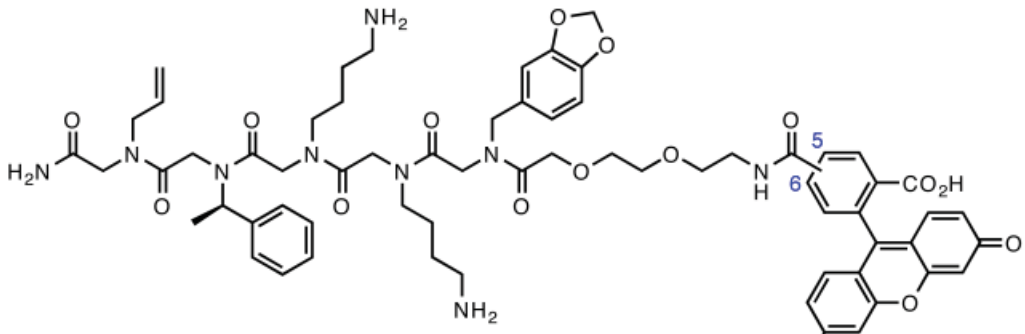
Appendix D: Peptoid Cell Uptake Studies

Abstract

Peptoids 5-FAM (**1**) and 6-FAM (**2**), previously reported by the Kodadek group, were synthesized and their cell uptake properties evaluated in HeLa cells using confocal laser scanning microscopy. A dual imaging laser system was used to image the peptoids and a DNA-binding nuclear stain simultaneously. The results demonstrate that the peptoids 5-FAM (**1**) and 6-FAM (**2**) are not cell permeable in HeLa cells.

D.1 Summary

Peptoids are a class of *N*-alkylated poly-glycine oligomers that have recently been exploited for the rapid synthesis of protein-targeted combinatorial libraries in an effort to discover new bio-active compounds with superior cell permeability and protease sensitivity as compared to traditional peptide scaffolds.[1-3] Recently, the Kodadek group has demonstrated that these compounds are often cell permeable and can be utilized as molecular recognition domains.[4] A peptoid was shown to be effective as a transcriptional activation domain for GST-KIX in a HeLa cell reporter gene assay.[5] The peptoid domain which bound a GST-KIX fusion protein with a K_d of 11.6 μM was discovered from a library of 50,000 members screened against the murine CREB core KIX domain. A carboxyfluoresceinated version of this peptoid (mixture of **1** and **2**, Figure D.1) was used for KIX binding studies in comparison to a scrambled peptoid sequence, which was shown not to bind the GST-KIX domain. In an effort to evaluate the potential use of this peptoid activation domain for use with different polyamide DNA binding scaffolds, the exact peptoid-fluorophore conjugates (**1** and **2**, Figure D.1) from the Kodadek study were synthesized independently (not as a mixture) as shown in Figure D.2. The cell uptake properties of these peptoid-FAM conjugates were assessed using confocal laser scanning microscopy and dual imaging studies with DNA binding dyes were performed to provide unambiguous location of the cell nuclei. These studies were performed in HeLa cells and show that the peptoid was completely excluded from the cell interior as shown in Figures D.3 and D.4.



1, 5-FAM
2, 6-FAM

Figure D.1 Compound **1**, 5-FAM, and **2**, 6-FAM.

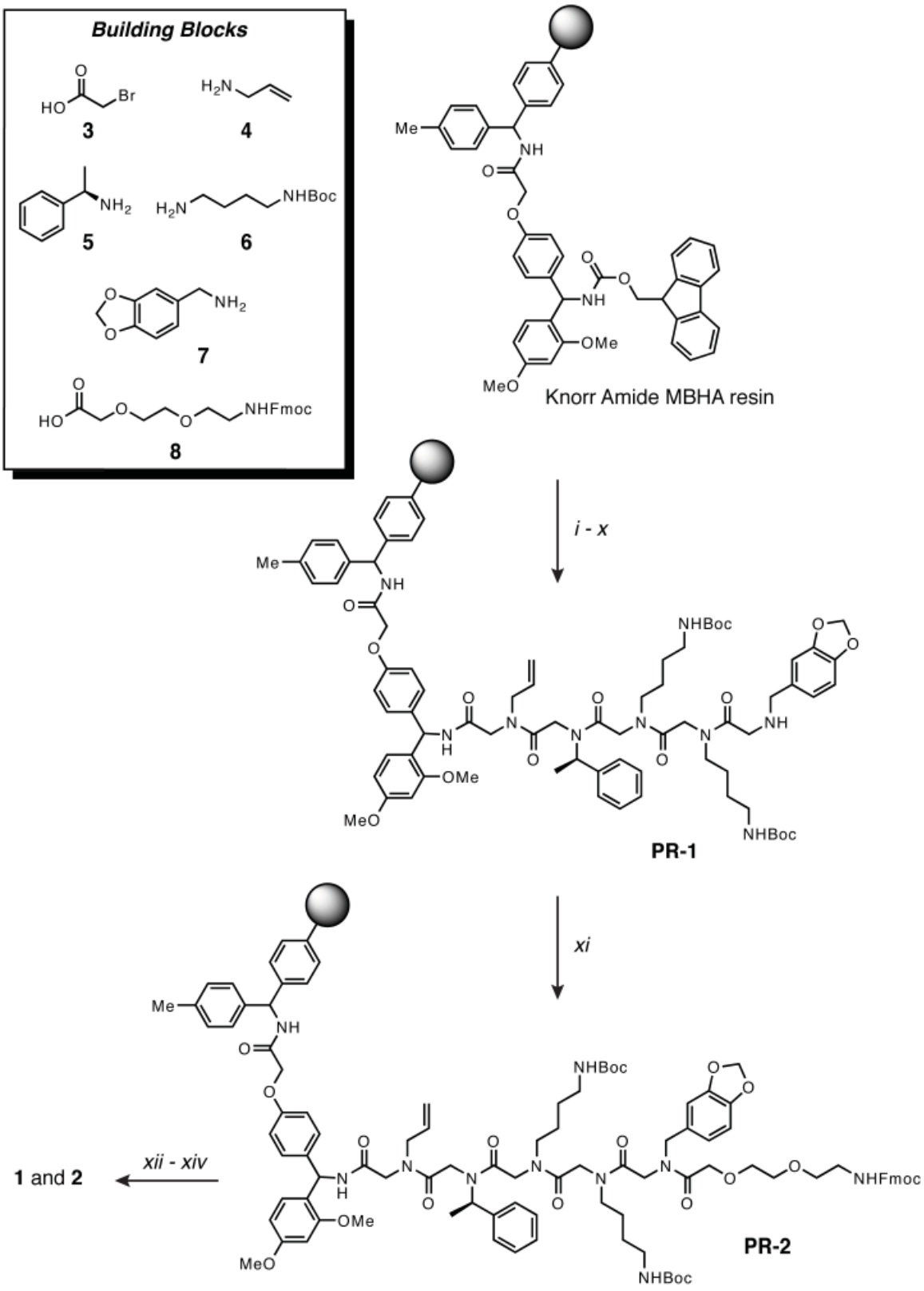


Figure D.2 Synthesis of compound **1** and **2**. For details, see Section D.2 Experimental.

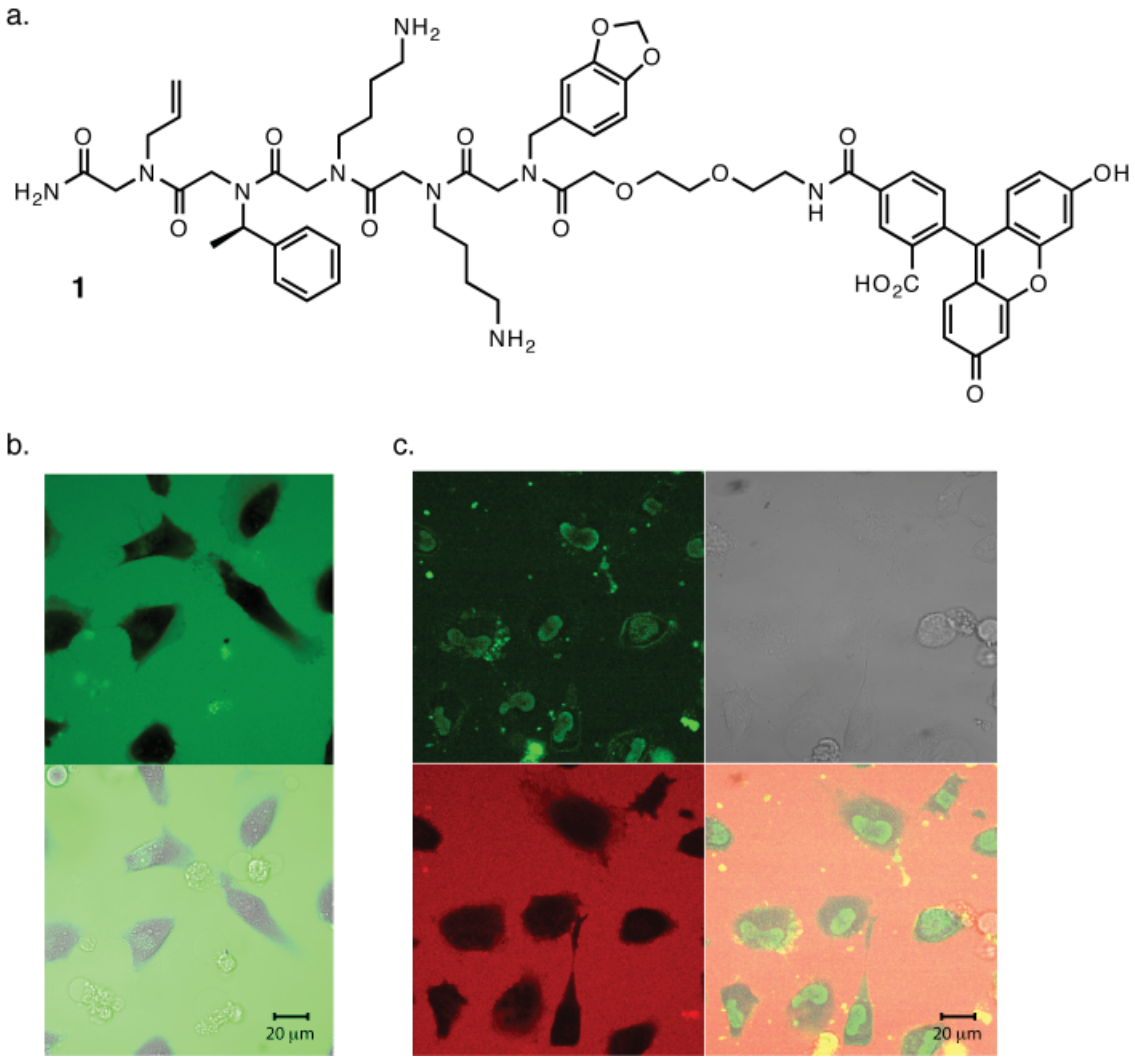


Figure D.3 HeLa cell uptake studies for compound **1** (5-FAM) using 2µM concentration. Dual image with Hoechst 33342. (2-Photon Laser l = 810 nm, 5% Power, BP480 - 520 nm filter.).

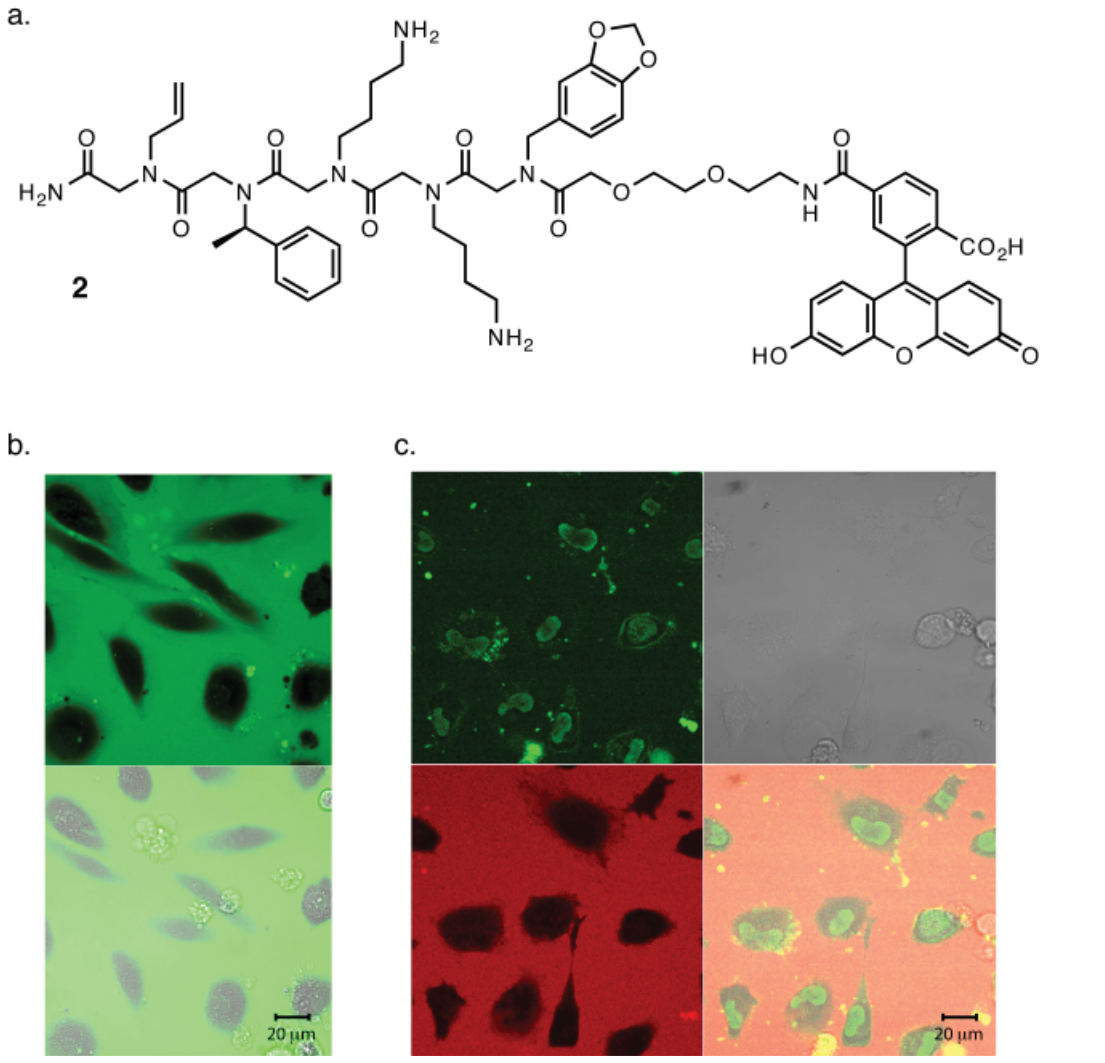


Figure D.4 HeLa cell uptake studies for compound **2** (6-FAM) using 2µM concentration. Dual image with Hoechst 33342. (2-Photon Laser l = 810 nm, 5% Power, BP480 - 520 nm filter.)

D.2 Experimental

D.2.1 Materials

D.2.1.1 Resin

Knorr Amide MBHA resin, Nova Biochem, 01-64-0459, A33927

Resin loading = 0.78 mmol/g

Amount = 200 mg

D.2.1.2 Stock solution preparation

Stock solution A

2 mL of piperidine in 8 mL of DMF.

Stock solution B

3.7 g of bromoacetic acid (**3**) in 14 mL of DMF.

Stock solution C

7 mL of *N*,*N*'-diisopropylcarbodiamide in 7 mL of DMF.

D.2.1.3 Amine solution preparation

Solution M1

300 μL of allylamine (**4**) in 1.7 mL DMF.

Solution M2

510 μL of (*R*)-(+)-α-methylbenzylamine (**5**) in 1.49 mL DMF.

Solution M3

1.5 g (1.53 mL) of *N*-Boc-1,4-butane diamine (**6**) in 2.47 mL DMF.

Solution M4

1.5 g (1.53 mL) of *N*-Boc-1,4-butane diamine (**6**) in 2.47 mL DMF.

Solution M5

500 μL of piperonylamine (**7**) in 1.5 mL DMF.

D.2.2 General bromoacetic acid addition procedure

Resin was treated with 2 mL of stock solution B followed by 2 mL of stock solution C. Next, the synthesis vessel was capped and microwaved for 15 sec on the low power setting followed by shaking and another 15 sec in the microwave. Next, the resin synthesis vessel was drained and washed with DMF (4 x 5 mL) followed by a final wash with 10 mL of anhydrous DMF.

D.2.3 General amine addition procedure

Resin was treated with the appropriate amine solution and agitated for 15 sec followed by 15 sec in the microwave on the low power setting. The reaction mixture was agitated for another 15 sec and microwaved again for 15 sec on the lower power setting followed by draining of the vessel and washed with DMF (4 x 5mL). Finally, a wash with 10 mL of anhydrous DMF was performed.

*D.2.4 Peptoid synthesis procedure (Synthesis of Resin **PR-1**)*

A solid phase synthesis vessel was charged with 200 mg of Knorr Amide MBHA resin and swelled

in DMF for 20 min with agitation. The resin was then washed 4 times with DMF, treated with 20% piperidine in DMF for 20 min (2 mL x 20 min each), washed with DMF (8 x 5 mL), and washed with 10 mL of anhydrous DMF.

1. General bromoacetic acid addition procedure.
2. General amine addition procedure using solution M1.
3. General bromoacetic acid addition procedure.
4. General amine addition procedure using solution M2.
5. General bromoacetic acid addition procedure.
6. General amine addition procedure using solution M3.
7. General bromoacetic acid addition procedure.
8. General amine addition procedure using solution M4.
9. General bromoacetic acid addition procedure.
10. General amine addition procedure using solution M5.

After step 10, the resin washed with DMF (5 x 5 mL), dichloromethane (10 x 5 mL), and then dried under high vacuum. This resin was called **PR-1**. Next, a very small aliquot of resin was cleaved using 95% TFA/H_2O for 1 h at room temperature. The solution was diluted with water, filtered, and checked by HPLC and ESI-MS. HPLC showed a single peak for product and the ESI-MS results are listed below.

Expected mass of product = 722.41

Found $[M+H]^+$ = 723.3, $[M+Na]^+$ = 745.4

*D.2.5 Procedure for first mini-PEG coupling (Synthesis of Resin **PR-2**)*

The resin, **PR-1**, from the peptoid synthesis procedure above was swelled in DMF for 30 min and drained. A separate vial was charged with 180 mg of Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-AEEA, **8**, CAS 166108-71-0, Peptides International), 244 mg of PyBOP, 92 μL of DIEA, and 1 mL of DMF. This reaction mixture was stirred at 23 C for 15 min and then added to the swelled resin **PR-1**. The reaction mixture was then put in a shaker at 37 C for 8 h. The resin washed with DMF (5 x 5 mL), dichloromethane (10 x 5 mL), and then dried under high vacuum to give resin **PR-2** (Yield = 351 mg of dry resin). Next, a very small aliquot of resin was cleaved using 95% TFA/H_2O for 1 h at room temperature. The solution was diluted with water, filtered, and checked by HPLC and ESI-MS. HPLC showed a single peak for product and the ESI-MS results are listed below.

Expected mass of product = 1089.55

Found $[M+H]^+$ = 1090.4, $[M+Na]^+$ = 1112.5

*D.2.6 Preparation of peptoid 5-FAM (**1**)*

The resin **PR-2** (20 mg) from the peptoid synthesis procedure above was swelled in DMF for 20 min and drained. Next, the Fmoc protecting group was removed by addition of 20% piperidine in DMF (2 x 4 mL for 10 min each). The resin was then washed with DMF (10 x 2 mL) then 5 mL of anhydrous DMF. Next, the deprotected resin was treated with a solution of 2 mg of 5-carboxyfluorescein succinimidyl ester (5-FAM-SE, Molecular Probes, C2210) in 300 μL of DMF. The reaction flask was covered with foil and agitated at 23 C for 1 h followed by 37 C for 1 hr. Next, the resin was drained, washed with DMF (10 x 1 mL), dichloromethane (10 x 1 mL), and dried under high vacuum. Next, the FAM labeled peptoid was cleaved from resin using 95% TFA/2.5% H_2O/2.5% triisopropylsilane for 1 h. The crude product was purified by preparative reverse phase HPLC eluting from 10% acetonitrile/90% (0.1% TFA-H_2O) to 50% acetonitrile/50% (0.1% TFA-H_2O) over 70 min. The pure product eluted at minute 47 to give 5-FAM (1.74 μmol). Extinction coefficient for 5-FAM is 75000 at pH = 9.0, λ_{max} = 498 nm.

1 (5-FAM): ESI-MS calculated for $[M+H]^+$: 1226.5, observed $[M+H]^+$: 1226.4

*D.2.7 Preparation of peptoid 6-FAM (**2**)*

Peptoid 6-FAM (**2**) was synthesized using the exact same procedure as for making 5-FAM except 6-carboxyfluorescein succinimidyl ester (6-FAM-SE, Molecular Probes, C-6164) was used. Purification was performed in the same manner and the elution time for the pure product was the same to give 6-FAM (1.13 μmol). Extinction coefficient for 6-FAM is 75000 at pH = 9.0, λ_{max} = 498 nm.

2 (6-FAM): ESI-MS calculated for $[M+H]^+$: 1226.2, observed $[M+H]^+$: 1226.2

D.3 Notes and References

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