Surface-Enhanced Hyper-Raman Spectra of Adenine, Guanine, Cytosine, Thymine, and Uracil

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ABSTRACT: Using picosecond excitation at 1064 nm, surface-enhanced hyper-Raman scattering (SEHRS) spectra of the nucleobases adenine, guanine, cytosine, thymine, and uracil with two different types of silver nanoparticles were obtained. Comparing the SEHRS spectra with SERS data from the identical samples excited at 532 nm and with known infrared spectra, the major bands in the spectra are assigned. Due to the different selection rules for the one- and two-photon excited Raman scattering, we observe strong variation in relative signal strengths of many molecular vibrations obtained in SEHRS and SERS spectra. The two-photon excited spectra of the nucleobases are found to be very sensitive with respect to molecule–nanoparticle interactions. Using both the SEHRS and SERS data, a comprehensive vibrational characterization of the interaction of nucleobases with silver nanostructures can be achieved.

INTRODUCTION

Raman spectroscopy is widely used for the sensitive characterization of molecular structure and interactions. In the structure elucidation of nucleic acids and their nucleotide building blocks, Raman spectroscopy, in resonance with electronic transitions in the UV, has been one of the most important tools.1−4 The possibility to enhance Raman signals of many chemical compounds in surface-enhanced Raman scattering (SERS)1−7 has been used to study the structure and interaction of nucleotides and nucleic acids off-resonance.8−13 Hyper-Raman scattering (HRS) is the two-photon excited analogue of Raman scattering and gives signals shifted relative to the second harmonic of the excitation wavelength.14−16

In the local fields of plasmonic materials the very low hyper-Raman cross-sections can be overcome,17−19 and sensitive probing of the interaction of the molecules with silver or gold nanoparticles, often used as plasmonic substrates, is enabled.20−22 SEHRS with its different selection rules compared to Raman and infrared absorption spectroscopy provides complementary chemical and structural information.15

HRS, due to the nonlinearity of the process, benefits even more from electromagnetic enhancement than Raman scattering, and it is possible to acquire spectra with cross-sections that are similar to two-photon fluorescence.23 While resonant hyper-Raman scattering in solutions of organic molecules is feasible,24 obtaining nonresonant hyper-Raman spectra from solutions of biomolecules without the help of surface enhancement is practically not possible because of the low cross-section of HRS. In addition to a higher electromagnetic contribution in SEHRS compared to SERS, also the chemical contribution in SEHRS enhancement can vary.22

SEHRS spectra of several chemical compounds such as pyridine,25,26 bipyridines,20 pyrazine,27 and adenine28 are known. Meanwhile, first SEHRS spectra of complex biological materials23 suggest a thorough assessment of the capabilities of SEHRS as a tool for microprobing of organic structures and materials. For example, it has been shown recently that the combination of SERS with SEHRS is more powerful for microenvironmental pH sensing than SERS alone.21,29

To explore further the potential of SEHRS for probing bioorganic samples, in this work, we report nonresonant SEHRS spectra of five important nucleobases, adenine, guanine, cytosine, thymine, and uracil. In order to characterize the nucleobases’ interaction with the plasmonic nanostructure used as SEHRS substrate, the spectra are obtained with different types of silver nanostructures and the SEHRS spectra excited with 1064 nm are compared with SERS data obtained at 532 nm excitation from the identical samples. The data are discussed before the extensive background of previous work on nucleobases carried out by means of Raman, SERS, and infrared spectroscopy.

EXPERIMENTAL SECTION

Synthesis of the Nanoparticles and Sample Preparation. Silver nitrate (99.9999%), hydroxylamine hydrochloride (99%), sodium hydroxide (p.a.), magnesium sulfate heptahydrate (99%), borax/sodium hydroxide buffer solution (pH 10), adenine (99%), guanine (99%), thymine (97%), uracil (99%),...
and cytosine (99%) were purchased from Sigma-Aldrich. Trisodium citrate dihydrate (99%) was purchased from Th. Geyer, and sodium chloride (99,6%) was purchased from J. T. Baker. All chemicals were used without further purification. All solutions were prepared using Milli-Q water (USF Elga Purelab Plus purification system).

Silver nanoparticles were prepared by chemical reduction of silver nitrate by citrate or hydroxylamine, respectively. For citrate reduced silver nanoparticles, 46 mg of silver nitrate was dissolved in 245 mL of water and heated to boiling with extensive stirring. A 5 mL aliquot of a 0.04 M sodium citrate solution was added dropwise, and the reaction mixture was kept boiling for ca. 1 h. For hydroxylamine reduced silver nanoparticles, 17 mg of silver nitrate, dissolved in 10 mL of water, was added rapidly to a 90 mL solution, containing 11 mg of hydroxylamine hydrochloride and 12 mg of sodium hydroxide. The reaction mixture was stirred for 30 min at room temperature.

For the SERS and SEHRS experiments, silver nanoaggregates were formed by the addition of sodium chloride or magnesium sulfate to the nanoparticle solutions and were mixed with stock solutions of the nucleobases to give a final sample concentration of $5 \times 10^{-5}$ M. Due to its poor water solubility, the guanine stock solution contained 0.001 M hydrochloric acid.

**Raman Experiments.** The SERS and SEHRS spectra were measured using an imaging spectrometer by microprobe sampling (10x objective). The experimental setup was described previously in ref 21. Briefly, hyper-Raman excitation at 1064 nm was provided by a mode-locked laser producing 7 ps pulses at a 76 MHz repetition rate, and its second harmonic was used for Raman excitation at 532 nm. The liquid samples were placed in microcontainers, and the Raman and hyper-Raman scattering were collected in confocal and epi-illumination microscope configuration. Typically, SERS spectra were accumulated for 1 s with a photon flux density of $1.4 \times 10^{27}$ photons cm$^{-2}$ s$^{-1}$, and SEHRS spectra for 40–60 s with $1.7 \times 10^{28}$ or $5.1 \times 10^{28}$ photons cm$^{-2}$ s$^{-1}$. Spectral resolution was 3–6 cm$^{-1}$, considering the full spectral range. The hyper-Raman spectra were background corrected using an automatic algorithm provided by ref 32.

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**RESULTS AND DISCUSSION**

Using high repetition rate mode-locked picosecond excitation at 1064 nm with photon flux densities ranging from $3.4 \times 10^{27}$ to $5.1 \times 10^{28}$ photons cm$^{-2}$ s$^{-1}$ and silver nanostructures prepared according to two different protocols as plasmonic substrates, it was possible to obtain high quality nonresonant SEHRS spectra of the five nucleobases (chemical structures in Figure 1). The overall SEHRS signals yielded in the experiments with citrate reduced silver nanoparticles were 5–10 times higher than those obtained with the hydroxylamine reduced nanoparticles. This is consistent with the SEHRS enhancement factors in previous experiments with the same nanostructures, pointing to specific properties of the nanoaggregates that are formed by the different nanoparticles.

Both one- and two-photon excited spectra with the two nanoparticle solutions exhibit characteristic vibrational bands of the investigated compounds, and the SERS spectra obtained here (spectra B and D in Figures 2–5) are in good agreement with similar studies reported previously, in refs 11–13, 28, and 33–36. We obtain very similar SERS spectra and very similar SEHRS spectra respectively with citrate and hydroxylamine reduced silver nanoparticles. This verifies the high reproducibility of the spectra. Nevertheless, as will be discussed in the following sections, small differences were observed, specifically regarding the intensity ratios for some bands. As will be shown, the molecules display qualitatively very different SERS and SEHRS spectra (compare, for example, spectra A and B in each of the Figures 2–6). One very obvious
The difference between SERS and SEHRS spectra common to all five nucleobases is the signal of the symmetric ring breathing mode, which is particularly enhanced in SERS but weak or medium compared to other bands in the SEHRS spectra. This mode is also very strong in the Raman but medium in the IR absorption spectra of the solid compounds (Raman and IR absorption spectra of the nucleobases in ref 37). Regarding the ring breathing mode, the SEHRS and the IR spectra show more similarity than SERS with IR spectra.

SEHRS Spectrum of Adenine. The SEHRS and SERS spectra of adenine are presented in Figure 2, and band assignments are given in Table 1. Both one- and two-photon excited spectra are in very good agreement with previously reported SERS23,33 and SEHRS spectra23,28 of adenine on silver substrates. In the SEHRS spectra (Figure 2A,C) the signal of the symmetric ring breathing mode (734 cm$^{-1}$), which is dominating the SERS spectra (compare with spectra B and D of Figure 2), is similar to those of the other bands. This band is also very strong in the normal Raman spectrum, but medium in the IR absorption spectrum of solid adenine (see, e.g., ref 37). Furthermore, the SEHRS data indicate a strong contribution from several bands associated with NH$_2$ and N9-H deformation modes, specifically the NH$_2$ rocking band at 1026 cm$^{-1}$, in-plane NH$_2$ scissoring vibrations at 1487, 1554, and around 1650 cm$^{-1}$, and the three bands at 564, 1141, and 1600 cm$^{-1}$, which can be associated with N9-H bending modes (see Table 1 for details).

The respective SEHRS (Figure 2A,C) and SERS (Figure 2B,D) spectra obtained with the two different silver nanostructures are very similar. Comparing the SEHRS spectra obtained with the different silver nanostructures (Figure 2A,C), we can observe small differences in the intensity ratios of the same bands. The differences in the SERS (Figure 2B,D) are very weak; a small band at 482 cm$^{-1}$ associated with an out-of-plane N9-H and NH$_2$ wagging appears only in the spectrum with citrate reduced silver nanoparticles (Figure 2B).

SEHRS Spectrum of Guanine. The SEHRS and SERS spectra of guanine with citrate and hydroxylamine reduced silver nanoparticles are shown in Figure 3. In adenine, the respective SEHRS and SERS spectra of guanine with the two types of silver nanostructures are very similar (compare Figure 3A with Figure 3C for SEHRS and Figure 3B with Figure 3D for SERS). The SEHRS spectra of guanine differ from the SERS spectra in the region between 1200 and 1700 cm$^{-1}$, e.g., in a pronounced SEHRS signal of the NH$_2$ scissoring at around 1570 cm$^{-1}$ and in
the absence of the in-plane NH bending mode at 1351 cm⁻¹ (Figure 3A,C). The most prominent differences, however, are found in the region below 800 cm⁻¹: The ring breathing mode at 660 cm⁻¹ is very strong in SERS (Figure 3B,D) but very weak in the SEHRS spectra (Figure 3A,C). Vice versa, the ring deformation modes at 572 and 517 cm⁻¹ are very strong in SEHRS and weak in the SERS spectra (see Table 2 for detailed band assignments). Unlike adenine, the SEHRS spectra of guanine (Figure 3) do not show comparable signals for the ring breathing mode and the ring deformation modes below 700 cm⁻¹ (compare, e.g., Figure 3A with Figure 2A).

**SEHRS Spectra of Uracil and Thymine.** Figure 4 and Figure 5 present the spectra of uracil and thymine, respectively. Previous SERS and DFT studies have shown that both pyrimidine bases interact with silver surfaces in their deprotonated forms, even at neutral pH. Therefore, also under the conditions of the experiments here, the spectra of uracil in Figure 4 and of thymine in Figure 5 must be those of the anions of the two nucleic acid bases. As for adenine and guanine discussed above, the SEHRS spectra of uracil (Figure 4A,C) and thymine (Figure 5A,C) differ greatly from their SERS spectra, this is observed for both types of silver nanoparticles. Tables 3 and 4 provide the band assignments for all spectra of uracil and thymine, respectively. In the SEHRS spectra we find strong signals due to the C=O stretching vibrations around 1600 cm⁻¹ and a relatively low intensity ring breathing band at 802 cm⁻¹ in uracil (Figure 4A,C) and around 780 cm⁻¹ in thymine (Figure 5A,C).

The spectra with citrate reduced silver nanoparticles (Figures 4A,B and 5A,B) were obtained by the addition of nucleobase solutions to the nanoparticle aggregates; the pH of the resulting mixtures was 7.5. It should be noted that it was not possible to obtain spectra of thymine and uracil under the same conditions (pH 7) with the hydroxylamine reduced silver nanoparticles (Tables 3 and 4 and Figures 4 and 5). The spectra with citrate reduced silver nanoparticles (compare panel B with panel D of Figure 5) could result from the different orientation at different pH, the pH of the resulting mixtures was 7.5. Therefore, also other differences in the spectra of uracil and thymine can be attributed to different orientation at different pH, the pH dependent differences in the spectra of uracil and thymine can also be caused by the presence of different tautomeric forms of the molecules: Thymine and uracil can be deprotonated at the

**Figure 5.** Surface-enhanced hyper-Raman (A, C) and surface-enhanced Raman (B, D) spectra of thymine obtained with citrate (A, B) and hydroxylamine (C, D) reduced silver nanoparticles: excitation, 1064 nm (A, C) and 532 nm (B, D); photon flux density, 1.7 × 10⁻⁷ photons cm⁻² s⁻¹ (A, C) and 1.4 × 10⁻⁷ photons cm⁻² s⁻¹ (B, D); acquisition time, 40 s (A), 100 s (C), and 1 s (B, D); scale bars, 5 cps (A), 300 cps (B), 1 cps (C), and 600 cps (D); thymine concentration, 5 × 10⁻⁵ M. Spectra with hydroxylamine reduced silver nanoparticles were obtained at pH 10.

**Figure 6.** Surface-enhanced hyper-Raman (A) and surface-enhanced Raman (B) spectrum of cytosine obtained with citrate reduced silver nanoparticles: excitation, 1064 nm (A) and 532 nm (B); photon flux density, 4.7 × 10⁻⁸ photons cm⁻² s⁻¹ (A) and 1.4 × 10⁻⁷ photons cm⁻² s⁻¹ (B); acquisition time, 40 s (A) and 1 s (B); scale bars, 10 cps (A) and 500 cps (B); cytosine concentration, 5 × 10⁻⁵ M.
N1 or N3 position, which leads to a tautomeric equilibrium between both deprotonated forms. The distribution of the two species depends on the temperature, the dielectric constant of the solvent, ionic strength, and pH of the solution\textsuperscript{13,43–45} and has been studied for uracil\textsuperscript{13} and thymine\textsuperscript{35} by means of SERS and DFT previously. According to the assignments proposed in ref 13, the N1$\rightarrow$C6 stretching at 1532 cm$^{-1}$ in the SERS spectrum of uracil is characteristic of the N1-deprotonated

Table 1. Raman Shift Values from SEHRS and SERS Spectra of Adenine with Hydroxylamine (AgHA) and Citrate (AgCit) Reduced Silver Nanoparticles and Assignment to Vibrations of the Adenine Molecule (Based on Reference 33)

| Raman shift/cm$^{-1}$ | SEHRS | SERS | plane | assignments\textsuperscript{a} |
|-----------------------|-------|------|-------|---------------------------------|
| AgHA                  | AgCit | AgHA | AgCit |                                 |
| 1641 m                | 1653 m | 1551 w | 1553 w | sciss NH$_2$, str C6$\rightarrow$N10, C5$\rightarrow$C6 |
| 1603 m                | 1600 m | 1461 m | 1464 m | str N3$\rightarrow$C4, N1$\rightarrow$C6, C5$\rightarrow$N7, N7$\rightarrow$C8, bend N9$\rightarrow$H |
| 1554 m                | 1554 m | 1397 m | 1398 m | sciss NH$_2$ |
| 1489 m                | 1487 m | 1372 m | 1372 m | str N7$\rightarrow$C8, bend C8$\rightarrow$H, sciss NH$_2$ |
| 1457 s                | 1461 s | 1332 s | 1329 s | str C5$\rightarrow$N7, N1$\rightarrow$C2, C2$\rightarrow$N3, C5$\rightarrow$C6, bend C2$\rightarrow$H |
| 1371 s                | 1374 s | 1274 m | 1275 m | bend C8$\rightarrow$H, N9$\rightarrow$H, str N7$\rightarrow$C8 |
| 1338 s                | 1335 s | 1249 m | 1251 vw | bend C8$\rightarrow$H, N10$\rightarrow$H11, str C4$\rightarrow$N9, N3$\rightarrow$C4, C6$\rightarrow$N10 |
| 1270 vw               | 1275 vw | 1249 m | 1251 vw | bend C8$\rightarrow$H, N10$\rightarrow$H11, str C4$\rightarrow$N9, N3$\rightarrow$C4, C6$\rightarrow$N10 |
| 1215 w?               | 1220 w? | 1136 m | 1135 m | str C8$\rightarrow$N9, bend N9$\rightarrow$H, C8$\rightarrow$H |
| 1141 s                | 1140 s | 1026 br | 1026 w | rock NH$_3$ |
| 1026 vw               | 1025 br | 965 s  | 960 w  | 5-ring def |
| 965 w                 | 964 w  | 920 w  | 920 w  | 6-ring def |
| 920 m                 | 920 m  | 792 w  | 792 vw | 6-ring def, wag C8$\rightarrow$H |
| 792 w                 | 790 m  | 734 s  | 734 vs | ring breath |
| 689 s                 | 689 m  | 649 m  | 631 m  | 5-ring def, wag C8$\rightarrow$H, N9$\rightarrow$H |
| 649 m                 | 652 m  | 564 s  | 563 w  | 6-ring def |
| 564 s                 | 564 s  | 564 s  | 563 w  | 6-ring def |
| 459 w?                | 463 w? | 459 w? | 459 w? | bend C2$\rightarrow$N10 |

\textsuperscript{a}vs, very strong; s, strong; m, medium; w, weak; vw, very weak; br, broad. \textsuperscript{b}Bend, bending; breath, breathing; def, deformation; rock, rocking; sciss, scissoring; str, stretching; wag, wagging; 5-ring, five-membered ring; 6-ring, six-membered ring.

Table 2. Raman Shift Values from SEHRS and SERS Spectra of Guanine with Hydroxylamine (AgHA) and Citrate (AgCit) Reduced Silver Nanoparticles and Assignment to Vibrations of the Guanine Molecule (Based on Reference 34)

| Raman shift/cm$^{-1}$ | SEHRS | SERS | plane | assignments\textsuperscript{a} |
|-----------------------|-------|------|-------|---------------------------------|
| AgHA                  | AgCit | AgHA | AgCit |                                 |
| 1664 m                | 1666 w | 1694 s | 1696 s | str C6=O, C5$\rightarrow$C6, bend N1$\rightarrow$H, sciss NH$_2$ |
| 1562 vs               | 1570 s | 1543 w | 1541 w | sciss NH$_2$, str C2$\rightarrow$N10 |
| 1455 s                | 1460 s | 1457 s | 1461 s | ring str C$\rightarrow$N, sciss NH$_2$, bend N1$\rightarrow$H |
| 1385 w                | 1376 w | 1382 s | 1388 s | ring str C$\rightarrow$N, C$\rightarrow$C, rock NH$_2$, bend N1$\rightarrow$H, N10$\rightarrow$H12, str C2$\rightarrow$N10 |
| 1289 m                | 1260 m | 1298 m | 1299 m | ring str C$\rightarrow$N, C$\rightarrow$C, bend C8$\rightarrow$H, rock NH$_2$ |
| 1229 m                | 1225 m | 1211 m | 1231 m | bend C8$\rightarrow$H, N9$\rightarrow$H10, str N5$\rightarrow$N7, N7$\rightarrow$C8 |
| 1051 vw               | 1097 m | 1055 vw | 1057 w | rock NH$_2$, ring str C$\rightarrow$N |
| 995 m?                | 1016 br? | 984 w  | 986 w  | 5-ring def |
| 865 m                 | 857 m  | 864 w  | 864 w  | 5-ring def, 6-ring def, wag N9$\rightarrow$H, N1$\rightarrow$H |
| 725 w                 | 726 w  | 653 w  | 656 vs | 660 vs | 6-ring def |
| 653 w                 | 656 w  | 572 s  | 570 s  | 574 w  | 6-ring def |
| 572 s                 | 570 s  | 517 vs | 520 m  | 519 m  | 6-ring def |
| 517 vs                | 517 vs | 459 w? | 463 w? | bend C2$\rightarrow$N10 |

\textsuperscript{a}vs, very strong; s, strong; m, medium; w, weak; vw, very weak; br, broad. \textsuperscript{b}Bend, bending; breath, breathing; def, deformation; rock, rocking; sciss, scissoring; str, stretching; wag, wagging; 5-ring, five-membered ring; 6-ring, six-membered ring.
tautomer. In the SERS spectrum measured with the citrate reduced silver nanoparticles (Figure 4B), this band is only very weak. This indicates that, in the case of the citrate reduced nanoparticles, the N3-deprotonated tautomer contributes more to the SERS spectra, supporting earlier findings.13

Particularly, the SEHRS data can provide valuable additional information about the interaction of the molecules with the silver nanostructures at the two different pH values, due to the different selection rules that govern the two-photon excited Raman process. Similar to the SERS spectrum, the SEHRS spectrum of uracil at pH 7 (Figure 4A) does not show a contribution at 1532 cm$^{-1}$. At alkaline pH (Figure 4C), a strongly enhanced band at 1532 cm$^{-1}$ appears in the SEHRS spectrum, clearly indicating the presence of the N1-deprotonated species. The signal is the strongest in the SEHRS spectrum and is more pronounced than in the SERS spectrum of this tautomer (Figure 4D). Also the band at 645 cm$^{-1}$ (Table 3) in the SEHRS spectrum at pH 10 (Figure 4C and Supporting Information Figure S1) can be related to a changed distribution of the uracil anion tautomers. In the SERS spectrum (Figure 4D), it is not visible as clearly.

Table 3. Raman Shift Values in the SEHRS and SERS Spectra of Uracil with Hydroxylamine (AgHA) and Citrate (AgCit) Reduced Silver Nanoparticles and Assignment to Vibrations of the Uracil Molecule (Based on Reference 13)

| Raman shift/cm$^{-1}$ | SEHRS | SERS |
|-----------------------|-------|------|
|                        | AgHA  | AgCit| AgHA  | AgCit |
| 1654 s                 | 1630 s| 1630 s| in    | str C4=O, C2=O |
| 1590 s                 | 1590 m| 1600 m| in    | str C2=O, C4=O, bend N1–H, C5–H |
| 1530 vs                | 1532 m| 1530 vv| in    | str C5–C6, C6–N1, bend C6–H |
| 1489 br?               | 1489 |      | in    | str C6–N1, C4–C5, C2=O |
| 1400 m                 | 1402 s| 1402 vs| in    | bend N1–H, C6–H, C5–H |
| 1374 m                 | 1372 m|        | in    | bend N3–H, C5–H, C6–H |
| 1275 w                 | 1279 s| 1279 s| in    | str N3–C4, C4–C5, C6–N1, bend N1–H, C5–C6–H |
| 1215 m                 | 1219 m| 1217 m| in    | bend N1–H, C6–H, C5–H, str C6–N1 |
| 1103 vv                | 1104 vv| 1098 vv| in    | bend C5–H, str C5–C6, C6–N1 |
| 1056 br                | 1040 br| 1050 br| in    | ring def |
| 1011 br                | 1011 br| 1047 br| out   | wag C6–H |
| 808 w                  | 804 w| 803 vs| in    | ring breath |
| 780 vw                 | 773 vw| 764 vw| out   | ring def |
| 643 m                  | 649 vv| 645 w| in    | ring def |
| 600 m                  | 600 m| 600 m| in    | ring def |
| 559 m                  | 561 m| 560 m| in    | ring def |
| 456 m                  | 440 m| 452 br| 448 br| in    | bend C2=O, C4=O |

*vs, very strong; s, strong; m, medium; w, weak; vw, very weak; br, broad. *Bend, bending; breath, breathing; def, deformation; str, stretching; wag, wagging.

Table 4. Raman Shift Values in the SEHRS and SERS Spectra of Thymine with Hydroxylamine (AgHA) and Citrate (AgCit) Reduced Silver Nanoparticles and Assignment to Vibrations of the Thymine Molecule (Based on References 35 and 12)

| Raman shift/cm$^{-1}$ | SEHRS | SERS |
|-----------------------|-------|------|
|                        | AgHA  | AgCit| AgHA  | AgCit |
| 1652 m                 | 1649 s| 1647 vs| 1649 s| in    | str C2=O, C4=O |
| 1600 vs                | 1601 vs| 1604 m| 1605 m| in    | str C2=O, C4=O |
| 1522 m                 | 1521 m| 1520 vv| in    | ring str |
| 1456 vw                | 1477 s| 1450 vw| in    | bend CH$_3$ |
| 1401 m                 | 1397 vs| 1400 s| 1396 vs| in    | bend N1–H, N3–H |
| 1347 m                 | 1352 s| 1350 vs| 1351 s| in    | bend CH$_3$, def C6–H |
| 1289 m                 | 1279 w| 1282 m| 1280 m| in    | ring str |
| 1217 m                 | 1220 m| 1219 s| 1221 m| in    | str C5–C9 |
| 1043 br                | 1034 br| 1031 vw| 1034 vw| out   | wag CH$_3$ |
| 821 w                  | 820 w| 808 vs| 786 s| in    | ring def |
| 776 w                  | 775 w| 785 vs| 786 s| in    | ring breath |
| 650 br                 | 630 w| 630 w| in    | C2=O, C4=O def |
| 589 w                  | 590 w| 587 m| 584 m| in    | ring def |
| 556 m                  | 556 m| 556 m| in    | ring def |
| 502 m                  | 501 m| 502 m| 498 w| in    | ring def |
| 452 m                  | 444 m| 452 w| 439 w| out   | ring def |

*vs, very strong; s, strong; m, medium; w, weak; vw, very weak; br, broad. *Bend, bending; breath, breathing; def, deformation; str, stretching; wag, wagging.
Figure S1, showing the same SEHRS spectra as Figure 4C, but for the citrate stabilized silver nanoparticles, illustrates that the differences observed in the spectra are indeed pH induced and are very similar for both types of silver nanoparticles. Analogous to this discussion of the contribution of the different tautomers to the uracil spectra at different pH, in the spectra of thymine at alkaline pH (Figure 5C,D) a new band due to the \( \nu \) stretching vibration at 1521 cm\(^{-1} \) appears, which is more intense in the SEHRS spectrum (Figure 5C and Supporting Information Figure S2) and indicates the presence of N1-deprotonated thymine. Similarly, the band around 650 cm\(^{-1} \) becomes more intense than in the spectra at pH 7.5 (Figure 5A,B). These differences between the spectra at different pH can be associated with the shift of the tautomeric equilibrium between the N1- and N3-deprotonated thymine.\(^{35}\)

It should be noted here that in the SERS spectra of uracil (Figure 4B) and thymine (Figure 5B) obtained with the citrate reduced nanoparticles, three additional bands can be observed at 900, 928, and 952 cm\(^{-1} \). These bands appear also in the SERS spectrum of citrate anions shown in ref 46. Since the same band pattern occurs in the SERS spectra of uracil and thymine, and only with citrate reduced nanoparticles (Figure 4B and Figure 5B), we conclude that they indicate coadsorption of citrate on the silver surface. In the SEHRS spectra (Figure 4A and Figure 5A, respectively), only a very weak band at 952 cm\(^{-1} \) can be observed. The weak citrate signals may present an additional advantage of SEHRS over SERS regarding the selective characterization of other analyte molecules as well.

**SEHRS Spectrum of Cytosine.** Due to the very low stability of the hydroxylamine reduced silver nanoparticles in the presence of cytosine, we were not able to collect SEHRS spectra of the molecule using these nanoparticles without a strong background contribution. Figure 6 shows the SEHRS and SERS spectra obtained with the more stable citrate reduced nanoparticles; Table 5 contains the assignments of the bands. The most obvious differences between the SEHRS (Figure 6A) and the SERS spectra (Figure 6B) are the pronounced signals of the C–N stretching mode at 1482 cm\(^{-1} \) and a relatively strong NH\(_2\) bending mode around 1590 cm\(^{-1} \). In accord with the SEHRS data of the other molecules, the ring breathing mode at 798 cm\(^{-1} \) shows a much smaller contribution to the spectra than in the SERS spectrum. Both spectra display the contributions from citrate as discussed above for the spectra of uracil and thymine, with a very small signal at 952 cm\(^{-1} \) in the SEHRS spectrum.

## CONCLUSIONS

We have discussed here nonresonant hyper-Raman spectra of the nucleic acid bases. In SEHRS experiments with silver nanostructures, at an excitation wavelength of 1064 nm, it is possible to obtain spectra of guanine, uracil, thymine, and cytosine, in addition to the SEHRS spectrum of adenine that has been reported before.\(^{23,28}\) In order to acquire more comprehensive vibrational information about the nanoparticle–nucleobase interaction and to interpret the SEHRS spectra, also one-photon excited SERS spectra of the same samples were acquired at a wavelength of 532 nm.

The spectra obtained with silver nanostructures that are stabilized by different molecular species at their surfaces are very reproducible qualitatively, in spite of different SEHRS and SERS enhancement factors of the different nanoaggregates. They suggest that the interaction of the molecules with the silver nanoparticles, e.g., at different pH values, is independent of the type of nanoparticles that are used. The SEHRS spectra of the nucleobases differ greatly from the SERS spectra, due to the different selection rules of the one- and two-photon excited Raman process. Specifically, they show several characteristics of infrared-active vibrations. The very strong ring breathing mode in SERS, which is often used to estimate the adsorbate orientation with respect to the surface, is relatively weak in the SEHRS spectra of all five molecules. As seen for the spectra of uracil and thymine with the silver nanostructures obtained at alkaline pH, the SEHRS spectra can provide additional information about the interaction of the molecules with the nanoparticle surfaces. For example, the N1–C6 stretching characteristic for N1-deprotonated uracil is more enhanced in SEHRS compared to SERS and therefore allows more sensitive determination of the tautomer involved in the interaction. As a further advantage of SEHRS we have observed a greater sensitivity with respect to the nucleobase molecules and fewer contributions by the bands of citrate ions that are known to stabilize the citrate reduced nanoparticles. This can be seen by the presence of citrate bands in the SERS spectra of uracil, thymine, and cytosine, in contrast to almost no contribution of citrate in the corresponding SEHRS spectra.

In conclusion, it was shown that the combination of one- and two-photon excitation allows a comprehensive vibrational spectroscopic characterization of the nucleobase–nanoparticle interactions for a whole set of nucleobases. The possibility to obtain the nonresonant SEHRS spectra at relatively low excitation intensities opens new possibilities for future SEHRS applications, specifically the investigation of biological samples, which generally profits from near-infrared excitation. The SEHRS spectra of the nucleobases will help to interpret SEHRS data obtained from more complex systems, such as spectra from cells. The high sensitivity of the two-photon excited Raman scattering enhanced by plasmonic metal nanoparticles with respect to the orientation and contact with the silver nanoparticle surfaces is a very promising approach for the characterization of nano-biointeractions.

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**Table 5. Raman Shift Values in the SEHRS and SERS Spectra of Cytosine with Citrate (AgCit) Reduced Silver Nanoparticles and Assignment to Vibrations of the Cytosine Molecule (Based on References 11, 47, and 36)**

| Raman shift/cm\(^{-1} \) | SEHRS | SERS | assignments\(^{56}\) |
|--------------------------|-------|------|-------------------|
| 1635 s                   | 1636 s| AgCit| str C2=O11         |
| 1587 s                   | 1591 w| AgCit| bent NH2         |
| 1547 w                   | 1544 vvw|       | str N3–C4–C547     |
| 1482 s                   | 1482 w|       | str C4–N847       |
| 1424 s                   | 1422 s|       | bend N1–H, C5–H, C6–H47 |
| 1387 w                   | 1373 w|       | bend N1–H, C5–H, C6–H47 |
| 1307 vs                  | 1307 vs|       | ring str C–N11     |
| 1240 w                   | 1248 w|       | ring str C–N11     |
| 1196 w                   | 1196 w|       | ring str C–N11     |
| 1103 w                   | 1103 w|       | C=O11             |
| 1036 m                   | 1038 w|       |                 |
| 798 w                    | 799 vs|       | ring breath13,47   |
| 703 w                    | 704 w |       | ring def56        |
|                         | 632 vw|       | ring def56        |
| 602 w                    | 600 w |       | bend C2=O11       |
| 562 w                    | 563 w |       | ring def57        |
| 433 w                    | 431 vw|       |                 |

\(^{54}\) vs, very strong; s, strong; m, medium; w, weak; vw, very weak; br, broad. \(^{56}\) Bend, bending; breath, breathing; def, deformation; str, stretching.
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Notes
The authors declare no competing financial interest.

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