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PAPER

Modeling of hyperbranched polyesters as hosts for the multifunctional bioactive agent shikonin†

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We employ fully atomistic molecular dynamics simulations to study in detail the mechanisms involved in the non-covalent association of the bioactive agent *Shikonin* with the commercially available hyperbranched polyesters (Boltorn®), in ethanol solutions. We examine effects of the (pseudo)generation of the hyperbranched polyester and mimic two different concentrations, under conditions corresponding to excess drug availability. The two mechanisms participating in the polymer/drug complexation are hydrogen bonding and spatial constriction of the drug molecules within the hyperbranched structure. Based on static, as well as on dynamic information obtained by the analysis performed, it is demonstrated that apart from the size of the polyester, factors like the degree of structural flexibility, the intrapolymer hydrogen bonding and the polymer concentration may affect decisively the polyester/shikonin associative behavior, as well as the behavior of the drug-molecules in the solution. The results from the present study offer a detailed picture of the relative importance of those parameters affecting the complexation, and may serve as a basis for the understanding of the behavior of more complex multi-polyester systems.

I. Introduction

The efficient delivery of drugs and other therapeutic compounds into the human body has attracted an increasing scientific interest during the past decade.[1–6] Recent studies demonstrate that a large percentage of newly developed pharmaceutical substances are not exploitable by the pharmaceutical industry because of poor bioavailability due to low water solubility and/or cell membrane permeability.[7,8] To this end, scientific efforts have been directed towards the design of drug delivery vehicles which can improve the therapeutic efficacy of the transferred material and reduce possible side-effects.[9] Various macromolecular compounds have been utilized in drug-delivery applications such as synthetic polymers, antibodies, hormones, lipids and liposomes.[10–14] Dendritic polymers represent a class of macromolecules which are increasingly being considered for drug and gene delivery purposes.[11,15] Their nanosized dimensions, their highly branched structures, the presence of a large number of modifiable surface functionalities combined with their low intrinsic viscosity and high solubility render them attractive candidates for a protected transfer and controlled release of therapeutic substances.[16]

Perfectly branched dendrimers have been proven to perform favourably as solubility enhancement agents for small hydrophobic compounds.[8,17] This effect is promoted either *via* covalent attachment of the active compounds onto the surface functional groups or through their encapsulation inside the inner cavities sustained by the combined action of steric hindrance and specific interactions.[18,19] Nevertheless, the tedious and expensive synthesis of regularly branched dendritic molecules prohibits their use in large-scale applications. Unlike dendrimers, non-regularly branched polymers with properties similar to those of their regularly branched counterparts can be easily synthesized *via* one step synthesis procedures and, therefore, represent economically promising products for biomedical uses.[20–22] Non-regularly branched systems have already been utilized in numerous such applications.[23,24] Among these polymeric materials, the commercially available hyperbranched aliphatic polyesters bearing the commercial name Boltorn® are considered promising candidates for the development of novel drug delivery systems as demonstrated in recent experiments.[25] Given the potential of these multifunctional polymers to act as solubility enhancement agents, their consideration as carriers for poorly water soluble but therapeutically active pharmaceutical compounds seems appealing.

In the majority of the experimental studies exploring the complexation capabilities between hyperbranched molecules and active guest compounds, factors such as the concentration, the topology, the flexibility and the size of the polymer were found to play a crucial role in the efficiency of the complex

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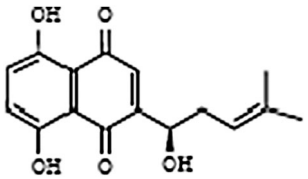

Fig. 1 Chemical structure of shikonin.

formation and the drug delivery processes. In most of the cases however, the effects of the above characteristics have not been adequately quantified, while the nature of the interactions responsible for polymer–drug association has not been thoroughly investigated. Molecular simulations have been proven an invaluable tool in extending our understanding of the mechanisms which operate at molecular levels and which essentially determine the physical behavior of such systems.[26,27] Particularly the fully atomistic molecular dynamics simulations have shed light into the driving forces responsible for the complexation of dendritic polymers with therapeutic substances.[28,29]

The aim of the present study is to examine pertinent parameters which are associated with the ability of the Boltorn® hyperbranched aliphatic polyesters to act as hosts for the pharmaceutical molecule *Shikonin via* fully atomistic molecular dynamics simulations.

Alkannin and Shikonin (A/S, Fig. 1) are optical antipodes of plant origin with a verified wide spectrum of antimicrobial, wound healing, anti-inflammatory and antioxidant activities.[30–32] Although the aforementioned antipodes were originally introduced as wound healing agents, recent studies on cancer chemotherapy revealed that A/S also exhibit antitumor activity.[33–36] Bearing in mind the frequent degradation of drugs' efficacy under conventional administration, we can surmise that this antitumor behavior could be optimized if A/S were administered through a drug delivery system analogous to the formulations described earlier. A/S and their derivatives are susceptible to transformations, such as photochemical decomposition, oxidation, polymerization and thermal degradation[30–32,37] and thus a drug delivery system could also enhance their stability. A/S have already been encapsulated in microcapsules,[38] cyclodextrins,[39] by our group. Preliminary results from recent experiments show that shikonin can be successfully incorporated in liposomes[40] and in liposome/hyperbranched polymer formulations, showing satisfactory *in vitro* release profiles.[41]

The present computational study addresses effects of concentration and topology/size of hyperbranched Boltorn® polyesters in their ability to associate with Shikonin. On account of analogous investigations,[42,43] these factors are anticipated to be of key importance in the associative behavior between these systems.

II. Description of the models

Fully atomistic models of Boltorn–shikonin complexes were constructed in ethanol solutions, as it is experimentally known that shikonin dissolves in this solvent.[44] Initial solvation of non-water-soluble pharmaceutical compounds in an organic solvent for the purpose of complex formation with the delivery agent is the first step prior to the production of solid precipitates which can later be used for the final pharmaceutical formulation.[45,46] We considered systems containing hyperbranched polyesters of the second and third (pseudo)generations (henceforth named H20 and H30, respectively). Taking into account the findings of previous simulational work on Boltorn® melts regarding the contribution of oxygen sites in hydrogen bonding interactions,[47] we opted to include in each solution (containing one polyester molecule) a number of shikonin molecules equal to the total number of oxygen atoms in the polyester. In this manner we could evaluate the propensity of hydrogen bonding between the polyester and the drug molecule, exploring thus the possibility of a maximum level of association.

Table 1 Characteristics of the simulated systems. The terms "high" and "low" refer to the concentration state of Boltorn–shikonin complexes

System notation	Box size/Å	Number of shikonin molecules	Number of ethanol molecules
H20_high	46.59	45	900
H30_high	59.75	93	1873
H20_low	57.69	45	1900
H30_low	74.04	93	4000

Based on an experimental work addressing the drug delivery potential of hyperbranched polyesters similar to those examined here,[48] we constructed models in which the weight fraction of the drug was comparable (henceforth this concentration is termed "low") or about 12% higher (henceforth termed "high") than the respective values of the above-mentioned study. In total, four ethanol solutions were generated possessing polymer–drug weight fractions of 14% and 26%, respectively. Details are presented in Table 1.

III. Simulation details

Atomistic models of the hyperbranched molecules (Fig. 2) were taken from an earlier work.[47] At the first stage of the construction of the polymer–drug models, shikonin molecules were inserted in a spherical shell of width approximately 5 Å further than the polymer's radius of gyration around the hyperbranched periphery (the maximum distance from the polymer's center of mass was larger than twice the radius of gyration of the hyperbranched molecule).

At the final stage of the preparation of the models, the polymer/drug complexes were solvated with explicit ethanol molecules in a cubic simulation cell. The interaction parameters for the energetic description of the polyester and the solvent were adopted from the AMBER force field[49] while for the shikonin molecule from the Generalized Amber Force field (GAFF).[50] Implementation of the aforementioned force field has been proven adequate for the description of dendritic polymers[51–53] and their complexes with drug compounds,[28] and in particular for the specific polyester molecules considered in the present study.[47] Partial charges were assigned according to the GAFF for shikonin (see ESI†) and for the other molecules according to the Gasteiger algorithm,[54] while all electrostatic interactions were calculated *via* full Ewald

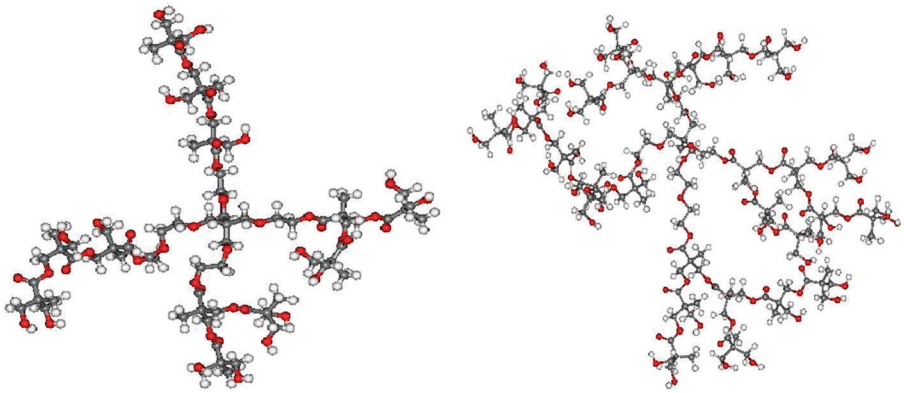

Fig. 2 Molecular structures of the 2nd (left) and the 3rd (right) (pseudo)generation aliphatic polyesters studied in the present work.

summation. van der Waals interactions were modelled by a 12–6 Lennard-Jones potential considering a cutoff radius of 10 Å. Hydrogen bonding interactions were described by a 12–10 potential term.[55]

Ensuing their construction, the systems in Table 1 were subjected to energy minimization by a combination of at least 150 000 steepest descent and conjugate gradient cycles. After completion of the energy minimization, 600 ps MD simulations were performed in the isothermal–isobaric ensemble (NPT, $p = 1$ atm, $T = 300$ K) at the end of which the density of the systems was stabilized. To allow models to adopt independent configurations and equilibrium distributions of shikonin and ethanol around the dendrimer, we performed a series of MD runs in the canonical ensemble (preserving the box-size that was determined from the preceding NPT runs), starting from 800 K and cooling in steps of 100 K until the temperature of 300 K was reached. At each temperature the systems spent at least 200 ps (in 1 fs steps). At the end of this procedure and at the $T = 300$ K run, energy, pressure (1 atm), average polymer dimensions and distributions of ethanol and drug molecules around the hyperbranched polymer have reached equilibrium. Using these systems as starting configurations we have conducted production runs of 4 ns in the microcanonical ensemble (NVE) with a time step of 1 fs and frame-saving frequency of 1 ps. During production runs all energetic components remained stable, the average pressure remained close to 1 atm and the temperature at 300 K. The length of the produced trajectories was 6 to 10 times longer compared to the timescales for the relaxation of the autocorrelation function describing fluctuations of the radius of gyration (see ESI†) of the hyperbranched molecules. In addition, examination of the mean square displacement of the centers of mass of all the molecular species (see Section VI below) showed that the polymers have diffused at distances considerably larger than their radii of gyration, while the drug and ethanol diffusivity have reached the hydrodynamic limit as well. Examples of equilibrated systems are shown in Fig. 3, for the higher concentration models.

As a check for the appropriateness of the utilized combination of the force field and the partial charge assignment procedure for the solvent and drug molecules, we have also simulated models of pure ethanol and shikonin at $T = 300$ K, adopting a similar combination of energy minimization and MD equilibration runs as the one described before. At the end of this procedure the average density was stabilized (at least for the ethanol sample where experimental data are available, the bulk density was reproduced within a deviation of less than 6%), while energetic contributions reached equilibrium as well. Ensuing the equilibration process, trajectories of 3 ns (for the shikonin) and 2 ns (for ethanol) length were performed in the NVE ensemble. To check whether the adopted force field parameters were effective in reproducing the experimentally observed miscibility of the shikonin in ethanol,

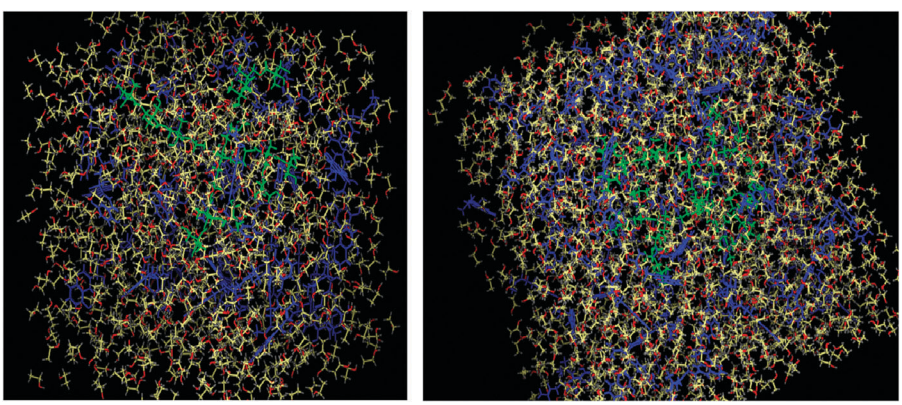

Fig. 3 Snapshots of systems H20_high (left) and H30_high (right). The hyperbranched polymer appears in green color whereas drug molecules are depicted in blue. Ethanol is shown in yellow (red dots correspond to oxygen atoms).

we have calculated separately and compared the solubility parameters of the two components. Calculation of the solubility parameter was performed through the determination of the cohesive energy density[56,57] according to eqn (1)

$$\delta = \sqrt{\frac{E_{coh}}{V}} \quad (1)$$

Here E_{coh} represents the cohesive energy of intermolecular origin and V the volume of the system. This calculation yielded a value of 13.10 ± 0.54 (cal cm^{-3})$^{0.5}$ for ethanol which is in close agreement with the experimental value of 12.70 (cal cm^{-3})$^{0.5}$,[58] while a value of 11.11 ± 0.52 (cal cm^{-3})$^{0.5}$ was calculated for the bulk shikonin. The similarity between the obtained values for the two systems is consistent with the experimentally observed miscibility of the two substances.[44]

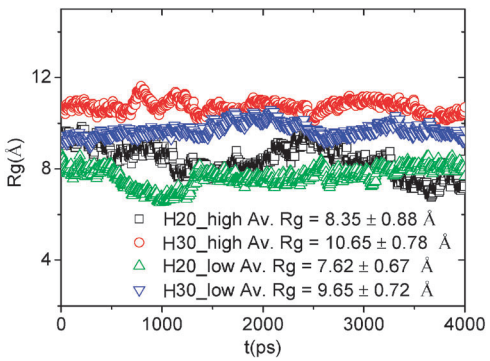

Fig. 4 Average radius of gyration of the polymer molecules as a function of time. The overall averages of the Rg values as well as the respective standard deviation are shown as well.

IV. Static features of the polyester/shikonin systems

The effect of the variation in concentration on the average size of the polymer is presented in Fig. 4, where the time evolution of the radius of gyration of the hyperbranched species is monitored.

For systems corresponding to polyesters of (pseudo)-generation 3, the average radius of gyration appears moderately higher in the high concentration system (approximately 10% higher than the lower concentration analogue), while for the systems of the polyester of (pseudo)generation 2, this difference is somewhat smaller. To collect more information regarding the relative spatial arrangement of the three components (which might relate to the average conformational state of the polymeric molecule) we have also constructed the density profiles of all the molecular species with respect to the center of mass of the hyperbranched polyester. Fig. 5 illustrates the density distributions of shikonin and ethanol molecules, as well as the profile arising from the hyperbranched molecule itself.

As can be verified in all cases, ethanol profiles reach the average bulk value at distances close to half the box size or shorter, well beyond the polymer's boundary, while both ethanol and shikonin molecules penetrate substantially within the polymeric structure. The hyperbranched polymer of the 2nd (pseudo)generation adopts a "dense core" conformation with a gradual drop in the distribution towards the periphery. A similar behavior is noticed for the higher (pseudo)generation polymer at high concentration. The behavior of the density profile in the "H30_low" system is somewhat different, reaching a maximum at approximately 4 Å with respect to the polymer center of mass. This is already an indication of a differentiation in the conformational behavior of the more "open" H30

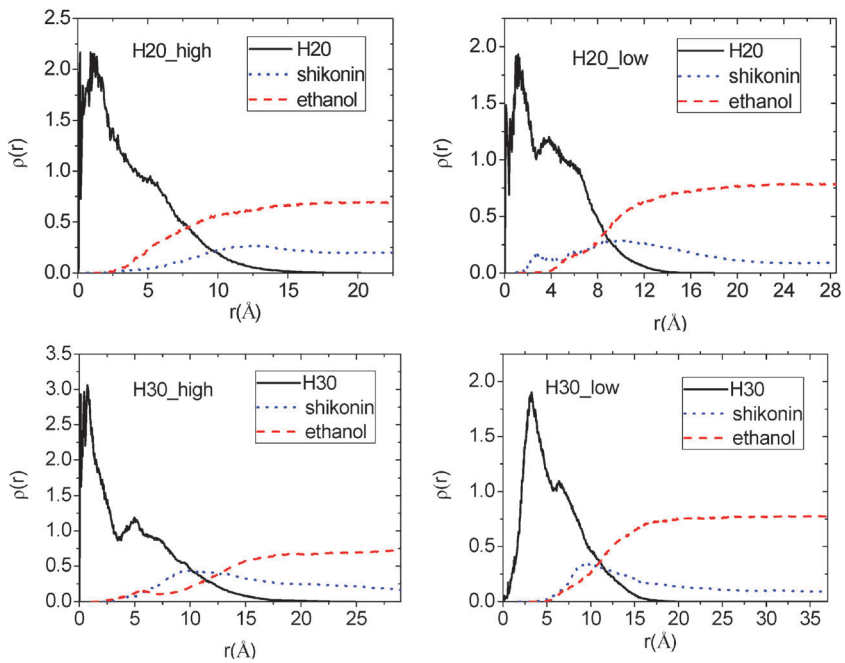

Fig. 5 Density distributions of hyperbranched, ethanol and shikonin molecules with respect to the center of mass of the polymer for all the examined systems.

Table 2 Average numbers of penetrating solvent and drug molecules into the boundaries of the polyester structure

System	N_{eth}	N_{drug}
H20_high	55.8	3.3
H20_low	39.9	3.9
H30_high	82.4	12.2
H30_low	76.0	7.5

polymer, upon increase in concentration. It is also noteworthy that in the "H20_low" system, drug molecules seem to penetrate very close to the center of mass of the polymer.

To quantify the degree of penetration of shikonin and solvent inside the polymeric interior we estimated the average number of penetrating solvent and drug molecules within the hyperbranched polyester boundaries. As boundary of the polymer we considered the distance from its center of mass at which the corresponding density profile has dropped as low as 0.05, *i.e.* practically close to zero. The so-estimated numbers of penetrating molecules are listed in Table 2.

In both, H20 and H30 systems, the number of penetrating solvent molecules increases upon increase of the concentration. The same applies for the penetrating drug molecules when passing from the "H30_low" to the "H30_high" system, while for the H20 systems this trend marginally reverses. Since in the studied systems the mechanisms involved in the polymer/drug association could only be related to non-bonded interactions and geometric restriction (*i.e.*, entrapment) of the latter within the hyperbranched structure, we can surmise that these mechanisms act differently depending on the (pseudo)generation of the polyester, at least at the low concentration levels.

One route for the rationalization of such differences is to consider whether the conformational characteristics of the host molecules in the studied conditions can promote or inhibit the residence of the guest moieties within their interior. For instance, it is known that the ability of the hyperbranched molecules for local rearrangements in order to accommodate guest molecules that can be sterically trapped or complexed with the polymer *via* non-bonded interactions is related to the degree of structural flexibility of the considered host system. As was demonstrated in studies involving complexes of dendritic molecules, higher flexibility usually promotes the formation and the stability of such complexes.[59,60]

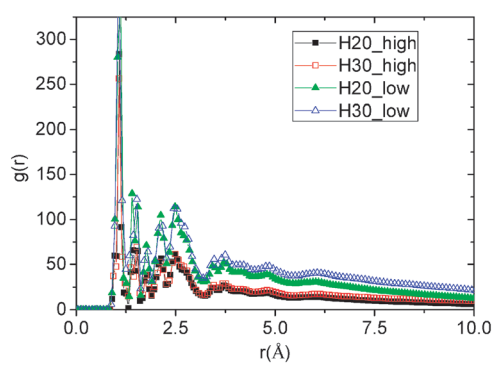

Fig. 6 Radial distribution functions of the hyperbranched components of the examined systems.

To obtain information regarding the degree of polymer flexibility in our systems, we calculated the total (*i.e.* taking into account all the atoms) radial distribution function of the polymer as illustrated in Fig. 6. Direct visual inspection of the spectra shows that the systems of low and high concentrations are grouped together. In particular, at distances corresponding typically to separations related to bending and to torsional angles (*i.e.* between 2 and 3.5 Å), the "high" concentration systems exhibit lower amplitude peaks, implying a lower degree of "structural coherence" and thus indicating a higher level of conformational flexibility.

Although this line of reasoning is consistent with the trend observed for the H30 systems (*i.e.*, increase of the number of drug penetrants as the concentration increases, Table 2), it appears that it cannot account for the behavior observed in the "H20_low" system. It is therefore necessary to consider additional parameters that may affect the associative capability between the drug and the polymeric host. To this end, we have examined another factor which is known to play a key role in drug/polymer complex formation, that is, hydrogen bonding.

V. Hydrogen bonding

Relevant experimental studies have linked the degree of conformational flexibility of the polymeric host with the potential for hydrogen bonding between active groups of the latter and the guest moieties.[61] In addition, experimental[62,63] as well as simulational efforts[47] in hyperbranched polyester melts similar to those studied here have demonstrated that intramolecular hydrogen bonding may alter the polymers' conformational characteristics as well as their local dynamic response.[47,64] Since these hyperbranched polymers do exhibit a tendency for hydrogen bonding, it is reasonable to assume that such interactions will affect both the conformational and the dynamic characteristics of the polymers' themselves as well as their potential to form non-covalent complexes with hydrogen-bonding-capable guest compounds.

V.1 Intramolecular hydrogen bonding in the polyesters

To examine the formation of internal hydrogen bonds in the hyperbranched polyesters in the solution state and in the presence of the drug molecules, we have calculated appropriate intramolecular spatial distribution functions between pertinent atomic pairs. Specifically, radial distribution functions (RDFs) between the hydroxyl oxygen (symbolized as OH henceforth) and hydroxyl hydrogen (HO), as well as between carbonyl oxygen (O) and hydroxyl hydrogen, have been examined. Identification of a hydrogen bond can be made through commonly used geometric criteria,[53,65,66] *i.e.*, the distance between the relevant hydrogen and oxygen atoms (typically close to 2–3 Å for the examined pairs) and the angle ϑ between the O–H$\cdots$O triplet. Here we have considered pairs forming angles $\vartheta \geq 120^{\circ}$. The so-calculated pair correlation functions are presented in Fig. 7.

In both the examined pairs, peaks indicative of hydrogen bond formation are present. Interestingly, the peaks corresponding to lower concentrations for both (pseudo)generations of the polyester exhibit a higher amplitude compared to those describing the higher concentration analogues.

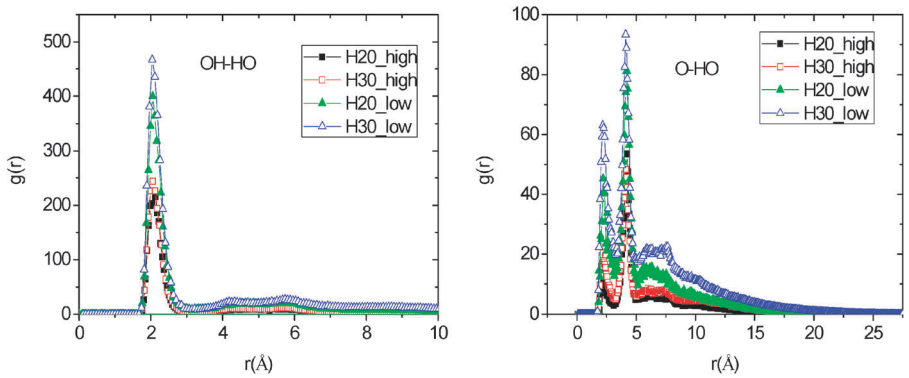

Fig. 7 Intramolecular pair distribution functions between hydroxyl oxygens and hydroxyl hydrogens (left) and carbonyl oxygens and hydroxyl hydrogens (right).

This behavior appears to be consistent with the smaller degree of flexibility observed for the lower concentration systems, since a higher level of intramolecular hydrogen-bonding would contribute towards a firmer internal structure. A less deformable environment restricts the motion of the polymer local structural units (*i.e.*, bonds, bending angles, torsions) reducing thus their probability to reorient in appropriate geometries in order to associate with guest moieties. It is also noteworthy to point out the appearance of the second peak in the O–HO pair (Fig. 7, right), which is consistent with the formation of a "double" hydrogen bond as has been observed in systems of bulk polyesters identical to those examined in the present work.[47,67]

V.2 Polyester–shikonin hydrogen bonding

To probe hydrogen-bond-mediated association of the polyester molecule with shikonin, in analogy to similar cases between dendritic polymers and small hydrophobic compounds,[68,69] we evaluated intermolecular pair distribution functions between pertinent atoms of polymers and drug molecules (again considering angles $\vartheta \geq 120^{\circ}$). Atomic pairs which manifested a clear hydrogen bonding formation were shikonin's hydroxyl oxygen (sOH) and polymer's hydroxyl hydrogen (pHO) as well as the drug's carbonyl oxygen (sO) and polymer's hydroxyl hydrogen (pHO). Fig. 8 shows the corresponding pair correlation functions.

A peak at approximately 2.2 Å can readily be resolved for both the examined pairs indicating hydrogen bond formation. The spectral features characterizing this maximum appear to be common for all the examined systems. This is not the case, however, for the broader maximum between 5 and 10 Å. Taking into account the chemical structure of shikonin (see Fig. 1), one possible explanation is to assume that this peak is related to the distance between the polyester's hydroxyl hydrogen forming the hydrogen bond (pHO) and the drug's oxygen (either sOH or sO) neighboring to the actual drug's oxygen (sOH or sO, respectively) participating in the hydrogen bonded pair. The fact that this peak appears more prominent in the low concentration systems could then be attributed to a more constricted microenvironment which would hinder rapid reorientational and/or translational motion of the associated drug molecules, contributing thus to a higher "persistence" of their local geometric characteristics (*i.e.*, orientation and location) with respect to the hyperbranched host. Alternatively (or even in a synergistic sense), the development of the second peak could be related to the presence of hydroxyl or carbonyl oxygens belonging to neighboring drug molecules near a hydrogen bond formed by another drug molecule and the polyester (snapshots of the systems indicating hydrogen-bond formation are shown in the ESI†). Both of the above-described mechanisms considered individually or combined appear consistent with the lower degree of structural flexibility of low concentration systems as argued earlier.

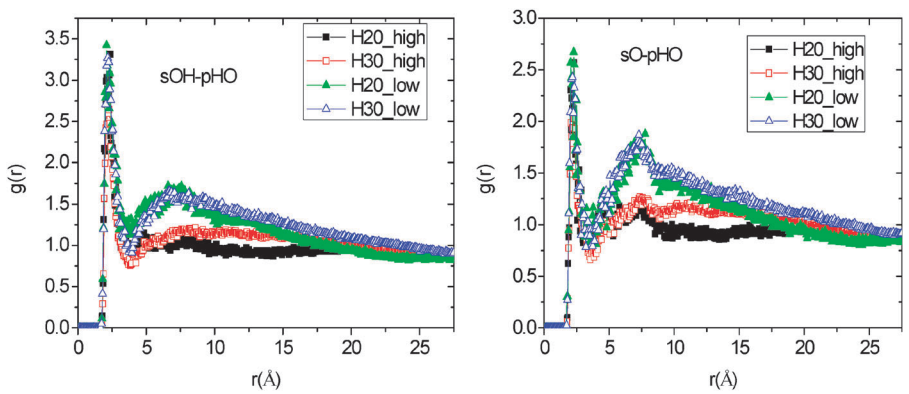

Fig. 8 Intermolecular pair distribution functions of hydrogen-bonding-capable pairs involving polyester hydroxyl-hydrogen atoms and shikonin's oxygens. Left: shikonin hydroxyl oxygen (sOH)–polyester hydroxyl hydrogen (pHO). Right: shikonin carbonyl oxygen (sO)–polyester hydroxyl hydrogen (pHO).

VI. Dynamics aspects of the polyester/shikonin systems

As has been demonstrated in earlier studies of systems comprised of hyperbranched polymers and guest compounds,[28,70] the specifics of association between the two components influence the dynamic characteristics of the individual materials participating in the complexes. On account of the differences observed in the associative behavior between the studied polyester/shikonin systems from the static properties, we therefore examined possible effects in relevant dynamic characteristics as well.

VI.1 Diffusive motion

Fig. 9 shows the mean square displacement of the centers of mass of the polymeric component of the examined systems. As anticipated, the polyester molecules in H20 systems diffuse faster compared to their H30 counterparts, due to their lower size and mass and the smaller number of drug molecules in the solution (the higher the number of drug molecules the higher the expected average viscosity of the solution).

Comparing the behavior of the "high" and "low" concentration systems for the same polyester, it becomes apparent that the H20 and the H30 systems do not exhibit the same trend in their diffusive motion. While for the H30 systems the polyester of the "high" concentration system appears to diffuse slower than the one in the "low" concentration analogue, the polymer diffusion in the H20 "low" system appears to take place at similar or slightly larger times compared to that in the H20 "high" concentration system. Intuitively, the presence of drug molecules is expected to retard the polymer's diffusive motion independently of the (pseudo)generation of the latter: on one hand the non-associated drug molecules would increase the average viscosity of the solution, while, on the other hand the association of a number of drug molecules with the polyester would increase the hydrodynamic radius of the latter resulting again in slower diffusion. It appears, however, that the presence of shikonin incurs a larger retardation of the polymer diffusion in the H20 "low" system. This effect should apparently be related to the specifics of association of the drug molecules with the polymer in this system, which might result in a larger hydrodynamic radius compared to that in the H20 "high" counterpart, and thus slower diffusion. A similar mechanism might also be in action in the H30 "low" system, but the degree of retardation of the diffusive motion of the polymer might not be high enough in order to observe a "reverse" situation regarding the relation between diffusion and concentration similar to that observed in the H20 systems.

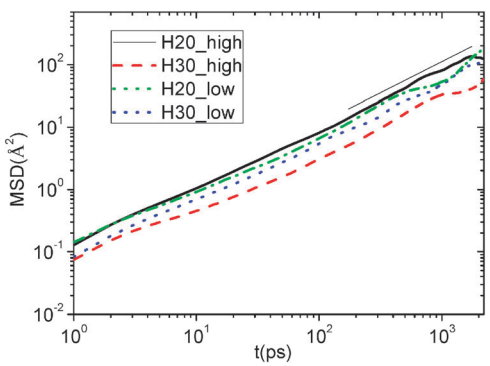

Fig. 9 Mean square displacement of the center of mass of the hyperbranched polyesters in the examined systems. The straight line corresponds to a slope of 1.

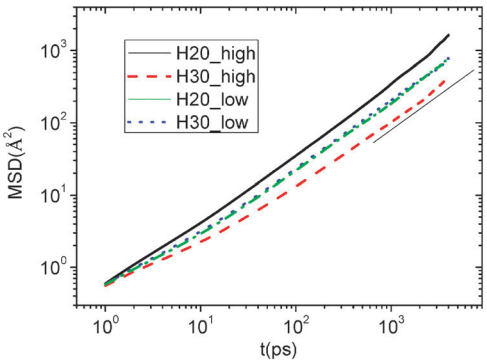

Fig. 10 Mean square displacement of the center of mass of the drug molecules in the systems studied. The straight line corresponds to a slope of 1.

If the above described effect is present in the H20 "low" system, it should also be reflected to the diffusive motion of the drug molecules as well. Fig. 10 illustrates the mean square displacement of the centers of mass of the drug molecules. In consistency with the behavior observed in Fig. 9 and the previous discussion, the drug molecules in the H20 "low" system diffuse slower compared to the H20 "high" case.

VI.2 Collective space–time correlated motion of the drug molecules

To further elaborate on the specifics of association between hyperbranched polyesters and shikonin and the effects of concentration and size of the polymer on the dynamics of shikonin, we have examined the time evolution of the cooperative spatial arrangements of the drug molecules in the solution. Since, as inferred from Table 2, drug molecules may reside either within or at the periphery of the hyperbranched molecule, the average behavior may actually reflect dynamic characteristics of both drug populations (*i.e.*, either the associated/geometrically-restricted or the "free" drug molecules). Nevertheless, it is possible to identify effects of the presence of the hyperbranched molecule on the drug molecules' spatiotemporal displacements, by resolving the characteristics of the latter at different timescales.

Specifically, to investigate the collective motion of shikonin molecules we have examined the *distinct* Van Hove correlation function (eqn (2)), which explores density fluctuations due to the collective motion of the neighbors around a particle (here a drug molecule).

$$G_{\mathrm{d}}(r,t) = \frac{1}{N}\left\langle \sum_{i}\sum_{j\neq i}\delta[r - |\boldsymbol{r}_i(t) - \boldsymbol{r}_j(0)|]\right\rangle \qquad (2)$$

In the former expression N represents the number of particles (*i.e.*, centers of mass of the shikonin molecules), while i and j stand for different drug-molecule indices. This property is proportional to the probability that a particle is at position **r**

at time t provided that a *different* particle was at the origin ($\mathbf{r} = 0$) at time $t = 0$. At $t = 0$, the distinct Van Hove function is proportional to the radial distribution function $g(r)$ of the examined particles. At large times and long separations, the position of each particle becomes uncorrelated to the position of another particle at earlier times, which leads to the gradual attenuation of the $g(r)$ peaks.

To facilitate a visual recognition of the rate at which the shell formed by the nearest neighbors loses coherence, we have normalized the y-axis with respect to the amplitude of the first-neighbor peak corresponding to the static case (*i.e.* at $t = 0$). Fig. 11 illustrates the behavior of $G_d(r, t)/G_{d,max}(r, 0)$ of the examined systems for a range of timescales.

As time lapses the peak indicating the spatial correlation with the nearest neighbors becomes broader reducing at the same time its amplitude. This indicates the gradual loss of spatial correlation with the surrounding drug molecules that had formed the original first neighbor shell. Since the longevity of such spatial correlations depends on the timescale related to the diffusion of the nearby drug molecules, lower concentration systems would be expected to exhibit a faster reduction of the first neighbor peak's amplitude, provided that no other factors that would inhibit free diffusion were present. In other words, a deviation from the expected behavior would indicate the presence of situations such as spatial constriction and/or specific interactions that would affect the drug molecules' cooperative diffusive motion.

To obtain information regarding the rate at which the loss of spatial correlation with the immediate drug molecule neighbors is realized, we monitored the ratio $G_{d,max}(r, t)/G_{d,max}(r, 0)$ which represents the relative drop in the amplitude of the first neighbor peak as time lapses. In this calculation we only considered times at which the peak was clearly discernible. The time dependence of the $G_{d,max}(r, t)/G_{d,max}(r, 0)$ ratio is illustrated in Fig. 12.

Evidently, the decay of the ratio in the H30 systems is lower compared to that in the H20 analogues. Focusing now on the relative rates corresponding to the two concentrations, the H20 "low" system assumes a lower rate compared to H20 "high", while the H30 "low" ratio reduces faster than in the H30 "high" case.

This behavior is consistent with the picture emerged from the self-diffusive motion of shikonin (Fig. 10), indicating that the collective spatial rearrangement of the drug molecules reflects the characteristics of the local microenvironment by incorporating sensitively the features of the self-motion. It is therefore straightforward to assume that as in the self-motion and as followed by the information emerging from the

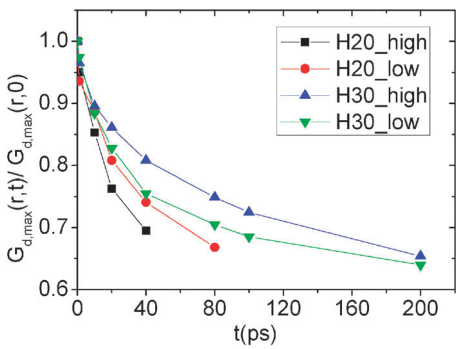

Fig. 12 The relative drop in the amplitude of the first neighbor peak in the distinct Van Hove functions of the centers of mass of the drug molecules as shown in Fig. 11, for the examined systems.

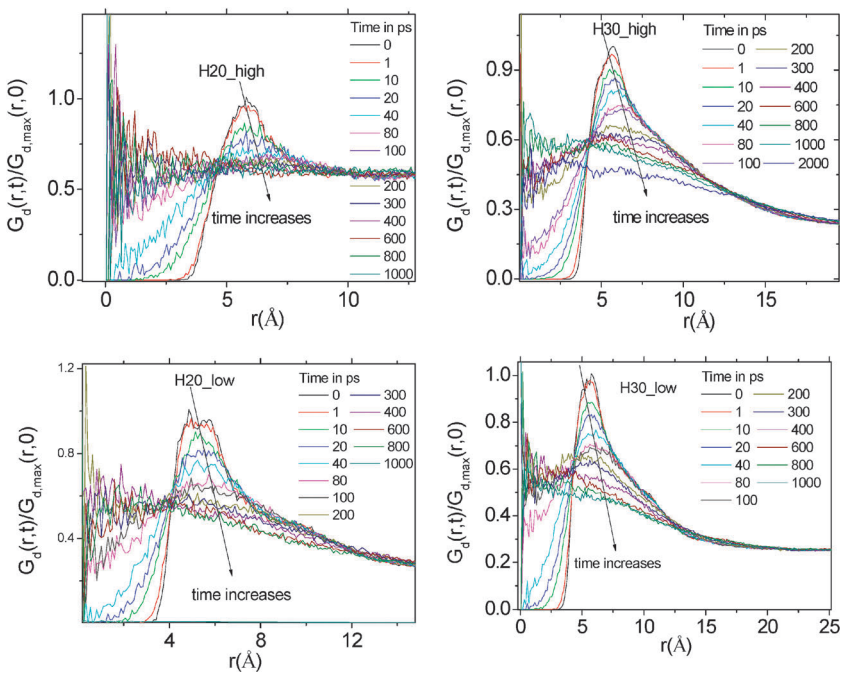

Fig. 11 Distinct Van Hove space–time correlation functions arising from the centers of mass of the drug molecules for all the examined systems, normalized to the value of the maximum at $t = 0$. The sequence of times (in ps) corresponding to the curves shown is: 0, 1, 10, 20, 40, 80, 100, 200, 300, 400, 600, 800, 1000 and 2000 (only for the "H30_high" system).

static/conformational characteristics of the systems, the mechanisms responsible for the observed features of the cooperative motion of the drug molecules are related to the spatial and dynamic restrictions imposed by the specific characteristics of the hyperbranched host (*i.e.*, its internal conformational state and the level of intra- and intermolecular hydrogen bonding) and to the characteristics of the drug clusters that can be formed either close to the polymer host, or in the bulk of the solution (see ESI†).

VII. Conclusions

In this work we have examined the specifics of the associative behavior between hyperbranched polyesters of (pseudo)-generations 2 and 3 and the multifunctional bioactive agent shikonin in ethanol solutions, mimicking systems of two different concentrations, namely 14% and 26% in polyester weight fractions. To our knowledge this is the first attempt to model potential hyperbranched host systems for this pharmaceutical molecule.

In summary, our findings show that between models of different polyester (pseudo)generations the larger size systems are more efficient in terms of drug loading. When comparing the degree of polymer/drug association in terms of concentration at constant polyester (pseudo)generation, it appears that the higher concentration in systems of the large (pseudo)-generation polyester and the lower concentration in those with the smaller (pseudo)generation polyester are more effective in associating with shikonin.

In more detail, as far as it concerns the ability of each polyester to associate with shikonin, it is shown that not only the size but also the specific conformational characteristics, as well as the solution's concentration, may affect the manner in which the polymer/drug complexes are formed. The higher (pseudo)generation systems can in general accommodate a larger number of drug molecules due to the higher number of hydrogen-bonding-capable sites (Table 2), but expectedly, their diffusional motion (which might affect their transport efficiency) is slower compared to the 2nd generation systems.

For systems of both (pseudo)generations the higher concentration models appear to possess higher structural flexibility, but this does not necessarily contribute towards more efficient association with the drug. This notion can be rationalized by the fact that the polyester/shikonin association appears to be a combined effect of structural features as well as intrapolymer and intermolecular hydrogen bonding with the drug. As was demonstrated in our case by examining static and dynamic properties, the reduced structural flexibility of the low concentration H20 system, which is associated with a high degree of intrapolymer hydrogen bonding, may result in a more efficient association of drug molecules compared to the higher concentration H20 system.

Although an increased level of internal hydrogen bonding is also present in the H30 "low" system (Fig. 7), it is possible that its more "open" structure compared to the H20 polyester is not as effective in maintaining a larger number of associated drug molecules compared to its higher concentration analog. This is corroborated by the observed faster diffusive motion of shikonin molecules (Fig. 10), as well as the faster cooperative motion of the drug molecules (Fig. 12), compared to those realized in the H30 "high" model. The cooperative diffusion of the drug molecules could also be affected by the formation of drug-clusters either close to the hyperbranched polymer or in the bulk. Based on the dynamic analysis performed (Fig. 11 and 12), it appears that not only concentration but also the size/topology of the hyperbranched polyester might affect their characteristics.

Although the systems we examined invoke a single polyester molecule, we believe that the effects observed and the relative importance of the different factors that can promote the polyester/shikonin association may apply as well in a more general situation where more than one polyester molecules are present. Actually, the present work may serve as the basis for a systematic investigation and a deeper understanding of the behavior of such multi-polymer/shikonin systems, which can be the subject of a future simulational and/or experimental study.

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