A stable ‘sandwich’ system of Surface-Enhanced Resonance Raman Scattering for the analysis of β-carotenes in a photosynthetic pigment-protein complex

Xiaochun Qin,†a Jiajia Zhu,b Wenda Wang,a,c Xiang Ding,b Kebin Wang,a Yan Fangb* and Tingyun Kuanga*

In plants, Photosystem I (PSI) is composed of a core complex and a membrane-associated antenna complex light-harvesting complex I that captures light and funnels its energy to the core complex. To obtain Raman structural information on β-carotenes embedded in the PSI core complex, a ‘sandwich’ system of roughened silver slice: target protein complexes: single silver nanoparticles was fabricated for Surface-Enhanced Resonance Raman Scattering (SERRS) measurements. This study provided a method to overcome spectral irreproducibility, which is the main drawback of Surface-Enhanced Raman Scattering/SERRS-based studies. The Raman spectra of β-carotenes embedded in the PSI core complex can be obtained at very low sample concentrations (1–5 μg Chl/ml) and high signal/noise ratios. The β-carotenes in the spinach PSI core complex were predominantly all-trans configuration. The membrane protein-mediated adsorption of silver nanoparticles induced the uniform distribution of a large number of single nanoparticles, which contributed to achieving highly reproducible SERRS spectra. This study is the first to apply single silver nanoparticle-based SERRS analysis in membrane proteins. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: carotenoid; carotene; Photosystem I; SERS; SERRS

Introduction

Carotenoids perform two crucial functions in photosynthetic organisms. First, they absorb light in the blue-green region, where chlorophylls (Chls) display little absorption, and transfer the energy to neighboring Chls. As a result, the absorption cross section for photosynthesis increases.[1,2] Second, under excess light, carotenoids protect the photosynthetic apparatus by directly quenching triplet-state Chl and singlet oxygen.[3–5] The regulation of light harvesting and photoprotection by carotenoids requires their close proximity and proper orientation to Chl molecules in photosynthetic pigment–protein complexes. Thus, knowledge of the structure and orientation of the carotenoids involved is important to understand the photosynthetic process completely.

Raman spectroscopy can provide detailed information on molecular vibrations and has been successfully employed in investigating the conformation and configuration of carotenoids. However, the Raman signal is usually very weak and requires enhancement. Resonance Raman scattering (RRS) is the most commonly used method to achieve enhancement for studying the structure of carotenoids. In the present study, we introduced another method, Surface-Enhanced RRS (SERRS), to obtain structural information on carotenoids in photosynthetic membrane proteins.

Raman scattering can be significantly enhanced (10² – 10⁶) if a molecule is attached or is placed within immediate proximity to a nanometer roughened metal (e.g. Ag, Au, or Cu) surface or metal nanoparticle colloid. This process is called Surface-Enhanced Raman Scattering (SERS).[6–8] SERS occurs when the laser excitation wavelength is in resonance with an electronic transition in the analyte and provides additional enhancement.[9,10]

A number of different photosynthetic pigment–protein complexes and membrane preparations have been examined by SERRS. These include reaction center complexes,[11,12] chromophores and spheroplasts from photosynthetic bacteria,[13,14] as well as PSI D1/D2/Cyt b559 reaction center complex from spinach.[12] SERRS spectroscopy can provide information on the photosynthetic membrane surface,[13,15,16] location, and orientation of chromophores within the membrane,[14] as well as on...
the conformation of carotenoids within the pigment–protein complexes. The major advantages of SERS/SERRS over RRS include high sensitivity and fluorescence-quenching properties. Much smaller amounts of material are needed for SERS/SERRS than for RRS because the latter needs high-concentration materials to minimize the fluorescence effect, whereas the metal surfaces used in SERS/SERRS are effective quenchers of fluorescence.

With almost 40 years of development, SERS/SERRS is gaining increasing attention in the fields of physics, chemistry, surface science, nanoscience, and biomedical science. SERS/SERRS is also proven to be a powerful technique for identifying target molecules. However, SERS/SERRS is strained by the inherent irreproducibility of spectra.

In most SERS/SERRS-based studies, silver colloid as active substrate is first prepared, and the analytes are assembled on this active substrate for further SERS/SERRS detection. The silver nanoparticle aggregates function as the hot spots where significant enhancement occurs. For biological macromolecules, although achieving high sensitivity is not difficult, achieving high reproducibility of SERS/SERRS spectra is challenging due to the heterogeneous substrates. For example, silver colloid is usually a mixture of particles with different sizes and shapes. The interaction between the active substrate and target macromolecules may also lead to different orientations on the substrate, conformational changes, and even denaturation, which also contributes to the irreproducibility. This drawback of irreproducibility is widely acknowledged. To some extent, SERS/SERRS would have been more extensively applied if highly reproducible spectra are easy to obtain.

The present study aimed to build a stable sandwich system of SERRS for analysis of β-carotenes in higher plant Photosystem I (PSI) core complexes. In plants, PSI is composed of a core complex of up to 15 subunits (PSI-A to -L, PSI-N, PSIO, and PSI-P) and a membrane-associated antenna complex light-harvesting complex I (LHCI) that captures light and funnels its energy to the core complex. About 96 Chlorophylls and 22 β-carotenes constitute the complicated pigments network in the PSI core complex of both cyanobacteria and higher plants. The crystal structure of pea PSI indicated 12 all-trans and 5 cis β-carotenes bound in its core complex, and therefore structural information of some carotene is still lacking due to the limitation of the resolution of its crystals. By designing a special ‘sandwich’ SERRS substrate, the β-carotenes in the PSI core complex of higher plants were measured to be mainly all-trans conformation, and in addition the irreproducibility problem of SERS/SERRS was also solved.

In this ‘sandwich’ system, roughened silver slice and silver colloid served as active substrates, and the PSI core complexes were isolated as previously described. The samples were frozen in liquid nitrogen and stored at −80 °C until use.

Three kinds of carotenoids (lutein, violaxanthin, and β-carotene) are bound in LHCl, whereas only one kind of carotenoid (β-carotene) is bound in the PSI core complex, and therefore the PSI core complex in high purity is indispensable for understanding the structure of β-carotenes within it correctly. The polypeptide composition of the PSI core complex was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 15% acrylamide gels. For electron microscopy, freshly prepared samples were negatively stained with 2% uranyl acetate and applied to glow-discharged carbon-coated grids. The images were analyzed using a Phillips CM100 electron microscope with an 80 kV accelerating voltage and 50,000× magnification.

**Preparation of nanometer roughened silver slice and silver colloid**

Silver slices (10 mm × 10 mm) were cleaned by ultrasonication for 5 min with acetone, ethanol, and distilled water sequentially, and then dried with filter paper. The clean slices were soaked in diluted nitric acid for 15 min, rinsed with distilled water, and dried with a stream of nitrogen gas. The treated silver slices were used immediately.

The colloidal silver nanoparticles for SERRS measurements were prepared by the aqueous reduction of silver nitrate (1.06 mM, 500 ml) with trisodium citrate [1% (w/v), 10 ml] using the method of Lee and Meisel. UV–visible spectra of the resulting silver colloid were recorded on a Shimadzu Model UV-2401PC UV–visible spectrophotometer. The absorption maximum was located at 413 nm. The size and shape of the colloidal silver nanoparticles were analyzed by a transmission electron microscopy (TEM) system (H-600, Hitachi).

**Preparation of the sandwich architecture**

The PSI core complex was diluted in buffer I (10 mM Tricine–NaOH, pH7.8) with 0.03% n-dodecyl-[β]-D-maltoside (DDM) (w/v) at 100 μg Chl/ml. To fabricate the ‘sandwich’ architecture for SERRS measurement, target proteins were mixed with buffer I and DDM to obtain a sample buffer, and then the resulting sample buffer were mixed with silver colloid to obtain a mixture. The ratio of silver colloid/sample buffer (v/v), and the concentration of DDM and sample in the final mixture were important factors that affected the reproducibility and sensitivity of SERRS spectra. For the mixture (1 μl) was poured onto the roughened silver slice (10 mm × 10 mm), which was then gently dried under a nitrogen stream. In this system, the bottom layer was the roughened silver slice, and the upper silver layer was produced by the interactions between the proteins in the middle layer and silver nanoparticles. In order to minimize the deformation of sample, which is caused by dehydration, it was measured right after drying.

**SERRS measurements**

All SERRS measurements were performed using a Renishaw RM2000 laser micro-Raman spectrometer equipped with a 514 nm argon ion laser. To minimize possible photodegradation, a laser line with a minimum power of 0.03 mW was delivered through a 90° angle to the stage (LEICA S/N244750 B2: 02) in the form of a 3 μm-diameter spot size. Our microscope was...
equipped with an objective lens (LEICA Germany 566027 50×) and an ocular lens (LEICA HC PLAN 10×/20). The grating was 1800/mm. The scattered signals were notch filtered, and a charge-coupled device camera was used to collect the Raman spectra. The typical scan time for each sample in this study was 10 s. The scan time was one, and the scan range was 800–1700 cm⁻¹. To assess the reproducibility of this approach, 10 replicate spectra were obtained from selected points on the sample matrix (Fig. S1). In addition, to learn the difference between different sandwiched samples, spectra from three sandwiched substrates were measured.

Low-temperature (77 K) SERRS measurements were done through the FDCS196-Freeze Drying System. The substrate was put into the sample chamber and then covered by sample window, and the temperature can be accurately controlled with the FDCS196-Freeze Drying System. SERRS measurements were performed at room temperature unless otherwise specified.

Results and discussion

Characterization of the sample of the PSI core complexes

Compared with the PSI complex (Fig. S2A, line 1), the PSI core complexes (Fig. S2A, line 2) had all LHCl antenna removed, demonstrating the high purity of the samples. The electron micrograph of the PSI core complexes showed that they were homogeneous monomers (Fig. S2B). The particles had an irregular round shape as well as long and short axes with lengths of about 15 and 10 nm, respectively. This structure was very similar to that of the PSI core complex revealed in other photosynthetic organisms.[26,32]

Characterization of roughened silver slice and silver nanoparticles

A wide nanoparticle size range inevitably induces the irreproducibility of colloidal SERS/SERRS spectra. The homogeneity of particles in the prepared silver colloid was examined by TEM. As shown in Fig. S3A and S3B, a small fraction of silver nanoparticles were long or short rod shaped. However, most silver nanoparticles were round with diameters of 80–100 nm. There is a specific relationship between the particle size and excitation wavelength, and the enhancement effect of differently sized nanoparticles significantly varies when they are excited at certain wavelengths.[33] Our result was in agreement with a conclusion that nanoparticles excited at 514 nm have a narrow size range of 80–100 nm.[34]

The silver slice looked very smooth, but it was nanoroughened when observed by SEM (Fig. S3C and S3D). The relatively homogeneous silver nanoparticles and nanometer roughened silver slice provided excellent active substrates for the fabrication of the ‘sandwich’ substrate.

Figure 1. (A) Effect of DDM concentration on the relative Raman intensity of the ν1 band. SERRS measurement was performed under the condition of the final sample concentration of 2.5 μg Chl ml⁻¹ and the silver colloid/sample buffer (v/v) ratio of 4/1 at room temperature. (B) Effect of the final sample concentration on the relative Raman intensity of the ν1 band. SERRS measurement was performed under the condition of DDM concentration of 0.006% (w/v) and the silver colloid/sample buffer (v/v) ratio of 4/1 at room temperature. (C) Effect of the silver colloid/sample buffer (v/v) ratio on the relative Raman intensity of the ν1 band. SERRS measurement was performed under the condition of DDM concentration of 0.006% (w/v) and the final sample concentration of 2.5 μg Chl ml⁻¹ at room temperature. (D) SERRS spectra of 10 points selected on the silver slice. Insert: ν1 band intensity of the 10 SERRS spectra after baseline correction. SERRS measurement was performed under the condition of DDM concentration of 0.006% (w/v), the final sample concentration of 2.5 μg Chl ml⁻¹, the silver colloid/sample buffer (v/v) ratio of 4/1 at 77 K.
Factors affect the reproducibility and sensitivity of SERRS spectra

For the Raman spectra of carotenoids, four main wavenumber regions called $v_1$, $v_2$, $v_3$, and $v_4$ (around 1530, 1120–1200, 1000, and 950 cm$^{-1}$, respectively) were observed, and these regions arose from conjugated C=C double-bond stretching vibrations, C–C single-bond stretches coupled either to C–H in-plane bending or C–CH$_3$ stretching, CH$_3$ in-plane rocking vibrations, and C–H out-of-plane wagging modes, respectively. Obtaining SERS/SERRS spectra with high reproducibility and high sensitivity may be affected by some factors. To determine the optimum conditions for the fabrication of SERRS active substrates, the effects of DDM concentration, sample concentration, the ratio of silver colloid/sample buffer (v/v), and temperature were examined.

Detergents are needed for membrane proteins in an aqueous environment. To determine the effect of detergents on SERRS analysis, DDM with various concentrations was added to the mixture of sample and silver colloid. The results showed that the relative Raman intensity of $v_1$ band decreased with increased DDM concentration (Fig. 1A). This finding can be attributed to SERS being a distance-sensitive method. More detergents surrounding the membrane proteins increased the distance between the target molecules and two kinds of substrates (nanoparticles and roughened silver slice). Notably, when the final DDM concentration was lower than 0.006% (w/v), some inhomogeneous aggregates appeared on the silver slice (Fig. 2A). Under this condition, SERRS spectra with high reproducibility cannot be obtained. However, with increased DDM concentration to 0.006% (w/v), aggregates did not form. As shown in Fig. 2B, single nanoparticles were homogeneously adsorbed onto the silver slice, which appeared very uniform. Only under this form were highly reproducible SERRS spectra obtained.

To determine the analytical sensitivity, samples with different concentrations (1–20 µg Chl/ml) were mixed with silver colloid and allowed to adsorb onto the roughened silver slice as described in the experimental section. The SERRS spectra were collected and the result showed that the SERRS signals became saturated at 2.5 µg Chl/ml. The intensity of $v_1$ band also decreased with lower or higher Chl concentration (Fig. 1B).

The ratio of silver colloid/sample buffer (v/v) ratio greatly affects the spectral features and reproducibility of SERS spectra when studying the SERS spectra of bacteria. Kahraman et al. found that the reproducibility of SERS spectra improves with increased silver colloid concentration. However, some spectral features begin to disappear during this process. Our result clearly showed that the intensity was stronger at 4:1 ratio than at 2:1 and 1:1 (Fig. 1C), and that more silver nanoparticles corresponded to higher signals. Precipitation by centrifugation was performed to increase the concentration of nanoparticles in the colloid. We found that large aggregations formed during centrifugation, and the heterogeneity of these aggregates rendered the achievement of reproducible spectra impossible. Therefore, colloid concentration by centrifugation was not adopted, and the silver colloid/sample buffer (v/v) ratio of 4:1 was used for further SERRS analyses.

![Figure 2](https://example.com/image2.png)

*Figure 2.* The distribution of nanoparticles on the roughened silver slice was observed by optical microscopy. Substrate were built under the condition of the final sample concentration of 2.5 µg Chl ml$^{-1}$, the silver colloid/sample buffer (v/v) ratio of 4/1, and DDM concentration of 0.003% (w/v) (A) or 0.006% (w/v) (B). Substrate were built by mixing silver colloid and the buffer I under the condition of the silver colloid/buffer (v/v) ratio of 4/1, and the final DDM concentration of 0.006% (w/v) (C). Only silver colloid was adsorbed onto the roughened silver slice (D).
The effect of temperature on the signal/noise ratios of SERRS spectra was examined by obtaining the spectra at 77 K, as shown in Fig. 1D. Compared with the SERRS spectra measured at room temperature (Fig. 4), the signal/noise ratios and intensity of Raman bands of the SERRS spectra measured at 77 K did not show significant improvement. Therefore, the SERRS spectra were obtained at room temperature in this study.

**Membrane proteins mediated the adsorption of nanoparticles onto the silver slice through the interaction with nanoparticles**

We next determined the reason for the nanoparticles being aggregated inhomogeneously (Fig. 2A) or separated homogeneously (Fig. 2B). We hypothesized that the distribution of silver nanoparticles was directly related to the sample. Under the condition of no PSI core complex attached into the system, many aggregations formed regardless of the presence (Fig. 2C) or absence (Fig. 2D) of DDM. This finding indicated that the uniform adsorption of individual silver nanoparticles onto the silver slice depended on the existence of samples.

The appearance of silver nanoparticles absorbed onto the silver slice at different conditions was observed by SEM. When the silver colloid was the only substance adsorbed onto the silver slice, most silver nanoparticles formed aggregates with different sizes. Only a very small fraction of the silver nanoparticles existed as single particles (Fig. 3A and 3B). We were thus prompted to determine whether the buffer had any

**Figure 3.** The distribution of nanoparticles on the roughened silver slice was observed by SEM. Silver colloid was poured onto the silver slice and dried under a nitrogen stream, and then observed by SEM at magnifications of 10K (A) and 50K (B). Substrate were built by mixing silver colloid and the buffer I under the condition of the silver colloid/buffer (v/v) ratio of 4/1, and the final DDM concentration of 0.006% (w/v), and the heterogeneous aggregates were observed by SEM (C and D). Substrate were built under the condition of the final sample concentration of 2.5 μg Chl ml⁻¹, the silver colloid/sample buffer (v/v) ratio of 4/1, and DDM concentration of 0.006% (w/v), homogeneous single nanoparticles were observed by SEM (E and F).
effect on the nanoparticle distribution. As shown in Fig. 3C and 3D, when the substrate was made under the condition of the silver colloid/buffer (v/v) ratio of 4/1, and the final DDM concentration of 0.006% (w/v), the inhomogeneous aggregates still existed. However, once the samples were added into the mixture, the single silver nanoparticles were uniformly distributed on the silver slice (Fig. 3E and 3F).

The characterization of the surface of a citrate-reduced colloid is optimized for the interaction between silver nanoparticles and proteins via electrostatic attraction.[37] Considering that the concentration of 0.006% (w/v) is close to the critical micelle concentration (CMC) of DDM in water [0.008%, (w/v)], we suggested that the interaction between the samples and nanoparticles greatly affected the distribution of nanoparticles. When the DDM concentration was lower than its CMC, the PSI core complexes aggregated and the nanoparticles also aggregated via the interaction between membrane proteins and nanoparticles. On the contrary, when the DDM concentration was greater than its CMC, the PSI core complexes separated homogeneously and the nanoparticles adsorbed onto membrane proteins accordingly to form the uniform distribution of single nanoparticles.

Therefore, we proposed that the PSI core complexes mediated the adsorption of single nanoparticles onto the substrate via the interaction with nanoparticles. Under the effect of the sandwich substrate, the interaction between silver nanoparticles and samples was so strong that the silver nanoparticles cannot aggregate, but the single nanoparticles distributed uniformly and spontaneously.

Figure 4. To learn the difference among different points and among different sandwiched samples, SERRS spectra of 10 points selected on the silver slice were measured. (A) No baseline correction or normalization was applied to the presented spectra. For better comparison, the baseline was applied to these spectra of sandwiched sample 1 (B), sandwiched sample 2 (C), sandwiched sample 3 (D). The \( v_1 \) band intensity of SERRS spectra form three sandwiched samples after baseline correction was shown in figure E and compared in figure F. SERRS measurement was performed under the condition of DDM concentration of 0.006% (w/v), the final sample concentration of \( 2.5 \mu g \text{Chl ml}^{-1} \), and the silver colloid/sample buffer (v/v) ratio of 4/1 at room temperature.
SERS spectra with high reproducibility can be achieved at certain conditions

Although irreproducibility is an inherent disadvantage of SERS/SERRS, SERS spectra with high reproducibility were obtained under certain conditions in this study. The process was as follows. Buffer I (7.44 μl), 0.2% DDM (1.31 μl), and the PSI core complex (1.25 μl) were mixed together to obtain a sample buffer (10 μl), and then the resulting sample buffer was mixed with silver colloid (40 μl) to obtain a mixture with a final Chl concentration of 2.5 μg/ml and a final DDM concentration of 0.006% (w/v). 10 μl of the resulting mixture was adsorbed onto the nanoroughened silver slice. Under this condition, the Raman spectra of the 10 selected points (Fig. 4A and 4B) and of any point in the sandwich system showed great reproducibility. Furthermore, to determine the difference among the different sandwiched samples, SERS spectra of the other two sandwiched substrates were obtained (Fig. 4C and 4D). The results indicated no clear distinction among them (Fig. 4E and 4F).

As shown in Fig. 4A–4D, the SERS spectra of the PSI core complex exhibited very strong Raman enhancement in v1–v4, revealing the refined structure of the embedded β-carotenes in the membrane protein complexes. The presence of the very strong v1 feature in the 1523.5 cm⁻¹ region suggested the dominance of \( \text{all-trans} \) β-carotene in the spinach PSI core complex because \( \text{cis} \) isomers generally exhibit higher v1 wavenumbers than \( \text{all-trans} \) isomer.\(^{[38-40]} \) The fingerprint region v2 is unique for each of the 14 isomers of β-carotene and is widely used as a key in predicting the configuration of β-carotenoids.\(^{[41-43]} \) For the SERS spectra of the spinach PSI core complex, only one major band at 1158 cm⁻¹ and three minor bands at 1189, 1214, and 1267 cm⁻¹ were detected, whereas the characteristic band at 1134 cm⁻¹ in \( \text{cis} \) isomers was undetectable in this region.\(^{[38,40]} \) By comparison with reported isomers, the β-carotenoids in the spinach PSI core complex can be concluded to have predominantly \( \text{all-trans} \) configuration. Up to now, Raman spectra of β-carotenoids in the PSI core complex of higher plants had not been reported, although Raman spectra of carotenoids in the PSI–LHCl complex have been determined by RRS.\(^{[44]} \) According to the pea PSI crystal structure, there are 12 \( \text{all-trans} \) and 5 \( \text{cis} \) β-carotenoids bound in its core complex. Why peaks indicating \( \text{cis} \) β-carotenoids were not presented in SERS spectra? The reason may be that most of \( \text{cis} \) β-carotenoids bound inside the core complex, and SERS is a distance–sensitive method, i.e. the electromagnetic field responsible for a large proportion of SERS enhancement exponentially decays with the distance between the target molecules and active substrates. As a result, the SERS signal decreases exponentially with increased distance.\(^{[45]} \) In addition, we suggested that some unknown carotenoids, which have not been resolved in the pea PSI crystal,\(^{[28,29]} \) had predominantly \( \text{all-trans} \) configuration.

The biocompatibility of SERS/SERRS substrates has been called into question. SERS/SERRS experiments have been performed on large molecules such as proteins, DNA and RNA. The adsorption of target macromolecules onto SERS/SERRS-active substrates may lead to different orientations on the substrate, conformational changes, and even denaturation.\(^{[23]} \) Silver electrode and colloidal silver particles have demonstrated good biocompatibility. With silver electrode as substrate, \( \text{cis} \) conformation of the native bacterial reaction center carotenoids is maintained, and no new peaks ascribing to heme denaturation of cytochrome b-559 in isolated PSII reaction center complex from spinach are observed during SERS experiments, suggesting that significant protein denaturation does not occur.\(^{[12]} \) With colloidal silver particles as substrate, the adsorbed chlorocatechol dioxygenase retains activity.\(^{[46]} \) and cytochrome \( c^{[47]} \) and cytochrome P450\(^{[48]} \) have been shown to retain native spin states. For these simple silver substrates, although these results are encouraging, a general rule cannot as yet be drawn, and every protein should be considered individually.\(^{[29]} \) However, sandwich substrates, such as Au: analyte: Ag,\(^{[50-51]} \) Au: analyte: Au,\(^{[51]} \) and Ag: analyte: Ag,\(^{[52]} \) show great potentials in maintaining the stability of biomacromolecules. The most common types of SERS/SERRS-active substrates are clusters of colloidal silver and gold particles, which are used in colloidal solution or dry on a surface.\(^{[53]} \) Large molecules are immobilized at similar orientation within the sandwich system due to complicated interactions with the nanoparticles.

Laser power is another factor that should be considered. Most photosynthetic membrane proteins are normally light sensitive, and their abilities to resist high light differ. Thus, to minimize possible photodegradation, the minimum power was used.

How to assess the structural stability and functional integrity? For large molecules containing chromophores, such as the haem-containing proteins and chlorophylls/carotenoids-containing proteins, special characteristics of the intrinsic chromophores are often used for the assessment. For photosynthetic pigment–protein complex, 77 K fluorescence spectroscopy is a useful and sensitive tool for monitoring the structural and functional integrity of the complex.\(^{[54]} \) Long-wavelength Chls (red Chls) are known to be bound in the PSI core complex. Given that the uphill energy transfer becomes improbable at 77 K, a significant part of the excitation is trapped on the red Chls. Generally, the 77 K fluorescence spectrum of the PSI core complex has a major emission peak at 720 nm because of red Chls, and a minor emission peak at 680 nm, which indicates that a fraction of energy absorbed by bulk chlorophylls does not transfer to red Chls. Any perturbation in protein structure may disturb the complicated pigment network; consequently, the peak at 680 nm rapidly increases and that at 720 nm blue-shifts. The 77 K fluorescence spectra of the isolated PSI core complex both in solution and on silver substrates were measured. As shown in Fig. S4, the spectrum of the PSI core complex on silver substrates peaked at 720 nm, corresponding to the spectrum of the core complex in solution. The emission peak at 680 nm of the PSI core complex on silver substrates increased by about 25% compared with the PSI core complex in solution. This result indicated that the silver substrates did not obviously modify the shape of the fluorescence spectra of the PSI core complex. Therefore, the experimental conditions used to obtain the SERS spectra did not significantly disturb the structure of the PSI core complex.

Construction of the sandwich substrate

For biological macromolecules, interactions with metal substrates may lead to different orientations on the metal surfaces and conformational changes. To date, the best substrates for maintaining the orientation and structure of proteins are sandwich substrates.\(^{[50,51]} \) Keating et al.\(^{[50]} \) reported a method of fabricating Au: protein: Ag sandwich substrate by adding protein: Au conjugates to silver nanoparticles aggregates. With this method, cytochrome c was immobilized in the Au: protein: Ag sandwich, and it retained not only its native conformation but
also the orientation of its cofactor heme with respect to the surface. Han et al. reported another method for preparing Au: protein: Au and Au: Ag sandwich substrates comprising a bottom gold monolayer, a middle protein layer, and an upper layer of gold or silver nanoparticle aggregates. In these sandwich substrates, complicated interactions between proteins and substrates enable the natural structure to be maintained. Therefore, sandwich substrates show great biocompatibility potential. However, the problem of irreproducibility is still not overcome by these sandwich systems. We believe that the aggregation ‘hot spots’ have an intrinsic disadvantage that contributes to irreproducibility. The silver nanoparticles are inclined to aggregate due to the strong and complicated interactions among them. On one hand, these aggregates function as SERS ‘hot spots’ with very high SERS enhancement. On the other hand, the aggregate sizes cannot be controlled; thus, enhancement is not the same among these different ‘hot spots.’

In the present study, we resolved the intrinsic problem of irreproducibility by designing a special sandwich SERRS substrate. The schematic of the sandwich structure is shown in Fig. 5.

The membrane proteins were harvested by the silver slice during building the ‘sandwich’ system. The silver slice, as the first layer of the structure, was hydrophobic. The strong interaction with the PSI core complexes induced the stable adsorption of the target membrane proteins onto it. The silver slice was nanoroughened and homogeneous, thereby enabling uniform distribution of the target proteins. From this point of view, the silver slice has a similar function to the gold monolayer in the aforementioned Au: protein: Ag sandwich substrate. The quantitative analysis of the adsorption of the PSI core complex onto the silver slice was performed as follows. Considering that the Chl concentration of the mixture containing the sample and silver colloid was $2.5 \mu g/ml$ and that each PSI core complex bound 96 Chl molecules, the number of PSI core complex in a 10 $\mu l$ mixture was $1.76 \times 10^{11}$ (i.e. $2.5 \mu g \cdot ml^{-1} \times 0.01 ml/893 g \cdot mol^{-1} \times 6.02 \times 10^{23}/96$). During the preparation of the sandwich substrate, the 10 $\mu l$ mixture formed a circle with a diameter of about 6 mm after it was dried and adsorbed onto the silver slice; its area was 28.26 $mm^2$. The area of each PSI core particle was about $1.5 \times 10^{-10} mm^2$ (15 nm $\times$ 10 nm). Hence, the maximum number of the PSI core complex needed to form the monolayer in the circle area was $1.884 \times 10^{11}$ (i.e. 28.26 $mm^2$ / $1.5 \times 10^{-10} mm^2$), which was very close to the number of PSI core complex in the 10 $\mu l$ mixture. The results showed that 2.5 $\mu g$ Chl/ml was the saturation point at which the largest enhancement was achieved, and that higher concentrations did not lead to increased Raman intensity as expected, but even decreased it to some extent. Higher concentrations can guarantee the formation of multilayers of the PSI core complexes; therefore, the saturation phenomenon suggests that only the sample layer, which is very close to the substrate, has an ability to be enhanced. In contrast, the middle sample layers contribute little to the enhancement and even decreases the Raman intensity for unclear reasons.

In addition, the adsorption of single silver nanoparticles was mediated by membrane proteins. There were about 160 nanoparticles in the area of $3 \mu m \times 4 \mu m$ (Fig. 3F), and the number of nanoparticles in the circle was $3.77 \times 10^6$ (i.e. $28.26 \times 10^6 \mu m^2 \times 160/12 \mu m^2$). Therefore, under the condition of Fig. 4, the ratio of silver nanoparticles: the PSI core complex was about 1: 476 (i.e. $13.77 \times 10^7/76 \times 10^{11}$). According to the experimental results, the measurement with laser of 3 $\mu m$ in diameter covers a sufficient area to average a number of active sites of membrane proteins. Therefore, the individual adsorption of a large number of individual silver nanoparticles onto the silver slice was the key to obtaining highly reproducible Raman spectra.

Thus, the greatest difference between our method and other SERS/SERRS-based methods was that our strategy for preparing active substrates for target proteins was based on two kinds of strong interactions, namely, between nanoroughened silver slice and membrane proteins, as well as between silver nanoparticles and membrane proteins. With the aid of these two interactions, the ‘sandwich’ substrate was constructed. Both the orientation with respect to the substrates and conformation of the PSI core complexes immobilized in the model were stable.

Conclusions

Until now, there is no standard method for the SERS/SERRS study of biological macromolecules. The main challenge with SERS/SERRS is the achievement of reproducible spectra, without which quantitative analysis is difficult. To our knowledge, this study is the first to apply single silver nanoparticle-based SERRS analysis in membrane proteins. Based on this method, SERRS spectra with high reproducibility and high sensitivity were obtained. However, this method may be restricted to membrane proteins because it depends on the intrinsic features of membrane proteins to realize the disaggregation and uniform distribution of single silver nanoparticles on the substrate. Spontaneous uniform distribution of single nanoparticles may be difficult to achieve when the method is used in other kinds of biological macromolecule. However, some linking molecules such as polylysine, poly(4-vinylpyridine), 4-(2-aminoethyl)aniline, 4-aminothiophenol, adene, and methamidoph can be used to immobilize the individual nanoparticles and consequently fabricate perfect substrates.

Acknowledgements

This work was supported by the National Natural Science Foundation (30900095) and the National Basic Research Program of China (973 Program, 2009CB118501, 2011CBA00901).
Supporting information

Supporting information may be found in the online version of this article.

References

[34] S. Nie, S. R. Emory, Science 1997, 275, 1102.