Flocculation and SERS Activation of Au Nanoparticles Using Cationic and Neutral Cresyl Violet Molecules

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The flocculates of gold nanoparticles (AuNPs), which are a few closely adjacent AuNPs in solution providing enormous surface enhanced Raman scattering (SERS) intensity, were successfully formed using oxazine dye molecules. We found that neutral cresyl violet (CV\(^0\)) molecules with two dihydro amino groups (–NH\(_2\)) chemisorb on Au surfaces via lone pair electrons at amino groups, whereas cationic CV\(^+\) molecules physisorb using electrostatic interaction between their positively charged amino groups and negatively charged AuNPs. Neutralization of CV\(^+\) gave appreciable peak shifts of their SERS bands, which also showed considerable spectral changes upon the deuteration of the amino groups, and thus assigned to vibrational modes of the amino groups. We found that diethyl substituent in amino groups in nile blue (NB), which has an oxazine structure similar to CV, sterically hinders chemisorption on AuNPs. Accordingly, NB\(^+\) physisorbed on as-prepared AuNP exclusively with electrostatic forces, whereas NB\(^-\) did not chemisorb on Au surfaces. Thus, the AuNP flocculates were readily formed using electrostatic and chemical interaction between target molecules and metal nanoparticles, albeit chemisorption was quite sensitive to bulky structure of substituent.

Keywords: Raman scattering; plasmons; chemisorption; physisorption; vibrations at surface molecules

I. INTRODUCTION

Excitation of localized surface plasmon (LSP) on metal nanoparticles or on metal nanostructures, enhances electric field at their surfaces or interfaces [1–4]. In particular, field enhancement by a factor of \(10^4\)–\(10^5\) is generated between the nanogap of closely adjacent metal nanoparticles by the illumination of light at a resonance wavelength with parallel polarization along the interparticle axis [1–11]. Single molecule detection using Raman scattering has been realized using this effect when the nanogap separation and the adsorption of target molecules is precisely adjusted. Metal nanoparticles immobilized on silicon or glass substrates have widely been used for this purpose; however probabilities for finding an optimum nanogap size with adsorbed molecules is quite small, that is much less than 1%. For this reason, it is not feasible to utilize the conventional SERS substrates in any practical applications. Also, adsorbed state such as molecular structure, orientation and electronic interaction with metal surfaces, which is prerequisite for the precise control of various catalytic properties or chemical reaction on metal nanostructures, has been scarcely elucidated in a vast number of SERS-related papers recently published.

To lift these issues, we have investigated to generate flocculates of silver and gold nanoparticles (AgNPs, AuNPs) linked by various target molecules in solutions [8, 12, 13]. This flocculation provides much higher probability to have appropriate gap sizes because they are primarily determined by the size of molecules, which are located at the nanogap. For this purpose, specific interaction between adsorbates and metal surfaces must be effectively utilized. For example, bulky surface residuals on AgNPs such as citrates, a-carbon or Ag\(_2\)O disturb adsorption of a cationic xanthene dye, like rhodamine 6G (R6G\(^+\)) or rhodamine 123 (R123\(^+\)). It is thus necessary to replace these residuals with Cl\(^-\) anions which generate negatively charged surfaces by forming AgCl or AgCl\(_2\) on AgNP surfaces. Accordingly, R6G\(^+\) or R123\(^+\) electrostatically adsorb on negatively charged AgNPs to generate flocculates providing huge SERS enhancement [12, 13], in contrast to faint adsorption of neutralized or negatively charged dye molecules. On the other hand, even neutral R123\(^0\) as well as R123\(^+\) cations adsorb on AuNPs, because the surface of AuNPs are covered by much less amount of negative charges and residuals [13]. Thus, we found a promising way to form metal nanoparticle flocculates linked by xanthene dye molecules, which provides enormous SERS activity.

Here we extended the flocculation method to oxazine dye, like cresyl violet (CV\(^+\), CV\(^0\)) in cationic and neutral state, while exploring the effect of charge on adsorbates to the flocculation. Also, we investigated the effect of steric hindrance to the adsorption using nile blue (NB) molecules, which possess a bulky diethyl amino group (–Netz\(_2\)) unlike compact dihydro groups (–NH\(_2\)) in CV molecules (Figs. 1a-1d). Extinction and SERS spectra of cationic CV\(^+\) and neutral CV\(^0\) molecules on AuNPs were measured in solutions to detect the flocculation of AuNPs with coupled LSP resonances and enhanced Raman intensity of adsorbates. Then, we evaluated SERS spectral deviations by neutralization while comparing with those by deuteration of their amino groups. Vibrational analysis of CV\(^+\) was also exploited using density functional theory (DFT) to assign the bands varied by neutralization. Thus, the effect of charge and steric hindrance of adsorbates were elucidated to form AuNP flocculates with enormous SERS enhancement exploiting oxazine dye molecules.

II. EXPERIMENTS

Detailed experimental procedures are the same as previously reported [8, 12, 13]. Briefly, AuNPs were prepared using the citrate reduction method reported by Frens [14]. Briefly, a trisodium citrate solution (16 mg in 1.6 mL so-
solution, i.e. 1% aq. solution) was added to a HAuCl₄ solution, and then the reacting solution was kept at 100°C for 5 min. The AuNPs thus prepared (called ‘as-prepared AuNPs’ were mostly spherical with a diameter of ca. 20 nm according to SEM measurements (data not shown). NaCl was used to substitute residual citrate on AuNPs with negatively charged Cl⁻ anions. Also NaCl eliminates steric hindrance of citrate anions for the adsorption of cationic dye molecules, and leaves larger amount of negative charge than as-prepared AuNPs [12]. To neutralize the dihydro amino groups in CV⁺ and NB⁺, these molecules were first incubated in a 10 mM NaOH solution, in which pH is higher than their pKa of ~ 9.5. Prepared neutral CV₀ and NB₀ molecules were then mixed with AuNP containing 10 mM NaOH solutions for adsorption.

In these experiments, the concentration of AuNPs was adjusted to be constant, which was estimated at ca. 1.9 × 10¹¹ particles/mL assuming that HAuCl₄ (20 mg = 0.10 mmol) was completely reduced by citrate salts to generate Au nanospheres with 20 nm in diameter (5.0 × 10⁵ atoms/particle), and being slightly corrected using a molecular extinction coefficient in literature [15]. Extinction spectra were measured for the sample solutions to characterize LSP resonances of isolated and adjacent AuNPs using a JASCO 540 uv-vis spectrophotometer with a conventional square quartz cell (10 mm in optical path). Here CV and NB concentrations higher than 1 × 10⁻⁷ M were used to detect definite features of coupled LSPs. SERS spectra were measured for the same solutions as those in extinction measurements using a modified Renishaw micro-Raman spectrometer with a thin layer solution cuvette composed of a cover slip, a ~ 1 mm thick rubber spacer, and a Si plate [8, 12, 13]. Sample solutions (100 µL) were illuminated using a He-Ne laser (632.8 nm and ~ 5 mW). In the present study, we adopted an external illumination using a conventional optics with a focal length of 10 cm to suppress laser power density less than 1 µW/µm² at the sample position. Scattered Raman signal was collected using a 50× objective, and transferred to the spectrometer. An accumulation time of 50 s was used to obtain SERS spectra with sufficiently good S/N ratio and reproducible wavenumber (~ ± 1 cm⁻¹) under the present experimental conditions, which is possibly reduced using higher laser power without damaging samples, e.g. 10 s with a power of 25 mW. A sharp long-pass edge filter transparent down to ~ 200 cm⁻¹ (Omega) was adopted to cut Rayleigh scattering.

To assign Raman bands shifted by neutralization of CV⁺ on AuNPs, amino groups (–NH₂) were deuterated (–ND₂) by dissolving these molecules in heavy water. AuNP dispersed solution and NaOH solutions were also prepared using heavy water before mixing with deuterated dye solutions. The final solution containing CV⁺, NaCl and AuNPs consists of water with a composition of 95% deuterium and 5% of hydrogen, which results in amino groups of 4D : 3D1H = 81 : 17 denoting replacement efficiency of two –ND₂ (4D), and one –ND₂ and one –NDH (3D1H) in CV (see also Fig. 1a). The ratio can be increased up to 96 : 4 by using a higher deuterium concentration of 99% (D) : 1% (H) in water. Still, the adopted concentration was sufficiently high to distinguish Raman peaks associated with amino groups from others as described in the following section. Namely, such deuteration gave rise to appreciable spectral shift for the peaks attributable to amino groups. Vibrational analysis was performed for CV⁺ with and without deuterated amino groups using a DFT method with a basis set of B3LYP/6-31+g(d,p) in Gaussian 03w. Scaling factor of 0.9887 was used to adjust calculated vibrational spectra to the observed values.

### III. RESULTS AND DISCUSSION

#### A. Extinction spectral changes upon the formation of AuNP flocculates by CV⁺, NB⁺, CV₀ and NB₀ molecules

A pronounced peak was observed at ~ 521 nm originated from the LSP of isolated AuNPs (Figs. 2a-2c), which accords with a theoretical position for an AuNP with a diameter of 20 nm [12]. By the addition of CV⁺ and NB⁺ molecules to AuNP dispersed solutions, coupled LSP peaks appeared at 580-750 nm (Figs. 2a, 2b, 3a, 3b, 4a and 4b) similar to the case for R123 molecules. This observation provides evidence that closely adjacent AuNPs were formed by the addition of CV⁺ irrespective of NaCl pretreatment of AuNPs [8, 12, 13]. We noted that the additional peak at ~ 676 nm at 6.0 × 10⁻⁷ M (CV⁺) on Cl⁻-treated AuNPs gradually shifted to longer wavelengths, for instance to 708 nm after 1 h incubation (Fig. 3a). This indicates a gradual increase of the particle number in each flocculate as theoretically predicted [12].

Neutral CV₀ and NB₀ were formed by precedent immersion of these cationic dyes in 10 mM NaOH solutions. The deprotonation of amino groups (–NH₂) was convinced
properties of NB molecules (Figs. 1b, 4a and 4c). Detailed adsorption nm for the flocculation of AuNPs induced by NB Note that coupled LSP peaks were observed at 600-800 nm for the flocculation of AuNPs induced by NB molecules adsorb only with chemical interaction, whereas CV molecules adsorb on Cl⁻-treated AuNPs, possessing negative charges, with electrostatic force, as we reported for xanthene dye molecules [12]. Accordingly, the results indicate that CV molecules adsorb on as-prepared AuNPs both with electrostatic and chemical interaction. This observation is in contrast to the result for NB⁺ on as-prepared AuNPs, which gave exactly the same SERS spectra as NB⁺ on Cl⁻-treated AuNPs (Figs. 6a and 6b). Hence, NB⁺ molecules adsorb on Au surfaces exclusively with electrostatic interaction. These distinct adsorption properties are probably caused by different steric hindrance of compact dihydro amino (–NH₂) groups in CV⁺ and bulky diethyl amino groups (–Net₂) in NB⁺ to the adsorption on AuNPs as discussed in the next section. 

Next, we discuss chemisorption of CV⁺ molecules on AuNPs using observed SERS spectra. CV⁺ molecules can chemisorb on Au surfaces via two amino groups, oxygen or nitrogen atoms in an oxazine ring (Figs. 1a and 1c). When they coordinate via lone pair electrons at oxygen or nitrogen atoms in an oxazine ring, CV⁺ molecules should be oriented vertical to the interparticle axis yielding exclusive enhancement in out-of-plane modes under the coupled LSP resonance [5, 12]. Also, residual citrate on AuNPs would sterically hinder vertical orientation due to its much smaller gap size compared to parallel or inclined orientations. In contrast, the inclined orientation of CV⁺ molecules allows chemisorption via two amino groups using both side of molecular plane as described in the next section. Some of SERS bands from CV⁺ molecules gave pronounced peak shifts from those of CV⁺ (> 5 cm⁻¹, which is large enough compared to reproducibility in our spectral measurements), such as 1496 (CV⁺)→1490 cm⁻¹ (CV⁺), 1184→1189 cm⁻¹, 1141→1153 cm⁻¹, 750→750 cm⁻¹, in addition to markedly enhanced peaks at 1585, 1153, 858 and 537 cm⁻¹ (Figs. 7a, 7b and Table I). Interestingly, most peaks shifted by the neutralization also showed spectral changes due to deuteration of amino groups. For instance, Raman bands shifted by the deuteration from 1496 cm⁻¹ (–NH₂) to 1500 cm⁻¹ (–ND₂), 1251→1245 cm⁻¹, 1184→1178 cm⁻¹, 1141→1136 cm⁻¹, 1144→1105 cm⁻¹, 943 cm⁻¹→952, 928 cm⁻¹, 830→808 cm⁻¹, 750→745 cm⁻¹, and 523→514 cm⁻¹ (Figs. 7a, 7b and Table I), which indicates noticeable contribution of amino groups to these bands. Thus, the Raman bands of CV⁺ shifted by the neutralization are associated with the amino groups by which CV⁺ molecules chemisorb on AuNPs. 

To further convince these arguments, we evaluated vibrational modes of CV⁺ molecules using a DFT method. First, optimized structures of CV⁺ and NB⁺ molecules

FIG. 2: Extinction spectra of (a) as-prepared AuNP suspension containing CV⁺, (b) Cl⁻-treated AuNP suspension containing CV⁻ and NB⁻ molecules and NaOH (10 mM), and (d) absorption spectra of 5 × 10⁻⁴ M CV⁺ (0 mM NaOH solution) and 5 × 10⁻⁴ M CV⁻ molecules in 10 and 20 mM NaOH solutions.

B. SERS spectra of CV⁺, NB⁺, CV⁻ and NB⁻ molecules adsorbed on AuNP flocculates

As evidenced by an appearance of coupled LSP peaks, CV⁺ and NB⁺ cations formed flocculates of Cl⁻-treated AuNPs and as-prepared AuNPs. These AuNP flocculates provided enormous SERS enhancement for these dye molecules located at their nanogap as shown in Figs. 5a and 6a. Interestingly, CV⁺ adsorbed on as-prepared AuNPs (Fig. 5b) showed two distinct SERS spectra that are composed of the spectra from CV⁺ on Cl⁻-treated AuNPs and CV⁻ on AuNPs (Figs. 5a and 5c). CV⁻ molecules adsorb on Cl⁻-treated AuNPs, possessing negative charges, with electrostatic force, as we reported for xanthene dye molecules [12]. Accordingly, the results indicate that CV⁺ molecules adsorb on as-prepared AuNPs both with electrostatic and chemical interaction. This observation is in contrast to the result for NB⁺ on as-prepared AuNPs, which gave exactly the same SERS spectra as NB⁺ on Cl⁻-treated AuNPs (Figs. 6a and 6b). Hence, NB⁺ molecules adsorb on Au surfaces exclusively with electrostatic interaction. These distinct adsorption properties are probably caused by different steric hindrance of compact dihydro amino (–NH₂) groups in CV⁺ and bulky diethyl amino groups (–Net₂) in NB⁺ to the adsorption on AuNPs as discussed in the next section.

Next, we discuss chemisorption of CV⁺ molecules on AuNPs using observed SERS spectra. CV⁺ molecules can chemisorb on Au surfaces via two amino groups, oxygen or nitrogen atoms in an oxazine ring (Figs. 1a and 1c). When they coordinate via lone pair electrons at oxygen or nitrogen atoms in an oxazine ring, CV⁺ molecules should be oriented vertical to the interparticle axis yielding exclusive enhancement in out-of-plane modes under the coupled LSP resonance [5, 12]. Also, residual citrate on AuNPs would sterically hinder vertical orientation due to its much smaller gap size compared to parallel or inclined orientations. In contrast, the inclined orientation of CV⁺ molecules allows chemisorption via two amino groups using both side of molecular plane as described in the next section. Some of SERS bands from CV⁺ molecules gave pronounced peak shifts from those of CV⁺ (> 5 cm⁻¹, which is large enough compared to reproducibility in our spectral measurements), such as 1496 (CV⁺)→1490 cm⁻¹ (CV⁺), 1184→1189 cm⁻¹, 1141→1153 cm⁻¹, 750→750 cm⁻¹, in addition to markedly enhanced peaks at 1585, 1153, 858 and 537 cm⁻¹ (Figs. 7a, 7b and Table I). Interestingly, most peaks shifted by the neutralization also showed spectral changes due to deuteration of amino groups. For instance, Raman bands shifted by the deuteration from 1496 cm⁻¹ (–NH₂) to 1500 cm⁻¹ (–ND₂), 1251→1245 cm⁻¹, 1184→1178 cm⁻¹, 1141→1136 cm⁻¹, 1144→1105 cm⁻¹, 943 cm⁻¹→952, 928 cm⁻¹, 830→808 cm⁻¹, 750→745 cm⁻¹, and 523→514 cm⁻¹ (Figs. 7a, 7b and Table I), which indicates noticeable contribution of amino groups to these bands. Thus, the Raman bands of CV⁺ shifted by the neutralization are associated with the amino groups by which CV⁺ molecules chemisorb on AuNPs.
FIG. 3: Temporal variation of coupled LSP spectra of AuNPs floculates formed by CV$^+$ and CV$^0$ molecules: (a) $6.0 \times 10^{-7}$ M CV$^+$ in 8 mM NaCl solution, and (b) $5.0 \times 10^{-7}$ M CV$^0$ in 10 mM NaOH solution.

FIG. 4: Extinction spectra of (a) as-prepared AuNP suspension containing NB$^+$, (b) Cl$^-$-treated AuNP suspension containing NB$^+$, (c) as-prepared AuNP suspension containing NB$^0$, and (d) absorption spectra of $5 \times 10^{-4}$ M NB$^+$ and NB$^0$ solutions.

were obtained, in which a pair of Co-N-Ca substituent are located in the oxazine molecular plane, here Co, N and Ca denote carbon in an oxazine ring, nitrogen and carbon in each amino group (Figs. 8a and 8b). Then, we calculated Raman spectra of CV$^+$ with optimized structures, which corresponded well to those experimentally observed spectra (Figs. 9a, 9b and Table I). Deuteration of the amino groups in CV$^+$ molecules yielded pronounced deviations in calculated peak positions such as $1496\rightarrow1500$ cm$^{-1}$, $1160\rightarrow1148$ cm$^{-1}$, $1122\rightarrow1088$ cm$^{-1}$, $1045\rightarrow1058$ cm$^{-1}$, $950\rightarrow957$ cm$^{-1}$, $759\rightarrow749$ cm$^{-1}$, and $525\rightarrow516$ cm$^{-1}$, which were precisely evaluated based on atomic displacements in each mode (Fig. 10, [17]). These spectral changes calculated for the deuteration are consistent with those experimentally observed shifts as summarized in Table I. CV$^+$ and CV$^0$ molecules possess $C_s$ symmetry (Figs. 1a and 1c), which allows bending $\delta_{N-H}$ to couple feasibly with various stretching and bending in oxazine ring with similar energy. Significant peak shifts by the deuteration are caused by notable contribution of amino groups to these Raman bands. Accordingly, the Raman bands of CV$^+$ shifted by the neutralization are
attributed to the amino groups, which is probably associated with chemisorption via lone pair electron at amino groups to Au surfaces. Also SERS bands at 1585, 1513, 858, and 537 cm$^{-1}$ apparently enhanced by the neutralization is presumably attributed to an orientation change from parallel to the inclined orientation of CV$^0$ molecules, which will be evaluated by more detailed DFT calculations including AuNPs to discuss SERS intensity of these bands under various orientations.

NB$^0$ containing solution gave prominent SERS enhancement, however, the observed spectra were almost the same as those for NB$^+$ as shown in Figs. 6a-6c. It is rather difficult to attribute the adsorption state of NB according to its particular structure consisting of two amino groups and a planar oxazine ring. We have two distinct explanations for the observations based on the molecular structure of NB. The first one is residual NB$^+$ instead of NB$^0$ adsorbed on AuNPs, which remains even in such alkaline solutions as evidenced by a shoulder peak at ~ 1630 nm (Fig. 4d and also see ref. [16]). The second explanation is that NB$^+$ and NB$^0$ do not adsorb via two amino groups (~NH$_2$ and ~N~), but adsorb via one ~NH$_2$ group and oxygen (or nitrogen) in the oxazine ring to link neighboring AuNPs. Note that the diethyl amino group does not chemisorb on Au surfaces in both of these interpretations. Indeed, marked steric hindrance at a diethy lamino group in NB$^0$, orienting vertical to the oxazine plane for free NB$^+$ or NB$^0$ molecules (Fig. 8b), disturbs chemisorption of lone pair electrons at the amino group. Re-orientation of ethyl substituent may eliminate

| TABLE I: Experimental SERS and theoretical vibrational wavenumber for CV$^+$ and CV$^0$ molecules (in cm$^{-1}$). |
|---------------------------------------------------------------|
|                  | CV$^+$ (~NH$_2$) | CV$^+$ (~ND$_2$) | CV$^0$ (~NH$_2$) | CV$^0$ (~ND$_2$) | Assignment$^{[15]}$ |
| Calc. | Exp. | Calc. | Exp. | Calc. | Exp. | Exp. | Exp. |идентифицирует |
| 1634 (88)* | 1643 m | 1634 (84) | 1643 m | 1636 s | 1637 s | 851 c- |
| 1564 (33) | 1585 vw | 1617 (42) | 1587 vw | 1585 m | 1585 m | 851 c |
| 1543 (373) | 1546 m | 1542 (241) | 1543 m | 1549 w | 1549 w | 851 c- |
| 1496 (193) | 1496 v | 1500 (110) | 1500 m | 1490 m | 1485 s | 851 c- |
| 1429 (967) | 1424 m b | 1411 (560) | 1427 m | 1439 s | 1437 s | 851 c- |
| 14157 (161) | 1403 v | 1413 (273) | 1403 sh | 1387 m | 1402 m | 851 c- |
| 1385 (479) | 1377 (600) | 1377 (600) | 1377 (600) | 1377 (600) | 851 c- |
| 1353 (114) | 1342 w | 1351 (216) | 1344 w | 1333 m | 1344 m | 851 c- |
| 1263 (8) | 1266 v | 1266 (2) | 1265 (21) | 1255 (41) |
| 1208 (219) | 1225 (283) | 1225 (283) | 1225 (283) | 1225 (283) |
| 1160 (120) | 1184 m | 1148 (45) | 1178 m | 1189 s | 1188 s | 851 c- |
| 1146 (16) | 1141 v | 1129 (2) | 1136 v | 1153 m | 1147 m | 851 c- |
| 1122 (12) | 1114 v | 1088 (10) | 1105 v | 1113 w | 1123 w | 851 c- |
| 1045 (74) | 1048 v | 1058 (58) | 1045 v | 1058 (58) | 1045 v | 851 c- |
| 1009 (21) | 998 v | 1023 (49) | 1008 v | 999 v | 1007 v | 851 c- |
| 950 (49) | 943 w | 957 (51) | 952 w | 953 w | 960 v | 851 c- |
| 880 (4) | 852 (11) | 928 w | 858 v | 855 v | 852 (11) | 851 c- |
| 841 (0) | 841 (0) | 803 w | 832 (0) | 803 w | 832 (0) | 851 c- |
| 835 (0) | 830 w | 832 (0) | 801 (1) | 801 (1) | 801 (1) | 851 c- |
| 832 (3) | 830 w | 832 (0) | 801 (1) | 801 (1) | 801 (1) | 851 c- |
| 759 (42) | 750 w | 749 (48) | 745 w | 759 w | 747 w | 851 c- |
| 677 (25) | 671 m | 672 (23) | 668 m | 673 m | 671 v | 851 c- |
| 598 (91) | 591 s | 595 (94) | 588 s | 591 s | 591 s | 851 c- |
| 569 (2) | 544 (3) | 548 v | 537 w | 534 w | 544 (3) | 851 c- |
| 534 (1) | 520 m | 520 (0) | 520 m | 520 (0) | 520 m | 851 c- |
| 525 (24) | 523 m | 516 (23) | 514 v | 519 w | 519 v | 851 c- |
| 494 (9) | 491 v | 489 (6) | 488 v | 488 m | 479 m | 851 c- |
| 468 (37) | 463 w | 458 (34) | 454 w | 450 v | 450 v | 851 c- |
| 342 (75) | 339 w | 335 (78) | 338 w | 344 v | 351 v | 851 c- |

*small contribution of $\delta_{\text{C} - \text{H}}$; $^1$Symbols of $\nu_{\text{C} - \text{H}}$, $\nu_{\text{NH}}$, $\delta_{\text{C} - \text{H}}$, $\delta_{\text{NH}}$, $\gamma_{\text{C} - \text{H}}$, $\gamma_{\text{C} - \text{H}}$, $\gamma_{\text{CCC}}$, $\gamma_{\text{NH}}$ denote stretching ($\nu$), in-plane bending ($\delta$) and out-of-plane bending ($\gamma$) of each group; $^2$Calculated intensity of Raman modes under off-resonances; $^3$Experimentally observed intensity: m (medium), w (weak), vw (very weak), s (strong), b (broad) and sh (shoulder). Blue and red numbers indicate marked peak shifts by deuteration of amino groups in calculated and experimental results, respectively.
the steric hindrance, however, coordination energy of lone pair electrons at amino groups, e.g. 20-40 kJ mol\(^{-1}\) is not enough large to compensate the re-orientation energy [18, 19]. A presumable re-oriented structure of NB\(^0\) is illustrated in Fig. 8c, in which two ethyl groups are located almost in the oxazine plane. It suggests such re-oriented structures, as well as those with vertically oriented ethyl groups, still have intrinsic steric hindrance for the chemisorption via lone pair electrons at nitrogen due to hydrogen atoms in diethyl groups. Details on adsorbed structure of NB will be further investigated using various oxazine molecules with distinct substituent and also using DFT calculations including Au atoms to evaluate the spectral variations for different adsorption structures.

In contrast to NB\(^0\), CV\(^0\) molecules with two dihydro amino groups adsorbed on Au surfaces without any serious steric hindrance as explained above. This type of chemisorption is also sensitive to surface charge of AuNPs; CV\(^+\) adsorbed on as-prepared AuNPs both with electrostatic and chemical interaction, whereas CV\(^+\) adsorbed only with electrostatic forces on Cl\(^-\)-treated AuNPs, which has significantly larger amount of negative surface charges than as-prepared AuNPs. Enhancement factor for CV\(^+\) and NB\(^+\) sitting at a nanogap of an AuNP flocculate.

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**FIG. 5:** SERS spectra of CV\(^+\) (a) on as-prepared AuNP, (b) on Cl\(^-\)-treated AuNP and (c) CV\(^0\) on as-prepared AuNP. CV\(^+\) concentrations in (a) and (b) solutions are 6 \times 10\(^{-7}\) M, while CV\(^0\) concentration in (c) is 1 \times 10\(^{-6}\) M.

**FIG. 6:** SERS spectra of NB\(^+\) (a) on as-p AuNP, (b) on Cl\(^-\)-treated AuNP, (c) NB\(^0\) on as-prepared AuNP. NB\(^+\) concentrations in (a) and (b) solutions are 8 \times 10\(^{-7}\) M, while NB\(^0\) concentration in (c) is 5 \times 10\(^{-6}\) M.

**FIG. 7:** SERS spectra of CV\(^+\) (a) and CV\(^0\) (b): Effect of deuteration of the amino groups. Concentrations of CV\(^+\) and CV\(^0\) in these experiments are 6 \times 10\(^{-7}\) M and 8 \times 10\(^{-7}\) M, respectively.

**FIG. 8:** Optimized molecular structures of (a) CV\(^+\), (b) NB\(^+\) obtained with DFT calculations. In addition, (c) presumable structure of NB\(^0\) with a re-oriented ethyl group located in the oxazine plane is illustrated, which is in contrast to almost vertical orientation of the ethyl group (b).
FIG. 9: Calculated Raman spectra of CV$^+$ with –NH$_2$ and –ND$_2$ (a, Upper part). Scaling factor of 0.9887 was used to adjust calculated peak positions to observed spectra. Experimentally observed SERS spectra of CV$^+$ with –NH$_2$ and –ND$_2$ are also shown (b, Lower part).

FIG. 10: Vibrational modes of CV$^+$ molecules: those with faint peak shifts (a, b), those with marked shifts (c-f) by deuteration of amino groups. Vibrational modes (g, h) are presumably assigned to those apparently enhanced peaks (g, h) by neutralization of CV$^+$. See also Fig. 9 and text.

was estimated to be $10^7$-$10^8$ normalized to that of a free CV$^+$ and NB$^+$ molecule in solution, which is similar to $1.2 \times 10^8$ for a cationic R123$^+$ molecule on an AuNP floc-culate. In this estimation, formation efficiency of AuNPs, flocculation efficiency, adsorption ratio of molecules at a nanogap and polarization of exciting laser were considered [5]. The observed enhancement factor is also consistent with $4 \times 10^8$ obtained by FDTD calculations [12].

C. Orientation of adsorbed CV$^+$ and CV$^0$ molecules on AuNPs

The observed results indicate that CV$^+$ molecules adsorb on as-prepared AuNPs with electrostatic forces and chemical interaction while linking them to form AuNP floculates. In contrast, NB$^+$ molecules adsorb on as-prepared AuNPs only with electrostatic forces, which is due to steric hindrance of ethyl substituent at amino groups. Electrostatic interaction does not inherently concern with orientation of adsorbed CV$^+$ and NB$^+$ molecules in AuNP floculates, because Coulomb force is determined solely by the distance of separated charges, here the distance between CV$^+$ and negatively charged AuNPs. Accordingly, CV$^+$ and NB$^+$ molecules are oriented parallel to the interparticle axis in average (Fig. 11a), while adsorbates may thermally change their orientation in a random way.

CV$^0$ molecules adsorb on AuNPs probably via lone pair electrons at amino groups. This was evidenced by noticeable peak shifts observed for Raman bands of amino groups, and also supported by an appearance of a $\nu_{\text{Au-N}}$ stretching band in R123$^+$ molecules [12] with the resembled structure to CV$^0$. An inclined orientation is inevitable for CV$^0$ to coordinate neighboring AuNPs (Fig. 11b), because their lone pair electrons are oriented almost vertical to the oxazine plane as shown in Figs. 1a and 8a. Hence, the diethyl amino group in NB molecules sterically hinders the chemisorption to AuNPs.
IV. CONCLUSION

From the observations in extinction and SERS spectra, we conclude that CV$^+$ molecules adsorb on AuNPs both with electrostatic and chemical interaction, whereas NB$^+$ molecules exclusively adsorb with electrostatic forces owing to considerable steric hindrance of ethyl substituent at amino groups. CV$^0$ molecules adsorb on AuNP via lone pair electrons at amino groups, which yield an inclined orientation to the interparticle axis of AuNPs. Thus electrostatic and chemical adsorption of oxazine dye molecules to AuNPs are realized by controlling charge and steric hindrance, which are indeed critical to form the flocculates providing enormous SERS intensity. The flocculation of metal nanoparticles using target molecules in solution is invaluable to apply single molecules SERS to various molecules.

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[16] Residual NB$^+$ cations in 10 mM NaOH solutions can affect extinction and SERS spectra. Based on proton dissociation of Brønsted acid, the concentration of residual protonated NB$^+$ (=HNB$^+$) is expressed by the following equation. $[\text{HNB}^+]/[\text{NB}^+] = K_a/[\text{H}^+] = K_a \times [\text{OH}^-]/K_w$. 
Here [NB\(^0\)], \(K_a\), [H\(^+\)], and [OH\(^-\)] denote concentration of deprotonated NB\(^0\), dissociation constant of HNB\(^+\), concentration of proton, and hydroxide ion, respectively. Using \(K_a = 10^{-9.5} \sim 10^{-10}\), \([\text{OH}^-] = 10^{-11.5}\) (at 10 mM NaOH), and \(C_a\) (total dye concentration, \(C_a = [\text{HNB}^+] + [\text{NB}^0]\)) = 5 \times 10^{-5} \text{ M}, the concentration of [HNB\(^+\)] and [NB\(^0\)] is 1.6 \times 10^{-6}-5 \times 10^{-7} \text{ M} and 5 \times 10^{-5} \text{ M}. Residual quantity of NB\(^+\) (=HNB\(^+\)), which is estimated to be 1.6 \times 10^{-6}-5 \times 10^{-7} \text{ M}, is enough large to form AuNP flocculates providing enormous SERS intensity. Residual NB\(^+\) was indeed detected in 10 mM NaOH solution as a shoulder peak at \(\sim 630\) nm in absorption spectra (Fig. 4d).

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