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A simple and efficient maleimide-based approach for peptide extension with a cysteine-containing peptide phage library

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ABSTRACT

Although peptide-based molecules are known to have therapeutic potential, the generation of phage focused libraries to optimize peptides is effort-consuming. A chemical method is developed to extend a maleimide-conjugated peptide with a cysteine-containing random-peptide phage display library. As a proof of concept, a 15-mer epidermal growth factor receptor (EGFR)-binding peptide was synthesized with a maleimide group at its C-terminus and then conjugated to the cysteine-containing library. After panning and screening, several extended peptides were discovered and tested to have a higher affinity to EGFR. This strategy can have broad utility to optimize pharmacophores of any modalities (peptides, unnatural peptides, drug conjugates) capable of bearing a maleimide group

Peptide-based molecules are a rich source of substrate for use in preclinical and clinical studies.[1] Phage-displayed peptide libraries have been a reliable supply for target-selective peptide discovery since the approach was first described in 1985.[2] A common strategy for peptide presentation is togenetically fuse up to 1×10^{11} randomized peptides to the N-terminus of the gene 3 proteins on M13 bacteriophage.[3] After multiple rounds of panning and screening selection, the desired phenotypes (such as target binding) can be correlated back to the genotypes coded within the encapsulated genome of the phage particle.

Peptides isolated from a primary phage display screen often do not have the optimal properties and functionality. Therefore, these peptides need to be optimized by modifying their amino acid components in subsequent steps. These modifications include, but are not limited to, randomization of amino acid sequences,[4] chemical modification of individual amino acids,[5–7]and alteration of the peptide structure by replacing and/or modifying any internal covalent bonds such as disulfide bridges.[8,9] As such, a wealth of diversity can be incorporated into peptides to expand their utility.

One common peptide modification is an extension with additional amino acids. Previous methods describe a genetic peptide extension approach with phage display by the generation of a second generation phage library containing a number of additional randomized amino acids flanking the parental sequence.[10,11] However, if multiple peptides are to be optimized, this type of extension can be time- and reagent-consuming. In addition, the genetic approach is limited to extension of the two termini of peptides.

Chemical extension of phase-displayed combinatorialpeptide libraries enable more complex elaborations. These approaches to extend peptides with phage libraries have thus been proposed as a more efficient and versatile solution. For example, a peptide functionalized with a thioester group was extended with a phage library containing an amino-terminal cysteine residue, in a process termed the native chemical ligation.[12] In another example, the N-terminal amines of the coat proteins of filamentous phage were converted into ketone groups, which then served as chemoselective sites for conjugation to alkoxyamine-functionalized peptides in oxime reaction.[13] A different chemical strategy modified the phage surface to display aldehydes, which then reacted with synthetic molecules containing a hydrazide functional group.[14] Conjugationof lysine, aspartic acid, glutamic acid, and tyrosine residues on the surface of M13 bacteriophage with functionalized fluorescent molecules has been used in bioimaging and drug delivery.[15] Additional chemical-based extension approaches with phage particles have been investigated with the use of other reaction-specific amino acids.[16] Glycan moiety was conjugated to phage particle by oxime ligation, following oxidation of an N-terminal serine or threonine residue on a peptide phage library.[17] A variety of molecules were conjugated to phage surface by sortase-mediated modification.[18] However, a common issue with these approaches is that their technical complexity obscures their advantages.

Here we report the development of a simple and efficient maleimide-based chemical extension method. An EGFR-binding peptide is used as a proof-of-principle model system. A maleimide

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functional group is added to the carboxy-terminus of the peptide, which will become the site of conjugation with the thiol group of the cysteine residue on a peptide phage library.

The large peptide diversity of a filamentous phage library **a** consists of a Cys–Asp sequence followed by 11 randomized non-cysteine residues (Fig. 1). This library is generated using the phage display vector fUSE55[19] and designed with codon-corrected trinucleotide cassettes to ensure that only a single cysteine residue exists on each unique peptide sequence in the library. For a control reaction, the phage library**b** is generated, comprising 20 randomized non-cysteine residues using the same vector (Fig. 1). Phage are processed with standard phage protocols.[20] The presence of single cysteine residues in library **a** and the absence of cysteine residues in library **b** are confirmed by DNA sequencing of at least 200 representative members of each library. The diversities of **a** and **b** are estimated to be 0.5×10^9 and 5×10^9, respectively. Due to technical limitation common to phage library production, these numbers are below the theoretical diversities of 10^{14} sequences for **a** and 10^{25} for **b**.

In our chemical extension strategy, library **a** is conjugated via their cysteine residue to maleimide-containing peptides. To evaluate the maleimide reaction efficiency, the commercially available maleimide-PEG_2-biotin molecule (Thermo Scientific # 21902) is used as an analytical reagent. The maleimide reaction is measured by the binding of biotin to streptavidin-coated plate (Thermo Scientific # 15121). Briefly, 1×10^{12} phage virions from library **a** are incubated with 0 or 1000 pmol of maleimide-PEG_2-biotin at 22 °C for 2 h. The biotin/phage conjugates are purified with disposable PD10 desalting columns (GE Healthcare # 17-0851-01) and applied on a streptavidin plate. Binding is detected by phage ELISA with horseradish peroxidase (HRP)-conjugated Anti-M13 monoclonal antibody (GE Healthcare #27-9421-01). As a negative control, the same reactions are performed with library **b**, which contains no cysteine residues. Reactions with library **a** produce higher binding to the streptavidin plate compared to the reactions with library **b**, suggesting that maleimide conjugation occurred preferentially with the cysteine-containing library **a** (Fig. 2A). A second approach is taken to quantitate the maleimide reaction. In this assay, we treat library **a** with maleimide-PEG_2-biotin or with DMSO solvent control. Each sample is applied on streptavidin-plate for binding, and the soluble, unbound fraction is used to infect K91 bacterial cells to determine phage titer. The comparison between the two reactions is then used to calculate the maleimide reaction efficiency with the following formula:

Reaction efficiency (%) = 100−[(A ÷ B) × 100].

A = number of unbound colony forming unitsfrom maleimide-PEG2-biotin reaction.

B = number of unbound colony forming units from DMSO reaction.

In eight independent experiments, the efficiency varies from 10% to 40%, with an average of 20% (Fig. 2B). Therefore, the diversity of the conjugated library is expected to be 1×10^8, which is approximately fivefold lower than expected initially. One possible explanation for this suboptimal conjugation reaction is the nature of the phage library, in which the cysteine residues may form internal or inter-phage disulfide bonds, instead of with the maleimide group. To investigate whether the conjugation reaction is biased toward any specific peptide sequences in the library, similar maleimide–PEG_2–biotin conjugation reactions are performed with library **a** and phage clones **c**–**f** of different peptide lengths and sequences. The streptavidin binding experiment shows that the biotin–phage conjugates are present in approximately equal amounts for all of the samples being analyzed (Fig. 2C). We concluded that there is no bias toward clonal sequences of different lengths from 5 to 26 amino acids.

The receptor tyrosine kinase EGFR is chosen as a model system to explore the chemical peptide extension approach. The activity of EGFR is regulated by the binding of its ligand, the epidermal growth factor (EGF). Our laboratory haspreviously identified an EGFR-binding cyclic peptide **1** (amino acid sequence: SEC-LPSKADWLECIL) (Fig. 3) that has the ability to displace the binding of EGF to EGFR (data not shown). A lysine residue is added to the carboxyl terminus of **1**, and a maleimide group is directly tethered to the amine group on the side chain of that lysine residue to create peptide **2**.

Peptide **2** is conjugated to library **a**. Briefly, 1×10^{12} phage virions are incubated with 1000 pmol of **2** in a total volume of 200 μL,

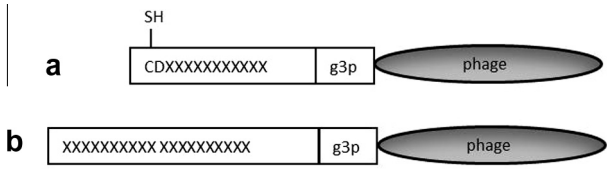

Figure 1. Phage libraries (a, b). 'CD' is Cys–Asp sequence. 'SH' is thiol group. 'X' is non-cysteine residue. 'g3p' is M13 gene 3 protein.

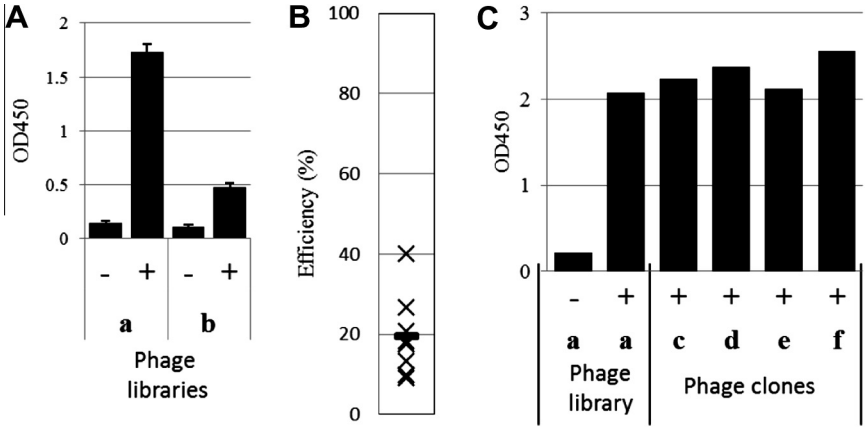

Figure 2. Maleimide conjugation efficiency with maleimide-PEG2-biotin reagent. (A) Phage library **a** reacts more with maleimide group. Libraries (a, b) are reacted with 0 pmol (−) or 1000 pmol (+) of maleimide-PEG2-biotin and tested for binding on a streptavidin-coated plate. (B) Percent conjugation efficiency as calculated from biotin-streptavidin binding and K91 bacterial cell infection. 'X' denotes eight independent experiments. Dash line denotes the average. (C) Length- and sequence-independence of the reaction. Phage library **a**, clones **c** (CDYYREPQVNKHW), **d** (CDVTKPDDNCYPV), **e** (CDVSLHKYYTDAGQSGEPAVSAETYF), **f** (CDAAN). Symbols '−' and '+' denote the absence and presence of maleimide-PEG2-biotin. Phage-binding ELISA assays are independently repeated at least three times.