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Chromatopanning for the identification of gallium binding peptides

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Highlights

- chromatography-based application of phage surface display for trivalent metal ions
- stable immobilization of gallium ions as a biopanning target
- highly stringent biopanning for the enrichment of specific bacteriophage clones
- successful identification of gallium binding bacteriophage clones

Abstract

This study is concerned with a chromatography-based approach (Immobilized Metal Ion Affinity Chromatography) for the recovery of gallium binding peptide sequences from a recombinant phage display library.

The here described methods apply the fundamental knowledge and methods of separation science and meet thereby the key requirement of the phage display technique of precise separation of target-binding bacteriophage clones from non-interacting bacteriophage during the biopanning.

During the chromatopanning called process, a total of 101 bacteriophage clones were identified of which in subsequent binding experiments, phage clones expressing the peptide sequences TMHHAAIAHPPH, SQALSTSRQDLR and HTQHIQSDDHLA were characterized to bind >10 fold better to a target that presents immobilized gallium ions than control phage, displaying no peptide sequence.

The performance of biopanning experiments in chromatographic systems is particularly suitable for demanding targets such as trivalent metal ions. We found, that the selection

process benefits immensely from the stable immobilization of the target metal ions during the entire biopanning process as well as the complete recovery of well interacting bacteriophage clones. Among others, this was possible due to an enhanced monitoring of process conditions and fractionation of eluates.

Keywords: Phage display, gallium, peptides, chromatopanning

1. Introduction

In its role as a high-tech metal, gallium has become almost ubiquitous in our everyday lives. It is used in LED's and photovoltaic elements. It is also an essential component of the semiconductor compounds gallium arsenide (GaAs), gallium nitride (GaN) and gallium phosphide (GaP). Gallium is of high importance for the electronics industry and the need for high-purity gallium for technological products is growing. At present, this demand is satisfied by gallium obtained from primary raw material sources, mainly as a by-product from aluminum and zinc mining [1]. However, the worldwide supply of gallium is not stable due to a growing demand, political uncertainties and the difficulty of processing gallium containing ores with higher complexity. For these reasons, the European Union has put the assured supply of gallium in the future at risk [2].

One strategy to avoid the shortage of Ga is the increase of recycling rates. In addition to the processing of end of life (EOL) products, waste from the semiconductor industry can be a productive source to produce high-purity gallium. Biotechnological methods could contribute to such innovations by providing solutions that include strategies for mobilization, complexation, concentration and selective separation of certain metals, some of which are already well practiced and in very promising [3].

Among these, biosorption, which is the passive interaction of biomass with certain ligands in aqueous solution, is very well studied [4, 5], especially with respect to the removal of heavy metals. Besides the often-discussed application for remediation purposes [6, 7], selective recovery of certain value metals by highly specific biomolecules is a promising approach. More so, as the use of peptides has several advantages, such as a high stability, target specificity, and affinity [8-11].

Phage display technology is considered to be a very effective tool for the identification of highly specific peptide ligands. It allows the presentation of a specific or random peptide on the surface of a bacteriophage that is encoded by the phage genome [12]. Phage peptide libraries contain a diversity of 10^9 different phage that express additional peptides based on

special genome modifications. Selection of specific, target-interacting phage is achieved using the so-called biopanning process, an affinity selection technique.

Identification of metal-ion specific phage using biopanning is challenging because phage display can only be applied for insoluble or immobilized targets. Therefore metal ions have to be immobilized on an appropriate target material such as ion exchange resins. In these approaches, metal-chelating molecules, *e.g.* nitrilotriacetic acid (NTA), iminodiacetic acid (IDA) or diethylamine (DEAE) are coupled to an appropriate carrier material like membranes or resins. These procedure relies on the affinity of transition metals to certain amino acids, in particular, histidine and cysteine, and are already being used for protein separation and purification in "Immobilized Metal Affinity Chromatography" (IMAC) [13]. This concept can be transferred to biopanning procedures. So far, different IMAC approaches for the identification of metal ion binding peptides have been described: NTA was used for immobilization of nickel [14, 15] and chromium [16], IDA for lead [17], and 1,4,7-Triazacyclononane (TACN) based ligands were used for nickel ions [18].

However, only a few studies describe the chromatographic methods using ion exchangers for biopanning. The pore size of the column material limits the passage of phage. Furthermore, pressure-sensitive resins, such as conventional sepharose, are not suited, since the flow-through of the phage requires higher pressure. Consequently, more stable materials such as cryogels or monoliths are much more appropriate [19, 20]. In fact, the implementation of biopanning experiments using the findings of separation sciences can yield significant advantages leading to more accurate selection. The success of phage display experiments depends largely on the respective process conditions. The use of Fast Protein Liquid Chromatography (FPLC) procedures allows working under controlled conditions metal loading and phage display library treatment as it can be monitored online. The system also allows the fractionation of all eluates and the application of concentration gradients.

In this study, chromatopanning for the identification of gallium binding peptide motives was applied. Phage Display against immobilized gallium ions requires an appropriate strategy for immobilization of the metal. Ga(III) IMAC was used for the purification of phosphoproteins and phosphopeptides [22-24]. In aqueous solutions, gallium is present in different speciation depending on pH value. Besides the trivalent gallium cation Ga^{3+}, gallium also occurs as a hydroxide complex at pH values above 1 [21]. The present study focuses on the Ga species Ga^{3+} and $Ga(OH)_4^-$, as these are most likely to be found the aqueous wastes of the semiconductor industry.

For the selection process, two different pH values were applied: acidic conditions at pH 3.8 or alkaline conditions at pH 8.5.

Gallium was immobilized with high stability on monolithic anion and cation exchanger materials. To our best knowledge this is the first time that high salt elution was used to recover metal ion binding phage clones from a biopanning experiment.

However, the success of biopanning experiments does not only depend on stable target immobilization. The selection process can be strongly influenced by unspecific interactions of the bacteriophage particle. Therefore, the influence of the bacteriophage capsid on the identification of gallium-binding peptides was also investigated.

2. Material and methods

2.1. Phage Display Library system

All biopanning experiments were performed using the commercially available random peptide library Ph.D.-12 (Ph.D.™-12 Phage Display Peptide Library Kit, New England Biolabs GmbH, Frankfurt am Main, Germany). The library is a derivative of the M13 KE bacteriophage vector, where random dodecamer peptides, which are connected by the linker sequence GGGS are N-terminally fused to minor coat protein PIII. A detailed description of the library can be found in the manufacturer's instructions.

The bacterial strain *E. coli* K12 ER2738 (F' *proA+B+ lacIq Δ(lacZ)M15 zzf::Tn10(TetR9)/fhuA2 glnV Δ(lac-proAB) thi-1*10 *Δ(hsdS-mcrB)5*) was used for the determination of infective phage particle concentrations as well as for phage particle amplification.

For the titration of infective phage particles a fresh culture of cells was prepared by growing the host strain in LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.5) up to an optical density (λ = 600 nm; OD_{600}) of 0.5 at 37 °C on a rotary shaker. Ten microliters of phage diluted in TBS (TRIS-buffered-saline with 50 mM TRIS-HCl, 150 mM NaCl, pH 7.5) were incubated for 5 min at room temperature with 200 µl of the fresh cells. Fifteen microliters of this incubate were mixed with 200 µl smelted TOP-agarose (LB medium containing 7 g/l agarose) and transferred to one well of 24-well-plate prepared with 1.2 ml IPTG-Xgal agar (LB medium containing 15 g/l Agar, 0.05 mg/ml IPTG (Isopropyl-β-D-thiogalactoside) and 0.04 mg/ml Xgal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside)) in each well. The plate was incubated overnight at 37 °C. The number of the infectious particles was determined by counting the blue colored plaques (plaque forming units (pfU)).

For the propagation of phage particles, a maximum of $5*10^8$ pfU per 30 ml of freshly grown host strain culture (OD_{600} 0.02) was used for amplification. The culture was incubated while shaking at 120 rpm for 4.5 h at 37 °C. Afterwards, cells were separated from the phage containing medium by centrifugation (10 000x g, 10 min, 4 °C). The supernatant was mixed with 6 ml PEG/NaCl solution (20 % (w/v) Polyethylene glycol 8000, 2.5 M NaCl) and

incubated over ice for at least 4 h to precipitate phage particles. Phage were sedimented by centrifugation (10 000x g, 30 min, 4 °C) and resuspended in 1 ml of TBS. For further purification, the phage suspension was again mixed with 200 µl PEG/NaCl solution and incubated for at least 1 h over ice. Phage were harvested by centrifugation (10 000x g, 30 min, 4 °C) and resuspended in a final volume of 200 µl TBS. Remaining impurities and cell debris were removed by a final centrifugation step of the phage suspension (10 000x g, 6 min, 4 °C).

2.2. Biopanning experiments

Here reported biopanning experiments were carried out to identify gallium binding peptides. A typical biopanning consist of three repetitive rounds, to gradually reduce the phage pool in search of the best binding phage clones. A commercial random peptide library was screened in a so-called chromatopanning process, where the biopanning process is integrated into a chromatographic system. This approach ensures online process monitoring, gradient elution and eluate fractionation.

Target preparation was carried out by immobilization of gallium ions on small monolithic ion exchange columns (CIM Disk Monolithic Column, BIA Separations d.o.o., Ajdovscina, Slovenia) for Äkta avant 25 FPLC system (GE Healthcare Europe GmbH, Freiburg, Germany).

Prior to each biopanning round, the system was disinfected by applying 40 column volumes (CV) NaOH/NaCl solution (1 M NaOH, 1 M NaCl); 80 CV ultrapure water ((Milli-Q® Direct, Merck KGaA, Darmstadt, Germany); 40 CV isopropyl alcohol (20 % (v/v) propan-2-ol) and 40 CV ultrapure water at a constant flow rate of 1 ml/min. Column equilibration to establish suitable binding conditions was done with application of 40 CV of the required buffer (see Tab. 1) at a flow rate of 1 ml/min. Gallium ions were applied to the system in form of $Ga(NO_3)_3$ diluted to a concentration of 10 mM in the equilibration buffer. Gallium was immobilized at a slower flow rate of 0.34 ml/min for 10 ml through the ion exchanger column. Unbound gallium was afterwards removed by washing with 40 CV of the appropriate buffer at a flow rate of 1 ml/min before decreasing to 0.34 ml/min for the phage display library application. Original phage library or subsequently enriched phage pools were diluted in the respective buffer to a final volume of 1 ml and applied in a repetitive recycling loop of 15 ml. Unbound and rather unspecific binding phage were removed by washing with 40 CV of the buffer at a flow rate of 1 ml/min. Good binding phage were eluted by applying 40 CV of the eluent (see Tab. 1) at a flow rate of 1 ml/min. The eluate was collected in fractions, 1 ml each. In a final step gallium together with the remaining tight bound phage were stripped by applying 40 CV 1 M HCl and fractionated as well.

The concentration of infectious bacteriophage in chromatopanning fractions was determined in phage titer experiments as described above. Gallium concentration was determined by inductively coupled plasma mass spectrometry (ICP-MS).

All solutions were prepared by using deionized water (Milli-Q® Direct, Merck KGaA, Darmstadt, Germany) and afterwards filtered through a 0.22 µm membrane filter (MF-Millipore™ Membrane Filter, Merck KGaA, Darmstadt, Germany) to remove remaining particles.

In order to achieve a strong and stable immobilization of the target metal, while keeping unspecific interactions of the phage particle as low as possible, two different protocols for the chromatopanning under various conditions were established.

2.2.1. Chromatopanning at pH 3.8

The method under acidic conditions (see Tab. 1) is based on common Ga-IMAC approaches for the purification of phosphopeptides and -proteins [22-24] as well as the earlier described protocol for a chromatopanning against lead [17]. It was applied for the initial three selection rounds of biopanning using the original Ph.D.-12 library and for subsequent competitive studies of putative gallium binding bacteriophage clones. Furthermore, M13 KE wildtype (Wt) bacteriophage were applied to characterize unspecific interactions. All steps were carried out at a pH of 3.8 in acetate buffer (0.014 M sodium acetate, 0.086 M acetic acid, 0.086 M NaCl). Metal immobilization was performed at the CIM® IDA disc possessing a column volume of 0.34 ml (BIA Separations d.o.o., Ajdovscina, Slovenia). It exhibits weak cation exchanger properties due to the chelator iminodiacetic acid (IDA). Phage elution was performed with 4 M $MgCl_2$.

2.2.2. *Chromatopanning at pH 8.5*

Chromatopanning under alkaline conditions (see Tab. 1) was established based on the experiments with the chromatopanning protocol for acidic conditions. It was used in competitive binding experiments with putative gallium binding phage clones obtained from the initial chromatopanning experiments. The influence of M13 KE Wt bacteriophage particles was examined as described above. The protocol differs mainly in the use of a phosphate buffer (0.095 M Na_2HPO_4, 0.005 M NaH_2PO_4) at a higher pH of 8.5 and the use of a CIM® QA with a column volume of 0.34 ml (BIA Separations d.o.o., Ajdovscina, Slovenia). The column provides strong anion exchanger properties by the representation of quaternary amine (QA). Phage elution was achieved with 4 M NaCl.

2.3. Single clone identification

Individual bacteriophage clones were further selected from the eluate and the stripping fraction of the third round of the initial chromatopanning and the two competitive

biopanning experiments in order to identify gallium binding peptide sequences. Single colonies of infected host bacteria were picked from titer plates and transferred to 50 µl TBS. The phage were allowed to diffuse overnight at 4 °C. Remaining agar and cells were removed by centrifugation (10 000x g, 10 min, 4 °C). Phage particles were stored for further characterization in 50 % (v/v) glycerol at –20 °C.

In order to identify the displayed peptide sequence of individual bacteriophage clones, the phage particle solution was used as the template for polymerase chain reaction (PCR) with the oligonucleotide primers 5'-GCAACTATCGGTATCAAGCT-3' (forward) and 5'-CCCTCATAGTTAGCGTAACG-3' (reverse). The PCR was performed using a Taq DNA Polymerase with ThermoPol® Buffer (New England Biolabs GmbH, Frankfurt am Main, Germany) under manufacturer's instructions with 120 sec and 95 °C initial denaturation; 35 cycles of 30 sec and 95 °C denaturation, 30 sec and 55 °C annealing and 45 sec and 72 °C elongation and a final elongation of 120 sec and 72 °C. The resulting PCR product was used as the template for a subsequent Sanger sequencing (GATC Biotech AG, Köln, Germany) carried out using the oligonucleotide primer 5'-CCCTCATAGTTAGCGTAACG-3'.

2.4. Single clone binding studies

Binding affinity towards gallium ions of the five most promising bacteriophage clones was investigated by performing single clone binding studies. Furthermore, the binding affinity of M13 KE Wt bacteriophage was determined as a control.

For this purpose, nitrilotriacetic acid (NTA) conjugated agarose was loaded with gallium and used as target material for phage binding. The target was prepared by washing 15 ml of PureCube NTA Agarose (Cube Biotech, Monheim, Germany) three times with 45 ml of ultrapure water to remove the ethanol used for storage. Subsequently, the agarose was then incubated for at least 2 h at room temperature in 15 ml of 10 mM gallium solution while vigorously shaking. This step was repeated with another 15 ml of 10 mM gallium. The gallium loaded NTA agarose was washed six times with ultrapure water and subsequently stored at 4 °C. The gallium loaded agarose had a gallium concentration of approximately 1.2 µg/µl sedimented material.

For binding studies, the gallium target material was washed two times with 45 ml of TBS-T (TBS, 0.5 % (v/v) Tween 20) and then resuspended in 15 ml of TBS-T. For each individual experiment 200 µl of the suspension were used. The target material was incubated with $3.75*10^{11}$ pfU phage in a total volume of 375 µl TBS-T overnight at room temperature while vigorously shaking. The supernatant was removed, and the agarose was washed ten times with 200 µl TBS-T. Bound phage were eluted by applying 200 µl glycine solution (0.2 M glycine, pH 2.2 adjusted with HCl) and incubation for 20 min at room temperature while

vigorously shaking. The phage containing supernatant was collected and the phage titer was determined in order to calculate the binding affinity.

3. Results

3.1. Immobilization of gallium ions

The aim of the study was the development of a biopanning procedure using immobilized gallium ions as target. A reliable immobilization of gallium ions is a prerequisite for all subsequent biopanning experiments. Therefore at first we developed a method for gallium ion immobilization using different carrier materials. In this study, two different ion exchanger materials were examined regarding a stable gallium immobilization during biopanning.

A method based on Ga-IMAC protocols for phosphopeptides and phosphoproteins was developed in 1999 by Posewitz and Tempst [24], whereas Nian et al. [17] described a chromatopanning experiment for the selection of lead ion binding peptides. Both approaches were used as a basis for the development of a protocol in which small amounts of gallium were bound to a monolithic IDA column.

In addition, another new chromatopanning method, which enabled the immobilization of higher amounts of gallium to a QA column was developed.

Both methods are here compared and evaluated in view of their suitability for the selection of gallium binding phage clones (see Fig. 1).

By applying the method under acidic conditions immobilization of 4.3 mg gallium on a column volume of 0.34 ml was achieved. By subsequent washing with acetate buffer about 3.1 mg of non-complexed gallium was removed from the system. Due to the interaction with the bacteriophage under constant buffer conditions, 0.1 mg gallium was removed from the column material. During the following washing step additional 0.7 mg gallium was released. During the first elution with 4 M $MgCl_2$ 0.1 mg gallium was co-eluted. Remaining gallium could be completely stripped off during the second elution with 1 M HCl.

Alkaline chromatopanning enabled the complexation of higher amounts of gallium. Negatively charged gallium hydroxide species are immobilized by the strong anion exchanger QA. This allowed the initial loading of 4.9 mg Gallium onto the column. Following washing steps removed 3.9 mg gallium. During the phage application, the subsequent washing step and the high salt elution gallium remained immobilized. Gallium could only be removed by hydrochloric acid treatment.

3.2. Biopanning experiments

A dodecamer random peptide phage display library with a complexity of 10^9 different phage clones was used as starting point for biopanning experiments. Three subsequent rounds of chromatopanning were carried out at slightly acidic conditions (refer Fig. 2). The eluates obtained after the first two rounds were amplified. Individual clones were picked from the high salt eluate fraction as well as from the stripping fraction of the third round. In summary, 101 putative gallium binding peptides were identified.

Out of these clones, 28 clones were selected as exhibiting the best gallium binding capacities and were used to generate a mini library (see Tab. 2) as reported elsewhere [25, 26]. The library was used in subsequent chromatopanning experiments at alkaline and acidic conditions in order to identify the most competitive phage clones. In this experiment, 5 different sequences were identified that were used for further analyses.

Sequences of all obtained clones were determined and the amino acid composition of the different phage pools was compared. Changes in the amino acid composition of the different phage pools were evaluated to evaluate the selection process from the initial biopannings to the competitive mini library experiments (see Fig. 3). Considered were (I) the naïve, original dodecamer library, (II) the pool of 101 clones identified after initial biopanning experiments, (III) the mini library consisting of 28 most promising gallium binders and the pool of 39 clones identified after competitive chromatopanning experiments, divided in (IV) 24 clones identified from eluate of competitive chromatopanning under alkaline conditions and (V) 15 clones identified from the experiment carried out under acidic conditions.

In addition, bacteriophage clones that do not present an additional peptide sequence on their surface (M13 KE Wt) were used for chromatopanning studies under acidic and alkaline conditions. The experiment was done in order to elucidate the influence of the capsid of phage particles on biopanning results.

3.2.1. Initial Biopanning

An amount of $4.75 \cdot 10^9$ pfU of the commercial phage display library Ph.D.-12 was used as starting pool for the initial chromatopanning experiments. Phage titers were determined for flow, wash, eluate and stripping fractions of all three chromatopanning rounds (see Fig. 2). A relative constant proportion of unbound phage, which has not interacted with the system after several repetitive loading cycles, remained in the flow through of all 3 rounds. On the one hand, the amount of unspecific or weakly bound phage, that were removed in the washing step decreased significantly between the first and third round of chromatopanning. On the other hand, the amount of eluted phage increased significantly (see Fig. 2).

Clones that were obtained after three biopanning rounds were picked from the stripping fraction as well as from the eluate. In total, 133 single clones were picked and further analyzed. Of these, 15 showed the genotype of M13 KE Wt and 110 had the genome structure of a library clone. The displayed peptide sequences of 101 positive phage clones were identified. Of these, only the sequences ANTELALANRKH, NYLPHQSSSPSR, SLPNLPPTYAKP, ASNHSIPTFPLK, NPMNNVAQNPGP, TLGLRPVPVATT, GSWNTFRAQPTI, SQALSTSRQDLR and HSACLGPSNLQC occurred twice, all other 83 sequences were unique.

To estimate the enrichment of specific phage clones through the initial chromatopanning, the amino acid composition of the naïve Ph.D.-12 library was compared to the pool of putative gallium binders after initial selection process (see Fig. 3). For this, the experimentally determined composition of the library, which was reported elsewhere [25] was compared with the experimental results of this work. Hydrophobic, aliphatic amino acids were generally enriched. Especially the number of leucine residues increased. Likewise, the acidic amino acid aspartic acid as well as asparagine and serine could be detected more often than in the naïve Ph.D.-12 library. All amino acids with aromatic, basic and sulfur containing side chains were depleted. A remarkable observation is the decline of lysine by more than 8 %.

3.2.2. Competitive biopanning experiment

After the successful selection of multiple phage clones by initial biopanning, further experiments for the identification of strong binding peptide sequences were performed. For this, a mini library (ML-12) consisting of equal amounts of the 28 most interesting phage clones was constructed (see Tab. 2).

ML-12 was used in single chromatopanning experiments under alkaline and acidic conditions to identify the most competitive clones for immobilized gallium under different biopanning conditions. A total of 48 clones were picked from both experiments. Twelve individual phage clones from eluate fraction and stripping fraction of the experiment under alkaline conditions, as well as 12 individual phage clones from eluate fraction and stripping fraction of the experiment under acidic condition were identified.

The distribution of clone variants varied within both experiments. GaBi_C3.108 (SQALSTSRQDLR) was most prominent in both experiments. A total of 17 clones were detected for alkaline conditions and 11 clones were detected for acidic conditions. GaBi_C3.15 (NYLPHQSSSPSR) and GaBi_C3.130 (NDLQRHRLTAGP) could also be detected in both experiments, but with lower frequency of only three and two clones under alkaline and one copy under acidic conditions. Two clones of GaBi_C3.129 (HTQHIQSDDHLA) were obtained from the alkaline chromatopanning experiment. This sequence was not detected in the acidic experiment under acidic conditions. In contrast, clone GaBi_C3.8 (TMHHAAIAHPPH) could

not be detected in alkaline chromatopanning, but at least 2 clones were identified in acidic chromatopanning experiments.

The relative amino acid occurrence within the phage pool was used as a first indicator for the enrichment of strong gallium binders (see Fig. 3). The amino acid composition differed from the initial distribution. However, only a few amino acids were actually enriched. Serine occurred most frequently within the identified clones. The relative abundance of arginine, glutamine, leucine and, aspartic acid increased as well. Valine, phenylalanine, tryptophan, glutamic acid, lysine and cysteine were not detected after competitive biopanning experiments. Methionine was only detected in clone GaBi_C3.8 (TMHHAAIAHPPH) and thereby only after chromatopanning under slightly acidic conditions.

3.2.3. Bacteriophage particle interaction

An important factor for the successful enrichment of highly selective binders in phage display is not only the stable target immobilization but also a minimized interaction of the bacteriophage capsid with the target material. It has to be ensured that the target specificity of the selected phage is based solely on its presented peptide and not on the phage particle itself.

M13 KE Wt does not display additional peptides on its surface and hence was used as control and compared with the peptide displaying phage. Both chromatopanning experiments were carried out with M13KE Wt bacteriophage and compared with the results obtained from the mini library experiments. The phage titer was determined for each step of the chromatopanning experiments. In Fig. 4, the resulting amount of phage bound to the target material is shown.

Bacteriophage with additional Ga-binding peptide sequences showed a stronger affinity to the target material than M13 KE Wt phage. A larger proportion remained attached to the material after phage application and even after washing more phage remained bound to the target material. High salt elution with $MgCl_2$ detaches M13 KE Wt bacteriophage more effectively from IDA immobilized gallium than NaCl elution from QA immobilized metal hydroxide. In fact, after the proportion of bound bacteriophage had already been reduced so drastically by the previous washing step, hardly any elution of M13 KE Wt phage with NaCl could be observed. By application of hydrochloric acid to both systems, the remaining bacteriophage could be stripped off completely.

The results obtained from experiments with M13 KE Wt bacteriophage were compared to the results from competitive biopanning experiments with mini library clones to define the influence of displayed peptide sequences. In general, chromatopanning under alkaline condition leads to stronger interactions of the tested bacteriophage clones than the more classical protocol carried out under acidic conditions. The ratio of initially applied phage to

the target material bound bacteriophage was slightly higher and the following washing step decreases the amount of immobilized phage much less when carried out in phosphate buffer.

Likewise, the experiments with M13 KE Wt phage, high salt elution with NaCl has been shown to be less effective than with $MgCl_2$ for peptide-displaying phage clones. The application of 1 M HCl in these experiments also leads to the removal of all remaining bacteriophage from the system (see Fig. 4).

3.3. Single clone binding studies

The interaction of individual clones was studied in independent binding experiments by using gallium-loaded NTA-agarose as target material. The relative binding to the gallium target was determined for each of the five clones obtained from competitive binding experiments. The results were compared to Wt phage. The binding was measured as number of bound phage per mass of target (total titer per µl agarose) (see Fig. 5).

All 5 tested clones showed better binding properties compared to the Wt. Clones C3.15 and C3.130 showed a relative binding factor of about 5.2 and 3.0 whereas the clones C3.8, C3.108 and C3.129 showed an increased binding factor of >10.0. Clone C3.129 showed a relative binding of > 14 compared to the Wt.

4. Discussion

4.1. Gallium ions as biopanning target

One key requirement for all biopanning approaches is the careful separation of target binding clones from non-interacting phage. This separation is comparatively simple for insoluble targets. In case of ions, a reliable immobilization of the target is essential to allow an appropriate selection. For biopanning against bivalent metal ions, the IMAC concept was often applied in previous studies, referred as chromatopanning [14, 17, 18, 20]. The IMAC concept was first presented by Porath et al. [27]. It is based on the affinity of bivalent transition metals for certain amino acids such as histidine. Since then, various standard protocols for protein purification have been developed based on these methods. Trivalent IMAC has been used for the enrichment of phosphoproteins and phosphopeptides [22-24, 28, 29] but was not applied for biopanning experiments yet.

In order to allow the identification of gallium binding peptides from phage display libraries, two protocols were developed for the chromatopanning against immobilized gallium ions (refer Tab. 2).

Both biopanning methods differ in the use of different column materials and the operation at different pH values. Traditionally, biopanning is performed in TBS or TBS-T at pH 7.5 [30].

Initially, the common IMAC method [13] was adapted for immobilization of Ga^{3+} in an FPLC system. Ga^{3+} is present in aqueous solution up to a maximum pH of 4.5. The proportion of trivalent ions decreases with increasing pH values in the benefit of monovalent and divalent gallium hydroxide complexes [21]. Therefore, a maximum pH value of 3.8 was chosen, stabilized with acetate buffer. Acetate buffer is known to interact with trivalent gallium ions, thus preventing gallium from precipitating with increasing pH values. Soluble Ga^{3+} is therefore available for immobilization on the weak cation exchanger IDA. However, small amounts of gallium were released from the system during bacteriophage treatment as well as during the high salt elution (see Fig. 1). As a consequence of the relatively poor gallium immobilization, only low amounts of gallium exist as interaction partners for binding bacteriophage, hence potential good gallium binders might get lost by gallium co-elution. On the other hand, low target availability is often discussed in literature to have a beneficial effect on selection stringency [31].

In the second method described in this study $Ga(OH)_4^-$ was attached to the strong anion exchanger QA. It was performed in phosphate buffer at pH 8.5. The alkaline chromatopanning system allowed a more stable complexation of higher amounts of gallium (refer Fig. 1). Almost no metal was released from the column material during subsequent process steps after initial gallium immobilization. This was achieved using quaternary ammonium as a stronger ion exchanger than IDA and the higher availability of immobilizable gallium hydroxide species [21]. An enormous advantage of this method is that the gallium complexation is neither affected by the buffer nor by the eluent. This allows a more accurate biopanning including an elution of interacting phage based on the binding properties to the target metal ion.

4.2. Phage clone selection

4.2.1. Enrichment of gallium binding phage clones

The enrichment of gallium specific bacteriophage from a dodecamer random peptide library was carried out in three consecutive selection rounds against immobilized gallium ions. This initial biopanning was performed using the protocol for acidic chromatopanning. The enrichment of putative binders for this target was evaluated by the titer of flow, wash, elution and stripping fraction (refer Fig. 2). During three biopanning rounds, bacteriophage clones interacting with the material were significantly enriched. Thus, non-specific and weakly binding clone variants are gradually removed from the phage pool. After three biopanning rounds, the phage pool was enriched with gallium-binding bacteriophage clones and individual clones with putative gallium-binding properties were identified. In summary, a total of 92 different clone variants were identified. Of these, only 9 occurred twice. All other 83 sequences were found once. Within the multitude of sequences found, no uniform binding

motif or recurring pattern in the distribution of the side chain functionalities could be determined comparable to those necessary for the recognition of inorganic surfaces in the phage display [32, 33].

However, the content of the amino acids G, A, V, I, L, P D, N and S was increased in course of the initial biopanning experiments, whereas the amino acids F, W, Y, E, H, K, R, Q, T, M and C decreased (see Fig. 3). Alterations in the amino acid composition are considered as a powerful indicator for the selection process during phage display. However, it should be noted that amino acid bias through multiple biopanning rounds is not only target dependent but also host strain dependent, too. It was reported earlier for phage display technology [34] that the amino acids P, F, Y, W, R, K, H, N, Q and T are more frequently displayed, while occurrence of G, A, V, I, L, D, E, S, C and M is reduced by host dependent amplification.

Hydrophobic aliphatic amino acids take a notable part in all examined phage pools. Already strongly represented within the naïve Ph.D-12 library, they are further enriched during initial chromatopanning. Although the importance of single side chain properties on the coordination of gallium ions is considered to be rather negligible [35], it cannot be excluded. Aromatic amino acids are consistently depleted during the initial biopanning. It can be assumed that the large side chains of aromatic amino acids inhibit the complexation of metals [8]. Amino acids with acid side chain functionality are considered to be interesting candidates for metal ion binding due to their negative charge under adequate conditions. In accordance with this, there was an observed increase in the presence of aspartic acid during the initial biopanning. Basic amino acids, especially histidine, are known for their outstanding complexing properties of transition metals such as nickel and copper [36]. Many standardized IMAC protocols for protein purification are based on this functionality. Although gallium should be predominantly trivalent, it is assumed that amino acids that offer nitrogen for metal binding also have good gallium complexing properties [37, 38]. Similarly, the increase of glutamine and serine in the phage pool can be explained by the initial biopanning. Both amino acids provide nitrogen or oxygen and thus have the requirements for an interaction with gallium. The sulphur-containing amino acids methionine and cysteine are rather challenging to propagate in the biopanning process due to the host dependency. For this reason, both amino acids are not strongly present in the initial pool of the phage library from the outset and, even with an existing target affinity, are difficult to establish themselves through the individual amplification steps between the biopanning rounds in the selection process. With this background, both amino acids occurred relatively frequently. This coincides with the well-known, good metal complexing properties, especially of cysteines [35].

The decrease in the proportion of threonine, asparagine and glutamic acid in the phage pool is not fully understood, especially since amino acids with analogous side chain functionalities

tend to accumulate. One possible cause could be that amino acids with similar functional side chain properties nevertheless differ greatly in their structure and therefore have a different influence on the stability of a complex with metal ions.

The results show that even if the increase and decrease of certain amino acid functionalities is a good selection indicator, it should not be considered separately from the side chain properties of individual amino acids.

Although certain functional groups are important for the chelation of metals, stable complexation can only be achieved by a proper steric arrangement of these groups. This is evident for inorganic solid target materials whose surface has a complex morphology that peptides can recognize for reliable detection [39, 40]. In fact, bacteriophage, which display metal ion-selective peptides, have already been reported elsewhere [16, 17], which shows that peptide structure plays a significant role for the stable metal ion bond in addition to the represented functionalities. This fact is also illustrated by the very different display of amino acids with similar functionality through continuous biopanning.

As the 92 clones identified in the initial biopanning experiment were not yet sufficient to determine the most suitable sequence motifs for gallium binding, a mini library consisting of 28 clones was constructed. ML-12 clones were chosen based on different criteria such as the frequency with which a clone type was identified after initial biopanning, auspicious pI of the presented peptide sequence, and its content of potentially metal-binding side chain functionalities, in particular basic, amidic and sulfur-containing amino acids, as well as their arrangement in the peptide.

Only the five clone variants GaBi_C3.8 (TMHHAAIAHPPH), GaBi_C3.15 (NYLPHQSSSPSR), GaBi C3.108 (SQALSTSRQDLR), GaBi_C3.129 (HTQHIQSDDHLA) and GaBi_C3.130 (NDLQRHRLTAGP) emerged from the competitive binding experiments (see Tab. 2).

GaBi_C3.8 (TMHHAAIAHPPH) was the only clone that was obtained from competitive binding experiments under acidic conditions. As the only clone it has a sulphur side chain. The sulphur functional group is the thioether group of methionine. In addition, it possesses a hydrophobic domain in the middle of the peptide, as well as 4 nitrogen-donating histidine residues and an oxygen-supplying threonine. Thus, the peptide provides all the necessary requirements for chelating the metal ion via a hexa-dentate complex, as already described elsewhere for typical gallium compounds [37, 38]. In the single clone binding experiment, the clone proved to be an outstanding candidate. It can be assumed that for GaBi_C3.8 (TMHHAAIAHPPH) there is a certain preference for a gallium ion target, as was available in the experiment with acidic conditions and in the single clone experiment, compared to the gallium hydroxide target in the experiment under alkaline conditions. GaBi_C3.15

(NYLPHQSSSPSR) could only be identified in the high salt eluates of the two experiments. This induces certain sensitivity to increasing ionic strengths. In general, this clone turned out to be a rather weak binder. The peptide sequence presented is very rich in serine and very hydrophilic compared to the other sequences. It is assumed that gallium complexation mainly takes place via the hydroxide groups of the serines. Some of these are directly adjacent in the peptide, which could have a sterically unfavorable effect on a stable binding. Clone GaBi_C3.108 (SQALSTSRQDLR) is very dominant under both acidic and alkaline conditions, thus it is the closest to a pH-flexible gallium binder. In the single binding experiment, the clone showed excellent binding properties. The presented peptide sequence is rich in serine, arginine and aspartic acid, which theoretically would meet the requirements for a hexadentate complexation with gallium adequate to clone GaBi_C3.8 (TMHHAAIAHPPH). The clone GaBi_C3.129 (HTQHIQSDDHLA), which performs best in individual experiments at pH 7.4 (see Fig. 5), could also only be identified after alkaline competitive chromatopanning and not after the experiment under acidic conditions. Its peptide sequence is very rich in histidine, aspartic acid and glutamine. The theoretical pI of this peptide is 5.7. Thus, the sequence was protonated in competitive chromatopanning under acidic conditions. This may have resulted in a less stable gallium binding compared to other clones in the experiment. Clone GaBi_C3.130 (NDLQRHRLTAGP) proved to be the worst binder in the individual experiment. Although the clone was still three times better at binding gallium than the wild type, we concluded this was unsuitable to qualify as a comparably better gallium binding peptide.

4.2.2. Impact of phage capsid to biopanning

In phage display technology, the bacteriophage serves as a vehicle for the selection of target-specific peptides; therefore the presented sequence motifs should generally have the decisive influence on the selection output. However bacteriophage capsid structure and composition as well as infection processes affect the selection during biopanning. The entire bacteriophage body is huge compared to the peptide sequence displayed. This is even more the case for the presentation of peptide motifs on a minor coat protein in much lower copy numbers in pIII libraries than on pVIII major coat protein [41].

In general, biomass is known to interact with different metals due to its very complex composition. This takes place passively via different functional groups, such as phosphates or carboxylates, which are present on the surface of biomass. Therefore, it can be assumed that the proteinogenic phage capsid also interacts with metals and thus interferes with the biopanning process [42]. The capsid has to be considered as an enormous disturbance variable for the selection of highly specific ligands. Non-specific attachment of the phage body to the target material and not by peptide based interactions can have a massive influence on the success of biopanning. Although unspecific interactions are rather weak

compared to the strong binding of specific sequence motifs, they often lead to the identification of false positive clones. As a result, the investigation of all identified peptides in sophisticated individual experiments is important.

In order to guarantee the success of biopanning experiments, the influence of the bacteriophage capsid must be kept as small as possible. For this purpose, the selection conditions can be adapted in such a way that capsid interactions are as low as possible and therefore only bacteriophage clones with a target specific peptide sequence on their surface are detected.

A strategy that is usually applied to avoid the enrichment of non-specific binders is the repetition of the biopanning process several times with intermediate amplification steps of the enriched phage pool (3-5 cycles). However, amplification is always host-dependent and leads to bias, so that not only unintentionally good binders can be lost between the biopanning rounds, but also the M13 KE Wt bacteriophage, which are also selected by unspecific attachment, is enriched in the pool.

Therefore, in this study two protocols for phage display based identification of gallium binding peptides were developed and compared. The first corresponds to the adapted chromatopanning for gallium ions and includes working at pH 3.8 in acetate buffer. The other is in accordance with the optimized process in phosphate buffer at pH 8.5 to reduce unspecific interactions.

The interactions of M13 KE Wt bacteriophage and mini library phage (ML phage) with the respective target material were investigated in individual experiments by applying the chromatopanning protocols under alkaline and acidic conditions (see Fig. 4). In general, the M13 KE Wt shows worse binding properties for both protocols in comparison to ML phage, thus proving the Ga-binding properties of the clones tested in the mini library.

The initial binding of M13 Wt bacteriophage is weaker in phosphate buffer at pH 8.5 than in acetate buffer at pH 3.8. However, the ML-12 clones interact much better with the target material in phosphate buffer than in the alternative system. Similarly, the binding is influenced during the subsequent washing step. By using phosphate buffer, considerably more M13 KE Wt phage is removed from the system, but interaction with mini library bacteriophage is much more stable. As a result, the ratio of bound ML-12 clones to M13 KE Wt under alkaline conditions is much higher than under acidic conditions thus indicating a strong reduction of non-specific binders in the optimized protocol.

The use of phosphate buffer is considered as to be responsible for this effect. Phosphate has both a high affinity for most polyvalent cations and charge-concealing properties with respect to proteins. It is assumed that phosphate, like citrate, competes for the complexation of gallium on the column material and can thus prevent non-specific binders from

accumulating. In addition, phosphate ions interact with the bacteriophage capsid and prevent unspecific electrostatic interactions with the target [43].

Elution of tightly bound phage occurred to be more successful by applying $MgCl_2$ rather than NaCl. This could be attributed to two possible reasons. The results indicate that specific phage clone interactions were much stronger at higher pH, consequently complicating the elution of tightly bound phage. Furthermore, $MgCl_2$ is considered to be a more efficient eluent due to a higher chloride proportion. Higher concentrations of kosmotropic salts decrease electrostatic interaction based adsorption by increasing entropy [29]. This leads to a considerable amount of residual phage after high salt elution in both protocols. These are recovered by co-elution of gallium while stripping with hydrochloric acid.

5. Conclusion

Here we report about the development and application of two chromatography-based biopanning methods for the identification of gallium binding peptides from a commercial phage display library. Two protocols for the chromatopanning against immobilized gallium species in an FPLC system were developed.

It has been shown that the immobilization of gallium hydroxide species at higher pH is stable and efficient. At lower pH, trivalent gallium ions could be offered for selection in biopanning.

Both methods were successfully applied to identify gallium binding peptide sequences.

Furthermore, the influence of unspecific interactions by the bacteriophage capsid on the selection of gallium-binding peptides was investigated. It was shown that the use of a phosphate buffer at pH 8.5 has a beneficial effect on the selection process, as the unspecific interactions of the bacteriophage capsid were considerably reduced during biopanning.

The use of highly saline eluents for the extraction of gallium-binding bacteriophage clones is well suited. A higher chloride ion concentration has a beneficial effect on the recovery of highly binding bacteriophages.

A total of five gallium binding peptide sequences could be identified and characterized as good binders. The peptide sequences of phage clone GaBi_C3.8 (TMHHAAIAHPPH), GaBi_C3.108 (SQALSTSRQDLR) and GaBi_C3.129 (HTQHISDDHLA) were displayed by the best gallium binders. The extent to which these sequences obtained by chromatopanning are suitable for the recovery of gallium from aqueous industrial waste will be further investigated, independently of the bacteriophage and under different conditions.

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Figure captions

Fig. 1: Immobilization of gallium during chromatopanning. Amount of gallium bound to monolithic column (0.34 ml) at slightly acidic and slightly alkaline conditions.

1 – initial immobilization; 2 – first wash; 3 – phage treatment; 4 – second wash; 5 – phage elution; 6 – stripping

Fig. 2: Bacteriophage titer during three rounds of chromatopanning against Ga immobilized on monolithic CIM®IDA disc under acidic conditions. Shown is the amount of bacteriophage in input, pass, wash, elution and stripping. The amount was normalized against the respective input in each chromatopanning round.

Fig. 3: Amino acid composition of different Ph.D.-12 phage pool. Shown is the percentage of each amino acid for the commercial Ph.D.-12 library (I); putative Ga binding phage clones (II); ML-12 (III) and competitive ML-12 clones obtained under alkaline conditions (IV) and acidic conditions (V).

Fig. 4: Interaction of bacteriophage with and without displayed peptide sequences with immobilized Ga during chromatopanning.

Fig. 5: Binding experiments with single clone amplicons and gallium loaded NTA agarose (25 single experiments for each clone). Shown is the relative binding in comparison to the M13 KE Wt control. The value was calculated from the amount of by Glycin-HCl eluated phage per µl Sepharose.

Tab. 1: Overview on experimental conditions during chromatopanning under acidic and alkaline conditions.

	Acidic chromatopanning (pH 3.8)	Alkaline chromatopanning (pH 8.5)
Buffer	0.1 M sodium acetate	0.1 M sodium phosphate
Column	0.34 ml CIM®IDA	0.34 ml CIM®QA
Elution	4 M $MgCl_2$	4 M NaCl
Stripping	1 M HCl	1 M HCl

Tab. 2: Mini library (ML-12) composition (III) and relative occurence of the single bacteriophage clones during initial chromatopanning (II) and competitive experiments under alkaline conditions (IV) and acidic conditions (V).

Clone	Peptide sequence	pI	ML-12 (III) Input concentration	Occurrence after Biopanning		
				II	IV	V
M13 KE Wt			0.00E+00 pfU	15/133	0/24	9/24
GaBi_C3.2	RVQPAHFNVMGQ	9.76	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.4	MVGTADGTLLDP	3.56	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.5	ANTELALANRKH	8.80	4.00E+11 pfU	2/110	0/24	0/24
GaBi_C3.8	TMHHAAIAHPPH	6.82	4.00E+11 pfU	1/110	0/24	2/24
GaBi_C3.14	GIVTNQHDSNAN	8.75	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.15	NYLPHQSSSPSR	5.08	4.00E+11 pfU	2/110	2/24	1/24
GaBi_C3.20	GLTFQVPWHANM	6.74	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.27	SLPNLPPTYAKP	8.31	4.00E+11 pfU	2/110	0/24	0/24
GaBi_C3.28	GHPMMPPKSEIR	8.75	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.52	ASNHSIPTFPLK	8.80	4.00E+11 pfU	2/110	0/24	0/24
GaBi_C3.72	NPMNNVAQNPGP	5.52	4.00E+11 pfU	2/110	0/24	0/24
GaBi_C3.77	TMAQGVAQRYGN	8.41	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.78	TLGLRPVPVATT	9.41	4.00E+11 pfU	2/110	0/24	0/24
GaBi_C3.86	SHQPGDQSPANN	5.06	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.90	DLINIDRNHSFR	6.75	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.102	LPKQCSLLTSAC	8.06	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.103	GSWNTFRAQPTI	9.75	4.00E+11 pfU	2/110	0/24	0/24
GaBi_C3.105	NFTLQAHPHKYP	8.61	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.106	STDHGSWQKSRA	8.49	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.107	VPQLHHLMPHFD	6.25	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.108	SQALSTSRQDLR	9.31	4.00E+11 pfU	2/110	17/24	11/24
GaBi_C3.115	TSMSQHFHVHRL	9.49	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.116	SPLTPPHAPETH	5.93	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.120	CPTDVRSGCMGT	5.82	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.124	IEMTRTNLNDVN	4.37	4.00E+11 pfU	1/110	0/24	0/24
GaBi_C3.129	HTQHIQSDDHLA	5.70	4.00E+11 pfU	1/110	2/24	0/24
GaBi_C3.130	NDLQRHRLTAGP	9.61	4.00E+11 pfU	1/110	3/24	1/24
GaBi_C3.131	DDTQNSQNMDTL	3.42	4.00E+11 pfU	1/110	0/24	0/24
GaBi_D3.1	HSACLGPSNLQC	6.72	4.00E+11 pfU	2/110	0/24	0/24