Clickity-click: highly functionalized peptoid oligomers generated by sequential conjugation reactions on solid-phase support†

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N-Substituted glycine peptoid oligomers were used as substrates for azide-alkyne [3 + 2] cycloaddition conjugation reactions and then elaborated through additional rounds of oligomerization and cycloaddition. This novel sequential conjugation technique allowed for the generation of complex peptidomimetic products in which multiple heterogeneous pendant groups were site-specifically positioned along the oligomer scaffold. Studies of a water-soluble estradiol–ferrocene peptoid conjugate demonstrated a potential application for the modular synthesis of biosensors.

Introduction

Techniques in bioconjugate chemistry have provided effective tools for endowing biomolecules with novel properties. Conjugation reactions are routinely employed to modify proteins and nucleic acids so that they incorporate fluorophores, ligands, chelates, radioisotopes, affinity tags, and numerous other groups.[1] When performed on solid-phase support, these reactions can be used to modify synthetic oligonucleotides and polypeptides with exceptional efficiency (reviewed in ref. 2). In many cases, solid-phase conjugation reactions can be adapted for automated protocols, allowing the development of novel combinatorial libraries and microarray applications.

Polypeptides, while capable of exhibiting an extraordinary range of bioactivities, often display poor pharmacological properties. For this reason, synthetic mimics of peptides have been the focus of vigorous development by medicinal and bioorganic chemists. A variety of oligomeric peptidomimetics have been introduced that begin to exhibit some of the structural and functional attributes of natural polypeptide species.[3] Further elaboration of peptidomimetic structures by means of conjugation reactions may lead to a greater range of capabilities for this promising class of molecules.

Our research focuses on developing techniques for enhancing the structural complexity of a family of peptidomimetics known as peptoids. These oligomers are comprised of *N*-substituted glycine monomer units and can exhibit a strong propensity to form stable secondary structures.[4] Peptoids are efficiently synthesized on solid-phase support to incorporate a specific sequence of chemically diverse monomer units.[5] Recently, molecular design approaches have been successful in generating peptoids that exhibit an array of biological activities.[6] These products may prove to be well-suited for biomedical applications due to their resistance to proteolytic degradation.[7] Our goal is to enhance the structural and functional capabilities of peptoids by developing new strategies for their chemical conjugation and ligation.[8]

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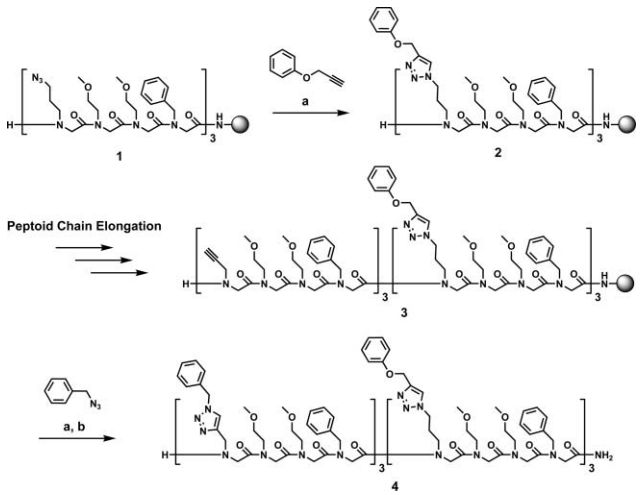
Cu(I) catalyzed azide-alkyne [3 + 2] cycloaddition reactions are gaining prominence as a versatile technique for conjugating reactants *via* 1,2,3-triazole formation.[9] These 'click chemistry' reactions have been shown to be regiospecific and compatible with a wide range of substrates and reaction conditions. For example, azide-alkyne [3 + 2] cycloadditions have been employed to link polypeptide chains, synthesize dendrimers and conjugate derivatives to the exterior of viral particles.[10–12] In a previous study, we demonstrated the advantages of using a click chemistry approach for multi-site conjugation of alkyne- or azide-containing groups onto peptoid scaffolds.[13] Bioactive ligands typically contain chemical functionalities that are incompatible with many solid-phase synthesis procedures. Owing to the broad orthogonality of azide-alkyne [3 + 2] cycloaddition reactions, we were able to conjugate diverse ligands containing biologically relevant chemical functionalities onto peptoid scaffolds with high efficiency. We seek to elaborate this procedure in order to sequentially conjugate multiple groups onto oligomer scaffolds through site-specific azide-alkyne [3 + 2] cycloaddition methods. In order to generate peptoid substrates that allow for the consecutive addition of heterogeneous pendant groups, we required a procedure for modifying reactive sidechain moieties and subsequently extending the oligomer scaffold. Herein, we report a technique to enhance the functionality and chemical diversity of peptoid oligomers through the convenient use of a sequential series of click chemistry and scaffold extension reactions. The capability for performing sequential cycles of conjugation will allow for the synthesis of complex modular structures in which specific functionalities are displayed in a site-directed fashion.

Results and discussion

Our efforts were initiated with the solid-phase synthesis of a linear peptoid dodecamer scaffold including three azidopropyl sidechains site-specifically positioned in the oligomer sequence (Scheme 1, compound **1**). Peptoid scaffolds were synthesized with high efficiency on Rink Amide resin using standard 'submonomer' synthesis protocols.[14] Azide-functionalized sidechains were conveniently integrated as *N*-substituents in the peptoid sequence using 3-azido-1-aminopropane[15] as a submonomer reagent. Following the synthesis and characterization of peptoid **1**, we were able to

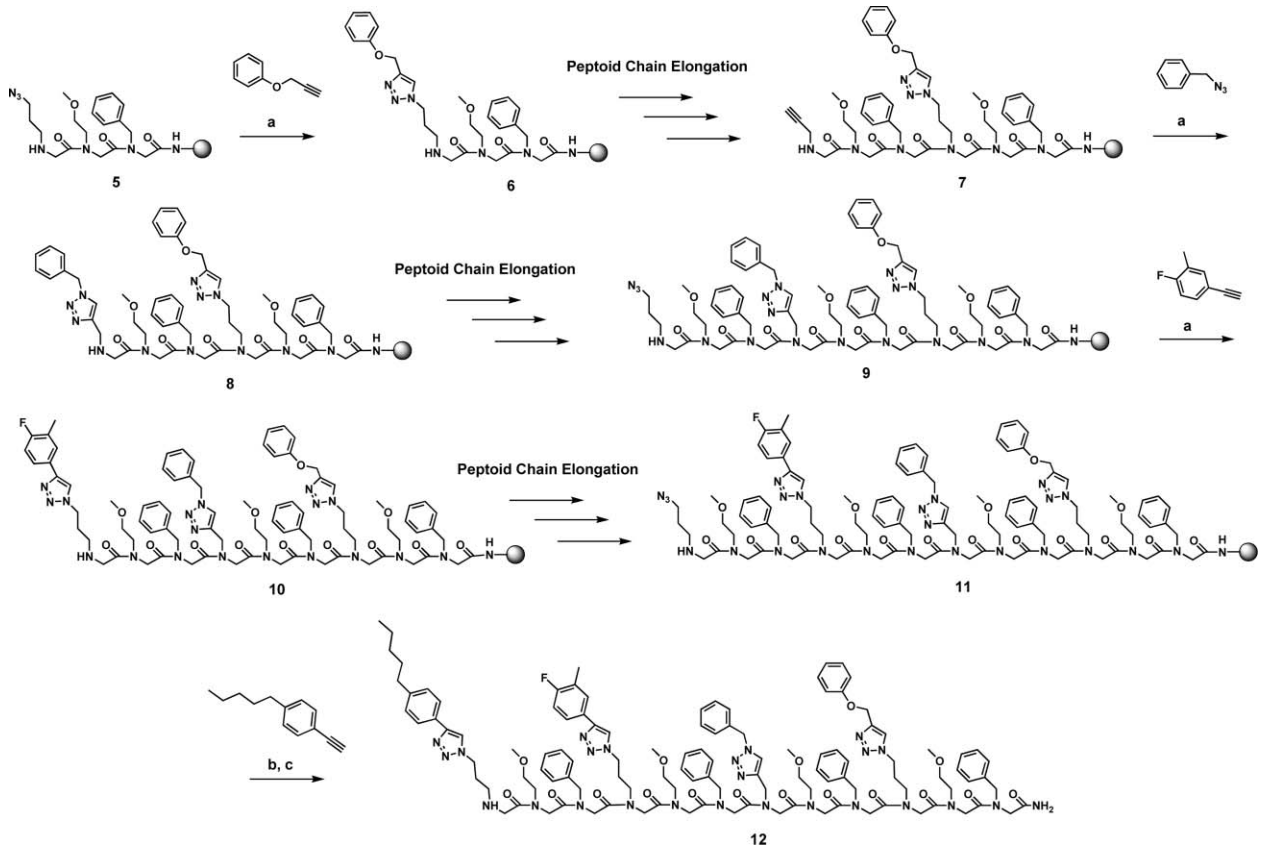


Scheme 1 Multi-site modification of peptoid sidechains by a sequential series of cycloaddition and oligomerization reactions. *Reagents and conditions*: (a) coupling partner (0.06 M), CuI (0.11 M), ascorbic acid (0.06 M) and DIPEA (0.14 M) in DMF–pyridine (7 : 3 v/v), rt, 18 h; (b) 95% TFA in H_2O, rt, 10 min.

conjugate phenyl propargyl ether to the three azide groups on the dodecamer scaffold. Trivalent conjugation was achieved by reacting **1** with phenyl propargyl ether in the presence of CuI, ascorbic acid and *N*,*N*′-diisopropylethylamine (DIPEA) in DMF–pyridine (7 : 3 v/v) at room temperature for 18 h. This reaction resulted in the formation of a 1,2,3-triazole linkage between the peptoid scaffold and the sidechain conjugates (Scheme 1, compound **2**).

We then investigated whether triazole linkages generated upon click chemistry cycloadditions are compatible with peptoid chain extension (Scheme 1, compound **3**). Following the synthesis of **2**, the click chemistry reagents were washed from the solid phase and 12 complete peptoid monomer addition cycles were executed. In order to allow for azide coupling, three propargyl sidechains were incorporated into the 24-mer peptoid scaffold **3**. Benzyl azide was conjugated to the three alkyne groups in a second round of click chemistry modification to generate **4**. All products shown in Scheme 1 were characterized and confirmed after each elongation and cycloaddition cycle using Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) and Liquid Chromatography/Mass Spectrometry sequencing techniques (LC/MS^2) (see also ESI†: Table **S-1**, entries 1–4; and Fig. **S-1** and **S-2**). Both azide and alkyne groups within the oligomer sequence were modified with equal efficiency (>95% conversion).

Using this approach, we were able to successfully generate multi-functionalized peptoid dodecamers that had been extended and modified through four sequential cycles of click chemistry (Scheme 2). Peptoid trimers containing terminal azide functionalities (Scheme 2, compound **5**) were synthesized on solid-phase support. Compound **6** was generated by reacting **5** with phenyl propargyl ether in the presence of CuI, ascorbic acid and DIPEA in 2-butanol–DMF–pyridine (5 : 3 : 2 v/v/v) at ambient temperature



Scheme 2 Sequential click chemistry performed on solid-phase support. All coupling partners were present at 0.06 M. *Reagents and conditions*: (a) CuI (0.11 M), ascorbic acid (0.06 M) and DIPEA (0.14 M) in 2-butanol–DMF–pyridine (5 : 3 : 2 v/v/v), rt, 18 h; (b) CuI (0.11 M), ascorbic acid (0.06 M) and DIPEA (0.14 M) in DMF–pyridine (7 : 3 v/v), rt, 18 h; (c) 95% TFA in H_2O, rt, 10 min.

for 18 h. The click chemistry reagents were washed from **6** and three complete rounds of peptoid monomer addition were conducted, generating peptoid hexamer **7**. Benzyl azide was allowed to react with **7** in a second cycle of click chemistry to afford peptoid **8**. The technique of sequential elongation and cycloaddition was repeated until we had synthesized peptoid dodecamers that were site-specifically modified with four distinct sidechain conjugates. Standard submonomer extension of peptoids **8** and **10** afforded peptoids **9** and **11**, respectively. Peptoids **10** and **12** were synthesized through the conjugation of 4-ethynyl-1-fluoro-2-methylbenzene and 1-ethynyl-4-pentylbenzene onto peptoids **9** and **11**, respectively. Fig. 1 shows overlaid analytical RP-HPLC spectra of the crude intermediates **6**, **8**, **10** and the product **12** following cleavage from solid-phase support. All products shown in Scheme 2 were characterized and confirmed after each elongation and cycloaddition cycle using RP-HPLC and LC/MS[2] sequencing (ESI†: Table **S-1**, entries 5–12; and Fig. **S-3**). All azide- and alkyne-containing coupling partners in Scheme 2 were conjugated with their respective alkyne and azide reactive sites on the oligomer scaffold with high efficiency (>95% conversion). The overall crude purity of final product **12** was found to be >75% as evaluated by RP-HPLC (Fig. 1, trace **12**).

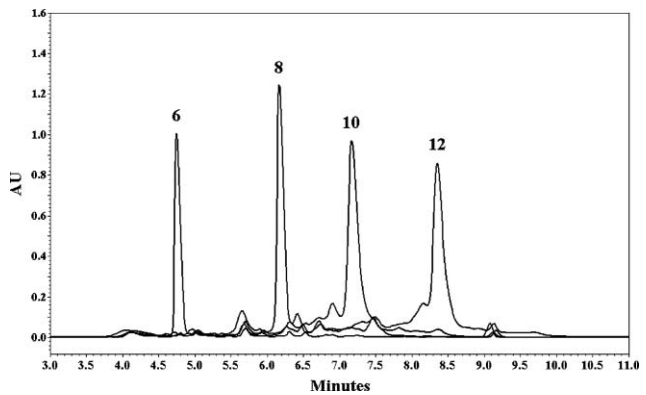


Fig. 1 Representative analytical RP-HPLC traces showing **6**, **8**, **10** and **12** following cleavage from solid support. Chromatographic analysis was performed on each of the crude products and are shown as overlaid traces.

We studied the feasibility of using the sequential click chemistry method to integrate multiple constituents suitable for the development of peptoids as biosensor platforms. This involved selection of a sensor moiety and a bioactive ligand as groups for conjugation. The reversible redox properties of ferrocene/ferrocenium have previously been exploited for biosensor applications.[16–18] Additionally, estradiol is a typical representative of a class of clincally important hormone ligands, leading to the study of estradiol conjugates for biomedical applications.[19] Reports have shown that stable organometallic hormone pharmacophores can be generated using a 17α-(ferrocenylethynyl)estradiol complex.[20] Utilizing the sequential click chemistry method, peptoid **13** (Fig. 2) was generated as a prototype sensor platform in which ethynylferrocene and 17α-ethynylestradiol were site-specifically positioned along the oligomer scaffold. Methoxyethyl groups were incorporated as the predominant sidechain in **13** in order to increase overall molecular hydrophilicity and impart water solubility to a compound incorporating two hydrophobic moieties.

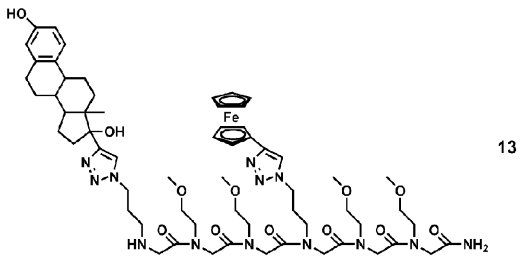


Fig. 2 Water-soluble bi-functionalized peptoid hexamer generated for biosensor applications.

To test the effect of triazole conjugation on ferrocene redox properties, compound **13** was purified to >96% as determined by RP-HPLC (Fig. **S-1**) and the electrochemical behavior of ethynylferrocene and **13** were compared using cyclic voltammetry (CV). Additionally, we evaluated the influence of the estradiol group on the redox potential of the neighboring ferrocene moiety by comparing the electrochemical characteristics of **13** with its azido-functionalized precursor **14** (ESI†: Fig. **S-4**). CV experiments were carried out at room temperature using previously described methods.[16] CV was performed on solutions of ethynylferrocene (0.5 mM), **13** (0.5 mM) or **14** (0.5 mM) prepared in water with NaCl (50 mM) as a supporting electrolyte, using a Ag/AgCl (KCl 3 M) reference electrode, a freshly polished glassy carbon working electrode, and a Pt wire counter electrode with a scan rate of 9.0 mV s^{-1}. Cyclic voltammograms of ethynylferrocene and **13** showed reversible redox couples of ferrocene/ferrocenium, as shown in Fig. 3. The values of the formal redox potential (E°) and the half-peak potential ($E_{p/2}$) of ethynylferrocene, **13** and **14** are shown in Table 1. As expected, the ferrocene core of **13** showed a significant decrease in redox potential when compared to ethynylferrocene. This is attributed to the altered electronic environment established by the extended conjugation of the ferrocene cyclopentadiene group with the 1,2,3-triazole ring.[21] Interestingly, E° and $E_{p/2}$ values for **13** and **14** were very similar, indicating that the redox potential of the ferrocene group is not affected by conjugation of a proximal bulky substituent. Because we seek to retain a similar relative signal intensity between sensor molecules that contain a variety of bioactive ligands, it is advantageous that the electrochemical properties of the conjugated ferrocene are not substantially diminished by the neighboring estradiol. Future studies will investigate the

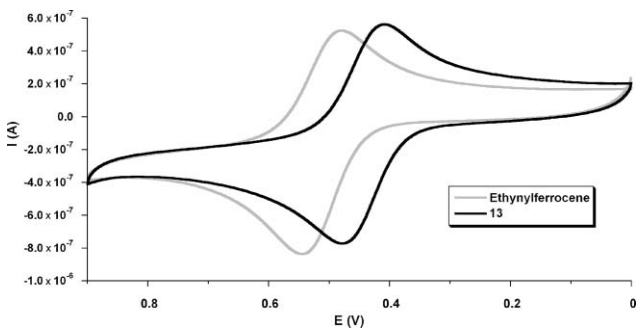


Fig. 3 Cyclic voltammetry curves of ethynylferrocene (0.5 mM) and **13** (0.5 mM) in water with NaCl (50 mM) as supporting electrolyte, a glassy carbon working electrode, Ag/AgCl reference electrode and a Pt wire counter electrode with a scan rate at 9.0 mV s^{-1}.

Table 1 Electrochemical data by cyclic voltammetry[a]

Entry	E°/V	$E_{p/2}$/V
Ethynylferrocene	0.512	0.525
13	0.442	0.459
14	0.442	0.456

[a] Cyclic voltammetry experiments were conducted on ethynylferrocene (0.5 mM), **13** (0.5 mM) or **14** (0.5 mM) in water with NaCl (50 mM) as supporting electrolyte, a glassy carbon working electrode, Ag/AgCl reference electrode and a Pt wire counter electrode with a scan rate at 9.0 mV s^{-1}.

development of electrochemical-based biosensors in which site-specifically positioned sensor groups are used to report a change in redox potential upon protein binding by suitably modified peptoid oligomers.[22]

Conclusion

A series of highly functionalized peptoid oligomers was generated utilizing a novel sequential click chemistry protocol. We have demonstrated the ability to precisely and efficiently position a variety of functional groups along a peptidomimetic scaffold. The sequential click chemistry method introduced here demonstrates that 1,2,3-triazole linkages are compatible with multiple rounds of peptoid chain elongation on solid-phase support. These techniques may prove suitable for similar sequential bioconjugation of polypeptides immobilized on a solid phase and may be amenable to automation. We have utilized this approach to begin development of peptoids functionalized with groups appropriate for biomedical applications, including moieties suitable for elaboration as constituents of biosensors and molecular imaging agents.

Experimental

General

Peptoid oligomers were characterized by analytical Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) using a C4 column (Peeke Scientific, Ultra-120, 5 μm, 120 Å, 2.0 × 50 mm) on a Beckman Coulter System Gold HPLC system. Products were detected by UV absorbance at 214 nm with a System Gold 166 detector. Data were analyzed with Beckman Coulter 32 Karat software version 5.0. Unless otherwise noted, linear gradients were conducted from 5 to 95% solvent B (0.1% TFA in HPLC-grade acetonitrile) over solvent A (0.1% TFA in HPLC-grade water) in 10 min with a flow rate of 0.7 mL min^{-1}. Subsequent chain elongation and average coupling yields were estimated by RP-HPLC using methods described previously by Jang *et al*.[13]

Additional characterization of peptoid oligomers was conducted using Liquid Chromatography/Mass Spectrometry (LC/MS). All peptoids described herein (mass range: 419–3783 Da) were analyzed using an Agilent 1100 Series LC/MSD Trap XCT equipped with an electrospray ion source. All LC/MS experiments were preformed in positive ion mode. Unless otherwise stated, all analyses were performed on peptoids cleaved from resin without further purification.

Peptoid synthesis

Synthesis of peptoid oligomers was conducted on Rink Amide resin (Novabiochem, San Diego, CA), using a modification of the standard submonomer synthesis procedures described by Zuckermann *et al*.[5] Peptoids were synthesized by manual techniques as well as automated procedures on a robotic workstation (Charybdis Instruments) with software program files written in-house. All reactions were conducted on solid-phase support at room temperature.

Typically, 100 mg of Rink Amide resin at a loading level of 0.55 mmol g^{-1} was swollen in 3 mL of dichloromethane (DCM) for 45 min before Fmoc deprotection. Multiple washing steps using *N*,*N*′-dimethylformamide (DMF) (4 × 2 mL) and DCM (3 × 2 mL) were performed between each synthetic procedure described below. All reactant equivalents are based on the resin loading level for a given amount of resin. The resin was Fmoc-deprotected by treatment with 20% piperidine in DMF (15 mL g^{-1} resin, 20 min). Deprotection reagents were washed from the resin and approximately 20 equiv. bromoacetic acid (1.2 M in DMF, 8.5 mL g^{-1} resin) and 24 equiv. diisopropylcarbodiimide (2 mL g^{-1} resin) were added. The bromoacetylation reaction mixture was agitated at room temperature for 20 min. Following washing, 20 equiv. monomer amine (1.0 M in DMF, 10 mL g^{-1} resin) were added and the reaction was agitated for 20 min. Bromoacetylations and monomer amine displacements were repeated until peptoid oligomers of the desired length were achieved.

Peptoid products were cleaved from the solid support by treatment with 95% trifluoroacetic acid (TFA) in water (40 mL g^{-1} resin) for 10 min. The TFA cleavage cocktail was evaporated under nitrogen. For characterization by RP-HPLC and LC/MS, peptoids were resuspended in 1 mL HPLC solvent (50% acetonitrile in water).

Polyfunctionalized peptoid 24-mer (4)

Linear peptoid dodecamers were synthesized with high efficiency using techniques described above. Compound **1** was allowed to react with 21 equiv. phenyl propargyl ether (0.06 M), 40 equiv. CuI (0.11 M), 20 equiv. ascorbic acid (0.06 M) and 50 equiv. DIPEA (0.14 M) in 20 mL DMF–pyridine 7 : 3 v/v (0.2 mL mg^{-1} resin) in a 20 mL scintillation vial (Wheaton Scientific, Millville, NJ), generating **2**. In order to completely dissolve the solid reactants, the vial was placed in a bath sonicator (VWR Aquasonic 75HT) and sonicated for 5–10 min. The vial was purged with gaseous nitrogen, tightly capped, sealed with Parafilm and shaken at room temperature for 18 h. Following completion of the reaction, the resin was transferred to a 10 mL fritted syringe (Torviq) and washed with DMF (7 × 3 mL), a Cu scavenger cocktail (DMF–pyridine 6 : 5 v/v, ascorbic acid 0.02 g mL^{-1}) (7 × 3 mL) and DCM (7 × 3 mL). The resin was then dried under nitrogen gas flow and approximately 3 mg was removed for characterization. Peptoid **2** was then elongated to a 24-mer (Scheme 1, compound **3**) through 12 rounds of monomer amine addition as described above. Compound **3** was added to 21 equiv. benzyl azide (0.06 M), 40 equiv. CuI (0.11 M), 20 equiv. ascorbic acid (0.06 M) and 50 equiv. DIPEA (0.14 M) in 20 mL DMF–pyridine 7 : 3 v/v (0.2 mL mg^{-1} resin) in a 20 mL scintillation vial. The vial was purged with gaseous nitrogen, tightly capped, sealed with Parafilm and was allowed to stir at room

temperature for 18 h, affording peptoid **4**. Following washing steps, the generation of **4** was confirmed using RP-HPLC (ESI†: Fig. **S-1**) and LC/MS (ESI: Table **S-1**, entry 4). The overall purity of **4** was found to be >35% as calculated by analytical RP-HPLC. Sequencing of peptoid **4** (ESI: Fig. **S-2**) was conducted using a MS/MS fragmentation technique as described in the ESI† on an Agilent 1100 Series LC/MSD Trap XCT equipped with an electrospray ion source. LC/MS^2 experiments were performed in positive ion mode.

Synthesis of multi-functionalized peptoid dodecamer (12)

Approximately 100 mg of peptoid **5**-bound Rink Amide resin was swollen in DCM for 45 min. The DCM was removed and the swollen resin was transferred to a 20 mL scintillation vial. Depending on the hydrophobicity of the respective coupling partner, the resin was suspended in either 20 mL 2-butanol–DMF–pyridine 5 : 3 : 2 v/v/v (0.2 mL mg^{-1} resin) or 20 mL DMF–pyridine 7 : 3 v/v (0.2 mL mg^{-1} resin). To compound **5** was added 21 equiv. phenyl propargyl ether (0.06 M) along with 40 equiv. CuI (0.11 M), 20 equiv. ascorbic acid (0.06 M) and 50 equiv. DIPEA (0.14 M) to generate peptoid **6**. In order to completely dissolve the solid reactants, the vial was placed in a bath sonicator (VWR Aquasonic 75HT) and sonicated for 5–10 min. The vial was purged with nitrogen, tightly capped, sealed with Parafilm and vigorously shaken at room temperature for 18 h. Following completion of the reaction, the resin was transferred to a 10 mL fritted syringe (Torviq) and washed with DMF (7 × 3 mL), a Cu scavenger cocktail (DMF–pyridine 6 : 5 v/v, ascorbic acid 0.02 g mL^{-1}) (7 × 3 mL), and DCM (7 × 3 mL). The resin was then dried under nitrogen gas flow and approximately 3 mg was removed for characterization.

Resin-bound peptoid trimer **6** was elongated to a hexamer using techniques as described (Scheme 2, compound **7**). Benzyl azide (0.06 M) was then coupled onto the terminal alkyne functionality of **7** using methods outlined above, generating compound **8**. The click chemistry reagents were washed from the resin and a small amount of compound (3 mg) was removed for characterization. This sequential click chemistry reaction method was repeated until four complete rounds of elongation and cycloaddition had been achieved (Scheme 2, compound **12**). Compound **12** was confirmed using RP-HPLC (Fig. 1) and MS/MS sequencing techniques described in the ESI† (Fig. **S-3**).

Synthesis of peptoid–ferrocene conjugate (13)

Approximately 100 mg of Rink Amide resin was swollen in DCM for 45 min. The DCM was removed and the resin was Fmoc-deprotected by treating it with 20% piperidine in DMF (15 mL g^{-1} resin, 20 min). Deprotection reagents were washed from the resin and peptoid trimers were generated that contained two methoxyethylamine monomers and a terminal azidopropyl moiety. The resin was transferred to a 20 mL scintillation vial and ethynylferrocene (21 equiv., 0.06 M), 40 equiv. CuI (0.11 M), 20 equiv. ascorbic acid (0.06 M) and 50 equiv. DIPEA (0.14 M) were added to the resin-bound peptoid in 20 mL 2-butanol–DMF–pyridine 5 : 3 : 2 v/v/v (0.2 mL mg^{-1} resin). The vial was purged with nitrogen gas, sealed with Parafilm and shaken at room temperature for 18 h. The click chemistry reagents were washed from the resin and a small amount of the peptoid–ferrocene conjugate was removed for characterization. This monofunctionalized peptoid trimer was then elongated to a hexamer with two additional methoxyethylamine monomers and a terminal azidopropyl moiety (compound **14**, see ESI†: Fig. **S-4**). The resin was transferred to a 20 mL scintillation vial and 17α-ethynylestradiol (21 equiv., 0.06 M), 40 equiv. CuI (0.11 M), 20 equiv. ascorbic acid (0.06 M) and 50 equiv. DIPEA (0.14 M) were added to the resin-bound peptoid in 20 mL DMF–pyridine 7 : 3 v/v mL (0.2 mL mg^{-1} resin). The vial was purged with nitrogen, tightly capped, sealed wirh Parafilm and shaken at room temperature for 18 h. Following completion of the reaction, the resin was transferred to a 10 mL fritted syringe (Torviq) and washed with DMF (7 × 3 mL), Cu scavenger cocktail (DMF–pyridine 6 : 5 v/v, ascorbic acid 0.02 g mL^{-1}) (7 × 3 mL) and DCM (7 × 3 mL). The resin was then dried under nitrogen gas flow and approximately 3 mg was removed for characterization. Peptoid **13** was purified to >96% purity as calculated by RP-HPLC (ESI†: Fig. **S-1**). ESI Table **S-1**, entry 13, shows calculated and observed mass values of the purified peptoid **13**.

Electrochemical analyses

Ethynylferrocene (1.05 mg, 5.0 μmol) was dissolved in 10 mL HPLC-grade water. NaCl (29.22 mg, 0.5 mmol) was added to the solution as a supporting electrolyte. Similarly, solutions of 10 mL water, 6.3 mg (5.0 μmol) peptoid **13** or 4.8 mg (5.0 μmol) peptoid **14** and 29.22 mg (0.5 mmol) NaCl were prepared. The following techniques were performed identically on 0.5 mM ethynylferrocene (50 mM NaCl), 0.5 mM **13** (50 mM NaCl) or 0.5 mM **14** (50 mM NaCl). Approximately 4 mL of solution was transferred to an electrochemical cell and subjected to cyclic voltammetry (CV) experiments. Cyclic voltammetry was conducted using a CH Instruments 600A Electrochemical Analyzer and CV curves were generated using software developed by CH Instruments. Current (μA) *versus* potential (V) was measured across a freshly polished CHI104 3 mm diameter glassy carbon disk working electrode (CH Instruments) with a Ag/AgCl (3 M KCl) reference electrode (CH Instruments) and a Pt wire reference electrode (scan rate = 9.0 mV s^{-1}).

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