Published as: *Chem Commun (Camb)*. 2010 August 21; 46(31): 5781–5783.

Synthesis of cyclic, multivalent Arg-Gly-Asp using sequential thiol-ene/thiol-yne photoreactions

Alex A. Aimetti^a, Kristen R. Feaver^a, and Kristi S. Anseth^{a,b}

^aDepartment of Chemical & Biological Engineering, University of Colorado, Boulder, CO 80309, USA. Kristi.Anseth@colorado.edu; Fax: +1 303-492-4341; Tel: +1 303-492-7471

^bHoward Hughes Medical Institute, University of Colorado, Boulder, CO 80309, USA

Abstract

A unique method has been developed for the formation of multivalent cyclic peptides. This procedure exploits on-resin peptide cyclization using a photoinitiated thiol-ene click reaction and subsequent clustering using thiol-yne photochemistry. Both reactions utilize the sulfhydryl group on natural cysteine amino acids to participate in the thiol-mediated reactions.

Peptides and their applications as potential therapeutics have been revitalized within the past decade. These biomolecules are capable of accessing both intra- and extracellular targets expanding the options relating to drug discovery.¹ Further, peptides bind to ligands via natural protein-protein interactions, which small molecules only attempt to mimic. This innate binding ability can cause the peptide to be more potent in comparison to a small molecule.² Initially, peptides, derived as fragments from whole proteins, were unable to perform at or near the level of the native protein. When these fragments are extracted from the protein, the short peptides are unordered and lack defined conformation leading to decreased activity.³ Further, peptides can be extremely unstable *in vivo* and are rapidly broken down by various proteases.⁴

Researchers have made great advances in addressing the inherent disadvantages related to peptide therapeutics. Peptide macrocyclization, including peptide stapling,^{5, 6} has rendered the biomolecules more potent in binding to their intended target. Additionally, peptides with a constrained conformation are more resistant to proteolytic degradation and are capable of achieving *in vivo* half-lives up to 24 hr.⁶ Macrocyclization has been reported, either on-resin or in solution, using a variety of ligation chemistries.

Additionally, multivalent interactions are known to play a critical role in many biological processes.⁷ The synthesis of multivalent peptides has further enhanced the interaction of individual ligands with their receptors. Multiple antigenic peptides (MAPs) were discovered by Tam et al.⁸ and utilized a branched lysine core. Although an elegant way to build multivalent peptides on the solid phase, the presence of single amino acid deletions made purification difficult. More recently, researchers have looked for highly efficient, chemoselective reactions to combine multiple peptides to a single core, or handle. “Click” reactions, defined by Sharpless,⁹ met the need for highly specific, rapid reactions between two functional groups in high yield for the synthesis of multivalent peptides. Recent reports exploit the Cu(I)-catalyzed Huisgen azide-alkyne 1,3-dipolar cycloaddition reaction as an

effective way to conjugate azido containing peptides to a core molecule with multiple alkyne handles.¹⁰ These peptide dendrimers exhibit enhanced potency in relation to their monomeric counterpart. However, there still remains a need for efficient reactions capable of achieving a molecule with a high density of peptide ligands.

Thiol-yne click chemistry¹¹ has emerged within the last year as a highly efficient, radical-mediated reaction between a thiol and alkyne. Two thiol groups can add across one alkyne making this a desirable reaction for the formation of multivalent peptides with increased peptide density. Photoinitiated thiol-yne chemistry has been used for various polymer applications,¹² as well as the formation of dendrimers.¹³

This contribution presents the use of sequential thiol-mediated (thiol-ene/thiol-yne) photochemical reactions for the facile synthesis of cyclic, multivalent peptides (Scheme 1). Recently, our group reported the first account of on-resin cyclic peptide formation using thiol-ene photochemistry.¹⁴ We extended this work to introduce a free thiol to the cyclic peptide for subsequent clustering using thiol-yne photochemistry. Arg-Gly-Asp (RGD), a peptide ligand of the $\alpha_v\beta_3$ integrin, was used as a model peptide to illustrate proof of concept. Biological activity of RGD derivatives was evaluated for their ability to prevent fibrinogen binding to glycoprotein IIb/IIIa (GPIIb/IIIa).

Our approach exploits the natural amino acid, cysteine, as the thiol source for sequential thiol-mediated photoreactions. Linear (**1**) and cyclic (**2**) RGD were synthesized on the solid phase using MBHA Rink Amide resin. **2** was cyclized using thiol-ene photochemistry as described previously.¹⁴ Briefly, Fmoc-C(Mmt)RGDSfK(Alloc)-resin was synthesized. The monomethoxytrityl (Mmt) was selectively deprotected on resin following an established protocol. The thiol-ene reaction was performed in the presence of photoinitiator (2,2-dimethoxy-2-phenylacetophenone: DMPA) and UV light (365nm, 20mW cm⁻²) for 3 hr. Upon reaction completion, the Fmoc group was removed and Gly, Gly, Cys(Trt) were subsequently coupled in a step-wise manner. **2** was isolated in 15% yield (calculated based on initial resin substitution) and characterized by MALDI-TOF and ¹H-NMR.

Various core molecules (n=1,2, or 3 where n indicates the number of alkynes) were prepared using traditional peptide chemistry. 4-Pentynoic acid was coupled to the ϵ -amino groups of Lys amino acids to introduce alkynes orthogonal to the peptide backbone. The thiol-yne photoreaction is capable of achieving clustered peptides where the valency is equal to 2n. All thiol-yne reactions were performed using unprotected peptides in solution. The reaction solvent was chosen based on peptide solubility at the required concentrations (~0.2M). **1** and **2** were used in water and dimethylformamide (DMF), respectively. The appropriate photoinitiator was selected based on its solubility in the reaction solvent; LAP, lithium phenyl-2,4,6-trimethylbenzoylphosphinate,¹⁵ was used for water while DMPA, 2,2-dimethoxy-2-phenyl acetophenone, was used with DMF. Table 1 shows the various multivalent RGD derivatives that were synthesized and their corresponding molecular weight. Linear RGD derivatives (**3** – **5**) were obtained in very good yields. Cyclic RGD multimers **6** and **7** were obtained in 48% and 11% yield, respectively. **8** (cyclic RGD hexamer) was not observed. We hypothesize the reason for decreased yields with increasing n relates to steric hindrances. The bulky macrocycles may prevent, or limit, the addition of 2 thiol containing peptides to 1 alkyne. During the formation of **7** the dominant product obtained contained 2 additions (as opposed to 4). ¹H NMR shows the presence of vinyl protons (vinyl sulfide) indicating single peptide addition to each alkyne (as opposed to double peptide addition to 1 alkyne). Although not explored in this contribution, this collective data indicates that increasing the spatial distance between alkynes or between the thiol and cyclic peptide may result in increased yield.

Studies were then performed to investigate the kinetics of the thiol-yne photoreaction. The formation of **4** was used as a model system to explore the reaction rate. Figure 1 shows sequential HPLC chromatograms corresponding to various time points of the reaction. The reaction is very rapid (~20min) and the desired product (**4**, peak A) was formed as the dominant peak. Peak C corresponds to a single peptide addition to a single alkyne as determined by ¹H NMR (Supporting Information Figure S2).

To evaluate the bioactivity of multivalent RGD derivatives formed *via* thiol-yne photochemistry, a competitive binding ELISA was performed. RGD has been shown to inhibit the binding of fibrinogen to GPIIb/IIIa.¹⁶ Table 2 shows the calculated IC₅₀ values for compounds **1** – **7**. As expected, cyclic RGD (**2**) formed by thiol-ene reaction exhibited enhanced potency relative to linear RGD (**1**). Further, **7** and **5** demonstrated a decreased IC₅₀ value of 1.5-2 orders of magnitude relative to their monomeric species.

In summary, this Communication presents a novel strategy for the formation of multivalent peptides using thiol-yne photochemistry. The reaction is very rapid (~20 min) and results in desired products obtained in relatively high yields for linear peptides (77-84%). Further, this work demonstrates that multiple thiol-mediated photoreactions (thiol-ene/thiol-yne) can be used sequentially to enhance peptide effects. This report has implications in the field of peptide chemistry and its application to peptide therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr. Ben Fairbanks and Cole DeForest for helpful discussions. This work was funded by the NIH (Grant RO1 DK076084), HHMI, and a Department of Education Graduate Assistance in Areas of National Need fellowship (A.A.A.).

Notes and references

- Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, Korsmeyer SJ. *Science*. 2004; 305:1466. [PubMed: 15353804]
- Hummel G, Reineke U, Reimer U. *Mol Biosyst*. 2006; 2:499. [PubMed: 17216031]
- Oh JE, Hong SY, Lee KH. *J Pept Res*. 1999; 53:41. [PubMed: 10195440]
- Tugyi R, Mezo G, Feller E, Andreu D, Hudecz F. *J Pept Sci*. 2005; 11:642. [PubMed: 15864815]
- Madden MM, Vera CIR, Song WJ, Lin Q. *Chemical Communications*. 2009:5588. [PubMed: 19753366]
- Schafmeister CE, Po J, Verdine GL. *J Am Chem Soc*. 2000; 122:5891.
- Mammen M, Choi SK, Whitesides GM. *Angewandte Chemie-International Edition*. 1998; 37:2755.
- Tam JP. *Proc Natl Acad Sci U S A*. 1988; 85:5409. [PubMed: 3399498]
- Kolb HC, Finn MG, Sharpless KB. *Angewandte Chemie-International Edition*. 2001; 40:2004.
- (a) Rijkers DT, van Esse GW, Merkx R, Brouwer AJ, Jacobs HJ, Pieters RJ, Liskamp RM. *Chem Commun (Camb)*. 2005:4581. [PubMed: 16158121] (b) Dijkgraaf I, Rijnders AY, Soede A, Dechesne AC, van Esse GW, Brouwer AJ, Corstens FH, Boerman OC, Rijkers DT, Liskamp RM. *Org Biomol Chem*. 2007; 5:935. [PubMed: 17340009] (c) Yim CB, Boerman OC, de Visser M, de Jong M, Dechesne AC, Rijkers DTS, Liskamp RMJ. *Bioconjugate Chem*. 2009; 20:1323.
- (a) Hoogenboom R. *Angew Chem Int Ed Engl*. 2010; 100:1002. [DOI: 10.1002/anie.201000401] (b) Lowe AB, Hoyle CE, Bowman CN. *J Mater Chem*. 2010; 20:1039. [DOI: 10.1039/b917102a]
- (a) Chan JW, Hoyle CE, Lowe AB. *J Am Chem Soc*. 2009; 131:5751. [PubMed: 19341293] (b) Fairbanks BD, Scott TF, Kloxin CJ, Anseth KS, Bowman CN. *Macromolecules*. 2009; 42:211. [PubMed: 19461871] (c) Hensarling RM, Doughty VA, Chan JW, Patton DL. *J Am Chem Soc*.

- 2009; 131:14673. [PubMed: 19778016] (d) Konkolewicz D, Gray-Weale A, Perrier S. J Am Chem Soc. 2009; 131:18075. [PubMed: 19947636] (e) Chan JW, Zhou H, Hoyle CE, Lowe AB. Chem Mater. 2009; 21:1579.(f) Yu B, Chan JW, Hoyle CE, Lowe AB. Journal of Polymer Science Part a-Polymer Chemistry. 2009; 47:3544.
13. Chen G, Kumar J, Gregory A, Stenzel MH. Chem Commun (Camb). 2009:6291. [PubMed: 19826698]
14. Aimetti AA, Shoemaker RK, Lin CC, Anseth KS. Chem Commun (Camb). 2010:1039/c001375g
15. Majima T, Schnabel W, Weber W. Makromolekulare Chemie-Macromolecular Chemistry and Physics. 1991; 192:2307.
16. Barker PL, Bullens S, Bunting S, Burdick DJ, Chan KS, Deisher T, Eigenbrot C, Gadek TR, Gantz R, Lipari MT, Muir CD, Napier MA, Pitti RM, Padua A, Quan C, Stanley M, Struble M, Tom JYK, Burnier JP. J Med Chem. 1992; 35:2040. [PubMed: 1597855]

Figure 1. Evolution of **4** (Linear RGD tetramer) determined by RP-HPLC. Peak A corresponds to desired product, Peak B to 3 additions, and Peak C to 2 additions.

Scheme 1^a

Synthetic route to form multivalent peptides using thiol-yne photochemistry.

^aConditions: Photoinitiator (chosen based on peptide solubility), $h\nu$ (365nm, 15 mW cm⁻²), 20 minutes. Note: Peptide cores with $n=2,3$ were synthesized with aminohexanoic acid (Ahx) linkers between alkynes. See Supporting Information for more detail. * indicates stereocenter generated by reaction.

Table 1

Calculated yields of multivalent RGD derivatives

Compound #	R	n	Initiator ^b	Calc. MW	[M+H] ⁺ Found	Purified Yield (%)
1^a	1	-	-	649.7	650.6	- ^a
2^a	2	-	-	1111.3	1112.2	- ^a
3	1	1	LAP	1638.8	1639.8	84
4	1	2	LAP	3373.6	3374.8	87
5	1	3	LAP	5108.5	5110.1	77
6	2	1	DMPA	2561.9	2563.2	48
7	2	2	DMPA	5219.9	5222.5	11
8	2	3	DMPA	7877.9	-	0

^aCompounds **1** and **2** correspond to linear H-CGGRGDS-NH₂ and cyclic H-CGGc[CRGDSfK(Alloc)]-NH₂, respectively. Formed *via* SPPS, isolated yields reported in Supporting Information.

^bType I initiators were chosen based on peptide solubility.

Table 2

IC₅₀ values for multivalent RGD corresponding to inhibition of fibrinogen binding to GPIIb/IIIa.

Compound #	R	n	IC ₅₀ (μM) ^a
1	1	-	15.4 ± 4.2
2	2	-	0.65 ± 0.1
3	1	1	5.65 ± 1.5
4	1	2	2.42 ± 0.4
5	1	3	0.76 ± 0.2
6	2	1	0.14 ± 0.03
7	2	2	0.012 ± 0.003

^aValues reported as mean ± SEM. Experiments were conducted in triplicate and repeated twice.