Laser desorption/ionization mediated by bionanostructures from microalgae

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Rationale: Organic matrices are the state-of-the-art ionization mediators in Laser Desorption/Ionization Mass Spectrometry (LDI-MS). Despite improvements in understanding matrix chemistry, interfering matrix-related signals complicate the analysis. Surface-assisted LDI techniques like desorption/ionization on silicon (DIOS) or nanostructure initiator mass spectrometry (NIMS) provide promising alternatives but rely often on elaborate materials.

Methods: We introduce nanopatterned biomineralized cell walls of microalgae as easily accessible biological surfaces that support the ionization of embedded molecules in LDI-MS. Microalgae cell walls were cleaned through oxidation and washing before pipetting on a stainless-steel matrix-assisted laser desorption/ionization (MALDI) target. Added molecules were efficiently ionized in positive and negative ionization mode in common MALDI sources. The method was rigorously validated by comparison with established MALDI experiments.

Results: Ionization of PEG600, D-sphingosine and raffinose was successfully mediated by nanostructured cell wall preparations from two different microalgae. Without any change in protocol, steric acid could be detected in the negative ionization mode. Ionization is also supported by commercially available celite, a material containing mineralized diatom cell walls. Characteristic ingredients of fresh coffee were detected in LDI-MS after pipetting it on celite without further sample preparation. Caffeine and saccharose were detected in positive and characteristic fatty acids in negative ionization mode. Detection limits were comparable to established MALDI experiments.

Conclusions: Bionanostructure-enhanced ionization allows the analysis of a diverse selection of analytes including polymers, sugars, amino alcohols, and organic acids without interfering matrix signals. We also show that celite, a commercially available porous material containing mineralized algal bionanostructures, supports LDI-MS.

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Matrix-assisted laser desorption/ionization (MALDI) plays a central role in the field of polymer and bioorganic analytics. A limitation of this powerful technique is the usage of an adequate matrix to support ionization. For larger molecules there are well-established matrices, like 2,5-dihydroxybenzoic acid (2,5-DHB)[1] or cinnamic acid derivatives,[2,3] with broad performance compared to MALDI.[4–6] In the last years advanced LDI techniques based on surface-assisted processes including desorption/ionization on silicon (DIOS)[5] or nanostructure initiator mass spectrometry (NIMS) were introduced.[8,9] Nanostructured surfaces can support ionization of embedded molecules through a rapid surface heating after laser irradiation, followed by desorption/ionization processes.[10,11] These techniques rely on elaborate synthetic materials[12,13] to assist ionization. We reasoned that processes could be simplified by exploiting nature’s ability to form nanopatterned materials through biomineralization. Some unicellular microalgae with their unique cell wall morphologies might be suitable suppliers for easy accessible nanopatterned material to support ionization processes. Among others, the impressive three-dimensional, nanostructured silica cell wall (frustules) of diatoms that are generated through biomineralization caught our attention. Diatom frustules are mainly composed of hydrated silica (SiO₂) tightly associated with peptides and polyamines[14] forming a species-specific scaffold with pores of diameters down to 40 nm (Fig. 1(E)).[15] But also the robust plate-like structures within the theca of some diatom frustules might exhibit patterns suitable to support LDI. These thecae are cellulosic-based exoskeletons with hundreds of spines that also exhibit nanometer shaped patterns (Fig. 1(C)).[16] Diatom cell walls are readily available, especially due to massive aquaculture activities focused on the generation of biofuels that produce the silicified material as a waste.[17] We also tested the potential of celite as a commercially available material...
containing bionanostructures. This material, also termed kieselguhr or diatomaceous earth, consists of mainly amorphous silicon dioxide from fossilized diatoms with remaining patterned particles (inserts Fig. 5).[18] Here we show that nanostructured biominerals and biopolymers from fresh and fossilized algae are an accessible alternative to the currently used synthetic surfaces. We explore the potential of bionanostructures from the diatom *Thalassiosira pseudonana*, the dinoflagellate *Prorocentrum minimum* and celite for the ionization of embedded molecules.

**EXPERIMENTAL**

**Materials and reagents**

PEG600 (average Mw 600, waxy solid), D-sphingosine and 2,5-dihydroxybenzoic acid (for MALDI-MS) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Raffinose pentahydrate was purchased from Alfa Aesar (Karlsruhe, Germany). ‘Brown’ celite and silica gel 60 were purchased from Fluka (Deisenhofen, Germany). ‘White’ celite and stearic acid were purchased from Applichem (Darmstadt, Germany). Acetonitrile (for HPLC – Super Gradient) was purchased from VWR International (Darmstadt, Germany). Ultrapure water was prepared with a micropure system (TKA, Niederelbert, Germany) from deionized water. All analytes and microalgae shells were dissolved or suspended, in the case of algae shells, in ethanol (gradient grade for liquid chromatography, Merck, Darmstadt, Germany). If not mentioned otherwise 1 mg/mL standard solutions were employed.

**Preparation of microalgae cell walls**

A common method for the preparation of biomineralized microalgal cell walls is the oxidation of organic material with a hypochlorite solution leaving the cleaned biomaterials that can be washed and dried.[19] To obtain pure microalgae cell wall preparations we thus mixed equal volumes (2 mL) of NaClO (12% free chlorine) and microalgal cultures in stationary growth phase that were concentrated by centrifugation to ca. 1/10 of their initial volume. The samples were heated to 75 °C (90 min) and again centrifuged. The remaining pellet was purified in four washing/centrifugation steps with 1.5 mL of acetonitrile and water (50:50; v/v). The resulting clean cell wall preparations were taken up in 300 μL ethanol and could be stored at 4 °C.

**Sample preparation**

Cell wall suspensions (0.7 μL in ethanol, prepared as described above) were pipetted on a stainless steel MALDI target and allowed to dry completely under ambient conditions. Celite (10 mg) was suspended in 300 μL ethanol and used directly. Dissolved analyte molecules in different concentrations were pipetted (0.5 μL) on these pre-treated targets and also allowed to dry under ambient conditions. The widely used commercially available matrix 2,5-dihydroxybenzoic acid (DHB) was used as a positive control. Samples were mixed with the matrix solution (10 mg of 2,5-DHB/mL ethanol) and pipetted on the target spot according to the standard dried-droplet method.[5]

**Mass spectrometry**

Measurements were performed on a MALDI micro MX mass spectrometer (Waters/Micromass, UK) equipped with a nitrogen laser (337 nm, 4 ns laser pulse duration). The pulse frequency was set to 5 Hz. The laser intensity was adjusted between 0 and 500 units, with 500 units corresponding to ca. 140 μJ. The accelerating source potential was set to 12 kV. If not mentioned otherwise, samples were measured in positive ionization in reflectron mode (reflectron voltage
5200 V). Mass data was recorded with the MassLynx software (Waters, UK). The chemical identities of the compounds encountered were confirmed by mass spectrometry on an LTQ ion trap instrument (Thermo Fisher, San Jose, CA, USA) with an AP-MALDI source equipped with a solid-state 355 nm NdYAG UV laser (MassTech, Columbia, MD, USA) and running Target 6 (MassTech) and Excalibur v.2.0 (Thermo) software for data acquisition. The UV laser was fired at 10 Hz and the pdf voltage was set to 35 V. The AP source was set to 255 °C for capillary temperature and 40 V for capillary voltage. Full-scan mass spectra were generated using m/Δm 30 000 resolution measured in positive or negative mode using the Orbitrap analyzer.

RESULTS AND DISCUSSION

Parameter optimization

Method development initially focused on the investigation of the ionization of polyethylene glycol (PEG600) using the two different bionanostructure-containing microalgal preparations. The laser energy was optimized by evaluating signal intensity of the m/z 613.3 ion of PEG600 (1 mg/mL ethanol). The typical ion series of this polymer enabled us to analyze the potential of the algae cell walls for ionization in a mass range from m/z 300 to 1000. Without further optimization we reached the same intensities of PEG600 signals using algal preparations to support ionization compared to the established 2,5-DHB matrix (arbitrary laser energy levels of 300 (p = 0.143) and 350 (p = 0.452) one-way analysis of variance (ANOVA)). A laser setting of 350 was used for further studies. We then tested the effect of different cell wall concentrations on spectral parameters. The introduced method proved to be very robust and high-quality spectra could be recorded if cell wall concentrations corresponding to 400 µg to 80 mg dry weight per mL ethanol were employed. A suspension of 25 mg cell walls per mL ethanol gave highest ion counts. No significant quantitative difference (p = 0.700; t-test) and no difference in standard deviation of measured intensities between T. pseudonana and P. minimum preparations could be observed. We reached full width at half maximum (FWHM) resolution of 1400 ± 400 for T. pseudonana and 1600 ± 300 for P. minimum (PEG600 at m/z 613.3). These results do not differ significantly from those obtained in 2,5-DHB (1300 ± 50 FWHM; p = 0.620; one-way ANOVA) but show an elevated standard deviation. Measurements were generally highly reproducible and comparable results were obtained with several different cell wall preparations from different batch cultures. In general, neither signal intensity nor resolution was negatively affected by bionanostructure-promoted ionization compared to the established matrix (Figs. 1(B), 1(D), and 1(F)). The direct measurement of pure PEG600 (1 mg/mL) without organic matrix or bionanostructures resulted in significantly (p < 0.01 for each comparison, factor 4–5) lower signal intensities.

Since both algal cell walls gave comparable results, further experiments were performed with the easy to obtain cell wall preparations of the fast growing diatom T. pseudonana. To investigate the limit of detection (LOD), different concentrations of PEG600 standard solutions were analyzed. It was possible to achieve comparable signal intensities for the polymer ion [HO\(\text{C}_2\text{H}_4\text{O}_2\)\(\text{H} + \text{Na}\)]⁺ between clean T. pseudonana cell wall preparations and 2,5-DHB if 5 ng of PEG600 was loaded on the target spot. A signal-to-noise ratio of 3:1 for this polymer ion was reached if 0.5 ng of PEG600 on target spot was ionized with clean T. pseudonana cell wall preparations, corresponding to a LOD of ca. 85 fmol (Fig. 2(A), insert). Consequently, we did not achieve the performance of NIMS with a reported detection limit in the attomolar range.[20] Nevertheless, our results are fully comparable to traditional matrices, where detection limits in the femtomolar range can be reached as well. Better instrumentation as well as selection of other diatom species used for the shell preparations could potentially further improve the performance of our method. The mass spectra of cleaned T. pseudonana and P. minimum cell walls clearly show that there are no interfering peaks in the lower mass range (Figs. 1(C) and 1(E)). Minor signals are attributed to minimal organic residues from the cell wall preparation process. In contrast, the mass spectrum of pure 2,5-DHB shows, besides three major matrix ions at m/z 137.0 [M – H2O + H]⁺, 154.0 [M]⁺ and 273.1 [2M – 2H2O + H]⁺, several other signals in the range between m/z 100 and 400 (Fig. 1(A)). The quotient of the analyte signal to the highest interfering signal was 0.3 in ionizations of PEG600 in 2,5-DHB matrix. In contrast, highly favorable values of 4.8 and 2.4 were

Figure 2. Mass spectra of low concentrated PEG600 standard solutions: (A) clean T. pseudonana cell wall preparations with 5 ng PEG600 on target spot. Insert shows mass spectrum of clean T. pseudonana cell wall preparation with 0.5 ng PEG600 on target spot; (B) 2,5-DHB (10 mg/mL) with PEG600 (5 ng on target spot).* signals belong to the potassium adduct m/z-cluster.
reached with identical concentrations of the analyte in ionizations supported by *P. minimum* and *T. pseudonana* cell walls, respectively (Figs. 1(D) and 1(F)).

**Detection of small molecules**

To test if the cell wall preparations are suitable to support ionization of a broad range of analytes, we applied the trisaccharide raffinose (Fig. 3) and the amino alcohol D-sphingosine (Supplementary Fig. S1, Supporting Information) to the cell wall preparations. In comparison with spectra recorded in the presence of the matrix, the application of bionanostructures leads to a significant reduction of background signals and simplifies spectra considerably. This is of special interest for molecules in the lower mass range. Supplementary Fig. S1(A) demonstrates this for D-sphingosine that was ionized in the presence of *T. pseudonana* cell walls. The characteristic peaks of D-sphingosine, m/z 282.3 [M–H2O+H]+, m/z 300.3 [M+H]+ as well as m/z 322.3 for the sodium adduct [M + Na]⁺, are clearly the three major signals in the spectrum. In comparison to the spectrum obtained in the 2,5-DHB matrix, the intensities of the [M–H2O]⁺ ion and the alkali adduct are more intense than the [M+H]⁺ ion (Supplementary Fig. S1, Supporting Information). The mass spectra in Fig. 3 show typical signals for raffinose with the fragment peak at m/z 365.1 that can be explained by the loss of anhydro-glucose and adduct formation with sodium [M–C6H10O5 +Na]⁺ and two additional signals at m/z 527.1 and 543.1 corresponding to the sodium and potassium adducts of raffinose, respectively. The direct comparison of mass spectra obtained with bionanostructures and 2,5-DHB shows a relatively increased intensity of the ion at m/z 365.1, whereas 2,5-DHB leads to the base peak at m/z 527.1 (Fig. 3(D)). The direct application of raffinose on *T. pseudonana* shells resulted in an enhanced ionization by a factor of 3 for the anhydro-glucose fragment compared to the direct measurement of that compound without any supporting matrix. We noticed an increase in ionization efficiency in the presence of diluted artificial growth medium from diatoms (Fig. 3(C)). This went ahead with increased background signals that presumably arise from the growth medium. To evaluate the effects of salts contained in the medium and to be able to work with analytical grade chemicals, we mixed the clean shell preparations with different amounts of seawater medium before they were pipetted on the target spot. The best results were obtained with a supplementation of a 30% medium and 70% ultrapure water mixture (Fig. 4(A)). An 8 times higher intensity for the characteristic fragment ion, compared to the measurement of the pure analyte, could be observed (Figs. 3(A) and 3(C)) and intensities were comparable to those in 2,5-DHB. Similar intense mass spectra of raffinose were recorded when the diluted artificial seawater medium was exchanged with a salt solution consisting of 400 mM NaCl and 30 mM MgSO4. No beneficial effect of different salinity on the ionization intensity of PEG600 could be achieved (Fig. 4(B)). We therefore conclude that additional salt ions can support the ionization of embedded polar analytes, similar to the initiator molecules in NIMS applications.

**Celite as commercial available source for bionanostructures**

Since the preparation of purified diatom cell walls requires some effort we tested if commercially available celite that contains mineralized bionanostructures from diatoms can be used as an alternative.
also support ionization. Figure 5 illustrates the ionization supported by pure silica gel (Fig. 5(A)) and two different commercially available celite preparations, ‘brown’ (Fig. 5(B)) and ‘white’ (Fig. 5(C)), which were suspended in ethanol (10 mg/mL) before application to the target. Under otherwise identical conditions as reported for the microalgal cell wall preparations, both celite products support the ionization of raffinose with results that are comparable to cleaned diatom frustules and traditional measurements in 2,5-DHB matrix. The composition of fossilized diatom frustules and the overall purity differ between the two commercial celites (inserts Figs. 5(B) and 5(C)). This is also reflected in obtained ion intensities and impurities in the mass spectra obtained with these celites as ionization mediators, with the purer white preparation being superior (Figs. 5(B) and 5(C)). We achieved an LOD (S/N ratio 3:1) for the characteristic fragment ion $[M-C_6H_12O_5+Na]^+$ below 990 fmol (0.5 ng) of raffinose on the target spot with white celite powder (Supplementary Fig. S2, Supporting Information). It is remarkable that celite can support the ionization of embedded molecules, despite the fact that it only represents a crude preparation of fossilized samples. Apparently, the presence of the nanopatterned structures in an otherwise amorphous material is sufficient to support ionization. This observation is supported by the comparison of amorphous silica gel, celite and clean *T. pseudonana* frustules as ionization mediators for raffinose. Intensities of analyte-related mass peaks increase with the amount of nanostructured surfaces. With respect to signal intensity and background signals best results can be obtained with clean *T. pseudonana* frustules but

**Figure 4.** Salinity dependent ionization intensity of analyte molecules: (A) raffinose (1 mg/mL) with 2,5-DHB or clean *T. pseudonana* cell walls in solutions of different salinity; (B) PEG600 (1 mg/mL) with 2,5-DHB or clean *T. pseudonana* cell walls in solutions of different salinity.

**Figure 5.** Comparison of desorption/ionization capacity of silica gel and celite: (A) silica gel with raffinose (1 mg/mL); (B) celite (brown) with raffinose (1 mg/mL); (C) celite (white) with raffinose (1 mg/mL). The inserts show electron microscopic pictures of the respective material.
celites represent a reasonable compromise if the effort for cell preparation shall not be undertaken. The observation that the finely powdered amorphous silica gel also slightly increases ionization efficiency compared to preparations on the target without any additional modifier is in accordance with reports on ionization enhancement by nanoparticulate silica.[22]

Bionanostucture supported negative ionization

To obtain further information about the mechanism of ionization, we explored the efficiency of the alga cell wall preparation in negative ionization mode. Therefore, we compared spectra obtained with cleaned microalgal cell walls with those obtained in 1,8-bis-(dimethylamine)naphthalene (DMAN) as a proton sponge matrix that is known to support negative ionization.[23] With stearic acid as analyte a good ionization can be observed for the microalgal cell wall preparation (Fig. 6). In both setups the deprotonated stearic acid at $m/z$ 283.3 ([M–H]$^-$) as well as the trimeric iron adduct at $m/z$ 905.7 ([3M + Fe$^{2+}$–3H]$^-$) can be observed. The bionanostructures thus universally support ionization in positive and negative mode and ionization does not exclusively depend on the transfer of protons by the help of embedded organic initiator molecules as it is observed in NIMS.[8] This notion is also supported by the fact that completely different bionanomaterials like the composite silicate/amine/protein diatom cell walls and the cellulose based P. minimum cell walls result in similar results.

We also tested a broad selection of diatom cell wall preparations from other species which resulted in comparable ionization (data not shown).

Figure 6. Mass spectra (negative ionization) of stearic acid: (A) clean P. minimum cell walls with stearic acid (1 mg/mL); (B) DMAN (10 mg/mL in ethanol) with stearic acid (1 mg/mL).

Figure 7. Mass spectra of direct applied coffee on white celite powder, 2,5-DHB and DMAN: (A) positive ionization of 1:10 (ethanol) diluted coffee supplemented with saccharose on white celite powder; (B) positive ionization of 1:10 (ethanol) diluted coffee supplemented with saccharose mixed with 2,5-DHB (10 mg/mL); (C) negative ionization of pure coffee on white celite powder; (D) negative ionization of pure coffee mixed with DMAN (10 mg/mL). Please note the different scales of the x-axis. All measurements were performed on the Waters Micro MX.
Investigation of coffee as complex sample

As a proof of principle experiment, we added freshly brewed coffee to white celite, as an easily accessible commercially available bionanostructure source. Under otherwise identical conditions as described above it was possible to detect characteristic coffee ingredients with positive and negative ionization from one single preparation (Fig. 7). For positive ionization, we characterized the [M + H]⁺ ion as well as the [M + K]⁺ ion of caffeine with m/z 195 and 233. In addition, we detected the [M–H]⁻ ion of the characteristic acids coumaric acid (m/z 163), caffeic acid (m/z 179), quinic acid (m/z 191) and ferulic acid (m/z 193) after changing to the negative ionization mode. The identity of the detected metabolites was supported by exact mass determination on an Orbitrap XL mass spectrometer coupled to an AP-MALDI source (Supplementary Table S1, Supporting Information). It was also possible to detect the [M + Na]⁺ ion (m/z 365.1) and the [M + K]⁺ (m/z 381.1) ion of saccharose after adding a sugar cube to the coffee (15 mg saccharose/1 mL coffee) before the application on celite. This prove of principle experiment demonstrates the validity of the introduced methodology but results might still be improved using the purified cell wall preparations instead of commercially available celite. To compare with established matrix techniques two preparations (in 2,5-DHB and DMAN) had to be prepared. In positive mode sugars and caffeine could be detected in similar intensities as H⁺ and Na⁺ (celite) and Na⁺ and K⁺ (2,5-DHB) adducts. An additional intensive saccharose fragment was detected in the matrix sample. In negative ionization mode signal intensities of caffeic acids were higher in the celite preparations compared to the matrix. These results show that bionanostructures can support the ionization of metabolites out of a complex sample using one preparation for positive and negative ionization mode and therefore could further benefit the progress in method development in the field of bioorganic analytics.

CONCLUSIONS

In conclusion, we successfully demonstrated the capability of bionanostructured surfaces derived from microalgae cell walls or fossilized celite to support ionization in LDI-MS. Best results can be obtained with purified diatom cell walls. Dependent on the analyte, addition of salt from the growth medium can increase signal intensity. Since diatom culturing and cell wall preparation is not a standard technique in analytical labs we also tested the efficiency of commercially available celite. The material contains mineralized diatom cell walls and can effectively support ionization as well. However, usage of the partially amorphous mineral preparations increases the background and reduces sensitivity compared to the optimized cell wall preparations. We thus present a natural, readily available and robust alternative to elaborate synthetic materials, such as semiconductor films, silicon substrates coated with Pt nanoparticles, or nanostructured carbon coatings used in the surface-assisted LDI-MS.[b] Resolution and sensitivity were competitive with established and well-developed matrices and a broad selection of analytes including polymers, sugars, amino alcohols, and fatty acids could be addressed in positive and negative ionization mode without further adjustments.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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