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Research paper

Gallium-binding peptides as a tool for the sustainable treatment of industrial waste streams

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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. 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.

1. 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 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 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).

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The implementation of biotechnological approaches in resource technology promises an innovative solution for the efficient and sustainable utilization of such raw materials (Hennebel 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 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 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 et al., 2018; Lederer et al., 2019). In addition to the use of specialized whole cells (Nguyen et al., 2013), certain metabolites like siderophores (Jain et al., 2019) or metallothioneins (Terashima et al., 2002), as well as specific metal binding proteins (Deblonde 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 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 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 et al., 2015), aluminum (Zuo et al., 2005), platinum (Cetinel et al., 2012), molybdenum (Cetinel et al., 2018), nickel and cobalt (Matys et al., 2017, 2020; Braun et al., 2018), gallium (Schönberger et al., 2019a, 2019b) and the rare earth elements neodymium (Sawada et al., 2016) and lanthanum (Lederer 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 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 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 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 et al., 2013; Mazhar 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 et al., 2019a, 2019b) 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.

2. 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 19 × 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 and 290 µM peptide (DGpeptides Co.,Lt; Hangzhou, CN, see Table XX) were used in the corresponding buffer. In addition, the experiments were repeated with arsenic ($NaAsO_2$, 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 PEG_{12} 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 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 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 1b
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 et al., 2004) and energy minimization using CHARMM 43b2 (Brooks 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 et al., 2008) with parameter and topology files generated at each stage. A cubic TIP3 (Jorgensen 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., 2017) force field. For equilibration steps, a harmonic constraint of 24 Kcal/mol/$Å^2$ 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 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 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 et al., 2018) suite. Hierarchical agglomerative clustering was used with clustering stopped when either 5 clusters or $\varepsilon = 4.0$ was reached. Root-Mean Square Deviation (RMSD) from starting structures was calculated and plotted (Supporting Information SI 4). LOcally EStimated Scatterplot (LOESS) smoothing (Cleveland and Devlin, 1988) was applied to plots with a smoothing parameter value, α, of 0.75.

3. Results and discussion

This work follows previous studies (Schönberger et al., 2019a, 2019b) and aims to further characterize the gallium-binding peptides

Table 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

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). 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 2 and Supporting Information SI 1A–J, SI 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 2). 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 2) 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

Table 2
Thermodynamic parameters for the interaction of peptides with gallium in the unbuffered and the acetate buffered system. The dissociation constant K_D 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

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 derivatives 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% and 40% (see Table 3). It is assumed that the loading efficiency is highly time-sensitive since the peptides and the pegylated material are significantly influenced by oxidation.

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 Fig. 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.

Table 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

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 (Fig. 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.

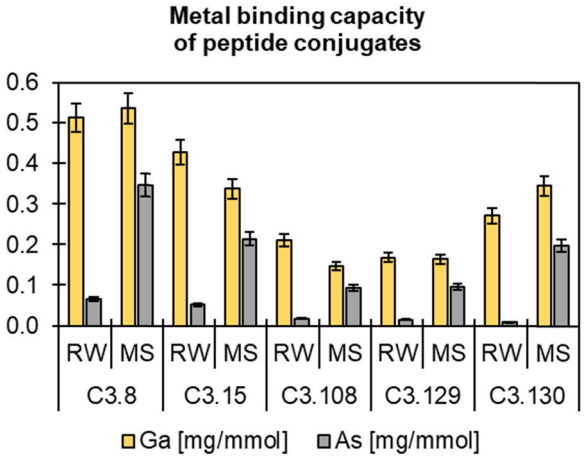

Fig. 1. Metal binding capacity [mg /mmol immobilized peptide] for gallium and arsenic of peptide conjugates obtained from batch experiments in three replicas with real wastewater samples (RW) and model solutions (MS). The presented values are corrected with the reference data from experiments for the 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).

The results for all peptide materials are shown in Fig. 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 Fig. 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.

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

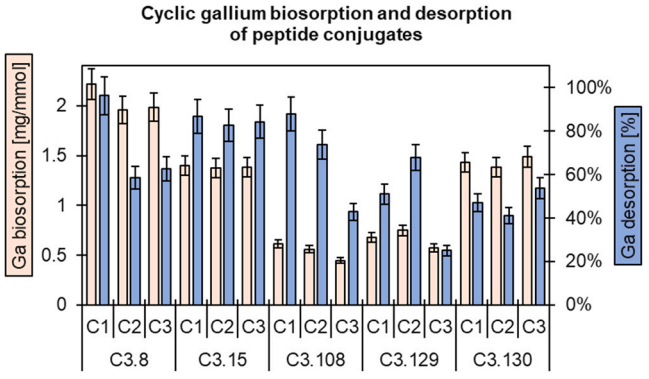

Fig. 2. Gallium-binding capacity [mg Ga/mmol immobilized peptide] and relative gallium desorption [%]. Data were obtained from chromatographic based mini column studies in three subsequent experiments (C1, C2, C3). The presented values are corrected with the reference data from experiments for the biosorption of gallium to pegylated polystyrene beads (0.084 mg/g Ga, 0.097 mg/g As). Citric acid (10%) was used for desorption.

algae-based biosorbents for removal of gallium from semiconductor manufacturing wastewater were developed (Li et al., 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 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 et al., 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) (Fig. 3).

4. Conclusion

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.

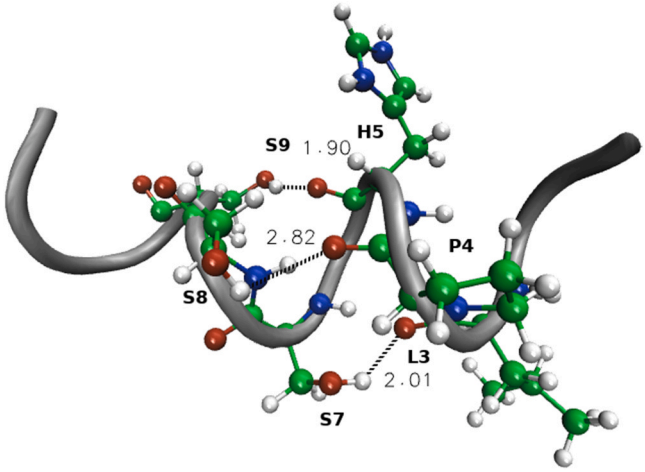

Fig. 3. Proposed structure for the non-immobilized peptide C3.15 (NYLPHQSSSPSR). The peptide center is stabilized by hydrogen bonds between L3 and S7, P4 and S8 and H5 and S9.

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.

CRediT authorship contribution statement

Nora Schönberger: Methodology, investigation, writing - original draft, writing - review & editing. **Corey Taylor:** Investigation (Model calculations), writing - original draft, writing - review & editing. **Martin Schrader:** Methodology (peptide immobilization). **Björn Drobot:** Methodology (ITC), writing - review & editing. **Sabine Matys:** Methodology (biosorption studies), Funding acquisition, writing - review & editing. **Franziska L. Lederer:** Conceptualization, writing - review & editing. **Katrin Pollmann:** Conceptualization, Funding acquisition, Project administration, writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125366.

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