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Short Communication

In situ surface-enhanced Raman scattering detection of biomolecules in the deep ocean

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ABSTRACT

In this study, we successfully developed a new type of surface-enhanced Raman scattering insertion probe (RiP-SERS) applicable to the deep sea using previously prepared Coccinella septempunctata-shaped SERS substrate, and successfully obtained the Raman spectrum of biomolecules in deep-sea cold seep vents. The experiment proved that the SERS substrate was not remarkably affected by the change in depth (pressure) in the deep sea; therefore, it had excellent pressure resistance. More importantly, Raman peaks of various biomolecules, including acetyl-CoA, β-carotene, and four amino acids, were successfully collected from the microbial communities at the seawater-sediment interface of cold seep vents. The successful application of SERS technology to deep-sea in situ biomolecule detection has added a new method for deep-sea biomolecule detection in the future. Meanwhile, the SERS substrate can withstand a complex deep-sea cold-seep environment (pressure, salinity, pH, and metal-salt presence), which also means that it can be applied to the detection of macromolecules in complex industrial systems.

1. Introduction

The deep-sea cold seep ecosystem is an environment without sunlight, and with high pressure and low temperature. This discovery was the first indication that chemosynthesis could replace photosynthesis as the primary productive force in the origin and development of ecosystems [1]. A natural interface is present between seawater and sediment, in which a large microbial community and rich organic matter are scattered [2]. Microbes play a key role in chemosynthesis, particularly in the anaerobic oxidation of methane by archaea, which can convert methane into nutrients such as acetic acid. Deep-sea organisms, especially microorganisms, are highly sensitive to changes in environmental factors, such as oxygen content and pressure changes [3], resulting in many inaccurate images in traditional sampling experiments. The particularity of deep-sea microbial living environments makes it almost impossible to achieve high-simulation culture in the laboratory [4]. Therefore, there is an urgent need to develop exploration technologies that can fully perform the in-situ study of deep-sea organisms.

Researchers have made attempts to achieve *in-situ* research and exploration of deep-sea organisms [5]. In 2007, the Monterey Bay Aquarium Research Institute developed a 1000 m-class environmental sample processor to conduct *in-situ* microarray detection of marine microorganisms and toxic substances [6]. The *in-situ* deep-sea microbial nucleic acid extraction device independently developed by Wang et al. obtained the continuous period of microbial nucleic acid in the sea test in 2019, revealing the structure of the deep-sea microbial community at 1000 m[4]. These studies have greatly promoted the development of deep-sea *in-situ* exploration technology. However, due to the lack of relevant technical methods, *in-situ* detection of chemical constituents at the seawater-sediment interface is rarely reported.

Laser Raman spectroscopy is a nondestructive, noncontact, and rapid detection technology that is widely used in the *in-situ* detection in deep-sea extreme environments [7,8]. However, the high detection limit and low sensitivity of Raman spectroscopy limits the possibility of detecting

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low concentrations of biomolecules in these environments [9]. SERS can increase millions of times the Raman signal intensity of molecules adsorbed on a rough metal surface [10,11]. Therefore, the in situ deep-sea Raman system can be combined with SERS technology to achieve deep-sea *in-situ* biomolecular detection.

In this study, we designed a new type of RiP-SERS and adjusted the SERS substrate at the Raman laser-focusing position (the distance was 3 ± 0.5 mm). At the cold seep vents of the *Formosa Ridge* in the South China Sea, we used ROV *Faxian* equipped with RiP-SERS system to successfully achieve the pressure resistance test for the SERS substrate. More importantly, we successfully used RiP-SERS to obtain Raman spectral data of bioinformation molecules in the seawater-sediment interface of *Haima* cold seep vents, and found that the marine-biome interface was rich in acetyl-CoA, β-carotene, and four amino acids. This is also the first study to obtain the spectral data of biomolecules *in situ* in deep sea using SERS technology.

2. RiP-SERS design and deployment

2.1. The design of RiP-SERS

2.1.1. Preparation and modification of the SERS substrate

The SERS substrate was prepared using the method reported in our previous study [12]. In brief, a layer of a silver nanofilm was plated on a quartz sheet, and the SERS substrate was obtained by a one-step calcination method. The heating rate for the annealing process was 10 °C/s. The substrate was heated up to 420 °C and held for 10 min in air before cooling to 20 °C. Then, 10^{-5} M 4-mercaptopyridine in ethanol (4-Mpy) was configured as a probe molecule, and the SERS substrate was soaked for 2 h and dried naturally to obtain the SERS substrate modified with 4-Mpy (M-SERSs).

2.1.2. Sea-going RiP system

The RiP system consists of four parts: a main titanium pressure housing, RiP-SERS, hydraulic filtration system, and oil-filled optical fiber. A complete Raman spectroscopic analysis system was installed in the main titanium pressure housing, which can resist the water pressure to a depth of 6000 m [13] The RiP-SERS specific structure was described in Section 2.1.3. The oil-filled optical fiber was used to transmit the excitation light and Raman signal to the main titanium-pressure housing with RiP-SERS.

The RiP system contained a custom-designed NRXNE-532-RA-SP spectrometer and a frequency-doubled Nd:YAG laser (532 nm) with an output power of 150 mW, both manufactured by Kaiser Optical Systems, Inc. (USA). An ADU-440A-BV-136 charge-coupled device (CCD) camera (Andor Technology, UK) with 2048 × 512 pixels, and a 27.6 × 6.9 mm image area was installed in the RiP system. The spectral range (100–4325 cm^{-1}) was split into two regions (100–2100 and 2100–4325 cm^{-1}) on the surface of the CCD; therefore, the spectral resolution of the RiP system was approximately 1 cm^{-1}. Before each deployment, the wavenumber and intensity calibrations of the RiP system were processed using neon and halogen lamps.

2.1.3. Design and preparation of RiP-SERS

The conceptual design of the new RiP-SERS system for *in-situ* SERS detection is shown in Fig. 1. The ROV *Faxian* is an underwater vehicle equipped with a RiP system for *in-situ* Raman exploration in the deep ocean, as shown in Fig. 1. The right side of the panel shows the structural

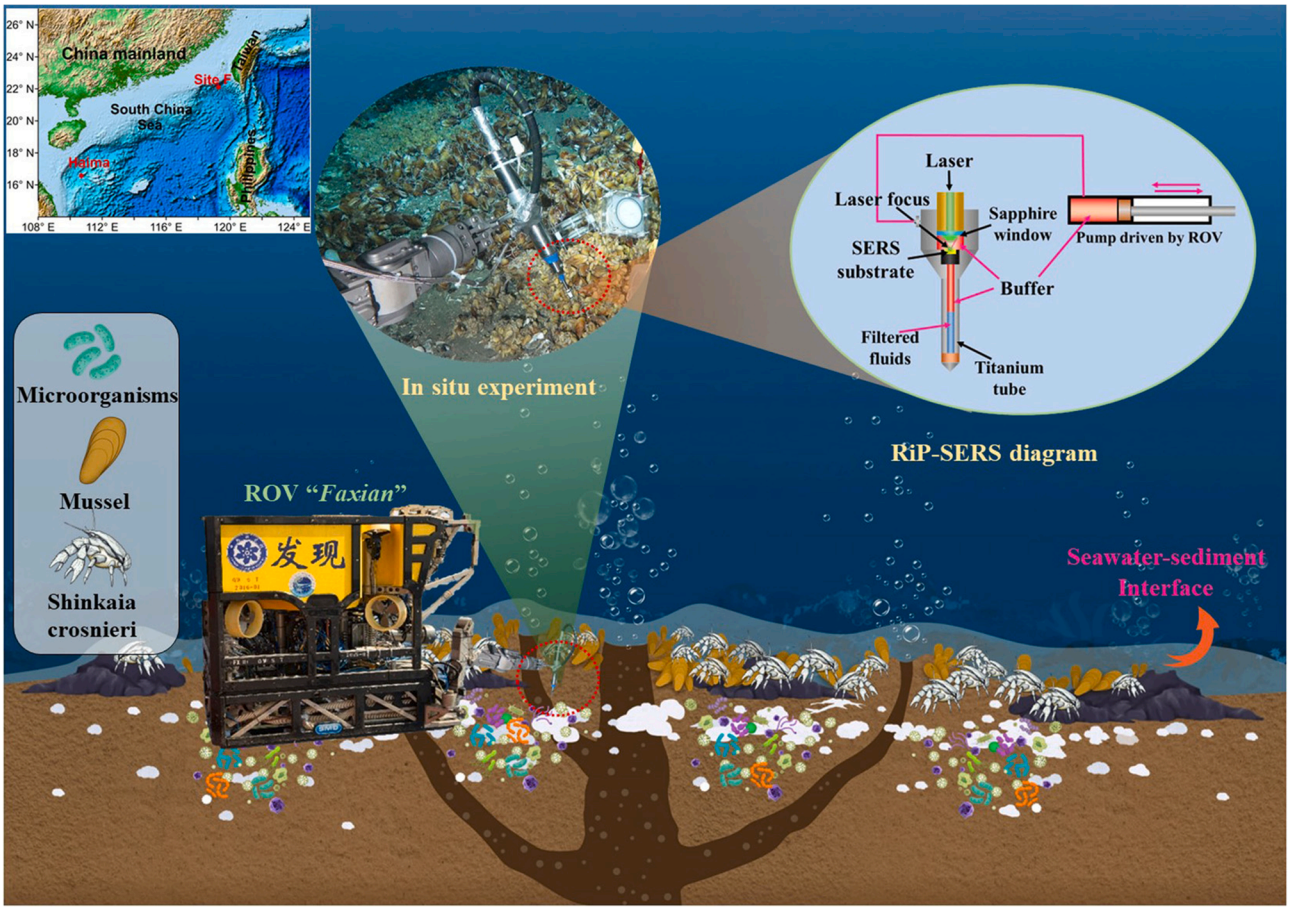

Fig. 1. Conceptual diagram of the in-situ SERS detection of the microbial community in deep-sea cold seep vents: Conceptual design of RiP-SERS on the right side of the panel; photo of in situ detection of RiP-SERS on left side of panel.

diagram of RiP-SERS, which contains a large cavity structure that is used for the adsorption of the SERS substrate and Raman laser sampling. Then, a new type of limiter was designed. The limiter is precise-machined and contains four 1-mm diameter water holes to facilitate the injection of the solution and cold seep fluid into the cavity. A groove was designed at the top of the limiter to place a fixed SERS substrate. M-SERSs were fixed in the groove, the focusing distance of the Raman laser was 3 ± 0.5 mm, and the limiter limit point ensured that the SERS substrate was located at the focal point of the Raman spot. Before ROV *Faxian* diving, the hydraulic pump was filled with a 10^{-5} M 4-Mpy solution. During the deep diving process, the ROV *Faxian* hydraulic drive was used to continuously inject 4-Mpy solution into the cavity of the RiP-SERS to avoid the pollution of the M-SERSs caused by seawater immersion. Deep-sea field fluid acquisition and in-situ SERS detection were also ROV-driven and the protective fluid was replaced with deionized water.

2.1.4. Raman spectra processing

HoloGRAM (Kaiser Optical Systems, Inc., Ann Arbor, MI, USA) and GRAM/Al (Thermo Fisher Scientific, Inc., Waltham, MA, USA) software were used to collect and process Raman spectra in the field exploration and simulation experiments, respectively. The exposure time was set to 4 s, and each spectrum was accumulated eight times. The Raman spectra of the samples in the simulation experiment were continuously acquired three times under the same temperature and pressure conditions. Baseline corrections were performed before processing each spectrum. The peak position, peak area, and full width at half maximum were determined using the peak fitting routine of the GRAM/Al.

2.2. The deployment of RiP-SERS

The RiP-SERS detector was subjected to two successful sea trials. On May 20, 2022, the deep-sea pressure test of the SERS substrate was conducted in the *Formosa Ridge* of the South China Sea (depth of 1180 m, temperature of 2–3 °C), and on July 8, 2022, the in-situ SERS detection was conducted in the cold seep vents of *Haima* in the South China Sea (depth of 1380 m, temperature of 2–3 °C). The RiP-SERS placement is shown in Fig. 1. The ROV *Faxian* drove the manipulator and operated the RiP-SERS to pump water at the marine-sediment interface of the microbial community. The RiP-SERS waited for 5 min to obtain the Raman spectra. The upper-left corner of Fig. 1 was an actual working photo of the *Haima* cold seep vents.

2.3. Materials

All reagents were purchased from Sigma-Aldrich. All reagents were of analytical grade and were used without further purification. Water used in all experiments was purified on a Millipore Q system. The configured solutions are aqueous unless otherwise stated.

3. Result and discussion

3.1. In-situ pressure SERS test in deep sea

As shown in Fig. 2a, when the ROV *Faxian* dives to 400 m, the characteristic peak of SO_4^{2-} (984 cm^{-1}) in seawater begins to appear [9]. Due to the low water pressure at the start of ROV dives, the protective fluid counteracts the seawater immersion under the action of gravity and pressure pump. However, when ROV dives below 400 m, water pressure increases and seawater was continuously immersed, resulting in the appearance of SO_4^{2-} characteristic peak. However, due to the supplementation of the protective solution, the characteristic SO_4^{2-} peak did not increase significantly, while the characteristic peak of 4-Mpy was retained. The experiment proved that the continuous replenishment of the 4-Mpy solution effectively prevented seawater erosion and pollution. Fig. 2b shows the dot-plot of the ratio between the peak intensity of the 4-Mpy characteristic peak (1052 cm^{-1}) [14] and the peak intensity of water as the depth changes. The results showed that the I_{Mpy}/I_{H2O} value was stable between 0.1 and 0.14, which confirmed that the SERS substrate had excellent pressure resistance and provided a theoretical basis for performing *in-situ* SERS detection in the deep sea.

3.2. SERS detection of deep-sea cold seep vents biome

In order to further confirm the feasibility of deep-sea in-situ SERS detection, ROV *Faxian* loaded RiP-SERS were used to extract solutions (1380 m depth) from the biomes in the *Haima* cold seep vents in the South China Sea, and the Raman acquisition spectrum was started after standing for 5 min. As shown in Fig. 3a, there was only the characteristic peak of sulfate (984 cm^{-1}) on the Raman spectrogram without SERS substrate. However, in the Raman spectrogram carrying SERS substrate, abundant characteristic peaks of biomolecular information appeared in the range of 600–1800 cm^{-1}. According to the obtained Raman spectra, in addition to sulfate at 984 cm^{-1}, there are also C-S, COO^-, C-N, P-O-C, and NH_3^+ etc. related vibration peaks (Fig. 3b and Table 1), which are can target biomolecules containing carboxyl, amino, phosphoric acid and other functional groups, such as amino acids and biological enzymes. Therefore, based on the analysis and detection of mussel epithelial cells by biologists [15], 10 most likely biomolecules (pyruvate, lactic acid, beta-carotene, acetyl-CoA, acetoacetic acid, proline, leucine, methionine, histidine and threonine) were selected as controls. The same SERS substrate as the deep-sea in-situ experiment was used to

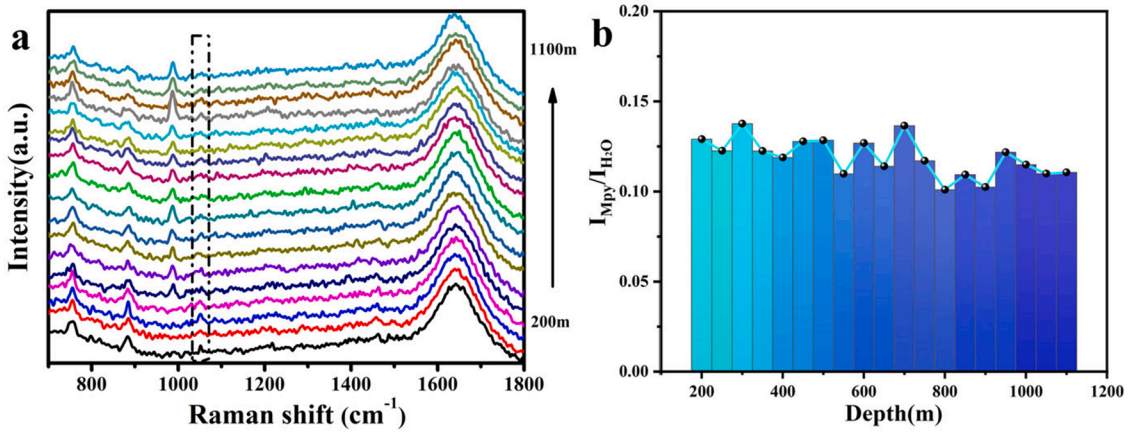

Fig. 2. (a) Raman spectra of M-SERSs versus the change of ROV diving depth; (b) Point plot of the ratio between the peak intensity of 4-Mpy characteristic peak (1052 cm^{-1}) and water peak intensity with depth change.

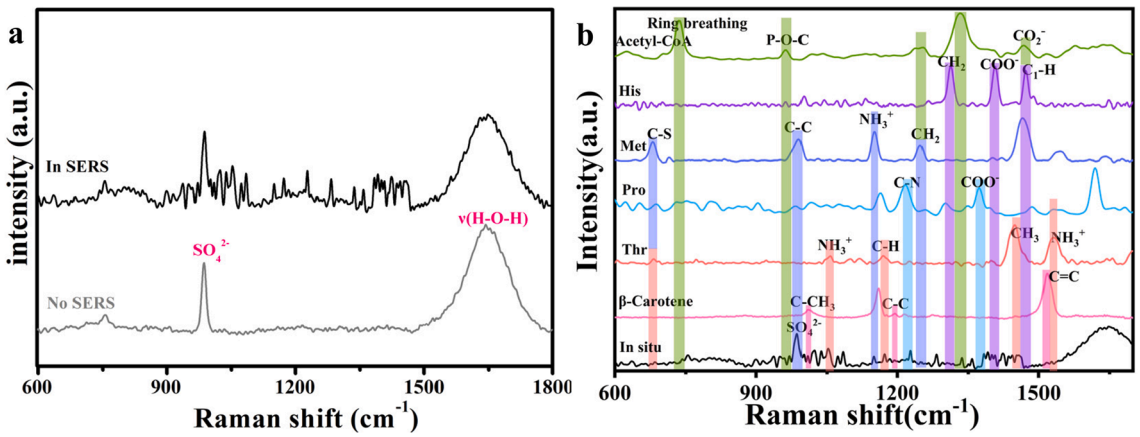

Fig. 3. (a) In situ Raman spectra from biomes in the Haima cold seep vent in the South China Sea; (b) Comparison of SERS spectra of various biomolecules with in situ Raman spectra.

Table 1
The SERS spectrum of each biomolecule corresponds to the peak position of the SERS spectrum in situ.

Biomolecule	Raman bands (in cm^{-1})	Ref.
Acetyl-CoA	724 (Ring breathe), 973 (POC), 1333, 1450 (CO_2^-)	This study
His	1306 (CH_2), 1410 (COO^-), 1482 (Ring stretching + C_1-H in-plane bending)	This study and [16]
Met	673 (C-S stretching), 995(C-C stretching), 1155 (NH_3^+ deformation), 1244 (CH_2 wagging)	This study and [16]
Pro	1226 (C-N stretching), 1393 (COO- symmetric stretching)	This study and [16]
Thr	1153 (NH_3^+ deformation), 1184 (C-H bending), 1456 (CH_3 asymmetric bending), 1539 (NH_3^+ deformation)	This study and [16]
β-carotene	1006 (ρ(C-CH_3)), 1189 (C-C polyene chain), 1512 (C=C polyene backbone)	This study and [17]

collect the spectrum of these 10 molecules (the concentration was all 10^{-4} M). The test result was compared with the *in-situ* SERS spectrum. These results demonstrated that corresponding characteristic peaks of β-carotene, acetyl-CoA, proline, methionine, histidine and threonine were observed in in-site Raman spectra, as shown in Fig. 3b. Therefore, we speculate that these five molecules are likely to be present in the water around the biome of the cold seep. Their corresponding characteristic peaks of biomolecules are described in detail in Table 1. Among them, acetyl-CoA and amino acids both contained amino groups, which confirmed that the SERS substrate had good adsorption ability for amino containing biomolecules and was universal in detection of amino containing molecules. That was consistent with what we reported previous [12].

The successful detection of a variety of amino acids, carotene and other chemical macromolecules confirmed that there are abundant biological nutrients at the marine-biome interface, which may be derived from cell metabolism or apoptosis. The acetyl-CoA is an important metabolic intermediate whose concentration reflects the general energy state of cells. The concentration of acetyl-CoA affects the specificity/activity of a variety of enzymes and helps control key cellular processes, including energy metabolism, mitosis, and autophagy[18]. It was very rare to detect acetyl-CoA in seawater, which means that acetyl-CoA was released into the extracellular environmental solution, presumably due to abnormal microbial death or microcell apoptosis. The experiment proved that SERS substrate could achieve the detection of low concentration biomolecules in deep-sea microbial community, further confirming the feasibility of in situ Raman detection of deep-sea microbial metabolites.

4. Conclusion

In conclusion, we successfully prepared a *Coccinella septempunctata*-like SERS substrate processed by one-step calcination into deep-sea cold seep vents at a depth of more than 1000 m and conducted *in-situ* SERS detection. In addition, we successfully achieved *in-situ* SERS detection in the deep sea and obtained biomolecular Raman spectral data from biomes in cold seep vents in the *Formosa Ridge* and *Haima*, South China Sea. The experiment proved that the performance of the SERS substrate was not affected by the depth (pressure) of the sea, which was suitable for *in-situ* experiments under the deep-sea high-pressure environment. Moreover, SERS substrates can complete the detection of biomolecules at low concentrations in the deep-sea microbial community, which provides a new way to obtain information on key biological metabolites in the deep sea in the future. This also means that it can be applied to the detection of macromolecules in complex industrial systems.

CRediT authorship contribution statement

Siyu Wang: Conceptualization, Methodology, Data curation, Writing – original draft. **Ruhao Pan:** Resources. **Wanying He:** Software, Resources, Formal analysis. **Lianfu Li:** Software, Resources, Formal analysis. **Yang Yang:** Funding acquisition, Project administration. **Zengfeng Du:** Funding acquisition, Project administration. **Zhendong Luan:** Funding acquisition, Project administration, Supervision. **Xin Zhang:** Funding acquisition, Project administration, Supervision, 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.

Data availability

No data was used for the research described in the article.

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