

# Ultrabroadband infrared nanospectroscopic imaging

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**Characterizing and ultimately controlling the heterogeneity underlying biomolecular functions, quantum behavior of complex matter, photonic materials, or catalysis requires large-scale spectroscopic imaging with simultaneous specificity to structure, phase, and chemical composition at nanometer spatial resolution. However, as with any ultrahigh spatial resolution microscopy technique, the associated demand for an increase in both spatial and spectral bandwidth often leads to a decrease in desired sensitivity. We overcome this limitation in infrared vibrational scattering-scanning probe near-field optical microscopy using synchrotron midinfrared radiation. Tip-enhanced localized light-matter interaction is induced by low-noise, broadband, and spatially coherent synchrotron light of high spectral irradiance, and the near-field signal is sensitively detected using heterodyne interferometric amplification. We achieve sub-40-nm spatially resolved, molecular, and phonon vibrational spectroscopic imaging, with rapid spectral acquisition, spanning the full midinfrared (700–5,000 cm<sup>-1</sup>) with few cm<sup>-1</sup> spectral resolution. We demonstrate the performance of synchrotron infrared nanospectroscopy on semiconductor, biomineral, and protein nanostructures, providing vibrational chemical imaging with subzeptomole sensitivity.**

nano-FTIR | chemical identification | bioimaging

**M**any properties and functions of natural or synthetic materials are defined by chemically or structurally distinct phases, domains, or interfaces on length scales of a few nanometers to micrometers. Characterizing these heterogeneities has long driven the development of advanced microscopy techniques, with notable advances in electron (1), photoemission (2), X-ray (3), and super-resolution (4) microscopies. Although extremely effective, these techniques have strict sample requirements: either they operate in vacuum, are limited by electron or X-ray beam damage, or rely on labeling with exogenous fluorophores.

In contrast, infrared (IR) vibrational spectroscopic imaging is minimally invasive, requires little sample preparation, is applicable in situ and under ambient conditions, and provides intrinsic chemical contrast and spectroscopic identification for a wide range of materials (5), including living cells (6) and tissues (7, 8). The spatial resolution, however, is diffraction-limited to 2–10  $\mu\text{m}$ , depending on wavelength. Moreover, high signal-to-noise spectra even at this low resolution are only possible with a source of high spectral irradiance (9). Subwavelength imaging has been achieved to some extent through point-spread function deconvolution (10, 11) and attenuated total reflection techniques (12). However, the micrometer-size wavelength of IR radiation, in general, has fundamentally limited its application for the characterization of essentially any mesoscopic, heterogeneous material where chemical information at the nanoscale is desired.

Infrared scattering-scanning near-field microscopy (IR *s*-SNOM) overcomes the diffraction limit by scattering incident light with the typically metallic tip of an atomic force microscope (AFM) scanning in nanometer proximity to the sample (13, 14). The conductive tip effectively acts as an optical antenna by localizing and scattering the IR optical field in the near-field region of its nanoscopic apex. Consequently, the spatial resolution of *s*-SNOM is independent of the incident IR wavelength and is determined to first approximation only by the radius of the tip apex, which is typically 10–20 nm.

IR *s*-SNOM nanospectroscopy and imaging with chemical contrast, however, has not yet achieved its full potential in terms of versatility and spectral coverage compared with conventional Fourier transform infrared (FTIR) techniques. Lasers are typically used as *s*-SNOM sources because they provide the high spectral irradiance that is necessary to obtain detectable signal levels from the nanoscopic probe volumes arising from the intrinsic high spatial resolution. Narrow-band continuous wave (CW) tunable IR lasers offer the highest spectral irradiance, but nanoscale spectroscopy can only be performed over the typically narrow tuning range of these lasers, which cover  $\sim 1$  vibrational mode (13, 14). Femtosecond-pulsed IR lasers, which trade both spectral irradiance and total power for increased spectral bandwidth, enable the investigation of one or multiple vibrational modes without tuning (15–17). Broadband and continuum sources have achieved bandwidths suitable for investigation of a significant portion of the IR region (18, 19), although the combination of sufficient power across the full mid-IR at high repetition rates remains difficult. Thus, the bandwidth of the broadest IR laser sources yet implemented for *s*-SNOM is only a fraction of the full IR range provided by a thermal source in conventional FTIR. For both CW and femtosecond-pulsed laser sources, the spectral tuning required to cover a broader spectral range necessarily makes the experiment prone to laser and sample drift, thereby limiting the spectral quality that can be obtained in *s*-SNOM.

Heated filament or global sources, which are the standard FTIR sources, have been implemented in *s*-SNOM investigations (20), but despite the large total flux, the extremely low spectral irradiance arising from the lack of spatial coherence in these

## Significance

Through direct measurement of intrinsic vibrational and electronic modes, infrared spectroscopy provides label-free, chemical characterization of molecules and solids. The long micrometer-sized wavelength of infrared light, however, has limited the application of this widely applied spectroscopic technique to ensemble studies, preventing nanoscale spatially resolved spectroscopy of heterogeneous materials. We overcome this limitation by combining scattering-scanning near-field optical microscopy with broadband infrared synchrotron radiation. Using this synchrotron infrared nanospectroscopy (SINS) technique, we achieve spectroscopic imaging over the entire midinfrared with nanometer spatial resolution and high sensitivity. With a spatial resolution 100–1,000 times better than conventional FTIR microscopy, SINS enables the investigation of nanoscale phenomena in soft matter, even under ambient and environmental conditions that are essentially inaccessible by other techniques.

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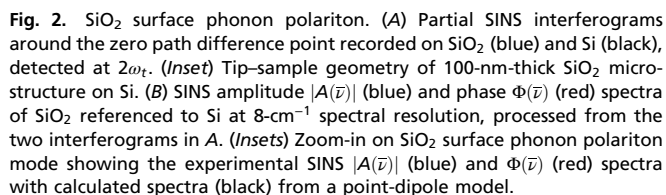
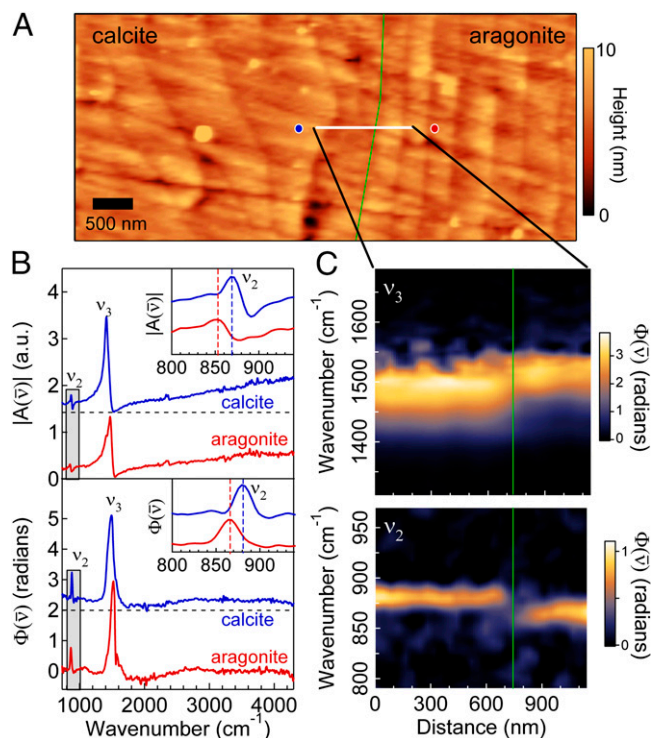


Fig. 3 *A* and *B* shows the topography and broadband SINS images of an  $\sim 100$ -nm-thick  $\text{SiO}_2$  microstructure on Si. Imaging contrast between the two materials integrated across this broad spectral range is primarily nonresonant and mostly represents the change in the nonresonant offset of the dielectric permittivity  $\epsilon_1$  between Si and  $\text{SiO}_2$ . Profiles extracted from these images show the spatial resolution to be better than 40 nm for the infrared signal (Fig. 3*B*, *Inset*).

**CaCO<sub>3</sub> Polymorph Heterogeneity in a *Mytilus edulis* Shell.** As a second example, we demonstrate the applicability of SINS toward the study of calcium carbonate (CaCO<sub>3</sub>) polymorph heterogeneity in a natural biological system. Like many bivalves, blue mussels (*M. edulis*) form a bimineralic shell that transitions during growth from calcite to aragonite as influenced by a number of environmental parameters (37). Fig. 4 shows AFM topography and SINS spectra from a polished *M. edulis* mollusk shell in the interface region between the outer calcite and inner aragonite (nacre) layers. The AFM topography (Fig. 4A) illustrates the different morphological structures of the two polymorphs, with the calcite crystals (Fig. 4A, *Left*) formed in large, irregular shapes and the aragonite (Fig. 4A, *Right*) crystals formed in polygonal tablets with widths of a few hundred nanometers.

**Fig. 3.** SiO<sub>2</sub> microstructures on Si. (A) AFM topography and (B) simultaneously recorded broadband SINS image. (Insets) Profiles of topography and IR response across the SiO<sub>2</sub> edge, as indicated by the white line in A and B, respectively. The distance between the dashed black lines is 40 nm. Spatio-spectral SINS linescan of (C)  $|A(\vec{r})|$  and (D)  $\Phi(\vec{r})$  along the white line in A and B. Each of the 30 spectra in the plot was acquired for 1 min at 16-cm<sup>-1</sup> spectral resolution. SINS (E)  $|A(\vec{r})|$  and (F)  $\Phi(\vec{r})$  spectra at three locations along the linescan in C and D. The position 1 spectra (blue) are offset for clarity by 2 and 1 in E and F, respectively, and the position 2 spectra (red) are offset by 1 and 0.5 in E and F, respectively. In E, the dashed lines are the maximum (1,130 cm<sup>-1</sup>) and minimum (1,264 cm<sup>-1</sup>) of the position 1 spectra, and in F the dashed lines are the peak positions of the position 1 (1,225 cm<sup>-1</sup>) and position 2 (1,155 cm<sup>-1</sup>) spectra.





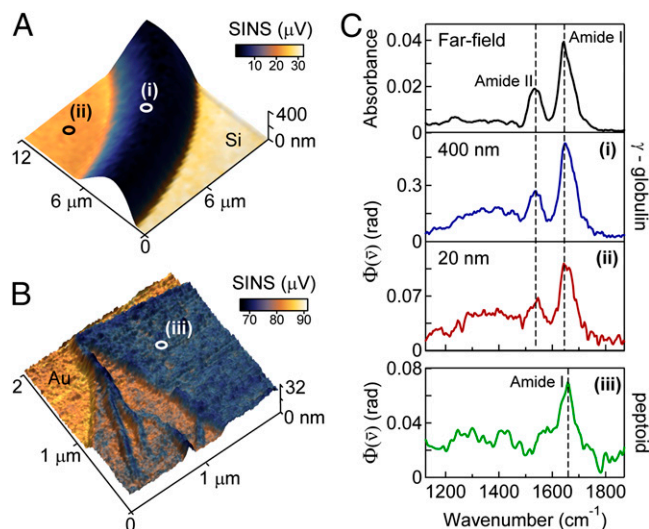
As shown in Fig. 4B, SINS  $|A(\bar{\nu})|$  and  $\Phi(\bar{\nu})$  spectra of calcite and aragonite readily distinguish the two  $\text{CaCO}_3$  polymorphs through the frequency of the  $\text{CaCO}_3$  vibrational modes in each material (38). Two vibrational modes localized on the  $\text{CO}_3^{2-}$  ion, namely the out-of-plane bending  $\nu_2$  and the doubly degenerate asymmetric stretching  $\nu_3$  modes, have IR vibrational resonances at different frequencies in each polymorph. The bandwidth of the synchrotron source allows both modes to be resolved simultaneously. Moreover, the broad bandwidth enables the assessment of any sample contamination, adsorbed water, or additional biominerals, such as apatite (39), that might have features in other spectral areas. The  $\Phi(\bar{\nu})$  spectra indicate that the  $\nu_2$  mode (Fig. 4B, *Inset*) in orthorhombic aragonite ( $880\text{ cm}^{-1}$ ) is observed at lower frequencies relative to that in trigonal calcite ( $865\text{ cm}^{-1}$ ), as expected and in agreement with laser-based *s*-SNOM (39), whereas the  $\nu_3$  mode in aragonite ( $1,520\text{ cm}^{-1}$ ) is resonant at a higher frequency relative to the calcite ( $1,495\text{ cm}^{-1}$ ). Although the  $\nu_3$  mode is stronger and has a larger difference ( $\sim 30\text{ cm}^{-1}$ ) in resonant frequency between the two materials, the vibrational shift is more evident for the  $\nu_2$  mode due to its narrower linewidth. Fig. 4C shows a spatio-spectral linescan of the interface region along the solid, white line in Fig. 4A. The  $\nu_2$  mode, in particular, reveals the sharp transition between calcite and aragonite, consistent with previous electron (40) and X-ray microscopy (41) investigations.

**Surface-Absorbed Dried Proteins and Peptoid Nanosheets.** SINS is minimally invasive and allows for the nondestructive study of soft matter, including synthetic- and biopolymers. IR  $s$ -SNOM

has recently been used for investigating protein organization and morphology, including membrane proteins (13, 42). The strongest vibrational signature of proteins arise from the Amide I (typical center frequency 1,620–1,700  $\text{cm}^{-1}$ ) and Amide II (1,500–1,560  $\text{cm}^{-1}$ ) modes, which have been previously used to identify the local density of proteins. These modes are known to shift in frequency and intensity as a function of protein secondary structure and can thus help identify a protein, as well as changes in its tertiary structure (43).

Spectroscopic identification of proteins requires resolving several modes simultaneously. SINS enables spectroscopic characterization of the complete fingerprint region, which spans several hundred  $\text{cm}^{-1}$ . Fig. 5 shows SINS images (Fig. 5A) and spectra (Fig. 5C) of a dried  $\gamma$ -globulin protein on a Si substrate.  $\gamma$ -Globulin is a naturally occurring immunological protein, which, as expected, aggregates in an amorphous film upon deposition. Thickness-dependent dielectric contrast is observed over tens of nanometers, corresponding to the penetration depth of the evanescent field from the tip. Fig. 5C compares  $\gamma$ -globulin SINS  $\Phi(\vec{r})$  spectra with a far-field FTIR spectrum of the same sample. The SINS peak positions and shapes of both the Amide I and Amide II peaks are nearly identical to far-field measurements that require averaging over a  $10 \times 10\text{-}\mu\text{m}^2$  area. Several smaller peaks are also observed in the  $1,200\text{--}1,400\text{-cm}^{-1}$  region, which result from the Amide III mode and the vibrations of amino acid side chains. Comparisons between  $\gamma$ -globulin and bovine serum albumin (BSA) (Fig. S3) reveal spectral shifts in the SINS spectra that are also observed in the far-field spectra, thus demonstrating that SINS shares the ability of far-field FTIR spectroscopy to determine both protein identity and distribution, but on the scale of tens of nanometers rather than several micrometers.

Fig. 5 also shows an SINS image (Fig. 5B) and spectrum (Fig. 5C) of a peptoid nanosheet. Peptoids, or *N*-substituted glycines, are synthetic, sequence-specific polymers that have a polypeptide backbone, but with the side chains attached to the nitrogen atom rather than to the  $\alpha$ -carbon as in peptides. Although this synthetic



polymer is chemically similar to naturally occurring proteins, it is readily distinguished by a change in lineshape of the Amide vibrational modes. With appropriate substitution of the side chains, these engineered biopolymers self-assemble into thin, 2D nanosheets that are only 2 molecules thick (44). For the peptoid nanosheet imaged here, short peptide sequences were inserted into the nanosheet-forming peptoid polymers to form peptide loops on the nanosheet surface, effectively increasing the bilayer thickness from 3 to 8 nm (45). The integrated IR response shows sensitivity to bilayer structures, wrinkles, and folds with a spatial resolution <40 nm as measured on a bilayer edge.

**Sensitivity.** SINS spectra in Fig. 5C (Lower) are collected from a region of  $\gamma$ -globulin 20 nm in thickness and of a peptoid double bilayer ~16 nm thick. These spectral measurements of thin films and nanosheets highlight the high sensitivity of SINS, which is facilitated by the optical antenna properties of the tip (46). From the measured thickness of the film, tip radius, and resulting spatial resolution, we can estimate the amount of protein probed. Assuming an average protein density (47) of 1.4 g/cm<sup>3</sup>, we establish that an upper bound of 150  $\gamma$ -globulin protein molecules are spectroscopically probed under the area of the tip, corresponding to subzeptomole sensitivity and approximately  $2 \times 10^5$  amino acid groups. A corresponding far-field synchrotron FTIR transmission microscopy analysis of the same protein sample in the same location shows that more than  $8 \times 10^5$  protein molecules are required to achieve a signal-to-noise equivalent to an SINS measurement of only 150  $\gamma$ -globulin protein molecules. This shows that the sensitivity of SINS exceeds that of far-field spectroscopy by more than a factor of 5,000. [With a field enhancement  $F \sim 2$ –3 for the Pt tips and an SINS signal intensity proportional to  $F^4 < 100$ , this large improvement in sensitivity is more than can be expected by local field enhancement alone and indicates that the near-field tip–molecule mode transfer (46) is enhanced by the optical antenna effects of the tip.]

## Perspective and Summary

The vibrational spectroscopic sensitivity achieved is a dramatic increase over far-field techniques and is comparable to laser-based *s*-SNOM. Although broadband IR laser source development continues, synchrotron IR radiation is currently the only source with the combined spectral irradiance, spectral coverage, and high repetition rate to measure near-field spectra over the entire mid-IR range within a single acquisition. In addition to the tip enhancement, the high sensitivity is achievable because of several advantages of the source, including minimal shot-to-shot fluctuations, high long-term power stability arising from top-off mode operations (48), and the exceptionally stable spectral distribution. These advantages enable effective signal averaging over minutes to hours, if necessary. Additional improvements to the stability of the *s*-SNOM apparatus, vibrational isolation, and mode quality of the beam are expected to further improve the sensitivity of SINS.

The broad spectral bandwidth enables more quantitative and accurate analysis of spectral position and lineshape compared with previous *s*-SNOM investigations. Real-time alignment of the interferometer before a series of scans is possible using the rapid-scan moving mirror in the interferometer, and aligning the interferometer on a resonant or nonresonant sample across the broader bandwidth ensures spectrally flat phase, improving the accuracy of the peak shape (49). Probe-induced peak shifts ranging from a few to several tens of cm<sup>-1</sup> have been observed in *s*-SNOM spectra relative to far-field spectroscopies as a result of, e.g., tip-sample coupling (21, 22), tip radius (16), and the spectral phase approximation (30). Other changes to lineshape are intrinsic to the sample, however, such as spectral narrowing in a subensemble of the sample (50). Simultaneous monitoring of two or more vibrational modes with SINS will enable improved quantification of and distinction between spectral signatures intrinsic to a material sample and spectral signatures resulting from probe-sample coupling.

In summary, we have developed an IR spectroscopic method that moves broadband FTIR spectroscopy beyond the realm of diffraction-limited spectro-microscopy. The near-field confinement of IR light results in tip-limited spatial resolution 2–3 orders of magnitude smaller than the diffraction limit, and the high spectral irradiance, bandwidth, and spatial coherence of synchrotron radiation enables high signal-to-noise vibrational spectroscopy across the entire mid-IR. The chemical identity of zeptomole subensembles within a sample can unambiguously be determined by spectral features of several vibrational modes. In the case of proteins or polymer systems, this opens the possibility for not only precise chemical identification but also investigation of the protein folding state. SINS has the same benefits as far-field IR methods to obtain label-free, detailed chemical and physical information, yet with drastically improved spatial resolution and sensitivity achievable only in the near field. This highly powerful combination provides access to a form of nanochemometric analysis with the investigation of nanoscale, mesoscale, and surface phenomena that were previously impossible to study with IR techniques. Moreover, the user facility aspect of SINS makes the *s*-SNOM technique widely available to nonexperts such that it can be broadly applied to biological, surface chemistry, materials, or environmental science problems.

## Materials and Methods

**SINS.** The ALS is a third-generation synchrotron operating in top-off mode to maintain a constant 500-mA current of 1.9-GeV electrons. Approximately 500  $\mu$ W of synchrotron infrared light (700–5,000 cm<sup>-1</sup>) at Beamline 5.4 is passed through a KBr beamsplitter (Thermo-Scientific) (Fig. S1, BS1). Half of the light is focused onto a platinum silicide (Nanosensors, PTSi-NCH) or platinum-coated silicon (Nanoworld, ARROW-NCPT) AFM tip with a custom parabolic mirror (N.A. ~0.4, Nutek). The other half is directed to the scanning mirror of a commercial FTIR spectrometer (Thermo-Scientific, Nicolet 6700) via a delay line. The entire system is enclosed and purged with nitrogen to remove water and carbon dioxide and to increase thermal stability. The beamsplitter (Fig. S1, BS2) of the commercial FTIR spectrometer has been customized to allow the HeNe laser to operate in its normal configuration, while having an open aperture for the synchrotron infrared beam. This modification allows the commercial instrument to operate without introducing unwanted interference artifacts into the *s*-SNOM signal. The use of the commercial FTIR spectrometer and software is advantageous because it uses the highly engineered and mature technology of traditional FTIR spectroscopy. The ability to perform rapid-scan measurements is particularly beneficial both in initial alignment and in avoiding phase drifts during a single acquisition. The rapid-scan mode can also be used to acquire low-resolution spectra simultaneously with topography. Back-scattered light and the light reflected off the moving mirror are combined on the KBr beamsplitter (Fig. S1, BS1) and focused onto an MCT detector (Kolmar KLD-0.1). To avoid saturation of the detector, a germanium wedge is used as a reflector in the delay line for the moving mirror. The double-pass arrangement allows for a reduction in signal from this arm by ~75%. The AFM (modified Innova, Bruker) is operated in noncontact mode and the tip-scattered signal is demodulated at the tip-tapping frequency (typically 250–300 kHz) with a lock-in amplifier (Zurich Instruments). Typical values of the lock-in time constant and mirror speed are ~200  $\mu$ s and 0.15 cm/s, respectively, which allow for a single interferogram to be acquired in ~4 s at 4-cm<sup>-1</sup> resolution. This speed can be increased up to 40 times faster at the expense of spectral resolution, signal-to-noise ratio, and/or limits on high-frequency spectral components. The demodulated signal is fed back into the commercial FTIR spectrometer and the data are acquired through commercial software (Omnic, Thermo-Scientific).

**Analysis.** Interferograms are postprocessed through custom programs with commercial fast Fourier transform analysis packages (Wavemetrics Igor). To remove the instrumental response from spectra, both the amplitude and phase spectra are referenced to a sample with flat spectral response, such as gold or silicon, obtaining  $|A(\bar{\nu})| = |A(\bar{\nu})|_{\text{sample}} / |A(\bar{\nu})|_{\text{ref}}$  and  $\Phi(\bar{\nu}) = \Phi(\bar{\nu})_{\text{sample}} - \Phi(\bar{\nu})_{\text{ref}}$ . The imaginary part of the SINS signal  $\text{Im}(\sigma_n(\bar{\nu}))$ , which is proportional to  $\Phi(\bar{\nu})$  to a good approximation for not too strong oscillators and/or large broadband nonresonant responses, approximates the absorption coefficient of materials measured in traditional FTIR measurements (17, 30).



**Far-Field IR Microspectroscopy.** Far-field IR transmission spectra of protein samples on silicon were measured with a synchrotron-based FTIR microscope (Thermo-Scientific, Nicolet 6700 spectrometer and Continuum microscope) on Beamline 5.4 at the ALS. The synchrotron source produces a diffraction-limited spot size (N.A. = 0.65), which is about 6  $\mu\text{m}$  in the region of the Amide I vibrational mode ( $\sim 1,640\text{ cm}^{-1}$ ). Spectra were averaged for 10 min and collected in the  $650\text{--}4000\text{ cm}^{-1}$  range with  $8\text{ cm}^{-1}$  resolution using a KBr beamsplitter and an MCT detector.

**Sample Preparation.** All samples were prepared on flat substrates suitable for AFM investigation and were investigated under ambient conditions.  $\text{SiO}_2\text{-Si}$  microstructures were purchased from MikroMasch (TGXYZ02), containing circular pillars of  $\text{SiO}_2 \sim 100\text{ nm}$  in height with a pitch of 5  $\mu\text{m}$  on a flat Si substrate. The *M. edulis* shell sample was provided by Pupa Gilbert (University of Wisconsin, Madison, WI) and was prepared by cutting with a jeweler's saw and polishing with alumina grit (Master Prep). Protein samples were prepared by Sun Choi (Lawrence Berkeley National Laboratory) by a recently developed printing process (51) using a 0.1% solution of bovine  $\gamma$ -globulin (Sigma-Aldrich) in deionized water on a silicon substrate. Finally, peptoid loop-displaying peptoid nanosheets

were prepared by Gloria Olivier (Lawrence Berkeley National Laboratory) by a previously described method (45) and dried onto a thermally evaporated gold substrate.

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