A Fluorescent Peptoid pH-Sensor

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ABSTRACT:

Peptoids, N-substituted glycine oligomers, can adopt stable three-dimensional structures and have found diverse application as peptide surrogates and as nanomaterials. In this report, we have expanded peptoid function to include pH sensing by coupling pH-induced peptoid conformational changes with fluorescence intensity changes. We report two new peptoids (2 and 3) that comprise carboxylic-acid functionalized side chains and undergo conformational rearrangement in response to pH. Peptoids 2 and 3 are also labeled at one side-chain with an environmentally sensitive fluorophore, 4-\(N,N\)-dimethylamino-1,8-naphthalimide (4DMN). The fluorescence intensity of 2 varies 24-fold over the pH range studied. These spectroscopic properties make 2 a sensitive, biocompatible pH sensor.
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INTRODUCTION:

Foldamers, organic molecules with stable, well-defined three-dimensional conformations,\textsuperscript{1-4} are attractive scaffolds for the development of biocompatible sensors. There are a number of reports of foldamers that undergo conformational changes in response to environmental stimuli, including pH,\textsuperscript{5-7} solvent composition,\textsuperscript{8-11} light,\textsuperscript{12} and ion binding.\textsuperscript{13-16} However, only a few of these couple such changes to a sensitive, fast, and inexpensive readout like fluorescence to develop robust sensors.\textsuperscript{6,10,11,13-15} Our studies have focused on the development of a fluorescent pH sensor based on a peptoid (N-substituted glycine) foldamer scaffold (Figure 1).\textsuperscript{17} The facile synthesis of diverse, sequence-specific peptoids\textsuperscript{18} has enabled their applications as putative therapeutic and diagnostic agents,\textsuperscript{19-25} molecular transporters,\textsuperscript{26} nanomaterials,\textsuperscript{27,28} antifreeze agents,\textsuperscript{29} and catalysts,\textsuperscript{30} to name a few. They are particularly attractive for use in biological contexts owing to their resistance to proteolytic degradation\textsuperscript{31} and their ability to cross cell membranes.\textsuperscript{32} The development of peptoid sensors has potential to expand the applications of this important class of molecule in diagnostics, imaging, and materials research.

We hypothesized that a solvatochromic fluorophore could be a useful probe of changes to peptoid conformation in response to a stimulus, enabling the development of a sensor. A peptoid with a well-studied pH-induced conformational rearrangement, 1 (Figure 1), was reported,\textsuperscript{5} but a strategy was needed to adapt it into an efficient pH sensor. The fluorescence emission intensity of 4-N,N-dimethylamino-1,8-naphthalimide (4DMN) is highly sensitive to the polarity of its local environment.\textsuperscript{33-35} Fluorescence emission is more intense when the dye is in a less polar environment. Recent work from our laboratory has established that this dye can be readily introduced into peptoids as a side chain, and it is a useful probe of peptoid structural features
within an amphiphilic helical structure.\textsuperscript{35} Importantly for sensor development, the fluorescence properties of 4DMN are unchanged over a wide pH range.\textsuperscript{36} These features make 4DMN an excellent candidate tool to couple fluorescence to pH-induced peptoid conformational changes.

In this report, we detail the synthesis and spectroscopic studies of fluorescent peptoid pH sensors that correlate dramatic changes in fluorescence intensity with pH-induced changes to peptoid conformation.

RESULTS AND DISCUSSION:

Design and synthesis of fluorescently-labelled peptoids

We identified peptoid 1, reported by the Kirshenbaum laboratory,\textsuperscript{5} as an excellent template for development of a fluorescent pH sensor because of its simple structure and dynamic CD spectral changes in response to pH. 1 comprises eight (S)-\(N\)-(1-carboxy-2-phenylethyl) glycine (Nscp) residues, and substantial changes in the circular dichroism (CD) spectral features of 1 were observed as the pH was changed. While no high-resolution structural data are available for 1, other peptoids with sterically bulky \(N\)-\(\alpha\)-chiral side chains adopt a compact secondary structure similar to the polyproline type I helix.\textsuperscript{37-42} At low pH, when side chains are neutral, 1 is hypothesized to adopt a similar structure. At high pH, a more extended secondary structure is expected as side chains are deprotonated. We hypothesized that peptoids 2 and 3, which include a fluorescent 4DMN moiety in place of one of the side chains of 1, could serve as fluorescent pH probes; pH-induced changes to the peptoid conformation were expected to modulate the local environment of the dye and therefore its fluorescence intensity.
We designed peptoids 2 and 3 to identify the dye placement that would maximize the changes in fluorescence in response to pH variation while minimally disrupting the secondary structure of the peptoid. Peptoid 2 replaces one Nscp residue with a 4DMN-modified residue (Ndmn) in position 4 of the 8-residue peptoid sequence. In previous studies, we have found that helical peptoids substituted with Ndmn in the middle of the sequence exhibit fluorescence emission that correlates well with the expected local polarity. However, the inclusion of the dye moderately disrupts the secondary structure. Because the N-terminus of peptoids often exhibits enhanced conformational freedom relative to other parts of the peptoids, installation of Ndmn at the N-terminal position (3) was anticipated to be minimally disruptive to peptoid structure, but the changes to the local environment of the fluorophore might be minimized.

Peptoids 1-3 were prepared on solid phase following the sub-monomer synthetic method (Scheme 1). Briefly, an amine functionalized resin was bromoacetylated, then the appropriate amine (either (L)-phenylalanine tert-butyl ester to generate the Nscp residue or amine 4 to generate the Ndmn residue) was added to displace the bromine. These two steps were repeated iteratively to build an eight-residue peptoid sequence-specifically. Finally, the N-terminus was acetylated. Peptoids were cleaved from the solid support by treatment with trifluoroacetic acid and purified by reverse-phase high-pressure liquid chromatography (HPLC). Peptoids were identified by mass spectrometry and purities were confirmed by analytical HPLC (see Supplementary Information).

**Fluorescence emission of 4DMN-functionalized peptoids at varied pH**

To evaluate the sensitivity of the fluorescent side chain to pH-induced changes in its local environment, we measured the fluorescence emission spectra of 10 µM aqueous solutions of
peptoids 2 and 3 at pH 2.2-11.9 (Figure 2). Both we and others have confirmed that when 4DMN is not conjugated to a peptoid, its fluorescence intensity is not influenced by pH (see Supplementary Information).\textsuperscript{36} Fluorescence changes that we observed, then, could be attributed to changes in the local environment of the dye. For both 2 and 3, we observed a marked decrease in fluorescence emission intensity and a red-shift in the emission $\lambda_{\text{max}}$ as the pH was increased. The intensity of the fluorescence emission of 2 at $\lambda_{\text{max}}$ is highest at pH 2.2 and lowest at pH 7. There is a 24-fold difference in fluorescence emission between these extremes, and the midpoint of this transition is at pH 4.5. As the pH was increased, the fluorescence emission $\lambda_{\text{max}}$ was red-shifted by about 7 nm for 2, with a midpoint of this transition at pH 5.4. The magnitude of the changes in fluorescence intensity and $\lambda_{\text{max}}$ over this range was much less dramatic for 3; there was only a 6.5-fold difference in fluorescence emission intensity between pH 2.2 and pH 7, and changes to $\lambda_{\text{max}}$ of 3 were minimal in the pH range measured. When the 4DMN fluorophore is not tethered to the peptoid, its fluorescence intensity is 41 to 141-fold less than the fluorescence intensity observed at $\lambda_{\text{max}}$ for 2 and 3 (see Supplementary Information). We did observe a small fluorescence intensity emission enhancement when the 4DMN fluorophore is equilibrated with 1 at pH 2.2. This enhancement is not observed at pH 8.0. These data suggest there may be some intermolecular dye-peptoid interactions when the peptoid is uncharged, but any effect on the fluorescence emission data of peptoids 2 and 3 should be minimal.

For both peptoids 2 and 3, the high fluorescence intensity at pH 2.2 relative to pH > 6 is consistent with placement of the dye in a less polar local environment at lower pH. The local environment of the dye can be modulated by two factors: the charge state of proximal residues, and the exposure of the fluorophore to the aqueous solvent as a result of the peptoid conformation. At pH $\leq 4$, the dye should be proximal to neutral, protonated $N$scp residues. At
low pH, the conformational ensemble of the peptoid is anticipated to include a compact secondary structure which may shield the fluorophore from the aqueous buffer. At higher pH, the lower fluorescence intensities and red-shifted $\lambda_{\text{max}}$ values observed are consistent with situation of the fluorophore in a more polar local environment. It is flanked by negatively charged Nscp residues. Additionally, charge-charge repulsion of the deprotonated Nscp side chains is expected to favor a more extended peptoid secondary structure that may allow greater exposure of the 4DMN moiety to the polar aqueous solvent. The midpoint of the transition for fluorescence intensity and for $\lambda_{\text{max}}$ is close to that observed for the CD spectral transition of 1 reported by the Kirshenbaum laboratory.\textsuperscript{5} These transitions are at a pH quite close to the pK\textsubscript{a} of the carboxylic acid functional group; this observation further supports the role of charge-charge repulsion in the conformational changes of the peptoids.

The range of fluorescence intensities and $\lambda_{\text{max}}$ values observed at different pH was greater for 2 relative to 3, indicating that the placement of the fluorophore in the middle of the peptoid sequence improves sensitivity of this probe. The difference in fluorescence emission intensities between 2 and 3 is most dramatic at pH < 4 when the peptoid charge is neutral; in this pH range, the fluorescence emission of 2 is at least 2.5-fold more intense than that of 3. At pH $\geq$ 6, when the Nscp residues will be deprotonated, fluorescence emission of 2 is 10-45% less than the fluorescence emission of 3. These results suggest that 4DMN is more exposed to the polar aqueous solvent when it is at the N-terminal position, and when situated in the middle of the peptoid, the charge of the flanking residues more greatly influences the local environment of the fluorophore. These observations are consistent with structural studies of helical peptoids that have shown more conformational freedom at the peptoid N-terminus than in the middle of the sequence.\textsuperscript{42}
To confirm that observed changes in fluorescence could be attributed to changes to peptoid secondary structure rather than potential peptoid association, we carried out experiments to evaluate intermolecular peptoid-peptoid interactions at pH 2.2 and pH 8.0 (see Supplementary Information for data plots). We reasoned that addition of non-fluorescent 1 to a fixed concentration of 2 would influence the fluorescence signal if peptoid intermolecular association was occurring. At pH 2.2, the addition of low concentrations of 1 has minimal effects on the fluorescence emission of 2. However, when 1 is at concentrations ≥ 50 μM, the fluorescence emission of 2 is moderately enhanced. These fluorescence data suggest that while peptoids may associate at higher concentrations, any such interactions at the concentrations used for analysis (10 μM) are minimal or do not affect the fluorescence signal. At pH 8, there is minimal change to the fluorescence of 2 upon addition of 1. It has been very challenging to identify peptoids that self-associate at the low concentration (10 μM) used in our fluorescence data analysis.10,11,43

**CD spectroscopy of 4DMN-functionalized peptoids**

To gauge the effects of introduction of the Ndmn residue on peptoid secondary structure, we acquired CD spectra for peptoids 1-3 at pH 2.1 and pH 8.0 (Figure 3). These data indicate that substitution of one Nscp residue in 1 with Ndmn minimally alters peptoid conformation. The spectral features of all three peptoids are essentially identical at pH 2.1. Peptoids’ spectra exhibit a global minimum at 195 nm and a local minimum at 210 nm. At low pH, inclusion of the Ndmn residue minimally impacts peptoid structure. At pH 8.0, the spectral features of all peptoids are dramatically changed from the spectra at low pH, indicative of a conformational rearrangement of the peptoids. Spectra of all peptoids at pH 8.0 include a broad negative peak with a minimum around 217 nm, and there is a significant decrease in the intensity of the minima relative to the
low pH spectra. At basic pH, the spectrum of 2 is slightly different than the spectra of 1 and 3; the spectrum of 2 includes an additional minimum at 197 nm, and the peak at 217 nm is less intense than the peaks in the spectra of 1 and 3. At pH 8.0, 2 likely has a slightly different conformational ensemble than 1 and 3.

**CONCLUSIONS:**

The fluorescent peptoids reported here correlate changes in their fluorescence intensity with pH-induced conformational changes to the peptoid. In particular, 2 is an attractive target for further development as a biocompatible pH sensor. It exhibits greatly different fluorescence intensities across the pH range studied. A protein functionalized with 4DMN has been used as an indicator of cellular pH. The favorable biostability and cell permeability properties of peptoids and the sensitivity and accessibility of fluorescence make 2 an especially promising candidate for analogous cellular imaging applications, and work in our laboratory is currently pursuing these aims.

The inclusion of environmentally sensitive dyes into peptoids also represents an important strategy to interrogate and clarify peptoid structure. Fluorescence spectroscopy data from Ndmn-functionalized peptoids is an inexpensive and fast technique that provides information about the polarity of residues surrounding the Ndmn side chain within the three-dimensional structure of the peptoid. Importantly, this information is complementary to that obtained by CD spectroscopy, a technique widely used to evaluate peptoid structure. Given that structural studies of peptoids at high resolution have been challenging, new low-resolution tools such as this one are valuable additions to deepen our understanding of sequence-structure relationships in this important class of foldamer.
EXPERIMENTAL PROCEDURES:

Materials

Reagents for the synthesis of 4 and for peptoid synthesis were purchased from commercial suppliers and used without further purification. Diisopropylcarbodiimide (DIC) was purchased from Anaspec (Fremont, CA). Fmoc-protected Rink amide resin (0.74 mmol/g) was purchased from Nova Biochem (San Diego, CA). All other reagents were purchased from ACROS organics (Morris Plains, NJ) in the highest purity available. Solvents were purchased from Fisher Scientific. Amine 4 was prepared according to previously published procedures. Phenylalanine-tert-butyl ester hydrochloride (used to generate the Nscp monomer) was purchased from AAPPTec (Louisville, KY), and neutralized before use. The ester was dissolved in a minimal volume of water, and 2.5 M NaOH solution was added to adjust the pH of the solution to 10. The ester was then extracted with CH$_2$Cl$_2$.

Buffer Preparation

Buffers were prepared by mixing varied volumes of 5 mM citric acid and 5 mM dibasic sodium phosphate solutions to achieve the desired pH.

Peptoid Synthesis and Purification

All peptoids were synthesized on solid phase at room temperature using a procedure adapted from the submonomer method of peptoid synthesis. Into a 25 mL glass vessel fitted with a coarse glass frit and a teflon stopcock, Rink amide resin (0.05 mmol) was added and rinsed twice with CH$_2$Cl$_2$. The resin was then allowed to soak in approximately 1.5 mL N,N'-dimethylformamide (DMF) for 30 minutes twice. The DMF was drained, and the resin was then
treated with 2.5 mL of a 20% 4-methylpiperidine solution in DMF and agitated on a wrist-action shaker twice for 10 min. at room temperature to remove the Fmoc protecting group. The resin was rinsed 7 times with approximately 2 mL DMF each.

Peptoid synthesis was carried out by alternating bromoacetylation and amination reactions eight times. The acetaldehyde/chloranil test was performed after each reaction to check for completion. Bromoacetylation was effected by adding 830 µL of a 1.2 M solution of bromoacetic acid in DMF (19.9 equiv.) and 200 µL DIC (25.5 equiv.) to the resin. The mixture was agitated on a shaker for 20 minutes, drained and washed 7 times with DMF. For the installation of the Nscp residue, phenylalanine-tert-butyl ester was added (1 mL of a 1 M solution in DMF, 20 equiv) to the reaction vessel and the mixture was agitated by shaking for 40 minutes. The solution was drained, rinsed with DMF 7 times, and then rinsed 3 times with CH₂Cl₂. For installation of the Ndmn residue, an equimolar amount of DIEA was added to the 1 M solution of 4 in DMF, and the solution was shaken with the resin 12 h.

Following the final amination reaction, peptoids were acetylated at the N-terminus by treatment with acetic anhydride (1.5 mL of a 1 M solution in DMF) and 50 µL N,N-diisopropylethylamine. This mixture was agitated on the shaker for 20 minutes, then drained and washed with DMF and CH₂Cl₂ (three times each). Peptoids were cleaved from the resin by equilibration with TFA/H₂O/triisopropylsilane (95:2.5:2.5) for 2 hours. The filtrate was collected and the TFA mixture was removed by evaporation.

Crude peptoids were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a Varian ProStar instrument equipped with a semi-preparative AAPPTec Spirit C18 column (5 µM, 10.0 mm x 25 cm) using a linear gradient of 30%-90% methanol (solvent B) in 0.1% aqueous TFA (solvent A) at a flow rate of 3 mL/min. Purified peptoids were lyophilized.
to afford white (1) or yellow (2 and 3) powders. The molar masses of purified peptoids were confirmed by electrospray mass spectrometry in negative ion mode using a Thermo LCQ Fleet mass spectrometer (see Table S1 in the supporting information).

Concentrated stock solutions of peptoids were made by dissolving the lyophilized peptoid in 5 mM citrate/phosphate buffer, pH 5 (3) or 0.1% TFA in acetonitrile (1 and 2). The concentrations of solutions of 2 and 3 were determined by measurement of the UV spectrum on a Shimadzu UV-3101PC scanning spectrophotometer and calculating concentration using the extinction coefficient reported for the fluorophore ($\varepsilon = 8800 \text{ M}^{-1} \text{ cm}^{-1}$ at 440 nm). Stock solutions of 1 were prepared by weighing 1-2 mg of 1 to the nearest hundredth of a millgram using a Mettler Toledo XS105 analytical balance and dissolving in an exact volume of solvent as calculated by mass.

**Fluorescence Spectroscopy**

Fluorescence spectroscopy was performed on a Horiba Fluorolog 3 spectrofluorometer equipped with FluorEssence software. All samples were recorded for 10 µM peptoid solutions, and emission spectra were collected from 440-670 nm in 0.5 nm increments. The excitation wavelength was set to 408 nm, and excitation and emission slit widths were set to 3 nm and 6 nm, respectively.

**Circular Dichroism Spectroscopy**

CD spectra were acquired using an Olis RSM Rapid Scanning Monochromator. The spectra were measured at 25 °C from 260-190 nm in 0.5 nm increments, and data were averaged for 3 seconds at each wavelength. Data were originally collected in millidegrees and then converted to per-
residue molar ellipticity (deg cm$^2$/dmol). For all spectra, approximately 40 $\mu$M peptoid solutions in 5 mM citric acid/5 mM sodium phosphate buffer with 40% CH$_3$CN (by volume) were measured in a circular quartz cell with a path length of 1 mm. A spectrum of the buffers was subtracted from peptoid solution spectra.

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SUPPORTING INFORMATION:

Mass spectrometry and HPLC characterization data for peptoids, supplementary fluorescence data as mentioned in the article text.
REFERENCES:

SCHEMES AND FIGURES:

Figure 1. Structures of peptoids prepared.

Scheme 1 Solid-phase synthesis of peptoids via the sub-monomer method.
Figure 2. Fluorescence emission of peptoids 2 and 3 show variation over the pH range studied. A) Fluorescence emission spectra of peptoid 2 at varied pH; B) Fluorescence emission spectra of peptoid 3 at varied pH; C) Change in intensity of fluorescence emission at $\lambda_{\text{max}}$ as a function of pH for peptoids 2 and 3; D) Change in $\lambda_{\text{max}}$ emission as a function of pH for peptoids 2 and 3. All fluorescence spectra were acquired at room temperature for samples at 10 $\mu$M in 5 mM sodium phosphate and 5 mM citric acid buffer, $\lambda_{\text{excitation}} = 408$ nm. *For panels A and B, emission traces at pH 3.0, 6.1, 8.0 are omitted for clarity, but data from these spectra are included in panels C and D.
Figure 3. Circular dichroism spectra of peptoids 1-3 at pH 2.1 and pH 8.0 indicate that the inclusion of the Ndnn residue has minimal effects on peptoid structure. Spectra were acquired at 25 °C for peptoids at approximately 40 μM in 5 mM sodium phosphate and 5 mM citric acid buffer with 40% CH₃CN.