Published in final edited form as:

Soft Matter. 2018 June 13; 14(23): 4762–4771. doi:10.1039/c8sm00908b.

Probing the self-assembly dynamics and internal structure of amphiphilic hyaluronic acid conjugates by fluorescence spectroscopy and molecular dynamics simulations

Denis Svechkarev^a, Alexander Kyrychenko^b, William M. Payne^a, and Aaron M. Mohs^{a,c,d}

^aDepartment of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE

^bInstitute for Chemistry, V. N. Karazin Kharkiv National University, Kharkiv, Ukraine

^cDepartment of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE

^dFred and Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE

Abstract

Polymeric nanoparticles are increasingly used as biocompatible carriers for drugs and imaging agents. Understanding their self-assembly dynamics and morphology is of ultimate importance to develop nanoformulations with optimal characteristics. To achieve better performance, it is vital to account on cargo-carrier interactions at the molecular level. We studied the self-assembly dynamics and reveal the internal structure of nanoparticles derived from a series of hydrophobically modified hyaluronic acid. Environment-sensitive ratiometric fluorescent probes provide valuable information about the nanoparticles interior morphology, and molecular dynamics simulations complement the overall picture with insight onto intramolecular and intermolecular interactions of the polymer, as well as its interactions with the small-molecule load. Van der Waals and π - π interactions of the hydrophobic side fragments play the leading role in self-assembly and loading of hydrophobic small molecules. Aliphatic substituents form more extensive hydrophobic domains, while aromatic moieties allow for more interaction of the loaded small molecules with the surrounding solvent.

TOC image

[†]Electronic Supplementary Information (ESI) available: [Detailed description of the fluorescent dye synthesis; ¹H NMR data for the fluorescent probes; spectral deconvolution of the dyes emission spectra in different media; additional data from the analysis of molecular dynamics simulation of multi-polymer octadecyl- and pyrene-substituted HA derivatives; and analysis of molecular dynamic data for the dye-polymer systems are provided in the Supporting Information]. See DOI: 10.1039/x0xx00000x

Conflicts of interest

There are no conflicts to declare.

Introduction

Soft nanomaterials, such as micelles, vesicles, and polymeric nanoparticles (NPs) have been investigated as promising carriers for drug delivery. They have a number of advantages over many inorganic nanomaterials, such as biocompatibility and structural tenability. However, due to the complex organization of polymeric nanoparticles, precise control of their colloidal properties, drug loading, retention and release ability, is challenging. Yet, these characteristics are critical for efficacious nanoformulations. Consequently, extensive research is being performed to address this problem.^{1,2}

Precise performance of nanomaterials ultimately depends on their internal structure and morphology. Various factors need to be taken into account to effectively predict and control the properties of nanomaterials. Recent results include exploring the interactions of the nanocarrier with the payload and the solvent,³ using the combination of diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, FE-SEM with multivariate classification methods,⁴ and exploring ways of synthetic control of soft NP size, molecular weight, and crosslinking density.⁵ However, a significant gap exists in our knowledge about the interplay between the polymer, the payload, and the surrounding solvent in soft nanoformulations.

Conventional methods to investigate colloidal materials, such as light scattering or microscopy, do not provide information about the internal structure of the NPs. Environment-sensitive organic dyes can be used to probe the nanoparticle's interior by monitoring the changes of the dye's spectral properties. Over the past decades, solvatochromic dyes have been extensively used, in particular, for biomedical applications.⁶ Such probes provide valuable information about protein dynamics and interactions with small-molecule fluorophores.⁷ Classic solvatochromic dyes such as Michler's ketone, 4-aminobenzophenone, coumarin 153, and Reichardt's dye were used to assess the Kamlet-Taft polarity parameters of cellulose and related materials,⁸ and dye-doped polymeric materials were effective for detection of polar additives in hydrocarbon blends.⁹

Ratiometric sensing has an important advantage over the conventional intensometric approach: the ratio of the intensity of the two emission bands is independent from (a) the probe's concentration in the sample and (b) fluctuations in the excitation intensity. This is particularly important for the analysis of complex samples. Among other ratiometric fluorophores, 3-hydroxyflavones exhibit the excited-state intramolecular proton transfer (ESIPT) and are extensively used as polarity sensors.^{10,11} Derivatives of 3-hydroxyflavone,

especially those with donor substituents in the 2 position of the chromone bicycle, were effectively used to probe the macrocycles, nanostructures and other host environments upon encapsulation in micelles,^{12–14} formation of host-guest inclusion complexes with cyclodextrins^{15,16} and cavitands.¹⁷

In light of 3-hydroxyflavones' ability to probe the internal structure of assembled materials, we have chosen to use this ESIPT fluorophore to characterize the self-assembly of hyaluronic acid (HA) that has been modified with a hydrophobic moiety. HA is a natural polymer composed of (1→4)-β linked D-glucuronic acid and (1→4)-β linked N-acetyl-D-glucosamine.¹⁸ Because of its versatility and biocompatibility, HA is a particularly interesting polymer for biomedical applications, including in vivo delivery of therapeutics (see, for example, Dosio *et al.*¹⁹ for a comprehensive review). In our own work, we have used amphiphilic HA to entrap fluorophores for image-guided surgery,^{20–22} pathogen biosensing,²³ and delivery of hydrophobic therapeutic compounds.^{24,25} In each of these cases, however, little is known about the internal environment of self-assembled HA and their interaction with dye or drug molecules.

Molecular dynamics (MD) simulation has become a powerful tool in studying of conformation behavior and self-organization of single-chain oligosaccharides and their ensembles, providing a complementary microscopic view to macroscopic experimental observations. In a first step to exploring HA amphiphiles internal structure, we recently reported on the self-assembly of individual HA strands that were modified with alkyl groups, containing either 6, 12, or 18 carbon atoms.²⁶ These MD simulations were based on the united-atom G53A6 force-field and its G53A6_{CARBO} modification, which were recently re-parameterized for hexopyranose-based oligosaccharides such as cellulose,^{27,28} glucose,^{29,30} sucrose,³¹ and chitosan.³² Through a combination of experimental techniques and MD simulations, we were able to demonstrate that the HA-alkyl chain supramolecular properties were closely related to alkyl chain length. Here we further validate this approach for HA polymer-dye interactions dependence on the nature of the hydrophobic ligands.

Based on the aforementioned amphiphilic HA study, limitations in the ability to simulate multiple amphiphilic HA chains, diversity in the chemical structure of the hydrophobic moiety, and absence of an entrapped molecule in the NP structure were evident. Here, we build upon the initial investigation of HA self-assembly by exploring several aromatic HA conjugates and their differences compared to aliphatic derivatives, to build a comprehensive picture of HA-derived polymeric NPs for delivery applications (Figure 1). On the first step, single-strand simulations of aromatic HA derivatives featuring increasing size of the aromatic moiety were performed to compare against alkyl substituents. Consequently, further insight on the spatio-temporal characteristics of amphiphilic HA self-assembly and the differences in the hydrophobic domains formed by aliphatic and aromatic moieties were obtained computationally using the lead representative derivatives – octadecyl- and pyrene-substituted HA. A series of 3-hydroxyflavone derivatives were prepared and used to interrogate the interactions of small molecule payload with the HA-derived NPs. In particular, these ratiometric fluorophores were designed to interact differently with the hydrophobic domains inside the nanoparticles and were used to characterize the polarity and hydration of the hydrophobic domains formed by representative HA derivatives. MD

simulations were used to investigate dye-hydrophobic domain interaction in assembled NPs. Ultimately, the combination of spectral studies and MD simulations of a series of HA amphiphiles and their interactions with a panel of ESIPT dyes were used to define the roles of van der Waals interactions, π - π stacking, and hydrogen bonding in the self-assembly of the polymeric NPs.

Experimental

Materials

N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (EDC), pyrenebutyric acid, 1,3-propyldiamine, 2-hydroxyacetophenone, 2-hydroxynaphthophenone and octadecylamine were purchased from Sigma-Aldrich (St. Louis, MO). N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), sodium methylate, hydrogen peroxide (30%), N,N-dimethylaminobenzaldehyde, N,N-diphenylaminobenzaldehyde, and hydrochloric acid (Certified ACS Plus, 36-38%) were purchased from Fisher Scientific (Pittsburgh, PA). Ethanol was purchased from UNMC internal supply. Sodium hyaluronate (HA) was purchased from Lifecore Biomedical (Chaska, MN).

Hyaluronic acid conjugation

Amphiphilic hyaluronic acid (HA) polymers were synthesized as described in previous reports.^{20,26} Briefly, 40-45 mg HA ($M_N = 10$ -20 kDa) was dissolved in 1:1 ultrapure water and DMF along with 30 mg of NHS and 30 mg of EDC. After mixing for 30 minutes to activate the HA carboxylic acid groups, 10 weight percent of hydrophobic reagent (octadecylamine or aminopropyl-pyrenebutanamide) was added to the HA solution and allowed to react for 24 hours. Samples were then removed and placed in 3500 MWCO dialysis tubing and dialyzed against 1:1 water and ethanol for 4 exchanges over 24 h, then against pure water for 8 exchanges over 48 h to remove any impurities. Finally, samples were frozen and freeze dried for later use. Structures of the obtained compounds were confirmed by ^1H NMR spectroscopy on a Bruker Advance 500 MHz NMR spectrometer at 25°C (Figure S1-S2). Samples were analyzed in DMSO- d_6 (99.9% D, Cambridge Isotope Laboratories). Data was processed in Mnova NMR (Escondido, CA). Hereafter, octadecyl-modified HA is referred to as ocdHA, and pyrene-butanamide substituted HA is referred to as pyHA.

Dye loading into nanoparticles

Four derivatives of 3-hydroxychromone bearing various substituents (Figure 2) were synthesized as described elsewhere.³³ Stock solutions containing 60 mg of ocdHA (in 40 mL of ultrapure water) and 60 mg of pyHA (in 40 mL of ultrapure water), as well as 6 mg of each dye (in 20 mL DMSO) were prepared. Solutions of the polymers and dyes were mixed together (10 mL of the modified HA solution + 10 mL of the dye solution) to obtain 8 systems containing every dye mixed with each of the polymers. The final solutions were thoroughly mixed on a vortex mixer and loaded into the 3500 MWCO dialysis bags. Samples were then dialyzed against ultrapure water with 8 exchanges at equal time intervals. Aliquots of 300 μL were collected during every dialysis change, and their spectra were immediately recorded. After dialysis, the samples were purified by SEC on PD10 columns,

then frozen and freeze-dried for further storage. Dynamic light scattering and TEM imaging data of the obtained nanoparticles is provided in ref. 23. Fluorescence spectra were recorded on a Horiba Jobin Yvon FluoroMax 4 spectrofluorometer in quartz micro cuvettes with the optical path length of 2.5 mm with excitation wavelength at 400 nm.

Molecular dynamics simulations

Molecular dynamics simulations were performed using GROMACS 4.6 with the united-atom GROMOS 53a6 force field³⁴ and the SPC water model.³⁵ The HA model and FF interaction parameters were adopted from the G53a6_{CARBO} united-atom FF,^{36,37} in which the original G53a6 FF version was recently extended to hexopyranose-based carbohydrate chains. The CH, CH₂ and CH₃ moieties are treated as a single, united interacting site. The bond length and angle parameters for 3-hydroxychromone dye molecules were optimized by density functional theory calculations at the B3LYP/cc-pVDZ level and adopted for the GROMACS 53a6 force field format. Partial charges needed for Coulomb interactions were derived from the B3LYP/cc-pVDZ electron densities by fitting the electrostatic potential to point (ESP) charges.

Each polymer model was simulated for 200 ns in case of single-strand systems and for 100 ns in case of all systems containing multiple polymer strands. A defined number of polymer chains (22 for self-assembly studies, and 4 in case of dye-polymer interaction simulations) in linear extended conformations were placed in rectangular cells of approximately 20 nm × 18 nm × 18 nm, filled with approximately $(1 \div 1.3) \cdot 10^5$ water molecules.

To keep the solution at the physiological ionic strength, 0.1 M NaCl was added to aqueous solution. All the MD simulations were carried out at a constant number of particles, constant pressure of $P = 1$ atm, and constant temperature $T = 303$ K (the NPT ensemble). The reference temperature of 303 K was kept constant using the velocity rescaling weak coupling scheme³⁸ with a coupling constant $\tau = 0.1$ ps. The initial atomic velocities were generated with a Maxwellian distribution at the given absolute temperature. Periodic boundary conditions were applied to all three directions of the simulated box. Electrostatic interactions were simulated with the particle mesh Ewald (PME) approach³⁹ using the long-range cutoff of 0.8 nm. The cutoff distance of Lennard-Jones interactions was also equal to 0.8 nm. The MD simulation time step was 1-2 fs with the neighbor list updates every 10 fs. All bond lengths in the HA chain were kept constant using the LINCS routine^{40,41}. The MD simulations were carried out using the GROMACS set of programs, version 4.6⁴². Molecular graphics and visualization were performed using VMD 1.8.6⁴³.

The analysis was performed using GROMACS tools. The radius of gyration, head-to-tail distance, number of polymer-polymer hydrogen bonds, number of polymer-solvent hydrogen bonds, and solvent-accessible surface area were extracted from the trajectory files and plotted using GraphPad Prism 7.0 (GraphPad Software; La Jolla, CA).

Results and discussion

Hydrophobic side fragments influence the self-assembly dynamics

Revealing the complex behavior of amphiphilic polymers during their self-assembly into nanoparticles starts from a better understanding of the dynamics of the polymer single strands. Indeed, numerous examples show that properties of the nanomaterials are influenced by the structure and properties of their constituents.^{26,44,45} In our previous work,²⁶ we explored the role of the hydrophobic chain length in a series of HA derivatives with aliphatic side substituents. It was shown that the size and conformational stability of the coils formed by single polymer strands containing hydrophobic ligands of various length are in good accordance with the sizes and polydispersity of the nanoparticles formed by such polymers. The van der Waals interactions between the alkyl chains play a major role in the hydrophobic packing of the polymer chains, with hydrogen bonds having no sensible role in formation or stability of the nanoparticles.

To expand our understanding of the role of various interactions in the self-assembly of the amphiphilic HA polymers beyond the van der Waals and H-bonding interactions, a series of computational atomistic models of amphiphilic 10 kDa (26 repeat units) HA derivatives were developed, which contained aromatic side substituents of various sizes. The derivative containing phenyl-butanamide fragments is further referred to as phHA, its naphthyl analog – npHA, and the pyrenyl analog – pyHA (Figure 3A). The substitution ratio was kept at ~30%, and the side substituents are located at the same randomly selected positions across all three models (Figure S3). Part of the carboxylic groups on the HA backbone are dissociated to reflect its dissociation state at physiological pH ($\alpha \approx 20\%$, $pK_a = 2.9^{46}$).

Simulation of these systems allowed studying the role of π - π interactions in the polymers self-assembly. The radius of gyration was chosen as a measure of compactness of the polymer. As can be seen from the Figure 3B, a larger size π -system of the pyrene fragment leads to a quick formation of a stable coil which is more compact than its counterparts. Formation of multi-layer π - π stacks of pyrene polycycles is illustrated in Figure 3C, where snapshots of final configurations are represented. At the same time, less extensive conjugated systems of phenyl and naphthyl moieties lead to less active interaction, thus allowing for greater conformational lability of the polymer chains.

Performance of the aliphatic and aromatic hydrophobic side substituents as drivers of the self-assembly of the amphiphilic HA nanoparticles was further scrutinized by comparing the behavior of the typical representatives of each type: octadecyl- (ocdHA) and pyrene-containing (pyHA) HA derivatives. Simulating full-size polymeric nanoparticles at the united-atom level that provides the necessary level of detail is still too resource-demanding. Thus, to allow investigation of intermolecular, in addition to intramolecular, interactions, systems containing 22 molecules of 10 kDa HA derivatives were developed. Such models represent the processes that occur inside the larger particles that are observed experimentally.

From the analysis of the corresponding MD trajectories, it becomes evident that π - π interactions play a very important role in self-assembly of the polymers, significantly

affecting their packing, stability and morphology of the resulting nanoparticles. Thus, the radius of gyration dynamics of the system consisting of pyHA polymer chains shows that they tend to form a distinctly tighter aggregate than the analogous ocdHA system (Figure S4A). It can be explained at least partially by a slightly greater volume that octadecyl chains occupy compared with the pyrene fragments. However, analysis of intramolecular and intermolecular contacts in these systems provides a more unambiguous evidence: there is at least 10-15% increase in the number of contacts in the pyHA system (Figure S4B). This shows not only a closer interaction between the hydrophobic aromatic fragments, but an overall tighter packing of the polymer chains. The fact that the increase of polymer-polymer contacts cannot be exclusively ascribed to π - π interactions is supported by the analysis of polymer-polymer hydrogen bonds (Figure S5A). Thus, a considerably larger number of intra- and intermolecular H-bonds are observed in pyHA system, in agreement with its smaller size and higher number of overall contacts.

Interestingly, the number of polymer-water hydrogen bonds is almost equal for both systems, with slightly more interaction between the solvent and the pyrene-substituted HA strands (Figure S5B). Taking into account the overall smaller size that the pyHA system maintained during simulation, it suggests a more significant hydration of the aggregates formed by the HA amphiphiles with aromatic hydrophobic ligands. This can be visually illustrated with the snapshots of the final configurations resulting from the 100 ns of MD simulations of both systems (Figure 4). In these snapshots, one can see that the long hydrocarbon chains form hydrophobic domains similar to the micellar cores, which are hardly accessible for water molecules (Figure 4A). On the other hand, the aromatic pyrene fragments form numerous multi-layer stacks, sometimes bringing together ten or even more pyrene units (Figure 4B). Such longitudinal structure of the aromatic hydrophobic domains leaves more opportunities for interactions with the solvent molecules. This particularly finds its reflection in the solvent-accessible surface dynamics: while the hydrophilic SAS remains almost the same for both systems, the hydrophobic SAS of the ocdHA system is consistently greater than that of the pyHA one (Figure S6).

Thus, comparative study of the aliphatic and aromatic fragments as side substituents on the HA backbone showed that larger hydrophobic ligands are more effective in driving self-assembly. Aromatic substituents' π - π interactions is an additional factor to provide efficient self-packing and conformational stability of the resulting nanoparticles. However, hydrophobic domains formed by stacks of aromatic fragments tend to allow for more interactions with the surrounding solvent molecules, whereas the hydrophobic domains formed as a result of hydrocarbon chains van der Waals interactions are less hydrated.

Fluorescent dyes probe the nanoparticle interior

To evaluate the above discussed findings and directly probe the internal morphology and cargo-carrier interactions of the nanoparticles formed by the representative amphiphilic HA derivatives in more detail, fluorescent dyes were used that are responsive to their microenvironment. Specifically, a series of 2-(4'-N,N-dimethylaminophenyl)-3-hydroxychromone (DMAF) derivatives were chosen for this purpose due to their unique spectral properties. Representatives of the 3-hydroxyflavone family of dyes exhibit the

process known as excited-state intramolecular proton transfer (ESIPT) that leads to emergence of two bands in their fluorescence spectra (Figure 5). The short-wavelength band is ascribed to the emission of the normal form (N*), where the proton remains on the 3-OH group, whereas the long-wavelength band is due to the fluorescence of the phototautomer (T*) – the product of the proton transfer to the 4-C=O group.⁴⁷ Multiple factors can influence the excited-state dynamics of ESIPT, such as polarity of the molecules microenvironment, external hydrogen bonding, temperature, etc.⁴⁸ The ratio of the emission intensity in both bands thus depends on these factors, and the dyes are often referred to as *ratiometric*. They are particularly convenient for sensing of complex systems, because the ratiometric analytical signal is independent from the dye's concentration. Particularly, 3-hydroxyflavone derivatives were successfully used as sensors of polarity^{49,50} and protic admixtures.^{51–53}

Four fluorescent probes were synthesized to assess the role of different interactions in the self-assembly and small molecule non-covalent entrapment into amphiphilic HA nanoparticles (Figure 2). DMAF was used as a parent dye in the series to provide a general reference response. An extra benzene ring was annealed to the DMAF core to obtain 2-(4'-N,N-dimethylaminophenyl)-3-hydroxybenzo[*f*]chromone (BfDMAF). This additional ring acts as a shield for the 4-C=O group and eliminates the possibility of external H-bonding to this site,³³ thus substantially decreasing the influence of intermolecular H-bonding on the ESIPT process. In 2-(4'-N,N-dioctylaminophenyl)-3-hydroxychromone (DOAF) and 2-(4'-N,N-diphenylaminophenyl)-3-hydroxychromone (DPAF), long octyl chains and additional benzene rings were introduced to enhance different types of hydrophobic interactions between the dyes and the hydrophobic side substituents on the HA backbone.

During dialysis, the probe dyes experience the change of solvent from the DMSO-water (1:1 v/v) system to pure water. Thus, their fluorescence spectra were first studied in DMSO-water binary solvent systems of different content (Figure S8, bottom). In pure DMSO, fluorescence spectra of all four dyes have some contribution of the emission of the anionic form^{54,55} that peaks around 530 nm, in partial superposition with the normal form emission peaking at ~480-490 nm, and the tautomer form with the emission maximum at ~570-580 nm. In the case of DMAF and DOAF, normal and anionic emissions dominate as the ESIPT concurs with and is partially disrupted by the external hydrogen bonding to the solvent. This effect is significantly less notable for BfDMAF due to the effect of the fused ring. The DPAF fluorescence spectrum reveals similar features, most likely due to steric hindering of intermolecular interactions by the additional phenyl moieties. Upon addition of water, a notable increase of the anionic emission intensity is observed for DMAF and DOAF, to a lesser extent for the latter, evidencing for further concurrence of the ESIPT process with external H-bonding with DMSO and water. In opposite, BfDMAF does not show any sensitivity to the polar admixture. Its overall fluorescence intensity slightly decreases, however, due to H-bond assisted fluorescence quenching.⁵⁶ The dramatic decrease of the fluorescence intensity of DPAF is clearly due to not only H-bond mediated quenching, but also to the aggregation. Unlike DMAF and its other analogs, it was visually evident that DPAF started aggregation at relatively low water concentrations, most likely due to a strong

role of increased π - π interactions contributed by additional phenyl moieties present in its molecules.

At equal contents of water and DMSO, aggregation is readily observed for all four compounds. As the fluorescence intensity continues to decrease, both due to aggregation and rising water content, the character of emission of the dyes evidences for the decrease of the polarity of their microenvironment, manifested by the rising contribution of the phototautomer fluorescence. This can be explained by the fact that the molecules surrounding each other in the aggregates create a lower-polarity medium compared to the bulk solution around the aggregate.^{57,58}

With the knowledge on the spectral behavior of the dyes in hand, it is now possible to study the process of their loading into the polymeric nanoparticles. Briefly, the hydrophobically modified HA derivatives dissolved in water-DMSO binary system were mixed with the dyes solutions in DMSO, and dialyzed against water. At every water change, a sample of each dialysate was taken and spectra were recorded (Figure 6), thus allowing to monitor the loading process in time.

Looking at the time evolution of spectra during dialysis, one can notice that the most drastic changes occur during the first hours of the process. The decrease of the emission intensity and overall hypsochromic shift of the fluorescence spectra suggests that dominant processes during this period are further aggregation of the dyes and DMSO diffusion from the dialysis bags with its subsequent replacement by water. However, an increase of intensity in the blue region compared to the free dye spectra in DMSO-water 1:1 (v/v) solution (shaded gray in the Figure 6) of the spectrum even at the beginning of the dialysis (0 hour time point) can be an evidence of notable interaction with the polymer's hydroxyl groups, especially in case of DOAF in ocdHA (due to expected high affinity of the octyl tails of the dye molecules to the octadecyl chains attached to the polymer's backbone). The same observation is true for most of the dyes in pyHA, which can be explained by the π - π interactions between the dye cores and the pyrene units in the polymer discussed in detail below. As the most notable spectral changes occur before the first solvent exchange is performed, spectra recorded at the 5 h time point reflect further aggregation and H-bonding interactions that are manifested in decrease of the overall fluorescence intensity. This is also supported by the short-wavelength emission of the normal form becoming more intensive in most cases.

To evaluate the state of the probe dyes inside the nanoparticles when the loading process is over, we analyzed their fluorescence spectra compared to the ones of the dye aggregates in water (Figure 7). It also provided us with information about the cargo-carrier interactions inside the nanoparticles, as well as insight about their inner morphology, particularly concerning the hydrophobic domains that encapsulate the loaded small molecules or their aggregates.

It can be noted that in all cases the loaded dyes experience higher polarity and/or hydrogen bonding ability of their microenvironment than they do in their aggregates: the long-wavelength tautomer emission at least partially gives way to the fluorescence of the anion and normal form (see Figure S8 for results of deconvolution of these spectra into their

individual components). Our hypothesis is that after being entrapped inside the nanoparticles, the dye aggregates partially dissociate as a result of their interaction with the hydrophobic domains formed by the side substituents of the polymers. The increase of the anionic emission evidence for external H-bonding with the polar groups of the polymer backbone. Similar observations have been described upon encapsulation of DMAF in γ -cyclodextrin.¹⁶ The rising intensity in the shorter-wavelength part of the spectrum is attributed to interactions with water molecules that affect the ESIPT, making it slower or completely disrupting it by breaking the intramolecular hydrogen bond in the dye molecules. Comparison of the dyes fluorescence in ocdHA and pyHA reveals that there is generally more contribution of the short-wavelength emitting components to the overall spectra of the dyes loaded into the aromatic HA derivative pyHA. Therefore, the dye-polymer interactions are stronger inside these nanoparticles, and/or the hydrophobic domains formed by the pyrene units allow for more interaction of the loaded dyes with surrounding water.

To shed more light onto the structure of the hydrophobic domains and the dye-polymer interactions inside the nanoparticles, model systems were designed and studied by means of molecular dynamics simulations. Two representative dyes with the same core but distinctly different character of hydrophobic substituents – DOAF and DPAF – were chosen, and systems containing two molecules of either of the dyes interacting with 4 strands of one of the polymers were simulated. Figure 8 represents the final snapshots of all four studied systems after 100 ns of the simulation. Distinct differences in the interactions between the dyes and polymers with hydrophobic substituents of various nature can be noted. In case of dye interactions with ocdHA, the hydrocarbon chains tend to “envelop” the dye molecule into the hydrophobic pocket formed around it (Figure 8A,C). There is no significant difference in the number of contacts between the hydrophobic fragments of the dye and the polymer in these cases (Figures S9-S12). Some difference is observed with the dyes interacting with the pyHA polymers. In both cases, π - π stacking is the main type of interaction that drives the inclusion of the dye molecules into the hydrophobic domains of the polymer aggregates. Interestingly, additional benzene rings of the DPAF apparently do not play a major role of this process: with a typical torsion angle value for the 2-phenyl fragment in the ground state being in the range of 15-25° for most flavones,⁵⁹ the rings at the nitrogen atom are rotated for additional 10-20°. However, octyl chains of the DOAF tend to additionally interact with the linkers that connect the pyrene units to the HA backbone, thus making the number of contacts between the hydrophobic fragments of the dyes and polymers larger than in the case of DPAF (Figures S9-S12).

How hydrophobic are the hydrophobic domains?

Spectral data acquired from the environment-sensitive dyes, and results from theoretical simulations, allow us to draw a better picture of the morphology of the hydrophobic domains that are responsible for loading and retention of the small-molecule cargo in the amphiphilic polymeric nanoparticles. A more detailed insight about the hydrophobicity of such domains was obtained from the analysis of the dye-water hydrogen bonding dynamics for all dye-polymer systems. It can be clearly seen that both dyes are more subjected to interactions with water when they are “loaded” into the pyrene-substituted HA derivative (Figure 9). It is true for the H-bonds between water and the dyes’ 4-C=O groups, and somewhat more

pronounced for the bonds between water and 3-OH groups of the dyes (Figures S9-S12). The intramolecular hydrogen bond responsible for the ESIPT process also remains intact slightly longer along the simulation trajectory in the case of ocdHA, which agrees well with the spectral data discussed above.

The results of the payload-solvent hydrogen bonding analysis is in good agreement with the findings discussed above, concluding that hydrocarbon chains are capable of forming more efficient hydrophobic domains. Interestingly, the overall higher hydration of aromatic domains calls for the analogy with the water content in its saturated solutions in hydrocarbons of various nature. For example, water content in benzene ($\sim 0.06\%$ ⁶⁰) is quite considerably higher than that in hexane ($\sim 0.009\%$ ⁶¹).

Conclusions

We explored the single-strand dynamics of amphiphilic hyaluronic acid derivatives bearing aromatic hydrophobic side fragments and compared their behavior with the analogs bearing aliphatic substituents studied previously. Self-assembly dynamics of the typical representatives of both types, octadecyl- and pyrene-substituted HA, was investigated using more complex computational systems containing 22 polymeric molecules. Four derivatives of 3-hydroflavone were designed and synthesized to be used as fluorescent probes to study their loading into the polymeric nanoparticles, and the internal structure of the latter. Using their ratiometric signal as an environment-sensitive response, the aggregation state of the payload, its interaction with the polymeric carrier, as well as polarity and hydration of the hydrophobic domains of the nanoparticles were studied.

Aromatic fragments stacking appears to be more significant as a driving force of the polymers self-assembly compared to the van der Waals hydrophobic interactions between the aliphatic hydrocarbon chains, as demonstrated in both single-strand and multi-molecule systems. However, the π - π stacks are more prone to be disrupted upon insertion of small-molecule payload containing aromatic fragments in their structures. The role of such interactions in the interplay between the payload and the carrier requires more exploration at larger scales.

Both aliphatic and aromatic fragments effectively form hydrophobic domains inside the nanoparticles. However, the domains formed by aliphatic chains are more spacious and envelop the payload with greater efficiency. At the same time, the domains formed by aromatic fragments have distinct longitudinal character.

Fluorescent response from the ratiometric probes showed that all four dyes are at least partially aggregated while being loaded into the nanoparticles from the DMSO-water (1:1, v/v) solution. The main spectral changes during dialysis are due to removal of most of DMSO during the first hours. Inside the nanoparticles, the dyes are in a partially aggregated state, showing signs of hydrogen bonding interactions with the polysaccharide units of the polymer, as well as with the surrounding water.

Molecular dynamics simulation of the dye-polymer interactions indicates extensive contact of the hydrophobic fragments in all cases. Most effective interaction is observed for the octyl

chains of the dye with octadecyl chains of the polymer. The latters also efficiently envelop the aromatic core of the flavonols, as well as interact with the phenyl rings of the diphenylamino group. In case of the pyrene-substituted HA derivative, the chromone core of all dyes appears inserted into the π - π stacks of the pyrene fragments. However, the 2-phenyl moiety and the phenyl rings of the diphenylamino group, being out of plane, have a potential to disrupt stacking to a certain extent at a larger scale.

Both spectral and simulation data lead to a conclusion that aliphatic hydrophobic domains are less hydrated, whereas the longitudinal character of the aromatic domains allows for more interaction of the payload with the surrounding solvent molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded in part by National Institutes of Health [grants R01 EB019449, P20 GM103480, P30 CA036727 (Fred & Pamela Buffett Cancer Center), 1S10RR17846, and 1S10RR027940], and the Nebraska Research Initiative. The authors acknowledge Holland Computing Center at the University of Nebraska for providing resources to perform molecular dynamics simulations described in this work.

References

1. Mitchell, GR., Tojeira, A., editors. Controlling the Morphology of Polymers. Springer International Publishing; Cham: 2016.
2. Vauthier, C., Ponchel, G., editors. Polymer Nanoparticles for Nanomedicines. Springer International Publishing; Cham: 2016.
3. Venier-Julienne MC, Benoît JP. Pharm Acta Helv. 1996; 71:121–128. [PubMed: 8810578]
4. Khanmohammadi M, Elmizadeh H, Ghasemi K. Iran J Pharm Res. 2015; 14:665–675. [PubMed: 26330855]
5. Martin HJ, White BT, Scanlon CJ, Saito T, Dadmun MD. Soft Matter. 2017; 13:8849–8857. [PubMed: 29143026]
6. Klymchenko AS. Acc Chem Res. 2017; 50:366–375. [PubMed: 28067047]
7. Loving GS, Sainlos M, Imperiali B. Trends Biotechnol. 2010; 28:73–83. [PubMed: 19962774]
8. Spange S, Fischer K, Prause S, Heinze T. Cellulose. 2003; 10:201–212.
9. Hubert C, Fichou D, Valat P, Garnier F, Villeret B. Polymer (Guildf). 1995; 36:2663–2666.
10. Demchenko AP, Duportail G, Klymchenko AS, Me Y. 2004; 1665:6–19.
11. Demchenko AP. FEBS Lett. 2006; 580:2951–2957. [PubMed: 16643906]
12. Sarkar M, Guha Ray J, Sengupta PK. Spectrochim Acta Part A Mol Biomol Spectrosc. 1996; 52:275–278.
13. Dennison SM, Guharay J, Sengupta PK. Spectrochim Acta Part A Mol Biomol Spectrosc. 1999; 55:1127–1132.
14. Sarkar M, Sengupta PK. Chem Phys Lett. 1991; 179:68–72.
15. Banerjee A, Sengupta PK. Chem Phys Lett. 2006; 424:379–386.
16. Organero JA, Tormo L, Sanz M, Roshal A, Douhal A. J Photochem Photobiol A Chem. 2007; 188:74–82.
17. Santos FS, Ramasamy E, Ramamurthy V, Rodembusch FS. Photochem Photobiol Sci. 2014; 13:992–996. [PubMed: 24901799]
18. Kogan G, Soltés L, Stern R, Gemeiner P. Biotechnol Lett. 2007; 29:17–25. [PubMed: 17091377]

19. Dosio F, Arpicco S, Stella B, Fattal E. *Adv Drug Deliv Rev.* 2016; 97:204–36. [PubMed: 26592477]
20. Hill TK, Abdulahad A, Kelkar SS, Marini FC, Long TE, Provenzale JM, Mohs AM. *Bioconjug Chem.* 2015; 26:294–303. [PubMed: 25565445]
21. Hill TK, Kelkar SS, Wojtynek NE, Soucek JJ, Payne WM, Stumpf K, Marini FC, Mohs AM. *Theranostics.* 2016; 6:2314–2328. [PubMed: 27877237]
22. Qi B, Crawford AJ, Wojtynek NE, Holmes MB, Soucek JJ, Almeida-Porada G, Ly QP, Cohen SM, Hollingsworth MA, Mohs AM. *Nanomedicine.* 2018; 14:769–780. [PubMed: 29325740]
23. Svechkarev D, Sadykov MR, Bayles KW, Mohs AM. *ACS Sensors.* 2018; 3:700–708. [PubMed: 29504753]
24. Hill TK, Davis AL, Wheeler FB, Kelkar SS, Freund EC, Lowther WT, Kridel SJ, Mohs AM. *Mol Pharm.* 2016; 13:720–728. [PubMed: 26824142]
25. Soucek JJ, Davis AL, Hill TK, Holmes MB, Qi B, Singh PK, Kridel SJ, Mohs AM. *Mol Cancer Ther.* 2017; 16:1819–1830. [PubMed: 28615298]
26. Payne WM, Svechkarev D, Kyrychenko A, Mohs AM. *Carbohydr Polym.* 2018; 182:132–141. [PubMed: 29279107]
27. Chen P, Nishiyama Y, Wohler J, Lu A, Mazeau K, Ismail AE. *J Phys Chem B.* 2017; 121:2244–2251. [PubMed: 28221796]
28. Chen P, Ogawa Y, Nishiyama Y, Bergenstrhle-Wohler M, Mazeau K. *Cellulose.* 2015; 22:1485–1493.
29. Plazinski W, Plazinska A, Drach M. *Phys Chem Chem Phys.* 2015; 17:21622–21629. [PubMed: 26226084]
30. Batista MLS, Prez-Snchez G, Gomes JRB, Coutinho JAP, Maginn EJ. *J Phys Chem B.* 2015; 119:15310–15319. [PubMed: 26572856]
31. Stortz TA, De Moura DC, Laredo T, Marangoni AG. *RSC Adv.* 2014; 4:55048–55061.
32. Naumov VS, Ignatov SK. *J Mol Model.* 2017; 23:244. [PubMed: 28748283]
33. Klymchenko AS, Pivovarenko VG, Demchenko AP. 2003:4211–4216.
34. Oostenbrink C, Villa A, Mark AE, Van Gunsteren WF. *J Comput Chem.* 2004; 25:1656–1676. [PubMed: 15264259]
35. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. *J Chem Phys.* 1983; 79:926.
36. Plazinski W, Lonardi A, Hnenberger PH. *J Comput Chem.* 2016; 37:354–65. [PubMed: 26525424]
37. Hansen HS, Hnenberger PH. *J Comput Chem.* 2011; 32:998–1032. [PubMed: 21387332]
38. Bussi G, Donadio D, Parrinello M. *J Chem Phys.* 2007; 126:014101. [PubMed: 17212484]
39. Darden T, York D, Pedersen L. *J Chem Phys.* 1993; 98:10089.
40. Hess B, Bekker H, Berendsen HJC, Fraaije JGEM. *J Comput Chem.* 1997; 18:1463–1472.
41. Hess B. *J Chem Theory Comput.* 2008; 4:116–122. [PubMed: 26619985]
42. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJC. *J Comput Chem.* 2005; 26:1701–18. [PubMed: 16211538]
43. Humphrey W, Dalke A, Schulten K. *J Mol Graph.* 1996; 14:33–38. [PubMed: 8744570]
44. Duan X, Li Y. *Small.* 2013; 9:1521–1532. [PubMed: 23019091]
45. Lombardo D, Kiselev MA, Magaz S, Calandra P. *Adv Condens Matter Phys.* 2015; 2015:1–22.
46. Cleland RL, Wang JL, Detweiler DM. *Macromolecules.* 1982; 15:386–395.
47. McMorro D, Kasha M. *J Phys Chem.* 1984; 88:2235–2243.
48. Sytnik A, Gormin D, Kasha M. 1994; 91:11968–11972.
49. Duportail G, Klymchenko A, Mely Y, Demchenko A. *FEBS Lett.* 2001; 508:196–200. [PubMed: 11718715]
50. Ercelen S, Klymchenko AS, Demchenko AP. *Anal Chim Acta.* 2002; 464:273–287.
51. Svechkarev DA, Bukatich IV, Doroshenko AO. *J Photochem Photobiol A Chem.* 2008; 200:426–431.

52. Kyrychenko A, Karpushina GV, Svechkarev D, Kolodezny D, Bogatyrenko SI, Kryshtal AP, Doroshenko AO. *J Phys Chem C*. 2012; 116:21059–21068.
53. Liu W, Wang Y, Jin W, Shen G, Yu R. *Anal Chim Acta*. 1999; 383:299–307.
54. Dereka B, Letrun R, Svechkarev D, Rosspeintner A, Vauthey E. *J Phys Chem B*. 2015; 119:2434–43. [PubMed: 25105471]
55. Protti S, Mezzetti A, Cornard JP, Lapouge C, Fagnoni M. *Chem Phys Lett*. 2008; 467:88–93.
56. Dereka B, Vauthey E. *Chem Sci*. 2017; 8:5057–5066. [PubMed: 28970892]
57. Svechkarev D, Kyrychenko A, Payne WM, Mohs AM. *J Photochem Photobiol A Chem*. 2018; 352:55–64. [PubMed: 29430162]
58. Faucon A, Benhelli-Mokrani H, Córdova LAW, Brulin B, Heymann D, Hulin P, Nedellec S, Ishow E. *Adv Healthc Mater*. 2015; 4:2727–2734. [PubMed: 26548458]
59. Todorova TZ, Traykov MG, Tadjer AV, Velkov ZA. *Comput Theor Chem*. 2013; 1017:85–90.
60. Karlsson R. *J Chem Eng Data*. 1973; 18:290–292.
61. Polak J, Lu BCY. *Can J Chem*. 1973; 51:4018–4023.

Figure 1. Logical concept and workflow of the presented study. Data on the single-polymer systems dynamics for aliphatic hydrophobic ligands is reported in our previous work²⁶.

Figure 2.
Structures of the 3-hydroxyflavone derivatives used in this study.

Figure 3.

Structure of the amphiphilic HA derivatives bearing aromatic side substituents (A); results of the MD simulations of the systems containing a single strand of 10 kDa HA with aromatic hydrophobic side substituents solvated by $\sim 1.1 \cdot 10^5$ water molecules: (B) time evolution of the radius of gyration of the system over the 200 ns trajectory, (C) final configurations of the polymers after 200 ns of simulations (water molecules are omitted for clarity of representation).

Figure 4. Snapshots of the systems consisting of 22 molecules of 10 kDa HA modified with (A) octadecyl (blue), and (B) pyrene butanamide (green) hydrophobic side fragments after 100 ns of MD simulation. The polymer backbone is colored light gray for clarity of presentation. The most visual examples of multilayer π - π stacking are circled in red.

Figure 5. (A) Protolytic equilibria of 3-hydroxychromone derivatives in the ground and excited states, and (B) fluorescence spectrum of DOAF in DMSO (black line) and its deconvolution into three individual components showing emissions of the normal (N*), anionic (A*) and tautomer (T*) forms.

Figure 6. Time evolution of the fluorescence spectra of the probes during their loading into the amphiphilic HA nanoparticles. Time points when the spectra were recorded during dialysis are given in the legend. Fluorescence spectra of the dyes in DMSO-water (1:1 v/v) mixture are given in light gray for reference.

Figure 7. Fluorescence spectra of the dyes loaded into the hydrophobically- modified HA nanoparticles. Spectra of the dye aggregates in water are given in light gray for comparison.

Figure 8.

Final snapshots after 100 ns of the MD simulations of two molecules of (A, B) DOAF (red) or (C, D) DPAF (orange) interactions with 4 molecules of 10 kDa ocdHA (A, C) or pyHA (B, D).

Figure 9. Analysis of the dye-water hydrogen bonding dynamics for the systems of 2 dye molecules and 4 polymer molecules. Histograms represent the frequency distribution for the last 50 ns of the simulation trajectories.