Mirror-image Phage Display: Aiming at the Mirror

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1. Introduction

For many years, researchers have been trying to rationally design drugs from scratch based on high-resolution three-dimensional structures of a given target molecule. Until now, however, this approach has worked only for a disappointingly small number of targets.[1] Thus, drugs are still almost exclusively identified by screening of very large substance libraries. What has improved, however, is the speed of screening compound libraries, which may contain several million different compounds.[2–4] But even with the help of recently developed high-speed and ultra-high-throughput techniques, such chemical compound libraries are very costly and expensive to screen. Maintainence and expansion of these compound libraries is demanding too.

Biological compound libraries have been known for almost two decades. Such libraries consist of nucleic acids, peptides, or proteins with variations in the sequence of their monomer building blocks. These variations are obtained by chemically synthesizing oligonucleotides using mixtures of different nucleotides for each synthetic step. For example, a randomized decamer oligonucleotide yields 4^{10}, that is, more than one million different substances.

A striking advantage of these libraries is their ability to be easily replicated. For example, can be DNA replicated by the polymerase chain reaction (PCR).[5] For replication of RNA, additional steps (reverse transcription and transcription) are needed. Screening of these biological libraries is achieved by enrichment of library members that show properties as close as possible to any desired attribute, for example, binding to a given target molecule. After several cycles of amplification and screening, one ends up with those molecules of the original library whose properties are very close to those desired. For RNA libraries, the respective method is well known as "SELEX"[6, 7] and the resulting molecules are known as aptamers.[8–11]

Since peptides cannot be replicated directly, more sophisticated methods were developed to link genotype (variations in the nucleotide sequence of the gene) and phenotype (the resulting variations in the amino acid sequence of the peptide product). Several solutions to this problem were achieved, for example phage display,[12] plasmid display,[13] polysome display,[14] and ribosome display.[15]

Such "biological" libraries do have some advantages over chemical compound libraries. They do not need high-throughput technologies for screening. These libraries can be easily obtained and screened with technology present in any molecular biology laboratory and they can consist of up to 10^{15} different molecules.

Thus, it is not surprising that SELEX and phage display in particular are techniques that are widely used in basic research, for example to determine epitope specificities of antibodies,[16] to characterize binding specificities of nucleic acid binding proteins,[17] and to identify potential binding partners of a given macromolecule.[18] Further, directed evolution of enzymes (proteins and RNAs) with altered or completely novel substrate specificities can be similarly done based on construction and screening of large libraries of the polymer to be optimized.[19–22] In addition, the stability of proteins can be optimized.[23, 24] Each amplification step may even contain an additional step introducing further variations to the enriched sublibraries, for example by error-prone PCR or in vitro recombination.[25]

There are, however, some striking disadvantages of nucleic acids and peptides identified from screening biological libraries that render the selected molecules practically useless for therapeutic and most diagnostic applications in living animals or humans. Biological polymers consisting of naturally occurring building blocks, like nucleic acids (consisting of D-enantiomers of nucleotides), but also peptides (built from L-amino acids) are prone to degradation by nucleases and proteases, respectively. Further, peptides often induce immune responses that impair single, and particularly multiple use in animals or humans. Biological compound libraries, however, necessarily consist of D-nucleotides and L-amino acids.

Newly developed mirror-image techniques[26, 27] circumvent these disadvantages and, thus, may be a way to make use of the

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advantages of biological libraries while avoiding their disadvantages. In this minireview we focus on one of these "spiegelmer" techniques and its applications: mirror-image phage display.[26]

2. Phage Display

Among the techniques that were developed to construct libraries of peptides with randomized amino acid sequences and to screen them for ligands for a given target, "phage display,"[12] is currently the most popular one.[16, 18, 28, 29] The peptide library is presented on the surface of bacteriophages ("phage display") as a fusion of a peptide and a phage coat protein. This technique is one of several ways to physically link the presented peptide (phenotype) to the DNA sequence coding for its amino acid sequence (genotype). Diversity can be introduced to the presented peptide or protein by combinatorial mutagenesis of the peptide-coding part of the fusion gene. Extremely large numbers of phages each displaying a different peptide can be constructed, replicated, selected, and amplified in a process called "biopanning" (Figure 1). The libraries are

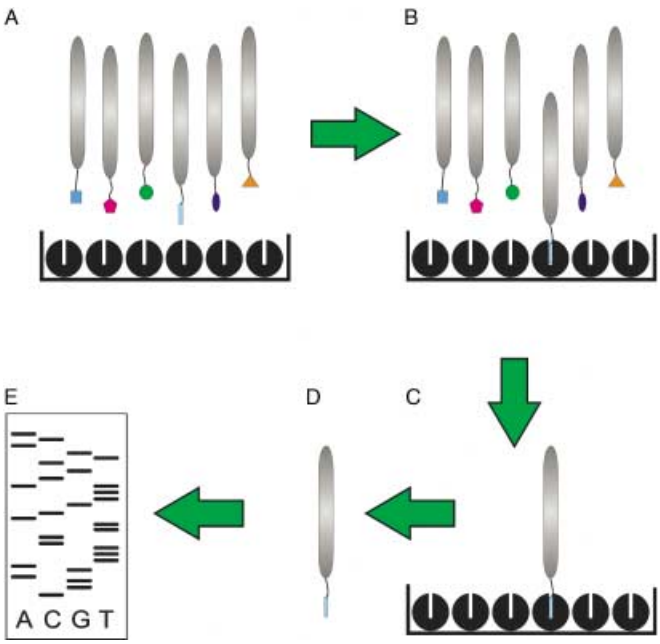

Figure 1. Scheme of a phage display screening procedure. A) A huge library of different peptides (symbolized by colored shapes) is presented fused with a coat protein found on filamentous phages (long grey symbols) and applied to a surface-immobilized target molecule (black round symbols). Some of the peptides will bind to the target (B). The rest are washed off (C). Peptides that show affinity to the target are eluted (D). The obtained sublibrary can be amplified and re-applied to the target (B) or the sequence of the displayed peptide can be determined by DNA sequencing of the fusion protein (E).

incubated with a target molecule either as an immobilized target or prior to capture of the complex on a solid support. As in affinity chromatography, noninteracting peptides and proteins are washed off and the interacting molecules are subsequently eluted. The hereby enriched population of phages displaying target-interacting peptides or proteins can be amplified by bacterial infection to increase the number of copies of the molecules. The screening/amplification process can be repeated to further enrich those library members with higher affinity for the target. The result is a final peptide population that is dominated by the sequences that bind the target best. Inherent to the technique is the fact that these peptides solely consist of the 20 proteinogenous L-amino acids.

3. Mirror-Image Phage Display

With the exception of glycin, all amino acids are chiral. The term chiral means that there are L- and D-enantiomeric forms that are not identical, but are related to each other like image and mirror image, or left and right hand.

Consequently, peptides and proteins are chiral. Two chiral molecules, for example, protein A and protein B, that tightly bind to each other will do so only if the correct enantiomers of both molecules are present. Thus, protein A would not bind to the mirror image of protein B. The mirror image of protein A, however, would bind to the mirror image of protein B. This principle has been demonstrated for the example of HIV protease and its substrate.[30]

This is the idea on which the method of mirror-image phage display[26] is based. During a "common" phage display approach a peptide library is screened to identify a peptide (e.g. by biopanning) that binds to a given target protein as it exists in a living cell. The target protein then consists of L-amino acids and is therefore labeled "L-target" (Figure 2 A). An inherent property of the phage display method is that the identified peptide consists of L-amino acids too, and thus is labeled "L-peptide".

To run a mirror-image phage display, biopanning is carried out against the mirror image of the target protein. This mirror-image

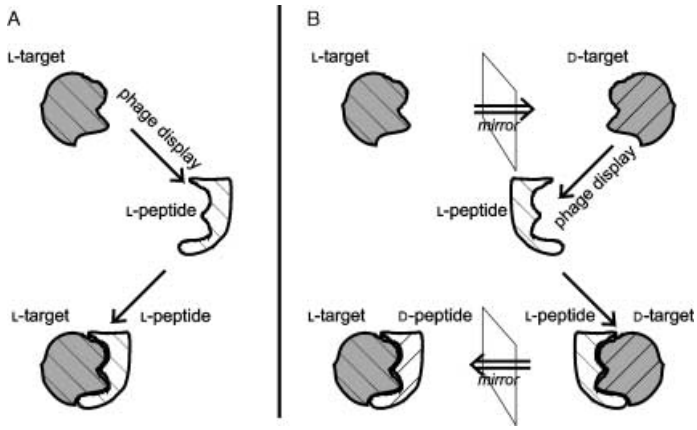

Figure 2. Schematic representation of the principles of common phage display (A) and mirror-image phage display (B). A) A phage displayed peptide library is screened for peptides that bind to a given target protein that consists of L-amino acids (L-target). Any identified peptide that binds to the L-target will consist of L-amino acids too (L-peptide). B) The phage-displayed peptide library is screened for peptides (L-peptide) that bind to the mirror image of the target protein (D-target). Any identified peptide will consist of L-amino acids (L-peptide) and will bind to the D-target. The mirror image of the resulting complex of D-target and L-peptide shows the complex between the original L-target and a peptide consisting of D-amino acids with the same sequence as the L-peptide. The resulting D-peptide is easily accessable by chemical synthesis.

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molecule has to be chemically synthesized from D-amino acids in the same sequence as the L-amino acids in the L-target. This molecule, called the D-target, is used as a bait for the biopanning procedure (Figure 2B). As in common phage display, one ends up with peptides that consist of L-amino acids. Such an "L-peptide" binds to the D-target. Thus, the "D-peptide", the mirror image of the L-peptide, will bind to the mirror image of the D-target, which is identical to the original L-target. The D-peptide is easily accessable by chemical synthesis of a peptide with D-amino acids in the same sequence as that of the amino acids in the L-peptide.

Mirror-image phage display[26] allows the use of phage display to ultimately identify peptides that bind specifically to a given target and consist solely of D-amino acids. D-amino acid peptides are known to be less protease sensitive,[31, 32] more resistant to degradation in animals,[33, 34] and less or even not at all immunogenic,[34] as compared to an L-amino acid peptide.[26, 35–37] Hence, D-peptides identified by mirror-image phage display may well be suited to diagnostic and therapeutic use in living animals and humans. The only remaining pitfall is that the mirror image of a given target molecule needs to be synthetically available, which restricts application of the method to smaller proteins. Many potential target proteins or protein domains, however, are well within the range of the technique.

4. Only A Few Examples So Far

The principle behind mirror-image phage display was developed and proven to be correct by Schumacher et al.[26] They used the mirror image of the c-Src homology 3 (SH3) domain of chicken Src kinase to screen for phage-displayed L-peptides that bind to D-Src-SH3. The peptide library was constructed from a randomized 10 amino acid peptide, flanked by either a serine or a cysteine residue on either side, and fused to the amino terminus of the minor coat protein of the bacteriophage fd. The library was designed to include a large proportion of sequences that support disulfide bond formation to increase the number of cyclic peptides. The peptides that were obtained after screening for binding to D-Src-SH3 did not contain polyproline sequences, as is typically found in L-SH3-domain-directed phage display screenings. Thus, the authors concluded that different binding modes exist for the binding of D-peptides and L-polyproline peptides to L-Src-SH3.

Interestingly, the binding site for one of the selected D-peptides partially overlaps with the site for physiological L-polyproline-peptide ligands of the L-Src-SH3 domain, as determined by nuclear magnetic resonance spectroscopic experiments with the D-peptide and L-Src-SH3 domain. A dissociation constant (K_D) of 63 μM for the interaction between the D-peptide and L-Src-SH3 was determined by competitive enzyme-linked immunosorbent assays (ELISA) and tryptophan fluorescence spectroscopic titrations. Indeed, the D-enantiomer of the identified cyclic peptide bound to L-Src-SH3 and the principle behind mirror-image phage display was proven to be correct.

The same research group published another potential application of the mirror-image phage display technique for viral targets.[37] The human immunodeficiency virus type 1 (HIV-1) gp41 protein promotes viral entry by mediating the fusion of viral and cellular membranes. As the full-length gp41 protein is too large to be synthesized as the D-enantiomer, a part of gp41 (IQN17) that resembles an important binding pocket for gp41 activity was used in its D-enantiomeric form as a bait for phage display screening. The phage-displayed peptide library used for this study was the same as that used in the above-mentioned investigation.

The screening procedure yielded several peptides that contained a consensus sequence that had conserved amino acids at four positions and were flanked by a cysteine residue on both sides of the sequence. The identified D-peptides were shown to be biologically active with respect to inhibition of membrane fusion, as measured by cell/cell fusion assays and HIV infection assays. IC_{50} values for viral entry in the micromolar range (11–270 μM) were determined by a recombinant luciferase assay. The D-enantiomer of one of the selected peptides inhibited HIV-1 infection in vitro and bound exclusively to the gp41 pocket (IQN17), as shown by structural investigations using X-ray crystallography and NMR spectroscopy. In addition, these structural studies showed direct interactions between the conserved residues of the D-peptide and IQN17. The authors of this study emphasized the possibility of rapid discovery of a new class of orally bioavailable drugs.

Cell-surface carbohydrates were used as targets in another mirror-image phage display application.[38] Carbohydrates on cell surfaces are part of molecular recognition processes, which are involved in various cell interactions. Such cell-surface carbohydrates play a role in bacterial and viral infections, inflammation, and cancer metastasis, and are thus promising therapeutic targets. The authors used the enantiomers 3-deoxy-α-L-manno-2-octulosinic acid (L-KDO) and L-sialic acid, as well as the respective disaccharide (L-sialo-L-KDO) as targets for the screening of a phage-displayed single-chain antibody library. Dissociation constants were determined by surface plasmon resonance to be in the nanomolar to micromolar range. Further, the authors postulated a polyvalent interaction between the selected peptides and the respective target.

In an effort to enable novel approaches to diagnosis and therapy of Alzheimer's disease (AD), we recently applied mirror-image phage display to identify D-peptides that specifically bind to amyloid peptide Aβ(1-42).[39] Aβ is the major component of the amyloid plaques found in the brains of people suffering from AD. So far, these amyloid plaques are only detectable *post mortem*.[40]

Aβ exists in a soluble overall helical structure[41] and an aggregated overall extended (β-sheet) structure.[42] The β-sheet conformation is found in the fibrils that build up amyloid plaques. These plaques are stainable with chromophores, such as Congo red and thioflavin, that show high affinity for β-sheet structures irrespective of the identity of the peptides contained therein.

To develop a novel and specific marker for AD amyloid plaques, we screened a randomized 12-mer peptide library presented on M13 phages for peptides with binding affinity for the mirror image of Aβ(1–42). Upon screening, we identified a clearly dominating amino acid sequence. The mirror image of

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this peptide (D-pep) was shown to bind Aβ(1–42) with a dissociation constant in the submicromolar range. Furthermore, in brain tissue sections derived from patients that suffered from AD, amyloid plaques were stained specifically with a fluorescence-labeled derivative of D-pep (Figure 3). D-pep is specific for Aβ plaques and did not stain amyloid deposits consisting of peptides other than Aβ.

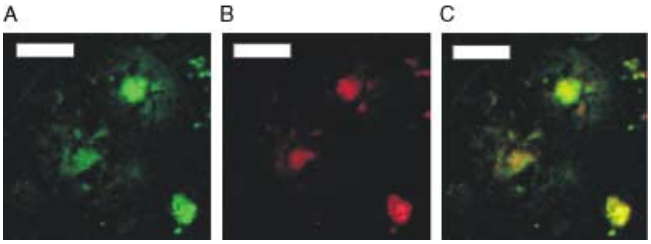

Figure 3. *Fluorescence microscopy images of a brain tissue section derived from a patient who suffered from AD. Co-staining was carried out with fluoresceinylated D-pep and an anti-Aβ(1–16) antibody detected with Cy3-labeled secondary antibody. The pictures were taken under two different filter conditions (A, fluorescein, excitation 450–490 nm, emission 515–565 nm; B, Cy3, excitation 510–560 nm, emission starting at 590 nm; C, superposition of the two pictures). The white bars indicate a length of 20 μm. The superposition clearly shows that amyloid plaque staining by D-pep is indistinguishable from that by anti-Aβ(1–16) antibody.*

D-pep may be a very suitable probe for the detection of amyloid plaques in living humans or animal models used to search for compounds that are suitable for AD therapy. D-pep may be synthetically combined with any marker employed for physiological detection or medical imaging techniques. Future studies are required to find out whether D-pep or derivatives thereof are able to cross the blood–brain barrier. The fact that D-pep is fully synthetic will simplify any variation of its structure.

5. Summary and Outlook

Mirror-image phage display[26] is one example of the so-called "spiegelmer" techniques.[27] Based on the use of perfect mirror images (enantiomers) of the original target molecules, mirror-image phage display delivers peptides consisting of D-amino acids. If these D-peptides have the expected properties with respect to immunogenicity and biostability, one might expect that mirror-image phage display has been applied for numerous targets since its invention. The fact that only a few examples of application of mirror-image phage display are described in the literature[26, 37, 38] leads to the inevitable question of why mirror-image phage display applications are rarely published. The advantages of the technique are quite clear: To search for a ligand for a given target, it is possible to start with a "biological" library that can easily be constructed and screened for binding to the target. One does not need high-throughput technologies for screening, and yet ends up with D-amino acid peptides that are considered to be rather protease resistant and mostly non-immunogenic[26, 31–37] as compared with L-amino acid peptides. The increased protease stability of D-peptides is also used in the so called "retro-inverso peptide" approaches, which use peptides consisting of D-amino acids with reversed amino acid sequence to mimic the original L-peptide.[43]

Overall, D-peptides derived from mirror-image phage display may be well suited to use for diagnostic and therapeutic applications in living animals and humans. The only disadvantage is the need for a given target molecule to be synthetically available, which restricts the technique to smaller targets. Many potential targets, however, are well within the range of the technique. The future will show whether spiegelmer techniques in general can be valuable tool for drug research. The comparatively low costs and the speed of the whole process may be of particular interest for the use of spiegelmers in the diagnosis and therapy of so-called "orphan diseases",[44] those diseases that affect only a small number of people, which makes this a nonprofitable target group for pharmaceutical companies.

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