Polydiacetylene-based ultrastrong bioorthogonal Raman probes for targeted live-cell Raman imaging

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Live-cell Raman imaging based on bioorthogonal Raman probes with distinct signals in the cellular Raman-silent region (1800–2800 cm⁻¹) has attracted great interest in recent years. We report here a class of water-soluble and biocompatible polydiacetylenes with intrinsic ultrastrong alkyne Raman signals that locate in this region for organelle-targeting live-cell Raman imaging. Using a host-guest topochemical polymerization strategy, we have synthesized a water-soluble and functionalizable master polydiacetylene, namely poly(deca-4,6-diyndiynedioic acid) (PDDA), which possesses significantly enhanced (up to ~10⁴ fold) alkyne vibration compared to conventional alkyne Raman probes. In addition, PDDA can be used as a general platform for multi-functional ultrastrong Raman probes. We achieve high quality live-cell stimulated Raman scattering imaging on the basis of modified PDDA. The polydiacetylene-based Raman probes represent ultrastrong intrinsic Raman imaging agents in the Raman-silent region (without any Raman enhancer), and the flexible functionalization of this material holds great promise for its potential diverse applications.
Advances in optical microscopy in the past decades have played a key role in the development of modern biological sciences\(^1\)–\(^4\). Raman scattering-based vibrational microscopy has been considered one of the most promising and powerful cell imaging tools in recent years\(^1\)–\(^3\), attributed to the many intriguing features it holds, including direct visualization of detailed molecular structure information, quantitative relationship between signal intensity and substance concentration, and narrow-band enabled multi-color imaging\(^4\)–\(^11\). However, in comparison with other widely applied imaging techniques, especially fluorescence imaging, one of the major obstacles in practicing Raman microscopy in the biological sciences is the intrinsically poor cross-section of Raman scattering of either endogenous cellular components or exogenous vibrational probes. Many techniques and strategies, such as surface-enhanced Raman scattering (SERS)\(^12\)–\(^14\),\(^18\)–\(^20\) and coherent anti-Stokes Raman scattering (CARS)\(^21\)–\(^24\), and stimulated Raman scattering (SRS)\(^25\)–\(^28\), have been developed to amplify the Raman signals of Raman probes or biomolecules. In particular, SRS imaging that can boost vibrational excitation represents a dramatic advance of detectability and imaging speed over conventional spontaneous Raman scattering. SRS also enjoys additional advantages, such as visualization of detailed distribution of biomolecules like lipids and proteins, pinhole-less three-dimensional optical sectioning, non-invasive observation and deep tissue penetration, and free from non-resonant background. However, to obtain effective SRS imaging in living cells requires coupling this optical imaging technique with intrinsic Raman-active molecular probes. Raman reporters with inherent strong Raman scattering intensity are still urgently needed to enable facile Raman imaging with high imaging quality.

Biological molecules in a cell, such as lipids, proteins, and nucleic acids are Raman active, exhibiting intense Raman signals in the regions of 400–1800 cm\(^{-1}\) and 2800–3100 cm\(^{-1}\). To efficiently eliminate interference of the endogenous cellular background, Bioorthogonal Raman probes with distinctive signals in the cell-silent vibrational region (1800–2800 cm\(^{-1}\)), including isotopes (C–D), azides, and triple bonds (C≡C and C≡N)-containing molecules, have been widely employed for labeled Raman imaging. Among the existing molecular Raman reporters, alkynes are the most promising candidates for live-cell imaging, because of their easy accessibility, minimal toxicity, and comparatively large Raman scattering cross-section. Since the initial demonstration of Raman bioimaging using 5-ethyl-2′-deoxyuridine (EdU)\(^30\), numerous alkyn-containing molecules have been exploited as bioorthogonal Raman probes for cell imaging by Raman microscopy\(^30\)–\(^36\). However, the Raman signal intensity of C≡C bond stretching is still far from ideal and the detection sensitivity is very limited for the existing alkynes\(^27\),\(^28\). Polydacetylenes, a class of conjugated polymers with an alternating ene-yne backbone structure, naturally endow the polymer a metallic appearance and shiny gold color, as displayed in Fig. 2d. Unlike most known polydacetylenes that are insoluble in common solvents, the propionic acid substituents of the PDDA give the polymer high solubility in aqueous solutions. Taking advantage of this aqueous solubility, PDDA can be isolated from the host–guest co-crystals as a pure polymer by extensive acid-base rinsing. After being extracted from its crystalline lattice, the isolated polymer shows very good solubility in pure water and DMSO and interestingly, forms transparent aqueous solutions showing a remarkable chromism in the range of pH 6–8 (Fig. 2c and Supplementary Fig. 2a, b). At basic conditions (pH > 8), the PDDA water solution is yellow in color and has a maximum absorption of 460 nm. Density function theory (DFT) calculations suggest that the two adjacent substituents are perpendicular to each other in their ionized forms, therefore the conjugation of the backbone is minimized in a twisted conformation (Supplementary Fig. 2c). At lower pH values (pH < 6), the solution turns red.
and the maximum absorption shifts to higher wavelengths. The red shift of the absorption corresponds to an increase of the backbone conjugation and planarity of PDDA, which may be induced by hydrogen bond-assisted inter-side chain assembly (Supplementary Fig. 2d).

Comprehensive characterization of PDDA in solution. The exceptional solubility of PDDA allows us to conduct a comprehensive exploration of the structure of PDDA and to assess the suitability of applying PDDA as a bioorthogonal Raman probe using solution-based methods, which has not yet been achieved by any other polydiacetylene. The Raman spectra of the polymer co-crystals and the polymer aqueous solution are similar, both displaying an intense peak corresponding to the $\text{C}≡\text{C}$ bond stretches (2045 cm$^{-1}$ for co-crystals and 2120 cm$^{-1}$ for solution) in the cellular silent region (Fig. 3a). The $\text{C}≡\text{C}$ bond Raman peak of the PDDA solution does not change upon irradiation under excitation with different wavelengths (785, 532, and 488 nm, Supplementary Fig. 3). The solution-phase $^1\text{H}$ NMR spectrum of the isolated polymer in D$_2$O shows only two broad proton peaks at 2.46 and 2.74 ppm, assigned to the $\alpha$ and $\beta$ methylene protons of the side chains of the polymer, Fig. 3b and Supplementary Fig. 4. The solution-phase $^{13}\text{C}$ NMR spectrum of PDDA does not show the two acetylene peaks at 64.7 and 77.5 ppm found in the monomer spectrum. Instead, two new broad peaks at 99.9 and 129.8 ppm, corresponding to the $\text{sp}^2$-carbon and $\text{sp}$-carbon of PDDA, respectively, emerge from the polymer spectrum, unambiguously proving the successful topochemical synthesis and complete dissolution of the polymer. Gel permeation chromatography (GPC) with basic aqueous medium as the mobile phase (Supplementary Fig. 5) suggests that the prepared PDDA has a number-average molecular weight ($M_n$) of 2.4 × 10$^4$ g mol$^{-1}$. Its molecular weight distribution (PDI) of 1.45 is much narrower than other polydiacetylenes reported to date (Supplementary Table 1), suggesting the superior polymer quality achieved by the host–guest co-crystal strategy.

Fig. 1 Polydiacetylene-based Raman probes for targeted live-cell Raman imaging. a Topochemical polymerization for the preparation of PDDA. b Overlaid Raman spectra of individual DMSO solution of PDDA and a series of representative Raman probes, including 1. ethynylbenzene; 2. EdU; 3. diphenyl phosphorazidate; 4. diphenylbutadiyne; 5. benzonitrile; 6. 5-bromopentanenitrile; 7. deca-4,6-diynedioic acid. The probe structures are shown in Supplementary Fig. 1. The table inset lists the concentration of Raman-active bonds ($\text{C}≡\text{C}$, $\text{C}≡\text{N}$, or azide) in each solution. The normalization is based on the absolute Raman intensity of each spectrum. c Schematic illustration of side chain modification of PDDA for subcellular organelle targeting Raman imaging.
Ultrastrong Raman intensity of PDDA. PDDA exhibits an exceptionally intense Raman peak at 2120 cm$^{-1}$ of PDDA in solution, resulting from the large polarizability of polydiacetylenes$^{46,47}$. In addition, the highly planar polymer backbone of PDDA further assists the stretching vibration of alkyne units in the backbone. To better calibrate the outstanding alkyne Raman intensity of PDDA, we normalized all the Raman spectra by C≡C bond concentration. The concentration of C≡C bonds in a PDDA solution was identical to the concentration of the repeating unit of PDDA. We used diphenylbutadiyne (DPY) as an internal standard to quantify the Raman intensity of PDDA, since the more commonly used EdU has a similar Raman peak at 2122 cm$^{-1}$ that overlaps with the alkyne peak of PDDA (~2120 cm$^{-1}$). DPY is a strong alkyne Raman tag, and its alkyne Raman peak (2220 cm$^{-1}$) can be clearly separated from that of PDDA. Figure 3c shows the Raman spectra of DMSO solutions of 50 mM DPY (100 mM C≡C bond) mixed with different concentrations of PDDA (displayed as concentration of C≡C bonds). The Raman intensity of PDDA shows a perfect linear concentration dependence (Fig. 3d), extending to when the C≡C bond concentration in PDDA solutions is as low as 1 μM, with a signal-to-noise ratio of over 10. Such a detection limit corresponds to a PDDA chain concentration of 8 nM, calculated based on the average polymerization degree of 120, which is far more sensitive than any other alkyne-containing molecule to date.

The Raman intensity of an alkyne-containing molecular Raman probe is typically evaluated by its relative Raman intensity versus EdU (RIE). We next calculated the RIE value of PDDA based on the intensities of the alkyne Raman peaks and the concentrations of the C≡C bonds of PDDA and DPY, using the below equation

$$\text{RIE}_{\text{PDDA}} = \text{RIE}_{\text{DPY}} \times \frac{I_{\text{PDDA}}/C_{\text{PDDA}}}{I_{\text{DPY}}/C_{\text{DPY}}}$$

(1)

where $C$ is the alkyne concentration in each solution, $I$ is the the alkyne Raman peak intensity of each solution. RIE$_{\text{DPY}}$ is the relative alkyne Raman intensity of DPY versus EdU as literature reported$^{31}$. We measured the RIE values of PDDA in DMSO solution at three different Raman excitation wavelengths (488, 532, and 785 nm), as shown in Supplementary Fig. 6a. The C≡C bond-normalized RIE value (RIE per C≡C bond, or RIE per repeating unit) were greater than 100 when measured at 785 nm Raman excitation. However, the C≡C bond-normalized RIE values exceeded $10^4$ when we measured the Raman intensity with a Raman excitation wavelength of 488 nm or 532 nm. Since PDDA had a maximum absorption at 476 nm in DMSO (Supplementary Fig. 6b), the Raman excitation at 488 nm, a wavelength close to its absorption maximum, was able to further enhance the Raman intensity through pre-resonance Raman scattering$^{48,49}$, resulting in a tremendously high RIE value of $2.3 \times 10^4$ (C≡C bond-normalized).

We have also investigated how RIE value changes with the chromism of PDDA. RIE values of PDDA in a pH range from 5 to 9 were measured using a series of Raman excitation wavelengths (488, 532, and 785 nm). The results showed that the RIE values of PDDA exhibited a prominent increase with decreased pH values when excited by the 532 nm laser, consistent with the change of the absorption of PDDA at 532 nm (Supplementary Fig. 6c). The change of the RIE values of PDDA was much weaker when excited by the 488 nm or 785 nm laser, or by the 853 nm and 1040 nm Pump/Stokes laser beams for SRS imaging (Supplementary Fig. 7). As a comparison, the C≡C bond-normalized RIE value of the monomer DDA was measured to be ~0.85 regardless the Raman excitation wavelength (Supplementary Fig. 8), clearly evidencing the synergistic enhancement of the Raman intensity by both extension of conjugation length and pre-resonance Raman scattering.

Figure 4 summarizes the C≡C bond-normalized RIE values of PDDA (measured in DMSO by 488 nm excitation) and other typical alkyne-based Raman probes. As a comparison, the C≡C bond-normalized RIE values of another class of conjugated polymer poly(phenylene ethynylene) (PPE) are reported to be less...
than 10⁻³⁶. In addition, the Raman intensity of polyynes containing directly conjugated C≡C bonds also increases nonlinearly with the increase of the molecular length²⁸. However, the synthesis of polyynes is highly difficult so that the length of polyynes is greatly limited⁵⁰. The C≡C bond-normalized RIE value of PDDA is over 300 times higher than that of dodecahexayne, the longest polyyne used as a Raman probe, demonstrating the enormous advantage of PDDA over other alkynes in terms of Raman intensity.

**PDDA-derivatives as super Raman probes.** The outstanding Raman sensitivity and the reactivity of propionic acid substituents of PDDA make it a potential precursor to a variety of bioorthogonal vibrational tags, through on-demand side chain modification using diverse biological targeting groups. We obtained three PDDA derivatives through modifications with different functional groups (Fig. 5), one with a tertiary amine (P₂, for lysosome targeting) and the other two with targeting peptides (P₃ for mitochondria and P₄ for nucleus targeting), for subcellular organelle targeting in living cells. The tertiary amine was coupled directly to the carboxyl group on PDDA side chains following a typical condensation procedure, and we used Mal-PEG-NH₂ as a linker to conjugate PDDA with the two targeting peptides (Supplementary Fig. 9). FTIR spectra of the PDDA derivatives (Supplementary Fig. 10) displayed the newly formed amide bonds on the side chain of PDDA as well as PEG-related peaks. The significant red shifts of the absorption peaks of the PDDA derivatives (Supplementary Fig. 11) also suggested the formation of amide groups, which reduced electrostatic repulsion of the side chain and resulted in a more planar polymer backbone in solution through intramolecular hydrogen bonds. In addition, the Raman spectra of the PDDA derivatives (Supplementary Fig. 12) each still exhibited a strong C≡C bond peak at 2120 cm⁻¹,
Targeted Raman imaging in fixed and living cells. To validate the superb Raman characteristics of PDDA-based vibrational tags, we performed hyperspectral SRS imaging of HeLa cells labeled with P2, P3, and P4. Figure 6a, b shows the schematic illustration of the hyperspectral SRS imaging system, which was implemented to perform molecular imaging in living cells. We used confocal laser scanning microscopy (CLSM) to image the intracellular distribution of the polymers within HeLa cells. After being co-incubated with standard fluorescence organelle tracking dyes, the fluorescence of the respective PDDA derivative overlapped precisely with that of the corresponding tracker (Supplementary Fig. 14), confirming the successful targeting of PDDA-derived tags to the specific sites. The cell uptake and targeted intracellular trafficking for PDDA-based macromolecular vibrational tags are therefore proved efficient and precise.

Supplementary Fig. 15, the cells stained with 50 μM of P4 clearly showed a good SRS imaging at a very low Pump/Stokes laser power setting of 10/30 mW, i.e., 10 mW for the Pump laser and 30 mW for the Stokes laser, an integration time of 10 μs, and based on an average of 10 imaging frames. As a comparison, the cells stained with EdU remained undetectable at this condition, and the SRS imaging on these cells could only be obtained at a much higher power setting of laser beams (Pump/Stokes 50/100 mW), a longer integration time of 40 μs, and based on a larger number of 50 imaging frames for averaging. This parallel SRS imaging study on cells stained with EdU and P4 unambiguously demonstrated how PDDA-based probes outperformed typical existing probes for SRS.

Figure 7 illustrated hyperspectral SRS imaging of living HeLa cells stained with the above PDDA-derived tags (P2, P3, and P4) at Pump/Stokes 10/30 mW, integration time of 40 μs, and with an average of 10 frames. The cellular distribution of the polymer tags and inherent lipids and proteins of cells were collected using 2120 cm$^{-1}$ for the C≡C stretching and 2850 cm$^{-1}$ for the C–H vibrations. The 2120 cm$^{-1}$ channel of the SRS images of living HeLa cells treated with different polymer tags clearly visualized the accumulation of the corresponding tag in lysosomes (P2, Fig. 7, first row), mitochondria (P3, Fig. 7, second row), and nuclei (P4, Fig. 7, third row) of the cells, respectively. In addition, we used commercial fluorescence organelle trackers to co-incubate PDDA-based probes in corresponding cells, and conducted SRS imaging of PDDA-based probes and two-photon fluorescence imaging (TPFI) of commercial organelle trackers on the same instrument. The parallel comparison between the TPFI images of the commercial trackers and the SRS images of the PDDA-based probes (Supplementary Fig. 16) clearly evidences the specificity of these conjugated probes to the desired targets.

On the other hand, the 2850 cm$^{-1}$ channel for the C–H vibrations clearly illustrated the healthy cell morphology and nuclear shape. The localization information of targeted organelles, together with the distribution of endogenic biomolecules such as lipids and proteins, provided a comprehensive cellular information by SRS imaging. To further confirm the biocompatibility of these probes, we studied the cytotoxicity of all PDDA-based probes using MTT assays (Supplementary Fig. 17). When the HeLa cells were treated with 50 μM of P2, P3, or P4, the cell viabilities remained above 90% for all three probes after 48 h of treatment. It should be noticed that when stained with P2 or P3, the cells just needed to be incubated with the probe (50 μM) for less than 6 h, and staining cells with P4 took a relatively longer treatment time of 48 h. In a more stressful condition, we treated the cells with 100 μM of PDDA-based probes, the cell viabilities after 48 h of incubation were all above 80%. The MTT experiment results clearly evidenced the biocompatibility of the PDDA-based Raman probes, and they are safe and applicable for Raman imaging of live cells.

In summary, we demonstrate that polydiacetylene derivatives with ultrastrong alkyne Raman signals can be used as intrinsic Raman reporters for live-cell Raman imaging. The C≡C bond-normalized RIE value of PDDA can reach 2.3 × 10^4, which is up to 10^4 fold higher than other existing alkynes. Functionalizing side chains of PDDA endows us a wide variety of polydiacetylene-based super vibrational tags that are advantageous as Raman-active nanomaterials for distinct imaging application. In addition, PDDA can be used in conjunction with other Raman enhancing techniques, such as SERS, to achieve even stronger Raman-active nanomaterials that are expected to break the existing sensitivity ceiling of Raman imaging. Developing PDDA-based multiplex Raman probes, as well as exploiting more applications of Raman imaging on the basis of PDDA derivatives is currently on the way.
Methods

Preparation of PDDA. In a typical run, 27.0 mg of host 1 was dissolved in 30 mL methanol with sonication to give a colorless solution. 19.4 mg of DDA was then added to the above solution and the mixture was centrifuged at 2400 × g for 10 min to remove possible dust impurities. The resulting solution was transferred to a crystallization dish and kept in 4 °C. The slow evaporation of the solvent generated white needle-like crystals in the crystallization dish. Heating the crystals in an oven set at 120 °C for 12 h turned the color of the crystals from pink to dark red then metallic gold. The final product was characterized with X-ray single crystal diffraction and Raman spectroscopy, which confirmed that the gold-colored crystals were PDDA-host 1 co-crystals. The crystallographic data have been deposited in the Cambridge Crystallographic Data Center (CCDC) with an accession number of 1900689.

X-ray single crystal diffraction. Crystals were selected and mounted on glass fibers using epoxy glue. The crystals were optically centered on a Bruker AXS Smart CCD diffractometer and diffraction data were collected using a Siemens graphite-monochromated Mo radiation tube. The unit cells were determined by a least-squares analysis using the SMART software package. The structures were solved and refined with standard SHELX procedures.

PDDA isolation. Dispersing the polymerized co-crystals in 0.1 M aqueous NaOH and extensive rinsing for 1 h yielded a yellow suspension, followed by removing undissolved host 1 by filtration. The basic PDDA solution was then acidified with hydrochloric acid to pH 1. The solution turned cloudy and was kept at 4 °C overnight. Red solid precipitated from the solution, which was collected and washed with dilute hydrochloric acid and methanol. Drying the solid in vacuum at room temperature gave dark red PDDA products. 

Nuclear magnetic resonance (NMR). All NMR spectra were recorded on an Agilent 400-MR 400 MHz spectrometer operated in the Fourier transform mode. Methanol-d4 and D2O were used as solvents. In case the PDDA polymer has a low solubility in D2O at neutral or acidic pH, the sample in D2O is basified with sodium carbonate to achieve a satisfactory polymer concentration for NMR measurements.

Fig. 7 SRS images of HeLa cells treated with 50 μM of P2, P3, and P4, respectively. Images shown from left to right are the alkyne (2120 cm⁻¹), lipids (2850 cm⁻¹), and merged images. Scale bar: 10 μm.

Fig. 6 Illustration of the SRS system and SRS images of fixed HeLa cells. a Schematic illustration of the instrumental setup of the SRS imaging system. EOM: electro-optic modulator; DM: dichroic mirror; GM: galvanometer; F: optical filters; PD: photodiode. b Stimulated Raman transition process for vibrational imaging of HeLa cells stained by PDDA-based tags. c SRS images (2120 cm⁻¹) of fixed HeLa cells treated with 50 μM of P2, P3, and P4, respectively. Scale bar: 10 μm. d SRS spectrum of the HeLa cell sample stained with P2 in the red box region in (c), the spontaneous Raman spectrum of P2 in aqueous solution shown as a reference.
Spontaneous Raman spectroscopy. Raman detection unit comprised of a spectrometer (Princeton Instruments Acton series SP-2300) and a liquid nitrogen cooled CCD camera (Princeton Instruments PyLoN1000R-xCcdCam) is used for Raman spectra measurements. A Sapphire SF 488 nm laser is applied to the sample with 2 mW laser power.

Relative Raman intensity measurements. 10 µL of the mixed solution of PDDA and DPY was sealed on a glass slide and then measured with a confocal Raman spectrometer. Optimized signal acquisition conditions for the DPY standard were applied. The peaks intensities of alkyne in PDDA and DPY were used to evaluate the relative Raman intensity. RIEPDDA could be calculated with Eq. (1). As we observed a linear relationship between relative IPEPDDA with CPEPDDA using DPY with fixed concentration as a standard, the above Eq. (1) could be simplified as

\[ \text{RIE}_{\text{PDDA}} = \text{RIE}_{\text{DPY}} \times \text{slope} \]  

(2)

All concentrations were quantified by the alkyne. The RIE values of the molecules containing multiple C≡C bonds were divided by the number of C≡C bonds prior to the comparison.

Cell culture for imaging. HeLa cancer cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). For SRS imaging experiments, PDDA-based Raman probes were incubated with cells in a glass-bottom petri dish (3.5 cm) and prepared with P3 and P4 were added to 1.5 mL DMEM culture medium containing 10% FBS and antibiotics (penicillin/streptomycin) to reach a final concentration of 50 µM (concentration based on C≡C in PDDA) and the used as a working solution. For lysosome-staining, the cells were incubated with the P2-containing culture medium for 2 h and then washed with 1× PBS for 3 times. Subsequently, 1 mL culture medium was added for confocal fluorescence imaging or two photon fluorescence imaging. Lyso-Tracker Red was then added to stain the cells following a standard protocol. The cells were then washed with 1× PBS for 3 times and 1 mL 1× PBS was added to immerse the cells for imaging. Similar procedures were conducted for P3 to stain mitochondrion and P4 to stain nucleus. The incubation procedure was done at 37 °C for about 30 min. After 10 min of incubation, the samples were washed with 1× PBS for 3 times and 1 mL 1× PBS was added to immerse the cells for imaging.

Stimulated Raman scattering imaging. The dual-output femtosecond laser (InSight DeepSee, Spectra-Physics) provided both pump (680–1300 nm, ~120 fs) and Stokes (1040 nm, ~220 fs) laser beams with a repetition rate of 80 MHz. The Stokes beam was modulated by a resonant electro-optical modulator (EO-AM-R-C2, Thorlabs) at 10.55 MHz with modulation depth about 95%. The temporal overlap between pump and Stokes pulse trains was ensured with a 1.5 m DMEM evan film. The pump beam was spatially overlapped with Stokes beam by a dichroic mirror (DMSP1000L, Thorlabs). Pump and Stokes pulses were linearly chirped to ~3 ps by 64 cm long SF57 glass rod. The hyperspectral image stack was obtained by scanning the relative time delay between pump and Stokes pulses. The two laser beams were guided into a laser scanning microscope equipped with a two-axis galvanometer (GV5002, Thorlabs). A x60 water immersion objective (NA 1.1, LUMFLN 60XW, Olympus) was used for all cell imaging, and the transmitted pump beam was collected with a high NA oil condenser and detected by a large area Si photodiode (S3994-01, Hamamatsu). Two shortpath filters (ET980SP, Chroma) were installed in front of the photodiode to completely block the Stokes beam. The SRS signal detected by PD was amplified by an in-house built 10.5 MHz resonant amplifier and demodulated by a digital lock-in amplifier (LIA, HF211, Zurich Instrument). The signal integration time for a pixel of 250×250 nm was 10 μs, the typical acquisition time for each frame with an area of 100 μm × 100 μm was around 1.6 s. To further increase the signal-to-noise ratio of the image, we averaged the results of 10 frames for the final image and the total time for each image in the manuscript was less than 30 s.

Data availability
The X-ray crystallographic coordinates for structures in this study have been deposited at the Cambridge Crystallographic Data Centre (CCDC), under a deposition number 1900689. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. All relevant data are available within the Article, Supplementary Information, Source Data file or available from the authors upon reasonable request.

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References
1. Kubitscheck, U. Fluorescence microscopy: from principles to biological applications (John Wiley & Sons, 2017).
2. Lichtman, J. W. & Conchello, J. A. Fluorescence microscopy. Nat. Methods 2, 910 (2005).
3. Min, W., Freudiger, C. W., Lu, S. & Xie, X. S. Coherent nonlinear optical imaging beyond fluorescence microscopy. Annu. Rev. Phys. Chem. 62, 507–530 (2011).
4. Abramczyk, H. & Brozek-Pluska, B. Raman imaging in biochemical and biomedical applications. Diagnosis and treatment of breast cancer. Chem. Rev. 113, 5766–5781 (2013).
5. Smith, R., Wright, K. L. & Ashton, L. Raman spectroscopy: an evolving tool (Cambridge University Press, 2017).
6. Freudiger, C. W. et al. Label-free biomedical imaging with high sensitivity by stimulated Raman scattering microscopy. Science 322, 1857–1861 (2008).
7. Caila-May, D., Zheng, X. S., Weber, K. & Popp, J. Recent progress in surface-enhanced Raman spectroscopy for biological and biomedical applications: from cells to clinics. Chem. Soc. Rev. 46, 2395–2401 (2017).
8. Okuno, M. et al. Quantitative CARS molecular fingerprinting of single living cells with the use of the maximum entropy method. Angew. Chem. Int. Ed. 49, 6733–6740 (2010).
9. Pezacki, J. P. et al. Chemical contrast for imaging living systems: molecular vibrations drive CARS microscopy. Nat. Chem. Biol. 7, 137 (2011).
10. Fu, D. et al. Quantitative chemical imaging with multiplex stimulated Raman scattering microscopy. J. Am. Chem. Soc. 134, 3623–3626 (2012).
11. Wei, L., Yu, Y., Shen, Y., Wang, M. C. & Min, W. Vibrational imaging of newly synthesized proteins in live cells by stimulated Raman scattering microscopy. Proc. Natl Acad. Sci. USA 110, 11226–11231 (2013).
12. Kneipp, J., Kneipp, H. & Kneipp, K. SERS—a single molecule and nanoscale tool for bioanalytics. Chem. Soc. Rev. 37, 1052–1060 (2008).
13. Haut, E. O., Wu, P. T., Dasari, R. R. & Lim, D. K. High resolution live cell Raman imaging using subcellular organelle-targeting SERS-sensitive gold nanoparticles with highly narrow intra-nanogap. Nano. Lett. 15, 1786–1787 (2015).
14. Nam, J. M., Oh, J. W., Lee, H. & Suh, Y. D. Plasmonic nanogap-enhanced Raman scattering with nanoparticles. Acc. Chem. Res. 49, 2746–2755 (2016).
15. Wang, X. et al. Quantitative SERS of biological samples using a spatiotemporal multiplexed microarray. J. Am. Chem. Soc. 134, 1819–1926 (2012).
16. Kim, J. et al. Smart SERS hot spots: single molecules can be positioned in a plasmonic nanojunction using host–guest chemistry. J. Am. Chem. Soc. 140, 4705–4711 (2018).
17. Khalil, M. & Nie, S. M. Single-molecule and single-nanostructure SERS: from fundamental mechanisms to biomedical applications. Chem. Rev. 117, 4841–4928 (2017).
18. Zumbusch, A., Holton, G. R. & Xie, X. S. Three-dimensional vibrational imaging by coherent anti-Stokes Raman scattering. Phys. Rev. Lett. 82, 4142 (1999).
19. Xie, X. S., Yu, J. & Yang, W. Y. Living cells as test tubes. Science 312, 228–230 (2006).
20. Arzumanyan, G. M. et al. Highly sensitive coherent anti-stokes raman scattering imaging of protein crystals. J. Am. Chem. Soc. 138, 13457–13460 (2016).
21. Krafft, C., Schie, I., Meyer, T., Schmitt, M. & Popp, J. Developments in spontaneous and coherent Raman scattering microscopic imaging for biomedical applications. Chem. Soc. Rev. 45, 1819–1849 (2016).
22. Chiang, I. et al. Video-rate molecular imaging in vivo with stimulated Raman scattering. Science 330, 1368–1370 (2010).
23. Wu, D., Yang, W. & Xie, X. S. Label-free Imaging of neurotransmitter acetylcholine at neuromuscular junctions with stimulated Raman scattering. J. Am. Chem. Soc. 139, 585–586 (2017).
24. Wei, L. et al. Super-multiplex vibrational imaging. Nature 544, 465 (2017).
25. Wu, F. et al. Supermultiplexed optical imaging and barcoding with engineered polynye. Nat. Methods 15, 194 (2018).
26. Wei, L. et al. Live-cell bioorthogonal chemical imaging: stimulated Raman scattering microscopy of viral probes. Acc. Chem. Res. 49, 1494–1502 (2016).
27. Yamashita, H. et al. Imaging of EdU, an alkyne-tagged cell proliferation probe, by Raman microscopy. J. Am. Chem. Soc. 133, 6102–6105 (2011).
31. Yamakoshi, H. et al. Alkynyl-tag Raman imaging for visualization of mobile small molecules in live cells. *J. Am. Chem. Soc.* **134**, 20681–20689 (2012).
32. Hong, S. et al. Live-cell stimulated Raman scattering imaging of alkynyl-tagged biomolecules. *Angew. Chem. Int. Ed.* **53**, 5827–5831 (2014).
33. Song, Z. L. et al. Alkyne-functionalized superstable graphitic silver nanoparticles for Raman imaging. *J. Am. Chem. Soc.* **136**, 13558–13561 (2014).
34. Wei, L. et al. Live-cell imaging of alkynyl-tagged small biomolecules by stimulated Raman scattering. *Nat. Methods* **11** (2014).
35. Ando, J. et al. Alkyne-tag SERS screening and identification of small-molecule-binding sites in protein. *J. Am. Chem. Soc.* **138**, 13901–13910 (2016).
36. Li, S. et al. Conjugated polymer with intrinsic alkyne units for synergistically enhanced Raman imaging in living cells. *Angew. Chem. Int. Ed.* **56**, 13453–13458 (2017).
37. Wegner, G. Topochemical polymerization of monomers with conjugated triple bonds. *Makromol. Chem.* **154**, 35–48 (1972).
38. Baughman, R. Solid-state synthesis of large polymer single crystals. *J. Polym. Sci., Polym. Phys. Ed.* **12**, 1511–1535 (1974).
39. Enkelmann, V. & Wegner, G. Crystallographic analysis of a topochemical polymerization. *Angew. Chem. Int. Ed.* **16**, 416 (1977).
40. Sun, A., Lauher, J. W. & Goroff, N. S. Preparation of poly(diododiacetylene), an ordered conjugated polymer of carbon and iodine. *Science* **312**, 1030–1034 (2006).
41. Lauher, J. W., Fowler, F. W. & Goroff, N. S. Single-crystal-to-single-crystal topochemical polymerizations by design. *Acc. Chem. Res.* **41**, 1215–1229 (2008).
42. Reppy, M. A. & Pindzola, B. A. Biosensing with polydiacetylene materials: structures, optical properties and applications. *Chem. Commun.* **42**, 4317–4338 (2007).
43. Alloisio, M. et al. Solution spectroscopic properties of polyDCHD-HS: a novel highly soluble polydiacetylene. *J. Chem. Soc., Perkin Trans. 2*, 146–152 (2001).
44. Xu, R., Schweizer, W. B. & Frauenrath, H. Soluble poly (diacetylene) using the perfluorophenyl-phenyl motif as a supramolecular synthon. *J. Am. Chem. Soc.* **130**, 11437–11445 (2008).
45. Luo, L. et al. Poly (diododiacetylene): Preparation, isolation, and full characterization of a very simple poly (diacetylene). *J. Am. Chem. Soc.* **130**, 7702–7709 (2008).
46. Perpète, M. A., Champagne, R. T. & Kirtman, B. Linear and nonlinear polarizabilities of polydiacetylene and polybutatriene chains: an ab initio coupled Hartree–Fock investigation. *J. Chem. Phys.* **107**, 2463–2480 (1997).
47. Materny, A., Chen, T., Vierheilig, A. & Kierfer, W. A review on linear and nonlinear resonance Raman spectroscopy of the conjugated system polydiacetylene. *J. Raman Spectrosc.* **32**, 425–445 (2001).
48. Hirakawa, A. Y. & Tsobo, M. Molecular geometry in an excited electronic state and a preresonance Raman effect. *Science* **188**, 359–361 (1975).
49. Wei, L. & Min, W. Electronic preresonance stimulated Raman scattering microscopy. *J. Phys. Chem. Lett.* **9**, 4294–4301 (2018).
50. Chalifoux, W. A. & Tykwinski, R. R. Synthesis of polyynes to model the sp-carbon allotrope carbyne. *Nat. Chem.* **2**, 967 (2010).

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**Author contributions**

S.T., H.L., and Z.L. contributed equally. S.T., H.L., and Z.L. designed and performed the experiments. H.T. and Y.C. participated some of the SERS work. M.Y. and Y.G. assisted with some of the synthesis and crystallization work. X.Y., S.W., and F.M designed the experiments. J.W.L., P.W., and L.L. conceived and obtained funding for the project, oversaw the research and wrote the paper. All authors discussed the results and commented on the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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