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Screening of Large Molecule Diversities by Phage Display

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Abstract: Molecules with tailored binding specificities are needed for many purposes such as the development of therapeutics, the detection and purification of biomolecules or in chemical biology for the study and manipulation of biological systems. With phage display technology, polypeptides with binding affinities to targets of interest can be isolated from billions of polypeptide variants with a modest amount of effort, time and cost. The technology was initially used for the generation and screening of peptide and antibody libraries and was later applied to many different protein scaffolds. More recently, chemically and structurally diverse molecule libraries were generated by chemically modifying phage-displayed polypeptides. In this article, the different classes of natural and non-natural structures that can be encoded and screened by phage display are reviewed with a special focus on bicyclic peptides that we routinely generate in our laboratory.

Keywords: Antibody · Combinatorial library · Cyclic peptide · Directed evolution · Peptide · Phage display · Protein scaffold

Introduction

In phage display, polypeptides are physically linked to their encoding DNA allowing their identification after extraction from large molecule libraries (>10^9 different polypeptide structures).[1] The technology was pioneered by G. P. Smith who first displayed a polypeptide on the surface of filamentous phage[2] and had later isolated linear peptides binding to an antibody paratope from a peptide phage library.[3] In the early 1990s, Winter and co-workers succeeded in displaying the variable regions of antibodies on filamentous phage[4] and showed that antigen-specific antibodies can be selected from libraries derived from mouse B cells.[5] Soon after, the first human antibody was isolated with this technique[6] and in 2003, the first phage-selected monoclonal antibody entered clinical use (adalimumab).[7] Later, phage display was applied to a large range of structurally diverse protein scaffolds and the number of building blocks used in phage polypeptide libraries was expanded beyond the 20 common amino acids by incorporating unnatural amino acids or by chemically modifying polypeptides on phage. Today, many polypeptide phage libraries are available that can be screened to generate binders. The choice of library is critical and needs to be based on the properties the binders require. As discussed in the following sections, the different polypeptide formats vary considerably in their properties such as stability, solubility, expression yield, access to chemical synthesis, immunogenicity, plasma half-life or tissue penetration.

Peptide and Protein Phage Libraries

Linear peptides were the first structures to be evolved by phage display and typically bind to their targets with rather weak affinities in the millimolar to micromolar range (Fig. 1a).[10] Significantly better binders are cyclic peptides that can be generated by connecting two cysteine residues flanking random peptides on phage, with a disulfide bridge (Fig. 1b).[10,11] Peptides are an attractive molecule format because they can be chemically synthesized and easily conjugated to functional groups such as fluorescent labels or affinity tags. If used as therapeutics, their small size allows tissue penetration, reduces the risk of immune reactions and potentially offers different application options. Antibodies have widely been used as scaffold to generate binders by phage display.[12] Their antigen-binding sites have evolved by nature to bind to diverse biological structures and selections with antibody phage libraries generally yield binders with high affinities. On the surface of phage, only fragments of the antibodies are displayed, such as the Fab fragment which is composed of one constant and one variable domain of each of the heavy and light chain, or the single-chain variable fragment (scFv) which is built of the variable domains connected by a flexible peptide linker (Fig. 1c).[13] After their isolation in phage selections, the antibody fragments can be genetically reassembled into full size IgGs or any other antibody format. Good binders can also be generated based on non-immunoglobulin protein scaffolds that are mutated in surface regions and screened by phage display. Many of these scaffolds were chosen or developed because of favorable properties including high expression yields in bacteria, high stability, redox-stability and low aggregation propensity. Protein scaffolds that combine more than one of these properties and that had yielded binders to many targets include the Z domain of protein A (affibody commercialized by Affibody), the lipocalin (anticalin commercialized by Pieris) and the ankyrin repeat protein (DARPins commercialized by Molecular Partners).[14] More specialized scaffolds are the zinc finger proteins used for the generation of binders to DNA to regulate gene expression[15] or the Kunitz domain applied for the evolution of specific serine protease inhibitors.[16]

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Phage Polypeptide Libraries with Unnatural Amino Acids

To incorporate special chemical functionalities into polypeptide libraries, unnatural or rare natural amino acids were inserted co-translationally into phage displayed peptides. Sandman *et al.* inserted selenocysteine into peptides on phage using a natural opal suppressing tRNA.[17] The selenocysteine can be used to covalently tether molecules to the polypeptide while leaving other nucleophilic groups of phage coat proteins unmodified.[18,19] Tian *et al.* applied an amber suppressor tRNA and a mutant tyrosyl-tRNA synthetase of *Methanococcus jannaschii* to display peptides with tyrosine derivatives (Fig. 2a).[20] In a similar approach based on the same amber suppression system, Liu *et al.* incorporated various tyrosine derivatives into a scFv antibody fragment and isolated from a phage library a scFv fragment with a sulfonated tyrosine in the paratope (Fig. 2a).[21] Hopefully, further technical developments will allow in the future the incorporation of several unnatural amino acids in a combinatorial fashion and the generation of chemically highly diverse phage-encoded polymer libraries.

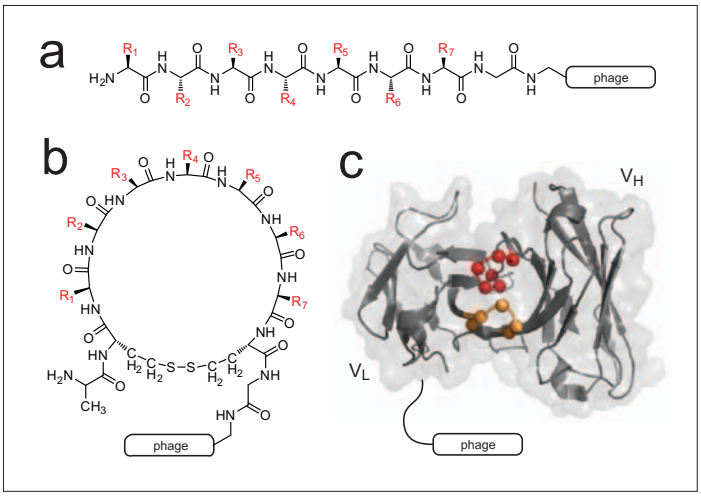

Fig. 1. Phage display peptide and antibody libraries that have yielded binders to numerous targets. Linear (a) and cyclic (b) peptide library Ph.D.-7 and Ph.D.-C7C commercially provided by New England Biolabs (NEB).[8] The side chains (R) of amino acids randomized in these libraries are highlighted in red. (c) Synthetic antibody scFv fragment library ETH-2.[9] Indicated in red and orange are the amino acid positions that are randomized in the complementarity determining region 3 (CDR3) of the variable heavy (VH) and light domain (VL). The unstructured linker connecting the VH and VL is not shown.

Chemical Modification of Phage-encoded Polypeptides

The co-translational incorporation of non-natural building blocks into phage peptides is limited to amino acids that are compatible with tRNA synthetases and that are accepted by the ribosome. To allow other chemical structures in phage peptide libraries, small molecules can be post-translationally linked in chemical reactions to phage peptides. Jespers *et al.* attached a fluorophore to a cysteine residue in the antigen-binding site of an antibody scFv fragment library to evolve an optical biosensor (Fig. 2b).[22] By native chemical ligation, Dwyer *et al.* linked a synthetic N-terminal fragment of a protein containing unnatural amino acids to a library of mutagenized C-terminal fragments of the same protein displayed on phage.[23] In a biological encoding system similar to phage display (mRNA display), Li and Roberts linked a pendant penicillin moiety to an encoded peptide library.[24] Selections with this hybrid peptide–drug library resulted in improved inhibitors of a *Staphylococcus aureus* penicillin binding protein 2. Tian *et al.* post-translationally tethered an alkyne-derivatized fluorophore to a *p*-azidophenylalanine which was, as described in the previous paragraph, co-translationally incorporated into a peptide on phage (Fig. 2b).[20]

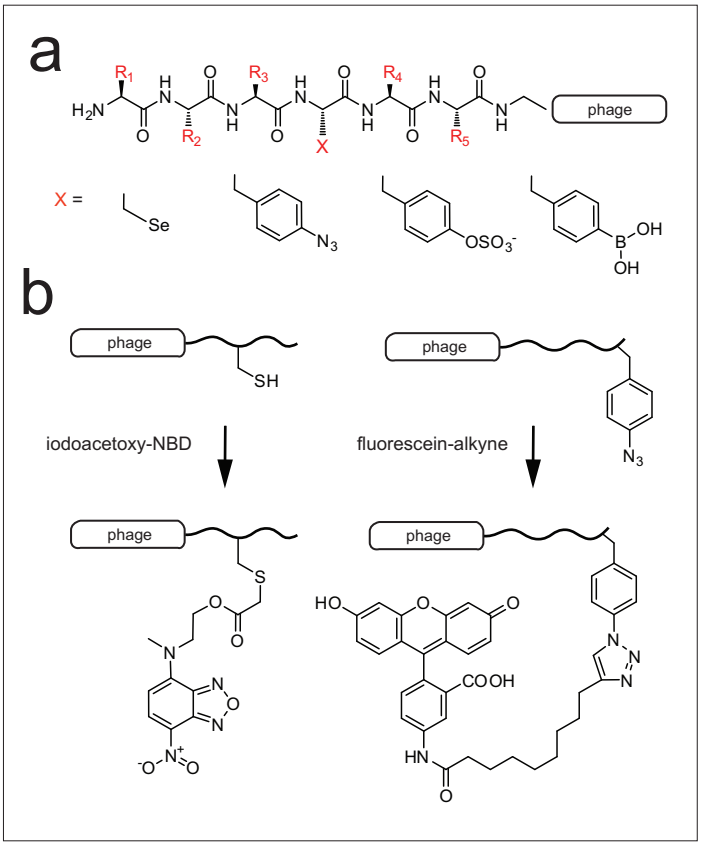

Fig. 2. Phage polypeptide libraries with synthetic building blocks. (a) Co-translational incorporation of unusual amino acids. Shown is an example of a phage peptide library with randomized amino acid positions (side chains R are indicated in red) and selenocysteine[17] or an unnatural amino acid in the indicated position (X). Amino acids from left to right: selenocysteine, *p*-azidophenylalanine, sulfotyrosine and 4-boronophenylalanine.[20,21] (b) Post-translational chemical modification of peptides on phage. Indicated are a iodoacetoxy-fluorophore (NBD) conjugate that is linked to a cysteine residue of a phage peptide[22] and a fluorescein alkyne derivate that is linked to the unnatural amino acid *p*-azidophenylalanine displayed on phage.[20]

Phage-encoded Bicyclic Peptides

Linear peptides displayed on phage can be converted into bicyclic structures by covalently linking three amino acids of the peptide to a small molecule in a chemical reaction.[23] For example, the thiol groups of two cysteine residues at either ends of the peptide and of a third one in the middle can be reacted selectively and quantitatively with the small molecule tris-(bromomethyl)benzene (Fig. 3a). First selections with bicyclic peptide libraries (Fig. 3b) were performed targeting the two serine proteases plasma kallikrein and cathepsin G and yielded potent inhibitors with binding affinities in the low nanomolar range (Fig. 3c).[25] The approach is now routinely applied in our laboratory and we have recently isolated bicyclic peptides against other target classes such as receptors (unpublished data).

Compared to monocyclic peptides, the bicyclic structures can potentially bind *via* two peptide loops to targets which could explain the higher binding affinities found for the bicyclic peptides. In fact, phage-selected bicyclic peptides frequently show consensus sequences in both of the two peptide loops suggesting that both of them participate in the binding. Due to their branched structures and the close proximity of the two peptide rings, the bicyclic peptides are conformationally more constrained than monocyclic peptides. A peptidic ligand with a rigid, pre-organized structure has the advantage that less entropy is lost upon binding, resulting in a higher binding affinity. The relatively constrained nature of bicyclic peptides may also increase their proteolytic stability which is important in therapeutic applications or in biological systems if applied as probes. In assays in human plasma performed *ex vivo* bicyclic peptides were found to remain active suggesting that they are rather stable.[25] To generate high affinity binders to different target classes, it is essential to screen large and structurally diverse bicyclic peptide libraries. In bicyclic peptides, basically two elements can be altered: the central small molecule core and the peptide loops. Since the central core cannot be genetically encoded and hence not easily varied in a combinatorial fashion, the peptide loops are preferentially altered in length and amino acid composition. We have recently generated large bicyclic peptide diversities with variable loop lengths and future phage selections will show which bicyclic peptide formats yield the best binders to different target classes.

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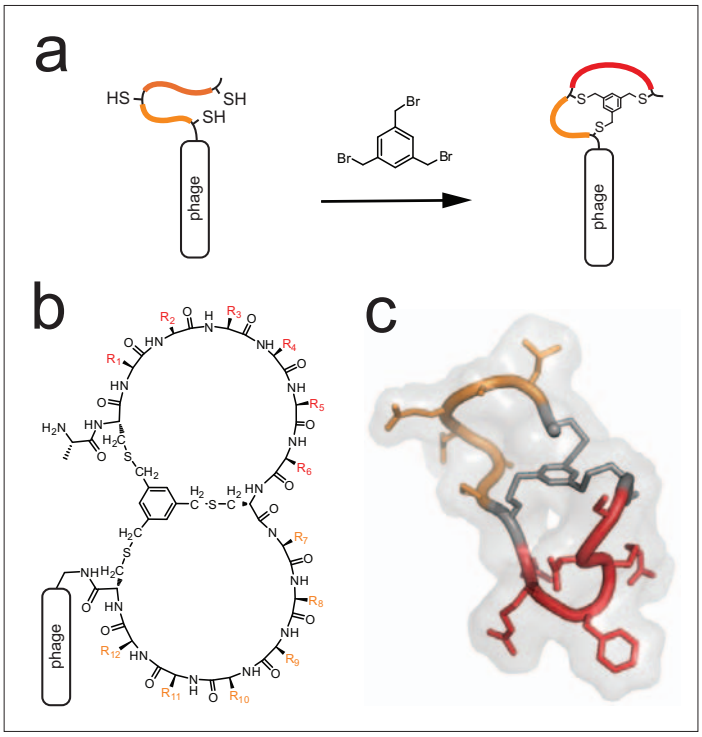

Fig. 3. Bicyclic peptides. (a) Linear peptides containing cysteine residues at both ends and another one in the middle are displayed on phage and chemically connected by reacting with tris-(bromomethyl) benzene. The chemical modification leads to branched, multicyclic structures. (b) Schematic representation of a bicyclic peptide phage library. The side chains (R) of the twelve amino acids are indicated with red (first loop) and orange (second loop). (c) Three-dimensional structure of a bicyclic peptide.

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