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Probing Peptide Amphiphile Self-Assembly in Blood Serum

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Abstract

There has been recent interest in designing smart diagnostic or therapeutic self-assembling peptide or polymeric materials that can selectively undergo morphological transitions to accumulate at a disease site in response to specific stimuli. Developing approaches to probe these self-assembly transitions in environments that accurately amalgamate the diverse plethora of proteins, biomolecules, and salts of blood is essential for creating systems that function *in vivo*. Here, we have developed a fluorescence anisotropy approach to probe the pH-dependent self-assembly transition of peptide amphiphile (PA) molecules that transform from spherical micelles at pH 7.4 to nanofibers under more acidic pH's in blood serum. By mixing small concentrations of a Ru(bipy)₃²⁺-tagged PA with a Gd(DO3A)-tagged PA having the same lipid-peptide sequence, we showed that the pH dependence of self-assembly is minimally affected and can be monitored in mouse blood serum. These PA vehicles can be designed to transition from spherical micelles to nanofibers in the pH range 7.0–7.4 in pure serum. In contrast to the typical notion of serum albumin absorbing isolated surfactant molecules and disrupting self-assembly, our experiments showed that albumin does not bind these anionic PAs and instead promotes nanofibers due to a molecular crowding effect. Finally, we created a medium that replicates the transition pH in serum to within 0.08 pH units and allows probing self-assembly behavior using conventional spectroscopic techniques without conflicting protein signals, thus simplifying the development pathway from test tube to *in vivo* experimentation for stimuli-responsive materials.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information

Experimental materials and synthesis schemes; HPLC chromatograms, ESI-MS spectra, CD spectra, pH- and time-dependent FA, CAC determination, and relaxivity data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

There has been emerging interest in the development of dynamically triggered diagnostic and therapeutic nanomaterials that preferentially accumulate at a disease site in response to specific physiological stimuli. For example, numerous studies have sought to design nanomaterials that exploit tumor-related stimuli such as the acidic extracellular pH, endogenous enzymes, or redox environments to release drugs at a tumor site.^{1–7} In contrast to carriers that fragment to release cargo in response to stimuli, a particularly intriguing notion is to create materials that transform into more slowly diffusing objects upon reaching an acidic tumor vasculature. This could serve as an effective way of achieving a higher concentration of an imaging, drug delivery, or radiotherapeutic agent at a tumor site compared to that in the pH-neutral normal tissue.^{8–10} In one example, Chien et al. have demonstrated that peptide–polymeric amphiphiles can be triggered to undergo a self-assembly transition *in vivo* from spherical micelles to aggregated assemblies using endogenous matrix metalloproteinases, leading to a higher concentration of material at the tumor site relative to that in the rest of the body.¹¹ Additionally, we have previously shown that peptide amphiphile (PA) molecules can be designed to transform from spherical micelles at a normal pH of 7.4 to bulky, more slowly diffusing nanofibers when the pH is reduced to 6.6 (as found in malignant tumors) in isotonic salt solutions that roughly mimic physiological conditions (150 mM NaCl, 2.2 mM CaCl₂)⁹ for use as cancer-targeting magnetic resonance imaging (MRI) contrast agents.¹²

One of the great challenges in developing biocompatible, dynamic systems that can transform morphologically *in vivo* is the difficulty in probing their self-assembly behavior in

blood serum. Serum contains variable amounts of salt concentrations, as well as proteins such as albumin and immunoglobulins, which bind to amphiphilic molecules, enzymes, and other molecules.^{13–16} Any spectroscopic technique for determining the self-assembly behavior of PAs in the presence of other proteins will require the addition of a chromophore to the molecular structure. However, even minor changes to the molecular structure of the PA can dramatically change the pH trigger of self-assembly. Indeed, we observed that the addition of just a single methyl group in the β -sheet sequence shifted the transition basic by 0.4 pH units.⁹ Thus, developing approaches for probing this transition in serum without significantly altering the self-assembly behavior is an essential prerequisite for understanding the influence of this pH-triggered self-assembly on *in vivo* biodistribution.

Here, we have developed a method to probe the pH-dependent self-assembly morphology of PAs in pure mouse blood serum without significantly changing their intrinsic self-assembly behavior. Conjugating a fluorophore with appropriate lifetimes and excitation and emission spectra to 1.5% of the PA molecules allows for distinguishing between spherical and nanofiber morphologies via fluorescence anisotropy (FA) in pure serum at a detection limit of 10 μ M. The molecular crowding of albumin promotes self-assembly of these anionic PAs into nanofibers in serum. Finally, a solution consisting of 150 mM NaCl, 2.2 mM CaCl₂, and 1.8 mM 20 kDa poly(ethylene glycol) (PEG) accurately simulated the ionic strength and crowded environment of pure serum and enabled characterization of self-assembly behavior using circular dichroism (CD) spectroscopy and critical aggregation concentration (CAC) measurements, replicating the transition pH values in pure serum to within 0.08 pH units.

EXPERIMENTAL SECTION

Chemicals and Reagents

All amino acids for PA syntheses were purchased from Anaspec Inc. Mouse serum and mouse serum albumin (MSA) were purchased from MP Biomedicals. The bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine-ruthenium *N*-succinimidyl ester-bis(hexafluorophosphate) fluorophore and all other chemicals were purchased from Sigma-Aldrich and used without further purification. All PAs were synthesized by the solid-phase Fmoc synthesis and purified via reverse-phase high-performance liquid chromatography (HPLC). Their purity was assessed by analytical HPLC (Supporting Information, Figure S1) and electrospray ionization mass spectrometry (ESI-MS) (Supporting Information, Figure S2).

CD Spectroscopy

Measurements were done on a Jasco-815 circular dichroism spectrometer using 0.5–1 cm path length quartz cuvettes. Concentrations ranging from 3 to 500 μ M of either pure PA or PA mixtures were prepared in 150 mM NaCl and 2.2 mM CaCl₂ (and 1.8 mM 20 kDa PEG in select samples) by dilution from a concentrated PA stock (0.5–1 mM, pH 9). Double deionized Milli-Q water was used for preparing all solutions. The solutions were then heated at 80 °C for 30 min in a water bath and gradually cooled to room temperature to prevent the PAs from remaining in kinetically trapped states induced by the lyophilization process. An Accumet XL15 pH meter (Fisher Scientific) coupled with an Orion Ross Ultra semimicro

electrode (8103BNUWP, Thermo Scientific) was used to adjust the pH of the solution to the desired value followed by collection of the CD spectra. Each trace shown was averaged over 3 accumulations and was baseline-subtracted using aqueous solutions containing salts only. All spectra are cutoff below 200 nm due to absorption by NaCl and CaCl₂. The same procedure was followed for the salt control samples with 3.0 and 4.0 mM CaCl₂.

CAC Using Pyrene 1:3 Method

For CAC measurements, a series of solutions of either pure PA or PA mixture with concentrations ranging from 100 nM to 700 μ M was prepared using serial dilutions in 150 mM NaCl and 2.2 mM CaCl₂ (and 1.8 mM 20 kDa PEG in select samples). The final concentration of pyrene in each solution was fixed to be 4.5 μ M. This was followed by pH adjustment of the solutions using careful additions of HCl or NaOH. One-hundred microliters of each solution was transferred to a 96-well plate, and the fluorescence emission of pyrene was monitored using a hybrid reader fluorimeter (BioTek Synergy H4) at room temperature. The excitation wavelength was set at 335 nm. The ratio of the intensities of emissions at 376 and 392 nm were then plotted as a function of the PA concentration (log scale). The CAC was determined from an abrupt change in the slope of the plot using the least-squares fitting technique.

Transmission Electron Microscopy (TEM)

TEM images were obtained using solutions of the 100 μ M PA mixture concentration in 1.5% (v/v) serum solution and a serum control. The PAs, however, were not heated in this case to avoid destroying/denaturing serum proteins. This was followed by pH adjustment using either HCl or NaOH. Five microliters of this solution was pipetted onto a Carbon Formvar grid (Electron Microscopy Sciences) and allowed to sit for 2 min before being wicked dry using filter paper. The samples were then negatively stained using 1 wt % uranyl acetate and imaged under a FEI Tecnai G2 Bio TWIN TEM system, operating at 100 kV. All TEM experiments were performed in duplicate.

Fluorescence and FA

Fluorescence and FA measurements were done using 20–100 μ M of PA mixtures (diluted from 1 mM PA stock solutions) in 150 mM NaCl and 2.2 mM CaCl₂ and various serum (MP Biomedicals) concentrations (0.75–100% v/v) diluted in the same salt buffer. The PA stocks were heated at 80 °C for 30 min in a water bath and gradually cooled to room temperature. All samples were then pH adjusted using HCl/NaOH solutions and transferred to a 96-well plate followed by collection of fluorescence emission from PA-Ru(bipy)₃ first parallel (I_{par}) and then perpendicular (I_{perp}) to the excitation polarization using a hybrid reader fluorimeter (BioTek Synergy H4) at room temperature. The excitation wavelength used was 458 nm, and the emission was monitored using a 640/20 nm filter. The same method was followed for control experiments involving serum background samples, dye-conjugated MSA, only 1.5% PA-(Ru(bipy)₃) in serum, and 100 μ M PA mixture in MSA and salt buffer. FA was calculated using the following equation:

$$FA = \frac{(I_{\text{par}} - I_{\text{perp}})}{(I_{\text{par}} + 2I_{\text{perp}})}$$

and plotted as a function of either pH or time.

Water Relaxivity (r_1) Measurements

Low-field spin–lattice (T_1) relaxation time measurements were carried out with various concentrations of PA2-Gd(DO3A) in 150 mM NaCl and 2.2 mM CaCl₂ solutions at different pH values and a standard Prohance control on a benchtop minispec mq20 (NF series, Bruker, Germany). It uses a permanent magnet to create a field (0.469 T) corresponding to a proton resonance frequency of 19.95 MHz. The sample temperature was kept at 40 °C. For all samples, the magnetic field was matched to the resonance circuit, and the durations were on the order of 2.8 and 5.6 μ s at full amplitude for $\pi/2$ - and π -pulses, respectively. The inversion–recovery pulse sequence was used to measure the ^1H T_1 relaxation times in the laboratory frame. In this pulse sequence, the bulk magnetization is inverted by a 180° radio frequency pulse and then allowed to recover to equilibrium via the T_1 relaxation process over a variable recovery time, before acquisition of the free-induction decay with 32 data points and 16 scans per point. A recycle delay time of 5 T_1 was used to allow the system to fully relax between FID acquisitions, and phase cycling was employed to eliminate signal artifacts. Relaxivity (r_1) values were then obtained from the slopes of $1/T_1$ vs PA2-Gd(DO3A) or Prohance concentration ([contrast agent]) plots using the following equation

$$\frac{1}{T_1} = \frac{1}{T_{1,d}} + r_1[\text{contrast agent}]$$

where $T_{1,d}$ corresponds to water proton relaxation time in the absence of the paramagnetic contrast agent.

RESULTS AND DISCUSSION

Model PA Systems

Palmitoyl-IAAAEEEEK(DO3A:Gd)-NH₂ or PA1-Gd(DO3A) and palmitoyl-MAAAEEEEK-(DO3A:Gd)-NH₂ or PA2-Gd(DO3A) (Table 1) were synthesized as our model PA systems (DO3A = 1,4,7-tris(carboxymethylaza)cyclododecane-10-azaacetyl amide). PA1-Gd(DO3A) was previously found to exhibit a concentration-independent transition from spherical micelles to nanofibers in 150 mM NaCl and 2.2 mM CaCl₂ at a pH of 6.0.⁹ To investigate how the pH-dependent self-assembly behavior in serum can be altered with the β -sheet propensity of the first amino acid next to the palmitoyl tail, the analogous PA2-Gd(DO3A), which features methionine in place of isoleucine, was synthesized. In our previous work, we found that altering the β -sheet propensity of this amino acid can tune the self-assembly transition pH from micelles to nanofibers in salt solutions.⁹ To distinguish between the spherical and nanofiber morphologies via FA, bis(2,2'-bipyridine)-4'-methyl-4-carboxybipyridine ruthenium(II) (Ru(bipy)₃) was conjugated to the lysine ϵ -amine group of

palmitoyl-IAAAEEEEK-NH₂ (PA1-Ru(bipy)₃) and palmitoyl-MAAAEEEEK-NH₂ (PA2-Ru(bipy)₃) via NHS ester linkers (Table 1). Chart 1 illustrates the chemical structures of all four PAs. For FA measurements, PA1-Ru(bipy)₃ and PA2-Ru(bipy)₃ were spiked into PA1-Gd(DO3A) and PA2-Gd(DO3A), respectively, at 1.5 mol % of the total PA concentration, and these mixtures are defined as PA1-mix and PA2-mix, respectively. pH-dependent absorbance measurements of PA1-Gd(DO3A) with the Arsenazo-III dyes showed that the Gd remained completely chelated to a pH of ~5.0 (Supporting Information, Figure S3).

Ru(bipy)₃ was chosen as the FA fluorophore for two reasons. First, it has an excitation wavelength of 458 nm, which is significantly red-shifted from that of serum proteins, thus minimizing serum autofluorescence. Second, the fluorescence lifetime of Ru(bipy)₃ (~400–500 ns) is sufficient to distinguish between the spherical micelle and nanofiber morphologies via FA. FA measures the extent of decorrelation of the polarized emission from a fluorescent dye with respect to the polarization of the excitation light, which linearly depends on rotational correlation time of the dye-containing rotating unit in solution and consequently its molecular weight.^{17,18} The FA value reflects an average molecular weight distribution of the entire ensemble of PA nanostructures. The unassembled, isolated PAs have molecular weights of ~1.8 kDa. A 10 nm spherical micelle formed from these PAs with an estimated aggregation number of 60–100 (~108–180 kDa)^{19,20} would have a different FA than a micron-sized nanofiber, which has a molecular weight 8 orders of magnitude larger and scaled according to length. For example, a 500 nm long fiber is expected to have a molecular weight of 5–9 MDa. The rotational correlation times of a spherical micelle and a nanofiber would be 50–100 ns and >1 ms, respectively.²¹

Phase Diagram of PA Mixture and Reversibility of Morphology Transition

Self-assembly behavior is known to be extremely sensitive to minor changes in monomer structure and to the presence of impurities. To ensure that the addition of 1.5% PA1-Ru(bipy)₃ to PA1-Gd(DO3A) does not significantly disrupt self-assembly behavior, a concentration–pH phase diagram was mapped for PA1-mix in a 150 mM NaCl and 2.2 mM CaCl₂ salt solution and compared with the phase diagram for pure PA1-Gd(DO3A) (Figure 1). CAC measurements via the pyrene 1:3 method²² were used to ascertain the single molecule to spherical micelle or nanofiber transitions at varying pH values (Supporting Information, Figure S4). CD spectroscopy was used to determine the pH points at which different concentrations (10–500 μ M) of the PA molecules transitioned from a random coil to a β -sheet secondary structure (Supporting Information, Figure S5). It has been previously shown that for pure PA1-Gd(DO3A) the random coil structure corresponds to either spherical micelles or isolated molecules and the β -sheet structures correspond to a nanofiber morphology.⁹ The CD transition point was defined to be the pH value at which the ellipticity at 205 nm rose from a negative value (random coil) to zero accompanied by the appearance of a minimum at 218–220 nm. The addition of PA1-Ru(bipy)₃ shifted the CD transition pH to more basic values by ~0.6 pH units. The CAC values, on the other hand, were found to be 5 $\times$ higher in the acidic region but comparable under basic conditions, relative to those of pure PA1-Gd(DO3A). This ~0.6 shift in the CD transition pH to more basic values was also observed when 1.5% of PA2-Ru(bipy)₃ was added to PA2-Gd(DO3A).

The transition was found to be rapid and reversible with respect to pH. This was tested by switching the pH of the PA mixture back and forth between ~5.0 and ~10.0 followed by collection of the CD spectra within 2–3 min (Supporting Information, Figure S6). The CD curves showed almost superimposable secondary structures for the acidic and basic pH values, indicating that the morphological transitions are reversible.

FA-CD Correlation

pH-dependent FA values of a 100 μ M PA1-mix in the same isotonic salt solution (Figure 2a) were then obtained. The inset in Figure 2a shows the fluorescence emission from PA1-Ru(bipy)₃ in the mixture upon excitation with 458 nm light that was exploited for FA measurements. The FA values increased from 0.066 to 0.115 as the pH was carefully lowered from 8.35 to 5.31. The onset of the self-assembly transition was defined by the data point that first showed a higher value relative to the constant FA at basic pH values. The end of this transition was defined as the pH point below which the FA became constant again. The transition profile measured via FA correlated well with that obtained from the pH-dependent CD spectra (Figure 2b,c). The FA transition onset at 6.98 is close to the most basic pH CD spectrum at 7.10 that started to deviate from a superimposable random coil morphology (Figure 2c). Also, the FA transition midpoint at a pH of 6.68 was close to our previously defined CD transition spectrum at a pH of 6.62. The close match of the transition pH is more clearly seen from the pH-dependent shift in the ellipticity value at 205 nm (Figure 2b).

FA in Pure Serum

The fluorescence of both PA mixtures was detectable in pure mouse serum above the serum autofluorescence background. Figure 3a shows this fluorescence emission from PA1-mix in blood serum along with the serum autofluorescence background. The fluorescence emission intensity increased with increasing amounts of PA1-Ru(bipy)₃ in serum (Supporting Information, Figure S7) and was found to be superimposable under acidic and basic conditions (Supporting Information, Figure S8) in our simulated salt solution. The ~30 nm red shift in the PA1-Ru(bipy)₃ fluorescence emission in serum is commonly observed for Ru(bipy)₃ in the presence of protein and lipid molecules due to changes in the dye's emission pathways.²³ For a 100 μ M PA1-mix, the FA in pure mouse blood serum was found to be pH-independent with a constant value of ~0.22, as shown in Figure 3a. This constant high value results from the formation of nanofibers immediately after addition of the serum to the PA mixture even at basic pH values. On the contrary, PA2-mix was found to transition in the pH range 7.1–7.4, as is evident from the increase in FA values from 0.09 to 0.23, also shown in Figure 3b. Comparison of the serum results with the FA of PA2-mix in our salt solution (Figure 3b) indicates that serum shifts the transition pH to more basic values. The stabilization of nanofibers is much stronger for PA1-mix, which goes from transitioning at pH 6.6 in salts (Figure 3b) to a constant nanofiber morphology. The fact that PA1-mix forms nanofibers across all pH values in serum and that PA2-mix still shows a transition is consistent with the fact that isoleucine has a stronger β -sheet propensity relative to that of methionine.

To confirm that this pH-dependent jump in FA values in the serum samples was actually reflecting changes in self-assembly morphology and not just the PA1-Ru(bipy)₃ or PA2-Ru(bipy)₃ single molecules bound to the 70 kDa serum albumin proteins, a control FA experiment was conducted with a sample containing only 1.5% PA2-Ru(bipy)₃ isolated molecules (no PA2-Gd(DO3A)) in serum (Figure 3b). The FA values remained constant at ~0.05 independent of pH. A second control where the Ru(bipy)₃ dye was directly conjugated to pure mouse serum albumin (MSA) also showed a constant FA value of ~0.04 over pH and time (MSA-dye control; Figure 3b and Supporting Information, Figure S9). Also, FA values obtained for the serum-only background ranged from 0.003 to 0.005, indicating that there is negligible contribution from serum autofluorescence.

The general increase in the absolute FA values in serum (~0.09–0.23) relative to salts (~0.06–0.11) is attributed to an increase in solution viscosity, which increases rotational correlation times and consequently FA.¹⁷ The transition in PA2-mix was rapid (~3 min after pH change) and reversible with a small hysteresis of 0.3 pH units, as shown in Figure 3c, further confirming that these morphologies are close to thermodynamic equilibrium in pure serum. The stability of the nanostructures in serum was tested for 100 μ M PA2-mix via FA over time and is shown in Figure 3d. At pH values of 8.01 and 6.50, the FA was constant at ~0.09 and ~0.23, respectively, over a period of 500 min. No further change in FA values was observed after 8 days, which highlights the stability of these self-assembled morphologies in serum.

FA in Diluted Serum

To elucidate the observed drastic influence of serum on the self-assembly behavior of PA1-mix, pH-dependent FA values were collected for the 100 μ M PA mixtures in solutions containing diluted serum concentrations (0.75–4% serum v/v in 150 mM NaCl and 2.2 mM CaCl₂) (Figure 4a). The transition onset and end points shifted to more basic values upon addition of greater concentrations of serum until no transition was observed in 4% serum. Similar to the values observed in the salt solution, the FA increased from ~0.065 to ~0.12 as the pH was lowered. At 4% serum, the FA was found to be constant at ~0.125, indicating nanofiber formation at and beyond this serum concentration. The pH-dependent transition at lower serum concentration again occurred reversibly within 3 min of pH adjustment, as shown in Figure 4b.

The self-assembly morphologies were further confirmed via conventional TEM (Figure 4c,d) of the 100 μ M PA1-mix at pH 6.85 (FA ~ 0.11) and 9.21 (FA ~ 0.06) in 1.5% serum. At pH 6.85, nanofibers having diameters of 12.3 ± 1.9 nm were observed. At a pH value of 9.21, a distinctly different spherical micellar morphology having diameters of 10.7 ± 1.4 nm was observed. This imaging data demonstrates that the higher FA values (~0.11) correspond to the nanofiber morphology.

Effect of Serum Albumin on Self-Assembly

The increased propensity for nanofiber formation in serum could be due to at least two probable factors: interaction with serum proteins and/or a higher ionic strength relative to that of our salt solution. Serum albumin (~67 kDa) typically constitutes ~75–80% of all

proteins in blood serum, having a concentration of 520–750 μM .¹³ It is well-known that serum albumin disrupts micelle formation via adsorption of isolated surfactant amphiphiles.^{24,25} A series of FA experiments was performed to determine if there was any binding interaction between the PAs and serum albumin. PA2-Gd(DO3A) was added to Ru(bipy)₃-labeled MSA at both acidic and basic pH values, and the FA was monitored over time. Furthermore, the FA was monitored over time when MSA was added to the fluorescent PA2-mix at both acidic and basic pH values (Supporting Information, Figure S10). No changes were observed in the FA values over a period of 5 h, indicating a lack of binding between the anionic PAs and MSA. Furthermore, if there was binding between isolated PA molecules and albumin, then we would expect the effective concentration of PAs in solution to decrease, and, consequently, according to our self-assembly phase diagrams (Figure 1), there should be a small shift in the observed transition to lower pH values. To probe the effect of pure MSA on the transition pH, pH-dependent FA measurements were performed on a sample composed of 100 μM PA1-mix, 7.8 μM MSA, 150 mM NaCl, and 2.2 mM CaCl₂ (Figure 5). This MSA concentration corresponds to the amount that would be present in 1.5% serum. The transition onset pH values was shifted more basic by 1.1–1.3 pH units with MSA and was relatively close to the transition observed (within 0.5 pH units) in 1.5% serum. Together, these experiments show that the addition of just MSA strongly induces fiber formation, not via binding to isolated PA molecules or micelles/nanofibers. The effect of adding just MSA closely replicates the change in transition pH in the complex serum mixture.

Furthermore, we showed that the increased propensity for nanofiber formation in serum is not due to the variability of ionic strength of serum relative to that of our salt solution. To determine the influence of salt concentration on the transition, the CD transition points of 100 μM PA1-mix in 150 mM NaCl solutions containing 3.0 and 4.0 mM CaCl₂ (Supporting Information, Figure S11) were measured. The CaCl₂ concentration was varied since divalent cations more drastically affect the critical coagulation concentrations of amphiphiles.²⁶ With 3.0 and 4.0 mM CaCl₂, the transition onset was shifted to more basic pH values by 1 to 2 pH units, respectively, close to the shifts observed in the 0.75 and 1.5% serum samples. If our observed shifts in the serum samples had been due to the greater Ca²⁺ concentration, then this would imply that pure serum would have to contain ~267 mM Ca²⁺. This incredibly high value is ~100 times higher than the known range of divalent salts in mouse serum.¹³ This further suggests that the observed transition shifts are primarily due to the presence of serum albumin, rather than the variability of ionic strength in serum.

Since there is no binding interaction between albumin and the PA nanostructures, the transition in forming nanofibers upon the addition of serum can be attributed to a macromolecular crowding effect. There exists a thermodynamic depletion force that drives macromolecules together and into more compact self-assembled structures when there is a high concentration of co-solutes in solution.^{27–29} This driving force arises due to an increase in entropy of the system, as a more compact self-assembled structure provides a larger volume for co-solutes to freely move.^{29,30} In this case, the high concentrations of albumin cause the PAs to form the more compact nanofiber morphologies, even at more basic values. This was further confirmed by achieving the same self-assembly behavior when albumin is replaced with PEG. We performed pH-dependent FA using 20 kDa PEG in place of MSA

(Figure 5). A PEG concentration of 26 μM , normalized to the MSA concentration of 7.8 μM by the ratio of their molecular weights, caused a comparable shift in the transition pH of 100 μM PA1-mix. Also, in 1.8 mM PEG, which corresponds to the molecular weight-normalized MSA concentration in pure serum, nanofibers formed exclusively irrespective of pH, consistent with pure serum.

Phase Diagram in Artificial Serum

Finally, a medium was created that accurately replicated the ionic strength and the crowded environment of pure serum and enabled characterization of our PA self-assembly behavior using CD and CAC measurements without interference from protein signals. This artificial serum solution contains 1.8 mM of 20 kDa PEG, 150 mM NaCl, and 2.2 mM CaCl_2 . A concentration–pH phase diagram (Figure 6c) was then constructed for pure PA2-Gd(DO3A) in this artificial serum solution using transition points obtained via concentration-dependent CD (Supporting Information, Figure S12) and pH-dependent CAC (Supporting Information, Figure S13) measurements. To evaluate the effectiveness of this simulated solution to mimic real serum, pH-dependent CD values were collected for various concentrations of PA2-mix in artificial serum (Supporting Information, Figure S14) and compared to the FA values obtained in pure serum (Supporting Information, Figure S15). Just as was observed in PA1-mix (Figure 1), the transition points of PA2-mix were shifted by ~ 0.5 units to basic pH values relative to that of pure PA. Remarkably, the pH transitions in pure and artificial serum agree within ~ 0.08 pH units. TEM images of a 500 μM PA2-mix in this artificial PEG serum confirm a nanofiber morphology at pH 5.5 and spherical micelle morphology at pH 8.2, in excellent agreement with the phase diagram (Figures 6a,b). Hence, this artificial serum precisely mimics real serum conditions and provides a screening medium for PA drug candidates *in vivo*.

Water Relaxivity (r_1) Measurements

The r_1 values of water protons in the presence of various concentrations of PA2-Gd(DO3A) at pH 5.5 and 9 were found to be 13.6 and 8.6 $\text{mM}^{-1} \text{s}^{-1}$, respectively, using a 0.5 T magnet (Supporting Information, Figure S17). These values were higher than those measured for a ProHance control standard (4.1 $\text{mM}^{-1} \text{s}^{-1}$).³¹ These r_1 values were close to the values previously measured for PA1-Gd(DO3A), which also show a similar increase in r_1 from micelles to nanofibers.⁹ This increase in r_1 from spherical micelles to nanofibers is indicative of the longer rotational correlation of the PA2-Gd(DO3A) tether when attached to larger molecular weight morphologies. While further optimization of the r_1 values can likely be achieved by rigidifying the E₄K tether, these vehicles already show enhanced r_1 values relative to those of FDA-approved contrast agents in current clinical use.^{31,32}

CONCLUSIONS

We have demonstrated an approach to successfully probe pH-dependent self-assembly transitions of peptide amphiphile materials in pure blood serum using fluorescence anisotropy. This method allowed the accurate determination of the formation of distinct peptide nanostructures in the presence of high concentrations of large molecular weight proteins, without altering the intrinsic self-assembly profile. We have shown that PA

molecules can be designed to transition from spherical micelles to nanofibers in the pH range 7.0–7.4 in pure serum. Moreover, we have created a medium that accurately simulates the ionic strength and crowded environment of blood serum, replicating the pH-triggered self-assembly behavior in pure serum. This technique promises to simplify the pathway from test tube to *in vivo* experimentation for this emerging class of smart stimuli-responsive materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Concentration–pH phase diagram of pure PA1-Gd(DO3A) (red) and the PA1-mix (blue) as determined via CD (solid diamonds) and CAC (hollow diamonds) measurements, respectively. All measurements were done in 150 mM NaCl and 2.2 mM CaCl₂.

Figure 2.

(a) pH-dependent FA of 100 μM PA1-mix. The inset shows the fluorescence emission from the PA1-Ru(bipy)₃ in the mixture. (b) pH-dependent ellipticity at 205 nm of 100 μM PA1-mix. (c) pH-dependent CD spectra of 100 μM PA1-mix. The color for each point in panels a–c corresponds to similar (within 0.02 pH units) pH values.

Figure 3.

(a) Fluorescence emission from 100 μ M PA1-mix in pure serum along with serum autofluorescence background. (b) pH-dependent FA of 100 μ M PA1-mix and PA2-mix in salts and pure serum. (c) pH reversibility of the morphology transition in 100 μ M PA2-mix in serum. (d) Time-dependent stability of spherical micelles and nanofibers in 100 μ M PA2-mix in serum via FA measurements.

Figure 4.

(a) pH-dependent FA of 100 μ M PA1-mix in 0–4% v/v diluted serum solutions. (b) Kinetics of the morphology switch of 100 μ M PA1-mix in 1.5% serum via time-dependent FA measurements. (c, d) TEM images of 100 μ M PA1-mix in 1.5% serum at pH 6.85 and 9.21, respectively.

Figure 5. pH-dependent FA of 100 μM PA1-mix in salts (150 mM NaCl, 2.2 mM CaCl_2), 1.5% serum, 7.8 μM MSA, and 26 μM and 1.8 mM PEG.

Figure 6.

TEM images of 500 μM PA2-mix in artificial serum at (a) pH 5.5 and (b) pH 8.2. (c) Concentration-dependent CD transition pH values of PA2-mix in artificial (blue) and pure mouse blood serum (red) overlaid on the phase diagram (faded orange) of 100 μM PA2-Gd(DO3A) in artificial serum.

Chart 1.
PA Structure and Design

Table 1

Synthesized PA Molecules

molecule	sequence
PA1-Gd(DO3A)	palmitoyl-IAAAEEEEK(DO3A:Gd)-NH ₂
PA2-Gd(DO3A)	palmitoyl-MAAAEEEEK(DO3A:Gd)-NH ₂
PA1-Ru(bipy) ₃	palmitoyl-IAAAEEEEK[Ru(bipy) ₃]-NH ₂
PA2-Ru(bipy) ₃	palmitoyl-MAAAEEEEK[Ru(bipy) ₃]-NH ₂