Chemoselective Immobilization of Peptides on Abiotic and Cell Surfaces at Controlled Densities

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Received March 25, 2010. Revised Manuscript Received May 1, 2010

We report herein a new and enabling approach for decorating both abiotic and cell surfaces with the extracellular matrix IKVAV peptide in a site-specific manner using strain promoted azide−alkyne cycloaddition. A cyclooctyne-derivatized IKVAV peptide was synthesized and immobilized on the surface of pancreatic islets through strain-promoted azide−alkyne cycloaddition with cell surface azides generated by the electrostatic adsorption of a cytocompatible poly-(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) copolymer bearing azido groups (PP-N3). Both “one-pot” and sequential addition of PP-N3 and a cyclooctyne-derivatized IKVAV peptide conjugate enabled efficient modification of the pancreatic islet surface in less than 60 min. The ability to bind peptides at controlled surface densities was demonstrated in a quantitative manner using microarrays. Additionally, the technique is remarkably rapid and highly efficient, opening new avenues for the molecular engineering of cellular interfaces and protein and peptide microarrays.

Introduction

Re-engineering of mammalian cell surface proteomes offers a valuable tool for investigating and directing biological processes governed by cell surface molecules.1 Genetic approaches have been commonly employed to introduce exogenous proteins or peptides to cell surfaces,1a,b,c but many cell types and proteins may be recalcitrant to genetic manipulation.2 Although cell surface proteins and peptides can be introduced through covalent tethering to native cell surface constituents1d,e or by the insertion of membrane-anchored proteins into the lipid bilayer,1f.g such processes may perturb cell physiology in unanticipated or undesired manners. Hence, a need exists for new chemical strategies to remodel cell surfaces molecularly,3 where the technique is characterized by fast reaction kinetics, the absence of a metal catalyst, and insensitivity toward air oxidation.4–8 To address these issues, Bertozzi developed a novel difluorenated cyclooctyne whose ring strain and electron-withdrawing fluorine substituents promoted [3 + 2] azide−alkyne cycloaddition without the need for a copper catalyst.8 Recently, Boons and co-workers have reported a strained dibenzocyclooctynol that reacts with azides at a much higher rate.5 Its efficiency is comparable to that of copper-catalyzed click chemistry and thus far has found utility in labeling cell surfaces,4–6 hydrogel formation,7 and dynamic imaging of living systems.8

Here, we describe a new and enabling approach for immobilizing peptides using cyclooctyne-promoted azide–alkyne cycloaddition (Scheme 1). First, we present a cytocompatible approach for re-engineering the surfaces of cells with an extracellular-matrix-derived peptide. Second, we demonstrate that strain-promoted cycloaddition affords a new immobilization strategy for the generation of peptide microarrays. Here, we utilized isolated pancreatic islets, which are multicellular aggregates responsible for regulating blood glucose levels that have been widely explored as a cell-based therapy for the treatment of diabetes. The clinical efficacy of islet transplantation remains limited by numerous challenges, including insufficient revascularization and impaired insulin secretion,5a which might be addressed through immobilizing bioactive peptides on the islet surface. The encapsulation of islets in hydrogels containing the extracellular matrix IKVAV peptide has been shown to improve islet function by inhibiting β-cell apoptosis and increasing insulin secretion,9,10 through interaction with integrin α6β1 expressed on islet cell surfaces.10 Therefore, IKVAV was used as a biologically relevant model peptide for functionalizing with cyclooctyne (CyO) and subsequently immobilizing on the surfaces of pancreatic islets.
The use of azide-cyclooctyne chemistry has notable advantages over other chemoselective cell surface engineering approaches, most notably that it is very efficient and that neither azide nor cyclooctyne exists naturally on the cell surface. However, a limitation of this approach is the amount of time it generally takes to generate cell surface azides through metabolic engineering approaches. Hence, the strategy reported here is remarkably rapid relative to other approaches for incorporating molecules on cell surfaces through copper-free click chemistry. This is anticipated to both open up new applications, particularly for cell transplant and in vivo applications, as well as to make the process of cell surface modification more convenient and less time- and resource-intensive.

The CyO-IKVAV conjugate can be immobilized on islets through strain-promoted cycloaddition with cell surface azides generated through the adsorption of a cytocompatible poly(l-lysine)-graft-poly(ethylene glycol) (PLL-PEG) copolymer bearing azido groups (PP-N3). To gain further insight into this strategy, CyO-IKVAV was conjugated to surface-bound PP-N3 in a microarray format. Significantly, we observed that the peptide surface density could be tailored through control of the peptide concentration and copolymer structure.

**Results and Discussion**

In a recent report, cyclooctyne was conjugated to peptides using amine groups (e.g., lysine residues and an N-termine) as the reactive nucleophilic partner. This strategy would be anticipated to interfere with the activity of IKVAV and other lysine-containing peptide sequences. Hence, an N-terminal cysteine was incorporated in the IKVAV sequence to enable the site-specific incorporation of cyclooctyne through a thiol conjugation strategy. Additionally, two glycine spacers and a Lys (Biotin) amino acid were introduced into the sequence to facilitate peptide detection with fluorescently labeled streptavidin.

Cyclooctyne derivatives cannot be directly incorporated into a resin-bound peptide owing to the instability of this group to trifluoroacetic acid and other scavengers, which are applied during cleavage from the resin. Thus, IKVAV was first synthesized on the resin; subsequently, the cyclooctyne derivative was attached using a chemoselective ligation reaction. Although various cysteine-functionalization strategies such as the thiol–ene reaction

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The surface density of cell-adhesive peptides, such as IKVAV, can have a significant effect on integrin-dependent cell behavior, including adhesion, differentiation, and survival. To explore this, we chose an amine-reactive NHS slide that would rapidly couple PP-N$_3$ to the substrate through nonmodified backbone lysine residues. First, serial dilutions (25, 12, 6, 3, and 1 $\mu$M) of PP-N$_3$ were printed on NHS-activated glass surfaces using a piezoelectronic noncontact printer. Biotin-hydrazine (50 $\mu$M) was printed as a fluorescence intensity standard. Each concentration was printed as six replicate spots, vertically from top left to bottom right with a measured average spot size of $\sim$100 $\mu$m. After being printed, the slide was incubated at 50 $^\circ$C for 1 h in a moisture-controlled chamber and then at room temperature for 12 h to ensure complete reaction. The slide was then thoroughly washed with buffer and blocked with 50 mM aqueous ethanolamine to prevent the nonspecific absorption of the primary amines. Strain-promoted azide–alkyne cycloaddition was performed by incubating the slide with CyO-IKVAV at 50 and 25 $\mu$M concentrations in phosphate buffer for 15 min. The detection of immobilized peptide was accomplished by incubating the slide with Alexa488-streptavidin (5 $\mu$g/mL) for 1 h, followed by fluorescence detection at 495 nm/519 nm (Ex/Em) with a Perkin-Elmer ProScanarray microarray scanner (Figure 2).

Immobilization on surface microarrays by strain-promoted cycloaddition is efficient, and the quantification of fluorescence intensity indicated that the immobilized peptide could be visualized on the solid support in less than 15 min. Control experiments (Figure 2C) confirmed that binding was dependent on strain-promoted cycloaddition. The surface-bound peptide density could be controlled by adjusting the concentration of either PP-N$_3$ or the CyO-peptide conjugate. As seen in Figure 3, PP-N$_3$ synthesized with PEG$_4$ spacer (PP4-N$_3$) or PEG$_{12}$ (PP12-N$_3$) presented $N_3$ at the terminus of PEG$_4$ (PP4-N$_3$) or PEG$_{12}$ (PP12-N$_3$) spacers incorporated at a similar grafting ratio (40–45%).

Figure 1. (A) Immobilization of the cyclooctyne-derivatized IKVAV peptide on islet surfaces (left). Representative confocal micrograph of islets serially treated with PP-N$_3$ and CyO-IKVAV, followed by detection with Cy3-streptavidin (right). (B) Control experiments demonstrating negligible nonspecific binding of the peptide to islet surfaces. PP-N$_3$+ control peptide 4 + Cy3-SA (left); PP-(OCH$_3$)$_3$ + CyO-IKVAV + Cy3-SA (right). The scale bar is 50 $\mu$m. (C) Islet viability demonstrates that the treatment of PP-N$_3$+CyO-IKVAV does not significantly ($p > 0.05$) influence islet viability relative to that of untreated controls.

Figure 2. Fluorescence micrograph of PP4-N$_3$ and PP12-N$_3$ printed at 25, 12, 6, 3, and 1 $\mu$M. Each concentration was spotted with six replicates, vertically from top left to bottom right with a measured average spot size of $\sim$100 $\mu$m. After being printed, the slide was incubated at 50 $^\circ$C for 1 h in a moisture-controlled chamber and then at room temperature for 12 h to ensure complete reaction. The slide was then thoroughly washed with buffer and blocked with 50 mM aqueous ethanolamine to prevent the nonspecific absorption of the primary amines. Strain-promoted azide–alkyne cycloaddition was performed by incubating the slide with CyO-IKVAV at 50 and 25 $\mu$M concentrations in phosphate buffer for 15 min. The detection of immobilized peptide was accomplished by incubating the slide with Alexa488-streptavidin (5 $\mu$g/mL) for 1 h, followed by fluorescence detection at 495 nm/519 nm (Ex/Em) with a Perkin-Elmer ProScanarray microarray scanner (Figure 2).

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the intermediate NHS coupling step supported enhanced immobilization. Consequently, greater binding of PP4-N\textsubscript{3} was observed in the subsequent strain-promoted azide–alkyne cycloaddition.

In summary, we report a novel approach to immobilizing peptides both on cellular and solid surfaces in a site-specific and chemoselective manner through strain-promoted azide–alkyne cycloaddition. In contrast to other peptide immobilization strategies, this method is robust under physiological conditions and does not require a metal catalyst, allowing the surfaces of viable mammalian cells and tissues to be decorated with peptide sequences without compromising cell viability. Additionally, this approach will overcome the limitations of conventional methods for surface bioconjugation, which are limited by low efficiency, selectivity, and harsh reaction conditions. Collectively, this work provides a versatile platform for immobilizing peptides and other CyO-containing biomolecules on both abiotic and cell surfaces with multiple potential biomedical and biotechnological applications.

**Acknowledgment.** This work was supported by grants from the National Institutes of Health (DK069275) and the Juvenile Diabetes Research Foundation. J.T.W. acknowledges the Whitaker Foundation for generous fellowship support.

**Supporting Information Available:** Syntheses of the cyclooctyne-maleimide linker (3), CK(Biotin)GGIKVAV (4), and CyO-IKVAV. Synthesis and characterization of PLL-g-PEG(N\textsubscript{3}) copolymers. Microarray printing, binding assay, and scanning. Islet isolation and culturing. Modification of the islet surface with CyO-IKVAV. Confocal microscopy. Assessment of islet viability. This material is available free of charge via the Internet at http://pubs.acs.org.