Multivalent Peptides as New Biomarker Probes for the Detection of Cancer Metastasis

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Multivalent Peptides as New Biomarker Probes for the Detection of Cancer Metastasis

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Abstract

Malignant tumor cells overexpress and release lipid vesicles called exosomes into the body fluids to facilitate their movement and metastasis to other parts of the body. It was found that an increased secretion of exosomes in the peripheral blood is correlated with lung cancer and melanoma metastasis. The hallmarks of exosomes are their highly curved surface (d = ~30-100 nm) that is distinct from other extracellular lipid vesicles and their enrichment with the anionic lipid phosphatidylserine (PS) in the outer leaflet of the membrane bilayer. These properties provide an opportunity in selectively targeting exosomes as potential cancer biomarkers. We have developed a novel, minimally invasive, peptide-based diagnostic tool to detect synthetic liposomes that could be used to detect exosome oversecretion in cancer patients. The successful development of this diagnostic tool for cancer metastasis will help in the proper diagnosis and therapy for cancer patients.
Introduction

Exosomes are a class of lipid membrane vesicles that are secreted by most cell types. They were previously believed to carry cell debris as part of normal cellular processes. Recent research has shown, however, that exosomes are secreted from live cells and carry proteins and RNAs, suggesting an important role in intercellular communication. The exact contents of an exosome depend on the cell of origin. Although the specific mechanism of information transfer via exosomes between the host cell that secreted the exosomes and the target cell is not yet understood, the overall result of exosome-mediated intercellular transfer is the incorporation of host cell RNA into the target cell resulting in functional protein production and therefore a change in the physiological state.

Exosomes are involved in stimulation and inhibition of the immune system, and a highly sought after clinical application addresses metastatic cancer. Although exosomes are naturally shed by normal cells, abnormal and increased secretion of these particles occurs in a diseased state. During cancer metastasis, malignant tumor cells spread from their original location to another site and are the cause of 90% of cancer-related deaths. Exosomes released by tumor cells are known to facilitate cancer metastasis (see Figure 1) and have been shown to express immune inhibitory effects, such as inducing T-cell apoptosis (cell death of white blood cells involved in immunity), blocking T-cell signaling as well as releasing pro-angiogenesis factors. Angiogenesis is a process that provides tumors with the necessary factors for growth and survival, such as blood, oxygen, nutrients, and waste removal. These features that are facilitated by exosomes may

![Figure 1. Secretion and function of exosomes in melanoma metastasis.](image)
provide an interesting account of the in-depth workings of cancer and lead to diagnostic and therapeutic approaches.

It has been shown that exosomes are present in human blood plasma. Furthermore, it has been suggested that by using a minimally invasive blood test, exosomes secreted by tumor cells could be detected. Their detection can be associated with changes in tumor progression regardless of tumor type or location and could be useful in providing diagnostic information and aid to therapeutic decisions.\textsuperscript{2} The blood of metastatic cancer patients has been shown to have a significant increase in exosomes\textsuperscript{5} of about 300-400\% when compared to normal volunteers.\textsuperscript{7,8} Clinical methods currently being used to classify metastatic patients are not accurate or reproducible, are often problematic, and, further, do not indicate valuable information, such as whether or not treatment is reducing metastasis.\textsuperscript{9} A clinical tool currently used to investigate cancer metastasis is the process of biopsy, which involves drawing tissue samples from the body organ of interest, which is a very invasive technique.

By using exosomes as biomarkers for metastatic cancer, it has been hypothesized that there will be a correlation between the amount of exosomes in the blood and tumor progression. This relationship, which would be determined periodically through analysis of minimally-invasive blood draws, could allow for earlier detection of metastasis as well as the ability to determine the effectiveness and responses to treatments at an earlier date.\textsuperscript{5} One hallmark of shed exosomes is the enrichment of the anionic phospholipid phosphatidylserine (PS) in the outer leaflet of the membrane.\textsuperscript{1} Since exosomes are very small and difficult to detect using conventional optical methods, this project aims to develop a new approach on detecting exosomes based on peptide-lipid interactions and advance the detection and understanding of using exosomes as biomarkers of cancer metastasis.
Bradykinin is a nine-residue cationic peptide (Figure 2) that was recently found by magnetic resonance studies to selectively bind to lipid vesicles that are enriched with negatively charged head groups. The amino acid sequence is RPPGFSPFR. This research group has also shown through NMR studies that the arginine residue serves as an anchor between the peptide and the membrane surface. The Yin Lab has previously shown by fluorescence assay that an analogue of bradykinin attaches to synthetic lipid vesicles that contain anionic phospholipids and whose sizes fall within the range of exosomes. Further, the Yin lab has demonstrated that the binding properties of the monomeric peptide (Peptide 1) to isolated exosomes and exosomes in whole blood plasma are statistically identical in rats (Figure 3). Finally, the Yin Lab synthesized an arginine-to-alanine (RtoA) mutant peptide (Peptide 5) as a negative control, which demonstrated no clear binding preference profile for either vesicle size or lipid composition. The small amount of binding that did occur could be due to the phenylalanine residues inserting into the membrane defects.

The diagnostic tools that were utilized are peptide-based molecules that bind with high affinity to lipid vesicles, which were used as synthetic models of exosomes. It has been
demonstrated that putting a biologically active molecule into a dendrimeric scaffold and making
the structure multivalent would render it to possess a higher affinity to its target. The Yin Lab
has previously performed fluorescence enhancement assays the monomeric peptide (Peptide 1)
and the trimeric peptide (Peptide 3), see Figure 4.\textsuperscript{10} Fluorescence enhancement relies on a
fluorophore changing its partitioning coefficient by switching from a polar, aqueous environment
to a nonpolar, phospholipid environment through binding, which leads to increased fluorescence
intensity.\textsuperscript{18} Importantly, even though the relative fluorescence enhancement decreases from the
synthetic lipid model to the exosomes, the decrease is small. Further, as the relative enhancement
increases, the difference will likely decrease. The synthetic lipid models used can be seen in

\textbf{Figure 4:} Fluorescence enhancement results from Peptide 1 and Peptide 3 and the structure
of Peptide 3
Figure 5, and have been designed to closely mimic a biological membrane. As the diameter decreases, the curvature increases, as does the number of defects along the outer membrane.

Peptide 1 showed an increase in fluorescence intensity that is directly related to the decrease in liposome size at any lipid composition. Peptide 3 showed increase in fluorescence intensity corresponding to increase in the amount of phosphatidlyserine (PS), with the highest preference for 30 nm LM3 and no size selection for LM1, indicating that multimerization of BK derivative resulted in an increased ability for curvature and PS sensing. However, Peptide 3 still showed a significant preference for 30 nm LM2 lipid vesicles, which contain 10% mol/mol of phosphatidylyserine.

The new proposed scaffolds will allow for the creation of dimeric (Peptide 2) and tetramer dendrimeric (Peptide 4) peptides. Dendrimeric peptides, named from dendrites, the branching projection of neurons, are branching peptides. These scaffolds will then be decorated with the peptide of interest, which will be tagged with a fluorescent molecule that will allow visualization of the binding affinity of the peptide to tumor-secreted exosomes using biophysical assays. It is the hope that this project will lead to a peptide-based, novel diagnostic tool that will create an invaluable impact to cancer diagnosis and therapy.

Procedure

Peptide Synthesis. The peptide bradykinin (BK) was synthesized with microwave assisted solid phase peptide synthesis (SPPS) on Rink Amide AM resin (KGRPPGFSPFR) with an amide at
the C-terminus (see Figure 6). The ε-amine of the lysine residues was protected with the orthogonal protecting group methyl-trityl (Mtt) that could be selectively removed without affecting the other protecting groups of the resin-bound peptide. The free amine of the lysine was selectively revealed by Mtt deprotection using 1% trifluoroacetic acid in CH$_2$Cl$_2$. The free amine was then converted to the azide, and following confirmation of conversion using the Kaiser test, the N-terminal Fmoc protecting group was removed and then conjugated with the fluorescent reporter molecule 6-$(N$-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid (NBD-Ahx). The peptide was then cleaved from the resin, purified by reverse-phase HPLC, and characterized by mass spectrometry.

Figure 6: Peptide synthesis route resulting in Peptide 1

Scaffold Synthesis. The synthesis of tetrameric alkyne functionalized scaffold of type A (Figure 7) could be completed with the formation of the amide bond between the benzoate alkyne (C) and the methyl-benzoate amine (B). The methylbenzoate amine fragment (B) was synthesized
from the reaction of \textit{t-butyl}-(2-bromoethyl) carbamate and methyl 3,5-dihydroxybenzoate (F) following \textit{t-butyloxy}carbonyl (BOC) deprotection of E. The benzoate alkyne (C) could be obtained from the hydrolysis of methylbenzoate alkyne of type D, which could be obtained by the conjugation of propargyl bromide with methyl 3,5-dihydroxybenzoate (F). F could be accomplished with the esterification of 3,5-dihydroxybenzoate of type (G). The methylbenzoate alkyne of type D is the dimer scaffold.

\begin{center}
\includegraphics[width=\textwidth]{dendrimer_diagram.png}
\end{center}

\textbf{Figure 7:} Retrosynthesis of the dendrimer scaffold.

\textit{Dendrimer and Dimer Synthesis.} The dendrimer and dimer were then synthesized from the appropriate scaffold, A and D respectively, and the functionalized peptide using copper-
catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC), more commonly known as ‘Click’ chemistry\textsuperscript{15}, see Figure 8. Following completion of the reaction, the peptide-small molecule conjugates were purified via reverse-phase HPLC and characterized by mass spectrometry.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Structures of a) dendrimeric Peptide 4 and, b) dimeric Peptide 2.}
\end{figure}

\textit{Liposome Preparation.} Three lipid models (LM) were prepared: LM1, LM2, and LM3, each with 2 mM total lipid concentration utilizing the method described by Morton\textsuperscript{15}. The models
contained varying amounts of palmitoyl-phosphatidylcholine (POPC), cholesterol, palmitoyl-phosphatidylethanolamine (POPE), and palmitoyl-phosphatidylserine (POPS). LM1 contained 70% POPC/15% POPE/15% cholesterol/0% POPS. LM2 contained 60% POPC/15% POPE/15% cholesterol/0% POPS, and LM3 contained 50% POPC/15% cholesterol/15% POPE/20% POPS. Following overnight incubation at 4°C in PBS buffer, pH 7.4, each model was extruded using the Liposofast LF-50 through a polycarbonate membrane with 400 nm, 100 nm, or 30 nm pore diameters to create three sizes for each LM.

**Fluorescence Enhancement.** 1 μM solution of the dimer (peptide 2) and dendrimer (peptide 4) were prepared in PBS, pH 7.4. The concentration was adjusted to exactly 1 μM by taking an absorbance reading using the UV-Vis spectrophotometer. The plate was prepared as follows. The first row contained the blank samples, including PBS and the appropriate liposome model. The second and subsequent rows contained the appropriate liposome sample and the peptide. Using the fluorimeter and the corresponding software, the blank was subtracted from the sample to give fluorescence enhancement due only to the peptide binding to the liposome.

**Results and Discussion**

Fluorescence enhancement results for Peptide 2 and Peptide 4 are reported in Figure 9. Peptide 2 preferentially binds to 30 nm liposomes. There is no statistical difference between the binding affinities of Peptide 2 to LM2 and LM3 30 nm vesicles, and the binding affinity to LM1 30 nm is greater than to LM2 and LM3 30 nm vesicles. The lack of preference for high levels of PS is evident, and is consistent with the selectivity trends observed in the monomeric peptide. Dendrimeric Peptide 4 does not bind to LM1, binds without size preference to LM2, and binds with increasing fluorescence intensity corresponding to an increase in the amount of PS.
Figure 9: Fluorescence enhancement due to Peptide 2 (left) and Peptide 4 (right).

Compared to Peptide 1 and Peptide 3, Peptide 4 is more selective for PS concentration but less selective for curvature. But, the fluorescence intensity is four-fold decrease for LM3 30 nm liposomes from Peptide 3 to Peptide 4.

The significant decrease in fluorescence from Peptide 3 to Peptide 4 contradicts Liskamp’s observation that increasing the number of displayed peptides will increase the binding affinity. This could be explained by recalling that bradykinin electrostatically interacts with the exosome by inserting the arginine residues, which are highly aromatic. The scaffold of Peptide 4 is not only cationic but is also highly aromatic because of the aromatic dendrimer scaffold, and could be inserting into the liposomal bilayer instead of the peptide. This would prevent fluorescence enhancement as a result of aromatic ring insertion in the bilayer and could significantly affect the fluorophore intensity.

Peptide 2 was proposed to test this hypothesis, but the results can neither be used to confirm or reject the theory for Peptide 4. Primarily, there is still significant binding between the peptide and the liposomal membrane, resulting in fluorescence enhancement that is much larger
than the monomer. This shows that the peptide is in fact binding and not the scaffold, in this case. The scaffold is very small compared to the peptide and with less aromaticity. If the scaffold were binding, it could be due to increased aromaticity, which is seen in Peptide 4 but not Peptide 2. This suggests that in order for the scaffold to not interfere with binding, the scaffold must contain less aromatic moieties than the peptide.

Conclusions

This paper reports on the synthesis and characterization of designed peptides that preferentially bind to highly curved lipid vesicles. Peptides 1, 2, 3, and 4 were designed from the peptide Bradykinin. We utilized solution phase 'Click' chemistry to prepare the trivalent peptide and the tetravalent dendrimeric peptide using the ε-azide on the Lys residue. Peptide 1 prefers highly curved liposomes that contain PS, and its curvature and PS sensing ability was further enhanced upon multimerization. Peptide 2 shows selectivity for vesicles with high curvature but no selectivity towards PS concentration. The trivalent Peptide 3 showed two-dimensional selectivity through its ability to discriminate variations in PS content as well as in its preference for lipid vesicles with the highest curvature. These observed properties were further enhanced in Peptide 4. Although the fluorescence enhancement due to the interaction of 30 nm LM 3 vesicles with Peptide 4 is weaker than the interaction with Peptide 3, improved selection for the highest curvature and PS content was achieved with Peptide 4.

We have developed a series of peptide probes that preferentially bind to highly curved synthetic lipid vesicles and to rat exosomes. We expect that further design modifications will result in a peptide probe with the potential to be a diagnostic tool in the detection of exosomes released into the blood of patients with metastatic cancer. The investigation of the exosome binding properties of these peptides on human blood plasma samples is ongoing. This project
may lead to a novel method to evaluate cancer progression and, ultimately, be translated from bench to bedside in order to help save lives.

**Experimentals**

*Chemistry General Methods*

All reactions were run under an inert atmosphere of N\textsubscript{2} gas. Reaction solvents were anhydrous and of HPLC quality. All other reagents were purchased from Sigma Aldrich and used without further purification. Yields were calculated for material judged homogenous by thin layer chromatography and nuclear magnetic resonance (NMR). Thin layer chromatography (TLC) was performed on Merck Kieselgel 60 F\textsubscript{254} plates, eluting with the solvent indicated, visualized by a 254 nm UV lamp, and stained with an ethanolic solution of 12-molybdophosphoric acid. Glassware for reactions was oven dried at 125 °C prior to use. Column flash chromatography was performed using Silica Gel Premium R\textsubscript{f} 60 Å, 200 x 400 mesh from Sorbent Technologies. Nuclear magnetic resonance spectra were acquired on a Bruker spectrometer; 300 MHz for \textsuperscript{1}H and 75 MHz for \textsuperscript{13}C. Chemical shifts for \textsuperscript{1}H NMR spectra are reported in parts per million (ppm) and referenced to the signal of residual nondeuterated chloroform at 7.26 ppm. Chemical shifts for \textsuperscript{13}C NMR and DEPT spectra are reported in parts per million (ppm) and referenced to the center line of the CDCl\textsubscript{3} triplet at 77.23 ppm. Chemical shifts of the unprotonated carbons (‘C’) for DEPT spectra were obtained by comparison with the \textsuperscript{13}C NMR spectrum. Mass spectrometry was performed at the mass spectrometer facility of the University of Colorado Boulder, Department of Chemistry & Biochemistry on a ESI-qTOF-MS (electrospray-triple quadrupole-time-of-flight mass spectrometer) from Applied Biosystems, PE SCIEX/ABI API QSTAR Pulsar Hybrid LC/MS/MS. Compounds with a ppm error less than 4% from HRMS were deemed pure. Compounds were named using ChemDraw.
Preparation of Methyl 3,5dihydroxybenzoate (F)

To a solution of 3,5dihydroxybenzoic acid (10 g, 0.064 mol) in MeOH (70 mL), sulfuric acid (2 mL) was added. The reaction mixture was refluxed (68 °C) for 25 hours and concentrated. The residue was dissolved in water and extracted with a 1:1 of CHCl₃ : t-butyl-OH mixture, dried over Na₂SO₄, and concentrated. The resulting residue was freeze dried overnight and then recrystallized from 100 mL of water at 70 °C. Methyl 3,5dihydroxybenzoate was obtained as a colorless crystal (7.447 g, 68.3%). \( R_f = 0.49 \) (30% EtOAc/DCM). \(^1\)H NMR (300 MHz, MeOD) \( \delta 6.92 \) (d, \( J = 2.28 \) Hz, 2H), \( 6.47 \) (t, \( J = 2.29 \) Hz, 1H), \( 4.88 \) (s, 2H), \( 3.85 \) (s, 3H). \(^{13}\)C NMR (75 MHz, MeOD) \( \delta 52.52 \) (CH₃), 108.23 (CH), 108.77 (CH), 133.03 (C), 159.80 (C), 168.67 (C). HRMS (ESI⁺) = calculated C₈H₈O₄ (M+Na⁺) = 191.0315, found = 191.0317.

Preparation of methyl 3-bis\{2-[(tert-butoxycarbonyl)amino]ethoxy\}benzoate\(^{17}\) (E)

To a solution of methyl 3,5dihydroxybenzoate (0.065 g, 0.425 mmol) in dry DMF (0.52 M) was added K₂CO₃ (0.264 g, 1.91 mmol) and tert-butyl(3-bromopropyl)carbamate (0.25 g, 1.12 mmol). The reaction mixture was stirred at 40°C for 16 hours. The mixture was then filtered and concentrated. The solution was taken up in ethyl acetate and washed with water (2x 10 mL) and
brane (1x 10 mL), dried over Na$_2$SO$_4$, and concentrated. Compound 2 was obtained as a yellow powder (0.312 g, 83%). $R_f$ = 0.77 (10% methanol in dichloromethane). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.17 (d, $J = 2.34$ Hz, 1H), 6.63 (t, $J = 2.33$ Hz, 1H), 4.03 (t, $J = 5.10$ Hz, 2H), 3.89 (s, 2H), 3.53 (dd, $J = 5.20$, 10.46 Hz, 2H), 1.45 (s, 10H). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 28.53 (CH$_3$), 40.13 (CH$_2$), 52.43 (CH$_3$), 67.67 (CH$_2$), 79.77 (C), 106.62 (CH), 108.23 (CH), 132.28 (C), 155.98 (C), 159.73 (C), 166.71 (C). HRMS (ESI$^+$) = calculated C$_{22}$H$_{34}$N$_2$O$_8$ (M+H$^+$) = 455.2390, found = 455.2388.

![Chemical Structure](image)

**Preparation of methyl 3,5-bis(2-aminoethoxy)benzoate**$^{17}$ (B)

Compound E (0.062 g, 0.137 mmol) taken up in 1:1 v:v TFA/DCM solution (0.08 mL:0.08 mL) and stirred at room temperature for 30 minutes. The mixture was concentrated under reduced pressure, and lyophilized overnight. Compound B was obtained as a yellow oil (7 mg, 20%). $R_f$ = 0.47 (10% MeOH/DCM), visualized with Ninhydrin stain. $^1$H NMR (300 MHz, MeOD) $\delta$ 7.30 (d, $J = 2.08$ Hz, 2H), 6.91 (t, $J = 2.00$ Hz, 1H), 4.31 – 4.25 (m, 5H), 3.90 (d, $J = 1.49$ Hz, 3H), 3.39 (t, $J = 4.77$ Hz, 4H). $^{13}$C NMR (75 MHz, MeOD) $\delta$ 40.14 (CH$_2$), 52.88 (CH$_3$), 65.70 (CH$_2$), 107.64 (CH), 109.68 (CH), 133.62 (C), 160.61 (C), 167.80 (C).
Preparation of methyl 3,5-bis(prop-2-yn-1-yl oxy)benzoate\(^{13}\) (D)

Methyl 3,5-dihydroxybenzoate (4g, 23.79 mmol) was dissolved in dry DMF (0.52 M) and K\(_2\)CO\(_3\) (8.22g, 59.5 mmol) was added. To this suspension, a solution of propargyl bromide in toluene (80 wt %, 5.13 mL) was added dropwise. Mixture was stirred at room temperature for 48 hours and then taken up in 50 mL EtOAc, washed with excess water (3x100 mL), then washed with NaHCO\(_3\) (3x50mL), and brine (3x50mL). Organic layers combined, dried with Na\(_2\)SO\(_4\), filtered, and concentrated under reduced pressure. The product was recrystallized from a 1:1 mixture of EtOAc/Hexanes (60 mL) by heating to 75°C and then lyophilized overnight. Compound D was obtained as a yellow powder (4.44 g, 77%). \(R_f = 0.54\) (50% Hexanes/DCM).

\(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 7.30 (d, \(J = 2.38\) Hz, 1H), 6.82 (t, \(J = 2.38\) Hz, 1H), 4.74 – 4.70 (m, 2H), 3.91 (s, 1H), 2.54 (t, \(J = 2.41\) Hz, 1H). \(\delta\) \(C\) (75 MHz, CDCl\(_3\)) 52.52 (CH\(_3\)), 56.28 (CH\(_2\)), 76.11 (CH), 77.36 (C), 78.10 (C), 107.68 (CH), 109.04 (CH), 132.31 (C), 158.66 (C). HRMS (ESI\(^+\)) = calculated C\(_{14}\)H\(_{12}\)O\(_4\) (M+H\(^+\)) = 245.0812, found = 245.0809
Preparation of 3,5-bis(prop-2-yn-1-yloxy)benzoic acid$^{13}$ (C)

Methyl 3,5-bis(prop-2-yn-1-yloxy)benzoate was taken up in dioxane/methanol (327.48 mL/128.52 mL, 14:5 v) and 4 N NaOH (64.29 mL) was added. Reaction was stirred at room temperature for 5 hours and then neutralized with 1 M HCl and concentrated. The mixture was dissolved in EtOAc and washed with NaHCO$_3$ (3x50 mL) and brine (3x70mL). The organic layers were combined, dried over Na$_2$SO$_4$, filtered, and concentrated under reduced pressure to give C as a yellow powder (0.247 g, 18%). R$_f$ = 0.29 (30% EtOAc/DCM). $^1$H NMR $^{13}$C NMR

HRMS (ESI$^+$) = calculated C$_{13}$H$_{10}$O$_4$ (M+H$^+$) = 231.0655, found = 231.0652.
**Preparation of methyl 3,5-bis(2-{[3,5-bis(prop-2-yn-1-yloxy)benzoyl]amino}ethoxy)benzoate (A)**

To a solution of 3,5-bis(prop-2-yn-1-yloxy)benzoic acid (0.1 g, 0.434 mmol), HATU (0.165 g, 0.434 mmol), HoBt (0.059 g, 0.434 mmol), and DIEA (0.172 mL) in THF (0.2 M) was added compound 3 (0.05 g, 0.197 mmol). Mixture was stirred at room temperature overnight and the solvent evaporated. The resulting oil was purified via flash SiO$_2$ column chromatography (5 x 15 cm, 10% MeOH/DCM) to yield A as yellow oil (59 mg, 44%). R$_f$ = 0.46 (10% MeOH/DCM). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.09 (d, $J = 2.32$ Hz, 2H), 7.03 (d, $J = 2.29$ Hz, 4H), 6.70 (t, $J = 2.29$ Hz, 2H), 6.56 (t, $J = 2.23$ Hz, 1H), 4.65 (d, $J = 2.40$ Hz, 8H), 4.07 (t, $J = 5.08$ Hz, 4H), 3.85 (d, $J = 2.43$ Hz, 3H), 3.79 (dd, $J = 5.29, 10.52$ Hz, 4H), 2.52 (t, $J = 2.39$ Hz, 4H). $\delta$ C (75 MHz, CDCl$_3$) 39.62 (CH$_2$), 40.97, 52.40 (CH$_3$), 56.18 (CH$_2$), 66.99 (CH$_2$), 76.19 (CH), 77.36 (C), 78.07 (C), 105.52, 106.87, 108.24, 132.16 (C), 136.62 (C), 158.75 (C), 159.51 (C), 166.55, 167.27 (C). HRMS (ESI$^+$) = calculated C$_{38}$H$_{34}$N$_2$O$_{10}$ (M+H$^+$) = 679.2292, found = 679.2287.
Preparation of Bradykinin Analog

The bradykinin analog Fmoc-NH-KGRPPGFSPFR-CO NH₂ (0.09 mmol) was synthesized using microwave assisted solid phase peptide synthesis on rink amide resin. The lysine residue was Mtt protected. Following synthesis, functionalization was carried out.

Mtt Deprotection

The lysine was first Mtt deprotected. The resin was washed with a cycle of MeOH and DCM (3x 5 mL, 3 minutes) and then DCM (2x5 mL, 3 minutes) while stirring, draining between washes. DCM (5 mL) was added and the resin was allowed to swell for 5 minutes. A mixture of 94:1:5 DCM/TFA/TIS (10 mL) was added to the resin and stirred for 3 minutes. After draining, the resin was washed with MeOH and then DCM (3x 5ml). The mixture of DCM/TFA/TIS was added, the resin was stirred, and washed until the resin no longer displayed a yellow color when in solution (10x). Following the last cycle of the DCM/TFA/TIS mixture, the resin was washed with MeOH and DCM (3x 5 mL), MeOH (5 mL), and lyophilized. The deprotection was confirmed using the Kaiser test.

Amine to Azide

The free amine of the lysine was then converted to an azide. The resin was swelled in a mixture of DCM (560 µL), MeOH (520 µL), diH₂O (490 µL), and Et₃N (2 µL) for two hours, and then drained. While the resin was swelling, NaN₃ (1.17 g, 18 mmol) was dissolved in diH₂O (2.7 mL) and DCM was added (5.4 mL) and cooled to 0°C. Tf₂O (605.3 µL, 3.6 mmol) was added dropwise to a vigorously stirred solution then allowed to heat to room temperature. The reaction was left to proceed for two hours. The DCM layer was then drained, and the aqueous layer was washed with DCM (2x2 mL), and the combined DCM layers were washed with saturated Na₂CO₃ (1x2mL). To the swelling resin was added all of the Tf₂N in the DCM layers and
allowed to stir for 5 minutes. Cu$_2$SO$_4$ (2.7 mg, 0.018 mmol) was taken up in diH$_2$O (30 µL) and then added to the resin mixture. The reaction proceeded for 24 hours at room temperature, after which the resin was drained and washed with 5 mL each of AcCN, DMF, H$_2$O, 0.1 M EDTA in H$_2$O (2x), H$_2$O (2x), MeOH, DCM, and MeOH. The peptide was lyophilized and the Kaiser test proved the success of the reaction.

**Fmoc Deprotection**

10 mL of a 1:4 v:v piperidine/NMP solution was made and 5 mL was added to the resin. The reaction was stirred at room temperature for 30 minutes, after which the resin was drained and washed with 5 mL of DMF, MeOH, DMF x2. 5 mL of the remaining piperidine/NMP solution was added to the resin and stirred for 30 minutes at room temperature. The resin was washed with DMF, (DCM, MeOH) x3 and lyophilized overnight. The Kaiser test confirmed successful deprotection.

**NBD-Ahx Labeling**

NBD-Ahx (44.25mg, 0.15mmol) and HATU (57mg, 0.15mmol) was added to the resin. NMP (2 mL) and DIPEA (.35 mL, 0.15mmol) were added and the mixture was stirred for two hours at room temperature. The resin was drained and washed with (DMF, DCM, MeOH) x3 and lyophilized overnight. Kaiser test confirmed labeling. The peptide was cleaved using 85/5/5/5 TFA/H$_2$O/Phenol/TIPS solution (7.5 mL) and stirring for one hour at room temperature. Following cleavage, the peptide was drained into a falcon tube and the frit tube was rinsed with 1 mL TFA and drained to the falcon tube. Cold Et$_2$O (45mL) was added to the falcon tube and kept at -20°C for 15 minutes, then vortexed and centrifuged for 10 minutes at 4500 rpm. The solution was decanted and cold Et$_2$O (20 mL) was added, vortex mixed, centrifuged for 10 minutes at
4500 rpm, and decanted. The resulting peptide was vacuum dried overnight and then purified by gradient reverse-phase HPLC.

**Preparation of Peptide 4**

MeOH and AcCN were purged with N₂ for at least 30 minutes prior to start of procedure. The TFA-salt of the peptide (15 mg, 0.0079 mmol) was taken up in MeOH (300 µL) and A (1.12 mg, 0.0017 mmol) was added in MeOH (70 µL). TBTA (7.02 mg, 0.013mmol) and tetrakis(acetonitrile)copper(I) hexafluorophosphate (24.68 mg, 0.066 mmol) were added directly. A few drops of MeCN were added to bring everything into solution, and then the reaction was stirred at room temperature for 20 hours. Then, the sample was quenched with water (1 mL) and lyophilized to dryness before purification by reverse-phase HPLC.

**Preparation of Peptide 2**

MeOH and MeCN were purged with N₂ for at least 30 minutes prior to start of procedure. The TFA-salt of the peptide (7 mg, 0.0037 mmol) was taken up in MeOH (225 µL) and D (0.368 mg, 0.0015 mmol) was added in MeOH (100 µL). TBTA (6.367 mg, 0.012mmol) and tetrakis(acetonitrile)copper(I) hexafluorophosphate (5.59 mg, 0.015 mmol) were added directly. A few drops of MeCN were added to bring everything into solution, and then the reaction was stirred at room temperature for 20 hours. Then, the sample was quenched with water (1 mL) and lyophilized to dryness before purification by reverse-phase HPLC.

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