Published in final edited form as:

Chembiochem. 2017 December 14; 18(24): 2395–2398. doi:10.1002/cbic.201700446.

A facile cyclization method improves peptide serum stability and confers intrinsic fluorescence

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Abstract

Peptides are a growing class of macromolecules used in pharmaceuticals. The path toward clinical use of candidate peptides involves sequence optimization and cyclization for stability and affinity. For internalized peptides, tagging is also often required for intracellular trafficking studies, although fluorophore conjugation has been shown to impact peptide binding, permeability, and localization. Here, we report a strategy based on cysteine arylation with tetrafluoroterephthalonitrile (4F-2CN) that simultaneously cyclizes peptides and imparts fluorescence. We show that 4F-2CN cyclization of an M2 macrophage targeting peptide yields in a single step a peptide with improved serum stability, intrinsic fluorescence, and increased binding affinity. We further demonstrate in a murine breast cancer model that the intrinsic fluorescence from the cyclized peptide is sufficient for monitoring biodistribution by whole organ fluorescence imaging and cell internalization by flow cytometry.

All-in-one

Tetrafluoroterephthalonitrile (4F-2CN) was used to cyclize a model peptide, M2 macrophage-binding peptide, imparting intrinsic fluorescence property, improved serum stability, and enhanced target binding affinity.

Keywords

peptide; cyclization; intrinsic fluorescence; tetrafluoroterephthalonitrile; M2pep

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Peptides are an important class of macromolecules in development of molecular diagnostics and therapeutics.^[1] Arrangement of amino acids with various side chains accommodates vast chemical space, enabling identification of unique peptide sequences that bind to desired targets. High throughput screening technologies (e.g. phage display libraries and one-bead-one-compound libraries) as well as computer-guided design are common methods for identifying potential sequences for downstream validation and characterization.^[2,3] However, lagging behind in the development pipeline is the ability to rapidly synthesize, test, and optimize the “hit” peptide candidates.

While an improvement in peptide synthesis technologies (e.g. flow-based systems and microwave-assisted heating) has greatly increased synthetic speed,^[4,5] necessary peptide modifications to improve biological stability, affinity, and tracking add greatly to the production time. Here, we present a simple strategy where commercially available tetrafluoroterephthalonitrile (4F-2CN) is used as a linker to cyclize a bioactive peptide, conferring fluorescence, increased binding affinity, and serum stability.

Cyclization of peptides can impart improvements in both serum stability and target binding and is often accomplished by disulfide formation between introduced cysteines.^[6] However, the disulfide bond is known to be reversible in serum, compromising the overall stability of the disulfide-based cyclic peptides compared to those cyclized via an irreversible chemistry such as lactamization.^[7] 4F-2CN contains multiple reactive fluorine atoms that can orthogonally undergo nucleophilic aromatic substitutions (S_NAr) with thiols.^[8] Furthermore, when reacting with cysteine, 4F-2CN reacts first with the thiol by S_NAr followed by cyclization with the primary amine; the resulting product exhibits fluorescence.^[9] We therefore hypothesized that 4F-CN can be used to simultaneously cyclize and impart intrinsic fluorescence to peptides if one of the cyclized cysteines is introduced at the N-terminus.

Our peptide of interest is M2pep(RY), a peptide that we previously reported binds specifically to M2 macrophages over M1 macrophages and is an effective ligand for targeting M2-like, tumor-promoting, tumor-associated macrophages (TAMs) in colon and breast cancer models.^[10,11] In this work, we use 4F-CN to successfully synthesize two cyclic M2pep(RY) analogs; **1** and **2**, where the linear precursors contain flanking Cys/Cys and Hcy(Homocysteine)/Cys respectively for cyclization (Scheme 1). The Hcy was used for comparison, because its N-terminal amine should be less favorable for additional cyclization.^[9] Indeed, MALDI-ToF MS confirmed that the amine group at the N-terminal cysteine in peptide **1**, possessing 2-aminoethanethiol structure, undergoes an additional S_NAr cyclization with the proximal fluorine whereas no reaction was observed for the N-terminal amine of homocysteine in peptide **2** due to its less favorable 3-aminopropanethiol structure (Figure S1). Notably, 4F-2CN cyclization proceeds efficiently under a basic conditions (Tris base in DMF) and tolerates the presence of TCEP, a reducing agent used to suppress disulfide bond formation, which implies redox stability of the cyclized product (Supplementary information: Experimental section).

Intrinsic fluorescence in peptides is preferred over fluorophore labeling, as several studies have reported changes in peptide activity and/or distribution resulting from fluorophore

conjugation.^[12–14] The intrinsic fluorescence properties of peptide **1** and **2** were therefore validated. In addition to characteristic peptide absorption around 220 and 280 nm, peptide **1** exhibits a unique, additional absorption band in the 350 – 475 nm range ($Abs_{max} = 423$ nm) whereas peptide **2** absorbs in the 310 – 400 nm range ($Abs_{max} = 362$ nm) (Figure 1a). The shift in the absorption range is expected due to the difference in the chemical identities of the two analogs at the linker region post-cyclization. Notably, when peptide **1** was excited at 420 nm, stronger fluorescence emission was observed in the 440 – 640 nm range compared to the emission by peptide **2** ($AUC_{ratio_{peptide1/peptide2, 440-680\text{ nm}}} = 20$) (Figure 1b). On the other hand, both peptide **1** and **2** only weakly emit fluorescence in the 380 – 680 nm range when excited at 350 nm. In addition, peptide **1** was confirmed to be excitable at 405 nm (corresponding to the wavelength of a violet laser typically used in flow cytometer or fluorescence microscopy) with detectable emission in the blue/green region (Figure S2) making it potentially useful for biological studies that require fluorescence readout.

We next evaluated applicability of the peptides for *in vitro* and *in vivo* biological studies. Binding activities of peptide **1** and **2** were first evaluated with bone marrow-derived, activated, murine M2 and M1 macrophages using flow cytometry in comparison to previously reported linear M2pep(RY) analog **3** (AcM2pep(RY)) (Figure 2a). All peptides were synthesized with a C-terminal biotin to enable validation via a secondary stain with streptavidin-Alexa Fluor 647. Both peptide **1** and **2** exhibit binding to M2 macrophages with selectivity over M1 macrophages (Figure 2b). These analogs also have enhanced binding activity compared to linear analog **3**. 4F-2CN cyclization of M2pep(RY) also improves its serum stability compared to the linear analog (Figure 2c).

The intrinsic fluorescence property of peptide **1** can also be used to monitor peptide binding and internalization via flow cytometry and confocal microscopy. M2 macrophages were incubated with the peptides at different concentrations and subsequently analyzed via flow cytometry with excitation wavelength of 405 nm and detection in the 425 – 475 nm bandpass filter. Despite comparable binding activities of peptide **1** and **2** on M2 macrophages (Figure 2b), only peptide **1**-incubated cells were detected with discernable signal following incubation above 100 μ M (Figure S3). No signal from peptide **2**-incubated cells was observed as expected from its weaker fluorescence property. For confocal microscopy, M2 macrophages were incubated with 100 μ M peptide **1** for 1 h, fixed, and imaged using excitation wavelength of 405 nm and detection in the 430 – 550 nm range. The internalized peptide **1** was successfully imaged by confocal microscopy (Figure 2d) which, together with the flow cytometry study, confirms that the 4F-2CN-Cys intrinsic fluorescence property is sufficient for monitoring peptide binding and internalization via both flow cytometry and fluorescence imaging modalities.

Finally, we investigated peptide **1**'s intrinsic fluorescence property for tracking the *in vivo* biodistribution of the peptide in a 4T1 breast tumor model. Tumor-bearing mice were intravenously administered with peptide **1** (200 nmol/mouse) and perfused after 20 min to collect organs for imaging under Caliper Xenogen IVIS. Highest fluorescence signal of peptide **1** was observed in kidneys due to renal clearance (Figure 3a). Accumulations of peptide **1** in tumor, liver, and heart were also observed with statistical significance over the PBS controls (Figure 3b). It is important to note that while we were able to obtain statistical

significance within this study, the overlap of peptide 1's excitation/emission with native cell and tissue auto-fluorescence may compromise the limit of detection of the peptide. Future studies on structure-activity relationship of 4F-2CN variants might result in peptide analogs with more desirable fluorescence property (e.g. longer excitation/emission wavelength). Following Xenogen analysis, tumor samples were cut into half for analysis of intratumoral distribution via flow cytometry and confocal microscopy. For flow cytometry evaluation, the tumors were homogenized into single cell suspensions and stained with PerCP anti-CD11b antibody to distinguish between macrophage (CD11b⁺) and non-macrophage (CD11b⁻) populations. Accumulation of peptide 1 in each respective population was measured based on its intrinsic fluorescence as performed in the *in vitro* binding study above. Statistical significance was observed in the comparison between CD11b⁺ populations with higher fluorescence signal in the peptide 1-treated samples versus the control samples (Figure 3c), corresponding well to the peptide ability to target TAMs. No difference in fluorescence signals between these treatment groups in the CD11b⁻ populations was observed mirroring the selectivity of the peptide. Accumulation of peptide 1 in TAMs was further evaluated from tumor tissue sections. Consistent with the flow cytometry analysis, accumulation of peptide 1 was detected in the TAM population (Red: F4/80⁺) in tumors (Figure 3d). These studies successfully demonstrate the use of peptide 1's intrinsic fluorescence in the analysis of organ biodistribution and intratumoral localization which are two important *in vivo* biological evaluations for optimization of peptide therapeutics.

In summary, we report a general strategy to simultaneously cyclize peptides, conferring fluorescence, serum stability, and increased binding affinity in a single step. The fluorescence property of the cyclized peptides is compatible with detection via flow cytometry and confocal microscopy as demonstrated in *in vitro* binding/uptake assays and in *in vivo* biodistribution studies of 4F-2CN-cyclized M2 macrophage-targeting peptide 1. This simple strategy mitigates the bottleneck of peptide development pipeline by speeding up the synthesis of cyclic, fluorescent peptides for downstream characterization and optimization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH 1R01CA177272. Chayanon Ngambenjawong was supported by an Anandamahidol Foundation Fellowship. Heather H. Gustafson was supported by the Cardiovascular Pathology Training Grant (5 T32 HL 007312-37). We thank Albert Yen for assistance with animal studies. Confocal microscopy was conducted using the W.M. Keck Microscopy Facility (supported by NIH S10 OD016240).

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Figure 1.

(A) Absorption spectra of peptide 1 and 2. Magnification on the region of interest (Right insert). Fluorescence emissions of the peptides at excitation wavelength of (B) 420 nm and (C) 350 nm.

Figure 2.

(A) Amino acid sequences of AcM2pep(RY) (Peptide 3). (B) Binding study with M1 and M2 macrophages detected with streptavidin-Alexa Fluor 647. (C) Serum stability of peptide 1 and 3. * denotes peak of the intact peptide (D) Confocal images of untreated (left) and peptide 1-treated (right) M2 macrophages.

Figure 3.

(A) Xenogen images of perfused organs from tumor-bearing mice injected with PBS (left) and peptide 1 (right). (B) Quantification of the fluorescence signal in each organ. * denotes statistical significance based on unpaired Student's t test ($P < 0.05$) (C) Intratumoral distribution of peptide in CD11b⁺ (macrophage) and CD11b⁻ (non-macrophage) populations. * denotes statistical significance based on unpaired Student's t test ($P < 0.05$) (D) Representative 4T1 tumor sections from PBS-treated (top) and peptide 1-treated (bottom) groups. Green: peptide, Red: F4/80 macrophage stain, Blue: DAPI.

XGYEQDPWGVRYWYGCkkk(K-Biotin)

X = Cys, Hcy

Scheme 1.

4F-2CN cyclization of M2pep(RY) with N-terminal Cys (Peptide **1**) and Hcy (Peptide **2**).