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HCN emission by a Polydesmid Millipede Detected Remotely by Reactive Adsorption on Gold Nanoparticles Followed by Laser Desorption/Ionization Mass Spectrometry (LDI-MS)

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Abstract

Hydrocyanic acid (HCN) is a well-known defensive allomone in the chemical arsenal of millipedes in the order Polydesmida. The presence of HCN in the headspace vapor of adult *Xystocheir dissecta* (Wood, 1867), a common millipede from the San Francisco Bay Area, was traced by laser desorption/ionization-mass spectrometry (LDI-MS). To accomplish this, the headspace vapor surrounding caged, live millipedes was allowed to diffuse passively over gold-nanoparticle (AuNP) deposits placed at various distances from the emitting source. The stainless steel plates with AuNP deposits were removed and irradiated by a 355-nm laser. The gaseous ions generated in this way were detected by time-of-flight mass spectrometry. The intensity of the mass spectrometric peak detected at *m/z* 249 for the $Au(CN)_2^-$ complex anion was compared to that of the residual Au^- signal (*m/z* 197). Using this procedure, HCN vapors produced by the live millipedes could be detected up to 50 cm away from the source. Furthermore, the addition of H_2O_2, as an internal oxygen source for the gold cyanidation reaction that takes place in the AuNP deposits, significantly increased the detection sensitivity. Using the modified H_2O_2 addition procedure, HCN could now be detected at 80 cm from the source. Moreover, we found a decreasing intensity ratio of the $Au(CN)_2^-/Au^-$ signals as the distance from the emitting source increased, following an exponential-decay distribution as predicted by Fick's law of diffusion.

Keywords Millipedes · Allomones · Hydrogen cyanide (HCN) · Gold nanoparticles (AuNP) · Laser desorption/ionization (LDI) · Hydrogen peroxide (H_2O_2) · Polydesmida

Introduction

Several groups of arthropods are known to release hydrocyanic acid for self-defense. The group best known for specializing in this type of chemical defense is the Polydesmida order of millipedes. They are detrivores (consumers of dead plant materials) that live underground and in shaded and humid habitats, such as under stones, fallen leaves and bark. Polydesmids are nocturnally active, and in central and northern California they breed during the rainy season. Generally, they have few predators, but some specialists have adapted to tolerate the millipedes' chemical defense. For example, ground beetles in the genus *Promecognathus* Chaudoir, 1846 can tolerate cyanide and preferentially prey upon *Xystocheir dissecta* (Wood, 1867) millipedes [https://www.kqed.org/science/1939811/this-millipede-and-beetle-have-a-toxic-relationship]. If left unmolested, polydesmids are gentle and peaceful creatures (Supplementary Material Fig. S1). However, their secretions can cause substantial skin damage to humans and on occasion even kill small birds and mammals that would prey on them (Hendrickson 2005). The production of HCN has been found to be due to the enzymatic degradation of cyanogenic organic molecules such as mandelonitrile (Eisner et al. 1975, 1978; Zagrobelny et al. 2008; Lechtenberg 2011). This process, called cyanogenesis, has been reported from many polydesmid millipedes (Guldensteeden-Egeling 1882; Schildknecht et al. 1968; Shear 2015; Zagrobelny et al. 2018; Blum and Woodring 1962; Duffey et al. 1977). One method employed for HCN detection involved the exposure of the body fluid squeezed from the

Dedication: This work is respectfully dedicated to the memory of Professor Jerrold Meinwald.

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millipedes onto filter paper strips infused with a benzidine acetate-copper acetate reagent (Eisner et al. 1967, 1975). The HCN precursor, mandelonitrile, which enzymatically dissociates into HCN and benzaldehyde, would turn the test paper blue. Other approaches for HCN detection used EPA methods such as gas chromatography- or liquid chromatography-mass spectrometry (GC- or LC-MS) procedures to show the presence of mandelonitrile or mandelonitrile benzoate in defensive secretions (Conner et al. 1977; Kuwahara et al. 2011). However, none of these methods are very practical for detecting the release of HCN directly from live plants or animals. Generally, highly skilled manipulations are required to obtain a sample of the defensive secretion from a source such as millipedes.

Many analytical methods, including electrospray-ionization mass spectrometry (Minakata et al. 2011), are available for the detection of HCN and cyanides (Ma and Dasgupta 2010). Recently, we described a technique for detecting HCN emanations from aqueous NaCN solutions, and plant tissues such as radish and cassava slices (Pavlov and Attygalle 2019), by adopting the classic cyanidation reaction for the extraction of gold from gold-bearing soils and rocks as a derivatization method for mass spectrometry. Briefly, colloidal gold (aqueous dispersions of gold nanoparticles) deposits were used to scavenge HCN vapors from air. The gold-cyanide complex formed either by direct or indirect contact with airborne HCN was shown to be highly amenable to laser ionization and subsequent detection by laser desorption/ionization mass spectrometry (LDI-MS). Herein, we extend the gold-nanoparticle LDI-MS method to trace HCN from defensive secretions of arthropods. We were interested in developing a remote detection procedure to monitor HCN emanating from artificial or animal sources. In addition, we wanted to obtain an estimate of the amount of HCN released by agitated polydesmids using the Californian *X. dissecta* millipedes as a model organism (Causey 1969). Another question we addressed was whether adding an internal oxygen source (such as H_2O_2) to the AuNP dispersion, instead of relying on ambient oxygen, would improve the sensitivity of the method and the range of detection of HCN.

Materials and Methods

Collection of millipedes Adult *Xystocheir dissecta* millipedes were collected from February to April in Californian oak woodland habitats by members of the Will laboratory at UC Berkeley (ESPM). Specimens were collected at the UC Forestry Russell Research Station and Save Mt. Diablo property at Curry Canyon Ranch, both in Contra Costa County in the San Francisco East Bay region of California. Millipedes were placed in plastic vials with moist leaf litter and soil from their habitats and sent to the laboratory in Hoboken, NJ immediately.

Chemicals Millipore deionized water (18.2 MΩ) was used in all experiments. Tetrachloroauric(III) acid trihydrate ($HAuCl_4 \cdot 3H_2O$, ACS reagent) was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA, USA). Sodium cyanide (99.0%) and sodium citrate (tribasic) dihydrate (ACS reagent) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2, 30%, Certified ACS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). "Museum putty" was purchased from Walmart (Secaucus, NJ).

Gold nanoparticles were prepared following the citrate reduction procedure described by Bastús et al. 2011) Briefly, an aqueous sodium citrate solution (2.2 mM, 15 mL) was heated while stirring until it boiled, when a solution of $HAuCl_4$ (25 mM, 100 μL) was added. A purple-colored AuNP solution (about 200 μM or 3×10^{12} ~10 nm size AuNPs per mL) was formed within 15 min, which was used for all experiments without further modification.

HCN detection experimental set-up A cylindrical cardboard tube (1.0 m x 7 cm) was capped at the ends. A rectangular window (7.0 cm x 6.0 cm) was cut at the top surface of one end to facilitate inserting millipedes or control samples (see Supplementary Material Fig. S2). Three large rectangular windows (7.0 cm x 6.0 cm) were cut in the tube to place the MALDI plates (Fig. 1). At 10 cm from one of the endcaps, a nylon mesh (1 mm x 2 mm perforations) was attached perpendicularly to the axis of the cylinder to form an enclosure for the millipedes to roam undisturbed. In the remaining volume of the tube, eight 96-spot steel MALDI plates (4.0 × 5.5 cm) were laid out horizontally along the tube, each spaced 10 cm from the next (Fig. 1). The plates were secured to the wall of the tube with "museum putty," a commercially available adhesive material commonly used to secure items on a flat surface preventing them from shifting. The plate nearest to the sample enclosure was placed in such a manner that the first column of sample spots lay on a line 16.5 cm from the marked geometric center of the wall of the enclosure. Three circular spots on column 1 of each sample plate were charged with 2 μL AuNP and 2 μL H_2O aliquots (spots A1, B1, C1), and three more spots on the same sample column (F1, G1, H1) - with 2 μL AuNP and 2 μL 5% H_2O_2 aliquots (Supplementary Material Fig. S3). The groups of spots charged with H_2O and H_2O_2 on each plate were symmetrical against the central plane of the cylinder. Seven plates were spotted with the same amount of AuNPs + H_2O or AuNPs and H_2O_2 before they were exposed to test vapors. The tube was tightly wrapped with transparent cellophane foil and sealed with adhesive tape, leaving only the opening for the arthropods and other samples exposed.

Initially, a test was performed by placing a plastic foil spotted with a 100-μL aliquot of a 5% aqueous NaCN solution at the center of the sample chamber (Fig. 1). The sample enclosure was immediately sealed and the whole set-up was allowed to stand at room temperature (25 ºC). When the test spots with deposited AuNPs had dried (about 30 min), the MALDI plates were taken out and analyzed by LDI-MS. Analogously, a control experiment was performed in a similar way as described above but with the plates exposed to volatiles emanating from dry leaf-litter and twigs without living millipedes. All determinations were repeated three times.

For trapping HCN released by millipedes, three adult specimens were placed into the sample enclosure and agitated by gently pinching with tweezers to stimulate them to release defensive secretions. The enclosure was immediately sealed, and the set-up was allowed to stand at room temperature (25 ºC) for about 30 min. Subsequently, the millipedes were removed, and the MALDI plates were analyzed by LDI-MS.

MS analysis LDI-MS experiments were conducted on a SYNAPT G2 MALDI-ToF mass spectrometer (Waters, Milford, MA, USA) equipped with a solid-state 355-nm Nd:YAG laser (pulse repetition frequency 1000 Hz). Data were recorded in the negative-ion-generating sensitivity mode over a range of *m/z* 50 to 1000 amu using the MassLynx 4.1 software (Waters Corp., Milford, MA, USA). Red phosphorus was used to calibrate the mass axis of the instrument (Pavlov and Attygalle 2012; Sládková et al. 2009). The energy of the laser beam was adjusted for optimum ion yield (typical setting 500 arbitrary units). The LDI source was operated with the sample plate at 10 V and extraction voltage at 10 V. For each sample, full-scale MS^1 spectra were acquired by moving the target plate on a pre-programmed spiral scan path (with a scan duration of 0.5 s and an overall cycle time of 0.524 s) from one sample location to the next. Data were acquired over a period of 3 min.

Results and Discussion

Using LDI-MS technique (Pavlov and Attygalle 2019), we demonstrated the presence of traces of HCN in the headspace volatiles of aqueous cyanide solutions and plant tissues such as apple seeds and peach pips, by placing drops of aqueous suspensions of gold-nanoparticles in the proximity of the HCN-emitting source. In the current study, the experimental set-up was modified for remote detection of HCN emanating from *X. dissecta* millipedes. Initially, to establish that HCN from a point source is dispersed linearly over several tens of centimeters, we used a cylindrical tube (Fig. 1 and Supplementary Material Fig. S2). One end of the tube housed the HCN source, while the remaining portion of the tube, where the MALDI plates with AuNPs were longitudinally positioned, served as the HCN dispersion chamber (Fig. 1). The negative-ion LDI mass spectra recorded from the AuNP deposits exposed to vapors emanating from leaf-litter control samples showed peaks at *m/z* 197, 394 and 591 for Au_1-, Au_2-., and Au_3- ions, respectively (Supplementary Material Fig. S4). In other words, no detectable chemical changes to the AuNPs were noted due to exposure to control vapors emanating from the leaf litter. On the other hand, when AuNPs were exposed to HCN vapor emanating from a NaCN sample, an additional intense peak at *m/z* 249 for $Au(CN)_2^-$ ions was observed in the spectra (Supplementary Material Fig. S5). The conversion of elemental gold to $Au(CN)_2^-$ by cyanide is attributed to the well-known Elsner reaction (Eq. 1)

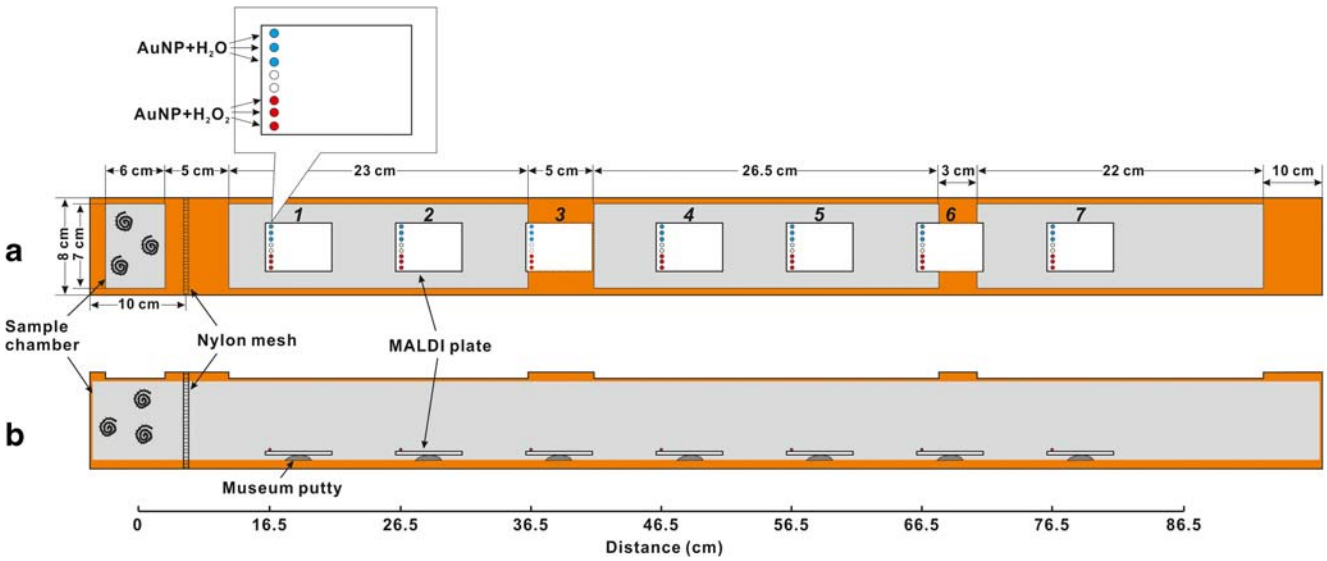

Fig. 1 Top (**a**), and cross-sectional view (**b**) of the set-up used for HCN collection. As shown in the expanded inset, three spots on each plate were charged with AuNP and H_2O (blue spots), and another three spots with AuNP and 2 μL of aqueous H_2O_2 (red spots)

(Yannopoulos 1991). Thus, the presence of a peak at *m/z* 249 can be used as a diagnostic maker to demonstrate that the cyanidation reaction has taken place (Supplementary Material Fig. S5).

$$4Au + 8CN^- + O_2 + 2H_2O \rightarrow 4Au(CN)_2^- + 4OH^- \quad (1)$$

The use of the intensity ratio of $Au(CN)_2^-$ to Au^- peaks as an indirect marker to evaluate the proximity to the HCN emanating source has been demonstrated previously by Pavlov and Attygalle 2019) Using this peak-ratio method, we have previously shown that HCN diffuses through air from the point of origin following an exponential-decrease trend predicted by Fick's law of diffusion (Fick 1855). However, our previous experiments involved only detection of short range diffusion (a few centimeters) within the area of a MALDI plate.

Using the cardboard tube to passively expose the volatiles to the AuNP deposits, we were able to demonstrate that the diffusion of HCN even over larger distances still followed the exponential-decrease model (Fig. 2). It is known that dry NaCN is odorless; but the reaction of NaCN with water produces a bitter almond odor that can be detected even by humans at a 1–5 ppm level (https://viceprovost.tufts.edu/ehs/files/Cyanides-SOP.pdf). Our experimental procedure detected HCN in this concentration level at a distance of about 40 cm away from the source (Fig. 2a). Although, our experiments were conducted in a closed chamber under highly controlled set of conditions, similar results can be expected under open-air environments if significant air currents can be avoided and passive diffusion can be achieved.

We hypothesized the detectability of HCN could be improved if we were to provide the oxygen required for Eq. 1 directly, without depending on atmospheric oxygen. We identified H_2O_2 as a potential oxygen source. Subsequently, we mixed H_2O_2 with aqueous AuNP drops and tested its efficacy as a homogeneous internal oxygen source that could promote the oxidation of Ag° to Ag^+. After AuNP spots made with or without admixing H_2O_2 were simultaneously exposed to a cyanide source, the intensity ratio of $Au(CN)_2^-$ to Au^- peaks in the mass spectra showed approximately a fivefold increase in sensitivity in those prepared by admixing H_2O_2 (Fig. S5). Each data point in the curves presented in Fig. 2 represents the average from three replicate determinations. Overall standard deviations were low, which indicated good reproducibility and low day-to-day variations (Supplementary Material Table S1 and S2).

Finally, we tested the improved preparation method using live millipedes as the HCN sample source (Supplementary Material Fig. S6). The addition of H_2O_2 resulted in tenfold increase in detection of HCN. The presence of millipedes releasing HCN could now be detected at distances of over 80 cm (Fig. 2b). Moreover, it was evident that three specimens of *X. dissecta* millipedes release about 20 times the amount of HCN as released by the aqueous NaCN solution. The bitter almond odor of NaCN can be detected even by humans at a 1–5 ppm level (https://viceprovost.tufts.edu/ehs/files/Cyanides-SOP.pdf). Our data showed that the HCN released by just three millipedes is about 20–50 ppm. This a significant concentration because inhalation exposure to 18–36 ppm levels for several hours can cause weakness, headache, confusion, and many other symptoms to humans.

As a notable aside, we observed from visual inspection of the sampling spots that H_2O_2 admixed with the AuNPs results in the additional benefit of causing the suspended material to be deposited much more uniformly, resulting in a more

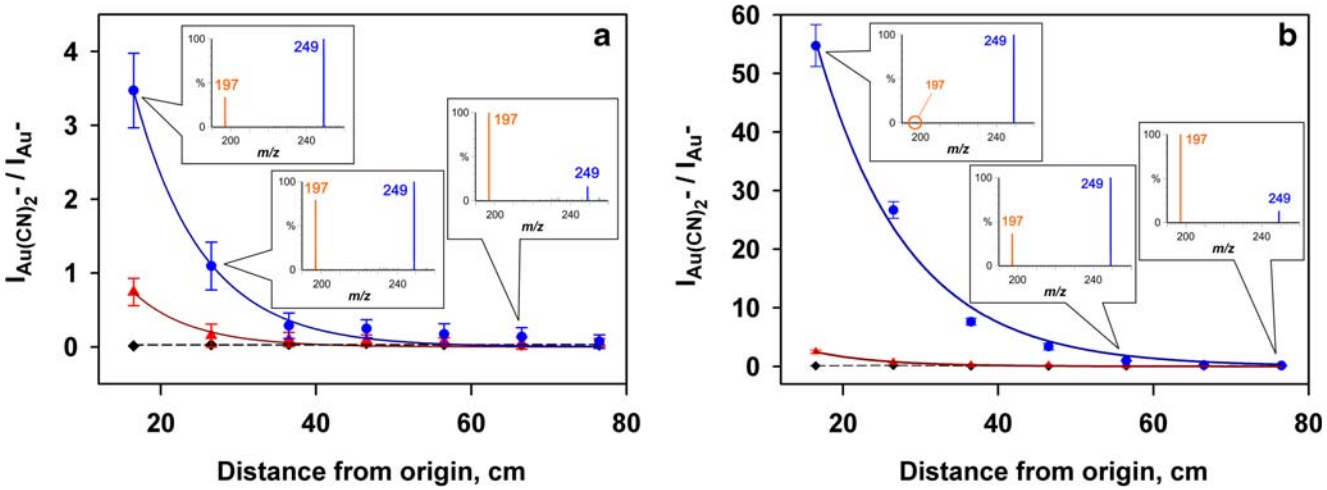

Fig. 2 Plots depicting the intensity ratios of the signals *m/z* 249, *m/z* 197 for $[Au(CN)_2]^-$, and Au^-, respectively, acquired from dry deposits of AuNPs (2.0 μL) placed at different distances from the geometric center of the sample-source cage. To each AuNP spot, either 2.0 μL of water (**a**) or 2.0 μL of 3% H_2O_2 (**b**) was added prior to exposure to the emanations from a blank (♦) ($N = 3$), 100 μL 5% NaCN solution (▲) ($N = 3$; $R^2 = 0.9479$ without H_2O_2; $R^2 = 0.9928$ with H_2O_2), or three agitated millipedes (●) ($N = 3$; $R^2 = 0.9932$ without H_2O_2; $R^2 = 0.9930$ with H_2O_2)

homogeneous sample spot. The homogeneity of a spotted deposit is, however, essential to obtain more reproducible spectra from acquisition to acquisition when the plate is moved to gain optimal signal intensities during mass-spectral recording.

Conclusions

An array of methods are available for the detection of HCN and cyanides (Ma and Dasgupta 2010; Randviir and Banks 2015). The novel experimental procedure described herein - using AuNP admixed with aqueous H_2O_2 - provides a way of detecting trace levels of HCN produced by living animals. The method can easily be adopted to reveal cryptic sources of HCN. In other words, AuNP laden strips could be used for field sampling. Once dried, the strips can be sent to a laboratory with LDI-MS facilities for analysis.

Millipedes are known to produce a variety of chemical repellents to defend themselves against predators (Jones et al. 2018; Kuwahara et al. 2002, 2011, 2015, 2017a, b, 2018). However, a cyanogenic defense system is found only in some millipedes. Thus, the presence or absence of a cyanogenic defense system is considered a phylogenetic marker (Shear et al. 2007). The described detection method should be useful not only to detect the presence of cryptic millipedes but also to score HCN presence as a character for phylogenetic investigations.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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