SELECTION OF PCB-BINDING PHAGES AS BIORECOGNITION ELEMENTS FOR THE DEVELOPMENT OF A BIOANALYTICAL DETECTION APPROACH.

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Introduction

Recently a lot of research effort is devoted to the development of rapid, accurate and cost-effective bioanalytical approaches, which allow a real time, *in situ* screening of chemical compounds in complex matrices. Affinity based biosensors are frequently used as a bioanalytical approach. Affinity based biosensors are very sensitive, selective and versatile since affinity-based biorecognition elements can be generated for a wide range of targets. Antibodies have long been the most popular affinity-based biorecognition elements. However, antibodies have some limitations: polyclonal antibodies are relatively cheap, but animals have to be immunized to obtain them. Moreover, specific antibodies require isolation and purification. Besides the ethical problems of the use of animals, it is also difficult to generate antibodies for toxic compounds or small compounds that cannot elicit an immune response. Another important disadvantage of polyclonal antibodies is the non-specific binding. These disadvantages can partially be minimized by using monoclonal antibodies, but hybridoma techniques are very expensive and time-consuming. Moreover, both poly- and monoclonal antibodies lose their binding properties under unfavorable environmental conditions and therefore cannot sustain the conditions in which environmental or food analyses are performed. Recent evolutions in biotechnology, nanotechnology and surface chemistry, have created the possibility to develop novel more robust affinity-based biorecognition elements that can overcome the limitations of antibodies. One such class of substitute antibodies which can be used as an innovative biorecognition element are phages[1-3]. Their outstanding characteristics, such as high affinity and specificity, fast, cheap and animal-friendly manufacturing process with low batch-to-batch variations and stability makes them appropriate to develop rapid, accurate and cost-effective bioanalytical approaches, which make real time and *in situ* measurements in complex matrices possible. The potential of landscape phages as biorecognition elements for food and environmental monitoring is already demonstrated for the detection of pathogens, like *Salmonella typhimurium*[4] and *Bacillus anthracis*[5-6] and the reporter protein β-galactosidase[7-8]. In this study the potential of landscape phages for the detection of small chemical compounds is explored, using PCBs as targets.

Materials and methods

PCB-binding phages were selected from the f8/8 landscape phage library[1]; each phage in the library displays an 8-mer peptide at all 4000 major coat proteins pVIII. Phages with a high affinity and specificity for the targets (a coplanar PCB 2,3',5,5'-Tetrachlorobiphenyl (PCB72) and a non-coplanar PCB 2',3',4',5,5'-Pentachlorobiphenyl (PCB86)) were isolated from this phage library using an affinity selection procedure, as illustrated in Figure 1. To allow an easy separation between bound and unbound phages in the selection procedure, the PCBs were immobilized on magnetic beads. Therefore, PCBs in a hydroxylated form were used. The presence of hydroxyl-groups enables the coupling of the PCBs onto carboxylic acid (COOH) conjugated magnetic beads, without changing their binding properties[9]. The first step in the selection procedure is a counter selection, to eliminate non-specific binding phages with affinity for the background. Phages bound to plain beads or tubes are removed in this counter selection step and the remaining phages are used in a selection step. In this selection step phages are incubated with beads coated with PCB and after washing away the unbound phages, the phages bound to PCB are eluted. This new phage pool is amplified in *E.coli* and used for a next round of affinity selection. After each round of affinity selection more phages with specific affinity for PCB will be obtained. After the fourth round of selection, the peptides displayed on the selected phages were identified by sequencing the recombinant gene gpVIII of the selected phages and their translation into the structure of fusion peptides. The binding properties of the selected phages were characterized with SPR.

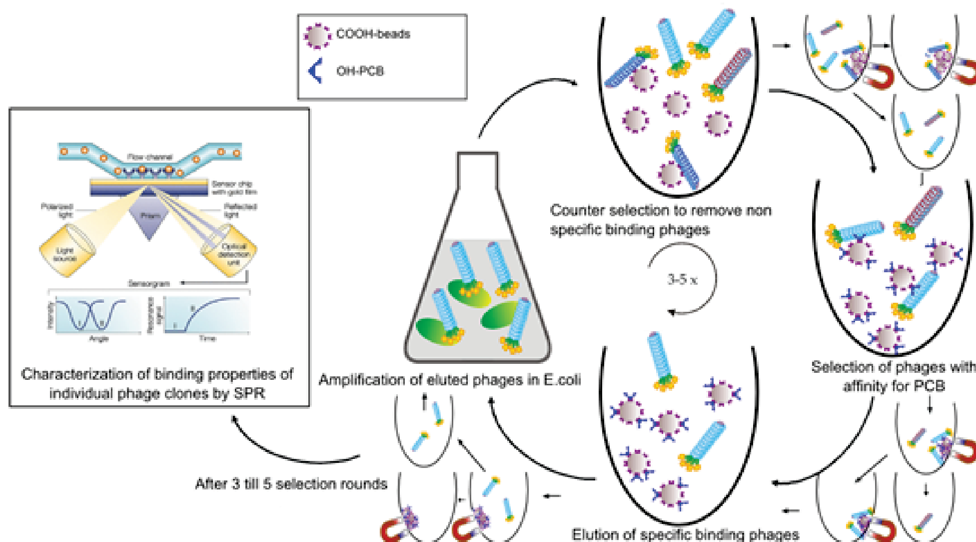

Figure 1: Selection of phages with affinity for PCBs

SPR enables the study of binding interactions in continuous real-time mode without labeling the binding components, by detecting changes in resonance angle caused by a variation of mass on a sensor chip surface. To characterize the binding interaction between the phages and PCB, the phages were immobilized on a sensor chip by amine coupling on COOH conjugated chips. Instead of the generally used CM5 chip, the CM3 chip was chosen, because of its shorter dextran linker, which can be advantageous when large particles like phages are immobilized. The binding of OH-PCB72, OH-PCB86 and PCB118 with the selected phages immobilized on the sensor surface was assessed, by injecting a concentration gradient of OH-PCB72, OH-PCB86 and PCB118 (from 10 µM till 400 µM) over the flow channel with immobilized phages (test flow channel). In order to assess the specific binding of the PCBs to the phages, the binding signal on the test flow channel was corrected with the background signal measured in the reference flow channel (where no phages were immobilized) and a solvent correction factor. The solvent correction was made to correct for bulk effects caused by DMSO[10], which has a high refractive index relative to water and can significantly affect the bulk response. Therefore, a calibration curve was generated, by plotting the difference in response between the reference flow channel and the test flow channel versus the reference response of 8 solutions containing DMSO between 4.5% -5.8%. Depending on the reference response of a sample, the solvent correction factor can be read from the calibration curve. This solvent correction factor, together with the background signal was used to correct binding signal and to obtain the specific binding of PCB with the phages.

Results and discussion:

Eight different peptides were identified on the surface of the selected phages with affinity for the two PCBs (Table 1).

Table 1: Peptide sequences displayed on selected phages

Peptide sequences	Number of phage clones	
	PCB86	PCB72
D S N K L S P E	3	2
D S N S K S S P(D)	1	3
D S S N K L N S(D)	2	0
L S F A A D R T	1	0
L S F A A E G E	1	0
D K N N L D N A	0	1
D K L A T Q N A	3	0
D S R S T G V A	1	0

Alignment of the peptides shows their striking similarities and divides them into four structural groups (families). The majority of the selected peptides contain the amino acid leucine; the frequency of leucine increased from 7.7% in the phage library to 9.4% in the selected peptides. This effect can be explained by involvement of hydrophobic leucine residues in binding of organic ligands. Besides leucine, the frequency of the hydrophobic amino acid phenylalanine also significantly increased from 1.8 % in the phage library to 3.1% in the selected phages. The aromatic phenyl group of phenylalanine probably also plays an important role in the interaction with phenyl groups of PCB (aromatic stacking). Using a bioinformatics program, RELIC (Receptor Ligand Contacts)[11], the selected phage clones were analyzed to identify for amino acid motifs. Three amino acid motifs were found in the selected phage clones, namely DSNxxS, SNKL and LSFAA.

The phage binding properties were assessed for each binding motif. Two phages, one which displayed peptide DSNKLSPE, which belong to the families DSNxxS and SNKL and another one with peptide LSFAADRT (family LSFAA) were characterized with SPR. During injection of PCB86 a specific binding of PCB to LSFAADRT and DSNKLSPE displaying phages was observed. A clear concentration dependent binding signal was observed for PCB86 with both tested phages (Figure 3). The ΔRU corresponding to the specific binding at the end of the injection of the PCB86 solutions was used to determine the dissociation constants (Kd). Therefore, the ΔRU values corresponding to the specific binding were plotted for various PCB86 concentrations (Figure 2). These data points were fitted by non-linear regression analysis with the help of the following equation: $y = Bmax * x / (Kd + x)$, to calculate the Kd. These Kd values were 59.83 +19.44 µM and 80.74 +21.44 µM for the binding of PCB86 with the DSNKLSPE and LSFAADRT displaying phages, respectively.

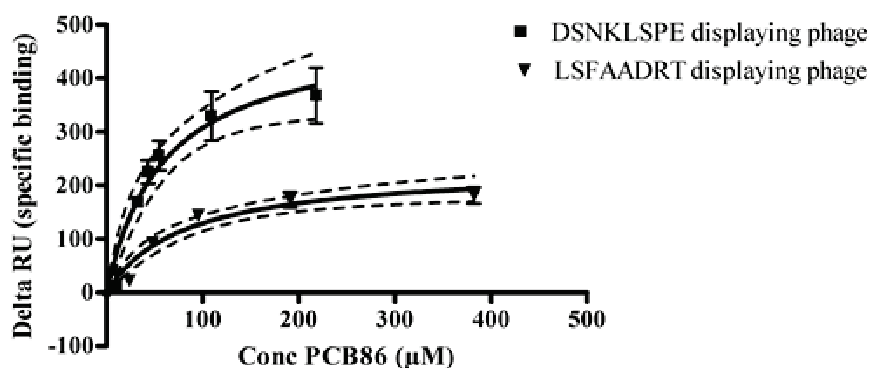

Figure 2: Specific binding of PCB86 with DSNKLSPE displaying phage (■) and the LSFAADRT displaying phage (▲) measured by SPR. The ΔRU values were obtained after injection of PCB86 solution over the test flow channel with immobilized phages and were corrected with the solvent correction factor obtained from calibration curve and the background signal measured in the reference flow channel. The curves were obtained by nonlinear regression analyses of the data points. The dotted lines represent the 95% confidence band.

Besides the binding with PCB86, the binding with PCB72 could also measured. For PCB72, no specific binding to LSFAADRT displaying phages was observed. This effect was expected, as the phages were not isolated during the selections with PCB72 (data not shown). However, no specific binding was observed to DSNKLSPE displaying phages (data not shown), which was expected to bind PCB72, since it was isolated in the selection procedure with immobilized PCB72. This can indicate that the phage has a low affinity for the soluble analyte PCB72, which couldn't be determined with SPR. However, the phage can demonstrate high affinity towards the immobilized analyte PCB72 due to its multimeric binding and avidity effect. No specific binding to the tested phages was observed also for another analyte, PCB118, demonstrating that the selected phages recognize PCB86 very specifically and not the general PCB scaffold. This specificity is striking, since there are only small differences between different PCB-congeners. The selection of phages which specifically recognize PCB86 and not the general PCB structure illustrates the potential of landscape phages to distinguish among molecules as

similar as PCB-congeners. Such selectivity can be required for development of biosensors that recognize one particular compound, but not its congeners. Although the specificity of the selected phages for PCB86 could be obtained, the Kds are still higher than the Kds observed for antibodies selected for PCBs[12]. Therefore a further enhancement of specific phage affinities towards PCB might be necessary for their use as biorecognition elements for food and environmental monitoring. The improvement of phage characteristics can be achieved by their further affinity maturation[13-14].

This study demonstrates that the large diversity of the recombinant landscape phage libraries, allow to develop specific phage-based biorecognition elements which can distinguish between molecules as similar as PCB-congeners. However, in spite of the huge potential of landscape phages and phages in general, there are only a few examples where phages were selected as biorecognition elements for food and environmental monitoring of chemical compounds[1,15-17]. This is due to the challenge of coupling the target chemical compounds onto a surface necessary for affinity selection of binding phages. As was demonstrated in this work, the recent evolution in surface chemistry and co-operation of experts in organic synthesis and biotechnology, create new opportunities for the development of phage sensors for food and environmental monitoring of chemical compounds.

Acknowledgements:
We sincerely thank Arnaud Verlinde (Biacore, Belgium) for advice regarding the measurement of on Biacore X100. This work was supported by the Institute for the Promotion of Innovation by Science and Technology in Flanders (Belgium) and by the Federal Public Service of Health, Food Chain Safety and Environment [RT 07/11 INVITRAB, RF6204 ERGOT].

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