Identifizierung und Charakterisierung von Gallium-bindenden Peptiden

Fakultät für Chemie und Physik
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DISSERTATION

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Datum, Unterschrift

Identification and characterization of gallium-binding peptides

Faculty of Chemistry and Physics

Technische Universität Bergakademie Freiberg

DISSERTATION

submitted in fulfillment of the requirements for the academic degree

doctor rerum naturalium

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submitted

by M.Sc. Biology Nora Schönberger (née Nowak)

born on 22nd August 1990 in Dresden

Freiberg, 04 June 2020

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Prof. Dr. Ross MacGillivray

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“We share deep admiration for evolution, a force of Nature that has led to the finest chemistry of all time, and to all living things on this planet.”

Frances H. Arnold

Content

Summary

The present work demonstrates how a peptide-based material can be obtained for the biosorptive recovery of metals from contaminated industrial wastewater. Starting with Phage surface display for the initial identification and optimization of gallium-binding peptides, all the following application-focussed experiments are based on chemically synthesized peptides.

Two chromatography-based biopanning methods for the identification of gallium-binding peptides from a commercial phage display library were developed. Five gallium-binding peptide sequences were identified and evaluated to show good gallium-binding properties.

Furthermore, the biosorption of free gallium and arsenic by gallium-binding bacteriophage clones was investigated. A large influence of the pH-value on the respective interactions was demonstrated.

Mutagenesis experiments were also carried out for a bacteriophage clone expressed peptide, in which a cysteine pair systematically replaced amino acids. Biosorption experiments with the resulting seven different bacteriophage mutants suggested a relationship between the rigidity of the peptide structure and the gallium-binding properties.

In isothermal titration experiments, the thermodynamics of the interaction between gallium and the peptides as chemically synthesized derivatives were characterized, independent of the bacteriophage. The peptides differed strongly in their interaction with gallium, and in some cases, the complex formation with gallium depended strongly on the surrounding buffer conditions.

The peptide with the amino acid sequence NYLPHQSSSPSR has particularly promising gallium-binding properties. Computer modeling suggests the probable structure of the peptide in aqueous solution and postulates a possible binding site for gallium.

The side-selective and covalent immobilization of the peptides on a polystyrene matrix led to the creation of a biocomposite for the biosorptive recovery of gallium. The sorption performance and desorbability of the peptide-based biosorption materials were determined in studies with model solutions and real waters from the semiconductor industry.

Chapter I.

THE APPLICATION OF PEPTIDES AS BIOREAGENTS IN RESOURCE TECHNOLOGY

The social changes of our time result not only in an increased but also in a changing demand for raw materials and thus constantly confront the traditional discipline of resource technology with new tasks. Due to the constant growth of the world population, increased quantities of water, land, energy, and raw materials are required to satisfy the basic needs of mankind. At the same time, technological progress is leading to major changes in lifestyle. In particular, developments in the high-tech sector and the associated entry into the digital information age have led to an enormous increase in demand for rare metals, which were previously of little or no economic relevance. These include, e.g., rare earth elements but also the metals gallium, indium, or germanium.

In addition, the general scarcity of fossil carbon sources and geopolitical irritations ("oil crisis"), but also a general increase in environmental awareness in society, which often goes hand in hand with the desire to reduce the CO_2 footprint of humanity, are leading to a boom in so-called low carbon technologies (Dodson et al. 2012). In particular, replacement technologies for energy generation such as photovoltaics and wind power plants, but also technologies for carbon fixation and storage as well as electromobility are increasing the global demand for other high-tech metals such as cobalt, antimony, scandium, or neodymium (Hagelüken and Meskers, 2013; Marscheider-Weidemann *et al.*, 2016).

Globalization and technological progress are thus exerting constant pressure on the world's raw material deposits. The continued prosperity of the industrialized nations depends to a large extent on satisfying this increased demand for raw materials. In Germany, for example, the high-tech sector has a large economic share (Marscheider-Weidemann *et al.*, 2016). As a result, the industry is massively dependent on the raw material supply chain, and there is a need for constant satisfaction of the demand for all raw materials to ensure economic growth. It is the task of modern resource technology to develop innovative strategies for providing all necessary raw materials.

The greatest challenge here is the uneven global distribution of natural resources on earth. As a result, the supply chain for individual key resources is dependent on a few countries. This creates the risk of uncertain supply situations in which the metal processing industry cannot be supplied with the required metals fast enough. In general, a change in the geopolitical atmosphere is considered more likely than a general depletion of primary resource deposits (European Commission and Ad-hoc Working Group, 2010). Therefore, strategies are needed to support the economy with non-imported raw materials, for example by developing alternative sources of raw materials. It is in accordance with the current zeitgeist to strive for

a technical solution that is as efficient as possible and, at the same time, environmentally and socially compatible.

Possible solutions are offered by the exploitation of raw material sources that are currently still little or even not used at all. These include not only demanding ore deposits and minerals that cannot be exploited yet because the intervention in the landscape cannot be justified or extraction cannot be carried out efficiently in terms of cost-benefit (mineral content not worthwhile or too demanding). Besides that, also secondary raw material sources are interesting. The alternative raw material sources with the greatest potential for the extraction of valuable high-tech metals include solid and liquid industrial waste, EOL products, landfills, metalliferous soils, low-grade ores, mining waste dumps and their wastewater (Dodson *et al.*, 2012). The extraction of raw materials from such secondary raw material sources using traditional metallurgical methods such as chemical precipitation, chemical coagulation, electrochemical technologies, ion exchange, and membrane technologies is often problematic. Their application is often associated with a high demand for chemical additives. These are expensive and lead to the accumulation of toxic waste. They also require high energy input and have a poor overall economic balance due to low efficiency (Dodson *et al.*, 2015). For this reason, the extraction of valuable industrial metals from such secondary raw material sources cannot currently be done in a cost-benefit-efficient manner. Consequently, they often remain untouched, which causes further problems for humans and the environment and is a real obstacle to the establishment of the desired circular economy. For this reason, the development of innovative processes is essential for the economically and ecologically justifiable processing of alternative raw material sources. Biotechnology provides a diverse and promising toolbox for the development of novel recycling strategies. Many concepts from nature are already well understood today and can be transferred into modern, socially relevant approaches. Therefore, the implementation of biotechnological methods in resource technology represents an important starting point that will help to close the loop to recycling management.

Utility of biotechnological approaches in resource technology

The utilization of biological systems in resource technology complements the repertoire of conventional metallurgical processes. At present, it fills a methodological niche by making metal sources accessible that cannot be efficiently exploited by classical metallurgical approaches. At the same time, it allows the mining industry to operate in a more environmentally friendly way. The implementation of biotechnological approaches is usually associated with the use of smaller quantities of chemicals and the release of less toxic by-products; it requires less energy and releases smaller amounts of carbon dioxide (Johnson, 2013).

The application of biotechnological processes for the extraction of metals is therefore considered as novel and innovative. However, the fact that the natural metabolism of some microorganisms can lead to the solubilization, transport, and deposition of metals and minerals

in the environment was already being used unconsciously in the 15th century to extract copper from mining drainage. Only much later, the microbial contribution was elucidated, which enabled the development of targeted biomining processes (Brierley, 1990). The generic term biomining covers processes that use biological systems for metal extraction from ores or waste materials (Johnson, 2014). The release of metals can occur directly through the metabolism of microorganisms or indirectly through their metabolites. In bioleaching, metals are solubilized by converting them from an insoluble metal species into a soluble form. Three different mechanisms can be distinguished. In redoxolysis, the metal release is achieved by electron transfer processes between mineral and cell or indirectly by oxidation of Fe(II) to Fe(III). Acidiolysis is achieved by the proton-mediated dissolution of minerals. Insoluble metal species are converted into soluble forms by acidification of the environment. Sulfur oxidizing microorganisms such as *Acidithiobacillus thiooxidans* consume elemental sulfur and produce biogenic sulfuric acid. In complexolysis, metal ions are converted by microbial metabolites such as organic acids or siderophores (Sethurajan, van Hullebusch and Nancharaiah, 2018).

The bioleaching of sulfidic ores by acidophilic chemolithoautotrophic bacteria has been best studied and is even implemented industrially. Here, Fe(II) or sulfur is used as an electron donor for microbial metabolism. The by-products Fe(III) or sulfuric acid causes the mineral dissolution. Non-sulfidic ores (oxides, phosphates, carbonates, and silicates) can be leached by the application of heterotrophic microorganisms. The formation of metabolites, such as organic acids, siderophores, or exopolysaccharides, can lead to mineral dissolution (Pollmann *et al.*, 2018).

In contrast to the metal solubilization during bioleaching in biooxidation processes, precious metals are enriched in the mineral by solubilizing undesirable components of the ore by oxidation. The process is primarily used for gold (Johnson, 2013).

Another possibility for biotechnological enrichment of minerals is the concentration of these minerals by bioflotation. Flotation is generally a separation process for particles, which is based on the different surface wettability of the components to be separated. This allows the particles to be transported through an aqueous medium by means of adhering gas bubbles to the surface where the particles can be collected. In mineral beneficiation, the surfaces of the components to be separated must be modified. For this purpose, chemicals are used, which either promote the hydrophobicity of the minerals as so-called collectors or act as depressants by accelerating the sedimentation of the particles through more hydrophilic surface properties. Microbial cells or cell components, their metabolites, and other biomolecules can recognize the surface of minerals and thus modify their properties. In bioflotation, they are used for mineral beneficiation as collecting or depressant flotation agents (Pollmann *et al.*, 2018).

In addition to the biological release of metals and mineral beneficiation, the recovery of raw resources from metal-containing solutions is a promising field of application for biotechnological resource management. One of the main challenges of modern resource technology is cost-effective and especially selective extraction from large-volume solutions with low metal concentrations. Biotechnology offers a large toolbox to complement common

techniques that contribute to the solution of this problem, some of which are already industrially established.

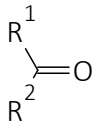
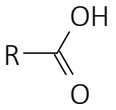
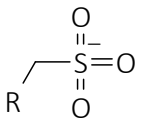
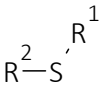
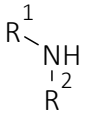
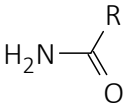
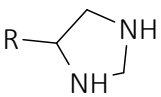
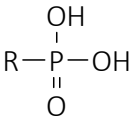
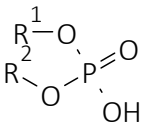
Precipitation processes are used for the removal and recovery of elements from metal-containing solutions. The precipitation of the metal can take place in the form of sulfides, hydroxides, and rarely carbonates. In rare cases, metals such as arsenic are co-precipitated by iron or aluminum ions. In any case, precipitation takes place by changing the ion equilibrium through the addition of precipitating agents. In general, the process is strongly dependent on the metal concentration and the pH value of the system. The major drawback of common precipitation processes is considered the sludge formation and the high amount of chemicals that must be used to adjust the pH accordingly. An alternative approach for the industry is bioprecipitation, where sulfate-reducing bacteria are used. Metal precipitation is carried out as a sulfide salt by biogenically produced sulfide and is influenced by various factors. Precipitation is also strongly pH-dependent and can be inhibited by various metal-chelating metabolites and media components. Since the bioprecipitation of different elements is pH-dependent, selective metal extraction can be achieved by adjusting the pH value (Sethurajan, van Hullebusch and Nancharaiah, 2018).

Another attractive option for the application of biological systems for metal recovery from solutions is biosorption. This process capitalizes the property of biomass or certain biomolecules to bind and concentrate certain ions or other molecules in an aqueous solution.

The sorption takes place on the surface of biomass and is mediated by a variety of functional groups (see table I-1) (Volesky, 2007). The underlying binding mechanisms can be physical adsorption mechanisms based on electrostatic forces and ion exchange or chemical adsorption mechanisms based on complexation, chelation, and micro surface precipitation (Das, Vimala and Karthika, 2007). The process is characteristically passive and, therefore, not dependent on metabolic activity and does so not require nutrient supply. This allows the application of biosorption in highly toxic environments. This advantage has often been considered for remediation, i.e., for the removal of toxic substances, e.g., heavy metals from contaminated systems (Volesky and Holan, 1995).

The special charm of biosorption is the cost-effective provision of sorption material from naturally occurring or waste materials. This advantage can also be used for the cost-effective recovery of valuable metals from aqueous systems. The application is especially useful when the use of more conventional methods is not possible. Classical pyro- and hydrometallurgical processes often fail due to raw material sources with polymetallurgical matrix in low concentrations. They also consume high amounts of energy, require additional chemicals, and generate further by-products (Dodson *et al.*, 2015). Biosorption is, therefore, an environmentally friendly alternative for recovering metals from industrial wastewater, leaching solutions, or mine water. Such raw material sources also pose a special challenge for potential biosorbent materials, as they often have a low pH value, contain various competing metal ions and other organic components that can interfere with the biosorption process. For an economical production of metals by biosorption, the biomaterials used must, therefore, meet the requirements of stability, element selectivity, effectiveness, and cost-efficiency

Table I-1. Relevant binding groups for biosorption (modified after Volesky, 2007)

Binding group		Ligand atom	Representation in biomolecules
Hydroxyl-		O	polysaccharides, uronic acids, sulfonated polysaccharides, amino acids
Carbonyl-		O	peptide bond
Carboxyl-		O	uronic acids, amino acids
Sulfhydryl-		S	amino acids
Sulfonate-		O	sulfonated polysaccharides
Thioether-		S	amino acids
Amine-		N	chitosan, amino acids
Secondary amine-		N	chitin, peptidoglycane, peptide bond
Amide-		N	amino acids
Imine-		N	amino acids
Imidazole-		N	amino acids
Phosphonate-		O	phospholipids
Phosphodiester-		O	teichoic acids, lipopolysaccharides

(Pollmann *et al.*, 2018). In previous studies, various materials for the biosorptive recovery of valuable metals have been discussed. Different fungi, bacteria, and algae, but also agricultural residues and individual biopolymers, especially polysaccharides such as cellulose, chitin, chitosan, and alginate, as well as proteins have been tested. The focus was mainly on materials that were abundant and cheap. Biosorbent materials were generally regarded in their native form. In particular, lignocellulose-based materials from agricultural residues can be used after minimal preparation and are therefore particularly cost-effective. However, such materials do not always offer the required mechanical stability and regenerability due to poor separability from the reaction mix. By immobilizing biomass on a suitable matrix, these disadvantages can be overcome, and biosorbent materials with improved porosity, capacity, and lifetime can be produced. However, the immobilization of biosorbents usually makes them more expensive. Furthermore, increased diffusion resistance through the immobilization matrix can lead to a reduced metal adsorption rate. In general, modifications should not compromise the advantageous properties or the environmental and social compatibility of the biosorbent (Dodson *et al.*, 2015).

Even though the biotechnological production of metals by biosorption is currently considered to play a promising and pioneering role in resource technology, the technology has hardly been industrially implemented and commercialized. This is often attributed to a lack of selectivity of the biosorbents in question. To overcome this obstacle, the use of sophisticated adsorbent materials has been discussed in many places. For this purpose, special, smaller biomolecules can be used, which are able to specifically recognize individual elements, bind them selectively, and thus remove them from solutions. A possible approach is the use of naturally occurring metabolites with specific metal-binding properties. In this context, different proteins (e.g., Maruyama *et al.,* 2007), metallothioneins (e.g., Terashima *et al.,* 2002), siderophores (e.g., Jain *et al.,* 2019) or organic acids (e.g., Inbaraj *et al.,* 2009) were investigated with respect to their suitability for application in biosorptive materials (reviewed by Pollmann *et al.,* 2018). A further approach consists in the direct development of suitable ligands. Peptides offer a particularly promising basis for such developments and have been investigated in many studies regarding their ability to selectively recognize inorganic surfaces and ions. Peptides are small and extremely flexible in their diversity due to the combinability of amino acids with different side-chain functionalities. This allows the configuration of specialized ligands by complementary adaptation to molecular architectures (Sarikaya, Tamerler, Daniel T. Schwartz, *et al.*, 2004).

One possibility for the development of application-specific peptide ligands is the directed molecular evolution.

Phage Surface Display for the recovery of inorganic binding peptides

Directed evolution is a process of protein engineering in which the natural selection process is imitated to obtain protein variants with specific properties. Alterations to the protein are achieved through iterative cycles of random mutagenesis, which leads to the generation of diversity at the genetic level. The result is a library of gene variants. The expression of the corresponding gene products allows high-throughput screening for certain desired properties at the protein level. The concept originated in Sol Spiegelman's experiments on the in vitro self-replicability of RNA oligomers, the so-called "Spiegelman's monster" in the 1960s (Mills, Peterson and Spiegelman, 1967). In the following years, the approach was transferred to in vivo selection for improved or even new enzymes in bacteria. With the idea of the Phage Surface Display (PSD) in 1985 by G.P. Smith (Smith, 1985), the concept of directed evolution experienced a major progression, as the method allowed for targeted adaptation by the variation of individual protein molecules and thus in vitro evolution on a molecular level (Bornscheuer *et al.*, 2019). In his work, Smith showed how foreign DNA fragments could be introduced into the genome of bacteriophage in a manner that enabled the corresponding gene product to be displayed as a fusion product on the bacteriophage particle surface during host-dependent particle assembly. This creates a close and very useful link between the phenotypic expression of a protein variant on a particle surface and the genotypic coding for this protein in the particle interior. The development was a milestone for science using directed molecular evolution and a springboard for further molecular display methods. In 1986 Freudl *et al.* and Charbit *et al.* showed how peptides could be expressed on the bacterial proteins OmpA and LamB in the Cell Surface Display (CSD) and thus be displayed on the surface of *Escherichia coli* cells (Charbit *et al.*, 1986; Freudl *et al.*, 1986). Another possibility is the cell-free display of peptides on nucleic acids or in the polysome display. The most common representative of these in vitro molecular display techniques is the ribosome display. It consists of a synthetic DNA library of a specific mutated gene. The in vitro transcription of the library produces a collection of the corresponding mRNAs. Due to the absence of corresponding stop codons during translation, these form a ternary complex with the translation product on the ribosome (Mattheakis, Bhatt and Dower, 1994).

However, the cell-dependent in vivo methods PSD and CSD are still the most common methods of molecular display today. However, they also have some disadvantages due to the cell dependency of the systems. These include limitations in the generatable complexity of libraries due to physiological limitations in the transformation efficiency of the host, but also the degeneracy of the genetic code. This allows a maximum complexity of 10^{10} variants and thus undercuts the presentation of all possible variants of a population starting at a sequence length of seven amino acids. A library presenting 12 amino acid peptides, therefore, contains only 0.00025 % of all theoretically possible variants. In addition, the complexity and composition of such in vivo systems are influenced by the host dependency in the expression of peptide variants. Both codon usage, the natural level of tRNAs in the host and individual

preferences in the expression and secretion of certain peptide products lead to a strong host influence on the composition of the library (Sarikaya, Tamerler, Daniel T. Schwartz, *et al.*, 2004). Nevertheless, the systems have proven to be particularly robust and easy to use. Prefabricated libraries are commercially available.

Ever since the first presentation of the method, the molecular display has been a rapidly growing technology with a major impact in the fields of immunology, cell biology, protein engineering, physiology and pharmacology, and in 2018 G.P. Smith was awarded the Nobel Prize in Chemistry for this achievement.

Regardless of their successful introduction to molecular display, bacteriophage have been useful tools in recombinant DNA technology since the 1970s. In particular, filamentous Ff bacteriophage of the Inoviridae group of class II viruses were widely studied as useful cloning vectors and became a model system in molecular biology. In this group, M13, fd and f1 are the best-characterized bacteriophage. They are male-specific coliphages that exclusively infect *Escherichia coli* cells that possess the conjugative F plasmid. The exact architecture of the bacteriophage particles is known and considered to be particularly robust against dramatic structural changes caused by the intervention of recombinant DNA technology (Petrenko, 2018).

The bacteriophage (see Figure I-1 for schema) have a covalently closed, single-stranded DNA genome. The phage genome is completely encapsulated by a capsid consisting of five different structural proteins. It codes eleven genes (pI - pXI). The corresponding gene products fulfill functions during DNA replication, particle assembly and as capsid proteins in the life cycle of bacteriophage. In addition, it has an intergenic region that contains the origin of replication for the synthesis of both the (+) and (-) strand as well as a hairpin that functions as a packaging signal.

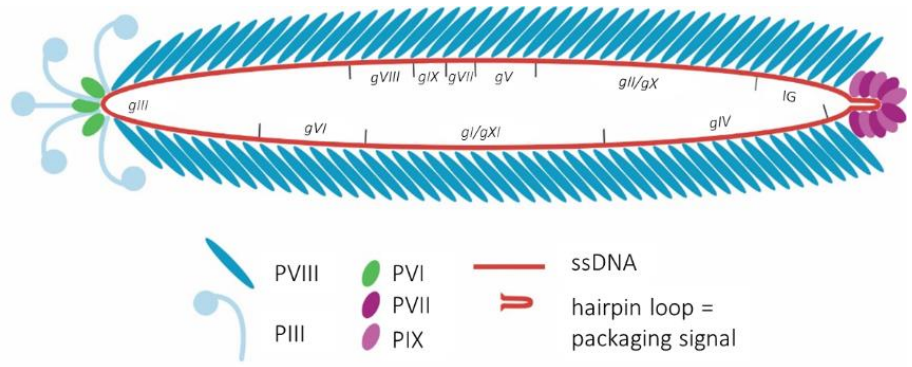


Figure I-1. Schematic representation of the Ff-type phage particle showing the arrangement of the capsid proteins and the orientation of the circular phage genome (modified after Trän, 2019)**.**

The phage genome is completely encapsulated by a capsid consisting of five different structural proteins. PVIII builds the elongated cylinder that forms the main structure of the particle. In this process, approximately 2700 PVIII units bind to the circular DNA molecule via the C-terminal domain, due to their positive total charge. The N-terminal protein domain forms

the particle surface. At the opposite ends of the filamentous particle, five copies each of the minor capsid proteins PVII and PIX form one pole and five copies each of PIII and PVI form the other pole. The infection of the *E. coli* host cells by the filamentous bacteriophage (see Figure I-2) is mediated by the interaction of the PIII minor capsid protein with the tip of the conjugative F-pilus. Depolymerization of the pilin subunits causes the pilus with the associated bacteriophage particle to be retracted into the inner bacterial membrane. The tip of the bacteriophage thereby encounters the membrane surface, integrating the capsid forming PVIII units into the bacterial membrane and translocates the phage genome into the cytoplasm. For the injection of DNA into the host cell, the host's own TolQRA complex is recruited by PIII. In the cytoplasm, the infectious ssDNA genome is converted by host-own enzymes into double-stranded supercoiled DNA, the so-called replicative form (RF-DNA). The RF-DNA serves as a template for the synthesis of the bacteriophage proteins PI-PXI and further (+) strands.

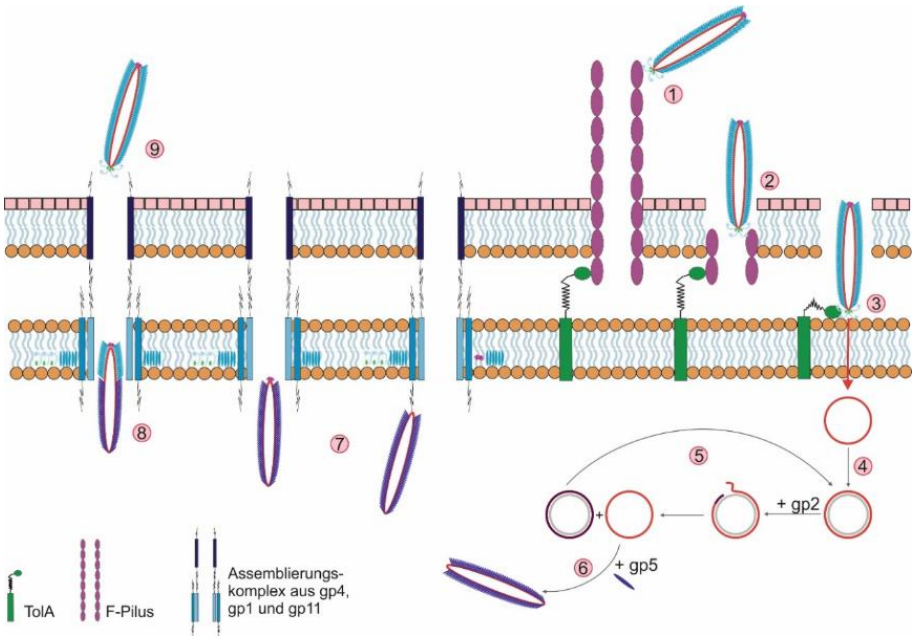


Figure I-2. Process of the host-dependent amplification of Ff-type bacteriophage in *Escherichia coli* with infection (1 - 3), reproduction (4 - 6) and assembly (7 - 9) (Trän, 2019).

The bacteriophage gene products PII, PV and PX, are involved in the replication of the phage genome. PII binds to the intergenic region of the phage genome and cleaves it endonuclease-like at a defined site. The resulting end serves as a primer for the synthesis of a new (+) strand in rolling circle replication by the host's own metabolism. PII is responsible for the circularization of the fully replicated phage genome. To prevent the conversion of the newly synthesized (+) strands into RF-DNA, it is recognized and bound by dimers of the protein PV and thereby recruited for the membrane-associated assembly complex. The protein PX is the translation product following initiation at codon 300 in gII. It has the same C-terminal domain as PII and probably has a regulatory function as a competitive inhibitor of PII in the replication of phage DNA.

The gene products PII, PVI, PVII, PVIII and PIX, form the capsid of the bacteriophage particle. After their biosynthesis, they remain in the cytoplasmic membrane until particle assembly. Particle assembly is a membrane-associated process involving the bacteriophage gene products PI, PIV and PXI, which form a complex at specific assembly sites for the construction and release of the particles. PI is integrated into the cytoplasmic membrane after its synthesis. It spans the membrane once completely and has an N-terminal region in the cytoplasm and a C-terminal region in the periplasm of the host cell. Several PI units join in the cytoplasmic membrane to form a transmembrane domain and thus form a channel through the inner host membrane. PXI is the translation product that follows initiation at codon 241 of gI and is thus homologous to the C-terminal domain of PI. PXI is also integrated into the cytoplasmic membrane, but the protein units are not able to form a channel comparable to PI. PIV is transported into the outer host membrane after its synthesis and anchored C-terminally in the membrane. There it forms oligomers of 10-12 subunits, while the N-terminal residue does not interact with each other in the periplasm. The oligomers form a large pore for the secretion of fully assembled bacteriophage particles. The periplasmic domain of PIV interacts with the periplasmic domains of PI and XI to form the assembly complex that spans both host membranes.

The assembly is initiated from the PV-DNA complex by the interaction of the N-terminal domain of PI with the packaging signal in the phage genome, which is not masked by PV units. Starting from the packaging signal, the tip of the bacteriophage particle is formed by the assembly of the minor capsid proteins PVII, PIX, with PVIII on the phage genome. The PV units are then subsequently replaced by units of the major capsid protein PVIII. The assembly of the cylindrical main structure of the particle is PI-mediated and ATP-dependent through the pore of the assembly complex. At the end of the DNA, the assembly process is terminated by the addition of the minor capsid proteins PIII and PVI. The finished bacteriophage particle is completely released from the host cell through the assembly pore. The biology of Ff-type bacteriophage is very beneficial for biotechnological exploitation (reviewed by Webster, 1996).

The life cycle, as well as the architecture and genetics of bacteriophage, are already well understood. Although the infection of bacteriophage has a major impact on the host cell metabolism, it does not lead directly to cell death. The release of the particles is lytogenic, continuously through the special membrane-associated assembly site during particle construction. This mechanism also makes the bacteriophage robust against molecular biological manipulations that lead to an enlargement of the phage genome. Actually, the size of the bacteriophage particle changes dynamically with the length of the genome to be packaged. Up to 12 kb of additional DNA codes can be placed on the phage genome without hindering the packaging (Marvin, 1998). The capsid itself has a clearly defined design that can be well exploited by nanotechnology. It is remarkably tolerant of drastic structural changes (Petrenko, 2018). Therefore, it is not surprising that the filamentous bacteriophage were the starting point for the development of Surface Display technology and are still the most frequently used to implement the method today.

A phage display is created by introducing an alien gene sequence, e.g., a peptide, into the phage genome in such a way that it is genetically fused with one of the capsid proteins. The protein chimera thus generated is incorporated into the bacteriophage during host-dependent particle assembly so that the foreign sequence is presented on the surface of the particle. A heterogeneous mixture of phage clones, each carrying a different foreign sequence, is called a phage display library. Phage display libraries can consist of natural peptides, random peptides or whole proteins or protein fragments (Petrenko, 2018).

In general, it is possible to use all five capsid proteins for the display of fusion products on the bacteriophage particle surface, but PIII and PVIII display systems are the most commonly used, and the fusion must take place on the periplasmic part of the respective capsid protein. Furthermore, the fusion product must be transportable through the inner membrane after biosynthesis in the cytoplasm; it must enter the assembly site from the periplasm and it must not interfere with the assembly itself (Webster, 1996).

In the presentation of peptides and protein fragments in PSD, a distinction is made between monovalent and multivalent PSD systems. Monovalent systems represent the foreign sequence on one or fewer copies of the capsid protein per bacteriophage particle. Multivalent systems represent the foreign sequence on more than one copy of the capsid protein per bacteriophage particle. If the phage genome contains only one copy of the capsid protein to which the foreign sequence is fused, the foreign sequence is displayed in the particle at all copies of the capsid protein. In hybrid systems, the phage genome contains two copies of the capsid protein: one contains the foreign sequence, and the other is unchanged. The expression of both copies is regulated by different promoter systems so that the wildtype copy of the capsid protein is generally more abundant in the particle than the fusion construct. In phagemid systems, the result is the same. It consists of the phagemid vector, which codes for the fusion product, and helper phage, which is used for the assembly of whole phage particles containing some copies of the modified capsid protein (Smith and Petrenko, 1997). The different PSD systems are suitable for different target selections. PIII systems show the foreign sequence only in a very small number of copies. Such systems are advantageous for the identification of high-affinity ligands. In contrast, strongly multivalent PVIII systems, which form a dense array of the foreign sequence on the particle surface, are more suitable for the selection of increased avidity of the interacting ligands (Petrenko, 2018).

The performance of PSD systems depends on the diversity of the peptides represented in the library. Due to the diversity, it is possible to mimic the evolutionary formation of biomolecules.

In a process called biopanning, the library is selected according to certain desired peptide variants (see Figure I-3). This is an affinity selection technique, in which the required quality of the peptide ligands depends on their recognition or interaction with a target.

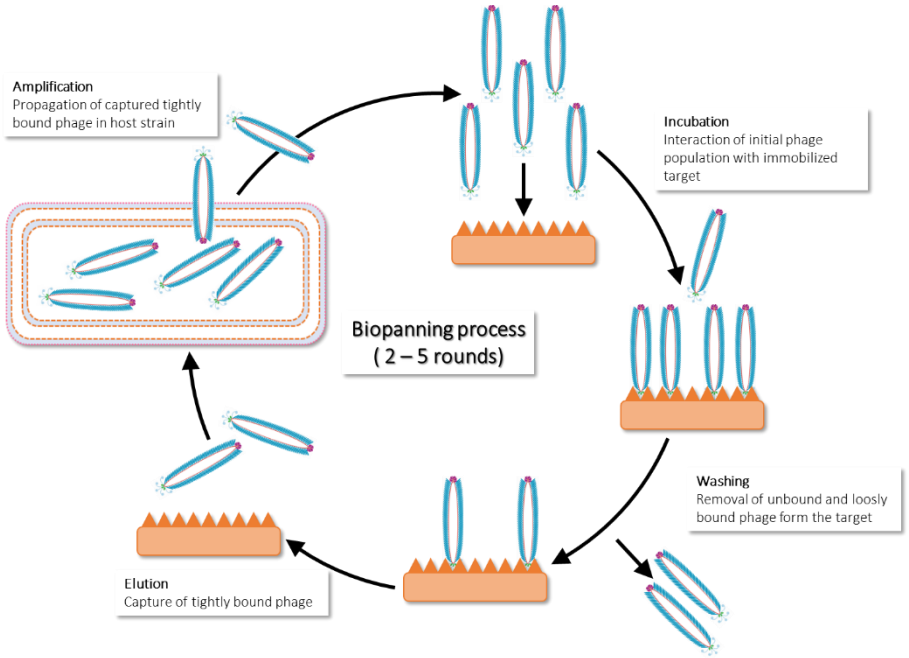


Figure I-3. Schematic biopanning process of a random peptide library against an immobilized target.

For singling out the peptide variants that interact with the target from those that do not have the desired property, it is essential that the target is immobilized. A starting population of phage clones, which usually corresponds to the naive PSD library, is given the opportunity to interact with the immobilized target in solution. Non- or weakly interacting clone variants are removed from the population by subsequent washing steps. The resulting subpopulation is separated from the target by elution and has greater fitness than the original population with respect to the desired peptide properties. The individual variants of the subpopulation are highly enriched by amplification. The amplified population can then be the starting point for further selection rounds. The fitness of the population of peptide variants can be successively increased in the subsequent biopanning rounds by increasing the stringency in the selection conditions with regard to the desired peptide property (Smith and Petrenko, 1997). The stringency of biopanning is determined by the amount of target offered and the solvent environment in which the selection takes place. The chemical composition of the solvent during the initial interaction, washing and elution significantly influences the relationship between target and bacteriophage clone (Sarikaya, Tamerler, Daniel T. Schwartz, *et al.*, 2004). Therefore, the success of a PSD experiment depends not only on the diversity of the library but also on the individual adaptation of the biopanning process conditions. Since its establishment 35 years ago, the PSD has developed into an important tool for tackling a wide range of challenges in the various fields of biotechnology. It has proved to be a particularly useful tool for the characterization of protein-protein interactions, enzyme specificity and inhibition, antibodies, receptor-ligand relationships, epitope and mimotope mapping, targeted cell and tumor targeting and other (reviewed in detail by Arap, 2005; Kehoe and Kay, 2005; Pande, Szewczyk and Grover, 2010; Wu *et al.,* 2016; Rahbarnia *et al.,* 2017).

The usability of Surface Display technologies is not limited to soft biological target materials. Brown and co-workers showed already a few years after the idea of the Surface Display was first described how inorganic binding peptides could be identified by the application of *E. coli* based CSD (Brown, 1992, 1997). Since then, the PSD, in particular, has been used for the identification of peptide ligands for a wide range of inorganic target materials. It is known from nature that proteins, together with inorganic compounds, can form hard biological materials such as teeth, bones, cartilage, mussels etc. in a controlled manner (Seker and Demir, 2011).

Such protein structures are highly interesting for nanotechnology and materials science and are a model for the development of biobased technologies for the selective recognition of surfaces and the controlled formation of innovative biocomposites. The targeted application of PSD technology allows the development of new peptide ligands that can perform technical functions.

In previous work, different peptide ligands for the recognition of defined inorganic surfaces were developed. Zuo *et al.,* for example, developed peptides that could be deposited on steel and aluminum to prevent surface corrosion (Zuo *et al.*, 2005). Meyers *et al.* developed titanium-binding peptides for medical applications on implants, where the peptides on the metal surface were supposed to mediate better integration into tissue (Meyers *et al.*, 2007). Furthermore, peptide chimeras that combined a titanium-binding peptide domain with an anti-microbial peptide domain to prevent infection of titanium implants were developed (Liu *et al.*, 2016). Whaley *et al.* developed GaAs-binding peptides that could selectively distinguish the surface of the semiconductor material from other material surfaces such as silica or gold (Whaley *et al.*, 2000). The selective recognition of different metal-containing particles by peptides was also reported. Umetsu *et al.* developed highly selective peptides that could discriminate between ZnO and ZnS (Umetsu *et al.*, 2005). Lederer *et al.* developed peptides for the resource-relevant differentiation of REE particles (Lederer *et al.,* 2017; Lederer *et al.,* 2019). The development of specific peptides for the binding of the different minerals hematite (Lower *et al.*, 2008), perovskite (Reiss *et al.*, 2006), sphalerite, chalcopyrite and enargite (Curtis *et al.*, 2009, 2017), which are highly relevant for resource technology, was also described.

In addition to the selective recognition of inorganic surfaces and particles, specialized peptides can take over other functions for nanotechnology. The unique structure and life cycle of the Ff type bacteriophage plays a special role. The filamentous particles can serve not only as a vehicle for the selection of inorganic binding peptides but also as a matrix for phage-originated nanocomposites. In various studies, it has been shown how specially selected metal-binding peptides can influence the size, shape and stability or the functionality of the nanoparticles. The peptide-mediated formation of nanoparticles from noble metals such as gold (Naik *et al.*, 2002; Slocik, Stone and Naik, 2005; Kim *et al.*, 2010), silver (Naik *et al.*, 2002), platinum (Kantarci *et al.*, 2005; Oren, Tamerler and Sarikaya, 2005; Seker *et al.*, 2007) or palladium (Pacardo *et al.*, 2009) was investigated particularly intensively. Here, the filamentous bacteriophage structure served as a carrier for the peptides that mediate NP formation. Another possibility is the peptide-controlled formation of biocomposites with special properties

for materials science, biomedicine and nanotechnology. For example, Kilper *et al.* created a novel bioceramic through the biomineralization of ZnO. Using the PSD technology, a five amino acid long ZnO-binding motif was identified, which was displayed on the surface of recombinant bacteriophage particles. In the presence of ZnO, the particles assembled to thin-layered bioceramics with remarkable properties (Kilper *et al.*, 2018).

Although PSD experiments are a well-established system for biological and chemical targets, special attention needs to be paid to the development of inorganically binding peptides. Often, PSD experiments cannot be performed according to a standard procedure and have to be specifically adapted for the development of peptides for specific inorganic targets. In this case, the consideration of the chemical and physical properties of the target material is crucial for the performance of a correct screening. It is necessary to ensure that the target material meets the experimental requirements during biopanning. The solvent environment must not unintentionally modify the target, and corrosive and erosive effects must be avoided. At the same time, incubation and washing conditions must avoid unspecific interaction with non-binders. Also, the eluents often have to be specially adapted to isolate the best binding clone variants (Sarikaya, Tamerler, Daniel T. Schwartz, *et al.*, 2004).

Recently, Matys *et al.* presented the method of biological elution, in which the most strongly binding and non-chemically elutable clone variants are solved by contact with host cells at the target (Matys *et al.*, 2020). The system takes advantage of the naturally high affinity of the minor capsid protein PIII for the pilus of the host cell, allowing the strongest binding variants to be amplified directly away from the target.

Metal ions represent a particularly challenging inorganic target. The selection against such substances, which actually only occur in dissolved form, requires the immobilization of these. At the same time, the biopanning environment must ensure the constant stable presentation of the immobilized metal ions for interaction with the clones of a bacteriophage population. The buffers for initial interaction, washing and elution should not cause unintentional leaching of the target material. Nian *et al.* first performed PSD experiments to obtain Pb^{+} binding peptides, selectively. He presented a novel and innovative approach in which he immobilized the target metal ions by chelating them on monolithic IDA columns. This allowed for a very well controllable biopanning against lead ions in a chromatographic system (Nian *et al.*, 2010). Yang *et al.* used an NTA resin for the immobilization of Cr^{3+}. By using the PSD for this target material, the identification of chromium(III) specific peptides was possible. By immobilizing the corresponding bacteriophage clones, a material for the discrimination between chromium(III) and chromium(VI) species in wastewater was obtained (Yang *et al.*, 2015). In another approach, Fe_2O_3-based magnetic microbeads were used for the immobilization of arsenic. The target material was used to identify arsenic(III) binding peptides. The peptides were further used to generate an As(III) sensitive biosensor (Yang *et al.*, 2018). Matys *et al.* presented the possibility of immobilizing metal ions on a planar functionalized sol material and isolated nickel and cobalt binding peptides (Matys *et al.*, 2020).

Gallium – Example of a high-tech metal

Gallium is declared a high-tech metal because of its industrial use in innovative technologies. It is a half-metal with interesting properties. The metal occurs at an extremely high temperature range from 29.76 to 2400 °C in a liquid aggregate state. It has a density anomaly comparable to water and is diamagnetic in the solid state but acts paramagnetic in the liquid state.

Gallium is of high economic relevance and is, therefore, declared as a strategic raw material (European Commission, 2014). Over 90 % of the gallium produced worldwide is used in electronic applications (Zhao *et al.*, 2012). Gallium is mainly used as a semiconductor in the compounds GaAs and GaN and less frequently as GaA, GaP or GaSb. In the form of these components, gallium is used in optoelectronics, LED elements, high-frequency technology and photovoltaics. Smaller quantities of the gallium produced worldwide are used as an alloying additive in dental technology and as a diagnostic agent in nuclear medicine.

In stark contrast to the enormous industrial importance of gallium, the raw material is not readily available. With about 14 ppm mass fraction in the earth's crust, the metal is considered rare. Gallium does not form its own ore deposits and naturally occurs only in combination with other elements. Mainly gallium is found esterified with aluminum and zinc. More than 90 % of the gallium produced primarily comes from bauxite as a by-product of aluminum production (Marscheider-Weidemann *et al.*, 2016). Pure gallium is obtained by electrolysis, which is very expensive and energy-intensive after several complex processing steps. At present, China is the main producer of primary gallium. The potential for supply bottlenecks of gallium to the industry has been assessed as high (European Comission, 2014). The dependence on the geopolitical climate of the supply chain, which is caused by the uneven global distribution of geological gallium, was considered a more pressing factor than the increasing scarcity of the resource itself (Ueberschaar, Otto and Rotter, 2017).

The pressure to recycle gallium from secondary raw material sources is correspondingly high. At present, the recovery of gallium from EOL products is considered to be difficult to implement (UNEP, 2011). Due to the low mass fraction in electrical and electronic equipment, gallium is lost through subsequently dilution steps during processing. In fact, only about 8% of the gallium produced worldwide is found in consumer end products. Most of the industrially used gallium is lost during production so that residues from the gallium processing industry are a much more abundant and important source for secondary gallium production (Ueberschaar, Otto and Rotter, 2017).

Aims and context of the present work

This dissertation was performed within the framework of the joint project "EcoGaIN - Gewinnung von Gallium aus Produktionsabfällen der Halbleiter-Industrie" (Extraction of Gallium from Production Waste of the Semiconductor Industry). It was financed within the framework of the r4 program "Innovative Technologies for Resource Efficiency - Extraction of Secondary Raw Materials and Mobilisation of Processing and Production Residues" and was

conducted in the period 06/2016 to 10/2019. The aim of the project was the development and implementation of strategies for the recovery of secondary gallium from a special source of raw materials, the industrial residues of the semiconductor industry. During the production of GaAs wafers, large amounts of solid and liquid wastes are generated, that contain considerable amounts of valuable materials - mainly Ga, As and SiO_2. Technologies for the treatment of these waste materials should be developed and integrated into real industrial processes for the implementation of a circular economy. Among other things, methods for the recovery of gallium and arsenic from low-concentration wastewaters should be developed in order to avoid the deposition of residual materials and to complete the recovery of valuable substances. These solutions are accumulated in considerable quantities and have different pH values between pH 3.0 and pH 9.0, depending on the process step. The metal content is below 0.002 %. In addition to gallium and arsenic, the wastewater also contains impurities with other metals such as iron or aluminum, silicon, fluorine and organic components, for example, surfactants. For this purpose, biotechnological processes for the selective sorption of gallium and arsenic from diluted rinsing solutions should be established.

The aim of the present work was the selection of gallium-binding compounds using PSD technology and the development of peptide-based sorption materials.

(i) Development of a method for the selection of Ga-binding bacteriophage clones using PSD technology.

(ii) Identification of gallium-binding peptides from the enriched bacteriophage population.

(iii) Establishment of test systems for the evaluation and quantification of the binding properties of the isolated clones.

(iv) Immobilization of the peptides for the possibility of phage-free application under practice-oriented conditions.

(v) Characterization of the generated biocomposites and testing of the biosorption efficiency using real wastewater samples.

The results of these studies are presented in Chapters II-IV. Chapters II and III have been published in scientific journals, and Chapter IV has recently been submitted for publication.

Chapter II reports on the identification of gallium-binding peptides. Methods for chromatography-based enrichment of gallium-binding peptide sequences from a recombinant phage library are presented. By developing binding assays, a total of five gallium-binding bacteriophage clones can be selected.

In Chapter III, the affinity and selectivity of these five bacteriophage clones for the biosorption of gallium and arsenic under different process conditions are investigated. A side-directed mutagenesis screening, a so-called "cysteine scanning," is used to optimize a less efficient adsorbing clone. Thus, the crucial relationship of peptide-target interaction based on peptide structure and amino acid position and composition can be demonstrated.

Chapter IV reports on the further characterization of gallium-binding sequences as peptides independently synthesized from the bacteriophage clone. Isothermal titration microcalorimetry is used to investigate the interaction of the free peptide with gallium and arsenic. In addition, the biosorption of side-directed and covalently immobilized peptides of gallium from model waters as well as real process water samples is studied.

Chapter V summarizes the insights gained in this work on the isolation of metal-binding peptides using phage surface display technology. Furthermore, conclusions are made about the development of peptide-based materials for the biosorptive recovery of metal ions from aqueous solutions.

Chapter II.

CHROMATOPANNING FOR THE IDENTIFICATION OF GALLIUM-BINDING PEPTIDES

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Author contributions

conceptualization, K.P. and F.L.L.; methodology, N.S. and F.L.; investigation, N.S and K.F.; writing—original draft preparation, N.S.; writing—review and editing, K.P., F.L.L., R.B. and S.M.; project administration, K.P.; funding acquisition, K.P. and S.M.

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.

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 (Frenzel *et al.*, 2017). 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 (European Comission, 2014).

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 (Pollmann *et al.*, 2018).

Among these, biosorption, which is the passive interaction of biomass with certain ligands in aqueous solution, is very well studied (Vijayaraghavan and Yun, 2008; Gadd, 2009), especially with respect to the removal of heavy metals. Besides the often-discussed application for remediation purposes (Kratochvil and Volesky, 1998; Gupta *et al.*, 2000), 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 (Steffens, 1990; Mejare and Bulow, 2001; DeSilva *et al.*, 2002; Sovago and Osz, 2006).

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 (Smith, 1985). 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) (Block *et al.*, 2009). 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 (Patwardhan *et al.*, 1998; Day *et al.*, 2013) and chromium (Yang *et al.*, 2015), IDA for lead (Nian *et al.*, 2010), and 1,4,7-Triazacyclononane (TACN) based ligands were used for nickel ions (Mooney, Fredericks and Hearn, 2011).

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 (Noppe *et al.*, 2009; Adriaenssens *et al.*, 2012). 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 (Posewitz and Tempst, 1999; Nuhse, Yu and Salomon, 2007; Novotna *et al.*, 2008). 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 (Wood and Samson, 2006). 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.

Materials 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 *Escherichia coli* K12 ER2738 (*F' proA+B+ lacIq Δ(lacZ)M15 zzf::Tn10(TetR9)/fhuA2 glnV Δ(lac-proAB) thi-110 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 $5 \cdot 10^8$ 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 Table II-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.

Table II-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

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 Table II-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 Table 1) is based on common Ga-IMAC approaches for the purification of phosphopeptides and -proteins (Posewitz and Tempst, 1999; Nuhse, Yu and Salomon, 2007; Novotna *et al.*, 2008) as well as the earlier described protocol for a chromatopanning against lead (Nian *et al.*, 2010). 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 Table II-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 $3.75 \cdot 10^{11}$ 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.

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.

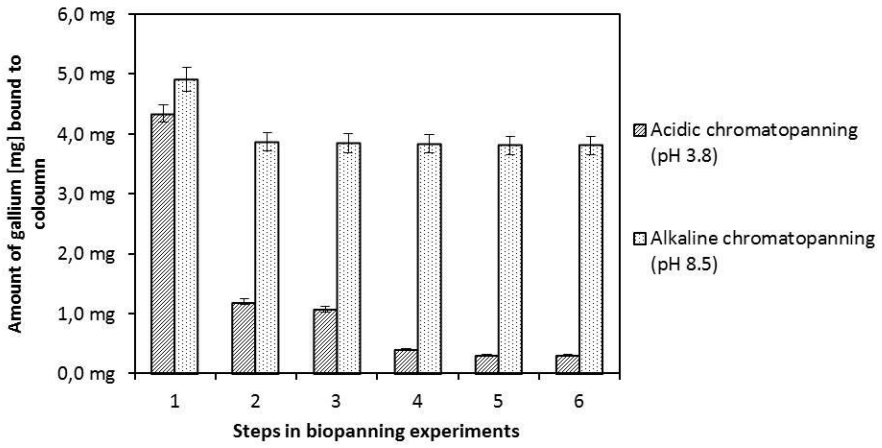

Figure II-1. Immobilization of gallium during chromatopanning. Amount of gallium bound to monolithic column (0.34 mL) at slightly acidic and slightly alkaline conditions. 1– initial immobilization; 2 – first wash; 3 – phage treatment; 4 – second wash; 5 – phage elution; 6 – stripping.

A method based on Ga-IMAC protocols for phosphopeptides and phosphoproteins was developed in 1999 by Posewitz and Tempst (Posewitz and Tempst, 1999), whereas Nian *et al.* (Nian *et al.*, 2010) 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 Figure II-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 Figure II-2).

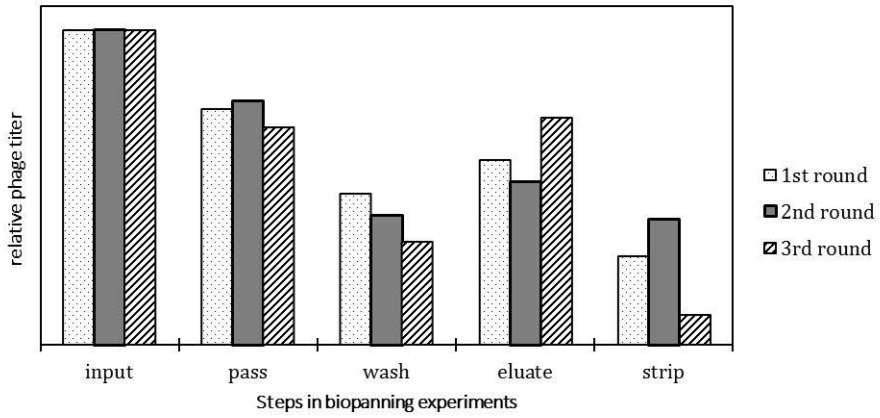

Figure II-2. Bacteriophage titer during three rounds of chromatopanning against Ga immobilized on monolithic CIM®IDA disc under acidic conditions. Shown is the amount of bacteriophage in input, pass, wash, elution and stripping. The amount was normalized against the respective input in each chromatopanning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

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 Table II-2) as reported elsewhere (Ploss *et al.*, 2014; Braun *et al.*, 2018). 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.

Table II-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)	Occurrence after Biopanning		
			Input concentration	II	IV	V
M13 KE Wt			0.00E+00 pfU	15/133	0/24	9/24
C3.2	RVQPAHFNVMGQ	9.76	4.00E+11 pfU	1/110	0/24	0/24
C3.4	MVGTADGTLLDP	3.56	4.00E+11 pfU	1/110	0/24	0/24
C3.5	ANTELALANRKH	8.80	4.00E+11 pfU	2/110	0/24	0/24
C3.8	TMHHAAIAHPPH	6.82	4.00E+11 pfU	1/110	0/24	2/24
C3.14	GIVTNQHDSNAN	8.75	4.00E+11 pfU	1/110	0/24	0/24
C3.15	NYLPHQSSSPSR	5.08	4.00E+11 pfU	2/110	2/24	1/24
C3.20	GLTFQVPWHANM	6.74	4.00E+11 pfU	1/110	0/24	0/24
C3.27	SLPNLPPTYAKP	8.31	4.00E+11 pfU	2/110	0/24	0/24
C3.28	GHPMMPPKSEIR	8.75	4.00E+11 pfU	1/110	0/24	0/24
C3.52	ASNHSIPTFPLK	8.80	4.00E+11 pfU	2/110	0/24	0/24
C3.72	NPMNNVAQNPGP	5.52	4.00E+11 pfU	2/110	0/24	0/24
C3.77	TMAQGVAQRYGN	8.41	4.00E+11 pfU	1/110	0/24	0/24
C3.78	TLGLRPVPVATT	9.41	4.00E+11 pfU	2/110	0/24	0/24
C3.86	SHQPGDQSPANN	5.06	4.00E+11 pfU	1/110	0/24	0/24
C3.90	DLINIDRNHSFR	6.75	4.00E+11 pfU	1/110	0/24	0/24
C3.102	LPKQCSLLTSAC	8.06	4.00E+11 pfU	1/110	0/24	0/24
C3.103	GSWNTFRAQPTI	9.75	4.00E+11 pfU	2/110	0/24	0/24
C3.105	NFTLQAHPHKYP	8.61	4.00E+11 pfU	1/110	0/24	0/24
C3.106	STDHGSWQKSRA	8.49	4.00E+11 pfU	1/110	0/24	0/24
C3.107	VPQLHHLMPHFD	6.25	4.00E+11 pfU	1/110	0/24	0/24
C3.108	SQALSTSRQDLR	9.31	4.00E+11 pfU	2/110	17/24	11/24
C3.115	TSMSQHFHVHRL	9.49	4.00E+11 pfU	1/110	0/24	0/24
C3.116	SPLTPPHAPETH	5.93	4.00E+11 pfU	1/110	0/24	0/24
C3.120	CPTDVRSGCMGT	5.82	4.00E+11 pfU	1/110	0/24	0/24
C3.124	IEMTRTNLNDVN	4.37	4.00E+11 pfU	1/110	0/24	0/24
C3.129	HTQHIQSDDHLA	5.70	4.00E+11 pfU	1/110	2/24	0/24
C3.130	NDLQRHRLTAGP	9.61	4.00E+11 pfU	1/110	3/24	1/24
C3.131	DDTQNSQNMDTL	3.42	4.00E+11 pfU	1/110	0/24	0/24
D3.1	HSACLGPSNLQC	6.72	4.00E+11 pfU	2/110	0/24	0/24

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 Figure II-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.

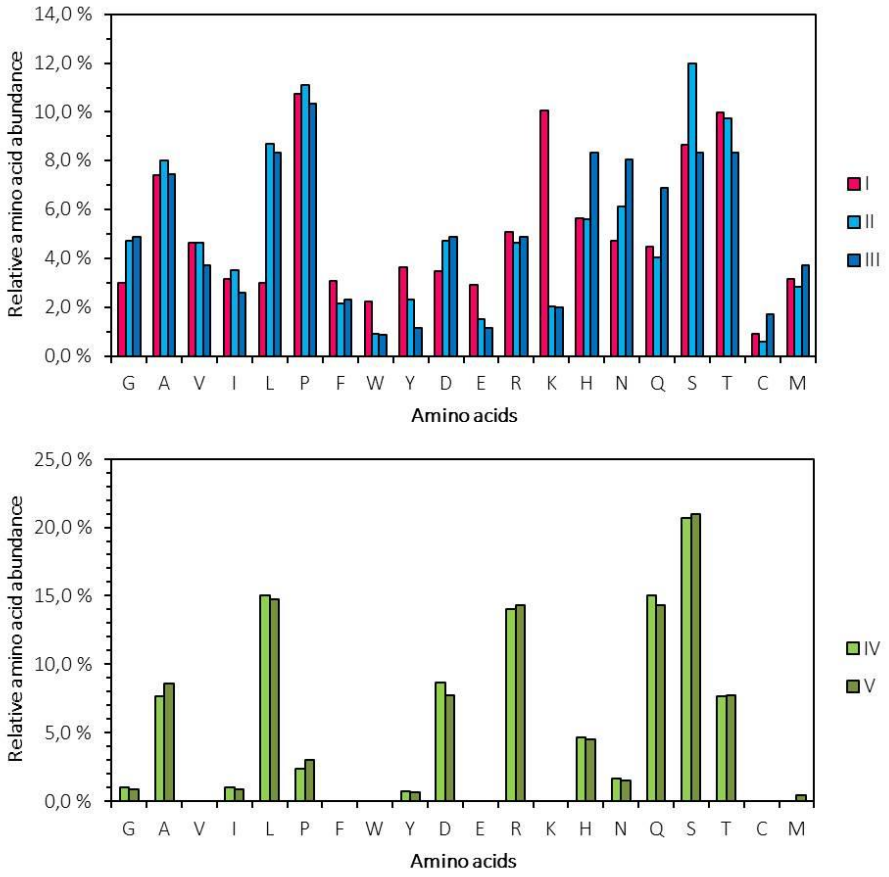

Figure II-3. Amino acid composition of different Ph.D.-12 phage pool. Shown is the percentage of each amino acid for the commercial Ph.D.-12 library (I); putative Ga-binding phage clones (II); ML-12 (III) and competitive ML-12 clones obtained under alkaline conditions (IV) and acidic conditions (V).

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 Figure II-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 Figure II-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 Figure II-3). For this, the experimentally determined composition of the library, which was reported elsewhere (Ploss *et al.*, 2014) 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 Table II-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. 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. C3.15 (NYLPHQSSSPSR) and 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 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 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 Figure II-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 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 Figure II-4, the resulting amount of phage bound to the target material is shown.

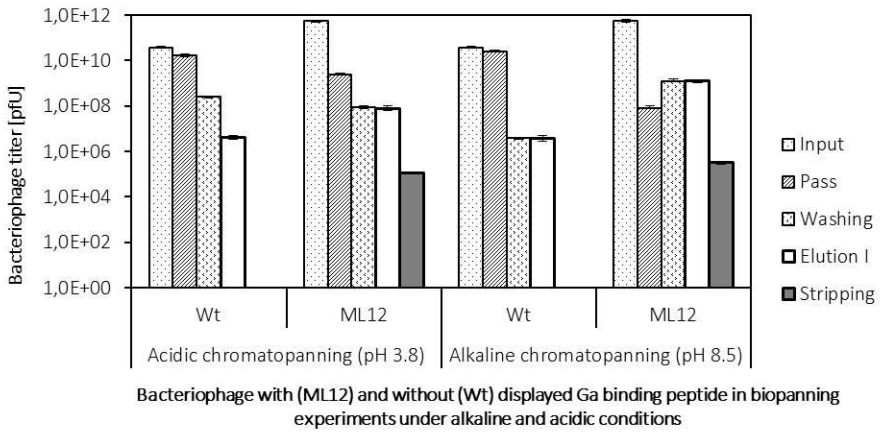

Figure II-4. Interaction of bacteriophage with and without displayed peptide sequences with immobilized Ga during chromatopanning.

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 MgCl2 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 Figure II- 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 Figure II-5).

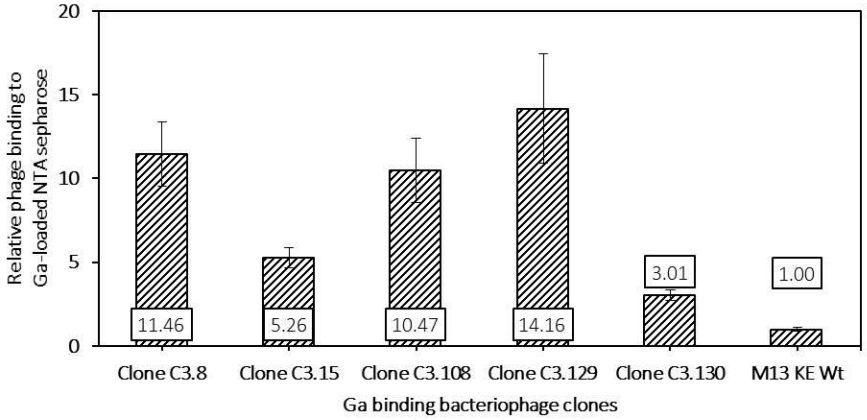

Figure II-5. Binding experiments with single clone amplicons and gallium loaded NTA agarose (25 single experiments for each clone). Shown is the relative binding in comparison to the M13 KE Wt control. The value was calculated from the amount of by Glycin-HCl eluated phage per µl Sepharose.

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.

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 (Patwardhan *et al.*, 1998; Noppe *et al.*, 2009; Nian *et al.*, 2010; Mooney, Fredericks and Hearn, 2011). The IMAC concept was first presented by Porath *et al.* (Porath, 1992). 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 (Andersson and Porath, 1986; Holmes and Schiller, 1997; Posewitz and Tempst, 1999; Nuhse, Yu and Salomon, 2007; Novotna *et al.*, 2008) 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 Table II-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 (Lawman, 2004).

Initially, the common IMAC method (Block *et al.*, 2009) 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 (Wood and Samson, 2006). 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 Figure II-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 (Smith and Petrenko, 1997).

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 Figure II-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 (Wood and Samson, 2006). 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 Figure II-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 (Curtis, MacGillivray and Dunbar, 2011; Lederer *et al.,* 2017).

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 Figure II-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 (Zanconato *et al.*, 2011) 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 (Shimazaki, Takani and Yamauchi, 2009),

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 (Sovago and Osz, 2006). 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 (Hochuli *et al.*, 1988). 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 (Kubíček *et al.*, 2010; Schmidtke *et al.*, 2017). 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 sulfur-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 (Shimazaki, Takani and Yamauchi, 2009).

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 (Sarikaya *et al.*, 2003; Artzy-Schnirman *et al.*, 2014). In fact, bacteriophage, which display metal ion-selective peptides, have already been reported elsewhere (Nian *et al.*, 2010; Yang *et al.*, 2015), 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 C3.8 (TMHHAAIAHPPH), C3.15 (NYLPHQSSSPSR), GaBi C3.108 (SQALSTSRQDLR), C3.129 (HTQHIQSDDHLA) and C3.130 (NDLQRHRLTAGP) emerged from the competitive binding experiments (see Table II-2).

C3.8 (TMHHAAIAHPPH) was the only clone that was obtained from competitive binding experiments under acidic conditions. As the only clone it has a sulfur side chain. The sulfur 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 (Kubíček *et al.*, 2010; Schmidtke *et al.*, 2017). In the single clone binding experiment, the clone proved to be an outstanding candidate. It can be assumed that for 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. 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 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 C3.8 (TMHHAAIAHPPH). The clone C3.129 (HTQHIQSDDHLA), which performs best in individual experiments at pH 7.4 (see Figure II-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 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 (Rakonjac *et al.*, 2011).

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 (Volesky and Holan, 1995). 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 Figure II-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 (Roberts *et al.*, 2015).

Elution of tightly bound phage occurred to be more successful by applying MgCl2 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 (Holmes and Schiller, 1997). 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.

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 bacteriophage.

A total of five gallium-binding peptide sequences could be identified and characterized as good binders. The peptide sequences of phage clone C3.8 (TMHHAAIAHPPH), C3.108 (SQALSTSRQDLR) and 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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Chapter III.

DIRECTED EVOLUTION AND ENGINEERING OF GALLIUM-BINDING PHAGE CLONES - A PRELIMINARY STUDY

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Author contributions

conceptualization, K.P. and F.L.; methodology, N.S.; investigation, N.S and C.Z..; writing—original draft preparation, N.S.; writing—review and editing, K.P., F.L., R.B. and S.M.; project administration, K.P.; funding acquisition, K.P. and S.M.

Abstract

The phage Surface Display technology is a useful tool to screen and to extend the spectrum of metal-binding protein structures provided by nature. The directed evolution approach allows identifying specific peptide ligands for metals that are less abundant in the biosphere. Such peptides are attractive molecules in resource technology. For example, gallium-binding peptides could be applied to recover gallium from low concentrated industrial wastewater. In this study we investigated the affinity and selectivity of five bacteriophage clones displaying different gallium-binding peptides towards gallium and arsenic in independent biosorption experiments. The displayed peptides were highly selective towards Ga^{3+} whereby long linear peptides showed a lower affinity and specificity than those with a more rigid structure.

Cysteine scanning was performed to determine the relationship between secondary peptide structure and gallium sorption. By site-directed mutagenesis, the amino acids of a preselected peptide sequence are systematically replaced by cysteines. The resulting disulfide-bridge considerably reduces the flexibility of linear peptides. Subsequent biosorption experiments carried out with the mutants obtained from cysteine scanning demonstrated, depending on the position of the cysteines in the peptide, either a considerable increase in the affinity of gallium compared to arsenic or an increase in the affinity for arsenic compared to gallium.

This study shows the impressive effect on peptide-target interaction based on peptide structure and amino acid position and composition via the newly established systematic cysteine scanning approach.

Introduction

The Interaction of biomolecules with metals is one of the most fascinating mechanisms in nature. The implementation of such mechanisms in technical applications is particularly promising and has been controversially debated in biomedicine (Farrell, 2002), biotechnological production (Benson, Wisz and Hellinga, 1998; Fushinobu, 2014; Happe and Hemschemeier, 2014) and nanotechnology (Tamerler and Sarikaya, 2009; Cohavi *et al.*, 2010; Drummy *et al.*, 2010; Care, Bergquist and Sunna, 2015). Their use for biotechnological applications in the resource technology (Dunbar, 2017) is considered to be particularly innovative. Highly specialized biomolecules are used for the selective biosorption of metals or metal-containing particles. In particular, naturally occurring metalloproteins are used, which can be recombinantly modified for technical usage (Pollmann *et al.*, 2018).

A major disadvantage of larger protein structures is their low selectivity for certain materials or metal ions. Therefore, the use of less complex biomolecules has become attractive. Especially siderophores and short peptides are promising candidates for biomining (Dunbar, 2017; Pollmann *et al.*, 2018). Actually, nature offers a wide range of such structures responsible for the recruitment of certain vital trace elements. Accordingly, naturally occurring biomolecules interact primarily with metals that are abundant in the biosphere (Hellinga, 1996). Metals that

occur rarely and are bound stable in ores do not play a role in the metabolism of most living organisms and therefore do not have naturally occurring ligands in the narrower sense.

For the production of highly specialized biomolecules that bind technological relevant metals, it is therefore important to find alternative approaches. One promising approach is the directed evolution of peptides. Inspired by natural evolution, which resulted in highly specialized biomolecules, the phage Surface Display technique systematically selects randomized peptide sequences for a certain target. This method has already been used to identify several peptides that interact specifically with metal-containing particles, surfaces or metal ions. Such peptides are mainly exploited for the development of biocomposites and nanomaterials and have as such a pioneering position in molecular biomimetics (Sarikaya *et al.*, 2003). However, several previous studies have shown that metal ion-binding peptides can also be used to differentiate between metals (Nian *et al.*, 2010) and even between different oxidation states of a metal (Yang *et al.*, 2015).

In this work the biosorption of gallium and arsenic on peptides was investigated. Gallium is considered to be a high-tech metal. Due to its important role in the electronics industry it is regarded as strategically critical (European Comission, 2018). Gallium is currently obtained mainly from primary raw material sources. The extraction from ores or minerals as a main product is not profitable. Therefore, it is primarily extracted as a by-product of aluminum during the processing of bauxite. This process is well established and highly efficient but entails a high energy and water consumption (Frenzel *et al.*, 2017). For these reasons and not at least, a rising awareness of the finiteness of primary raw materials sources the use of secondary resources is recently discussed. In this context, metal-containing wastewaters are an attractive source. For example, residues from the semiconductor industry accumulate during the production of GaAs wafers. Besides valuable amounts of gallium, these residues also contain large amounts of arsenic and other components in lower concentrations. However, as low metal concentrations, a complex metal matrix and a variable composition of other accompanying substances make the usage of such waters challenging for traditional metallurgic approaches metal binding peptide might be a suitable tool for the selective recovery of valuable metals from such solutions.

In an earlier study, we reported the identification of binding motifs of gallium-binding bacteriophage clones obtained from a commercial random peptide library. For the respective clones, a maximum of 14-fold better gallium biosorption compared to wildtype phage binding could be determined (Schönberger *et al.,* 2019).

In this work, the binding affinity of these clones for gallium compared to arsenic was investigated. The bacteriophage clones present gallium-binding peptide motifs that are 12 amino acids long and of linear structure. For a later application it is important that the peptides displayed on the bacteriophage are able to differentiate between gallium and arsenic with high affinity. The investigations on the chelation of metals by organic molecular structures showed that a lower entropy in the molecular structure of the ligands led to a more stable complexation (Hancock and Martell, 1988; Vallet, Wahlgren and Grenthe, 2003). However, there was a

possibility that the displayed dodecameric peptides could be too long and flexible to sorb the gallium ions constantly equally well and stable.

In the present study one selected phage clone was optimized for its interaction with gallium ions. Using systematically altered site-directed mutagenesis, each amino acid of the peptide was replaced at least once by a cysteine. In order to bring the functional groups involved in the interaction into closer contact for a collective complexation of the target metal ion, an additional amino acid was exchanged for cysteine at a distance of 4 amino acids. The so-called cysteine scanning is very suitable for reducing the entropy of the peptides presented on the bacteriophage and systematically validating the position of the disulfide-bridge in order to identify optimized metal-binding peptide sequences.

Materials and Methods

2.1 Handling of phage display library clones

The gallium-binding bacteriophage clones, that were investigated here, originated all from the commercial random peptide library Ph.D.-12 (Ph.D.™-12 Phage Display Peptide Library Kit, New England Biolabs GmbH, Frankfurt am Main, Germany) (see Table III-1).

Table III-1. Bacteriophage clones used in this study: their name, origin, displayed peptide sequence, as well as the theoretical isoelectric point of the corresponding peptides.

Name	Sequence	pI	Origin
C3.8	TMHHAAIAHPPH	6.82	
C3.15	NYPLHQSSSPSR	5.08	
C3.108	SQALSTSRQDLR	9.31	
C3.129	HTQHIQSDDHLA	5.70	
C3.130	NDLQRHRLTAGP	9.61	
M1: H_1C_1/Q_6C_2	CTQHICSDDHLA	5.05	This work; SDM experiment of clone C3.129
M2: T_2C_1/S_7C_2	HCQHIQCDDHLA	5.70	
M3: Q_3C_1/D_8C_2	HTCHIQSCDHLA	6.25	
M4: H_4C_1/D_9C_2	HTQCIQSDCHLA	5.97	
M5: $I_5C_1/H_{10}C_2$	HTQHCQSDDCLA	5.05	
M6: Q_6C_1/L_11C_2	HTQHICSDDHCA	5.70	
M7: $S_7C_1/A_{12}C_2$	HTQHIQCDDHLC	5.70	

pI: isoelectric point, C: clone, M: mutant, SDM: side-directed mutagenesis.

For the propagation of individual clones the bacterial host strain *Escherichia coli* K12 ER2738 mL*mcrB)5*) was used. 30 mL of LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.5) were inoculated with *E.coli* and cultivated to an optical density of ~ 0.02 (λ = 600) at 37 °C while shaking. The culture were infected with $1.5 \cdot 10^3$ pfU of the respective phage clone and incubated at 30 °C with vigorous shaking overnight. 10 mL of the pre-culture were used to infect 100 mL LB medium. The propagation batch was incubated for additional 7 h at 30 °C

while vigorous shaking. The phage particles were purified from culture supernatant as described elsewhere (Smith and Scott, 1993). The purified phage particles were resuspended in 0.5 mL TBS (TRIS-buffered-saline with 50 mM TRIS-HCl, 150 mM NaCl, pH 7.5) and were diluted to a final concentration of $5 \cdot 10^8$ pp/µl.

The phage particle concentration was determined by spectrophotometric quantitation as described by Scott, 2001 (Carlos F Barbas *et al.*, 2001). The concentration is calculated based on the absorption of purified phage particles at 269 nm and 320 nm. It is considered that the size of filamentous bacteriophage is proportional to the number of nucleotides in the phage genome and therefore correlates with the protein content of the phage particle. This corresponds to about 6 times of the DNA content. Using a molar extinction coefficient for the capsid, the concentration of physical particles can be calculated. (see Equation 1).

$$\text{Phage particles per microliter} = \frac{(A_{269\,\text{nm}} - A_{320\,\text{nm}}) * 60^{13}}{[\text{nucleotides in the phage genome}]} \quad (1)$$

A nucleotide number of 7270 bases was calculated in case of Ph.D.-12 library phage particles as well as mutated progeny of clone C3.129 and 7222 bases in case of control phage without peptide insert (M13 KE Wt).

2.2 Site-directed mutagenesis experiments

The phage clone C3.129 was used as template for site-directed mutagenesis experiments. Each amino acid of the displayed peptide sequence HTQHIQSDDHLA was replaced at least once by a cysteine. In order to allow the formation of disulfide-bridges within the displayed peptide, an additional amino acid was exchanged with cysteine at intervals of 4 amino acids (see Table III-2).

Table III-2. Primer design for site-directed mutagenesis screening for clone C3.129 (HTQHIQSDDHLA).

Clone	T_A	Pol	SDM Primer_forward (5'-3') & SDM Primer_reverse (5'-3')
M1	59 °C	Ph	CATATTTGTAGTGATGATCATCTTGCG / CTGCGTACAAGAGTGAGAATAGAAAGGTAC
M2	59 °C	Ph	ATTCAGTGTGATGATCATCTTGCGGGTG / ATGCTGACAATGAGAGTGAGAATAGAAAGG
M3	61 °C	Q5	CAGAGTTGTGATCATCTTGCGGGTGGA / AATATGACACGTATGAGAGTGAGAATAGAAAG
M4	61 °C	Q5	AGTGATTGTCATCTTGCGGGTGGAGGT / CTGAATACACTGCGTATGAGAGTGAGAATAG
M5	64 °C	Q5	GATGATTGTCTTGCGGGTGGAGGTTCG / ACTCTGACAATGCTGCGTATGAGAGTGAG
M6	65 °C	Q5	GATCATTGTGCGGGTGGAGGTTCGGCC / ATCACTACAAATATGCTGCGTATGAGAGTGAGAATA GAAAGGTAC
M7	62 °C	Ph	CATCTTTGTGGTGGAGGTTCGGCCGAA / ATCATCACACTGAATATGCTGCGTATGAGAGTG

T_A: annealing temperature, Pol: DNA polymerase.

Replicative form (RF) phage DNA was isolated from infected bacterial host cells. For this purpose, 5 mL LB medium was inoculated with *E.coli* cells and infected with approximately $5 \cdot 10^{10}$ pfU of the C3.129 clone as described above. The propagation batch was incubated at

30 °C with vigorous shaking overnight. The preparation of the RF DNA was performed using the PureYield™ Plasmid Miniprep System (Promega, US) according to the manufacturer's instructions. The purified DNA was dissolved in water and used as template for site-directed mutagenesis PCR.

The Q5® Site-Directed Mutagenesis Kit (New England Biolabs GmbH, Frankfurt am Main, Germany) was used according to the manufacturer's instructions. Mutagenesis primer pairs were designed to anneal back-to-back at the template DNA. For this purpose, the free online software NEBaseChanger™ (New England Biolabs GmbH, Frankfurt am Main, Germany) was used (see Table III-2). However, to generate the mutants M1, M2 and M7 (see Table III-1) it was necessary to carry out the site-directed mutagenesis PCR under usage of the Phusion® High-Fidelity PCR Kit (New England Biolabs GmbH, Frankfurt am Main, Germany). The cycling conditions have been selected with 30 sec and 98 °C initial denaturation; 25 cycles of 10 sec and 98 °C denaturation, 30 sec annealing (temperature according to Table III-1) and 225 sec and 72 °C elongation and a final elongation of 120 sec and 72 °C. The resulting PCR product was subjected to a reaction with the provided Kinase-Ligase-DpnI (KLD) enzyme mix to circularize the PCR product and remove template DNA.

The resulting plasmids were used to generate the mutant phage particles (M1-M7). For this purpose, *E.coli* host cells were made chemically-competent (Laboratory of Jasper Rine) by washing a freshly grown bacterial culture (OD_{600} ~ 0.45) subsequently with ice-cold 0.1 M magnesium chloride solution, 0.1 M calcium chloride solution and 0.1 M calcium chloride solution with 15 % glycerol. The DNA was introduced into the cells by heat shock at 42 °C for 30 sec. The transformed cells were cooled down on ice, mixed with ice-cold SOC medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) and incubated for 30 min at 37 °C.

The resulting culture was mixed with liquid TOP-agarose (LB medium containing 7 g/l agarose) and transferred to an IPTG-Xgal agar plate (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)). The plate was incubated overnight at 37 °C. Single transformants appeared as blue plaques on the plate. They were picked and transferred to individual tubes containing TBS and incubated at 4 °C overnight to allow the bacteriophage particles to diffuse out of the agar while preventing any undesirable further propagation of the phage by residual *E.coli* cells. Remaining agarose and cells were removed from the solution. The phage particle containing supernatant was analysed for successful mutagenesis by Sanger sequencing (GATC Biotech AG, Germany) using the oligonucleotide primer 5'-CCCTCATAGTTAGCGTAACG-3'.

2.3 Biosorption experiments

The interaction of all bacteriophage clones with free metal ions in aqueous solution was investigated in biosorption experiments. Pilot experiments with different solutions have shown that the biosorption of metal ions on bacteriophage particles can best be investigated in low concentrated millimolar metal salt solutions (result not shown). A total of six different gallium

and/or arsenic containing solutions with a metal concentration of approximately 3 mM were prepared (see Table III-3).

Table III-3. Composition of gallium and arsenic-containing buffer solutions.

Metal concentration	Concentration of buffer components	pH
2.8 mM Ga	0.0947 M Na_2HPO_4 0.0053 M NaH_2PO_4	8.5
2.7 mM Ga	0.0995 M CH_3COOH 0.0005 M $NaCH_3COO$	3.2
3.1 mM As	0.0947 M Na_2HPO_4 0.0053 M NaH_2PO_4	8.5
2.6 mM As	0.0995 M CH_3COOH 0.0005 M $NaCH_3COO$	3.2
1.2 mM Ga 1.3 mM As	0.0947 M Na_2HPO_4 0.0053 M NaH_2PO_4	8.5
1.5 mM Ga 1.4 mM As	0.0995 M CH_3COOH 0.0005 M $NaCH_3COO$	3.2

Solutions were kept at pH 3.2 using a sodium acetate buffer or at pH 8.5 using a sodium phosphate buffer. In each individual experiment, 500 µl gallium solution was mixed with $5 \cdot 10^{10}$ phage particles and incubated overnight at 4 °C while shaking. Phage particles were precipitated together with bound metal ions by the addition of 100 µl ice-cold PEG/NaCl solution (20 % polyethylene glycol 8000, 2.5 M NaCl). The supernatant was thoroughly removed, and phage particles and metal ions were resuspended in 1 mL TBS. The metal content of the solution was determined by inductively coupled plasma mass spectrometry (ICP-MS). Each Ph.D.-12 library clone and each mutant was tested with all six metal solutions in 6-fold redundant experiments.

Results and Discussion

3.1 Experimental context

Industrial wastewaters are an attractive secondary resource for high-tech metals. Such waste streams often have a complex composition with varying pH values. The mixtures are a challenge for any technology aiming a selective metal recovery. The use of conventional methodologies is not efficient and therefore unprofitable. Metal selective peptides derived from natural models could provide a solution.

In our study we intended the development of metal selective peptides that can be used for the selective Ga removal from low concentrated wastewater streams from the semiconductor industry. These waters are characterized by a complex and variable composition with varying pH values depending on the process stage from which they emerged. In addition to valuable amounts of gallium, the solutions always contain considerable amounts of arsenic. In addition to a variable matrix of other constituents, such solutions contain approximately 4 mg/l gallium and arsenic with a pH ranging from 3.0 - 4.2 or > 8.0.

Within the scope of the project, Ga selective peptide motifs are selected using the phage Surface Display technology and used as functional compounds for the development of biosorptive composites. The present study focused on the investigation of the biosorption capacity of different peptide presenting phage clones for dissolved gallium and arsenic.

The composition and pH value of metal solutions has great influence on metal speciation. With increasing complexity, the determination of such species becomes more elaborate. For less characterized complex solutions, such as those resulting from semiconductor production, the speciation remains unknown. Therefore, the biosorption of gallium and arsenic in simplified model solutions was investigated. In order to match the pH range of real industrial wastewater, acetate-buffered solutions with a pH value of 3.2 and phosphate-buffered solutions with a pH value of 8.5 were applied.

Although it was not possible to determine the exact composition of the metal speciation in these synthetic solutions, justified assumptions can be made on the basis of speciation studies for gallium and arsenic in aqueous solution, that were reported elsewhere (Cullen and Reimer, 1989; Wood and Samson, 2006). It can be expected that at pH 3.2 a certain amount of gallium will be present as Ga^{3+}, but also of $Ga(OH)^{2+}$ and $Ga(OH)_2^{+}$. At pH 8.5 a dominance of the complex $Ga(OH)_4^{-}$ can be expected. Arsenic predominates at pH 3.2 as arsenate ion in the compound $H_2AsO_4^{-}$. At pH 8.5 mainly $HAsO_4^{2-}$ can expected.

3.2 Original phage clone characterization

Phage clones used in this study were obtained in experiments performed with the commercial Ph.D.-12 library (New England Biolabs GmbH, Frankfurt am Main, Germany) and used for further investigations. In total, five different gallium-binding peptides were identified (referred to Table III-1). In order to determine the sorptive behavior of those five phage clones, six different model solutions at pH 3.2 or 8.5 were used. Each solution contained approximately 3 mM gallium or arsenic or 3 mM gallium and arsenic in combined solutions (see Table III-3).

A defined amount of phage particles was incubated in the model solutions. Phage particles were co-precipitated with bound metal, thus determining the interaction of bacteriophage clones with metal ions in solution. In addition to the gallium-binding bacteriophage clones, a clone corresponding to the wildtype (Wt), i.e. which did not present a peptide sequence, was also carried as a control experiment. This reference shows the influence of the peptide sequences of the clones. The precipitation experiments showed a great influence of the pH on the biosorption efficiency for both elements gallium and arsenic, as well as on the binding affinity of the bacteriophage clones for gallium in comparison to arsenic (see Figure III-1 and -2, as well as Tables III-4 and -5).

Table III-4. Relative biosorption of the clones increased above wild-type control. The values were normalized to the wild-type biosorption of gallium or arsenic (Wt = 1).

Clone	Relative biosorption of Ga (increase above Wt)		Relative biosorption of As (increase above Wt)	
	Phosphate buffered (pH 8.5)	Acetate buffered (pH 3.2)	Phosphate buffered (pH 8.5)	Acetate buffered (pH 3.2)
C3.8	2.06 ± 0.52	1.83 ± 0.69	1.46 ± 0.12	4.58 ± 0.27
C3.15	2.21 ± 0.76	1.27 ± 0.37	0.27 ± 0.08	1.09 ± 0.34
C3.108	1.10 ± 0.09	1.01 ± 0.25	1.32 ± 0.15	2.41 ± 0.3
C3.129	1.22 ± 0.22	1.13 ± 0.22	1.06 ± 0.3	1.51 ± 0.28
C3.130	1.83 ± 0.34	1.31 ± 0.41	1.08 ± 0.23	2.46 ± 0.43

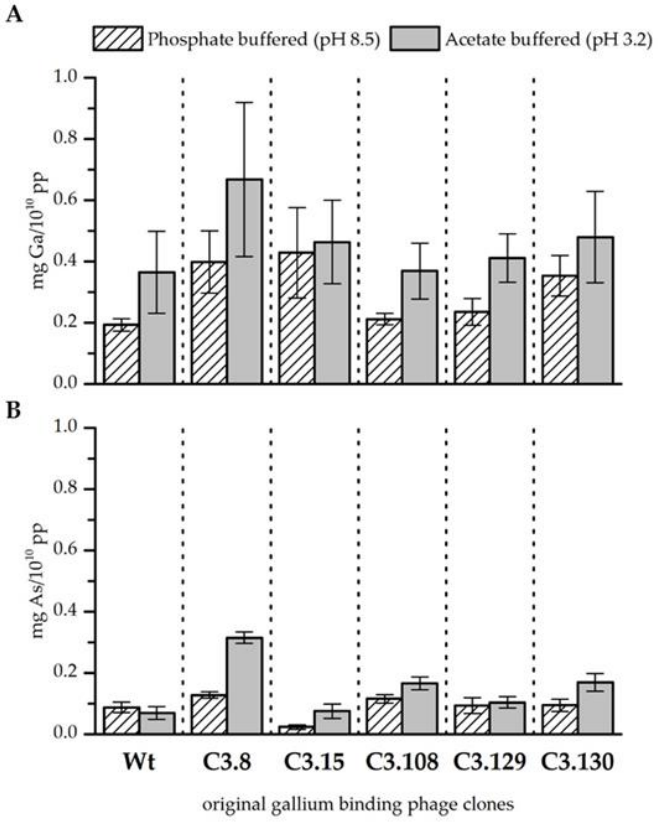

Figure III-1. Biosorption of gallium (A) and arsenic (B) to original gallium-binding phage clones and wild-type phage control at pH 8.5 in phosphate buffered solution and pH 3.2 in acetate buffered solution. Results are expressed as the amount of sorbed gallium (mg) to 10^{10} phage particles (pp). Error bars represent the standard error.

In general, gallium was bound better by the bacteriophage at pH 3.2 than at pH 8.5 when it was solely present in the solution. The wildtype phage control indicates that especially at lower pH, unspecific binding of gallium to the phage capsid takes place. However, the binding of gallium that occurs through the displayed peptides of the gallium-binding phage clones is higher at pH 8.5 than at pH 3.2 (see Figure III-1A). In summary, it was found that clones C3.8 (TMHHAAIAHPPH), C3.15 (NYLPHQSSSPSR) and C3.130 (NYLPHQSSSPSR) bound gallium at pH 8.5 considerably better than the wild-type control. Since the clones differ from

the wildtype only in the displayed peptide sequence, this result indicates an interaction between the peptide and gallium.

The displayed peptides differ strongly in their amino acid composition and their arrangement so that no uniform binding domain or mechanism could be identified. However, the investigation of different chemical gallium ligands indicates that gallium is preferentially complexed in hexadentate structures by nitrogen and oxygen (Kubíček *et al.*, 2010; Schmidtke *et al.*, 2017). In principle, all five sequences meet such functional requirements for the complexation of gallium on the phage particle. At pH 3.2, gallium is partial present as Ga^{3+}. Thus, it acts as hard Lewis acid and can form complexes with the nitrogen and oxygen atoms of the displayed peptides. At pH 8.5, gallium occurs as $Ga(OH)_4^-$. The stability constant of this complex is described to be very low (Hacht, 2008). It can, therefore, be assumed that single hydroxide ligands might be substituted in favor of building a more stable complex. Depending on the affinity of the displayed peptides for gallium this might be the case. However, further studies of gallium-peptide interactions have to be done to resolve the binding mechanism. Nevertheless, there are indicated differences in the biosorption performance of the individual displayed peptides. Phage clones C3.8 (TMHHAAIAHPPH), C3.15 (NYLPHQSSSPSR) and C3.130 (NDLQRHRLTAGP) showed the highest biosorption of gallium at both pHs. These sequences differ mainly by the presence of large hydrophobic amino acids. The secondary structure of these peptides is characterized in particular by the inflexible and structure-giving character of proline. The Phage clones C3.108 (SQALSTSRQDLR) and C3.129 (HTQHIQSDDHLA) showed lower biosorption for gallium. The higher flexibility of these peptides due to the absence of secondary structure stiffening amino acids in the peptide might be a reason for this (Vallet, Wahlgren and Grenthe, 2003).

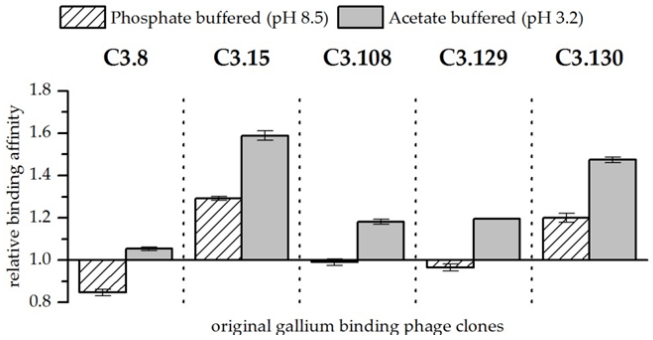

Figure III-2. Relative binding affinity of original gallium-binding phage clones for gallium compared to arsenic at pH 8.5 in phosphate buffered solution and at pH 3.2 in acetate buffered solution. Results are expressed as the fold increase above the biosorption of wild-type bacteriophage particles. The values were normalized to the wild-type phage affinity for gallium compared to arsenic (Wt = 1). Error bars represent the standard error.

Arsenic, if present solely in the solution, was sorbed by the bacteriophage as well. However, less arsenic was bound than gallium, although the concentration of the two elements in all solutions was similar. It is noticeable that the wildtype control sorbed arsenic slightly better at pH 8.5. The opposite is true for the gallium-binding clones tested. In particular, the clones C3.8 (TMHHAAIAHPPH) and C3.108 (SQALSTSRQDLR) bound noticeable more arsenic at both

pH values than the wildtype control. Clone C3.8 stands out with its remarkable binding of arsenic, that is four times more than the control phage at pH 3.2. Clone C3.15 (NYPLHQSSSPSR) is noteworthy because it sorbs very little arsenic at both pH values. Arsenic is present at pH 3.2 and 8.5 as arsenate in the compounds $H_2AsO_4^-$ and $HAsO_4^{2-}$ respectively. The coordination sphere of arsenic is covalently saturated in these compounds. This makes direct complexation by the peptides unlikely. It is therefore assumed that the interaction of bacteriophage clones is mainly based on electrostatic interactions with protonated groups. This assumption is supported by the fact that arsenic preferably interacts with those displayed peptides that have a higher isoelectric point than those that have a lower one (see Table III-1).

When both elements are present in the solutions, they compete for sorption to the bacteriophage. Biosorption experiments with solutions at pH 3.2 and 8.5 and approximately equimolar concentrations of arsenic and gallium showed differences in their affinity for the respective elements. These results are of particular interest because gallium-binding peptides should be able to distinguish between gallium and arsenic for later application. In order to better assess the effect of the peptides presented on the bacteriophage, the wildtype phage was again used as a reference. Figure III-2 shows the affinity of gallium-binding clones for gallium in comparison to arsenic and in relation to the biosorption to the wildtype control. In general, it was found that at pH 3.2 the peptides have a higher affinity for gallium compared to arsenic than at pH 8.5. Furthermore, at pH 3.2 all displayed peptides show a preference for interacting with gallium compared to arsenic. As already discussed, the Ga3+ ion occurring at low pH has a considerable advantage for the formation of a complex compared to arsenate. It is therefore assumed that the greater affinity is the result of chelating effects. At pH 8.5, only the phage clones expressing the peptides C3.15 (NYLPHQSSSPSR) and C3.130 (NDLQRHRLTAGP) showed a considerable better biosorption of gallium compared to arsenic (see Figure III-2). In the case of the other peptides, even a slightly increased affinity for arsenic could be observed. Therefore, it is unlikely that these peptides are selectively binding gallium. It is assumed that the compact molecular structure makes a direct binding of gallium difficult. However, substitutions of hydroxide ligands by peptides that bind gallium with a very high affinity is possible due to ligand substitution effects.

3.3 Site-directed mutagenesis experiments

The experiments with original library phage clones indicated that there is a relationship between selectivity and metal affinity and the secondary structure of the represented peptides. Long, flexible amino acid chains show poorer biosorption performances and lower specificity for gallium recognition. The fact that cyclic ligands can often form more stable complexes than open structures is known as the macrocyclic effect (Cabbiness and Margerum, 1969). Accordingly, the biosorption of such peptides might be optimized by providing a more rigid secondary structure. In order to test this hypothesis, a peptide motif was specifically modified which, due to its flexible structure, was suspected of being less able to sorb gallium.

It has already been reported elsewhere that the targeted exchange of individual amino acids in a peptide by alanine can lead to considerable improvements (Lederer *et al.,* 2017) or deteriorations (Cetinel *et al.*, 2012) in the functionality of the peptide. Alanine scanning enables the systematic replacement of amino acids in a peptide sequence. Alanine is usually used as a substitute because its methyl group is inert and is therefore excellent for testing the functionality of individual amino acids in a peptide.

However, in this work, an alternative approach was chosen. The influence of structural changes on the selective recognition of metal ions of one gallium-binding peptide sequence was investigated. For this purpose, two amino acids with a distance of four amino acids were systematically replaced by cysteine (see Table III-1). It was examined whether the systematic introduction of disulfide-bridges, that would have a major influence on the secondary structure of the peptides and thus their flexibility, leads to a change in the biosorption of gallium and arsenic and whether an optimization of the sequence can perhaps be achieved by the changes. The clone C3.129 (HTQHIQSDDHLA) was chosen for cysteine scanning. The peptide sequence presented on the clone promised interesting possibilities for interaction with gallium and in previous studies (Schönberger *et al.,* 2019) a high affinity of the clone for an immobilized gallium target could be demonstrated. Nevertheless, only a low biosorption of free gallium ions could be observed in this work. Therefore, it was assumed that a more rigid secondary structure could have a beneficial effect on the complexation of gallium. All seven derivates of C3.129 were obtained by site-directed mutagenesis. The mutant phage particles display modified peptide sequences that are circularized due to cysteine derived disulfide-bridges.

3.4 Mutant phage clone characterization

The precipitation experiments were repeated for all seven mutants of the C3.129 clone. Figure III-3 shows the biosorption of gallium and arsenic at both pH values. The biosorption of gallium at pH 8.5 has been notably improved by cysteine scanning, not only with respect to the wildtype control phage, but also with respect to the original phage clone C3.129. The results for pH 3.2 indicate diverse effects. Major improvements were achieved by the changes in the mutants M1, M3 and M5. It is assumed that especially with the displayed peptides of these mutants the improved gallium complexation might be achieved by macrocyclic effects. The biosorption of gallium by the mutant M2 has considerably decreased. The substitution of the hydroxyl amino acids at positions 2 and 7 in the mutant M2 resulted in a remarkable reduced biosorption of gallium. This indicates that the hydroxyl side chain functionality of serine and threonine at these positions is decisive for the complexation of Ga^{3+}. The biosorption of arsenic at pH 3.2 could be increased by cysteine scanning of all mutants. A possible reason for this effect could be the very good interaction between arsenic and cysteines (Stöhrer, 1991). At pH 8.5 the sorption performance for arsenic was rather reduced. It is assumed that the more complex secondary structure restricted the possibilities for deposition on the peptide surface via electrostatic interactions.

Table III-5. Relative biosorption of the clones increased above wild-type control. The values were normalized to the wild-type biosorption of gallium or arsenic (Wt = 1)

Clone	Relative biosorption of Ga (increase above Wt)		Relative biosorption of As (increase above Wt)	
	Phosphate buffered (pH 8.5)	Acetate buffered (pH 3.2)	Phosphate buffered (pH 8.5)	Acetate buffered (pH 3.2)
C3.129	1.22 ± 0.22	1.13 ± 0.22	1.06 ± 0.3	1.51 ± 0.28
M1	2.69 ± 0.41	2.15 ± 0.15	0.53 ± 0.07	0.78 ± 0.13
M2	2.81 ± 0.34	0.78 ± 0.14	0.87 ± 0.24	1.87 ± 0.19
M3	2.62 ± 0.19	1.80 ± 0.54	0.71 ± 0.08	1.04 ± 0.09
M4	3.52 ± 1.02	0.99 ± 0.12	0.74 ± 0.25	1.74 ± 0.13
M5	2.08 ± 0.59	2.10 ± 0.11	0.71 ± 0.08	1.59 ± 0.18
M6	1.76 ± 0.46	0.83 ± 0.1	0.52 ± 0.07	1.57 ± 0.29
M7	1.58 ± 0.25	1.02 ± 0.21	0.85 ± 0.23	1.43 ± 0.14

In experiments in which gallium and arsenic were present together in the solution, the affinity of bacteriophage for gallium compared to arsenic could be increased by the cysteine scanning (see Figure III-4). Exceptions are the mutants M2 and M5. The respective changes in the amino acids increased the affinity of the mutants for arsenic compared to gallium.

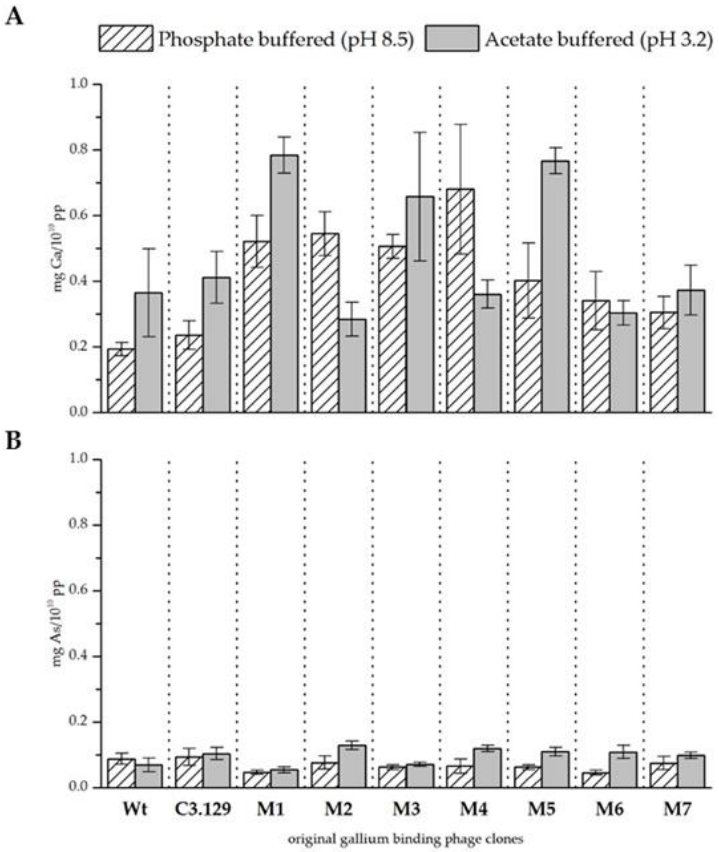

Figure III-3. (A) Biosorption of gallium and (B) arsenic to mutant gallium-binding phage clones, original clone C3.129, and wild-type phage control at pH 8.5 in phosphate buffered solution and pH 3.2 in acetate buffered solution. Results are expressed as the amount of sorbed gallium (mg) to 10^{10} phage particles (pp).

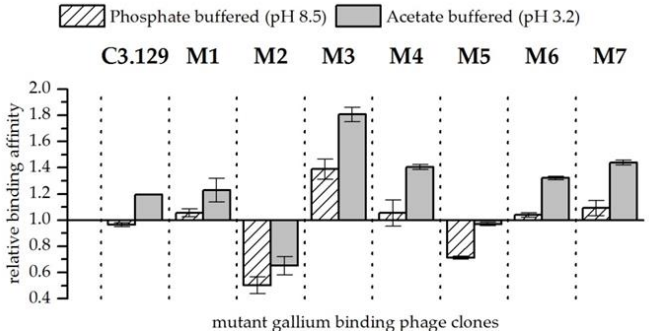

Figure III-4. Relative binding affinity of mutant gallium-binding phage clones for gallium compared to arsenic at pH 8.5 in phosphate buffered solution and at pH 3.2 in acetate buffered solution. Results are expressed as the fold increase above the biosorption of wild-type bacteriophage particles. The values were normalized to the wild-type phage affinity for gallium compared to arsenic (Wt = 1). Error bars represent the standard error.

A highly increased affinity for gallium with arsenic at both pH points could be observed in the biosorption behavior of mutant M3. It can be speculated that by closing the disulfide-bridge between the amino acids at positions 3 and 8 a pocket is created which is able to complex Ga^{3+} with a high stability. However, more sophisticated structural analyses of synthetic peptides would be necessary to verify such an assumption. In addition to the strongly enhanced biosorption of gallium compared to arsenic from mutant M3, an improvement in gallium affinity at pH 3.2 for mutants M4 and M7 could also be observed. Interestingly, the mutants M3 and M4 each substituted aspartic acid once, and mutant M7 concealed both aspartic acid groups probably by the formation of a peptide ring that is displayed on the bacteriophage. Possibly the acidic side chain functionality impeded the specific recognition of Ga^{3+}.

Conclusions

In this study, the biosorption of dissolved gallium and arsenic by gallium-binding bacteriophage clones was investigated. A large influence of the pH-value on the respective interactions could be determined. Many of the identified bacteriophage clones actually present peptide sequences that can sorb the gallium at pH 3.2 and pH 8.5

Experiments with arsenic have shown that arsenate ions were less well bound due to its chemical properties. In solutions containing both elements gallium and arsenic could be shown, that gallium might be complexed due to chelate effects and that the stable coordination sphere of arsenate allows only electrostatic interactions.

Since structural effects of the peptides were suspected to be involved in the specific binding of gallium, one of the gallium-binding peptide sequences was specifically modified. Site-directed mutagenesis experiments were performed to systematically replace amino acids with a cysteine pair. The modified peptides were presented on the bacteriophage. Biosorption experiments with the resulting bacteriophage mutants showed that the formation of a macrocyclic peptide structure has positive effects on the complexation of gallium. Furthermore,

amino acids with hydroxyl side chain functionality were shown to be particularly important for the binding of gallium.

We conclude that the phage Surface Display is a suitable tool for the identification of metal binding peptide sequences. By applying the tool to less abundant metals, the spectrum of natural occurring metalloproteins could be extended. Furthermore, cysteine scanning has proven to be a very helpful complement for the engineering of peptides, that were obtained by directed evolution. It could be applied for the closer characterization of the structure-related interactions between peptide sequences and gallium.

However, the results reported here primarily show the behavior of different bacteriophage clones that display peptides. Bacteriophage particles are practically not applicable. The production is problematic due to a high mutation rate of the phage and the large-scale industrial application of genetically modified organisms in wastewater treatment is not accepted. Since it is not possible to predict whether the peptides will behave exactly as they do on bacteriophage, experiments will now be conducted to characterize synthetic peptides. Suitable sequences will be immobilized on a suitable carrier and used for the recovery of gallium from industrial wastewater.

Acknowledgements

We like to thank Sabrina Beutner and Stefanie Bachmann for their valuable support in sample analysis by ICP-MS measurement. We also thank our project partner Freiberger Compound Materials for providing data on industrial wastewater compositions.

Chapter IV.

GALLIUM-BINDING PEPTIDES AS A TOOL FOR THE SUSTAINABLE TREATMENT OF INDUSTRIAL WASTE STREAMS

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In order to embed the published article into the thesis the in-text references were adjusted to the format of the thesis.

Author contributions

Methodology, Investigation, writing (original draft preparation, review and editing) – N.S.; Investigation (Model calculations), writing (original draft preparation, review and editing) – C.T.; Methodology (peptide immobilization) – M.S.; Methodology (ITC), writing (review and editing) – B.D.; Methodology (biosorption studies), funding acquisition, writing (review and editing) – S.M.; Conceptualization, writing (review and editing)- F.L.L.; Conceptualization, funding acquisition, project administration, writing (review and editing) – K.P.

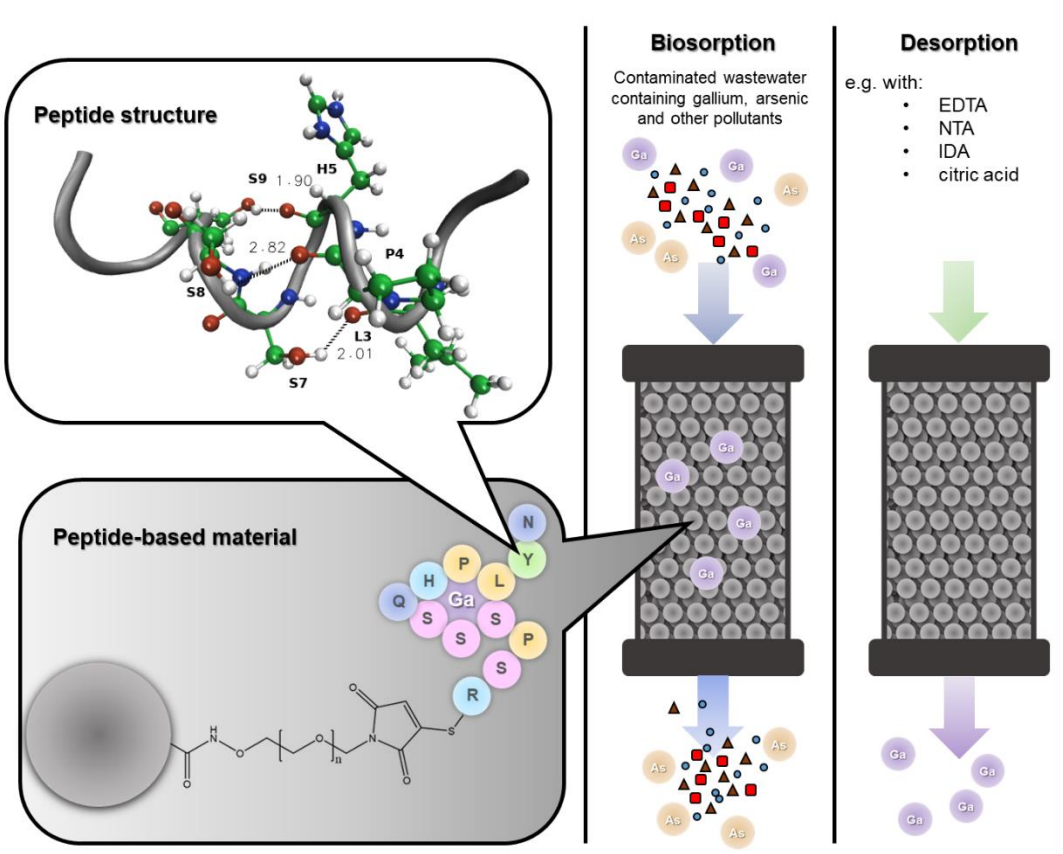
Textual and graphical abstract

Here we provide a proof of principle for an application-oriented concept for the peptide-based recovery of gallium in industrial wastewater, which was supported by biosorption studies with a real wastewater sample. We investigated the interaction of the gallium-binding peptides TMHHAAIAHPPH, NYLPHQSSSPSR, SQALSTSRQDLR, HTQHIQSDDHLA, and NDLQRHRLTAGP with gallium and arsenic through different experimental and computational approaches.

Data obtained from isothermal titration microcalorimetry indicated a competitive influence by the presence of acetate ions with an exothermic contribution to the otherwise endothermic peptide gallium interactions. For peptide HTQHIQSDDHLA, a stabilizing influence of acetate ions on the metal peptide interaction was found. Peptide NYLPHQSSSPSR showed the highest affinity for gallium in ITC studies.

Computational modeling of peptide NYLPHQSSSPSR was used to determine interaction parameters and to explain a possible binding mechanism.

Furthermore, the peptides were immobilized on polystyrene beads. Thus, we created a novel and exceptionally robust peptide-based material for the biosorption of gallium from an aqueous solution.



Introduction

The rapid development of new technologies and their global spread has resulted in a growing demand for raw materials. The high-tech sector, in particular, requires a large number of different elements in large quantities and high purity. The efficient and sustainable provision of such raw materials for the industry is a challenge for modern resource technology. Innovative supply strategies must be found. This is necessary especially for those elements which are considered critical (e.g. cobalt, tungsten, indium, germanium or gallium) because they are subject to high demand due to their great importance for the economy, but whose (future) supply situation is assessed as uncertain (Mathieux, Ardente et al. 2017). Particularly desirable is the further technological development for the extraction of high-tech metals from raw material sources that are currently still difficult to access. In addition to ores with low metal content and a complex matrix, secondary raw material sources include end-of-life products or residues from the metal processing industry. These raw material sources are particularly challenging because common methods of primary raw material exploitation often are not efficient enough for secondary resources (Dodson, Parker et al. 2015). Nevertheless, such secondary material streams are not only a potential ecotoxicological burden but also a rich source of raw materials for valuable industrial metals, which is why their exploitation is desirable in more than one aspect. One major obstacle in such recycling procedures is the selective recovery of low concentrated metals from complex solutions (Reuter 2011).

The implementation of biotechnological approaches in resource technology promises an innovative solution for the efficient and sustainable utilization of such raw materials (Hennebel, Boon et al. 2015). These approaches are based on the natural interaction of microorganisms as well as their metabolites with metals, resulting in metal transformation, complexation or adsorption (Pollmann, Kutschke et al. 2018). For the targeted extraction of individual resources, however, these concepts still lack selectivity for certain valuable elements especially when they are in competition with less expensive conventional approaches (Pollmann, Kutschke et al. 2018). A potential solution is provided by modern biotechnologies that enable e.g. the development of tailor-made biomolecules for the selective recovery of valuable high-tech metals (Braun, Bachmann et al. 2018, Lederer, Braun et al. 2019). In addition to the use of specialized whole cells (Nguyen, Lee et al. 2013), certain metabolites like siderophores (Jain, Fan et al. 2019) or metallothioneins (Terashima, Oka et al. 2002), as well as specific metal binding proteins (Deblonde, Mattocks et al. 2020) that act as naturally occurring metal chelators, can also be transferred into technical applications. Regarding this, the development of specialized biomolecules for certain applications through the directed evolution of metal-binding peptides using Phage Surface Display (PSD) technology is very promising (Braun, Bachmann et al. 2018). The use of small peptides for the biosorption of valuable metals has decisive advantages. Due to their offset amino acid sequence, short-chained peptides can be developed into highly specific ligands for individual ions (Sarikaya, Tamerler et al. 2004). The peptides remain robust and can easily be synthesized chemically or biologically.

Previous studies focused on the identification of different metal-binding peptides. For example, sequences for the recognition of chromium (Yang, Zhang et al. 2015), aluminum (Zuo, Ornek et al. 2005), platinum (Cetinel, Dincer et al. 2012), molybdenum (Cetinel, Shen et al. 2018), nickel and cobalt (Matys, Lederer et al. 2017, Braun, Bachmann et al. 2018, Matys, Schönberger et al. 2020), gallium (Schönberger, Braun et al. 2019, Schönberger, Zeitler et al. 2019) and the rare earth elements neodymium (Sawada, Asada et al. 2016) and lanthanum (Lederer, Curtis et al. 2017) have been reported using PSD technology. Recently, a study was published in which magnetic adsorbents with lead-binding peptides previously identified using PSD technology (Nian, Kim et al. 2010) were produced (Xu and Yoo 2020).

For various reasons, the direct application of phage particles in industrial processes is not practicable as the filamentous particles exhibit insufficient physical and chemical stability for the application. In addition, the phage particles must be propagated by amplification in a host organism; during this step, mutations can be introduced and the permanent presence of the peptides on the phage surface cannot be guaranteed (Lederer, Braun et al. 2019). Furthermore, phage particles with complex surface structures displaying numerous functional groups are very large in comparison to the displayed functional peptide sequence alone, and can lead to unwanted unspecific interactions, especially in the interaction with metal ions.

Therefore, the focus of the present work is on the development of a peptide-based material for the recovery of gallium from contaminated wastewaters from the semiconductor industry. The production of the technologically highly demanded GaAs wafers generates large quantities of various metal-containing wastes. The targeted processing of these wastes can provide additional economic and ecological value (Ueberschaar, Otto et al. 2017).

Especially interesting for the treatment by a biotechnological process are waste streams with a low metal content that cannot be treated with conventional methods (Plaza Cazón, Viera et al. 2013, Mazhar, Ditta et al. 2019). These are accumulated in large quantities and vary in their specific composition and pH value depending on the process step in the wafer manufacturing.

In previous studies (Schönberger, Braun et al. 2019, Schönberger, Zeitler et al. 2019) we have developed gallium-binding peptides that are able to bind gallium under such challenging conditions as those mentioned above. Based on a dodecamer peptide library (Ph.D.™-12 Phage Display Peptide Library Kit, New England Biolabs GmbH, Frankfurt am Main, Germany), PSD experiments against immobilized gallium in acetate-buffered environments were performed. Extensive competitive and single binding experiments with the enriched bacteriophage pool resulted in five different gallium-binding peptide sequences. In further biosorption studies, these peptides were characterized and optimized with regard to their gallium selectivity. However, the Ga-binding of single, independent peptides was not studied.

The work presented here aimed to investigate the individual gallium-binding peptides independently of the bacteriophage particle and to develop an application-oriented material for use in gallium recovery.

We applied isothermal titration microcalorimetry (ITC) as a tool for the thermodynamic characterization of metal-peptide interactions. ITC verified that the identified gallium-binding peptides selectively recognize gallium independent of phage particle.

To make the peptides usable for the recovery of gallium, they were immobilized covalently and site-selective on a polystyrene matrix and tested in biosorption studies with synthetic solutions and real industrial wastewaters.

Methods

2.1 Peptides

Peptides with the sequences TMHHAAIAHPPH, NYLPHQSSSPSR, SQALSTSRQDLR, HTQHIQSDDHLA and NDLQRHRLTAGP were selected earlier by PSD technology and identified as Ga-binding motifs in previous studies (Schönberger et al., 2019a, 2019b). The peptides were obtained as chemically synthesized trifluoroacetic acid (TFA) salts with a purity >95 % (GL Biochem, Shanghai, China; DGpeptides, Hangzhou, China). Aliquots of 3 mM peptide stock solution were prepared by dissolving TFA salts in water and used for all experiments.

2.2 Isothermal titration microcalorimetry (ITC)

ITC experiments were performed with five different chemically synthesized gallium-binding peptides to characterize their metal complexing properties. A MicroCal Peaq-ITC (Malvern Instruments, Worcestershire, UK) with 200 µl sample cell and 40 µl titration syringe was used to determine the thermodynamic parameters of the peptide-gallium interactions. The thermograms were recorded in 19x 2 µl injection steps and evaluated with the MicroCal PEAQ-ITC Software V 1.3 (Microcal-Malvern Panalytical, Malvern, UK). The heat change in the sample cell was measured in relation to a water-filled reference cell, which was obtained by stepwise titration of a gallium solution to a peptide solution in the sample cell.

The experiments were performed in 150 mM NaCl at pH 3.0, adjusted with either 80 mM acetate buffer (77.2 mM acetic acid, 2.8 mM sodium acetate, pH 3.0) or 1 mM HCl.

For the titration, 3.6 mM gallium (Ga(NO3)3-xH2O, Alfa Aeser Kandel AG, Landau, Germany) and between 160 - 290 µM peptide (DGpeptides Co.,Lt; Hangzhou, CN, see Table IV-3) were used in the corresponding buffer. In addition, the experiments were repeated with arsenic (NaAsO2, Merck KGaA, Darmstadt, Germany) as the titrant. To determine the background heat, control experiments were performed in which the corresponding metal solution was titrated in buffer without peptide.

2.3 Preparation of peptide conjugates

Peptides were immobilized on amino-functionalized polystyrene beads (H10002, Rapp Polymere, Tübingen, Germany), with a particle size of 75 - 150 µm and a capacity of 0.97

mmol·g-1 via a bifunctional Polyethylene glycol (PEG) linker molecule. Chemically synthesized gallium-binding peptides (GL Biochem, Shanghai, China; DGpeptides, Hangzhou, China) with an additional C-terminal cysteine were used. A 0.1 g/mL suspension of the material in water was made. The beads were washed 3 times with water and once with borate buffer before they were equilibrated for one hour at 4 °C in borate buffer (50 mM sodium borate, pH 8.0). The beads were transferred to fresh borate buffer. Succinimide maleimides heterobifunctional PEG12 crosslinkers (SM(PEG)12, Pierce Biotechnology, Rockford, Illinois, US) were freshly dissolved in borate buffer, and were incubated with the beads (~ 1.5 mM crosslinker per gram of beads) for one hour at room temperature. After incubation, the beads were thoroughly washed with water to remove excess linker molecules and subsequently equilibrated in conjugation buffer (50 mM sodium phosphate, 50 mM NaCl, 50 mM EDTA, pH 7.2) for 15 min. Meanwhile, 2.5 mM peptide per gram of bead was dissolved in conjugation buffer and reduced by the application of 50 % (w/v) TCEP agarose (Tris(2-carboxyethyl) phosphine, immobilized on agarose CL-4B, Merck KGaA, Darmstadt, Germany) for 2 min at room temperature. The freshly reduced peptide was added to the beads. The conjugation occurred overnight at 4 °C while shaking. After thoroughly washing with water 10 times to remove unconjugated peptides, the peptide conjugates were lyophilized. Successful peptide conjugation, as well as the stability of the conjugates, was analyzed by the ninhydrin reaction. For this, the reagent (3 % ninhydrin (w/v) in ethanol: acetic acid (15:5)) was mixed with 1 mg/mL peptide conjugate and incubated for 3 min at 99 °C. After cooling the samples to room temperature, primary amino groups were detected by determination of absorption at 570 nm. To determine the peptide loading of the beads the range between maximum and minimum adsorption was assessed. For this, the ninhydrin detection was determined for untreated and for pegylated resin. When calculating the peptide loading of the resin, the number of primary amino groups present in each conjugated peptide was taken into account. To determine the stability of the peptide conjugates, the ninhydrin reaction was repeated after storage of the materials in water for one week and for two months at 4 °C; after resuspension of the dried conjugates after long-term storage at -20 °C, and after each biosorption experiment.

2.4 Biosorption studies

Biosorption of gallium and arsenic to the lyophilized peptide conjugates was studied in batch experiments as well as continuous column experiments at room temperature.

For biosorption studies, a synthetic model solution (MS: 0.2 mM Ga, 0.2 mM As, 150 mM NaCl, 1 mM HCl, pH 3.0) and process water from a wafer manufacturer (RW: 0.2 mM Ga, 0.2 mM As, pH 3.0) were used. The exact composition as well as other contaminants can be found in Table IV-1a.

In the batch experiments, the biosorbent dose was 100 mg/mL, and the contact time 24 h in an overhead shaker with very thorough shaking. After incubation, the remaining metal content in the supernatant was determined by ICP-MS.

In continuous column experiments, approximately 0.7 g of the peptide conjugates were packed in mini-columns (1 mL Empty Bio-Scale™ Mini Cartridges, Bio-Rad, Feldkirchen, Germany)

and washed in an FPLC system (Äkta pure protein purification system, GE Healthcare; Freiburg, Germany) for metal loading and unloading.

The columns were first rinsed with water. The same 2 mL wastewater sample was applied in a recycling loop for 20 CV to allow the peptide conjugates the biosorption of gallium. The desorption was performed with 10 CV 10 % (w/v) citric acid. The experiment was conducted in three consecutive cycles (see Table IV-1b for specific experimental conditions). The metal content of the different fractions was determined by ICP-MS to analyze the loading capacity with gallium and arsenic as well as the metal desorption.

Table IV-1a. Main contaminants in industrial wastewater from wafer manufacturer (pH 3.0). Concentrations were determined by Inductively coupled plasma mass spectrometry (ICP-MS, NexION™350X, Perkin Elmer).

	Ga	As	Na	K	Ca	P	Fe	Mg	Al
Concentration [mM]	0.22	0.18	0.15	0.13	0.07	0.06	0.03	0.02	0.01

Table IV-2b. Specific experimental conditions of continuous column experiments for the investigation of biosorption of peptide conjugates at room temperature.

	Volume		Flow rate	Contact time	Biosorbent dosage
H_2O	20 mL	20 CV	2 mL/min	-	-
RW	2 mL	20 CV	0.5 mL/min	40 min	350 mg/mL
10 % (w/v) citric acid	10 mL	10 CV	0.5 mL/min	20 min	-

2.5 Model calculation of peptide C3.8

In preparation for molecular dynamics simulation, computer modeling was used to predict the possible structure of peptide C3.15 by using UCSF Chimera (Pettersen, Goddard et al. 2004) and energy minimization using CHARMM 43b2 (Brooks, Brooks III et al. 2009) using Steepest Descent (SD) and conjugate gradient (CONJ) minimization.

The system was solvated and neutralizing ions were added with CHARMM-GUI (Jo, Kim et al. 2008) with parameter and topology files generated at each stage. A cubic TIP3 (Jorgensen, Chandrasekhar et al. 1983) water box with edge distance of 10 Å was generated to ensure complete coverage of the peptide. Na+ and Cl- counter ions were added to a concentration of 150 mM. Periodic boundary conditions were used.

All simulations were conducted with CHARMM 43b2 using the CHARMM36m(Huang et al., 2016) force field. For equilibration steps, a harmonic constraint of 24 Kcal/mol/Å2 was first applied to the peptide backbone atoms. This was followed by short minimizations of 50 steps each (SD/Adopted Basis Newton-Raphson) to relax the structure. An equilibration simulation

of time step 1 fs was run for 250 ps at 303.15 K. The Verlet (Verlet 1967) integrator and SHAKE (Ryckaert, Ciccotti et al. 1977) algorithms were used in concert with the Nose-Hoover (Hoover 1985) thermostat to ensure a constant temperature.

Constant-pressure production simulations were run with Langevin dynamics and time step of 2 fs for a total of 30 ns with restarts every 1 ns. GPU-accelerated simulations were performed with the CHARMM/OpenMM (v7.3.1) interface (Eastman, Swails et al. 2017). A constant pressure of 1 atm was maintained with the Monte Carlo barostat included with OpenMM. Pressure Particle Mesh Ewald summation (Ewald 1921) was used with non-bonded interaction cut-offs set at 11 Å. The Leapfrog (Van Gunsteren and Berendsen 1988) integration algorithm was used. SHAKE was again switched on. Each simulation was performed with three replicates to ensure a significant hypergeometric p-value.

Post-processing of trajectories was conducted using CPPTRAJ (Roe and Cheatham 2013) the Ambertools (Case, Cerutti et al. 2018) suite. Hierarchical agglomerative clustering was used with clustering stopped when either 5 clusters or ε=4.0 was reached. Root-Mean Square Deviation (RMSD) from starting structures was calculated and plotted (SI 4, supporting information). LOcally EStimated Scatterplot (LOESS) smoothing (Cleveland and Devlin 1988) was applied to plots with a smoothing parameter value, α, of 0.75.

Results and Discussion

This work follows previous studies (Schönberger, Braun, et al. 2019; Schönberger, Zeitler, et al. 2019) and aims to further characterize the gallium-binding peptides resulting from PSD experiments for a future technical implementation in wastewater treatment.

3.1 Interaction studies of free peptides in solution

ITC is a well-suited method for the thermodynamic characterization of metal-peptide interactions. The experimentally determined heat change during the interaction of peptide and metal provides information on various reaction parameters such as stoichiometry, binding constant, enthalpy and entropy. These thermodynamic quantities can be well interpreted in terms of probability and stability for the formation of a complex between peptide and metal and allow cautious conclusions to be drawn about the corresponding binding mechanisms (Wilcox 2008). The reaction environment is crucial for ITC experiments. Ideally, the matrix within which the interaction between peptide and metal is determined should have only a low background heat during the experiments and preferably does not interact with one of the components to be investigated (Wilcox 2008).

Table IV-2. Thermodynamic parameters for the interaction of peptides with gallium in the unbuffered and the acetate buffered system. The dissociation constant KD and the stoichiometry N as well as the binding enthalpy ΔH, entropy -TΔS and Gibbs free energy provide information on binding affinity and mechanisms.

Peptide (Cell)	c (M)	Metall (Inj.)	c (M)	Buffer	T (°C)		N	Binding site model	K_D (M)	ΔH (kJ/mol)	ΔG (kJ/mol)	-TΔS (kJ/mol)
C3.8	2.6e-4	Ga	3.6e-3	80 mM NaAc, 150 mM NaCl, pH 3.0	25.2	Binding (weak)	1	Single	1.47E-04	-30.4	-21.9	8.51
C3.8	2.6e-4	Ga	3.6e-3	150 mM NaCl, pH 3.0	25.2	Binding (weak)	1	Single	1.90E-03	40.5	-15.5	-56
C3.15	1.6e-4	Ga	3.6e-3	80 mM NaAc, 150 mM NaCl, pH 3.0	25.1	Binding	1	Single	2.28E-06	91.9	-32.2	-124
C3.15	1.6e-4	Ga	3.6e-3	150 mM NaCl, pH 3.0	25.1	Binding	1	Single	3.13E-05	174	-25.7	-200
C3.108	2.9e-4	Ga	3.6e-3	80 mM NaAc, 150 mM NaCl, pH 3.0	25.2	Binding	1	Single	1.51E-05	177	-27.5	-204
C3.108	2.9e-4	Ga	3.6e-3	150 mM NaCl, pH 3.0	25.2	Binding	1	Single	3.51E-05	195	-25.4	-221
C3.129	1.7e-4	Ga	3.6e-3	80 mM NaAc, 150 mM NaCl, pH 3.0	25.2	Binding (weak)	1	Single	4.78E-04	-76.8	-19.0	57.9
C3.129	1.7e-4	Ga	3.6e-3	150 mM NaCl, pH 3.0	25.2	no Binding	1	Single	1.07E-07	-0.434	-5.54	-5.11
C3.130	1.6e-4	Ga	3.6e-3	80 mM NaAc, 150 mM NaCl, pH 3.0	25.2	Binding	1	Single	1.95E-06	33.6	-32.6	-66.2
C3.130	1.6e-4	Ga	3.6e-3	150 mM NaCl, pH 3.0	25.2	Binding	1	Single	2.58E-05	75.9	-26.2	-102

An aqueous medium with an ionic strength of 150 mM NaCl and a pH of 3.0 was chosen as the matrix for peptide and titrant. The pH value was adjusted in two different setups by either 1 mM HCl (unbuffered system) or 80 mM acetate buffer (buffered system). The test conditions thus reflect the environment under which the peptides were originally selected during phage display. At the same time, the low pH value meets the subsequent application conditions of the industrial wastewater.

The data obtained from ITC (see Table IV-3 and supporting information, SI 1 A-J, 2) were fitted using the MicroCal PEAQ-ITC Software V 1.3 (Microcal-Malvern Panalytical, Malvern, UK) for a one binding site model for all obtained integrated heats. The results show clearly how the individual peptides differ from each other in their interaction behavior with gallium and arsenic.

The thermograms of arsenic titration to the peptide solutions do not differ from background measurements. The competitive titration experiments confirmed this finding. Based on the ITC studies, gallium substitution by arsenic could be excluded; however, an unspecific and temporary attachment of arsenic ions on the surface of the complexes could not be excluded. The Gibbs free energy was identified as negative for all five gallium-peptide interactions studies, implying that the binding of gallium to the biomolecule is favored, whereas no interaction with arsenic was detected for any peptide. There are approximately three orders of magnitude between the affinities for gallium of the individual peptides (see Table IV-3). Furthermore, the effect of the acetate buffer is clearly shown. Acetate buffer was used to simulate the conditions in the selection process of the peptides in the PSD. With its carboxylic group, the acetate could complex metal ions like gallium and is likely to act competitively with the peptide. The control experiments in which gallium was titrated in acetate buffer indicate such an interaction (see Supporting information, SI 1). It is assumed that the majority of the heat measured in the control experiment is generated by the interaction of free gallium ions with acetate. The interaction studies of the peptides C3.8, C3.15, C3.108 and C3.130 indeed showed a higher affinity ($1/K_D$, cf. Table IV-3) for gallium in the absence of acetate ions than in the acetate-buffered environment. This effect is also reflected in the minor enthalpy change and Gibbs free energy of the respective peptides.

The highest affinity for gallium under both conditions, in buffered and unbuffered environments, was shown by peptide C3.15 and C3.130.

Overall, the binding of gallium to the peptides is endothermic. However, in the case of peptide C3.8, a reversal of the enthalpy change from an endothermic to an exothermic reaction course could be noticed due to the competitive effect of the acetate ions for gallium. Peptide C3.8 shows a comparatively low affinity for gallium in the ITC studies, making the effects of the acetate ions on the heat change in the experiment particularly obvious. This observation leads to the conclusion that a variety of influences lead to the heat change in the reaction cell: The titration of gallium into the cell already occurs in acetate buffer, but the interaction of free gallium ions with acetate also leads to an endothermic reaction contribution (see Supporting information SI 1, Control experiments). The peptide molecules compete with acetate for interaction with gallium, displacing it. This provides an exothermic reaction contribution, but

the binding of gallium to the peptide is again exothermic. Similar effects have already been discussed for the interaction of calmodulin and europium(Drobot et al. 2019).

In our series peptide C3.129 showed the worst binding properties for gallium in the acetate-buffered experiment. The dissociation constant was very low compared to the other tested peptides and even no interaction between peptide C3.129 and gallium was observed in the unbuffered system. This finding can be interpreted as a decisive stabilizing effect of acetate ions on the peptide complex. Furthermore, this poor affinity is a good example of the fact that high-affinity motifs identified from PSD experiments cannot necessarily be transferred to free peptides in solution.

The peptides studied here are 12 amino acids long and do not show any complex structure. Nevertheless, it can be assumed that the biomolecules behave differently when they are free in solution or bound to bacteriophage or polystyrene beads.

However, these findings clearly show, that some of the selected peptides provide specific and high affinity gallium binding and are therefore suitable as a basis for the selective recovery of gallium from real wastewater.

3.2 Biosorption studies with peptide polystyrene conjugates

For industrial applications, a reliable immobilization of peptides while keeping their functionality is necessary. Hence, the chemically synthesized peptide derivates were immobilized covalently and in a site-selective manner on polystyrene beads. The peptide conjugates were investigated for their metal-absorbing properties at conditions that are close to reality. For this purpose, the composites were tested regarding their ability to recover Ga from process wastewater from a wafer manufacturer and compared to studies carried out using a synthetic model solution.

3.2.1 Covalent and site-selective immobilization of peptides

The PEG spacer-mediated conjugation ensured sufficient flexibility of the peptides on the matrix and prevents steric hindrances due to lack of space on the matrix surface. The use of the PEG linker has further decisive advantages to produce a functional gallium biosorbent. The covalent conjugation of the peptides via a cysteine located at the C-terminus of the peptide sequence ensures the stable surface presentation of the peptides adequate to the original presentation at the bacteriophage particle. Furthermore, the immobilization of the peptides on the polystyrene matrix is site-selective and thus creates a homogeneous material.

The successful immobilization of the peptides as well as the stability of the generated materials under different conditions was demonstrated by the ninhydrin reaction and accompanying control experiments. The peptide loading of each conjugate was between 20-40 % (see Table IV-2). It is assumed that the loading efficiency is highly time-sensitive since the peptides and the pegylated material are significantly influenced by oxidation.

Table IV-3. Immobilization of gallium-binding peptides on polystyrene beads. Shown is the relative peptide load [%] and the total load [mmol·g-1] of the material with peptide. The values correspond to an estimate based on control experiments with ninhydrin reagent.

Peptide conjugate	Load [%]	Load [mmol·g^{-1}]
C3.8	21.1	0.204
C3.15	22.4	0.217
C3.108	39.8	0.386
C3.129	36.2	0.351
C3.130	33.6	0.326

High stability of the material, and long-term storage capabilities are important requirements for an industrial application. The produced materials were stable over at least 2 months while stored at 4 °C in water as demonstrated by the ninhydrin reaction. In addition, the resuspension of the peptide conjugates after long-term storage as a lyophilized powder at -20 °C did not influence the stability of the conjugates (see supporting information; SI 4). The measurements of the materials after individual biosorption experiments also showed no changes beyond the error range of single values in parallel determinations. The peptide conjugates proved to be sufficiently stable for a technical application for the biosorption of gallium from real sample waters.

3.2.2 Interaction of peptide conjugates with gallium

Samples of industrial wastewaters (RW) and synthetic model solutions (MS) were used in batch biosorption studies to investigate the gallium-binding capacity of the peptide conjugates. Pegylated but not yet peptide-conjugated polystyrene beads served as a reference. The control material showed weak and probably non-specific biosorption of gallium and arsenic in comparable amounts. The metal biosorption of the peptide conjugates was corrected for non-specific interactions with the material by using the respective reference values (see Figure IV-1).

Overall, gallium biosorption for MS and RW was comparable in all individual experiments indicating a specific gallium-binding by the individual conjugates. Especially the immobilized peptides C3.8, C3.15 and C3.130 showed a significantly better biosorption of gallium compared to the immobilized peptides C3.108 and C3.129. Overall, the peptide conjugates C3.8 and C3.15 with 0.51 and 0.43 mg Ga/mmol immobilized peptide, demonstrate the best metal binding capacities for gallium in both tested samples, RW and MS.

A slightly lower gallium biosorption in the experiments with RW samples could occur due to the more complex composition compared to MS. The real water sample contains various accompanying contaminants, including other trivalent metal ions and organic surfactants that

compete for or interfere with gallium biosorption, which may result in a reduced metal binding capacity.

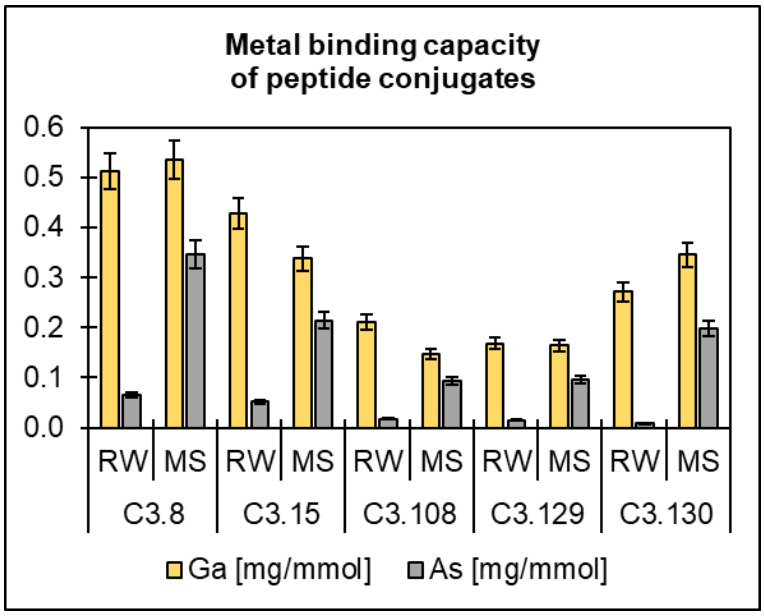


Figure IV-5. Metal binding capacity [mg /mmol immobilized peptide] for gallium and arsenic of peptide conjugates obtained from batch experiments in three replicas with real wastewater samples (RW) and model solutions (MS). The presented values are corrected with the reference data from experiments for the biosorption of gallium and arsenic to pegylated polystyrene beads (RW: 0.097 mg/g Ga, 0.096 mg/g As; MS: 0.093 mg/g Ga, 0.088 mg/g As).

3.2.3 Interaction of peptide conjugates with arsenic

Besides Ga, the industrial wastewater contains 0.2 mM equimolar concentrations of As. Application of the specific peptide for Ga recovery requires a high selectivity towards Ga. Therefore, the binding of As to the peptide was investigated. All materials showed low As binding capacity thus proving a high selectivity of peptides (Figure IV-1). The distinct differences in the biosorption of arsenic from RW and MS are due to the different composition of the solutions. The RW samples are much more complex and contain tensides and other impurities. These prevent the unspecific surface interaction of arsenic on the material.

The ITC studies on the interaction of peptides with arsenic also suggest that arsenic cannot be specifically complexed by peptides and that biosorption, if any, is likely to be non-specific on the peptide surface or related to bound gallium.

From this point of view, the peptide conjugates C3.15 and C3.130 appear particularly efficient because these materials bind little arsenic in relation to adsorbed gallium in RW sample.

3.2.4 Continuous experiments

Due to the high costs of biomolecules, especially of peptides, regeneration and reusability of constructed materials are essential for applications. For this, column experiments with immobilized peptides were performed. Columns were packed with peptide conjugates and

used in a chromatography system. Citric acid was used for desorption. The results for all peptide materials are shown in Figure IV-2. In particular, the peptide materials C3.8, C3.15 and C3.130 showed high Ga-binding capacities.

All columns were repeatedly loaded with the metal solution, resulting in an increased loading compared to the batch experiments (see Figure IV-2). In particular, the conjugates C3.8, C3.15 and C3.130 were efficiently loaded with gallium that is comparable to the results of the batch experiments.

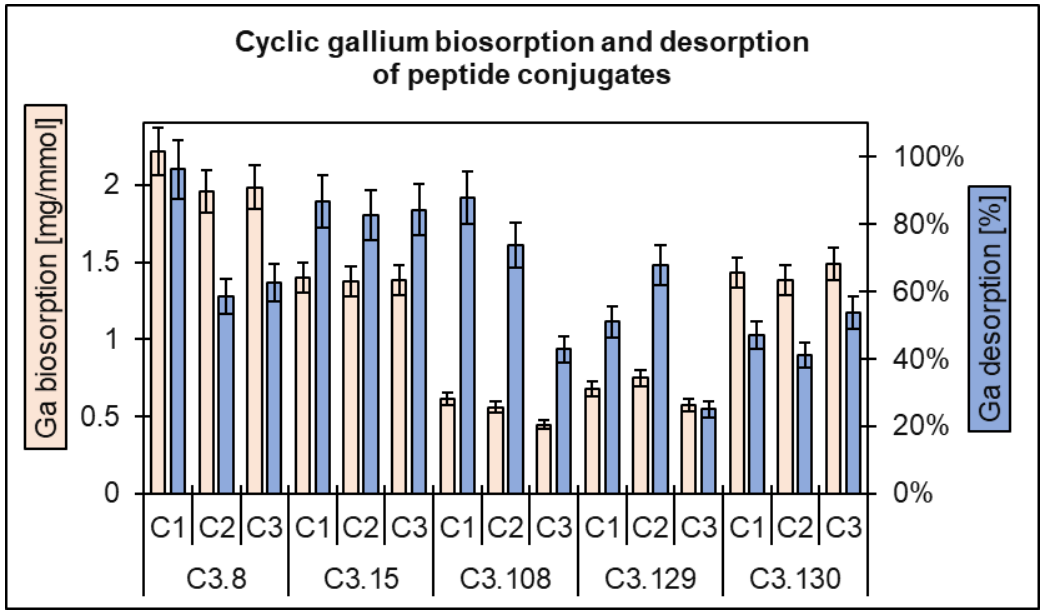


Figure IV-6. Gallium-binding capacity [mg Ga/mmol immobilized peptide] and relative gallium desorption [%]. Data were obtained from chromatographic based mini column studies in three subsequent experiments (C1, C2, C3). The presented values are corrected with the reference data from experiments for the biosorption of gallium to pegylated polystyrene beads (0.084 mg/g Ga, 0.097 mg/g As). Citric acid (10%) was used for desorption.

When considering the individual results for Ga biosorption and desorption in the individual cycles, however, some obvious differences can be observed.

As in the batch experiments, the peptide conjugates C3.108 and C3.129 showed the lowest metal binding capacity. This capacity decreased with each loading cycle. At the same time, the amount of gallium that could be recovered decreased. This suggests that after each cycle a quantity of residual gallium remained on the material, thus reducing the metal binding capacity after each experiment. Perhaps a stronger desorption agent, such as EDTA, could help to keep the metal binding capacity of these peptide conjugates constant over many loading cycles.

The peptide conjugates C3.8, C3.15 and C3.130 showed consistently good metal binding capacities for gallium during the three consecutive loading cycles. Nevertheless, a reduction of the discharge efficiency could be observed for the material C3.8 after the first desorption. For the C3.130 material, this was comparatively constant but very low with about 50 % relative desorption. Only the peptide conjugate C3.15 showed a constantly high regeneration rate

while keeping a consistently high metal binding capacity using citric acid as eluent. These results qualify the material as the most suitable candidate for further experiments.

By using alternative eluents, other peptides might also prove suitable. Very strong eluents such as EDTA are often discussed in this context. However, their cost-benefit factor and environmental compatibility as well as possible interference with the electrolysis for the final obtainment of high-purity gallium is considered problematic.

The biosorption characteristics of the peptide conjugates presented here are comparable to other application-oriented studies conducted by other researchers. Terashima *et al.* achieved a Ga recovery of about 0.06 mg/g wet material with a composite based on a metallothionein fusion construct immobilized on chitopearl resin (Terashima et al. 2002). Close to 10 times higher binding of gallium for the peptide material C3.15, for example, was achieved which demonstrates that the use of more specific ligands for individual raw materials can also lead to a more effective yield. However, due to the great diversity of both materials, direct comparisons of the two studies is difficult. In another study algae-based biosorbents for removal of gallium from semiconductor manufacturing wastewater were developed (Li, Shadman, and Ogden 2018). The material could achieve significantly higher gallium recovery with up to 38.5 mg/g wet material. Nevertheless, any comparison to the present work has to be made very carefully, as all experiments were performed with a synthetic sample water containing only gallium. A special selectivity of the material for gallium compared to other contaminations contained in real wastewater could not be demonstrated, so that a use under practical conditions would be doubtful.

3.3 Model calculation for peptide C3.15

Peptide C3.15 (NYLPHQSSSPSR) has experimentally proven to be a suitable ligand for the directed recovery of gallium. The relationship between the flexibility of peptide structures and the biosorption success by the respective peptide was previously discussed (Schönberger, Zeitler, et al. 2019). In this context, better recognition of metals by organic components was linked to a more rigid structure of the corresponding ligand (Hancock and Martell 1988; Vallet, Wahlgren, and Grenthe 2003).

Unbiased Molecular Dynamics (MD) simulation can be used to predict energetically favorable conformations assumed by a peptide in a corresponding environment and thus elucidate some indication regarding the most likely conformation of the compound. Simulations of the peptide C3.15 demonstrated the lowest intrinsic flexibility among the five peptides discussed. Simulations predict that hydrogen bonds formed between hydroxyl groups of the serine side chains at position 7-9 with backbone amide groups of amino acids at position 3-5 stabilized the central region of peptide C3.15. The molecule reached the conformation quickly, within 10 ns simulation time, as polar interactions between serine hydroxyl side chains and other residues formed and largely remained for the duration of all simulations. In addition, the two prolines at positions 4 and 10 likely reduce the flexibility of the peptide, assisting the stability of these interactions. It can be assumed that the low flexibility and the resulting secondary

structure of the peptide also promotes the very efficient complexation of Ga-ions by the peptide C3.15.

The exact binding position of gallium with C3.15 is yet to be determined. However, the hydrogen bond predicted to be formed between the backbone carbonyl of L3 and sidechain hydroxyl of S7 is, of the three formed, the most transient. It is present in ~30% of simulation frames vs 69% of frames for similar interactions between H5/S9 and 54% for P4/S8 (supporting information, SI 5).

Since desorption with citric acid was determined to be almost complete in the case of C3.15, weak binding of the metal by the hydroxyl groups in the peptide center must be assumed. It is proposed that the relative stability afforded to the overall peptide structure in this area by sidechain and backbone interactions coupled with the relative instability of the L3/S7 interaction and availability of nearby lone-pair electron donors should lead to gallium-binding in this position. Previous work on the complexation of gallium by organic ligands confirms octahedral geometry is likely, typical of transition metal binding in solution (Kubíček et al. 2010; Schmidtke et al. 2017). It is therefore possible that Ga interacts with the center of the peptide, and forms interactions with the lone-pairs of electrons in the backbone carbonyl atoms of L3 and P4 as well as the lone-pair of electrons in the hydroxyl oxygen of S7. Gallium coordination should then be satisfied by nearby water molecules. This idea is supported by the exothermic energy conversion observed in the ITC experiment (see supporting information, SI 1).

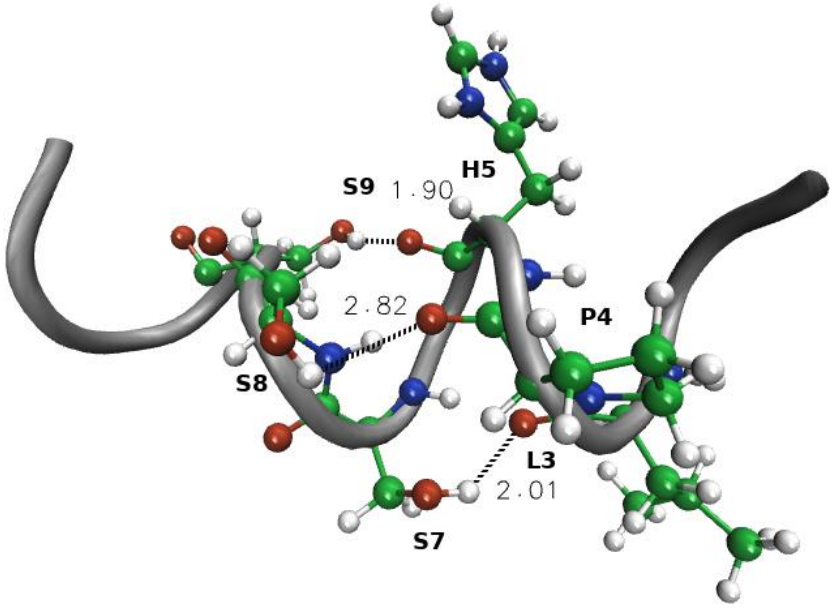


Figure IV-7. Proposed structure for the non-immobilized peptide C3.15 (NYLPHQSSSPSR). The peptide center is stabilized by hydrogen bonds between L3 and S7, P4 and S8 and H5 and S9.

Counclusion

The present study concentrated on the investigation of Ga-binding properties of peptides as well as their immobilization on polystyrene beads and thus construction of peptide-based biosorbents for the recovery of gallium from wastewater.

The ITC and biosorption studies carried out here clearly show that the peptide sequences displayed on the bacteriophage were able to bind metal independently from an immobilization anker such as a bacteriophage capsid or a polystyrene matrix under various conditions.

Furthermore, the peptides retained their functionality after immobilization on a carrier material. Regarding biosorption and desorption, the results interestingly demonstrate that the peptide with the highest affinity for gallium is not necessarily the most suitable for technical applications.

The presented methodology is novel and has yielded a proof of principle for Ga recovery, but principally the technology can be transferred to other elements, thus covering a broad range of applications in industry.

However, there are some barriers that currently hinder the application of peptide-based materials, including environmentally friendly and cost-effective peptide production, optimization of peptide-conjugates as well as general process optimization and up-scaling.

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Chapter V

CONCLUSION

The aim of the presented work was the identification and characterization of gallium-binding peptides for application in metal recovery from low concentrated industrial wastewater. For this purpose, a method for the selection of Ga-binding peptides using PSD technology was developed. Biopanning was performed for a commercial bacteriophage library against application-relevant gallium ion species. To optimize the biopanning process and to isolate and enrich specific binding phage clones, the selection was performed as so-called chromatopanning. Gallium was made available immobilized in a chromatographic system on a monolithic ion exchanger material. The biopanning was performed in three consecutive rounds within an acetate buffered system. From the enriched bacteriophage population, 133 individual bacteriophage clones were selected and analyzed with respect to the presented peptide sequences. 101 putative gallium-binding peptide motifs were identified. The number of peptide motifs was reduced to five by competitive binding experiments: The candidates C3.8 (TMHHAAIAHPPH), C3.15 (NYLPHQSSSPSR), C3.108 (SQALSTSRQDLR), C3.129 (HTQHIQSDDHLA) and C3.130 (NDLQRHRLTAGP) remained as the most promising peptides. These five isolated peptides were first examined displayed on bacteriophage in various binding studies and quantified with respect to their gallium and arsenic adsorbing properties. In further experiments, the interaction between metal and peptide was evaluated independently of the bacteriophage particle. For this purpose, ITC experiments with free peptides in solution were performed, and peptides were immobilized on polystyrene for biosorption studies.

The results of all these different binding experiments clearly show that the peptides behave unlike each other under different conditions. These differences are evident between peptides displayed on bacteriophage particles, free peptides and those immobilized on polystyrene, as well as in terms of affinity and interactions with different target materials. The peptides C3.108 (SQALSTSRQDLR) and C3.129 (HTQHIQSDDHLA) showed excellent adsorption properties to immobilized gallium ions on bacteriophage particles. The biosorption of free gallium ions from solution was much less effective. For the peptides presented on the bacteriophage as well as for the free and polystyrene-immobilized variants, only a poor interaction with gallium ions was detected. For the peptide C3.129 (HTQHIQSDDHLA) the worst results were obtained. The modifications to the peptide-induced by cysteine scanning led to an improved biosorption of gallium. This indicates a close relationship between the flexibility of the peptide structure and the ability to bind gallium ions in solution. On the other hand, a more flexible peptide structure seems to be beneficial for adsorption to larger targets, such as NTA-immobilized gallium. The peptides C3.8 (TMHHAAIAHPPH), C3.15 (NYLPHQSSSPSR) and C3.130 (NDLQRHRLTAGP) are characterized by particularly good biosorptive properties for

free gallium in solution. The peptide C3.8 (TMHHAAIAHPPH) stood out particularly for its versatility. Regardless of the presentation form and the environmental conditions, the peptide consistently showed very good experimental results. Nevertheless, the peptide C3.15 (NYLPHQSSSPSR) with its comparatively weaker experimental binding results is the most suitable candidate for the development of an application. The results on continuous biosorption clearly showed that the very good biosorption of gallium by peptide C3.8 (TMHHAAIAHPPH) results in poor desorption of the metal. However, this is essential for a later economic application of the biocomposites produced here.

Obtained insights for the selection of metal-binding peptides in biopanning experiments

The Phage Surface Display (PSD) is a method for the isolation of highly affine and selective peptide ligands. This versatile tool has been widely used for different targets since the first report on this method and has been further developed.

Nevertheless, the experimental implementation of the Phage Surface Display still has many obstacles to overcome, as the experimental setup has to be adapted to a concrete selection goal. It must always be defined in advance what the peptide ligand should bind under certain conditions. The old principle "you get what you screen for" applies here (Schmidt-Dannert and Arnold, 1999).

The Phage Surface Display is mainly used for selection against organic, biological targets such as biomolecules, cell components or entire cells. There are commercially available phage libraries that achieve good results with well-stablished standard protocols. Such experiments are performed under standard physiological conditions and require hardly any modifications or experiment-specific adaptations.

However, the experimental requirements for the isolation of peptide ligands for inorganic targets represent a different case. While standard physiological conditions are usually required for biological systems, conditions for inorganic systems vary greatly depending on the target and its desired functionality. Depending on the environment (pH value, temperature, ionic strength, other agents such as buffer salts or detergents), the state of inorganic targets sometimes changes considerably. Among other things, the possible formation of oxide layers on the surface of the material, which conceal the actual target, different crystallographic facets, and chemical and physical modifications during incubation with biological agents in biopanning must be taken into account (Sarikaya, Tamerler, Daniel T Schwartz, *et al.*, 2004).

Accordingly, the biopanning conditions must correspond to the working environment in which the peptides are to be used later. This is the more difficult, the more sensitive the target reacts to changes in the surrounding environment, and the more versatile the peptide must function under different conditions.

Metal ions as a target for biopanning experiments represent its own special case. As such, they are very small, soluble, and strongly impactable targets. Their speciation and accessibility strongly depend on the solution in which the metals are dissolved. They form different speciations depending on the pH value and on the presence of other ions; they form hydration spheres depending on the ionic strength, and they are sensitive to the smallest changes in the surrounding environment. In addition, the immobilization of the metal ions on a suitable matrix is crucial for biopanning. Only by this, it is possible to separate strong binders and such clones that do not or does only weakly interact during the selection process. For metal ions, the immobilization is usually done by chelation on functionalized surfaces. Binding sites on the metal ions are occupied by the chelator, and a balance must be kept between the stability of the target immobilization and the freedom and thus accessibility of the metal ions.

This also reveals the greatest weakness of the procedure, namely that biopanning against immobilized metals results in exactly that: Binders are isolated that recognize exactly this target, immobilized metal ions but not necessarily free metal ions in solution. The chelation leads to the occupation of binding sites on the metal ion that cannot be recognized by the peptide ligands. This can lead to the selection of weak ligands or to ligands that are only capable of cooperative complexation together with the chelator molecule. It is, therefore, important to match biopanning conditions as closely as possible on the later application conditions. In any case, evaluation of the isolated binders under application conditions is necessary.

Besides the form and availability of the target, the choice of the peptide library also has a significant influence on the quality of peptide selection. The selection result depends both on the correct handling of the library but also on the initial selection of a suitable library. Peptide libraries are usually purchased commercially since the preparation of individual libraries is extremely time-consuming. Different PIII and PVIII systems can be used for this purpose. PIII libraries present random peptide sequences in a maximum of five copies on the minor capsid protein PIII. PVIII libraries present random peptide sequences in a considerably higher number of copies on the major capsid protein PVIII. Such systems are usually well suited for the accurate recognition of complex inorganic surfaces such as mineral particles. The higher copy number of a peptide sequence on the phage surface allows the emergence of avidity effects (Petrenko, 2018). This is less advantageous for the recognition of very small target molecules, such as metal ions. In this case, the use of PIII system promises better results with single ligands that have a high affinity for a target molecule.

The form of the peptides presented also has an influence on the selection success. While long-chain complex peptide structures can be advantageous for selective recognition of complex surfaces, only a few amino acids are required for tiny targets such as metal ions. In addition, the macrocyclic effect comes into play, in which a tighter and rather rigid peptide structure for binding proves to be beneficial compared to more open and flexible peptides.

Furthermore, biopanning success also depends on the design of experiment. The number of biopanning cycles and amplification steps carried out has a significant influence on the

selection result. The selection of peptide ligands is not only guided by the stringency of biopanning and experimental conditions, but also by the host's preferences to produce different bacteriophage clones. Thus, during the PSD, both good binders and clones that can be produced well by the host organism are enriched. Depending on the desired selection result, the question must be answered whether only one really good clone should be found for a certain application or whether a statistical survey of possible peptide motifs should be carried out for a more fundamental understanding of the interaction between peptide and target. Accordingly, it must be considered how many selection and amplification rounds should be performed for an optimal result. A good way to increase the selection success in a few biopanning rounds is the stringency of biopanning. Often an approach is used in which the stringency of selection is increased from experiment to experiment. This can be achieved, for example, by lowering the target availability, increasing detergent concentrations, or changing the pH value. The danger of not stringent biopanning conditions lies in the selection of a very high number of different clones that only weakly interact with the target and must then be sorted out in complex binding experiments. If procedures are too stringent, very good clones can be lost due to host-related bias, and more dominant "false friends" can emerge.

In summary, it can be concluded that biopanning experiments for difficult inorganic targets such as metal ions require careful planning. The experimental procedure must be carefully considered to ensure selection success. It is advantageous to characterize a larger clone population after the final biopanning-round to be able to assess the selection success. In any case, the identified putative binders must be evaluated in detailed binding experiments that imitate the later application conditions.

Conclusions for the development of peptide-based materials for the biosorptive recovery of metal ions from aqueous solutions

In order to answer the question whether the PSD is suitable for the identification of high-affinity and selective peptide ligands, not only the result of this work should be considered, but also the path that was taken to achieve this result. In general, the hard principle "you get what you ask for" applies to directed molecular evolution (Schmidt-Dannert and Arnold, 1999). However, it is not always easy or even possible to ask the right questions.

It can certainly be generalized that the PSD does not lead to clear results quickly and easily. On the contrary, the amount of work is enormous, and many influencing factors must be taken into account to achieve the most accurate results. It is a biological system, which, by its very nature, is highly prone to errors. The isolation of peptide ligands in the PSD is always controlled in two ways: by the affinity of the target and the bias of the phage host. In addition, the massive size discrepancy between the phage capsid as the vehicle for the comparatively tiny peptide sequence presented always plays a role during selection. This is accompanied by the

influence of the unspecific target interaction by the phage capsid, which inevitably leads to the selection of some unsuitable peptide ligands.

Nevertheless, the evaluation of the suitability of a method raises the question of possible alternative approaches. It is reasonable to use naturally occurring ligands and optimize them for certain applications. Biomolecules have a natural affinity to metals, which is related to the presence of functional groups on the surface of these. Metals have important functions in the metabolism of all living organisms. Correspondingly, accurate systems exist for regulating the use and availability of individual metal ions in living systems. It is due to the enormous complexity and diversity of life that only a fraction of these available ligands is known to date. Elaborate screening processes must, therefore, often precede the utilization of natural ligands to bind metal ions. An alternative is the rational design of ligands and their subsequent synthesis. This requires extensive knowledge on the interaction mechanisms. Such insights can usually only be gained from very extensive experimental investigations and require solid basic research in the respective field. For some metals, there exists certainly enough knowledge that allow such an approach. Less well studied metals, such as gallium, preclude rational design approaches without first establishing experimental foundations.

PSD experiments can be a contribution to create such fundamentals and to optimize and shorten later screening processes. It is useful to characterize the inherent binding mechanisms between the metal ion and the selected peptide. The thermodynamics of the interaction can provide important information. Furthermore, it is interesting to know which amino acids or functional groups are crucial for the interaction and how the peptide structure might change due to the interaction with the metal ion. Spectroscopic investigation methods such as CD, IR- and Raman spectroscopy or NMR spectroscopy can answer the question and provide data for the calculation of an optimized peptide structure.

In the application-oriented development of metal-binding peptide ligands, it is not only important to identify a suitable sequence, but also to be able to use this sequence independently of the bacteriophage. It is not necessarily the case that the peptide sequence selected on the phage will retain its functionality in a later application concept. In general, bacteriophage particles are rather unsuitable for technical implementation. The filamentous particles are sensitive to shear stress, and their host-dependent production is susceptible to mutations. Even though bacteriophage particles are not classified as GMOs per se, the social acceptance of their use in industry is uncertain. Since the technical use of bacteriophage clones is not feasible, the selected metal-binding peptides must be provided by other means.

The peptides can be provided by chemical or biological synthesis. Chemical synthesis is carried out, for example, as solid-phase synthesis. It is a long-established process that can be easily automated. Chemical modifications of the peptide, e.g., for cyclization, tags for immobilization or the introduction of protective groups, can easily be carried out. Individual methods can be easily established and optimized. Despite these many advantages, chemical synthesis is not considered to be particularly environmentally friendly. The use of harsh chemicals produces toxic waste, and peptide purification consumes large amounts of solvents

that produce toxic waste. In addition, the production of larger amounts of the peptide is expensive, as the savings potential through up-scaling is rather low. Chemically synthesized peptides are very good in small quantities for development work and basic research as well as pharmaceutical applications, but an alternative approach is needed to provide larger quantities of peptide for technical use.

A possible alternative is the biological synthesis of peptides. This is now possible in vivo and in vitro. However, the most common approach is still the heterologous expression of recombinant proteins. In contrast to chemical solid-phase synthesis, the heterologous expression is very complex in method development. Once a functioning system for production and purification exists, the method is suitable for the cost-effective and environmentally friendly provision of large quantities of peptides. The main challenge in the biological synthesis of small peptides is that it is not possible to produce pure peptides. These are too small to be isolated from the cells, are susceptible to post-translational modifications and can have toxic effects on cell metabolism. Therefore, the heterologous expression of recombinant peptides usually is conducted as fusion products. Such a fusion product has the function to increase the mass of the peptide and to facilitate the purification of the peptide product. To produce pure peptides, the final cleavage of the tag is necessary. Alternatively, the tags can also be advantageously used for the immobilization of the peptides. The presentation of the peptide ligands on such a fusion partner can be done adequately to present the peptide in the PSD. This is advantageous because, in this way, the peptide functionality selected on the bacteriophage can most likely be preserved.

Nevertheless, this approach has its pitfalls since the size discrepancy between peptide and fusion tag has a similar effect during the presentation on the bacteriophage capsid. The selectivity of the metal-binding peptides could be reduced by immobilization via protein tags through unspecific interactions with metals. In addition, there is a risk of biosorption of metals to immobilization matrices like cellulose or chitin.

An alternative to the use of fusion tags is chemical conjugation on inorganic matrices. Such biocomposites exhibit high metal selectivity and material stability. The necessary flexibility of the immobilized peptides can be ensured by using linker molecules. The decisive factor for this approach is that the functionality of the selected peptide ligands is also retained by the conjugation on the carrier material. The side-selective immobilization of the peptides is crucial for this.

The aim of this work was selective recovery of gallium at the lowest possible process costs. The peptide-based material to be used for this purpose is not only complex in its development but also comparatively expensive in later technical implementation. The costs of the biocomposite can be reduced by optimizing of the production process. Nevertheless, to be used profitably, the material must be able to withstand as many cycles of loading and desorption as possible. In this thesis, only the foundation has been created. Further optimization and upscaling of the method are required before a technical implementation in industrial processes can be considered. For this purpose, a stable and, at the same time,

inexpensive material with a high selective sorption performance is required. The gallium must be completely desorbable after each loading. For desorption, it should be possible to use chemicals that are inexpensive and environmentally friendly and do not interfere with the electrolysis for the recovery of high-purity gallium.

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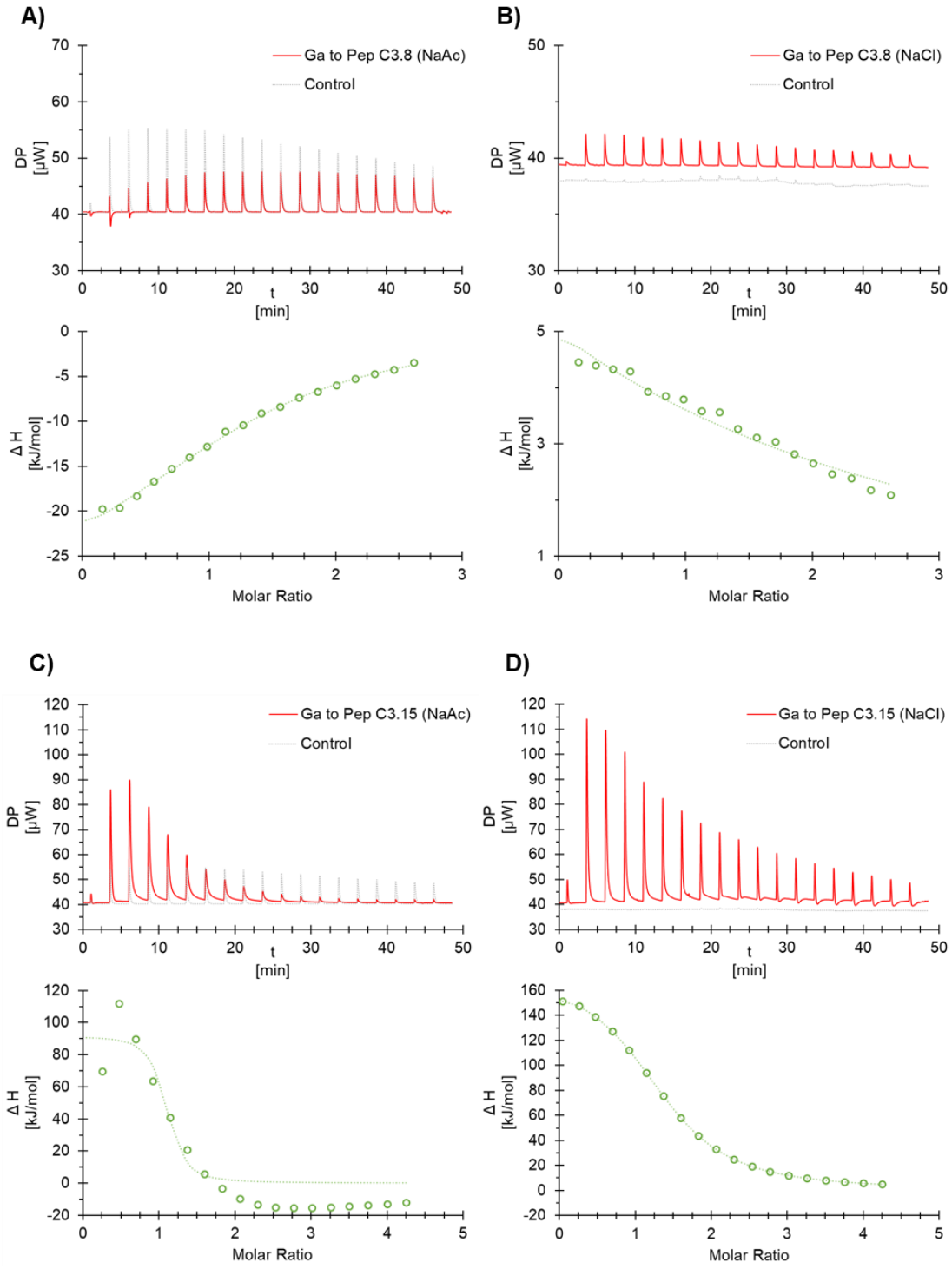
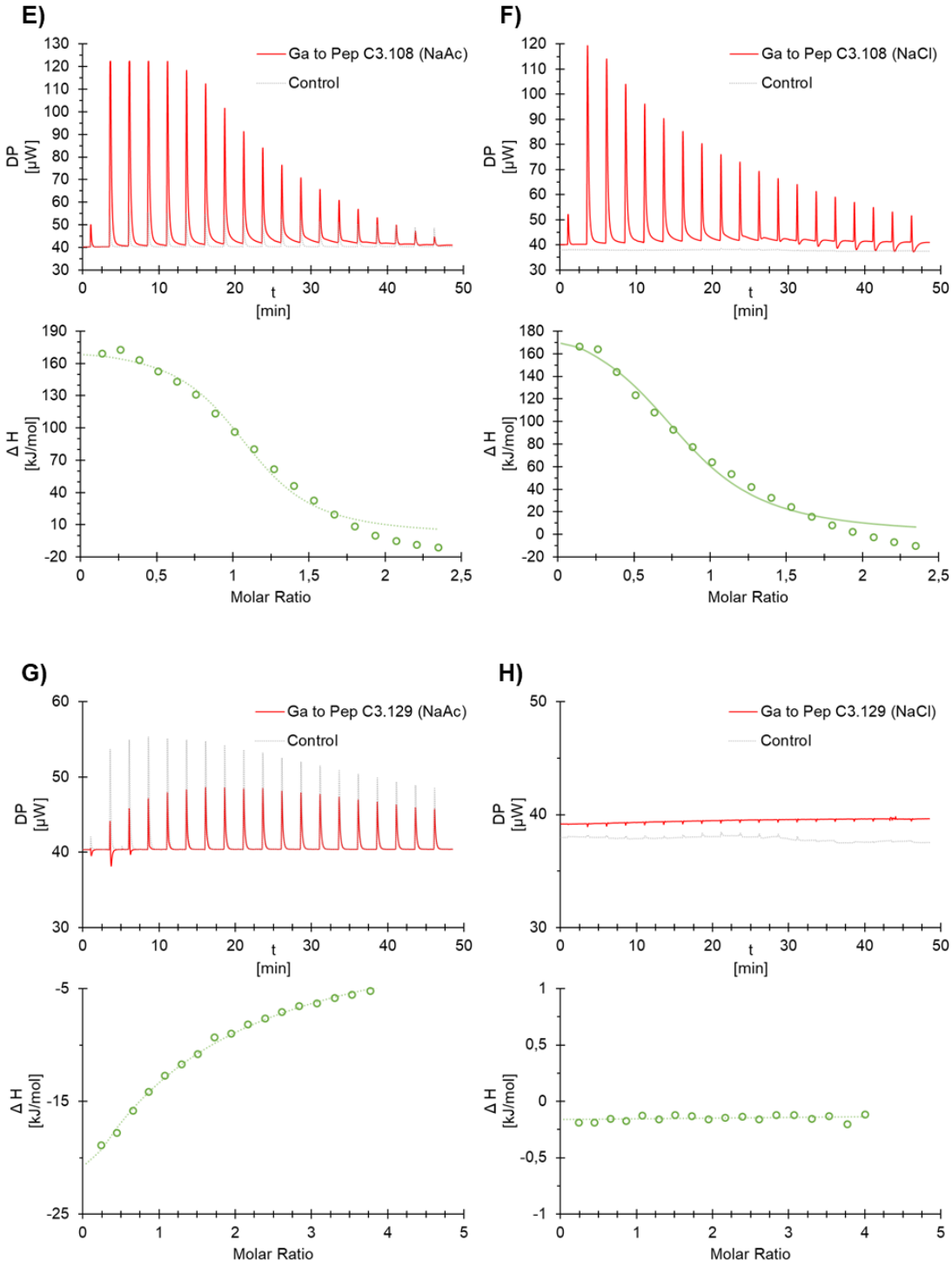
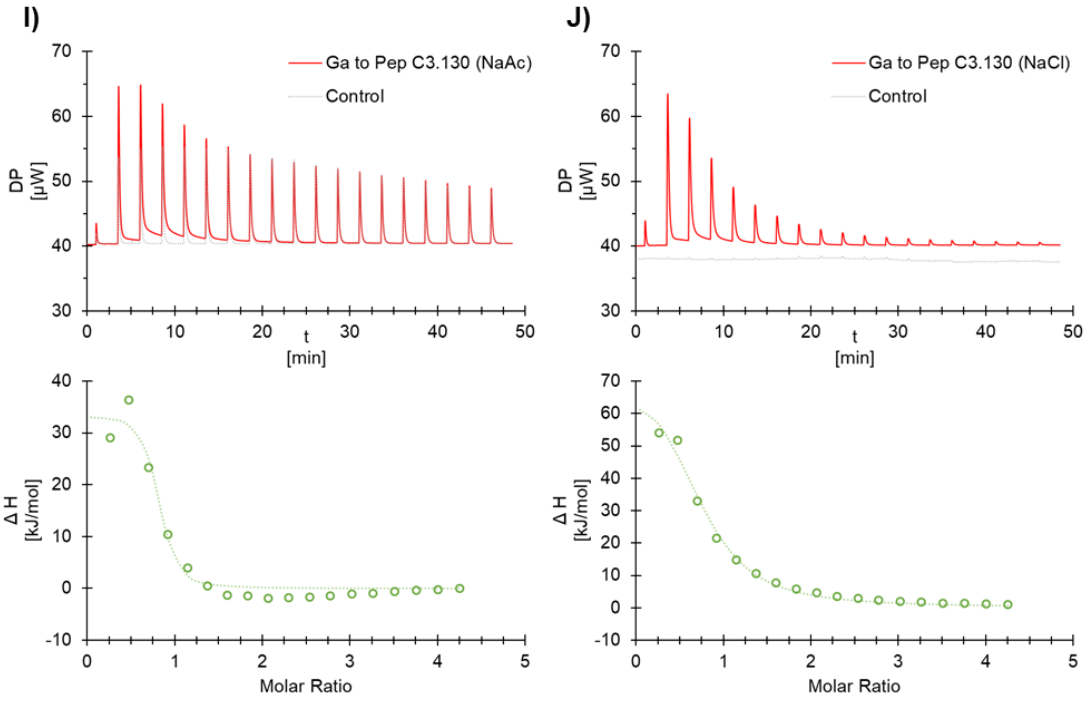
APPENDIX

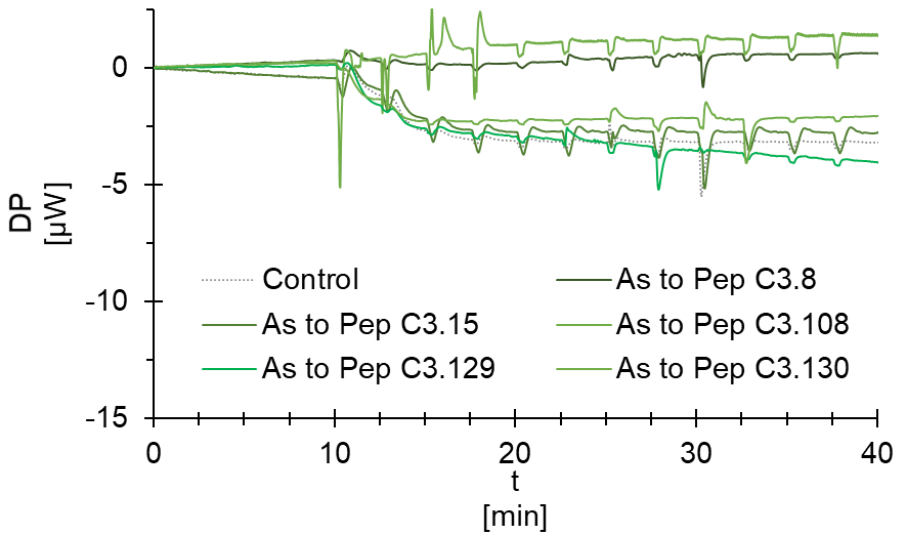
Supporting information for Chapter IV




E)
Ga to Pep C3.108 (NaAc)
Control
DP [µW]
t [min]
ΔH [kJ/mol]
Molar Ratio
F)
Ga to Pep C3.108 (NaCl)
Control
DP [µW]
t [min]
ΔH [kJ/mol]
Molar Ratio
G)
Ga to Pep C3.129 (NaAc)
Control
DP [µW]
t [min]
ΔH [kJ/mol]
Molar Ratio
H)
Ga to Pep C3.129 (NaCl)
Control
DP [µW]
t [min]
ΔH [kJ/mol]
Molar Ratio



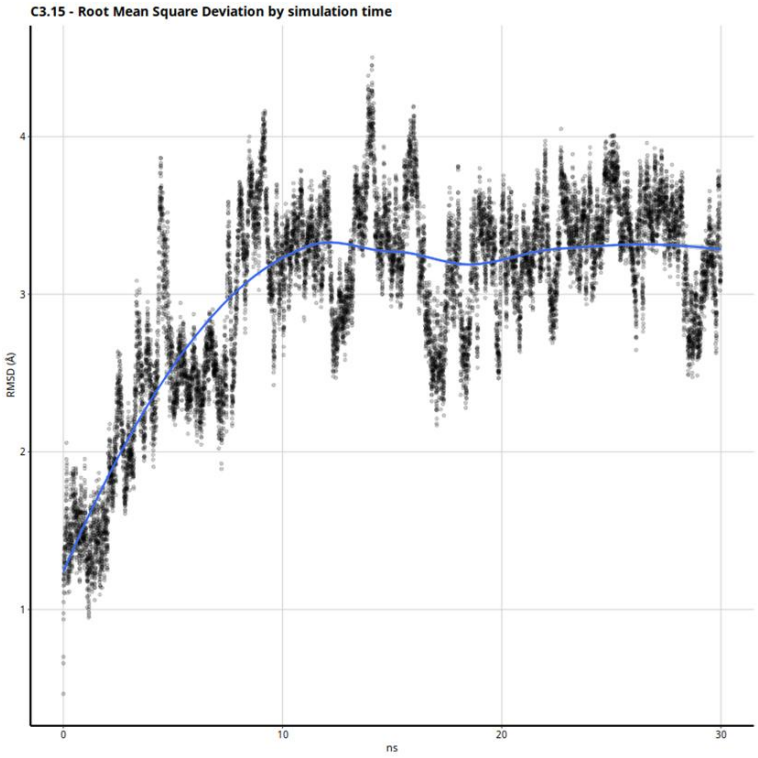
SI 1. ITC titration thermogramms and integrated heat plot for one binding site model of interaction studies of 3.6 mM gallium with A) 0.26 mM peptide C3.8 in acetate-bufferend environment at pH 3.8, B) 0.26 mM peptide C3.8 in unbuffered environment at pH 3.8, C) 0.16 mM peptide C3.15 in acetate-bufferend environment at pH 3.8, D) 0.16 mM peptide C3.15 in unbuffered environment at pH 3.8, E) 0.29 mM peptide C3.108 in acetate-bufferend environment at pH 3.8, F) 0.29 mM peptide C3.108 in unbuffered environment at pH 3.8, G) 0.17 mM peptide C3.129 in acetate-bufferend environment at pH 3.8, H) 0.17 mM peptide C3.129 in unbuffered environment at pH 3.8, I) 0.16 mM peptide C3.130 in acetate-bufferend environment at pH 3.8, J) 0.16 mM peptide C3.130 in unbuffered environment at pH 3.8. The control data set was generated by titrating 3.6 mM gallium into the cell with the appropriate buffer without peptide. The data set was used to subtract the background heat.



SI 2. ITC titration thermogramms of experiments were 0.17 mM arsenic were used to titrate the peptide solutions. The control data set was generated by titrating 1.7 mM arsenic into the cell without peptide.

SI 3. Detection of Peptide conjugation by Ninhydrin reaction. Shown are adsorption values at 570 nm of Ninhydrin-stained Polystyren beads after peptide conjugation. Beads ware stored in water at 4 °C. Ctrl 1 - unconjugated PS beads (max load; amine groups: 0.97 mmol g-1); Ctrl 2 - capped PS beads (zero load; amine groups capped with acetic anhydride); Ctrl 3 - pegylated PS beads (zero load; no free amine groups due to pegylation)

	direct after conjugation			7 days after conjugation			63 days after conjuagtion			lyophilized		
Ctrl 1	0.381	0.368	0.399	-	-	-	-	-	-	-	-	-
Ctrl 2	0.007	0.011	0.009	-	-	-	-	-	-	-	-	-
Ctrl 3	0.014	0.009	0.015	-	-	-	-	-	-	-	-	-
C3.8	0.069	0.089	0.076	0.065	0.072	0.086	0.076	0.063	0.090	0.061	0.088	0.078
C3.15	0.088	0.082	0.079	0.072	0.080	0.074	0.099	0.089	0.082	0.069	0.100	0.102
C3.108	0.171	0.132	0.139	0.153	0.155	0.169	0.149	0.143	0.161	0.153	0.139	0.132
C3.129	0.121	0.142	0.139	0.133	0.149	0.143	0.144	0.119	0.135	0.121	0.133	0.148
C3.130	0.118	0.132	0.123	0.145	0.139	0.115	0.144	0.110	0.145	0.115	0.108	0.158



SI 4. Peptide C3.15 average Root-Mean-Square Deviation (RMSD) relative to starting structure. The RMSD of C3.15 can be seen to stabilize at approximately 10 ns, indicative of stable secondary-structure formation.

SI 5. Top-10 most frequent hydrogen bonds formed between residues in C3.15. Acceptor, DonorH and Donor refer to respective interaction partner residues, Frames is a count of the number a frames where the hydrogen bond was present, Frac is the proportion of total frames where the hydrogen bond was present, AvgDist and AvgAng are respective distance and angle criteria for each hydrogen bond.

#Acceptor	DonorH	Donor	Frames	Frac	AvgDist	AvgAng
TYR_2@O	GLN_6@HN	GLN_6@N	10306	0.6871	2.8521	162.0373
HSP_5@O	SER_9@HG1	SER_9@OG	9809	0.6539	2.74	163.2984
PRO_4@O	SER_8@HG1	SER_8@OG	8095	0.5397	2.7543	161.1583
LEU_3@O	SER_7@HN	SER_7@N	7514	0.5009	2.862	159.304
PRO_4@O	SER_8@HN	SER_8@N	6822	0.4548	2.8676	155.0694
ASN_1@O	HSP_5@HN	HSP_5@N	5329	0.3553	2.8688	158.5391
LEU_3@O	SER_7@HG1	SER_7@OG	4601	0.3067	2.7679	161.1498
HSP_5@O	SER_9@HN	SER_9@N	2024	0.1349	2.8786	149.7203
SER_8@O	SER_11@HG1	SER_11@OG	1437	0.0958	2.7275	161.677
SER_8@O	SER_11@HN	SER_11@N	1346	0.0897	2.8922	152.748

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List of abbreviations

As	Arsenic
BB	Borate buffer
CB	Coupling buffer
CO2	Carbon dioxide
Cr	Chromium
CRM	Critical raw materials
CSD	Cell surface display
C-terminal	means the carboxyl terminal end of a protein or peptide
CV	Column volume
DEAE	Diethylaminoethanol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EOL	End-of-life (product)
Fe	Iron
Fe_2O_3	ferric oxide
FPLC	Fast protein liquid chromatography
Ga	Gallium
$Ga(NO_3)_3$	Gallium nitrate
$Ga(OH)^{2+}$, $Ga(OH)_2^{+}$, $Ga(OH)_4^{-}$, $Ga(OH)^{4-}$	Gallium hydroxide ions
GaAs	Gallium arsenide
GaN	Gallium nitride
GaP	Gallium phosphide
GaSb	Gallium antimonide
$H_2AsO_4^{-}$	Dihydrogen arsenate
$HAsO_4^{2-}$	Hydrogen arsenate
HCl	Hydrogen chloride
ICP-MS	Inductively coupled plasma mass spectrometry
IDA	Iminodiacetic acid
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl-β-D-thiogalactoside
ITC	Isothermal titration calorimetry
kb	Kilobase
Kd	Dissociation constant
LB	Lennox broth
LED	Light-emitting diode
MD	Molecular Dynamics
$MgCl_2$	Magnesium chloride

ML	Mini library
mRNA	Messenger ribonucleic acid
N	Nitrogen
Na_2HPO_4	Sodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NTA	Nitrilotriacetic acid
N-terminal	means the amino terminal end of a protein or peptide
O	Oxygen
OD_{600}	Optical density at a wavelength of 600 nm
Pb	Lead
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfU	Plaque forming unit
ppm	Parts per million
PSD	Phage surface display
QA	Quarternary Amine
REE	Rare earth elements
RF-DNA	Replicative form of DNA
S	Sulfur
SiO_2	Silicium dioxide
ssDNA	Single stranded DNA
TACN	1,4,7-Triazacyclononane
TBS	Tris(hydroxymethyl)aminomethan buffered saline
Wt	Wild type
Xgal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside
ZnO	Zinc oxide
ZnS	Zinc sulfide

Amino acids

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
Q	Glutamine
E	Glutamic acid
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine

K	Lysine
M	Methionine
F	Phenylalanine
P	Proline
S	Serine
T	Threonine

Nucleobases

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

List of chemicals

2-Propanol (≥99,8 %, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Acetic acid (ROTIPURAN® ≥95,9 %, p.a., Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Agar (BD Difco™ dry culture media, granulated agar, Fisher Scientific GmbH, Schwerte, Germany)

Agarose (Pierce™ Agarose, Thermo Fisher Scientific, Schwerte, Germany

Amino-methyl polystyrene resin (H10002, Rapp Polymere, Tübingen, Germany)

Calcium chloride ($CaCl_2$, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

CIM Disk (Monolithic Column, BIA Separations d.o.o., Ajdovscina, Slovenia)

di-Sodium hydrogen phosphate dihydrate (Na_2HPO_4, p.a., Merck KGaA, Darmstadt, Germany)

Ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA, ≥99 %, p.a., ACS, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Gallium(III) nitrate hydrate ($Ga(NO_3)_3 \cdot xH_2O$, 99,9 % trace, Alfa Aeser Kandel AG, Landau, Germany)

Glucose (p.a., ACS, anhydrous, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Glycine (PUFFERAN® ≥99 %, p.a., Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Hydrochloric acid (HCl, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Isopropyl-ß-D-thiogalacto-pyranoside (IPTG, ≥99 %, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Magnesiumchlorid-Hexahydrat ($MgCl_2$, p.a., Merck KGaA, Darmstadt, Germany)

Magnesiumsulfat-Heptahydrat ($MgSO_4$, p.a., Merck KGaA, Darmstadt, Germany)

Ninhydrin (ACS reagent, Alfa Aeser Kandel AG, Landau, Germany)

PEG-Linker (heterobifunctional PEG_{12} crosslinkers ($SM(PEG)_{12}$, Pierce Biotechnology, Rockford, Illinois, US)

Ph.D.™-12 Phage Display Peptide Library Kit (New England Biolabs GmbH, Frankfurt am Main, Germany)

Phusion® High-Fidelity PCR Kit (New England Biolabs GmbH, Frankfurt am Main, Germany)

Polyethylene glycol sorbitan monolaurate (TWEEN® 20, for molecular biology, viscous liquid, Sigma Aldrich, Merck KGaA, Darmstadt, Germany)

Potassium chloride (KCl, ≥99 %, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

PureCube NTA Agarose (Cube Biotech, Monheim, Germany)

PureYield™ Plasmid Miniprep System (Promega GmbH, Walldorf, Germany)

Q5® Site-Directed Mutagenesis Kit (New England Biolabs GmbH, Frankfurt am Main, Germany)

Sodium (meta)arsenite ($NaAsO_2$, Merck KGaA, Darmstadt, Germany)

Sodium acetate trihydrate (≥99,5 %, p.a., ACS, ISO, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Sodium chloride (NaCl, ≥99,5 %, p.a., ACS, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Sodium dihydrogen phosphate dihydrate (NaH_2PO_4, p.a., Merck KGaA, Darmstadt, Germany)

Sodium hydroxide (NaOH, ≥98 %, p.a., ISO, in pellets, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Sodium tetraborate decahydrate (ACS reagent, ≥99.5%, Merck KGaA, Darmstadt, Germany)

TCEP agarose (Tris(2-carboxyethyl) phosphine, immobilized on agarose CL-4B, Merck KGaA, Darmstadt, Germany).

TRIS Hydrochlorid (TRIS-HCl, PUFFERAN® ≥99 %, p.a., Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Tryptone (Bacto™ Tryptone, Fisher Scientific GmbH, Schwerte, Germany)

X-β-Gal (5-Bromo-4-chloro-3-indoxyl-β-D-galactoside,≥99 %, BioScience Grade, Carl Roth GmbH + Co. KG, Karlsruhe, Germany)

Yeast extract (Bacto™ Yeast Extract, Fisher Scientific GmbH, Schwerte, Germany)

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Bergakademie Freiberg. Ich möchte außerdem besonders **Herrn Dr. Frank Haubrich** für die Leitung des Projekts danken. Ich danke zudem **Herrn Dr. Thomas Reinhold** und **Freiberger Compound Materials** für die Bereitstellung verschiedener Realwasserproben. Ich möchte mich bei **Herrn Aaron Weigelt** für die Verwaltung des Projekts von Seiten der Technischen Universität Bergakademie Freiberg bedanken. Nicht zuletzt danke ich dem **Bundesministerium für Bildung und Forschung** für die Finanzierung des Projekts.

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Curriculum vitae

Personal information

Name	Nora Schönberger, née Nowak
Date of Birth	22 August 1990
Family status	married one daughter, born 14 November 2019

Scientific carrier

Since 06. 2020	Scientist at Helmholtz Centre Dresden-Rossendorf, Helmholtz Institute Freiberg for Resource Technology, Biotechnology Department working in the WIPANO project *NORA - Effiziente Herstellung von bifunktionalen Peptiden*
08. 2016 – 09. 2019	Research assistant / PhD candidate at TU Bergakademie Freiberg, Institute for Nonferrous Metallurgy in the joint project *EcoGaIN - Gewinnung von Gallium aus Produktionsabfällen* working at the Helmholtz Center Dresden- Rossendorf, Helmholtz Institute Freiberg for Resource Technology, Biotechnology group PhD project: *Identification and characterization of gallium-binding peptides*
05. 2015 – 03. 2016	Master Student Helmholtz Centre Dresden-Rossendorf, Helmholtz Institute Freiberg for Resource Technology, Biotechnology Group Topic of the master thesis: *Selection of cobalt- and nickel-binding peptide sequences using the phage surface technique*
08. – 09. 2014	Research trainee Dresden University of Technology, Chair of General Biochemistry Assignment: *Enzyme purification from a white rot fungus*
02. – 07. 2013	Bachelor student at Dresden University of Technology, Chair of Microbial Diversity Topic of the Bachelor thesis: *Analysis of a new selection system for Methanosarcina acetivorans and presentation of an antigen*
02. – 11. 2012	Student assistant at Dresden University of Technology, Chair of Plant Physiology Assignment: *Isolation of phytohormones*

Education

since 2016	PhD studies at TU Bergakademie Freiberg, Faculty of Chemistry and Physics
2013- 2016	Study Master Biology, Technical University of Dresden Degree: Master of Science
2010 -2013	Study Bachelor Biology, Technical University of Dresden Degree: Bachelor of science

2009 - 2010	Professional school for paramedics, Wilthen Qualification: Paramedic
2001 - 2009	Pestalozzi-Gymnasium Dresden Degree: Abitur (General qualification for university entrance)

Part-time jobs

2011 – 2012	Service International Congress Center and Maritim Hotel Dresden
2012 – 2014	Employees gastronomy Club Passage, Dresden
2013 – 2015	Service, waiter CrashIce Event Service GmbH
2014 & 2016	Beverage Catering Filmnächte am Elbufer; Dresden
2014 - 2016	Customer Happiness, Cyberport, Dresden

Skills

Language

German	Mother tongue
English	Good written and spoken
French	Expandable written and spoken
Russian	Basics

Biological working techniques

Cultivation	aerobic and anaerobic
Chromatography	FPLC, HPLC
Molecular biology	reparation, purification and detection of DNA, RNA and proteins; versatile practical experience with PCR (including qPCR, RT-PCR); Recombinant manipulation of prokaryotic and eukaryotic systems and bacteriophage; Heterologous expression of recombinant peptides and protein fragments
Phage surface display	Biopanning against metal ions and inorganic surfaces
Biophysics	Experience with Raman spectroscopy, DLS and microcalorimetry

List of publications

Publications in first authorship

Schönberger, N., Matys, S., Flemming, K., Lehmann, F., Lederer, F.L., Pollmann, K. (2017*)* 'Development of metal ion binding peptides using phage surface display technology', *Solid State Phenomena*. doi: 10.4028/www.scientific.net/SSP.262.591.

Schönberger, N., Braun, R., Matys, S., Lederer, F.L., Pollmann, K. (2018) 'Peptidbasierte Rückgewinnung von Gallium aus Abwässern der Halbleiterindustrie', *Chemie Ingenieur Technik*. John Wiley & Sons, Ltd, 90(9), pp. 1287–1288. doi: 10.1002/cite.201855340.

Schönberger, N., Braun, R., Matys, S., Lederer, F.L., Pollmann, K. (2019) 'Directed evolution and engineering of gallium-binding phage clones-a preliminary study', *Biomimetics*, 4(2), p. 35.doi: 10.3390/biomimetics4020035.

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Schönberger, N., Zeitler, C., Braun, R., Matys, S., Lederer, F.L., Pollmann, K. (2019) 'Peptide-based recovery of gallium', in *Proceedings of the 35th European Peptide Symposium*, pp. 133–135. doi: 10.17952/35eps.2018.133.

Schönberger, N., Taylor, C., Schrader, M., Drobot, B., Matys, S., Lederer, F.L., Pollmann, K. (2021) 'Gallium-binding peptides as a tool for the sustainable treatment of industrial waste streams', *Journal of Hazardous Materials* 414, 125366. doi:10.1016/j.jhazmat.2021.125366.

Co-authored publications

Braun, R., Matys, S., Schoenberger, N., Lederer, F., Pollmann, K. (2017) 'Simplified expression and production of small metal binding peptides', *Solid State Phenomena*. doi: 10.4028/www.scientific.net/SSP.262.447.

Matys, S., Lederer, F.L., Schönberger, N., Braun, R., Lehmann, F., Flemming, K., Bachmann, S., Curtis, S.B., MacGillivray, R.T.A., Pollmann, K. (2017) 'Phage display - A promising tool for the recovery of valuable metals from primary and secondary resources', *Solid State Phenomena*. doi: 10.4028/www.scientific.net/SSP.262.443.

Braun, R., Bachmann, S., Schönberger, N., Matys, S., Lederer, F.L., Pollmann, K. (2018) 'Peptides as biosorbents – Promising tools for resource recovery', *Research in Microbiology*, 169(10), pp. 649–658. doi: 10.1016/j.resmic.2018.06.001.

Matys, S., Schönberger, N., Lederer, F.L., Pollmann, K. (2020) 'Characterization of specifically metal-binding phage clones for selective recovery of cobalt and nickel', *Journal of Environmental Chemical Engineering*, 8(2), p. 103606. doi: 10.1016/j.jece.2019.103606.

Braun, R., Schönberger, N., Vinke, S., Lederer, F.L., Kalinowski, J., Pollmann, K. (2020) 'Application of Next Generation Sequencing (NGS) in Phage Displayed Peptide Selection to Support the Identification of Arsenic-Binding Motifs', *Viruses*. 12: 1360. https://doi.org/10.3390/v12121360

Contributions to conferences

Schönberger, N., Matys, S., Lederer, F.L., Pollmann, K. (2017) 'Selection of Gallium-Binding Peptides Using Phage Display Technology' Oral presentation at the 6th International Symposium on Biosorption and Biodegradation/Bioremediation 25 – 29 June 2017, Prague, Czech Republic

Schönberger, N., Matys, S., Lederer, F.L., Pollmann, K., Stelter, M. (2017) 'Selection and providing of gallium-binding peptides' Poster presentation at the 22. International Biohydrometallurgy Symposium 24 - 27 September 2017, Freiberg, Germany

Schönberger, N., Matys, S., Lederer, F.L., Pollmann, K. (2018) 'Development of Ga-binding Peptides using Phage Display Technology' Oral poster presentation at the 4th Euro Bio-inspired Materials 2018, 19 - 22 March 2018, Potsdam, Germany

Schönberger, N., Braun, R., Lehmann, F., Flemming, K., Matys, S., Lederer, F.L., Pollmann, K. (2018) 'Peptide based recovery gallium' Poster presentation at the 35. European Peptide Symposium, 26 - 31 August 2018, Dublin, Ireland

Schönberger, N., Braun, R., Lehmann, F., Flemming, K., Matys, S., Lederer, F.L., Pollmann, K. (2018) 'Peptidbasierte Rückgewinnung von Gallium aus Abwässern der Halbleiterindustrie' Poster presentation at the ProcessNet-Jahrestagung und 33. DECHEMA-Jahrestagung der Biotechnologen, 10 - 13 September 2018, Aachen, Germany

Schönberger, N., Braun, R., Matys, S., Lederer, F.L., Pollmann, K. (2019) 'EcoGaIN - Rückgewinnung von Gallium aus Abfällen der Halbleiterindustrie' Oral presentation at the r^4 - Nachwuchsforscherkongress 2019 26 - 27 February 2019, Pforzheim, Germany