Efficient optical plasmonic tweezer-controlled single-molecule SERS characterization of pH-dependent amylin species in aqueous milieus

Wenhao Fu  
Hong Kong University of Science and Technology

Huanyu Chi  
Hong Kong University of Science and Technology

Xin Dai  
Hong Kong University of Science and Technology

Hongni Zhu  
Hong Kong University of Science and Technology

Vince St. Mesias  
Hong Kong University of Science and Technology  https://orcid.org/0000-0002-6206-8014

Wei Liu  
The University of Hong Kong  https://orcid.org/0000-0002-5294-7963

Jinqing Huang (jqhuang@ust.hk)  
The Hong Kong University of Science and Technology  https://orcid.org/0000-0001-6865-8528

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Abstract

It is challenging to characterize single or a few biomolecules in physiological milieus without excluding the influences of surrounding molecules and micro-environments. Here we utilize optical plasmonic trapping to construct a dynamic nanocavity, which reduces the diffraction-limited detection volume and provides reproducible electromagnetic field enhancements for high-throughput single-molecule surface-enhanced Raman spectroscopy (SERS) characterizations in crowded aqueous environments. Specifically, we study human Islet Amyloid Polypeptide ( amylin, hIAPP) under different physiological pH, where two types of low-populated transient species of hIAPP are characterized as β-sheet-containing structures among its predominant helix-coil monomers, driving irreversible amyloid developments even after a post-adjustment of pH. Our results might provide profound mechanistic insight into the pH-regulated amyloidogenesis and open a new door to investigate complex biological processes at the single-molecule level.

Introduction

By removing ensemble averaging, single-molecule techniques discern the signal of individual molecules to unveil hidden details and revolutionize our understandings of physics, chemistry, and biology \(^1\)–\(^5\). Of particular interest is the characterization of single or a few biomolecules, such as intrinsically disordered proteins (IDPs), in an aqueous milieu containing hydrogen-bonding, electrostatic, and hydrophilic-hydrophobic interactions like the complex biological systems. For example, human Islet Amyloid Polypeptide (hIAPP) is a typical IDP that lacks stable secondary structures in the secretory granules of pancreatic β-cells at around pH 5.5 and millimolar concentration \(^6\). Whereas, hIAPP misfolds and assembles in certain extracellular compartments at pH 7.4 and micromolar concentration \(^5\), generating low-populated, heterogenous, and transient species in a dynamic equilibrium mixture to induce cytotoxicity and initiate the growth of amyloid fibrils \(^7,8\), which are commonly found in type II diabetes patients \(^9\). However, the molecular mechanism of the amyloid formation, especially at the early stage, remains unclear since it is challenging to probe these rare species without excluding the influences of surrounding molecules and micro-environments \(^10\). Although significant advancements have been achieved by single-molecule fluorescence methods, the structural determination is limited by fluorophore labeling, and the single-molecule scheme is restricted to ultra-dilution and/or molecular immobilization because the diffraction-limited detection volume cannot be further reduced \(^11,12\).

Upon excitation of the localized surface plasmonic resonance (LSPR) in metallic nanostructures, surface-enhanced Raman spectroscopy (SERS) can overcome the optical diffraction-limit and recognize endogenous molecular vibrations \(^13,14\) for chemical bond imaging \(^15\), catalytic reaction monitoring \(^16\), and DNA sequencing at the single-molecule level \(^17\). Since water generates weak Raman signals as background, it is feasible to operate in aqueous conditions. Nevertheless, the applications of single-molecule SERS are mainly reported at ultralow concentrations (aM-nM) and/or conducted on dry states without solvents \(^18–20\), while the characterization of a single biomolecule involving in molecular
interactions from aqueous environments at relatively high concentrations (µM-mM for physiological protein assembly and enzyme activity) remains challenging\textsuperscript{11}, considering the following issues: (i) Poor spatial control of the SERS active-detection volume\textsuperscript{13}. (ii) Low stability of the plasmonic substrates suspended in liquid (e.g., nanoparticle colloids in Brownian motion)\textsuperscript{21}. (iii) Practical difficulties in fabricating precise nanostructures and locating biomolecules in the highly confined local electromagnetic field at the nanoscale\textsuperscript{5,22}.

Recently, there has been an emerging trend to integrate optical trapping and optical plasmonic trapping techniques in SERS detection systems\textsuperscript{17,23–25}, which enables the mechanical manipulation of nanostructured substrates in solution environments to improve the measurement reproducibility, stability, and efficiency\textsuperscript{26,27}. Optical tweezers utilize the tightly focused laser beam to exert optical force to attract objects at focus against Brownian motion\textsuperscript{28}. The position control of the trapped objects can be further enhanced and confined near plasmonic metallic nanostructures beyond the diffraction limit of the laser beam, known as optical plasmonic trapping\textsuperscript{28–32}, which is a promising tool to address the above challenges. Whereas the accessibility of existing optical plasmonic trapping-integrated SERS platforms is limited by the complicated fabrication of precise nanostructures, the special instrument to locate plasmonic nanocavity, and the potential perturbation of molecular immobilizations on the trapped objects. Hence, it awaits further developments to exploit the power of optical plasmonic trapping to facilitate the single-molecule SERS detections in complex aqueous milieus.

Here, we developed a convenient optical plasmonic tweezer-coupled SERS platform for efficient single-molecule SERS characterization of biomolecules in aqueous milieus. We constructed a simple plasmonic junction between silver nanoparticle (AgNP)-coated silica microbeads to exert optical plasmonic trapping force upon excitation to control an AgNP in close contact of the coated AgNP at the junction, creating a dynamic and reproducible SERS-active nanocavity to sandwich the freely diffusing biomolecules in confined detection volume. After the verification of its single-molecule sensitivity by the bi-analytes SERS approach (BiASERS), we studied the pH-dependent structural transition of Tyrosine and the conformational features of hIAPP species under two physiological pH conditions (pH 5.5 and pH 7.4) in microfluidic flow. We characterized two types of rare hIAPP species among the dominating monomers at the early stage during the irreversible long-term development of amyloid fibrils under pH 7.4 and a post-adjustment from pH 7.4 to pH 5.5, which might provide critical mechanistic insight into the pH-regulated amyloid aggregations. With high-throughput capability, this platform holds the promise to advance the solution-phase single-molecule vibrational characterization methods and probe a single biomolecule among overflowing background molecules in complex physiological environments.

Results

Creating dynamic nanocavity by optical plasmonic trapping for efficient single-molecule SERS detections in solutions
To develop an optical plasmonic tweezer-coupled SERS platform, we constructed a simple plasmonic junction between two AgNP-coated silica microbeads and placed it at the focus of two laser beams to trap an AgNP, which could form a SERS-active nanocavity among the trapped AgNP and the coated AgNP as illustrated in Fig. 1a. Supplementary Fig. 1 shows the experimental set-up combing a 532 nm (2.7 mW) excitation laser and a 1064 nm trapping laser (8.0 mW) via stereo double-layer-pathways in a microscope for simultaneous optical manipulations and spectroscopic measurements. First, silica microbeads were functionalized with amino groups to chemically coat AgNP in sparse distribution (Supplementary Fig. 2), which were further bonded with the 5’-thiol-modified oligonucleotide L1 and the 5’-thiol-modified oligonucleotide L2, respectively. Next, the oligonucleotide L3 was added to tether L1 and L2 by complementary base pairing to generate AgNP-coated bead assemblies, giving a nanogap of around 20 nm. These bead assemblies were then deposited on the bottom of a microfluidic chamber, as visualized in the microscopic images of Fig. 1b (zoom-in) and Supplementary Fig. 3 (zoom-out). Before the spectroscopic measurements, the solutions of analyte molecules and AgNP were injected into the microfluidic chamber in adjacent channels under a consistent flow rate. Upon laser irradiation, a freely diffusing AgNP could be trapped at the plasmonic junction of an AgNP-coated bead dimer to form a dynamic nanocavity among the trapped AgNP and the coated AgNP, sandwiching the analyte molecules in the confined detection volume with a further enhanced local field for sensitive SERS characterizations. Moreover, the AgNP could be trapped with the 1064 nm trapping laser (on state) and released without it (off state), which enables dynamic switching of the trapped AgNP for high-throughput sampling. (Fig. 1)

As a proof of concept, we switched the 1064 nm trapping laser between on and off states and utilized the 532 nm excitation laser to conduct continuous SERS measurements at the plasmonic junction of an AgNP-coated bead dimer in 500 nM Nile Blue A (NBA) solution, where the formation of dynamic nanocavity could be controlled by the optical plasmonic trapping of an AgNP in close contact of the coated AgNP at the junction. The trapping laser was switched 12 times continuously and 60 real-time SERS spectra with 1 s integration time per spectrum were recorded accordingly in Supplementary Fig. 4. By taking the time-averaged SERS spectra at each switching state, Fig. 1c demonstrates a regular pattern that the characteristic peak of NBA at ~ 1645 cm⁻¹ (ring stretching)³³ was emerged at on states and vanished at off states. Moreover, the histogram analysis of the peak intensities at each state gives the relative standard deviations (RSD) within 20%. These results verify the reproducibility of creating the dynamic nanocavity on the optical plasmonic tweezer-coupled SERS platform. To further evaluate the effectiveness and robustness of the platform, numerical simulation using FDTD method was carried out (Supplementary Note.1), which shows the electric field distribution of the plasmonic junction from the AgNP-coated bead dimer upon excitation in Supplementary Fig. 6. Since the trapping force is proportional to the square of electric field in Rayleigh regime²⁴,³⁴, a freely diffusing AgNP is attracted towards the highest-intensity in the plasmonic junction. However, the size of the nanogap between AgNP-coated beads is determined by the oligonucleotide linkers (20 nm), which is smaller than the diameter of AgNP (70 nm). Consequently, the trapped AgNP is confined against the coated AgNP in the plasmonic junction with the action force of optical plasmonic trapping and the reaction force at the contact surface as shown in Fig. 1d. The numerical simulation gives a time-averaged total force of 8.8 pN upon the
excitation of 532 nm and 1064 nm laser beams at the plasmonic junction. Moreover, the trapping potential distribution with respect to the Z positions in Fig. 1d indicates the depth of potential well at the equilibrium position as $3.5 \times 10^{-18}$ J, which is sufficient to overcome the thermal energy of Brownian motion ($k_B T = 4 \times 10^{-21}$ J at room temperature) for the stable AgNP trapping\textsuperscript{35,36}. Besides, optical plasmonic trapping requires less laser power to exert stronger force in comparison with optical trapping, which could avoid potential thermal damages to protect biological samples\textsuperscript{37}. More importantly, this platform enables the mechanical confinement of the trapped AgNP at a nanoscale precision since it is no longer subject to the optical diffraction limit. As shown in Fig. 1e, the dynamic nanocavity among the trapped AgNP and the coated AgNP in the plasmonic junction significantly enhances the localized electric field and provides the enhancement factors up to $10^9$, which is adequate to detect a single molecule\textsuperscript{38}.

To verify the single-molecule sensitivity and identify a single molecule in crowded environments, we exploited bi-analytes SERS approach (BiASERS)\textsuperscript{39} in the solution phase on the optical plasmonic tweezer-coupled SERS platform. Methylene Blue (MB) and NBA were chosen as the bi-analytes partner because of their comparable Raman cross-section and fair detection probability under 532 nm excitation. Equimolar solutions of each dye ($10^{-8}$ M) were mixed and injected into the sample channel in adjacent of the AgNP channel inside the microfluidic chamber. Similar to the above procedures, we switched the 1064 nm trapping laser between on and off states and utilized the 532 nm excitation laser to conduct the SERS measurements under on states at the plasmonic junction of an AgNP-coated bead dimer. Figure 2a displays the SERS mapping out of 3600 spectra continuously collected from dynamic nanocavity in time series, showing the distinct spectral features in the regions of 590–610 cm\textsuperscript{-1} and 1620–1660 cm\textsuperscript{-1}. Statistical analysis of the data indicated that ~5% spectra are considered to be above the cutoff of noise and classified into the catalog of ‘single-MB event’, ‘single-NBA event’, or ‘dual-MB and NBA event’, based on the characteristic Raman peaks of MB or NBA at a high concentration of $10^{-5}$ M in Supplementary Fig. 7. Specifically, the appearance of the Raman peak at 1650 cm\textsuperscript{-1} accompanied by the emergence of a peak at 600 cm\textsuperscript{-1} represents the detection of ‘single-NBA’ event (Fig. 2b blue), while the appearance of a peak at 1630 cm\textsuperscript{-1} and the vanish of the peak at 600 cm\textsuperscript{-1} represents the detection of ‘single-MB event’ (Fig. 2b red)\textsuperscript{33}. ‘dual-MB and NBA event’ refers to the presence of the dual peaks at around 1640 cm\textsuperscript{-1} (Fig. 2b black). Representative full spectra of each event are shown in Supplementary Fig. 8, and the fluctuation of peaks position was analyzed in Supplementary Fig. 9 to facilitate accurate identification among different events. The statistical histogram in Fig. 2c is predominated by ‘single-dye-event’ (NBA or MB), suggesting that the signals were highly likely originated from a single molecule at the dynamic nanocavity. Furthermore, we repeated the BiASERS experiments by increasing the concentration of MB by 10 times ($10^{-7}$ M) while keeping the concentration of NBA ($10^{-8}$ M) unchanged. Most of the spectra contain the characteristic peaks of MB at 1630 cm\textsuperscript{-1} with a large number of MB as the overflowing background molecules. Nevertheless, we still captured a few spectra in which the subtle peaks of NBA at 1650 cm\textsuperscript{-1} emerged from the predominant peaks of MB at 1630 cm\textsuperscript{-1} (Fig. 2d), implying the presence of an NBA molecule in the MB-dominated nanocavity\textsuperscript{40}. As the low-populated species, NBA was differentiated from the dominating MB at the single-molecule level. Hence, the optical plasmonic tweezer-
coupled SERS platform could achieve the single-molecule SERS detection and differentiation among overflowing background molecules in aqueous environments.

**Monitoring the dynamic pH-dependent structures of tyrosine in solution**

To further verify the aqueous stability and compatibility, we employed the optical plasmonic tweezer-coupled SERS platform to characterize tyrosine (Tyr) in different pH environments, considering that the side chains of amino acids dictate the protein structure–function relationships while their hydrogen bonding and electrostatic interactions are affected by environmental factors, such as pH\(^{41}\). The real-time SERS spectra of Tyr were monitored by the dynamic nanocavity during the change from acidic to basic conditions, as presented in Fig. 3a. At the beginning, the microfluidic chamber was filled with 50 µM Tyr solution at pH 1, generating the characteristic peak at 1620 cm\(^{-1}\) as the in-plane ring stretching (\(v_{8a}\) mode) of Tyr at +1 charged state\(^{42,43}\). Then 2 M NaOH was added through a side channel to adjust the environments from pH 1 to pH 13, meanwhile SERS spectra were continuously collected. There is a downshift of \(v_{8a}\) mode to 1602 cm\(^{-1}\) along with the gradual conversion of Tyr to -2 charged state\(^{42,43}\) and a change in the intensity ratio of Tyr doublet located at ~854 cm\(^{-1}\) and ~830 cm\(^{-1}\), which is assigned to the Fermi resonance between the symmetric ring-breathing (\(v_1\)) and the overtone of the out-of-plane ring deformation (2\(v_{16a}\))\(^{44}\). The intensity of 830 cm\(^{-1}\) increases and the ratio of I\(_{830}\)/I\(_{854}\) changes from approximately 1:1 to 2:1\(^{43,44}\). These differences are attributed to the increase of negative charge on the phenolic hydroxyl group (pKa = 10.5) upon the adjustment from acidic to basic conditions\(^{44}\). Other changes, such as the increasing intensity of the peak at 1071 cm\(^{-1}\) (C-N stretching) and the peak shifting from 1153 cm\(^{-1}\) to 1161 cm\(^{-1}\) (C-C-N asymmetric stretching), are also observed and consistent with literatures\(^{45}\). On the other hand, there is no obvious signal at 930 cm\(^{-1}\) (C-COO\(^-\) stretching), 1390 cm\(^{-1}\) (COO\(^-\) symmetric stretching), and 1090 cm\(^{-1}\) (amino group vibration) related to Ag-Tyr interactions via the carboxylate and amino groups\(^{46,47}\), owing to the attachment of the nucleotides on the AgNP surface as a protected layer for Tyr. Although the oligonucleotide linkers might be liable to depurination and cleavage at extreme conditions, the geometry and interparticle distance between the AgNP-coated beads were fixed upon deposition in the microfluidic chamber. Moreover, at the midpoint of the pH adjustment process, the spectra measured with a long integration time (10 s) show the spectral broadening of \(v_{8a}\) mode at 1610 cm\(^{-1}\) in Fig. 3a (green), while the spectra acquired with a short integration time (1 s) exhibit sharp and fluctuated peaks in the range of 1620 to 1602 cm\(^{-1}\) in Fig. 3b, indicating the dynamic snapshots of single or a few Tyr molecules with distinct charge states at the dynamic nanocavity analogues to the previous single-molecule SERS studies\(^{47}\).

To understand the chemical environment of Tyr in aqueous solutions, we compared the SERS spectra of Tyr with the calculated Raman spectra of various Tyr-H\(_2\)O clusters in Fig. 3c. Using density functional theory (DFT) simulations, the construction of Tyr-H\(_2\)O clusters was initially based on B. Hernández’s work by involving seven water molecules in the vicinity to coordinate with the amino group (pKa = 9.2), the carboxyl group (pKa = 2.2), and the phenol hydroxyl group (pKa = 10.5) of Tyr (Supplementary Note. 2)\(^{48}\), then their structures were further optimized at +1 and -2 charged states to match the experimental
results at pH 1 and pH 13, respectively. Supplementary Fig. 10 presents possible hydrogen-bonds between Tyr and water molecules and the charges of Tyr nitrogen and oxygen accordingly. The electrostatic potential (ESP) mapping\(^4^9\) in Figs. 3d and e were generated from the interaction energy between a unit charge on 0.01 a.u. iso-surface and the Tyr molecule, demonstrating the charge distribution of Tyr with the electrophilic moieties in red and the nucleophilic moieties in blue. These results indicate that both the phenol hydroxyl group and the carboxyl group of Tyr become negatively charged after the deprotonation at pH 13, which depolarizes the whole molecule and increased the Raman activity of in-plane ring stretching to account for the downshift and enhancement of \(v_{\text{8a}}\) mode\(^4^3,\)\(^4^5\). Moreover, the hydroxyl group serves as both hydrogen-bond donor and acceptor in acidic and neutral environments, while its oxygen is exposed as a sole hydrogen-bond acceptor under basic conditions. The loss of hydrogen and the increase in negative charges on phenolic oxygen lead to the enhanced intensity at 836 cm\(^-1\) and the unequal intensity ratio of the Tyr doublet\(^4^4\). With rich pH-dependent vibrational characters, Tyr could be an indicator to reveal the structural detail and micro-environment of biomolecules in solution by the optical plasmonic tweezer-coupled SERS platform.

**Characterizing transient species of hIAPP under different physiological pH**

With efficiently reduced detection volume and integration time, we utilized the optical plasmonic tweezer-coupled SERS platform to probe the SERS signal of hIAPP at two physiological pH conditions (pH 5.5 and pH 7.4) in the manner of small-size sampling to overcome the ensemble and temporal averaging of heterogeneous species\(^2^3\). As an amyloidogenic IDP, free hIAPP monomers possess the propensity to assemble into heterogeneous aggregates and amyloid fibrils\(^1^0\). Its ensemble oligomers in dilute solutions and amyloid fibrils in solid forms are well characterized by SERS studies\(^5^0,\)\(^5^1\). Yet, it is unclear how the environmental factors affect hIAPP intermediate species and aggregation pathways\(^5^2,\)\(^5^3\). Specifically, low pH is documented as the inhibitor against amyloid formation\(^6\). The CD spectra of 100 µM hIAPP in the PBS buffer of pH 5.5 at different incubation times (0 h, 2 h, 24 h) present a persistent negative peak at around 203 nm in Fig. 4a (black, green, blue), indicating that the ensemble conformation is dominated by random coils\(^6\). Whereas, the CD spectrum of hIAPP after the 24 h incubation under pH 7.4 displays a broad peak with negative ellipticity centered at 218 nm in Fig. 4b (blue), suggesting the formation of \(\beta\)-sheet structure\(^6\). Furthermore, we incubated hIAPP at pH 7.4 for 2 h, then adjusted the pH back to 5.5 for continuing incubation to obtain the CD spectra in Fig. 4c. Interestingly, the \(\beta\)-sheet structure was still detected at \(t = 24\) h after the post-adjustment of pH from 7.4 to 5.5, implying that the critical intermediate species associated with amyloid developments already formed before the pH adjustment at the timepoint of \(t = 2\) h. However, no considerable change was observed in the CD spectra of hIAPP at pH 7.4 from \(t = 0\) h to \(t = 2\) h, regarding as the macroscopic lag phase of hIAPP fibrillation\(^5^4\). Thus, to distinguish the low-populated species buried under the ensemble averaging in quantity and time, we utilized the dynamic nanocavity to characterize the structural features of hIAPP in a small sample size at different incubation time-points under pH 5.5, pH 7.4, and post-adjustment of pH from 7.4 to 5.5 conditions.
Following the same experimental procedure in the previous Tyr section, 10 µM hIAPP solution was injected into the microfluidic chamber with AgNP-coated bead dimers deposited at the bottom, which is adjacent to the AgNP channel to allow the formation of the dynamic nanocavity and sandwich hIAPP species for SERS characterizations. This concentration mimics a near-physiological milieu involving moderate intra- and inter-molecular interactions that are crucial to initiate the aggregation process and stabilize the transient intermediates of hIAPP\textsuperscript{55–57}. Although it is below the detection threshold of spontaneous Raman spectroscopy (Supplementary Fig. 11), we have obtained the clear SERS signals of hIAPP on the optical plasmonic tweezer-coupled SERS platform. Figure 4d shows the representative SERS spectra of hIAPP under pH 5.5 incubation at \( t = 0 \) h (black) and \( t = 2 \) h (green). The characteristic peaks are assigned to protein backbones including amide I band (1656 cm\(^{-1}\)), CH\(_2\) deformation (1450 cm\(^{-1}\)) and amide III band (1250 cm\(^{-1}\) and 1287 cm\(^{-1}\)), as well as specific residues such as Phe (1006 cm\(^{-1}\), 1585 cm\(^{-1}\)) and Tyr (830 cm\(^{-1}\) and 850 cm\(^{-1}\), 1605 cm\(^{-1}\))\textsuperscript{58,59}. The details of peak assignments are listed in Supplementary Table. 1. To better analyze protein secondary structures, we conducted the second derivative analysis\textsuperscript{59} for the amide I region from 1550 cm\(^{-1}\) to 1750 cm\(^{-1}\) in Fig. 4e to plot Fig. 4f. The spectra at \( t = 0 \) and \( t = 2 \) h exhibit the identical amide I band centered at 1656 cm\(^{-1}\), which are attributed to the persistence of the helical and disordered hIAPP\textsuperscript{50,51}. It is further supported by the amide III bands at 1250 cm\(^{-1}\) and 1287 cm\(^{-1}\), assigned to random coil and \( \alpha\)-helix structures\textsuperscript{58}, respectively. Since hIAPP has four positive charged sites (\( \alpha\)-amino group, K1, R11, H18) at pH 5.5, as illustrated in Fig. 4g, the electrostatic repulsions would give rise to the swollen coil conformation and plausible inhibitory effect on fibril development\textsuperscript{6,60,61}. The co-existing \( \alpha\)-helical structural component could be attributed to the moderately amphipathic segment 8–16 (Fig. 4g, dashed box), based on previous studies\textsuperscript{60,62}. In addition, the Tyr doublets with the value of \( I_{830}/I_{854} \approx 1 \) in the SERS spectra of hIAPP under pH 5.5 (Fig. 4d) imply that the C-terminal Tyr residue acted as both hydrogen-bond donor and acceptor in exposure to water molecules\textsuperscript{44,59}, in consistent with the extended and coil-rich conformations of hIAPP in the physiological acidic environment\textsuperscript{63,64}.

The representative SERS spectra of hIAPP under pH 7.4 incubation at \( t = 0 \) h and \( t = 2 \) h are shown in Fig. 4h, followed by the plots of their amide I region from 1550 cm\(^{-1}\) to 1750 cm\(^{-1}\) in Fig. 4i and the corresponding second derivative spectra in Fig. 4j, respectively. At the beginning, the spectrum of hIAPP at pH 7.4 (black) demonstrates the amide I band at 1656 cm\(^{-1}\), which is attributed to the helix-coil structure and similar to the initial conformation at pH 5.5. However, after 2-hours incubation at pH 7.4, we observed three types of SERS spectra of hIAPP from the small-size sampling measurements at the dynamic nanocavity with 1 s accumulation time: the predominant type and two rare types. The most frequently appeared spectrum (Fig. 4h, green) exhibits a typical amide I band at 1656 cm\(^{-1}\), representing the predominant helix-coil structure of hIAPP at 7.4. Intriguingly, two distinct types of spectra were obtained occasionally and reproducibly, as shown in Fig. 4h, purple and red. The type I of rare spectra (purple) features the broaden amide I band at 1668 cm\(^{-1}\), attributed to an emerging partial \( \beta\)-sheet conformation\textsuperscript{51}. Moreover, the intensity ratio of the Tyr doublets \( I_{830}/I_{854} \approx 1 \) in the type I of rare spectra
indicates the exposure of the C-terminal Tyr-37 in an extended conformation\textsuperscript{44,59}. While the type II of rare spectra (red) shows the characteristic amide I band at 1655 and 1674 cm\textsuperscript{-1}, implying the co-existence of helix-coil and β-sheet structures\textsuperscript{50,51}. In addition, the Tyr doublets with the intensity ratio $I_{830}/I_{854} > 1$ suggest that the C-terminal Tyr-37 slightly shifted toward hydrogen donating as interrelated to its surrounding chemical environment (i.e., buried hydrophobic core, as shown in Fig. 4k)\textsuperscript{59,65,66}. As evident from these spectral features, the type I and type II of rare spectra might be putatively assigned to the intermediate species of hIAPP containing the partial β-sheet with the C-terminal in extended or bent conformations, respectively. Since His-18 (pK\textsubscript{a3} = 6.0) deprotonated at pH 7.4, it reduces the electrostatic repulsion of the whole hIAPP molecule to promote intra- and inter-molecular interactions\textsuperscript{61}. As a result, the hIAPP transient species with a bent C-terminal could be stabilized by the special hydrogen bonding environment near His-18 and the ring stacking between aromatic residues, which is indicated by the distinct change of Tyr doublets in the type II of rare spectra (red)\textsuperscript{43,67}. This assignment is supported by the previous studies that the interaction between His-18 and Tyr-37 would minimize the entropic cost of forming a transient β-sheet in the amyloidogenic region (roughly S20-S29, Fig. 4k, dashed box)\textsuperscript{60,68–70}. Although existing in a low population, the two β-sheet-containing transient species of hIAPP were directly differentiated from the dominating helix-coil species of hIAPP at the early lag phase of the amyloid formation at pH 7.4, which reveals the influence of pH on the structural conversions of hIAPP and demonstrates the effectiveness of our SERS platform in characterizing a few heterogeneous proteins involving in molecular interactions under physiological pH environment.

After the 24 h-incubation at pH 5.5, the SERS spectrum of hIAPP in Supplementary Fig. 12 demonstrates similar spectral characteristics to the spectra at $t = 0$ and $t = 2$ h, indicating that most hIAPP maintained the helix-coil structure in consistent with the ensemble CD spectroscopy analysis. In contrast, hIAPP fibrils were observed and confirmed by AFM image (Supplementary Fig. 13) at $t = 24$ h of the incubation at pH 7.4. No obvious SERS signal of hIAPP was detected at the nanocavity, due to the size limit\textsuperscript{50}. We then performed the bulk characterization of hIAPP fibrils using AgNP colloids to serve as a reference. Supplementary Fig. 14 clearly demonstrates the amide I band at 1672 cm\textsuperscript{-1} and amide III band at 1226 cm\textsuperscript{-1}, which are attributed to the ordered β-sheet structure in hIAPP fibrils\textsuperscript{51,71}. Furthermore, identical amide I band at 1674 cm\textsuperscript{-1} and amide III band at 1226 cm\textsuperscript{-1} were observed from the SERS spectra of hIAPP incubated at pH 7.4 for the first 2 h then at pH 5.5 for the remaining 22 h. The SERS spectrum and AFM image (Supplementary Fig. 14) imply the formation of hIAPP fibrils with the ordered β-sheet structure. More importantly, the spectral features of hIAPP further verifies the assignments of the type I and type II of rare spectra of hIAPP under pH 7.4 incubation at $t = 2$ h to its β-sheet containing transient species. Since amyloidogenesis is driven by nucleation\textsuperscript{60,72}, the pH-regulated formation of these intermediates might slightly shift the dynamic conversions of hIAPP species in amyloid-competent and alternative conformations among the predominant helix-coil structures to facilitate the subsequent aggregation even after the post-adjustment of pH from 7.4 to 5.5. Figure 4l illustrates the influence of pH on the amyloid developments of hIAPP, based on the small-size sampling results on our SERS platform. At $t = 0$ h, hIAPP possesses helix-coil conformations, which is preserved without apparent aggregation.
during the incubation under pH 5.5 for 24 h. The pH 7.4 environment maintains the predominant helix-coil hIAPP in the macroscopic lag phase, but induces the formation of the low-populated transient species of hIAPP containing the partial β-sheet with the C-terminal in extended or bent conformations during 2 h-incubation, which eventually converts into amyloid fibrils. The slight shift in the equilibrium between different hIAPP species at the early stage of incubation under pH 7.4 subsequently results in irreversible amyloid formations even after the post-adjustment of pH from 7.4 to 5.5. It is worth noticing that the environmental pH plays a vital role in the structural conversions of hIAPP, where His-18 (highlighted in yellow in Fig. 4g and 4k) is deprotonated in the change from pH 5.5 to pH 7.4 to reduce its electrostatic repulsive interactions. However, the formation of low-populated hIAPP transient species drives the subsequent amyloid development irreversibly and independently upon pH post-adjustments, which is highly likely mediated by the C-terminal residues (Tyr-37, highlighted in cyan in Fig. 4g and 4k) in addition to His-18. Thus, the previous strategies that focused on a single site of hIAPP might not be optimal in blocking hIAPP amyloidosis, whereas targeting its on-pathway transient species could be a promising strategy. Furthermore, the direct structural characterizations of the early-stage transient species of hIAPP involving in intra- and inter-molecular interactions at physiological concentrations would provide profound mechanistic insights to understanding the protein folding and assembly in amyloid formation, which also showcases the power of the optical plasmonic tweezer-coupled SERS platform in detecting low-populated species in heterogeneous mixtures.

Discussion

In summary, we utilized optical plasmonic trapping to construct a dynamic nanocavity, which reduces the diffraction-limited detection volume and generates reproducible SERS enhancements for efficient single-molecule characterizations in solution. By switching the trapping laser between on and off states, an AgNP was trapped and released at the plasmonic junction of an AgNP-coated silica microbead dimer, respectively, which enables efficiently and continuously high-throughput detections at the well-defined location under microscopic visualization. As verified by BiASERS experiments, this optical plasmonic tweezer-coupled SERS platform could identify the low-populated species among overflowing background molecules with the single-molecule sensitivity. Moreover, we monitored the real-time structural transitions of Tyr upon the changing pH conditions and analyzed its pH-dependent vibrational characters from two distinct charged states as an environmental indicator. Furthermore, we characterized the conformations of hIAPP incubated under two physiological pH (pH 5.5 and pH 7.4), where the different species of hIAPP were in a dynamic mixture and difficult to be detected in previous ensemble and temporal averaging measurements. Having the ability to conduct small-size sampling at the confined detection volume of the dynamic nanocavity, two types of low-populated hIAPP transient species were differentiated from its predominant monomers at the early stage of pH-induced amyloidosis at pH 7.4, which were assigned to the partial β-sheet with the C-terminal in extended or bent conformations. Such a slight shift in the equilibrium between different hIAPP species stimulated the irreversible amyloid developments even after the post-adjustment of pH from 7.4 to 5.5. Hence, the direct structural characterizations of the heterogeneous hIAPP transient species could reveal their intra- and inter- molecular interactions and
provide profound mechanistic insights to understand amyloid formation processes in patients with type II diabetes.

This optical plasmonic tweezer-coupled SERS platform offers a new strategy to address the challenge of characterizing a single molecule from the crowded and heterogeneous mixtures in aqueous milieus. Since both optical plasmonic trapping and SERS techniques are surface-sensitive relying on nanostructured substrates, the integration overcomes the optical diffraction limit to confine the position of the plasmonic nanocavity and reduce the SERS active-detection volume for consistently high SERS enhancements. Therefore, this platform is able to detect the freely diffusing biomolecules at the single-molecule level without molecular immobilization or solution dilution, which holds great potential to unveil various molecular behaviors and interactions in complex biological systems.

Methods

Materials

Silica beads were purchased from Spherotech Inc. Silver nitrate (≥ 99.0%), trisodium citrate (≥ 99.0%), Tris (≥ 99.9%), sodium chloride (≥ 99.0%), (3-aminopropyl) triethoxysilane (≥ 98.0%), Nile blue A (≥ 99.0%), methylene blue (≥ 99.0%), and tyrosine (≥ 99.0%) were purchased from Sigma-Aldrich. hIAPP sample containing disulfide bridge between Cys-2 and Cys-7 and amidated C-terminal was purchased from GL Biochem (Shanghai) Ltd. hIAPP was dissolved in HFIP to dissociate any potential small aggregates then lyophilized. Samples were rehydrated in PBS buffer immediately prior to use.

Oligonucleotides of sequences 1, 2 and 3 (L1, L2, and L3) were customized from BGI Genomics (Hong Kong). L1 and L2 were functionalized with thiol group (-SH) at 5’ end.

The sequences of L1, L2, and L3 are as follows (from 5’ to 3’).

L1: HS-AAAAAAAAAAATCTCAACTCGTA
L2: HS-AAAAAAAAAAACGCATTCAAGGAT
L3: AGAGTTGAGCATATCCTGAATGCG

Preparation of AgNP-coated silica microbeads

AgNP-coated silica beads were prepared according to the protocols reported previously\textsuperscript{23}. Briefly, AgNP were synthesized by adding 1.0 mL trisodium citrate solution (0.1 M) to 50 mL boiled AgNO\textsubscript{3} solution (1 mM) and boiling for 16 min under constant stirring. After cooling down to room temperature, the synthesized AgNP (70 nm) colloid was washed with Milli-Q water for three times to remove the excess reducing agent. Meanwhile, 1 mL silica beads (1.26 µm, 5.0% w/v) was dried overnight at 60 °C and redispersed in 500 µL anhydrous ethanol awaiting surface modification for AgNP coating. Next, 1 mL ethanol solution containing 0.2% (3-aminopropyl) triethoxysilane (APTES) was added to the bead
suspension (final concentration 0.1%) and reacted for 24 h at room temperature under continuous stirring. The resulting solution was then purified by centrifuging with distilled ethanol at 1500 × g for three times and discarding the supernatant. The remaining pellet was further dried at 60 °C to remove the ethanol. Followed by adding 4 mL Milli-Q water to redispense, the silica beads with the amino groups modified surface were obtained. Lastly, silica beads with sparsely and uniformly coated AgNP were prepared by adding the dispersed APTES-modified beads to the AgNP colloids in an AgNP colloids:APTES-modified silica beads ratio of 995:5 (v/v) under agitation and reacting for 10 min at room temperature.

Construction of Oligonucleotide-linked AgNP-coated bead assembly

Thiolated oligonucleotides L1 and L2 were used to conjugate with AgNP-coated beads, while L3 with the complementary sequence to part of L1 and L2 was used for hybridization. A mixture of 13.5 µL SDS solution (1% v/v), 135 µL phosphate buffer (100 mM, pH 7.4) and 200 µL L1 or L2 solution (30 µM) was firstly added to 1 mL aforementioned AgNP-coated beads suspension. Over a period of 30 min, 110 µL NaCl solution (2 M) was added in a stepwise manner. After overnight incubation at room temperature, the solution was centrifuged three times (1500 × g), and the supernatant was removed to eliminate residual oligonucleotides and unmodified metal-coated silica beads. Then the precipitate was redispersed in 100 µL PBS buffer (10 mM phosphate, 150 mM NaCl, pH 7.4) to obtain the L1 or L2 conjugated AgNP-coated beads. Next, 100 µL of L1 conjugated and L2 conjugated AgNP-coated beads were mixed with the addition of 50 µL L3 solution (30 µM) to allow hybridization at room temperature overnight. Finally, the oligonucleotide-linked AgNP-coated bead assemblies were obtained. The construction of oligonucleotide-linked AgNP assemblies follows the same protocol except that the AgNP-coated beads suspension were replaced with AgNP colloids.

Instrumental setup and dynamic SERS measurements

The instrumental setup is illustrated in Supplementary Fig. 1. A 532 nm excitation source (MLL-III-532-50 mW, CNI, China) was directed into a built-in optical tweezers system with a 1064 nm trapping laser and bright field microscope imaging system (LUMICKS, Netherlands). The 532 nm and 1064 nm laser beams were combined by a dichroic mirror and collimatedly focused into a microfluidic chamber through a 60× water immersion objective (Olympus, LumplanFLN60x, N.A. = 1.2). The backscattered light was reflected by a 750 nm long-pass dichroic mirror and filtered by a 532 nm notch filter before entering a spectrometer (IsoPlane SCT-320, 1200 lines/mm, Teledyn Princeton Instrument, United States) with a liquid nitrogen-cooled charge-coupled device (CCD) camera (400B eXcelon, Teledyn Princeton Instrument, United States) at a spectral resolution of 2 cm⁻¹ for spectroscopic measurements. Prior to the measurements, AgNP-coated bead assemblies were flowed into the microfluidic chamber for overnight deposition. Next, the sample solution and the AgNP solution were flowed into the microfluidic chamber in adjacent channels. The concentration of the AgNP solution was carefully diluted to avoid forming aggregates. A very few amounts of AgNP would diffuse into the adjacent sample channel for optical plasmonic trapping, thus the chance of trapping multiple AgNP simultaneously was kept at minimum. During the measurements,
the power of 532 nm excitation laser was 2.7 mW and the power of 1064 nm trapping laser was 8 mW. Under the microscopic visualization, the microfluidic chamber was moved by a piezo-controlled micro-stage to place the junction of a deposited AgNP-coated bead dimer at the focus of the laser beams. By switching the 1064 nm trapping laser between on and off states, the freely diffusing AgNP was trapped and released at the plasmonic junction, forming the dynamic nanocavity among the trapped AgNP and the coated AgNP for high-throughput SERS measurements. The SERS spectra were collected at the junction of the AgNP-coated bead dimer in sample solutions and the background spectra were acquired in reference solutions. All presented spectra were obtained upon the subtraction of the background accordingly and smoothed by Savitzky–Golay filter.

**Declarations**

**Data and materials availability:**

All data are presented in the main text and the supplementary information. The raw data are shared in DataSpace at: [https://doi.org/10.14711/dataset/](https://doi.org/10.14711/dataset/).

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**Author contributions:**

Conceptualization: HC, WF, WL, JH

Methodology: HC, WF, XD

Investigation: WF, XD, HZ

Visualization: WF, VM

Supervision: WL, JH

Writing—original draft: WF, HC

Writing—review & editing: WF, XD, VM, WL, JH

**Competing interests:**

Authors declare that they have no competing interests.

**Supplementary information**

Supplementary information for this article is attached.
References


**Figures**

**Figure 1**

**Dynamic nanocavity controlled by optical plasmonic trapping.** a, Schematic illustration of the optical plasmonic tweezer-coupled SERS platform. Top: Off state that has the 532 nm excitation laser (green) for
SERS measurements. Bottom: On state that has the 532 nm excitation laser (green) and the 1064 nm trapping laser (red) to form the dynamic nanocavity among a trapped AgNP and two coated AgNP at the plasmonic junction of an AgNP-coated microbead dimer. b, Brightfield image of an oligonucleotide-linked AgNP-coated silica microbead dimer by a conventional optical microscope. c, The time-averaged SERS spectra over 5 s recorded at an AgNP-coated bead dimer in 500 nM NBA solution by the 532 nm excitation laser with the switching of the 1064 nm trapping laser between on (red) and off (black) states for 12 times. d, Calculated trapping potential along the Z-axis. The red dashed line represents the virtual trapping potential within the plasmonic junction of an AgNP-coated microbead dimer, where the trapped AgNP is confined against the coated AgNP. Insert: The confinement of the trapped AgNP due to a balance between the action force of optical plasmonic trapping ($F_{OPT}$) and the reaction force at the contact surface ($F_R$). e, FDTD simulation of the E-field ($|E/E_0|$) distribution at the dynamic nanocavity among the trapped AgNP and the coated AgNP in the plasmonic junction.

**Figure 2**

**BiASERS analysis of MB and NBA at dynamic nanocavity.** a, Time-series SERS mapping from 3600 SERS spectra of $10^{-8}$ M MB and NBA mixture solution. Integration time: 1 s per spectrum. Only the spectra above the noise level were shown. b, Representative spectra of ‘single-MB event’ (red), ‘single-NBA event’ (blue), and ‘dual-MB and NBA event’ (black). c, Histogram of ‘single-MB event’, ‘single-NBA event’, and
‘dual-MB and NBA event’ from 3600 spectra. **d**, Representative spectrum of ‘dual-MB and NBA event’ showing the signal of low-populated NBA emerged from the predominant signal of MB at the NBA/MB ratio of 1:10.

**Figure 3**

**pH-dependent structural transitions of Tyr.** **a**, Real-time SERS spectra of Tyr in the change from pH 1 (black) to pH 10.5 (blue), and pH 13 (red). Integration time: 10 s. **b**, Representative SERS spectra of Tyr at pH 10.5 with 1 s integration time, showing spectral fluctuations arisen from Tyr in distinct charge states. **c**, Comparison of SERS spectra (upper panel) and simulated Raman spectra (lower panel) of Tyr at pH 1 (red) and pH 13 (black), respectively. The SERS spectra are adopted from (a). The simulated Raman spectra and electrostatic potential are generated from DFT calculations on Tyr in +1 charged state as NH$_3^+$CH(CH$_2$C$_6$H$_4$OH)COOH (d) and -2 charged state as NH$_2$CH(CH$_2$C$_6$H$_4^-$)COO$^-$ (e), respectively. **d, e**, ESP values of Tyr in +1 charged state and -2 charged state mapped on 0.01 a.u. van der Waals surface. The unit of ESP values is in atomic units (a.u.).
Figure 4

Structural characterizations of hIAPP incubated under different pH conditions. a, CD spectra of hIAPP during the incubation under pH 5.5. b, CD spectra of hIAPP during the incubation under pH 7.4. c, CD spectra of hIAPP during the incubation under pH 7.4 for the first 2 h and then adjusted to pH 5.5 for the following 22 h. d, Representative SERS spectra of hIAPP at $t=0$ (black) and $t=2$ h (green) incubated under pH 5.5. e, Enlarged Amide I region of (d). f, Secondary derivative spectra of (e). g, Properties of

\[
\text{NH}_3-\text{KCNTATCATQRLANFLVHSNNFGAILSSSTNVGSNTY-CONH}_2
\]

\[
\text{NH}_3-\text{KCNTATCATQRLANFLVHSNNFGAILSSSTNVGSNTY-CONH}_2
\]
hIAPP residues at pH 5.5: Positively charged residues in red, hydrophobic residues in cyan, and residues with helical potency in the dashed box. h, Representative SERS spectra of hIAPP at $t = 0$ (black) and at $t = 2$ h (dominating spectrum, green; type I rare spectrum, purple; type II rare spectrum, red) incubated under pH 7.4. i, Enlarged Amide I region of (h). j, Secondary derivative spectra of (i). k, Properties of hIAPP residues at pH 7.4: Positively charged residues in red, His-18 highlighted in yellow as the only deprotonated residue upon the change of physiological pH from 5.5 to 7.4, hydrophobic residues in cyan, and amyloidogenic residues in the dashed box. l, Cartoon illustration of the hIAPP amyloid developments affected by physiological pH environments (pH 5.5 and pH 7.4).

**Supplementary Files**

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