Combined analysis based on crystalline sponge method

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Abstract

The crystalline sponge (CS) method was developed as an X-ray crystallographic molecular structure analysis method that can be performed without the need for crystallization of the analyte. CS has strong molecular recognition properties and a highly flexible framework. The amount of analyte can be reduced to sub-milligram level. These features of the crystalline nano-space allow for the determination of the absolute structure of a trace analyte. In this review, we focus on the discovery of the CS method and its applications to biosynthetic products in combination with NMR spectroscopy. We also describe some examples of the CS method that is used mainly in combination with mass spectrometry (MS). Both approaches demonstrate the potential of microanalysis to determine the molecular structure of an unknown sample. Finally, we mention the use of a crystalline "nano-surface" rather than a crystalline nano-space in MS, which can detect small metabolites as well as post-translation biomolecules.

Key words

crystalline sponge, X-ray crystallography, mass spectrometry, structure determination, laser desorption ionization
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Introduction

Recently, the crystalline sponge (CS) method has attracted researchers who are engaged in the characterization of molecular structures by X-ray crystallography because it abolishes the need for analyte crystallization. Many successful analyses have been demonstrated for trace amounts of samples, including metabolites, bioactive chemicals, and synthetic organic compounds containing highly reactive molecules. There is no doubt that the application of the CS method has spread widely from chemistry to biology in the field of molecular structure analysis.

Herein, we briefly describe the unique characteristics of nano-spaces constructed by the CS framework and the applications of the CS method in combination with NMR analysis in metabolite analysis and biosynthetic pathway determination. In addition, we focus on the applications of the CS method in combination with laser desorption ionization-mass spectrometry (LDI-MS). After that, we describe surface-assisted LDI (SALDI). SALDI method was developed for the accumulation of trace amount of an analyte through non-covalent interactions using the surfaces of nanoparticles or meso/nanoporous materials, when the crystalline nano-space is lacking. Chiral compounds and polypeptides from post-translational modification can be characterized by SALDI-MS.
**Crystalline Sponge**

In 2013, Inokuma, Fujita, and co-workers\(^1\) developed the crystalline sponge (CS) method. They demonstrated that sample molecules in such solvents liquid as isoprene, guaiazulene, and so on were encapsulated in nanopores, and the molecular structures of the samples in the CS frameworks were determined. Surprisingly, the molecular structure of the samples including liquid or oil at room temperature has been determined. In general, the crystallization of an organic molecule is often carried out by controlling the solubility of sample in solvent which is depended on the temperature or the ratio of poor/good solvents. Nanopores are constructed by the complexation of zinc halide and tridentate triazine ligand TPT (2,4,6-tris(4-pyridyl)-1,3,5-triazine), as shown in Scheme 1. The nanopores are occupied by molecules of a crystallization solvent, such as dichloromethane or methanol, after a CS is synthesized. When the CS is immersed in a cyclo- or linear alkane solvent for one week, the crystallization solvent molecules in the nanopores are exchanged with alkane solvent molecules (Scheme 2). This step is important for CS to encapsulate analyte molecules effectively. Basically, the crystallization solvent is incorporated into the nanopores of the crystalline framework by non-covalent interaction, such as hydrogen bonding, \(\pi\)-\(\pi\), or CH-\(\pi\) interaction.

After the solvent exchange, the CS is activated to encapsulate the analyte molecule. Single-crystal X-ray structure analysis is applicable when a crystal with good crystallinity suitable for X-ray structure analysis is available. It should be emphasized that the shape of the analyte is not a much concern. However, a solvent that does not degrade CS and is suitable for the analyte is necessary.

In 2013, Inokuma, Fujita, and co-workers reported the crystal structure of guaiazulene (Fig. 1(a) in orange) encapsulated CS, which has a melting point of
approximately 30 °C. Two Crystallographically independent analytes were observed in one pore, one of which is shown as thermal ellipsoids in Fig. 1(b), consistent with the reported azulene derivative in conventional single-crystal X-ray structural analysis. In addition, the synthesized CS has an achiral C2/c space group before analyte encapsulation, but after the encapsulation of a chiral analyte, its space group changes to a chiral space group such as P2₁. The absolute structure was determined by Flack parameter analysis. In fact, the chemical structure of santonin was determined by the CS method and the absolute structure was validated on the basis of the Flack parameter of 0.092(18) (Fig. 2(a)). Many chemical structures have been determined by the CS method, some of which are summarized in Table 1.

The CS method was developed as an X-ray crystallographic molecular structure analysis method that can be performed without the need for analyte crystallization. Although this method is a powerful tool for molecular structure determination, the CS method developed by Fujita et al. is still rare. We are of the opinion that two unique properties are required for a material to be recognized as a CS: molecular recognition ability and high crystallinity. These properties have been independently investigated in host-guest chemistry and single-crystal X-ray structure analysis, respectively. The molecular structure of a solvent was determined in a coordinated 3D porous network in the 1990s. The solvent did not coordinate to metal ions, that is, the solvent was loosely packed in the crystal framework and not part of the coordination framework. Furthermore, it was important to determine the coordination framework, but at that time, it was difficult to determine the molecular structure of solvent except for the molecules that were solvated by the metal. This was partly because the sensitivity of the X-ray detector and the power of the X-ray source were not sufficient at that time. Owing to the improvement of X-ray detectors and X-ray sources, even if disordered models need to
be resolved by X-ray structure analysis, the molecules of interest could be analyzed including liquid or oily samples at room temperature. It became possible to determine both structure and chirality. For example, such aromatic compounds as benzonitrile, anthracene, and benzyl cyanide were encapsulated in CS and their molecular structures were resolved, including disordered components.\(^9\) In the same way, the absolute structure of menthol acetate, a fragrance compound, was determined by encapsulation of its molecules in different zinc halide frameworks. In all cases, the Flack parameters were smaller than 0.06, which were sufficiently small to establish the absolute structure.\(^10\) For highly volatile analytes such as (+)-\(\alpha\)-pinene, the Flack parameter is relatively large at ca. 0.2. However, it was difficult to determine the absolute structure of pinene because the amount of pinene was small and overlap of cyclohexane solvent and the disorder was observed.\(^11\) Encapsulation methods for volatile compounds have improved. One approach is to expose CS to the saturated vapor of an analyte at room temperature for 7 days. Another approach is to expose CS to a pentane/cyclohexane solution of an analyte for 7 days at \(-30^\circ\text{C}\) to slowly promote solvent evaporation.\(^12\) An advantage of encapsulation at low temperature is that crystal degradation caused by the nucleophilicity of N-containing compounds is suppressed. The conditions for immersion of CS in several micrograms of caffeine sample at 4 °C for 2 days were mild, and caffeine was successfully encapsulated in CS without crack formation. The molecular structures of N-containing heterocycles associated with pharmaceuticals were investigated by the CS method under improved conditions, as shown in Table 1.\(^4\) As shown here, the CS method can determine the molecular structures of various kinds of analytes.
“Dynamic” structure analysis using crystalline sponge

In CS, an analyte occupies only part of a unit cell constructed by the TPT and zinc halide coordination network containing nanopores. Therefore, the analyte requirement can be reduced compared with a single crystal obtained only from the analyte molecule. When the CS crystal size is approximately $0.1 \times 0.1 \times 0.1 \text{ mm}^3$, the chemical formula and the density of the crystal can be determined by X-ray structure analysis. In addition, the chemical formula gives the weight ratio of the analyte to the CS framework. In general, the analyte weight is estimated to be less than 1 $\mu$g for a 5 $\mu$g single crystal of CS. This is an important point when using CS. In general, if the amount of analyte is less than 0.1 mg, the range of analytical methods available for characterization and the accuracy of the analysis would be reduced. To increase accuracy, measurement time would have to be increased significantly. However, it is often difficult to extend the measurement time due to the CS decomposition, so that the structural characterization of the analyte would likely fail. Because the CS method requires only a sub-µg amount of analyte, it is possible to widen the scope of candidates for structural analysis to include trace analytes, such as biosynthetic products and metabolites, as well as main products and by-products of organic synthesis. Fujita, Abe, and co-workers were able to not only elucidate the molecular structure of astellifadiene, a new terpene compound obtained from genome mining, but also determine the biosynthetic pathway.\textsuperscript{13}

In another example, single-crystal X-ray structure analysis was applied to an unknown analyte that was extracted and separated from a natural product. In the X-ray structure analysis, raw reflection data are organized as $hkl$-indices on the basis of structure factors and Fourier-transformed to produce an electron density map. However,
the electron density cannot be uniquely determined by this process due to the lack of a phase parameter. Precise measurement of weak reflections is essential for phase determination. Even if an X-ray diffractometer equipped with a high-power X-ray source that can detect low-intensity diffraction was developed and the phase was determined, the assignment of elements often remained uncertain. To solve this problem, MS or elemental analysis is required, which generally gives the chemical formula. In a study by Fujita and co-workers, 1D and 2D NMR measurements were carried out in combination with the CS method to overcome the difficulty of X-ray structure analysis of an unknown sample. In general, NMR analysis mainly provides information on connectivity and spatial relationships between $^1$H and $^{13}$C atoms. The structure of sesquiterpene synthesized by red macroalga was determined by NMR analysis coupled with CS method. The connectivity pattern of the prespatane (C$_{15}$H$_{24}$) structure was resolved by NMR analysis and reported 20 years ago. However, the absolute structure of the prespatane determined by the CS method differed from that originally reported. Finally, it was confirmed that the absolute structure determined by single-crystal X-ray structural analysis was consistent with $^{13}$C and $^1$H NMR observations by performing COSY, HMQC, HMBC, and NOESY measurements, as shown in Fig. 3. This approach demonstrated the analytical possibilities of NMR spectroscopy and the CS method. This method allowed us to determine the molecular structure of an unknown sample, eliminating uncertainties with regard to the absolute structure determined by NMR measurement alone.

In another example, the structure of a boron-containing organic compound was difficult to assign by $^1$H NMR measurement because of signal broadening by the $^{11}$B nucleus. The stereochemistry of the product prepared by metal-free diboration was
determined by the CS method and its reaction selectivity for the diboration was evaluated. Using the crystalline nano-space, the CS method is capable of such potential applications as trace biomaterial characterization and unknown sample characterization, and will be applicable to other fields as a sample characterization technique.

We propose the following criteria for CS with molecular recognition properties: The CS should encapsulate molecules having three or more types of skeletons; there should be three types of functional groups in one skeleton, such as a polycyclic aromatic compound (the number of rings is 3); and there should be an electrophilic/nucleophilic functional group. In addition, CS should have nanopores and good crystallinity.

**Application as “space” in MS**

As has been discussed, the CS method is not limited to X-ray structure analysis, and it is clear that the combination of different methods of analysis and the CS method would provide details of molecular structure. The combined use of MS and the CS method was firstly reported in 2015. Stilbene derivatives were encapsulated in CS nanopores, and one CS suitable for X-ray structure analysis was selected for collection of diffraction patterns for structure analysis. After diffraction measurement, a single crystal was subjected to LDI. Imaging MS revealed that areas of detected ion peaks were almost superimposable on an optical image of CS. The detection sensitivity for most ionization methods in MS is very high, generally below nmol order; this was confirmed by showing that CS can visualize hotspot areas, as shown in Fig. 4(a). However, it should be noted that the solubility characteristics of stilbenes vary by
functional group, namely, -H, -Me, or -Cl. This leads to the problem that the matrix does not mix well with the analyte under the same conditions.

Molecular ions derived from stilbenes were clearly detected in the 1D mass spectra. At the same time, the proton adduct of TPT, which is a component of CS, was observed, as shown in Fig. 4(b). This indicates that the ionization of CS has an important role; the ionization enables CS to absorb laser energy and transfer it to the analyte, so that CS can ionize an encapsulated molecule as a “matrix”. This is called “CS-LDI-MS”. The encapsulation in CS nanopore is a molecular recognition phenomenon that has a few limitations. One limitation is the difficulty of predicting whether an analyte can be encapsulated or not before encapsulation. Obviously, analyte size is one of the important factors for encapsulation. A CS pore measures approximately $15 \times 10$ Å$^2$; as such, analyte size is limited to that size. It was reported that molecules having $\pi$-conjugation, linear alkyl chains$^{22}$, or bulky skeletons$^{23}$ could be encapsulated.

Although the encapsulation process is still difficult to understand, it seems that the electron-deficient property of TPT strongly affects the encapsulation. In this regard, the effect of ionization of the same electron-rich skeleton was examined. Four 1,3-benzodioxole derivatives having electron-rich catechol skeletons were selected for encapsulation, and the CSs were subjected to LDI.$^{24}$ The physical states of the 1,3-benzodioxole derivatives were different, i.e., liquid, oil, or powder, under laboratory conditions. Regardless of physical state, however, the four 1,3-benzodioxoles were soluble in CS-containing cyclohexane. In conventional methods, an analyte was prepared depending on its physical state. In the CS method, one CS suitable for X-ray structure analysis was selected and diffraction patterns of the CS were collected. After that, CS-LDI-MS measurement of the CS was carried out. In the 1D mass spectra,
molecular ions of only safrole and piperonyl acetone analytes were detected, although the proton adduct of TPT was observed in all cases.

The ion peak at $m/z$ 108.0 was observed in the four mass spectra, and it was found that it originated in the 1,3-benzodioxole skeleton (Fig. 5). The ion peak at $m/z$ 105.0, which was also apparent in the four mass spectra, was assigned to a fragment of TPT. It implied that ion peaks in the lower mass range were not contaminated by the fragmentation of TPT or zinc halide in CS-LDI except for the proton adduct of 4-cyanopyridine as a fragment of TPT. The authors did not mention the reason for the ionization difference among them. Single-crystal X-ray structure analysis revealed the presence of $\pi-\pi$ interaction in only piperonyl acetone contained in CS, and the presence of halogen-CH interaction was confirmed in all cases (Fig. 5). It was experimentally confirmed that the difference in ionization was dependent on the presence of interaction, which is the key to ionizing an analyte encapsulated in the pore. It is natural to expect that the weak interaction between an analyte and a matrix is the key to ionization, but author pointed out the relationship between interaction and ionization on the basis of molecular coordinates. In a very recent report of the measurement of diphenyl ene compounds having different linear chain lengths in CS by CS-LDI MS, a unique fragment pattern as well as molecular ion was observed. This fragment pattern was unique to a straight-chain aliphatic hydrocarbon and observed by electron impact method (EI), a hard ionization method.\(^22\) In an earlier work by the same authors, a comparison of case in the presence and absence of interactions for CS-LDI was reported.\(^23\) The authors encapsulated 5-, 11-, and 15-membered cyclic hydrocarbons and succeeded in X-ray structure analysis, as shown in Fig. 6. Single-crystal X-ray structural analysis revealed the absence of interaction between large cyclic hydrocarbon analytes and the CS framework, and the presence of $\pi-\pi$ interaction for the 5-membered cyclic
compound. Molecular ion was detected in the presence of interactions, whereas no ions derived from analyte were observed in the absence of interactions. It was experimentally confirmed that the ionization of an analyte strongly depends on the presence/absence of interactions. CS could be used as a new material for revealing the environment of a molecule as well as the molecular structure in LDI-MS.

In MS, CS was confirmed to be used as the matrix in LDI. At this time, there are no clear advantages over other methods that do not use CS as an MS application. However, as the same amount of sample is used for CS and LDI-MS, the relationship between interaction and ionization can be easily investigated by conducting experiments. We expect the applications of CS will increase further.

Application as “surface” in MS

There is no doubt that CS is an attractive material whose crystalline nano-space has molecular recognition properties as well as “matrix” which can absorb laser energy and transfer it to analyte in laser desorption ionization. On the other hand, if weak interaction with the target surface is used instead of crystalline nano-space interaction, surface-assisted LDI-MS, SALDI-MS becomes possible. For example, metal-organic frameworks (MOFs) are very similar to CS in terms of crystallinity and the presence of nanopores for absorbing molecules.25–27 Huang, Shih, and co-workers firstly reported28 the use of MOFs for SALDI, and cage-type MIL-100(Fe) MOFs were used as the matrix in SALDI to detect nonpolar polycyclic aromatic hydrocarbons (PAHs) as shown in Fig. 7. MIL-100(Fe) is composed of Fe(III) ion and benzene-1,3,5-tricarboxylate, which are assembled into a zeolite architecture of the MTN (Mobil Thirty-Nine) type.
Its framework is relatively stable; it is not damaged by heating at 250 °C for 12 h. Fe(III) ion acts as a Lewis acid and lies on the surface of pore, and pore size is sufficiently large for small organic molecules to pass. Because of these characteristics, Fe(III) ion can interact with PAHs based on electron acceptors and donors. Although MIL-100(Fe) provides good ion peak reproducibility, MIL-100 with other metals show different thermal stabilities and are difficult to use. MIL-100(Fe) is very similar to CS regarding possessing crystallinity and nano-space, but their space group symmetries are markedly different in single crystal structural analysis. In general, a crystal has a periodic structure in nano-space, and its periodic pattern can be classified into a specific space group symmetry on the basis of molecular position and packing pattern. If a porous host framework belongs to a higher symmetry, the analyte in the pore has to belong to the same symmetry space group, or else the molecular structure of the analyte cannot be determined due to mismatch of molecular symmetry. In fact, many MOFs including MIL-100 belong to the very high symmetry space group $Fd\bar{3}m$, whereas CS belongs to the low symmetry space group $C2/c$. This subtle difference is a critical factor for determining whether the molecular structure can be elucidated or not. No interaction between analyte and matrix has been observed directly due to the difficulty of evaluating the presence of interaction. SALDI should be regarded as proceeding through attractive non-covalent interactions rather than molecular recognition for structure determination as seen in the CS application. However, in any case, SALDI has been successfully applied in many fields.

In SALDI, the material surface was not limited to crystalline nanopore, and a surface is also present on nano-particles, metal nano-film and meso-pore. The surface has fascinated to modified by chemical reaction to introduce interaction site such as electrostatic,29 hydrophobic,30 hydrogen interaction and so on. An approach that uses
nanoparticles is convenient for the preparation of both matrix and analyte and setting up on a target plate. In addition, the particle surface is easy to functionalize depending on the chemical and physical properties of the analyte. For example, Russell and co-workers examined the utility of size-selective (2, 5, and 10 nm) Au nanoparticles (AuNPs). They detected phosphopeptides that are difficult to ionize by matrix-assisted LDI (MALDI) and enhanced the signal-to-noise ratio because of effective energy transfer (Fig. 8).

Metal nanoparticles could be chemically modified and used to detect target analytes such as peptides obtained from post-translation proteins.

On the other hand, gold and silver films were used for the detection of biomolecules by immunoaffinity. Granzow and co-workers employed a thin gold surface for detecting biomolecular interactions using the surface plasmon resonance (SPR) technique, and directly ionized an affinity-captured analyte, myotoxin A, by LDI (Fig. 9).

SALDI is frequently coupled with SPR because SPR features high sensitivity, real-time analysis, and label-free detection. SALDI can provide chemical information of an analyte, although the SPR sensor only shows differences in optical change. As an interesting example of coupling of SPR and SALDI, Masson and co-workers compared MS and SPR imaging of mouse kidney. They pointed out that SPR provided quantitative and kinetic information of the protein transfer process and MS furnished specific molecular information related to tissue histology. They showed that the two techniques offered complementary information (Fig. 10).

As shown above, the use of surface as an ionization field in MS has been developed for detecting trace amount of an analyte, such as a post-translation peptide or a small organic compound including metabolites, and selective detection is achieved.
depending on the functional group.

Summary and Perspective

In this review, we focused on the crystalline sponge (CS) and its unique features and described the important characteristics of the CS method. CS possesses two characteristics, molecular recognition and crystallinity, both of which are effectively combined into one material. The CS method can be used for the determination of molecular structures of biosynthetic compounds including metabolites as well as small organic compounds, because only a trace amount of analyte is required and no analyte “crystallization” is necessary. The CS method is not only a molecular structure determination method but also a new approach for evaluating molecular interactions and their functions in reactions. In the future, the ionization mechanism of LDI-MS using the CS method will be uncovered. We hope that further studies using nanoparticles or porous material surfaces will be conducted to enhance sensitivity, simplicity, and selectivity. A basic understanding of LDI is vital to open doors to new types of matrices.
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Table 1. List of chemical structure determined by crystalline sponge method.
Scheme Captions

Scheme 1. (a) Synthesis of crystalline sponge, and crystal structure constructing 3D porous networks packing view along with (b) $b$-axis and (c) (101) directions showing large pore shape.

Scheme 2. Experimental procedure for obtaining crystalline sponge (a) and preparing of activated CS to encapsulate analyte (b).
Scheme 1.

(a) $3 \text{ZnX}_2 + 2 \text{TPT}$  \hspace{1cm} (X = Cl, I)

\[ [(\text{ZnX}_2)_3(\text{TPT})_2 \cdot \text{G}]_n \]

(G = crystallization solvents)

(b) Channel direction:

(c)
Scheme 2.
Figure Captions

Fig. 1  The crystal structure of guaiazulene encapsulated crystalline sponge (a) ORTEP drawing (30% probability level) of the guaiazulene in (b) (left) and its chemical structure (right)

Fig. 2 (a)  The crystal structure of santonin encapsulated crystalline sponge, (b) ORTEP drawing (30% probability level) of the santonin picked up as red square in (a) (left) and its chemical structure (right)

Fig. 3 (a) Molecular structure analysis with NMR in $^1$H, $^{13}$C, COSY, HMBC, NOESY and so on (left), and observation for a part of molecular structure in X-ray analysis (right).  (b) Molecular structure determined by NMR and X-ray analysis, respectively.  (c) Revised structure based on NMR and X-ray analysis (left) and previously reported structure (right).

Fig. 4  (a) Crystalline sponge on the target plate, optical images (top column), superposed of both optical and IMS (middle column), IMS image at stilbene molecular ion (b) 1D mass spectra for stilbene derivatives, dashed line indicate molecular ion position, respectively, and the asterisk shows the TPT molecular ion position.  $^{21}$

Fig. 5  1D mass spectra obtained from 1,3-benzodioxole derivatives in CS-LDI MS (a) safrole, (b) piperonyl methyl ketone, (c) piperonyl acetone, and (d) piperonylnitrile. $^{24}$

Fig. 6  (a) observation of $\pi-\pi$ interaction between pentamethylcyclopentadiene and CS
frameworks and its 1D LDI mass spectrum, the absence of interaction between CS frameworks and (b) zerumbone or (c) muscone, and their 1D LDI mass spectra.  

Fig. 7  SALDI-MS procedure to mixing MIL and PAHs, and laser irradiation providing molecular ion from PAHs.  
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Fig. 8  Size selective gold nanoparticle and LDI-MS for phosphorylated peptides.  
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Fig. 9  BIA/MS of myotoxin a retained within the confines of individual flow cells.  
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Fig. 10  Comparison IMS, H&E stain, SPR image for mouse kidney section.  
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Fig. 1

The crystal structure of guaiazulene encapsulated crystalline sponge (a) ORTEP drawing (30% probability level) of the guaiazuelene in (b) (left) and its chemical structure (right).
Fig. 2  (a) The crystal structure of santonin encapsulated crystalline sponge, (b) ORTEP drawing (30% probability level) of the santonin picked up as red square in (a) (left) and its chemical structure (right)
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Fig. 4
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Fig. 8
Fig. 9  Biomolecular interaction analysis and MS of myotoxin a retained within the confines of individual flow cells.
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Graphical Index

Widely acceptable molecular structure analysis
"Crystalline Sponge"