Peptoids advance multidisciplinary research and undergraduate education in parallel: Sequence influences peptoid-lipid interactions

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ABSTRACT

Peptoids are versatile peptidomimetic molecules with wide-ranging applications from drug discovery to materials science. An understanding of peptoid sequence features that contribute to their interactions with lipids will be advantageous to expand the functions of peptoids in these varied fields. Further, these topics capture the enthusiasm of undergraduate students who participate in the preparation and study of diverse peptoids in laboratory coursework and/or in faculty-led research. Here, we present the synthesis and study of 21 peptoids with varied functionality, including 19 tripeptoids and two longer oligomers. We monitored fluorescence spectra for ten of the tripeptoids and observed differences in spectral features correlated with peptoid flexibility and relative positioning of chromophores. Interactions of representative tripeptoids and two longer peptoids with sonicated glycerophospholipid vesicles were also evaluated using fluorescence spectroscopy, revealing an increase in emission intensity at the λ_{max} in the presence of lipids. We also summarize here the advantages of engaging students in peptoid-based projects as an effective strategy to advance both research and undergraduate educational objectives in parallel.

INTRODUCTION

The quantification of drug concentrations partitioned between the aqueous phase and the plasma membrane (PM) is a critical measurement to evaluate bioavailability and toxicity in the development of new pharmaceuticals.[1] The main structural feature of the PM is a lipid bilayer composed mostly of glycerophospholipids. Interactions between putative drugs and the lipid bilayer may change the packing of the lipid acyl chains, altering the structure of the PM, in turn changing the activity of membrane-bound proteins.[2–4] Studies of small molecule drugs and bioactive peptides in the presence of PMs or model PMs have revealed some structural motifs that promote PM-drug interactions.[5–8] For example, one strategy commonly employed to enhance membrane permeability of peptides *in vitro* and *in vivo* is *N*-methylation.[9–11]

The related *N*-substituted glycine oligomers, peptoids, are a valuable template scaffold for the development of new potential therapeutics.[12,13] A number of researchers have explored peptoids' utility as antimicrobial agents,[14–17] and their efficacy in this context likely depends on their selective interactions with microbial PMs. Additionally, peptoids have been explored as inhibitors of membrane-bound VEGF receptors[18] and ubiquitin receptors,[19] to name a few. Peptoids are attractive for these applications; many peptoids, including large combinatorial libraries, have been prepared by the robust sub-monomer method[20] in which diversity originates from the wide array of commercial or synthetic primary amines available for site-specific incorporation of side chain functionality. Further, they are resistant to proteolytic degradation.[21] Although peptoids have been shown to have higher cell permeability than peptides,[22] there are few quantitative studies of peptoid–PM interactions.[14,23] Advancing peptoid-based therapeutics[12,24] and antimicrobial coatings[14,25,26] will require an enhanced understanding of how peptoid sequence and structural features modulate their interactions with PMs.

We speculated that we could address the scientific challenge of understanding peptoid-PM interactions while simultaneously addressing the educational challenge of teaching

undergraduates synthetic and analytical research skills. Peptoid research is a rich platform to advance undergraduate learning. Because the applications of peptoids extend to a range of scientific disciplines, students with widely varied interests can find ways to engage with peptoid topics. Importantly, syntheses and spectroscopic studies of peptoids have historically employed many approachable techniques, a critical consideration for students with nascent laboratory skills. In this report, undergraduates executed all of the experimental work (Figure 1): they prepared an array of 21 diversely-functionalized peptoids (**A–U**, Figure 2), then evaluated peptoid structures and lipid interactions of 10 representative examples using fluorescence emission spectroscopy in the presence and absence of small unilamellar vesicles (SUVs) composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) as model PMs.

Undergraduate participation in research activities has been classified as a high-impact educational practice and is endorsed by such agencies as the President's Council of Advisors on Science and Technology (PCAST)[27] and the American Chemical Society Committee on Professional Training (ACS-CPT).[28] Their experiences in research projects have been shown to advance undergraduate learning and to build students' resilience and confidence.[29,30] Indeed, experiments that engage students in peptoid synthesis or the related synthesis of arylopeptoids in educational laboratory settings have been shown to yield positive learning outcomes.[31–34] In our own experience, students are highly motivated when their work has an immediate application, and this project provides just that—seventeen students enrolled in an introductory organic chemistry laboratory course prepared most of the peptoids detailed here for study of their interactions with model PMs. The remainder were prepared by undergraduates working in faculty-led research experiences over the summer or academic year.

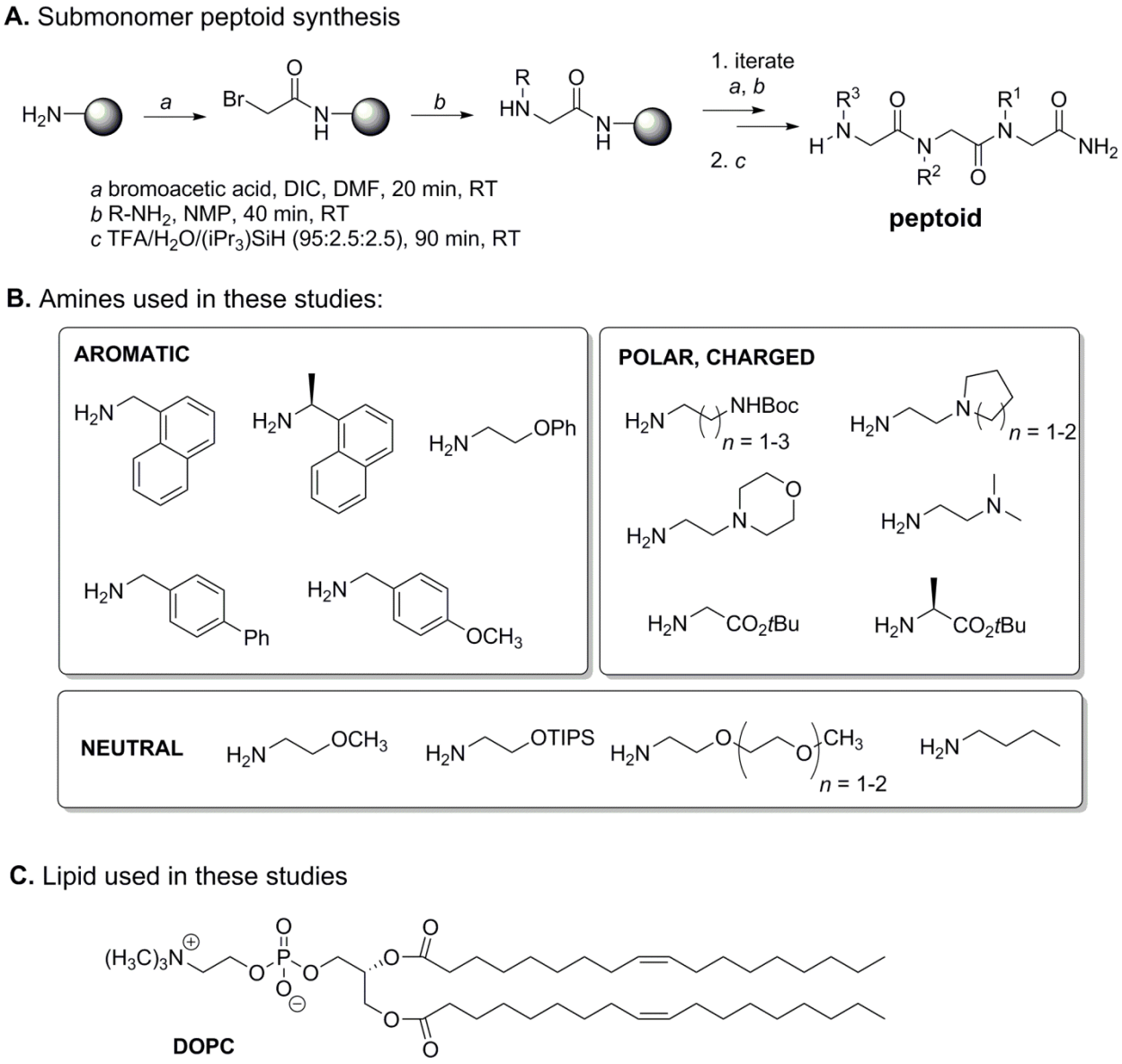

Figure 1. Structures of molecules used in these studies. A) Submonomer synthesis method for preparation of peptoids. B) Amines with varied functionality were used to install side chain functionality sequence-specifically in an array of 19 tripeptoids and two longer peptoid sequences. C) Structure of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) lipid used to prepare small unilamellar vesicles as PM mimics.

Peptoid synthesis experiments are an excellent match with undergraduate organic chemistry laboratory courses, and we have implemented a five-week peptoid synthesis experiment module in an introductory chemistry laboratory course that closely parallels those previously described[31–33] (Table 1, refer to detailed student instructions and instructor materials

on pages S4 through S18 in the Supporting Information). The introduction of peptoids offers an opportunity to engage students with diverse interests; we assigned students to present brief literature reports that summarized the applications of peptoids across multiple sub-disciplines to provide context for their hands-on work. Additionally, peptoid synthesis reactions contextualize concepts addressed in typical introductory organic chemistry lecture courses, including acylation and nucleophilic substitution reactions. The techniques and mechanisms also parallel those typically used for chemical synthesis of peptides. In our class, students researched relevant reaction mechanisms and prepared brief presentations of these. Peptoid synthesis also showcases multi-step synthesis, another central concept in organic chemistry courses that can be challenging to incorporate into laboratory coursework. In addition to reinforcing lecture topics, peptoid synthesis provides a platform to introduce modern research techniques less commonly seen at the introductory level, such as combinatorial chemistry and solid phase synthesis.[33,35–38]

Fluorescence spectroscopy is a highly approachable experimental technique for undergraduate students to study properties of molecules they have synthesized. Peptoid structure comparisons using fluorescence spectroscopy (among other techniques) have been carried out by undergraduate researchers in the Fuller and Stokes laboratories, including in the study of two previously reported peptoids also used in this work, **T** and **U** (Figure 2).[39] Peptoids **T** and **U** are water-soluble 6- and 15-residue peptoid sequences, respectively. Both have postulated amphiphilic helix structures, but they exhibit different fluorescence emission features in aqueous buffer. **T** has a single emission peak with λ_{max} at 340 nm, while **U** has the 340 nm emission peak as well as a broad emission peak at 392 nm attributed to an excited state dimer (excimer). The ratio of these two peaks in fluorescence spectra of **U** is concentration-dependent, and this is one of several experimental observations that is consistent with the self-association of **U** in aqueous solution. The interactions of **T** and **U** with benzo[*a*]pyrene[40] and with silica surfaces[41] have also been evaluated using varied spectroscopic techniques, including fluorescence spectroscopy.

Table 1. Timeline for laboratory activities in tripeptoid synthesis experiments

Week	Lab lecture topics	Student presentation topics	Lab technique demonstrations	Student lab tasks
1	• Peptoids and their utility • Sub-monomer peptoid synthesis • Solid-phase synthesis • Combinatorial chemistry • Protecting groups • Introduction to syringe		• Synthesis vessel usage	• Swell resin • Deprotect resin • Identify amines for use, do calculations for preparation of at least one amine solution • Draw target product in ChemDraw, generate molecular weight
2	• Understanding peptoid-lipid interactions using SHG spectroscopy	• Mechanism: Fmoc removal • Peptoid applications in the literature	• Chloranil test	• Bromoacetylation reaction • Amine displacement reaction • Bromoacetylation reaction • Prepare amine solution
3		• Mechanisms: bromoacetylation reaction, amine displacement reaction • Peptoid applications in the literature		• Amine displacement reaction • Bromoacetylation reaction • Amine displacement reaction
4	• Introduction to LC-MS instrumentation and techniques	• Mechanisms: chloranil test, cleavage from resin, side chain deprotection • Peptoid applications in the literature	• LC-MS sample preparation	• Cleave tripeptoid from resin • Prepare sample for LC-MS analysis
5	• Introduction to LC-MS data analysis			• Analysis of LC-MS data

We reasoned that fluorescence emission spectroscopy would provide an efficient and straightforward technique for undergraduate students engaged in faculty-mentored research to survey peptoid spectra in the presence and absence of model PMs. All peptoids were designed

to include at least one naphthalene-functionalized residue to provide a fluorescent handle for these analyses. Several previous studies have monitored changes to the fluorescence emission spectrum of a small molecule or peptide upon binding to model PMs—typically, an increase in quantum yield and a blue-shift of the λ_{max} were observed.[42,43] Measurement of these changes has allowed researchers to quantify the amount of fluorescent small molecule or peptide that partitioned into lipid membranes.[42] From our spectroscopic data, we expected to extract more qualitative comparisons; we would identify correlations that relate peptoid sequence features with spectral changes.

This experimental strategy offers the advantage of being able to survey a large number of peptoids to discern trends that contribute to peptoid-lipid interactions. Quantitative measurement of interactions between peptoids and model PMs have been made using X-ray reflectivity,[14] and isothermal calorimetry (ITC) has been used to study α-peptide/β-peptoid chimera peptidomimetics' affinity for model PMs.[23] Additional information about peptoid-lipid interactions has been explored using imaging of cells exposed to peptoids.[17,44] However, all of these studies are less straightforward for undergraduate researchers than fluorescence spectroscopy.

Here, we report both the preparation and study of 21 water-soluble peptoids. We monitor the effects of peptoid sequence on the fluorescence emission spectra at neutral and acidic pH. Additionally, we describe changes to the peptoids' fluorescence spectra in the presence of model PMs. From these, we identify important correlations between peptoid structure and spectral features. These correlations inform the design of future experiments that will quantify peptoid-lipid interactions with the long-term goal of developing a predictive model for peptoid-PM affinities. In parallel, we offer perspectives from our own experiences about how engaging undergraduate student researchers in all aspects of these projects can advance both research and educational goals.

MATERIALS AND METHODS

Materials

Reagents for peptoid synthesis were purchased from commercial suppliers and used without further purification. Alanine-*tert*-butyl ester hydrochloride and glycine-*tert*-butyl ester hydrochloride were purchased from AAPPTec (Louisville, KY). Before use, the ammonium salt was dissolved in a minimal amount of water, and 2.5 M NaOH was added to adjust the solution pH to 10. The neutralized amine was then extracted with CH_2Cl_2, and the organic solution was dried over $MgSO_4$, then concentrated by rotary evaporation . 2-((triisopropylsilyl)oxy)ethan-1-amine was prepared according to the reported procedure, and spectroscopic properties were identical to those reported.[45] 2-(2-methoxyethoxy)ethan-1-amine and 2-(2-(2-methoxyethoxy)ethoxy)ethan-1-amine were prepared by the laboratory of Ronald Zuckermann according to reported procedures.[46] Fmoc -protected Rink amide resin was purchased from EMD Millipore. Solvents were purchased from Fisher Scientific unless otherwise specified. Chloroform (HPLC grade) was purchased from Honeywell Burdick and Jackson and was dried over 4 Angstrom molecular sieves (Alfa Aesar) prior to use in the preparation of dried lipids as detailed below. Peptoids **T** and **U** were prepared as previously described.[39]

Tripeptoid Synthesis

Solid-phase tripeptoid syntheses were executed by undergraduates, most commonly in the introductory organic chemistry laboratory course. Detailed student procedures and instructor materials for implementing peptoid synthesis experiments as laboratory course experiments is included in the Supporting Information. Peptoid syntheses were done in syringes fitted with coarse frits purchased from Torviq (Niles, MI). The syringe plunger was removed to add Fmoc-Rink amide resin (0.1 mmol) to the syringe barrel, then the plunger was replaced. Reagents solutions or wash solvents were drawn into or pushed out of the resin using the syringe plunger. Syringes were capped during resin swelling and reaction times.

A syringe charged with Fmoc-Rink amide resin was washed with DMF three times, then allowed to swell for at least 10 min in the capped syringe. The DMF was removed from the syringe, and the resin was treated with 2 mL of a 20% 4-methylpiperidine solution in DMF for 15 mins. The resin was washed twice with DMF, then equilibrated with another 2 mL of a 20% 4-methylpiperidine solution in DMF for 15 mins. The resin was drained, then washed with DMF ten times. A mixture of 1.7 mL of 1.2 M bromoacetic acid in DMF and 0.4 mL DIC was drawn into the syringe. The syringe was capped and the reaction was allowed to proceed for 20 mins with occasional manual agitation of the syringe. The reaction solution was expelled from the syringe, and the resin was washed with DMF (4 times) and NMP (4 times). To the syringe was added 2 mL of a 1 M solution of the appropriate amine in NMP. The syringe was capped, then subjected to occasional manual agitation. After 40 mins, the reaction mixture was expelled from the syringe, and the resin was washed with DMF (8 times). A few resin beads were removed and subjected to the chloranil test to confirm the presence of the secondary amine (blue-green beads). The bromoacetic acid/DIC and amine displacement reactions were iterated twice more using the appropriate amines to complete the peptoid synthesis. Again, reaction completion at each step was monitored using the chloranil test to assess the presence or absence of a secondary amide. The resin was then washed with CH_2Cl_2 five times, then the syringe plunger was removed, and the resin was allowed to dry open to air in the hood for at least 10 min. The syringe was capped, clamped upright to a support in the hood, and 5 mL of a solution of 95% TFA/2.5% H_2O/2.5% triisopropylsilane were added to the resin. After 60-90 mins, the cap was removed, and the filtrate was collected into a clean, tared vial. TFA was removed by evaporation.

Peptoid Purification and Identification

Crude tripeptoid **O** was purified by column chromatography using a Hypersep Si column (500 mg/3 mL) using 5% methanol in dichloromethane, then 10% methanol in dichloromethane to effect complete elution from the column. The remaining tripeptoids were purified reverse-phase

high performance liquid chromatography (RP-HPLC) on an Agilent Technologies 1260 Infinity II system equipped with a Polaris C18-A column (250 x 10.0 mm, 5 µm) using a 10 minutes linear gradient of 30%-90% methanol (solvent B) in 0.1% aqueous TFA (solvent A) at a flow rate of 4.7 mL/min. Peaks eluted were detected by absorbance at 220 nm. All data were visualized with OpenLab CDS software.

Peptoids were identified by electrospray mass spectrometry in positive ion mode using a Thermo LCQ Fleet mass spectrometer. High resolution mass spectral data were collected for ten representative tripeptoids using an Agilent 1260 Infinity II LC with 6230 TOF MS detector (electrospray ionization, positive ion mode) and were within 5 ppm of expected values. These data are tabulated in the Supporting Information, Table S1. Purified peptoids were lyophilized to white powders.

Buffer Preparation

Phosphate buffered saline (PBS buffer) was prepared with 50 mM sodium phosphate (Fisher Scientific) and 100 mM sodium chloride (Alfa Aesar) using ultrapure 18 M*ohm water (ThermoScientific Barnstead Micropure). The pH of the PBS buffer was adjusted to 7.4 with sodium hydroxide (Macron) or hydrochloric acid (EMD). PBS buffers were stored at 4 °C. Acetate buffer (pH 5.0) was prepared with 50 mM sodium acetate (EM Science) and 183 mM sodium chloride (VWR) to maintain total ionic strength at 215 mM.

Peptoid Stock Preparation

Peptoid stocks solutions (1-2 mM) were prepared by diluting lyophilized peptoid into HPLC-grade methanol. Stock concentrations were determined by UV spectroscopy using experimentally-determined extinction coefficients at 266 nm as tabulated in the Supporting Information, Table S2. All peptoids contained naphthalene residues which exhibit strong

adsorption at 266 nm. Peptoid solutions for spectroscopic studies were prepared by diluting the methanol stock solution into the appropriate buffer.

SUV Preparation

Following standard procedures,[47] we prepared dried lipid films (2.62 mg/mL) from stock 1,2-dioleoy-*sn*-glycero-3-phosphocholine (DOPC) dissolved in chloroform (25 mg/mL) purchased from Avanti Polar Lipids (Alabaster, AL). The lipids were evaporated under a gentle stream of nitrogen gas (ultra high purity, Matheson) and vacuum dried overnight to remove residual chloroform. Dried lipid aliquots (7.85 mg) were stored at -20 °C. SUVs were prepared by reconstituting dried lipid films with 3 mL PBS buffer (lipid concentration = 3320 μM) followed by vortexing to mix and bath sonication for 20 minutes until solutions were clear. Average diameter and polydispersity of SUVs made by this procedure were determined by Dynamic Light Scattering (90Plus Particle Size Analyzer, Brookhaven Instruments Corp). Sonicated SUVs had a mean diameter of 111.9 ± 2.2 nm and the polydispersity was 0.269 ± 0.005.

SUVs made in the appropriate buffers were subsequently studied by fluorescence spectroscopy. Fluorescence measurements were complete within 2 h after sonication to address the potential that SUVs prepared by sonication may not be stable over long periods of time.[48] Equal volumes of sonicated SUVs and aqueous peptoid solutions were added together, mixed by vortexing, and allowed to equilibrate to final concentrations of 1660 μM lipid and 100 μM peptoid. For measurements of peptoid-lipid mixtures at pH 5.0, SUVs were initially formed by sonication in PBS pH 7.4 until clear, as described above, then diluted into pH 5.0 acetate buffer to the concentrations reported above. This ensured that acidic conditions would not destabilize the lipid bilayers when SUVs were formed during sonication.[49]

Fluorescence Spectroscopy

Fluorescence emission spectra of tripeptoids **A**, **B**, **C**, **D**, **I**, **M**, **N**, **O**, **R**, and **S** were acquired on a Molecular Devices SpectraMax i3x plate reader instrument, and experimental setup and data extraction were done with SoftMax Pro 6.5.1. Solutions for analysis were pipetted into black, flat-bottom Thermo Scientific Microtiter 96-well plates. Total solution volume in each well was 150 μL, and three wells were filled from each solution. The data reported are the average of these three replicates. Emission spectra were collected from 300-500 nm in 1 nm increments. The excitation wavelength was set to 270 nm, and excitation and emission slit widths were set to 9 nm and 15 nm, respectively.

For the fluorescence spectra of **T** and **U**, a Horiba Fluorolog 3 spectrofluorometer was used. Samples were excited at 270 nm and emission was scanned from 300-500 nm in 1 nm increments with excitation and emission slit widths set to 6 nm and 3 nm, respectively. All experiments were conducted with 50 mM phosphate buffer, pH 7.4 with no added NaCl.

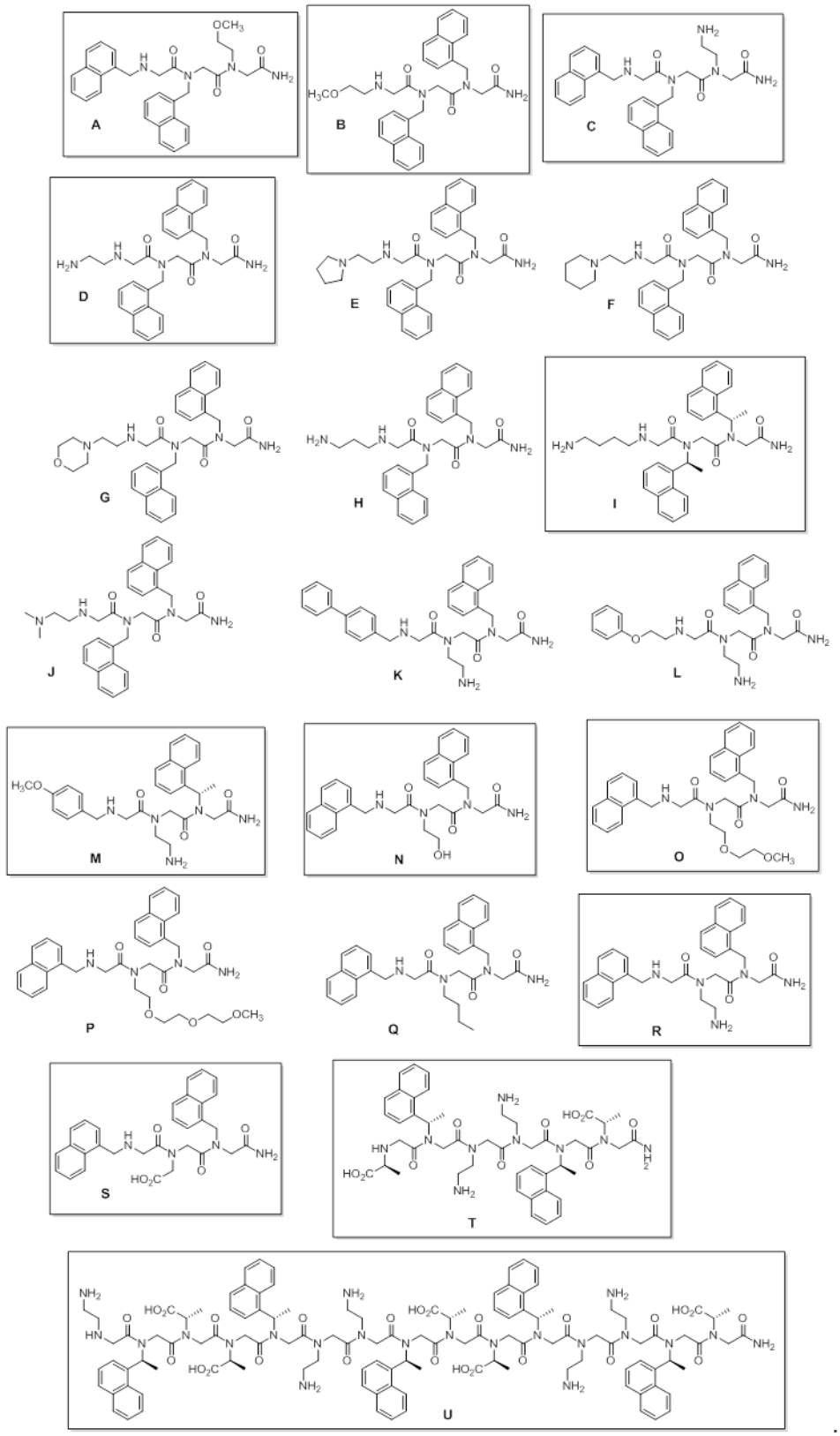

Figure 2. Structures of all peptoids prepared in this work. Boxed structures were studied by fluorescence spectroscopy as detailed in the text.

RESULTS AND DISCUSSION

Peptoid Design

An array of tripeptoids were designed for preparation by undergraduate students enrolled in an introductory organic chemistry laboratory course (Figure 2). Tripeptoids were chosen because they could be prepared in a reasonable timeframe in instructional laboratories (Table 1). We speculated that small peptoids would likely be more water soluble, enabling their later study by optical methods without observing scattering effects.[50,51] Additionally, in considering peptoids as potential drugs, aqueous solubility was important for delivery of hydrophobic molecules to target receptors within the body.[1] Lastly, tripeptoids enabled us three positions to vary functionality to begin to survey which residues contribute to peptoid-lipid interactions. Each peptoid comprised at least one naphthalene-functionalized side chain, which was essential to give each peptoid a fluorescent chromophore. Other appended functionalities were varied to include other aromatic groups (peptoids **K**, **L**, **M**) that might contribute to the optical properties of the molecule and/or moieties that modulated peptoid solubility in water and net charge: neutral polar groups (peptoids **A**, **B**, **N**, **O**, **P**), charged polar groups (peptoids **C** – **M**, **R**, **S**).

Two sequences prepared included (*S*)-1-naphthylethyl side chains in place of the more common 1-naphthylmethyl side chains (**I**, **M**). Admittedly, the substitution of the (*S*)-1-naphthylethyl side chains in **I** and **M** was accidental; students mis-read the bottle labels and used the "incorrect" amine (a reasonably frequent error of an inexperienced chemist). Nonetheless, this "mistake" provided an opportunity to diversify our sequences. Tripeptoid **I** in particular is a close analog of isomeric tripeptoids **C**, **D**, and **R**. Further, the process of working backwards to validate the unintended sequence and discover the origin of this substitution was an excellent learning experience for the students.

Two additional, longer peptoids were also prepared for study, **T** and **U** (Figure 2). Their inclusion in these studies enabled preliminary investigation of peptoid length contributions to

peptoid-lipid interactions. **T** and **U** are both water-soluble and have putative amphiphilic helix structures.

Tripeptoid Synthesis and Educational Considerations

Peptoids were prepared on solid support by the submonomer synthesis approach[20] (Figure 1A) in which each peptoid residue was installed in two synthetic steps. Fmoc-Rink amide resin was deprotected, then subjected to DIC-mediated acylation with bromoacetic acid. The bromine was subsequently displaced by reaction with an excess of a primary amine bearing the functionality corresponding to the *C*-terminal peptoid residue. Alternating treatment of the resin-bound intermediate with bromoacetic acid/DIC and primary amines was repeated two more times to install site-specifically each residue. Completion of reactions was monitored by subjecting a few beads to the chloranil test; blue-green beads indicated the presence of a secondary amine. The tripeptoid was liberated from the solid support with concomitant removal of side chain functionality protecting groups by treatment with strong acid.

In the laboratory course setting, each student was charged with preparing one unique peptoid, and 17 of the peptoids (**A** – **Q**, Figure 2) were initially prepared in this fashion. Data from liquid chromatography-mass spectrometry (LC/MS) experiments allowed students to estimate crude purities and verify if their syntheses were successful. Of the 17 tripeptoids prepared in the laboratory course, 13 were successfully prepared in > 85 % crude purity, two were prepared in lower crude purity, and two were not identified from the students' syntheses. The low-purity and unsuccessful sequences were subsequently re-made successfully by independent undergraduate research students along with peptoids **R** – **U**. Detailed student procedures and instructor materials used in this course are included in the Supporting Information on pages S4 through S18. The high level of success in tripeptoid preparation by undergraduate students highlighted the robustness of the solid-phase submonomer synthesis methods in the hands of novice experimentalists.

Students who were entirely unfamiliar with the equipment and techniques involved in solid phase synthesis were able to execute these experiments reliably.

Before their use in spectroscopic studies, peptoids were purified by reverse-phase high performance liquid chromatography (RP-HPLC), and their identities were confirmed by mass spectrometry. RP-HPLC purification was carried out outside of the laboratory course setting owing to the time-consuming nature of this more technically demanding procedure.

Fluorescence Spectroscopy of Peptoids

Selection of Peptoids for Study

From our library of 19 tripeptoids prepared, we selected ten for further study by fluorescence spectroscopy in the absence and presence of SUVs: **A**, **B**, **C**, **D**, **I**, **M**, **N**, **O**, **R**, and **S**. These were chosen to enable us to correlate tripeptoid structural features (*e.g.*, residue identity, conformational flexibility, and residue ordering) with spectroscopic observations. Peptoids included polar groups that were expected to influence peptoid net charge: **A**, **B**, **N**, and **O** all comprise a polar, uncharged side chain whereas **C**, **D**, **I**, **M**, and **R** include a basic side chain, and **S** contains an acidic side chain. Peptoids **C**, **D**, and **R** are all isomers; comparisons across this series enabled analysis of the effects of specific residue positioning on spectroscopic features. Peptoids **I** and **M** include (*S*)-1-naphthylethyl side chains; these are known to favor the *cis* amide conformation strongly.[52] We expected that **I** and **M** would allow us to investigate the influence of conformational flexibility on fluorescence spectral features and/or lipid interactions.

Fluorescence Emission of Tripeptoids in Buffers at Varied pH

From previous studies with **T** and **U**, we learned that fluorescence spectra of peptoids with multiple chromophores can change with peptoid conformation. We collected fluorescence emission spectra for ten representative tripeptoids to investigate structural changes that might emerge. A summary of fluorescence data is shown in Table 2. Spectra were acquired in two

different buffers--at pH 5.0 (Figure 3A, 3B) and at pH 7.4 (Figures 3C and 3D). These two pH conditions were chosen in anticipation of the peptoid-vesicle studies (vide infra). Human blood and other bodily fluids are commonly modeled with pH 7.4 PBS buffer, while low pH conditions were chosen to mimic the acidic gastrointestinal environment.

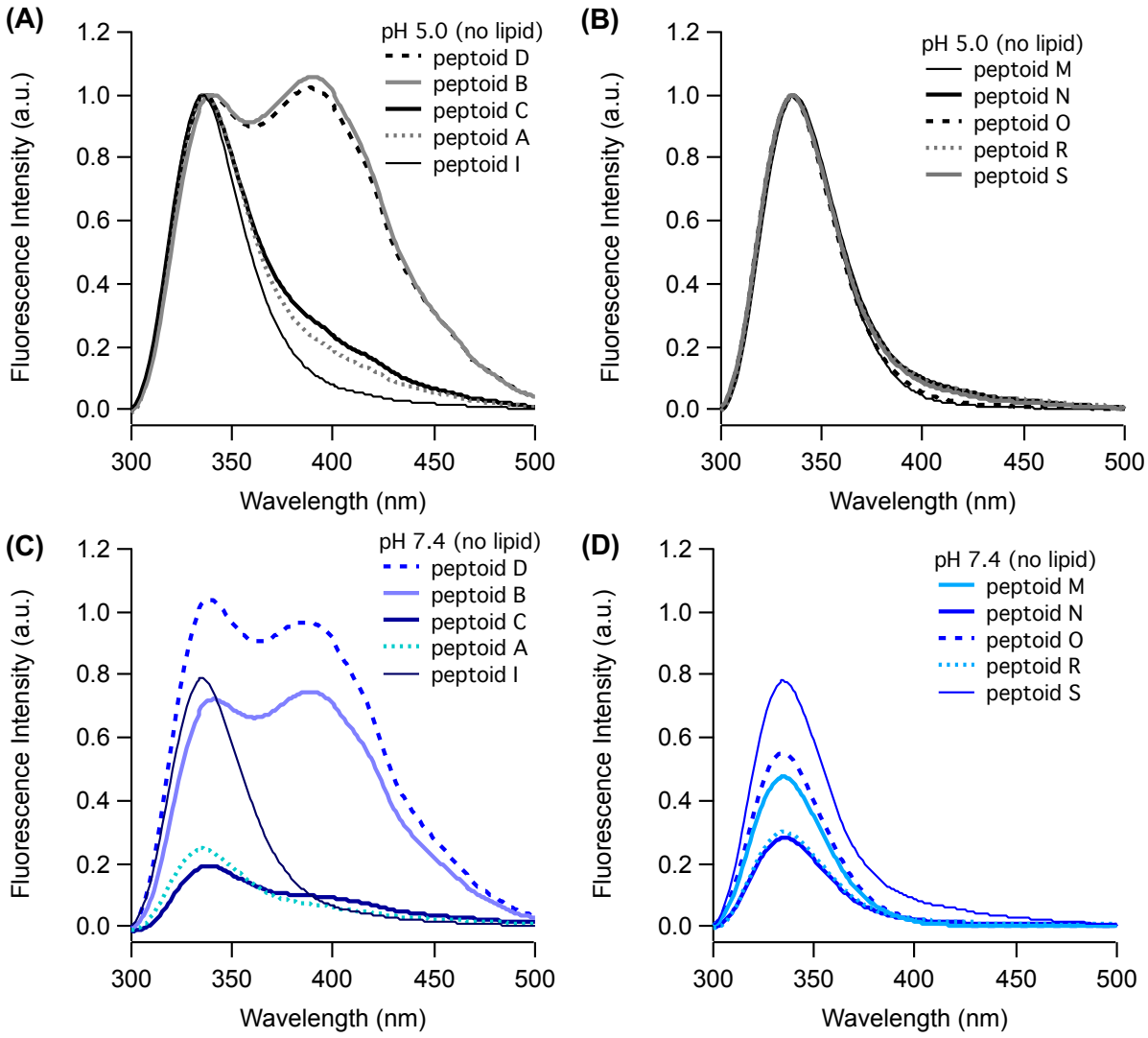

Figure 3. Fluorescence emission spectra of tripeptoids **A**, **B**, **C**, **D** and **I** (panels A and C) versus **M**, **N**, **O**, **R**, and **S** (panels B and D). Experiments were conducted at pH 5.0 (A) and (B) and at pH 7.4 (C) and (D). Dissolved peptoid concentrations were maintained at 100 μM. Data were normalized to spectra collected for each peptoid sequence at pH 5.0.

Table 2. Summary of fluorescence emission observations for tripeptoids

Tripeptoid	λ_{max}[a]	$I_{5.0}/I_{7.4}$[b] (no lipid)	$I_{5.0}/I_{7.4}$ (with lipid)	I_{lipid}/I_{buffer}[c] (pH 5.0)	I_{lipid}/I_{buffer} (pH 7.4)
A	335 nm	4.04	6.88	2.68	1.58
B	341 nm	1.38	1.12	1.39	1.72
	387 nm	1.42	0.98	0.71	1.02
C	338 nm	5.21	6.70	2.34	1.81
D	339 nm	0.96	0.90	1.71	1.84
	389 nm	1.07	0.82	0.45	0.98
I	335 nm	1.27	1.02	0.97	1.21
M	335 nm	2.10	2.00	1.26	1.32
N	335 nm	3.53	3.62	1.83	1.78
O	335 nm	1.81	1.57	1.39	1.61
R	335 nm	3.31	4.42	2.13	1.59
S	335 nm	1.28	1.17	1.25	1.36

[a] λ_{max} emission in pH 7.4 buffer in the absence of SUVs. [b] Ratio of fluorescence emission intensity at λ_{max} for solutions of peptoid at pH 5.0 and pH 7.4; [c] Ratio of fluorescence emission intensity at λ_{max} for solutions of peptoid in the presence and absence of 16.6-fold excess of SUVs. Data for excimer peaks are shaded in gray.

Emission Spectral Feature Comparisons. We noted that for all peptoids, the major spectral features observed were not changed as solution pH was varied, although intensities of peaks did change (*vide infra*). The majority of the tripeptoids' fluorescence emission spectra included a single peak with λ_{max} at 335 nm at pH 7.4 (**I**, **M**, **N**, **O**, **R**, and **S**). This is a typical spectral feature for emission of naphthalene-comprising molecules in water.[53,54] However, the spectra of **A** – **D** had notably different features. The emission spectra of **A** and **C** exhibited a shoulder around 390 nm. The λ_{max} observed for the spectrum of **C** was also slightly red-shifted to 338 nm (pH 7.4). Most strikingly, the emission spectra of **B** and **D** each had two peaks: for **B**, these were at 341 nm and 387 nm, and for **D**, these were at 339 nm and 389 nm. The broad, red-shifted peaks in the spectra of **B** and **D** are characteristic of naphthalene excimer emission wherein two naphthalene chromophores positioned close to one another (usually < 4 Å) form an excited state dimer (excimer).[55] For **B** and **D**, the intensity of this excimer peak relative to the monomer emission peak increased slightly with a decrease in pH. In contrast, the shoulder observed for peptoids **A** and **C** was slightly less intense at lower pH.

The excimer emission observed for **A** – **D** was correlated with specific structural features of these tripeptoids. In all of these, the two residues bearing 1-naphthylmethyl side chains were adjacent; in **A** and **C**, they were both on the *N*-terminus of the molecule, whereas in **B** and **D** they were both on the *C*-terminus. We speculated that the proximity of these two residues to one another enabled the tripeptoids to adopt a conformation in which the two naphthalenes are close enough in space to form an intramolecular excimer. Intramolecular excimers were observed in analogous water-soluble bichromorphoric molecules.[53,54] These results suggested that the conformation that enables this excimer is more favorable when the two naphthylmethyl groups are attached to the two *C*-terminal residues. When the two naphthylmethyl residues are in non-adjacent residues, the conformation that enables excimer emission was not favored (e.g., **N**, **O**, **R**, **S**). Given the relatively low concentrations at which these studies were carried out (100 μM), we expected that intermolecular association of chromophores was unlikely. To confirm this, we noted that the ratio of excimer to monomer emission intensities does not change for peptoid **D** at lower concentrations (Supporting Information Figure S1).

Interestingly, the emission spectrum of **I**, which is structurally quite similar to **D**, did not include a peak attributed to excimer emission. Although both **D** and **I** comprised two naphthalene-functionalized *C*-terminal residues, the (*S*)-1-naphthylethyl side chains in **I** conferred less conformational flexibility than the 1-naphthylmethyl side chains in **D**. We inferred that the enhanced conformational mobility of **D** was necessary for the molecule to adopt a conformation that enables intramolecular excimer formation. In analogous studies, rigid scaffolds that bear several naphthalene chromophores do not exhibit excimer fluorescence; indeed, the absence of the excimer peak has been used as an indicator of structural rigidity.[56,57]

From these comparisons, we recognized that the emission spectral features are most influenced by the relative positioning of the naphthalenes (*i.e.*, whether or not they are on adjacent residues) and the relative flexibility of the backbone. This was most clear in the comparison of the spectral features of isomeric peptoids **C**, **D**, and **R**. Changes to the emission spectra were

attributed to the relative positioning of the 1-naphthylmethyl-functionalized residues and the accessibility of conformations that enabled excimer formation. By contrast, the charge or other properties of the polar side chains did not have a significant impact on emission spectral features. For example, the spectral features of **A** and **C** are similar to one another, as discussed above, despite their different polar side chains (neutral vs. basic). Lastly, less conformationally flexible peptoids did not exhibit excimer emission, as discussed above.

Emission Intensity Comparisons. For nearly all tripeptoids, we observed that fluorescence emission intensities were higher at pH 5.0 than at pH 7.4 ($I_{5.0}/I_{7.4} > 1$, Table 2). Other researchers have shown that neutral amines quench naphthalene fluorescence either inter- or intramolecularly.[54] At lower pH, we likewise observed greater fluorescence efficiency because the peptoids were more positively charged. The proximity of the naphthalene to the ionizable secondary amine at the *N*-terminus of the tripeptoid also influenced the $I_{5.0}/I_{7.4}$. Peptoids **C**, **A**, **N** and **R** displayed the greatest $I_{5.0}/I_{7.4}$; these had naphthylmethyl side chains in the *N*-terminal position, closest to the secondary amine that could most efficiently quench fluorescence. In contrast, when the two naphthalenes are further from the *N*-terminus, spectral intensities are less sensitive to pH changes (e.g., **B**, **D** and **I**).

The intensities of excimer emissions had different sensitivities to pH. As pH was lowered, the intensities of the excimer shoulders decreased for both **A** and **C**, but increased slightly for the excimer peaks of **B** and **D**. We hypothesized that the conformation that enabled the excimer formation was more strongly favored at lower pH for **B** and **D**.

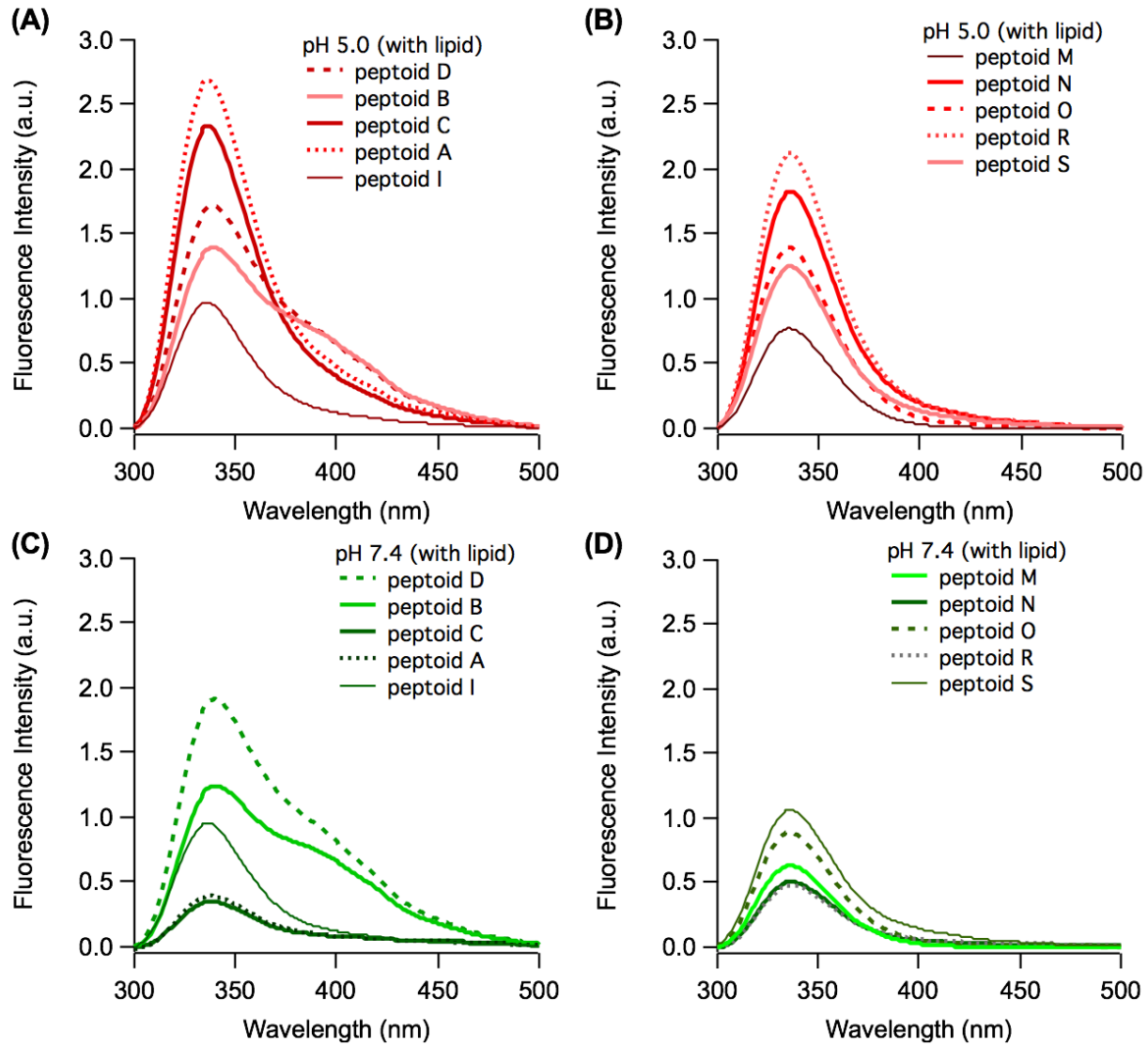

Figure 4. Fluorescence emission spectra of tripeptoids **A**, **B**, **C**, **D** and **I** (panels A and C) versus **M**, **N**, **O**, **R**, and **S** (panels B and D) in the presence of SUVs composed of DOPC. Experiments were conducted at pH 5.0 (A) and (B) and at pH 7.4 (C) and (D). Dissolved peptoid concentrations were maintained at 100 μM. A lipid-to-peptoid molar ratio of 16.6 : 1 was used for all peptoid sequences. Data were normalized to spectra collected for each peptoid sequence at pH 5.0 in the absence of SUVs.

Fluorescence Emission of Peptoids in the Presence of SUVs

To evaluate peptoid-lipid interactions, we chose to use SUVs as model PMs because of their optical compatibility and because they are straightforward to prepare by sonication.[58] SUVs are a common model PM to probe fluorophore-lipid interactions.[42,59–61] Because >50% of the

glycerophospholipids found in eukaryotic PMs are zwitterionic and in the fluid phase,[62] we chose to use SUVs composed of DOPC (structure shown in Figure 1). We acquired fluorescence spectra of ten representative tripeptoids in the presence of 16.6-fold excess SUVs, shown in Figure 4 (refer to Table 2 for summarized data).

Introduction of SUVs into solutions of aqueous peptoids resulted in highly complex mixtures for analysis; our fluorescence spectra convoluted lipid-associated peptoids and peptoids partitioned in the aqueous phase. Using only steady-state fluorescence emission spectroscopy, we were unable to quantify adsorbed chromophores, elucidate a binding mechanism, or determine the orientation or location of bound peptoids (*e.g.*, at the periphery, inside the SUV, or spanning the membrane). Despite these limitations, fluorescence emission spectra still provided qualitative insight into peptoid-lipid interactions and highlighted important considerations for evaluating spectral comparisons, as detailed below.

Peak Intensities Change with SUVs. In the presence of SUVs, fluorescence spectra of **A**, **C**, **I**, **M**, **N**, **O**, **R**, and **S** exhibited a single λ_{max} around 335 nm. For these tripeptoids, fluorescence emission intensities at the λ_{max} increased when SUVs were present ($I_{lipid}/I_{buffer} > 1$). These changes were most significant at pH 5.0 for **A**, **C**, and **R**, as reflected in the highest I_{lipid}/I_{buffer} values. This result was consistent with localization of the fluorescent tripeptoids in the more nonpolar environment of a lipid membrane. In analogous studies, increased fluorescence emission intensities and blue shifts in λ_{max} were previously observed for coumarin,[60] quinine[42] and tryptophan-containing peptides[43] in the presence of SUVs, and these spectral changes were associated with the molecules' binding to the lipids. To our surprise, the overall charge on the tripeptoid did not correlate with changes to I_{lipid}/I_{buffer}. For example, peptoids **A** and **C** had different net charges (+1 and +2, respectively) due to their different polar side chains, but exhibited similarly high I_{lipid}/I_{buffer} values.

The spectral changes with added lipid for **B** and **D** were more complex. **B** and **D** exhibited I_{lipid}/I_{buffer} = 1.39 and 1.71 for the lower wavelength peaks at 341 nm and 339 nm, respectively, which was consistent with their binding to SUVs. However, there was an accompanying change in the excimer peaks, which decreased in intensity upon addition of lipid at pH 5.0 (I_{lipid}/I_{buffer} < 1). For **B** and **D**, I_{lipid}/I_{buffer} = 0.71 and I_{lipid}/I_{buffer} = 0.45, respectively, for the excimer peaks. We hypothesized that **B** and **D** underwent a conformational rearrangement effected by the presence of lipids that disfavored excimer formation.[61,63] Similarly, we noted that the excimer shoulder observed for **A** and **C** disappeared from the spectra of these tripeptoids with lipids. Interestingly, the intensity of the fluorescence emission of the structurally similar but less conformationally mobile tripeptoid **I** exhibited no change (within error) when SUVs were added.

These results highlighted the importance of considering the complexity of the system when making spectral comparisons. It was clear that both conformational changes and varied binding interactions contributed to spectral changes, but overall tripeptoid charge did not correlate well with intensity changes. From an educational standpoint, we recognized that the complexity of the data offered an opportunity to challenge student researchers to consider a range of factors that contributed to our analysis. In doing so, they learned more about peptoid conformation and detailed effects of side chain identity on overall peptoid charge, for example.

Effects of pH with SUVs. Fluorescence emission intensities in the presence of lipids were generally higher at pH 5.0 than at pH 7.4 ($I_{5.0}/I_{7.4}$ > 1). Consistent with our observations in the absence of SUVs, we also observed the highest $I_{5.0}/I_{7.4}$ for **A**, **C**, **R** and **N** in the presence of SUVs. As such, we still attributed this trend to higher fluorescence quenching by neutral amines at higher pH. The lowest $I_{5.0}/I_{7.4}$ was observed for **D**, although structurally similar **B** and **I** also exhibited minimal changes in fluorescence intensities as a function of pH in the presence of SUVs. In the case of **D,** $I_{5.0}/I_{7.4}$ = 0.82 for the excimer emission peak and $I_{5.0}/I_{7.4}$ = 0.90 for the lower wavelength emission in the presence of SUVs. Lastly, although the origin of this effect is not certain, we also noted that the range of I_{lipid}/I_{buffer} values was narrower at pH 7.4 than at pH 5.0.

*Fluorescence spectra of longer peptoids **T** and **U***

To explore the effect of peptoid length on spectral changes in the presence of SUVs, the fluorescence emission spectra of 6- and 15-residue peptoids (**T** and **U**, respectively) were also monitored in the absence and presence of 100-fold excess SUVs composed of DOPC (Figure 5). In the absence of lipid, the emission spectrum of **T** included a single peak and was similar to that observed for the majority of the tripeptoids. The spectrum of **U** in the absence of lipids also included the excimer peak observed for tripeptoids **B** and **D**.

In the presence of SUVs, the fluorescence spectra of **T** and **U** resembled their fluorescence spectra in the organic solvent methanol.[39] At pH 7.4 in the presence of SUVs, the intensity of the single emission peak of **T** at 340 nm remained unchanged (within error). We expected that **T** would have a single major conformation owing to its two (*S*)-1-naphthylethyl side chains and two additional *N*-α-chiral substituents that make amide bond isomerization unfavorable.[52] Similarly, the fluorescence emission of the less conformationally flexible **I** showed little change in the presence of SUVs at pH 5.0 ($I_{lipid}/I_{buffer} \approx 1$). Again, the difference in net charge on the peptoids (+1 for **T**, +2 for **I**) did not correlate with spectral changes effected by lipid addition. This observation again highlighted that conformational flexibility was a likely contributor to spectral changes.

Of the peptoids studied here, **U** was the longest and most hydrophobic, and we expected that it would have greatest affinity for lipids. The spectrum of **U** changed dramatically upon addition of SUVs: the 340 nm emission peak increased 1.3-fold and the excimer peak disappeared. Because the excimer emission was previously correlated with self-association of **U**,[39] we theorized that the addition of SUVs to **U** decreased its self-association, either on its own or upon binding to model PMs. Longer peptoids with bulkier, more hydrophobic substituents were previously shown to penetrate bacterial membranes more readily[14] and adsorb to large unilamellar vesicles (LUVs) at higher surface concentrations compared to shorter sequences.[23]

Peptoid length appeared to significantly impact peptoid-lipid interactions, warranting further investigations in future studies.

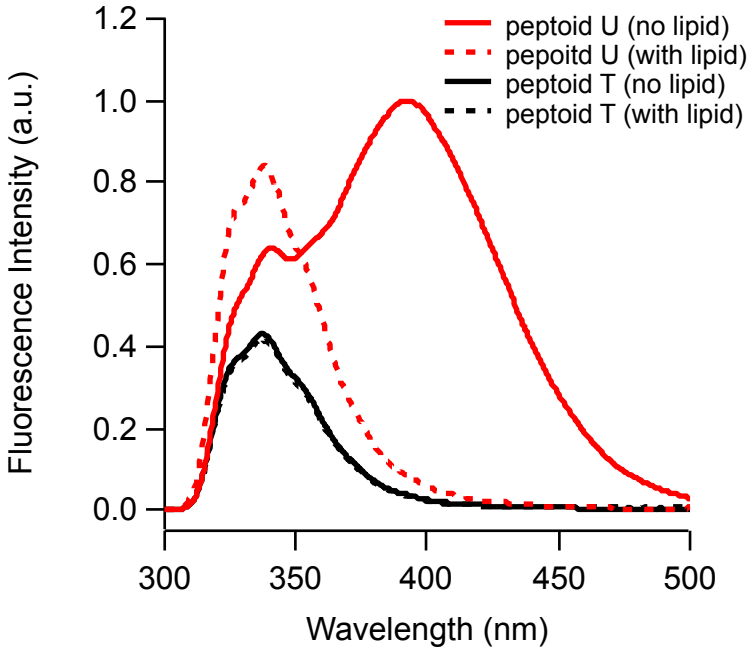

Figure 5. Fluorescence spectra of peptoids **T** (black) and **U** (red) in the absence (solid lines) and presence (dashed lines) of SUVs composed of DOPC. Experiments were conducted at pH 7.4. Dissolved peptoid concentrations were maintained at 40 μM and a lipid-to-peptoid molar ratio of 100 : 1 was used.

Table 3. Summary of fluorescence emission observations for longer peptoids

Peptoid	λ_{max} [a]	I_{lipid}/I_{buffer} [b] (pH 7.4)
T	338 nm	0.97
U	340 nm	1.30
	392 nm	0.04

[a] λ_{max} emission in pH 7.4 buffer in the absence of lipid vesicles.[b] Ratio of fluorescence emission intensity at λ_{max} for solutions of peptoid in the presence and absence of 100-fold excess of SUVs. Data for excimer peak are shaded in gray.

CONCLUSIONS

The comparative spectral data discussed here constitute an initial evaluation of peptoid sequence features that modulate their interactions with lipids. This is a key first step in our long-

term aim of developing predictive insights about peptoid-lipid affinities; such insights will facilitate the design and implementation of peptoid therapeutics or applications of peptoids as biosensors or antibacterial coatings where they might encounter cell membranes. In particular, we highlight our initial and surprising observation that overall peptoid charge may not dominate these interactions. Further, we note that considering inter- and intramolecular fluorophore interactions were important in the analysis of spectra collected both in the presence and absence of model PMs. In the absence of lipids, peptoid sequence influences spectral features, most substantially by conferring access to a conformation or aggregate that enables excimer fluorescence. In the presence of lipids, we observe changes in fluorescence intensities, suggesting that all ten tripeptoids interact with SUVs. However, extracting trends was complicated by spectral changes attributed to conformation and/or aggregation state changes upon interaction with SUVs.

The initial set of results motivates additional studies that will provide a more detailed picture of peptoid-lipid interactions than the one provided by steady-state fluorescence spectroscopy. For example, we intend to evaluate peptoid interactions with immobilized model PMs using second harmonic generation (SHG) spectroscopy. Use of SHG spectroscopy will allow us to detect low (and biologically relevant) concentrations of peptoids adsorbed to immobilized lipids and distinguish between membrane-bound and aqueous-phase peptoids without a separation step or an extrinsic tag; we will quantify the thermodynamic forces which drive peptoid-lipid interactions. In addition, we will monitor peptoid interactions with other lipid components and compositions that more closely represent of the diverse membranes found in cellular systems.

Through this work, we also demonstrate that undergraduate researchers can be important contributors to advancing understanding in peptoid science. Even without much experience as experimentalists, undergraduates can reliably execute approachable techniques, including solid-phase synthesis and fluorescence spectroscopy. We prioritize developing exercises or creating venues that cultivate students' understanding of the broader context for their individual contributions to the project when engaging students in research activities. In our experience,

students are motivated by working to achieve a “big picture” goal that addresses an important challenge that they recognize (here, the need for new pharmaceuticals). We accomplish this in the laboratory course through student presentations on peptoid articles from the literature. In the faculty-mentored research laboratory, we address this goal in group meeting discussions of ongoing research and current literature and by preparing undergraduate students to attend and make presentations at research events, including scientific conferences.

We advocate for the incorporation of peptoid synthesis experiments into instructional laboratories for investigators who need access to large numbers of diversely-functionalized peptoids. Enrolled students are an under-utilized “workforce,” and the synergistic research and educational benefits from engaging students in research modules are compelling. Students trained in these research-based courses are also excellent candidates to continue in faculty-led research, including at the graduate level.

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