Physical crosslinking of hyaluronic acid in the presence of phospholipids in an aqueous nano-environment

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Hyaluronic acid and phospholipids are two components in the synovial joint cavity that contribute to joint lubrication synergistically. Molecular dynamics simulations were performed and hydrogen bonds in hyaluronic acid were analyzed to identify specific sites that are responsible for its physical cross-linking. Two molecular masses of hyaluronic acid, 10 kDa and 160 kDa, were considered. We use molecular dynamics simulations and the small world network approach to investigate dynamic couplings using a distance map applied to oxygen atoms in a chain of hyaluronic acid in the presence of phospholipids and water. The distance characterizing the coupling can be defined in various ways to bring out the most evident differences between various scenarios of the polymer chain conformation. We show herein a physical distance understood as H-bond length and classes of these distances which are defined in a coarse-grained picture of the molecule. Simulation results indicate that addition of phospholipids has little influence on hyaluronic acid crosslinking. However, longer chains and addition of lipids promote appreciably long lasting (resilient) networks that may be of importance in biological systems. Specific sites for hydrogen bonding of phospholipids to hyaluronic acid have also been identified.

Introduction

Hyaluronic acid (HA) (Fig. 1) is a widely distributed biopolymer present in several locations in the body, such as skin, synovial fluid, lungs, eyes, etc. It plays an important role in synovial joints, where its synergistic action with common phospholipids (PLs) (and other biomolecules) contributes to the outstanding lubrication of articular cartilage (AC). It fulfills several roles in synovial fluid (SF), such as a shock absorber, viscosity modifier, etc. Efficient shock absorption results due to its crosslinking behavior, as the polymers create dense networks that contribute to the viscoelastic properties of the bulk fluid. HA in natural systems forms cross-linked networks primarily due to hydrogen bond formation involving oxygen and nitrogen atoms and hydrophobic contacts between carbon atoms. Under pathological conditions alterations in SF composition occur; the concentration of PLs can increase threefold while the polydispersity of HA is typically reduced. These changes lower the system’s functionality and can lead to higher rate of cartilage wear.

In recent years it has become increasingly clear that understanding the outstanding lubrication properties of synovial joints requires an investigation of lubrication synergies at the nanoscale, i.e., how different molecular species interact together to produce low friction as well as high load bearing capacity and low wear. To date, several synergistic pairs have been identified, including lubricin with cartilage oligomeric matrix...
protein\textsuperscript{3} and hyaluronan with phospholipids.\textsuperscript{1,2} A central concept is “hydration lubrication” which was first emphasized by Klein and describes how water, when strongly bound to surfaces under confinement, remains fluid under high pressure and allows sliding motion with low energy dissipation.\textsuperscript{17}

To understand the nature of the interaction between HA and PLs, which is crucial for proper interpretation of joint lubrication nanoscale effects,\textsuperscript{5,18} one must obtain information on specific inter- and intra-molecular bonding in HA-HA and HA-PL systems on several length scales, including the atomicistic description. The aim of this study is to elucidate specific hydrogen bonds and hydrophobic contacts between HA molecules (and PLs\textsuperscript{18}) for long and short chains in the absence and presence of PLs. The properties of long chains mixed with PLs play a pivotal role in the nanotribology of joint lubrication.\textsuperscript{2,19,20} Interactions between HA and PLs are complex in nature and may involve both hydrogen bonding (H-bonds) and hydrophobic interactions. Some studies suggest that HA molecular mass seems to be a key factor in the process, as HA of molecular mass below 160–170 kDa creates different functional forms as compared to those of higher molecular mass.\textsuperscript{21} However, it seems rather unlikely that all the structural arrangements presented in ref. 21 occur in the natural systems. Nevertheless, structural differences may be responsible for the reduction of lubrication in dysfunctional joints.

The analysis of biopolymers uses methods that describe the molecule in either an atomistic or coarse-grained framework, which brings out the most important features that follow from the full atomic representation. The small world networks’ theory\textsuperscript{22} can be used as a tool to take us to such a coarse-grained representation of biopolymer properties including dynamics. There are examples of the effective use of this method in polymer science.\textsuperscript{23–25}

Small world networks in general allow the study of the topology of a complex dynamical system by representing it as an undirected graph. Such a graph consists of a set of nodes with a set of edges that connect pairs of nodes. From the physical point of view a node represents an element of the system, and the physical distances between the elements determine the interactions.

In the description of polymers, structural units (SUs) are typically defined as the repeated building blocks of a chain, and these would then constitute the nodes. The connection between the SUs is represented by the edges in the small word network and the distance between them is considered. Central quantities of the network are the characteristic path length and the clustering coefficient. Such an approach has successfully been used in studies of proteins.\textsuperscript{26} Reducing larger structures to single units in this way is characteristic of coarse-grained methodology.

Although we use the small world networks’ approach in this work, we also propose some modifications. In the SUs all oxygen atoms are numbered from 1–11 (see Fig. 1). This scheme of numbering is repeated for each SU in a polymer. According to this, in our analysis each single node represents groups of oxygen atoms assigned with the same number in the repeating SUs. This modification is justified due to the periodicity in the structure of HA. After this division of oxygen atoms into classes we perform an analysis, not on single distances between atoms, but on average distances between groups of atoms, each of them belonging to two different classes across all identical SUs. This distance $D_{ij}$ between two classes $i$ and $j$ can be calculated by the following formula:

$$D_{ij} = \frac{1}{N^2} \sum_{k=1}^{N} |i_k - j_k|$$  \hspace{1cm} (1)$$

where $l$ is a variable denoting atoms in class $i$, and $k$ is a variable denoting atoms in class $j$. The number $N$ denotes the number of units building the HA molecule.

**Simulation details**

All atom molecular dynamics simulations were performed using the AMBER03 force field\textsuperscript{27} to evaluate interactions between the hyaluronic acid macro-ions and phospholipid molecules. The AMBER03 potential function describing interactions among the particles is given by:

$$E_{\text{total}} = \sum_{\text{bonds}} k_b (R - R_{eq})^2 + \sum_{\text{angles}} k_\theta (\theta - \theta_{eq})^2$$

$$+ \sum_{\text{dihedrals}} \frac{V_2}{2} [1 + \cos(n\phi - \gamma)] + \sum_{i<j} \left[ \frac{A_{ij}}{R_{ij}^{12}} - \frac{B_{ij}}{R_{ij}^6} \right] + \frac{q_i q_j}{\varepsilon R_{ij}}$$  \hspace{1cm} (2)$$

The quantities in formula (2) are defined as follows: $k_b$ and $k_\theta$ are the force constants for the bond and bond angles, respectively; $R$ and $\theta$ are bond length and bond angle; $R_{eq}$ and $\theta_{eq}$ are the equilibrium bond length and bond angle; $\phi$ is the dihedral angle and $V_n$ is the corresponding force constant; the phase angle $\gamma$ takes values of either $0^\circ$ or $180^\circ$. The nonbonded part of the potential is represented by a short-range repulsive term ($A_{ij}$), a London dispersion terms ($B_{ij}$) and interactions between partial atomic charges ($q_i$ and $q_j$). $\varepsilon$ is the dielectric constant. HA and PL structures were downloaded from PubChem. The lipid used for this study was dipalmitylphosphatidylcholine (DPPC). The downloaded hyaluronic acid macro-ion was modified to obtain longer chains by using the YASARA Structure Software. The final molecular masses of HA considered in this study were 10 and 160 kDa (consisting of 27 and 432 SUs). For the large HA chain we only consider one chain, but for the small HA chain our simulation box contains 16 chains. Thus, the concentration of HA subunits is the same in the two cases considered. Additionally the TIP3P water model was used.\textsuperscript{28} For the isobaric–isothermal ensemble, all atom simulations were performed under the same conditions: temperature 310 K (physiological), pH = 7.0 and 0.154 M NaCl aqueous solution, with a time step of 2 fs. The Berendsen barostat\textsuperscript{29} and thermostat with a relaxation time of 1 fs were used to maintain constant pressure and temperature. Then, the DPPC solution was mixed and added to a concentration of $c_{\text{DPPC}} = 2 \times 10^{-7}$ M, i.e., much higher than the cmc (critical micelle concentration) of DPPC $c_{\text{cmc}} \sim 5 \times 10^{-10}$ M.\textsuperscript{30} The final concentration of polymer was
$c_{HA} = 10^{-6}$ M (the SU concentration was kept constant by increasing the total number of chains for the 10 kDa case to 16), which is much higher than the average in SF. However, we are focused on the dense network region of SF, rather than on the overall concentration. Two molecular masses of HA were investigated, and in both cases the situations of HA in water alone and HA with phospholipid in water were considered.

**Hydrogen bond identification**

A hydrogen bond is formed between two oxygen atoms if: (i) the distance between the hydrogen and adjacent oxygen atoms is smaller than $R_{O\cdot H} = 2.6$ Å; and (ii) the distance between two neighboring oxygen atoms is less than $R_{O\cdot O} = 2.8$ Å. YASARA assigns at most one hydrogen bond per hydrogen atom, picking the one with lowest energy if two acceptors are available. Here we divide the hydrogen bonds inside HA into two sets. First, nearest neighbors (NN) are the atoms from the two closest SUs in the linear chain order (separated by $<1$ nm along the chain). Further neighbors (FN) are bonds between atoms that are separated by more than one SU ($>1$ nm along the chain). This distinction has been made to look at two different mechanisms: (a) chain stiffness, related to NN bond formation, (b) physical crosslinking of the HA network, related to FN.

In our small world network analysis we did not consider a single SU in its entirety, as is common in these kinds of methods, but instead their chemically active subparts – all oxygen atoms that are present in the whole molecule. According to formula (1), a single edge between two nodes (sets of oxygen atoms assigned with the same natural number) represents a set of distances between oxygen atoms belonging to these two nodes. Due to the distinction of hydrogen bonds inside HA into NN and FN sets, our analyses will be performed twice i.e. in a set of NN and then in a set of FN, respectively. We can get a single value for each edge by calculating the average over all distances belonging to this class.

The small world network distance between nodes (classes of oxygen atoms) is one relationship between them. Apart from sets of distances we can consider other interactions between nodes. It results in construction of many graphs that differ only in the values assigned to each edge. These edge values have different physical meaning according to the chosen definition of interaction. In this work we consider only Euclidean distances between oxygen atoms and the number of contacts between them. Finally, the values assigned to an edge are an average distance and an average number of contacts between oxygen atoms belonging to the two different classes considered in the graph.

From such graphs, as defined above, we obtain characteristic parameters describing the molecular arrangements. Initially we calculate the network distance between two nodes. It is defined as the number of edges along the shortest path connecting them. We mark it by $d_{ij}$, where indices $i$ and $j$ denote two classes. After calculation of all distances, we obtain the characteristic path length of the network. This quantity is defined as the average network distance between two nodes calculated over all pairs of nodes:

$$L = \frac{2}{N(N-1)} \sum_{i=1}^{N-1} \sum_{j=i+1}^{N} d_{ij}$$

The characteristic path length $L$ of the network is a global property that reflects the efficiency of the network. A smaller value is due to increased randomness in the network.

The second quantity of the network is a clustering coefficient, which characterizes a local structure of the network. If we first select node $i$, then we have some number $k_i$ nodes that are connected to the i-th node. Then we can find the number $E_i$ of edges that actually exist between these $k_i$ nodes. The maximum number $M_i$ of edges that can exist between these $k_i$ nodes is expressed by:

$$M_i = \frac{k_i(k_i - 1)}{2}$$

The ratio between $E_i$ and $M_i$ gives the value of the individual clustering coefficient of the $i$-th node. The clustering coefficient of the network is the average of all individual clustering coefficients:

$$C = \frac{1}{G} \sum_i E_i / M_i$$

where $G$ is the total number of nodes in the network (in our case $G = 11$). A larger value of the cluster coefficient should be interpreted as a larger tendency for clustering.

From the molecular dynamics simulations we utilize two parameters for further analysis. One is the Euclidean distance between oxygen atoms, which were established during simulations. The second quantity is the number of contacts between oxygen atoms (number of H-bonds). These two quantities, after division into classes, produce two kinds of maps: distance maps and contact maps. Additionally, we distinguish between NN and FN neighbors. For each case we perform the small world network analysis and calculate the $L$ and $C$ parameters as described above.

**Hydrophobic interactions**

Hydrophobic atoms are identified and assigned an atom type from the groups defined as: type 1 for carbon atoms with three or more bound hydrogen atoms (–CH3), type 2 for carbon atoms with two hydrogen atoms or one hydrogen atom plus three carbon atoms bound (–CH2–, HCC3). In our study of the HA molecule only the NAG moiety creates hydrophobic contacts – carbon atoms C6 and C8 near atoms O10 and O11, respectively. For each of the resulting hydrophobic interactions, a knowledge-based potential was extracted from high-resolution X-ray structures in the PDB files (MicroSoft), and the favorable interaction was determined.

**Results**

The final structures of HA chains with PL are presented in Fig. 2. Nearest neighbors form 42% of all H-bonds in long
chains and 55% for short chains. Addition of PLs caused the number of H-bonds inside the HA network to decrease by 5% for long and by 10% for short chains. H-Bonds between nearest neighbors decreased between 2–4% for both types of HA chains after addition of PLs. The reduction in number of H-bonds for FN were greater, dropping by 8% and 16% for long and short chains, respectively.

Upon addition of PL the number of hydrophobic contacts decreased by 10% for both cases. Atoms of short HA chains create 30% more H-bonds and 60% more hydrophobic contacts inside the polymer than long HA chains. The organization of water between molecular species is more complex, however in this work we will focus only on single molecule bonding.

**Bonding inside HA chains**

Fig. 3 and 4 show the total number of H-bonds for all cases for FN and NN (contact maps). The corresponding data in terms of average bond length is presented in Fig. 5 and 6 (distance maps). By comparing Fig. 4a and c as well as Fig. 6a and c one notices that hydrogen bonding between NN are similar for short and long HA chains, where only a few possible pairs of interactions are favored (red color in Fig. 3 and 4 and blue color in Fig. 5 and 6), which differs significantly compared to FN H-bonds that are less selective (compare Fig. 3 and 4 as well as Fig. 5 and 6). This is an influence of the local stiffness of the HA chain. The presence of PL leads to marginal decrease in the number of NN H-bonds as shown in Fig. 4b and d, where the difference in number of H-bonds in absence and presence of PLs are shown. However, in case of FN the presence of PLs has higher impact (Fig. 3b and d).

The length of the HA chain affects both FN and NN H-bonds. Fig. 3a and c show that there is rather small chain length dependence for FN bonding sites, except for bonding between oxygen atom O2 with atoms O10 and O11, where the total...
number increases 2–4 folds for the longer chain. There are several pairs in NN that are exclusive for each HA chain. Namely, O2–O10/O11 for short HA and pairs O4–N, O6–O8 for long HA (and several minor pairs O1–O9, O8–O11 etc.) as presented in Fig. 4. This, however, does not influence the average H-bond length as presented in Fig. 5 and 6.

Using the algorithm described in the methods section, we apply the following coding for the small world network distance between two classes in the graph. If there is direct connection between a pair of atom classes we assign it as 1. Next, if there is one class along the path between two nodes in the graph we code this as 2, and so on. During the calculations leading to the values reported in Tables 1 and 2 we average the interactions associated with the two oxygen atoms of the carboxyl group and the rest of the oxygen atoms (both of them show up almost identical H-bonding distribution).

Calculations using the small world network, performed only for intramolecular hydrogen bonds, are presented in the Tables 1 and 2. The values describe the characteristic path length L and clustering coefficient C of the network. The network was created according to the description provided in the Introduction, and values were calculated according to formula (3)–(5). These parameters provide a quantitative measure of the overall characteristics of the maps.

The results of the calculations for distance maps (Table 1) lead to the conclusion that FN interactions are characterized by a smaller L and larger C, signifying a higher tendency of clustering for FN interactions.

Results of the small world network calculations for the contact maps (parameters L and C in Table 2) are characterized by less regular changes, and thus less straightforward to interpret. However, in all cases clustering coefficients are greater for FN than for NN. It express that specific clusters are present in the network. The L parameter in case c is slightly smaller for FN than for NN. In the other case (a, b and d) this parameter is slightly greater for FN than for NN.

In this analysis we can see that the influence of phospholipids is different in the case of short chains and long chains. For instance, for FN (Table 2) the C parameter is the same for all cases, suggesting similar clustering tendency. However, for short chains (case a and b) the characteristic path length is smaller in the presence of phospholipids. This tendency is opposite to the case of long chains where L is larger in presence of phospholipids.

Fig. 7 and 8 show the duration of H-bonds and hydrophobic contacts evaluated from the MD simulations. Hydrophobic contacts show similar longevity to FN H-bonds, reflecting the dynamics of the chain. Hydrophobic interactions are much weaker than H-bonds, thus breaking H-bonds is the key factor governing cross-linking (as expected from the mainly hydrophilic nature of HA).

### Table 1

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<tr>
<th>Case</th>
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<th>C for NN</th>
<th>L for FN</th>
<th>C for FN</th>
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<td>(c)</td>
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<td>(d)</td>
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<td>0.43</td>
<td>1.24</td>
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</table>

### Table 2

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<th>C for NN</th>
<th>L for FN</th>
<th>C for FN</th>
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<tbody>
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<td>0.39</td>
<td>9.04</td>
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<tr>
<td>(b)</td>
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<tr>
<td>(c)</td>
<td>9.42</td>
<td>0.37</td>
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<tr>
<td>(d)</td>
<td>9.38</td>
<td>0.40</td>
<td>9.45</td>
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</table>
HA bonding with water

We have also examined H-bonds between HA oxygen atoms and water molecules, see Fig. 9. There were no significant differences between long and short chains. The addition of PLs likewise did not have any large influence on the H-bonding with water. The oxygen atoms from the carboxyl group (5 and 6) show the highest propensity for hydrogen bonding with water. Additionally, we looked at the water bridges for the closest atoms in the nearest SU and those further away (further than 1 dimer). Fig. 10 shows H-bonds of HA mediated by a single water molecule. Water can bind up to 4 atoms inside the HA chain. Most of them are H-bonds between further neighbors. Although, most of them are between FN, there are cases of three-site water H-bonding where two important H-bonds form between NNs. Namely, atoms 2-5-N create 14% and atoms 2-9-10 create 24% of all NN 3 sites H-bonds.

HA bonding with PL

Finally, we have determined the specific bonding between HA and PLs and their durations. Results are presented in Fig. 11 and 12.

Discussion

Since HA combined with PLs is one of the most important synergistic pairs for the function of the articular cartilage in terms of nanoscale shock absorption\textsuperscript{13} and boundary lubrication\textsuperscript{2,3} we have focused on providing understanding of their interactions. This is motivated by the fact that while phospholipids and hyaluronan interact and form self-assembly structures in bulk and at interfaces\textsuperscript{11,34} the driving force for the self-assembly process remains poorly understood. Some progress has, however, been made and it was revealed that hyaluronan primarily interacts with phospholipid headgroups and this interaction is amplified by the presence of calcium ions\textsuperscript{11}.

Our results show how creation of HA inter- and intra-molecular contacts depends on molecular mass. Fig. 3–6 show that while interactions between further atoms do not significantly differ between the two molecular masses, inspection of NN H-bonds does yield a distinction. Shorter chains, on the one hand, create more H-bonds with NN atoms and this contributes to higher stiffness in these chains. On the other hand, longer chains are prone to more crosslinking. Further analysis (by applying density functional theory) can be performed to understand the nature of this process. Fig. 5 and 6 show that FN create on average slightly shorter H-bonds than NN. On the other hand, Fig. 7 and 8 show that long chain networks seem to last longer than networks formed by short chains, and here the hydrophobic effect plays an important role (Fig. 8). The comparatively short-lived networks formed by short chain HA may be of less significance than the more stable networks formed by long chain HA with respect to viscoelastic and shock absorption properties, cf. Fig. 2 for visual inspection.

Addition of PLs can slightly enhance the network stability, as evidenced by their extended longevity. Fig. 11 shows that short HA chains create longer lasting H-bonds with PLs as compared to longer HA chains. Taking into account that PLs are absorbed into the network, they may to some extent act as agents responsible for sustaining the network. Related to this, it has been observed that shorter HA chains affect the structure of...
Langmuir PLs layers more than the longer chains. Fig. 9 and 10 show that water can form water bridges between specific atoms, thus being an additional agent influencing network stability. Both direct H-bonds between atoms and such bonds mediated by water bridges contribute to crosslinking and stiffness of the chain. Such water bridges are short lived; however they may function as temporary assistants maintaining network properties. For long chains that adopt a compact conformation in the simulation box, phospholipids tend to accumulate at the surface of the volume defined by the HA chain. For the more open network formed by shorter HA chains, the simulation snapshots (Fig. 2) suggest that phospholipids also enter the interior of the volume occupied by the chains. 

PLs, in forms of vesicles and bilayers, have been shown to provide conditions for efficient boundary and mixed lubrication mechanisms. Association between HA and DPPC occurs, and our simulations provide information on hydrogen bonds between DPPC and different oxygen classes (Fig. 12), where a preference for direct hydrogen bonding between the phosphate group of DPPC and classes 1, 2, 8, 10 and N of HA has been found. Bonding between other oxygen atoms is mediated by water molecules, therefore water is important for both facilitating formation of the HA network (Fig. 9 and 10) and for HA–PL interactions.

The current understanding is that effective boundary lubrication in the synovial joint area is due to hydration repulsion generated by water interacting strongly with e.g. lubricon, phospholipid bilayers and HA present at the cartilage surface. It is also known that changes occurring in abnormal (osteoarthritic) synovial fluid cause the system to lose its properties. In particular, the concentration and polydispersity of HA decreases whereas the concentration of PL drastically increases. This leads to dramatically different performance of the SF. The viscosity of such unhealthy fluid can decrease drastically, reducing the ability to absorb sudden pressure changes.

Conclusions

This work presented molecular dynamics simulations of the HA–PL system and our data was discussed in relation to previously reported biolubrication effects. One key finding of our study is that H-bonding inside the hyaluronic acid network is relatively little affected by its molecular weight. However, for large molecular weight HA (160 kDa) H-bonds between NN make up 42% of all H-bonds, whereas the corresponding number for short chains (10 kDa) is 55%. The presence of PL decreases the number of HA H-bonds by 5–10%, and the largest effect is observed for FN interactions. The number of hydrophobic contacts between the structural units of HA is also decreased by 10% in the presence of PL. H-Bond formation between NN are more selective than between FN (Fig. 3–6), which influences of the local stiffness the HA chain. The clustering coefficient for intramolecular FN H-bond interactions is found to be larger than for NN, signifying increased clustering tendency for the FN (Tables 1 and 2).

The dynamics of the network structure is mainly dictated by H-bond interactions and low molecular weight HA networks are less stable than networks formed by long HA chains (Fig. 7b), which is expected to negatively affect viscoelastic and shock absorption properties. This is consistent with studies showing that long chain HA is essential for the proper function of synovial joints. H-Bonds between HA and water are found for each class of oxygen atoms, but most are formed between water and the oxygen atoms of the carboxylic acid group, whereas the concentration of PL classes 5 and 6, but also oxygen classes 8 and 11 form many H-bonds with water (Fig. 9). Our data also provide information on the nature of HA–PL interactions, and specific sites (oxygen classes 1, 2 and 10 and to a lesser extent classes 8 and N) on the HA-chain that form direct hydrogen bonds with PLs have been identified (Fig. 12).

Based on the small world network concept we have proposed a simple but robust numerical method of analysis of polymers that are built of identical SUs, whose structure is predominantly determined by a single kind of intramolecular interaction (in our case predominantly intramolecular H-bonding). In order to do so we considered H-bonding sites rather than structural units as nodes. We have used this method to describe HA molecules under various chemical and physical conditions and identified specific hydrogen bonding sites inside HA molecules. This method distinguishes, in a quantitative way, between topological characteristics of the polymer molecule and its associated structures. This work presents a first well-defined step toward building a realistic model of interactions between pairs of SF components.

Conflicts of interest

There are no conflicts to declare.
Acknowledgements

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