after photobleaching, \( f(t) \), was defined as the ratio \( [F(0) – F(t)]/[F(0) – F(\infty)] \), where \( F(t) \) is the time dependence of the fluorescence recovery, \( F(0) \) and \( F(\infty) \) are the residual fluorescence of the bleached spot and the recovered fluorescence at equilibrium, respectively. Fluorescence recovery was measured by averaging the fluorescence intensity within the 30 × 30 pixels area. The Axelrod relation for lateral diffusion allows the extraction of the time constant, \( \tau_d \), from the normalized fluorescence recovery curve with time, \( f(t) \) [11, 12]:

\[
f(t) = \sum_{n=0}^{\infty} \frac{-k^n}{n!} \left[ 1 + n \left( \frac{2t}{\tau_d} \right) \right]^{-1}
\]

where the constant \( k \) indicates the efficiency of the bleaching.

The plane diffusion coefficient \( D \) is evaluated from

\[
\tau_d = \frac{\omega^2}{4D}
\]

where \( \omega \) is the half width of the normalized Gaussian intensity profile of the spot caused by bleaching at the initial time and at a depth equal to \( e^{-2} \).

Molecular Dynamics Simulations

Simulation Details

The computational study was carried out using an IBM System p5 575 computer at CASPUR (Consorzio Interuniversitario per le Applicazioni di Super calcolo Per Universita e Ricerca, Rome, Italy). MD simulations were performed using the GROMACS 3.3.1 program [13, 14] with the GROMOS 45A4 force field, developed for the explicit-solvent simulation of hexopyranos-based carbohydrates [15]. Aliphatic CH, CH2, and CH3 groups were treated with the united-atom convention and the simple point charge (SPC) water model was applied. Parameters and atomic partial charges for carboxylate and amide groups were set according to the GROMOS 45A3 force field. Sodium ions were considered as counterions of carboxylate groups. The Ryckaert-Bellemans function was used to describe the torsional potential energy terms of hexadecyl chains. All simulations were carried out in the NPT ensemble, with the leapfrog integration algorithm [17] using a 2-fs time step, periodic boundary conditions, and the minimum image convention. The SHAKE procedure [18] was applied to constrain all bond lengths with a relative geometric tolerance of 10⁻⁴. Temperature was controlled by the Berendsen’s coupling algorithm [19] using a time constant of 0.1 ps with solvent and solute separately coupled. The pressure was maintained at 1.0 bar by weak coupling to a pressure bath via isotropic coordinate scaling [20] with a relaxation time of 0.4 ps. Electrostatic interactions were calculated using the particle-mesh Ewald method [20] and nonbonded interactions were accounted within a cutoff of 1.4 nm.

Hydrogel Models

HA and HYADD4 oligosaccharides were simulated with a glucuronate residue (U) at the nonreducing terminus and an N-acetylglucosamine residue (A) at the reducing terminus. Only the β anomer were considered. Preliminary MD simulations of the
tetrasaccharide (UA), corresponding to two HA repeating units, in a cubic box of 1525 water molecules with two Na\(^+\) ions were carried out to validate the force field. Trajectories in the space of \(\Phi (O_1-C_1-O_\alpha-C_\alpha)\) and \(\Psi (C_\beta-O_\beta-C_\gamma-C_\delta)\) glycosidic dihedral angles for the 1 → 3 and 1 → 4 glycosidic linkages (with \(X = 3\) and 4, respectively) during 10 ns of MD simulation were compared with the recent results of Almond et al.\(^{21}\) in MD simulations of HA oligomers in aqueous solution. Good agreement was obtained in terms of the most populated conformational domains.

Six oligosaccharides, \((\text{UA})_3-\text{(UA)}-\text{(UA)}\), where U indicates the glucuronic residue bearing the hexadecyl-amide group, were located in a cubic box of edge length 8.7 nm at maximum distance from each other. The box was solvated with 21120 water molecules and 42 Na\(^+\) ions were added at the positions with the most favorable electrostatic potential by replacing 42 water molecules. The whole assembly was energy minimized and pre-equilibrated at 298 K and 1 bar for 3 ns. A cycle of simulated annealing was then performed for a total time of 1.6 ns, with the following sequence: heating from 298 to 370 K at 1 bar in 200 ps, 300 ps at 370 K and 1 bar, heating from 370 to 393 K at 2 bar in 200 ps, 300 ps at 393 K and 2 bar, cooling from 393 to 370 K at 2 bar in 200 ps, 350 ps at 370 K and 1 bar, cooling from 370 to 298 K at 1 bar in 50 ps. This procedure was adopted in order to mimic the autoclaving process for the preparation of HYADD4 samples. After the simulated annealing, a cluster of two oligosaccharides, stabilized by contacts between hexadecyl chains, was obtained. The following MD simulation was carried out for a total trajectory time of 50 ns, with the first 5 ns for equilibration. The atomic coordinates were saved every 2 ps for analysis.

A corresponding simulation was performed for a HA hydrogel model, where six \((\text{UA})_3\) oligosaccharides were considered in a cubic box with water and 48 Na\(^+\) ions. The simulating approach was the same as for the HYADD4 model, with a total trajectory time, after the simulated annealing, of 50 ns.

HA and HYADD4 hydrogel models correspond to a polysaccharide concentration of approximately 5 wt.-%.

### Results and Discussion

As pointed out in the work by Pusey and Van Megen\(^{22}\) gels, colloidal glasses, strongly interacting systems, or particles entrapped in a network cannot be considered ergodic systems when they are probed by visible light. This implies that the value of a property measured by an ensemble averaging over the configurations of the systems does not correspond to the value of the same property measured by averaging over time. For solutions, the treatment of the second-order or intensity autocorrelation function, \(g_2(t, t)\), relies on the equivalence between the two averaging processes and the data collection is carried out by averaging over time.\(^{23}\) In nonergodic systems, this approach is not valid and a direct ensemble averaging is needed.\(^{24}\) This is a general feature of systems having a dynamic arrest of at least some motion modes. In the case of a gel, the chains are hindered in their translational motion due to the presence of physical or chemical crosslinking points, although movements of chain segments within crosslinks are still allowed. In photon correlation spectroscopy, this is reflected by a decay of the autocorrelation function of the intensity of the scattered light to a nonzero value over long periods of time.

In this case, at a determined value of the scattering vector \(q\), which is defined by the incident wavelength \(\lambda_0\), the scattering angle \(\theta\), and the refractive index of the medium \(n\) as

\[
q = \frac{4\pi n}{\lambda_0} \sin \left(\frac{\theta}{2}\right)
\]

the normalized dynamic structure factor \(f(q, t)\) can be obtained from the experimentally accessible \(g_2^{(2)}(q, t)\) according to

\[
f(q, t) = 1 + \frac{\left(\frac{g_2^{(2)}(q, t)}{\beta} - 1\right)}{\gamma}^{1/2} - 1
\]

where \(Y\) is the ratio between the intensity of the scattered light averaged over the configurations, \(\langle I(q)\rangle_E\), and the time-averaged intensity of the scattered radiation, \(\langle I(q)\rangle_T\),

\[
Y = \frac{\langle I(q)\rangle_E}{\langle I(q)\rangle_T}
\]

and \(\sigma^2\) is the amplitude of the \(g_2^{(2)}(q, t)\) intensity autocorrelation function at \(t = 0\),

\[
\sigma^2 = g_2^{(2)}(q, 0) - 1 = \frac{\langle I^2(q)\rangle}{\langle I(q)\rangle^2} - 1
\]

\(\beta\) is the instrumental coherence factor\(^{25}\) accounting for the finite, although small, scattering volume determined by the detector aperture and it is obtained by extrapolating at \(t = 0\) the \(g_2^{(2)}(q, t)\) of a standard of monodisperse latex particles (see Experimental Part).

Equation (1) gives the correct evaluation of the dynamic structure factor \(f(q, t)\), and is obtained by detection of the second-order autocorrelation functions averaged over time and over the configurations \(g_2^{(2)}(q, t)\) and \(g_2^{(2)}(q, t)\), respectively. This is accomplished by collecting the intensity correlation function with a cuvette containing the gel when fixed and during rotation.\(^{24}\)

The dynamic and structural features of HYADD4 hydrogels were assessed by this modified DLS method. When probing such systems with light, the baseline of the \(f(q, t)\) correlation function maintains a value larger than zero, even for delay times longer than seconds. However, over a shorter time course (tens of microseconds), the system loses part of its correlation due to the presence of local or segmental chain motions that are still active in the assembled polymer network. This feature clearly emerges
in Figure 1 when plotting the dynamic structure factor \( f(q,t) \) for HYADD4 hydrogel concentrations ranging from 0.3% to 0.8% (w/v). A progressive flattening of the \( f(q,t) \) curves in response to an increase of concentration indicates the progressive strengthening of a dynamically arrested state.

On this basis, DLS study of the properly detected autocorrelation function can represent a useful tool for investigating the dynamic features of HYADD4 hydrogels.[25–29]

Extraction of significant structural and dynamic parameters of the hydrogels from \( f(q,t) \) is accomplished by processing the normalized dynamic structure factor by a stretched exponential model developed for describing the internal dynamics of colloidal gels.[30] In this model, the average mean square displacement of the network elements is described by:

\[
\langle \delta^2(t) \rangle = \frac{3k_BT}{K} \left\{ 1 - \exp \left[ -\left( \frac{t}{b\tau_c} \right)^p \right] \right\}
\]  

(4)

\( K, b, \) and \( p \) are constants linked to the fractal properties of the network and to the integration procedure and \( k_BT \) has the conventional meaning. \( \tau_c \) represents the time required to relax the overall cluster and is related to the cluster spring constant \( k_c \). The validity of Equation (4) extends over a time frame of approximately 10 \( \tau_c \).

The normalized dynamic structure factor \( f(q,t) \) (see Equation 1), is expressed in terms of the mean squared displacement by

\[
f(q,t) = \exp \left[ -q^2 \left\{ \langle \delta^2(t) \rangle / 6 \right\} \right]
\]  

(5)

Insertion of Equation (4) into Equation (5) allows the evaluation of an average characteristic length, \( \delta \), and an average relaxation time, \( \tau = b\tau_c \), of the hydrogel domain by fitting the experimental \( f(q,t) \) according to Equation (6) in a range of time shorter than 10 \( \tau_c \):

\[
f(q,t) = \exp \left\{ -q^2 \left[ 1 - \exp \left( -\left( \frac{t}{\tau} \right)^p \right) \right] \right\}
\]  

(6)

As an example, the fittings of \( f(q,t) \) at the lower and higher concentration are shown in Figure 2.

With this procedure, the parameters describing the hydrogel dynamic behavior can be accessed. A summary of the values of the characteristic hydrogel lengths, \( \delta \), and of the relaxation times as a function of the concentration is given in Table 1.

The correlation length at the nanoscale level, \( \delta \), characterizing this hydrogel decreases with increasing concentration. The dynamics displayed by HYADD4 hydrogels, with correlation times ranging from some

<table>
<thead>
<tr>
<th>HYADD4 concentration (w/v)</th>
<th>( \delta ) (nm)</th>
<th>( \tau ) (( \mu s ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>24 ± 2</td>
<td>208 ± 3</td>
</tr>
<tr>
<td>0.4</td>
<td>18 ± 2</td>
<td>188 ± 4</td>
</tr>
<tr>
<td>0.5</td>
<td>16 ± 1</td>
<td>130 ± 3</td>
</tr>
<tr>
<td>0.6</td>
<td>14 ± 1</td>
<td>112 ± 2</td>
</tr>
<tr>
<td>0.7</td>
<td>15 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>0.8</td>
<td>13 ± 1</td>
<td>74 ± 1</td>
</tr>
</tbody>
</table>
hundreds to tens of microseconds, can be associated to motion of chain portions about an equilibrium position.

As HYADD4 hydrogels are stabilized by physical junction domains, melting should be a way to pass from an arrested dynamic regime to the sol state. We studied the correlation function behavior as a function of temperature at a concentration of 0.3% (w/v), the lowest concentration of the HYADD4/water system in the gel state. A gradual change toward an ergodic behavior, characteristic of the sol state, was observed. However, at 65 °C the system is still strongly correlated and the $f(q,t)$ does not recover the usual shape expected for the sol state over a time span of 0.1 s.

In order to probe the gel texture at a mesoscopic scale and to study the diffusional behavior inside the network, we used the fluorescence recovery after photobleaching method (FRAP). The data obtained by the FRAP method were analyzed in terms of the free volume theory.[31] According to this theory, which was originally developed for solute diffusion in a liquid,[32] solute displacements are described by a set of jumps into holes provided by the solvent redistribution due to thermal random fluctuations occurring at equilibrium. This theory was adapted to the solute diffusion in gels.[33] In this case, the random fluctuations of voids are also due to displacements of polymer chain sections. However, in gels with high water content, this contribution is negligible and the free volume, $v_F$, allowing diffusion of the solute is:

$$v_F \propto (1 - \varphi)v_{F,W}$$  \hspace{1cm} (7)

where $\varphi$ is the polymer volume fraction and $v_{F,W}$ is the free volume per molecule of water. The main contribution of the polymer chains to solute diffusion in the gel is to hinder or allow the solute passage according to the segmental motions of the chains between crosslinks. In this regard, a solute molecule can diffuse according to the product of the probability of finding a void in the solvent times the probability of finding an effective opening of the polymer chains in the gel. In a diffusion experiment of a solute with known dimensions, the network sieving effect can be used for the determination of the average mesh dimension. These considerations lead to a link between the diffusion coefficient of the solute in the gel phase and $\varphi$:

$$\frac{D_g}{D_0} \propto \exp \left[ -A \frac{\varphi}{1 - \varphi} \right]$$  \hspace{1cm} (8)

where $A$ is a constant linked to the structural features of the polymer chains in the gel and the molecular dimension of the solute.

Linearization of this equation yields:

$$\ln \left( \frac{D_g}{D_0} \right) = S + A \left[ \frac{1}{H} - 1 \right]$$  \hspace{1cm} (9)

$p$ describes the sieving effect and $H$ is the hydration of the gel, that is, the volume fraction of water.[34] According to the “free volume” theory,[35] the parameter $S$ in Equation (9) is zero in the absence of a sieving effect, whereas $S$ has negative values when this effect is present.

Although this approach has been subjected to much criticism, mainly due to the fact that the sieving effect of the polymer is often considered independent from the polymer volume fraction, Equation (9) is a useful tool in diffusion experiments in gels for the evaluation of the average mesh size.

In HYADD4 hydrogels with concentrations of 0.3–0.8% (w/v), the linear trend was maintained over the entire concentration range, with a nonzero intercept $S$, see Figure 3, indicating that a sieving effect was found in studying the diffusion of monodisperse FITC-labeled dextran with molecular weights of 500 and 250 kDa. Dextran with a molecular weight of 20 kDa was not observed to undergo a sieving action by hydrogels over the same HYADD4 concentration range, see Figure 3.

The hydrodynamic coil dimensions of dextran fractions showing a sieving effect of the hydrogels by FRAP experiments can be compared with the mesh size, $\delta$, of HYADD4 hydrogels studied by DLS. A comparison between the hydrodynamic radius of dextran fractions and mesh size of HYADD4 hydrogels determined by DLS is shown in Table 2.

The right-hand column indicates that in the presence of 250 and 500-kDa dextrans the observed sieving effect can be explained by an average mesh size smaller than the hydrodynamic dimensions of the polysaccharide coils. Diffusion coefficients of FITC-dextran fractions with different molecular weights in HA solutions were measured by the FRAP method[37,38] and provided information on transient network structure of this polymer in solution.

Figure 3. Log of the normalized diffusion coefficient of dextran in HYADD4 hydrogels as a function of the inverse hydration degree. Dextran 20 kDa (■); dextran 250 kDa (●); dextran 500 kDa (●). Solid lines are the fits according to Equation (9).
At a HYADD4 concentration of 0.8% (w/v), a diffusion coefficient of $6 \times 10^{-8}$ cm$^2$/s was determined for the 500-kD FITC-dextran, in agreement with the value obtained in HA solutions at the same concentration.[37] This suggests that in HYADD4 hydrogels, the structure of the physical network is similar to that obtained in solution.

An MD simulation study of a HYADD4 hydrogel model was performed to clarify the interactions and structure of the junction zones. The choice in favor of an atomic-level description of the hydrogel components instead of a coarse-grained picture of the system allowed us to model the interaction pattern, both intramolecular and intermolecular, present in polysaccharides, involving hydrogen bonds between hydroxyl groups, and, in the case of HA, amide and carboxylate groups. The dynamics of these interactions is affected by water molecules, acting as a competitor for hydrogen bonds, and only an explicit description of the solvent could take this effect into account.

The self-assembly of HYADD4 oligosaccharide chains in a network was observed in the MD simulation during the first 10-ns trajectory following simulated annealing. The system evolves in a cluster of five interconnected chains, with different junction zones between couples of chains, two of which directly involve the hexadecyl moieties. The network connectivity can be traced by considering the matrix of the mean smallest distances between pairs of repeating units. Figure 4A shows this matrix, obtained with a time average of 1 ns, in the trajectory interval between 29 and 30 ns. Color coding has been used to indicate the distance value. In Figure 4A, the disaccharide (UA) or (U^'A) has been considered as a residue (i.e., the HA repeating unit as it is or grafted by the hexadecyl group) and 48 total residues have been included, belonging to the 6 oligosaccharide chains. Along the diagonal of the matrix, the six oligosaccharide chains, reported in an arbitrary sequence and labeled with integer numbers from 0 to 5, are also indicated. The junction zones between chains are represented by out of diagonal yellow-orange domains.

Examination of Figure 4A reveals two extended contact regions, stabilized by hydrophobic interactions, between pairs of chains (3–4 and 1–5), where the central (U^'A) residues are also involved. Three other junctions, driven by interchain hydrogen bonds, arise between the terminal residues of the oligosaccharides (pairs 1–2, 2–3, and 2–5) propagating the connectivity to five out of six chains of the system. This cluster is in contact with its periodic image, thus giving a percolating network. It should be noted that the interresidue distance matrices calculated in a time window of 1 ns along the trajectory for times longer than 20 ns are similar to that shown in Figure 4A. Only local minor differences involving pairs of isolated residues on different chains can be found, indicating a quite well-defined structural arrangement for a space resolution of about 1 nm (the length of a HA repeating unit) and a time resolution of 1 ns.

Figure 4B shows the matrix of the smallest distances between pairs of repeating units for the HA hydrogel model, obtained as for the HYADD4 system. A lower number of contacts with a smaller extension than those found in the HYADD4 model can be observed at the same trajectory time. This result is in agreement with the higher gelling ability of HYADD4 with respect to HA due to the hydrophobic junction domains.

### Table 2. Comparison of the hydrodynamic dimensions of dextran fractions and HYADD4 hydrogel pore size.

<table>
<thead>
<tr>
<th>Dextran-FITC molecular weight (kDa)</th>
<th>$R_H$</th>
<th>Sieving effect</th>
<th>$\delta^b/2R_H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>17</td>
<td>+</td>
<td>0.5</td>
</tr>
<tr>
<td>250</td>
<td>10</td>
<td>+</td>
<td>0.8</td>
</tr>
<tr>
<td>20</td>
<td>~5</td>
<td>–</td>
<td>1.6</td>
</tr>
</tbody>
</table>

$^a$Hydrodynamic radius, data from Gibbon et al.[35] and Bolle et al.[36]. $^b$The value of $\delta$, determined by DLS, taken at an intermediate concentration of HYADD4 hydrogel of 0.5% (w/v) is 16 nm (see Table 1).

At a HYADD4 concentration of 0.8% (w/v), a diffusion coefficient of $6 \times 10^{-8}$ cm$^2$/s was determined for the 500-kD FITC-dextran, in agreement with the value obtained in HA solutions at the same concentration.[37] This suggests that in HYADD4 hydrogels, the structure of the physical network is similar to that obtained in solution.

An MD simulation study of a HYADD4 hydrogel model was performed to clarify the interactions and structure of the junction zones. The choice in favor of an atomic-level description of the hydrogel components instead of a coarse-grained picture of the system allowed us to model the interaction pattern, both intramolecular and intermolecular, present in polysaccharides, involving hydrogen bonds between hydroxyl groups, and, in the case of HA, amide and carboxylate groups. The dynamics of these interactions is affected by water molecules, acting as a competitor for hydrogen bonds, and only an explicit description of the solvent could take this effect into account.

The self-assembly of HYADD4 oligosaccharide chains in a network was observed in the MD simulation during the first 10-ns trajectory following simulated annealing. The system evolves in a cluster of five interconnected chains, with different junction zones between couples of chains, two of which directly involve the hexadecyl moieties. The network connectivity can be traced by considering the matrix of the mean smallest distances between pairs of repeating units. Figure 4A shows this matrix, obtained with a time average of 1 ns, in the trajectory interval between 29 and 30 ns. Color coding has been used to indicate the distance value. In Figure 4A, the disaccharide (UA) or (U^'A) has been considered as a residue (i.e., the HA repeating unit as it is or grafted by the hexadecyl group) and 48 total residues have been included, belonging to the 6 oligosaccharide chains. Along the diagonal of the matrix, the six oligosaccharide chains, reported in an arbitrary sequence and labeled with integer numbers from 0 to 5, are also indicated. The junction zones between chains are represented by out of diagonal yellow-orange domains.

Examination of Figure 4A reveals two extended contact regions, stabilized by hydrophobic interactions, between pairs of chains (3–4 and 1–5), where the central (U^'A) residues are also involved. Three other junctions, driven by interchain hydrogen bonds, arise between the terminal residues of the oligosaccharides (pairs 1–2, 2–3, and 2–5) propagating the connectivity to five out of six chains of the system. This cluster is in contact with its periodic image, thus giving a percolating network. It should be noted that the interresidue distance matrices calculated in a time window of 1 ns along the trajectory for times longer than 20 ns are similar to that shown in Figure 4A. Only local minor differences involving pairs of isolated residues on different chains can be found, indicating a quite well-defined structural arrangement for a space resolution of about 1 nm (the length of a HA repeating unit) and a time resolution of 1 ns.

Figure 4B shows the matrix of the smallest distances between pairs of repeating units for the HA hydrogel model, obtained as for the HYADD4 system. A lower number of contacts with a smaller extension than those found in the HYADD4 model can be observed at the same trajectory time. This result is in agreement with the higher gelling ability of HYADD4 with respect to HA due to the hydrophobic junction domains.
Conclusion

HYADD4 is a modified hyaluronate carrying 1 to 3 hexadecyl side chains every 100 sugar residues. Such a small structural change has a dramatic effect on the physical properties of aqueous solutions containing a low concentration of this polymer. At a concentration as low as 0.3% (w/v) HYADD4 in aqueous PBS the system forms a gel, while unsubstituted hyaluronic acid forms viscous solutions at concentrations that are ten times higher. In this study, we investigated the factors leading to this behavior. Characterization of the gel phase provided an evaluation of the average mesh size and the relaxation time of the network at different polymer concentrations. The findings from this study suggest that the hydrophobic interactions occurring between the alkyl side chains may be the driving force for the aggregation processes. This hypothesis was confirmed by the results of an MD simulation describing the HYADD4 system.

A number of issues still require further evaluation to evolve our understanding of the HYADD4 behavior at the gel point, such as: (1) the possibility of a hierarchical process characterized by self-assembled intermediate structures, leading to such aggregation, and (2) the dynamic behavior of water as well as of the polymer chains in the formed network. Such investigation could use quasi-elastic incoherent neutron scattering experiments combined with a MD study devoted to the analysis of the dynamics of water in HYADD4 hydrogels.

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