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## Use of surface-enhanced Raman scattering for detection of cancer-related serum-constituents in gastrointestinal cancer patients

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### Abstract

Laser-mediated surface-enhanced Raman scattering (SERS) has industrial and biological applications. We have developed a rapid and simple method for generating silver nanoscale hexagonal columns (NHCs) on the surface of a phosphor bronze chip for measurement of SERS spectra. This was used to detect SERS spectra from blood samples obtained from patients with gastric cancer, colorectal cancer, or benign diseases ( $n = 12$  each) using a low intensity helium-neon red laser beam with a 632.8-nm wavelength; the intensity of the SERS spectra was compared among the patient groups. The peak heights of SERS spectra from patients with benign diseases were significantly lower than those from patients with gastric or colorectal cancer, whereas those from patients with gastric cancer and colorectal cancer did not differ significantly. Thus, SERS using NHC chips holds promise for the easier and faster detection of cancer-related serum-constituents as biomarkers.

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*Key words:* Biomarker; Cancer screening; Nanoscale hexagonal columns; Proteomics; Surface-enhanced Raman scattering

### Background

Early diagnosis is an important factor in the improvement of survival of patients with cancer. Among the easy and minimally invasive approaches for investigation, blood tests are more widely applied and give a comprehensive overview of organ systems. For diagnosis of cancer from blood samples, a tumor marker,<sup>1–3</sup> cancer-related nucleic acids,<sup>4,5</sup> and circulating tumor cells (CTCs)<sup>6–9</sup> have been targeted.

The tumor marker has been already implemented practically, and is widely used because of its ease of implementation. However, it needs to be used in combination with other tests,

because its sensitivity is generally low.<sup>10,11</sup> Furthermore, using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR), we have previously shown that cancer-related mRNA is related to the prognosis of patients with esophageal cancer.<sup>12,13</sup> The detection of cancer-related nucleic acids in blood is specific and highly sensitive,<sup>14,15</sup> but its use is controversial because of the lack of a standard procedure.<sup>16</sup> Moreover, we have reported that circulating tumor cells (CTCs) indicate the prognosis of patients with gastric cancer.<sup>6</sup> CTCs are reported to be useful for diagnosis, estimation of prognosis, and judgment of treatment efficacy for patients with breast,<sup>7</sup> prostate,<sup>8</sup> lung,<sup>9</sup> and colorectal cancer;<sup>17</sup> however, the preparation of samples and the analytic procedure required for analysis of CTCs are relatively complicated in comparison with a routine clinical blood test. Additionally, the sensitivity and specificity of the technique vary;<sup>18,19</sup> thus, CTC analysis is not widely used.

Surface-enhanced Raman scattering (SERS) using a laser beam is a method widely used in industrial microanalysis, and has been applied in various fields, including detection of poisonous substances<sup>20</sup> and biological research.<sup>21</sup> Moreover,

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recently, SERS has been used for cancer diagnosis.<sup>22–26</sup> Although blood analysis using SERS has an advantage in that it may detect very small amounts of constituents, including circulating tumor cells,<sup>22</sup> nucleic acid,<sup>23</sup> ribonucleic acid,<sup>24</sup> proteins,<sup>25</sup> and lipids<sup>26</sup>, it requires several hours or more to prepare nanoscale hexagonal columns (NHCs) for SERS and for the measurement procedure.

The aim of this prospective preliminary study was to confirm the differences in SERS spectra targeting cancer-related serum-constituents among serum samples from patients with cancer and those from individuals without cancer, to develop a simple and rapid blood test for cancer diagnosis. To this end, we have developed a rapid (requiring a few minutes) and simple methodology for generating silver NHCs for SERS on the surface of a chip made with phosphor bronze. Moreover, we standardized this chip by measuring the SERS spectra of control serum, avidin, and globulins. We then used these silver NHC chips to detect SERS spectra generated from cancer-related serum-constituents in patients with gastric and colorectal cancer and compared these spectra to those of individuals with benign diseases.

## Methods

### Study design

In this prospective study, we studied patients who underwent surgery for gastric cancer, colorectal cancer, and benign diseases at the Digestive Disease Center, Showa University Northern Yokohama Hospital, between January 2013 and April 2013. The SERS spectra of each serum sample from these patients were analyzed. We hypothesized that a positive signal defined by an adequate cut-off level would be detected in 20% and 80% of the samples from the patients with benign disease and cancer, respectively. A sample size of 10 individuals in each group would have a power of 80% at  $\alpha$  of 0.05 to detect a significant difference. Therefore, we studied 12 patients in each group, in order to include dropout due to sample failure.

### Patients

The study was approved by the Institutional Review Board of the Showa University, Northern Yokohama Hospital (No. 1212-02). We explained the study protocol to patients before they gave written informed consent. This study was registered with the University Hospital Medical Information Network in Japan, number 000009818.

We studied 36 patients who underwent surgery for gastric cancer, colorectal cancer, or benign diseases. The inclusion criteria were as follows: (i) presence of carcinoma histologically proven from endoscopic biopsy (patients with gastric and colorectal cancer); (ii) absence of malignant disease by computed tomography (patients with benign diseases); (iii) clinical solitary tumor; (iv) no prior treatment of endoscopic resection, chemotherapy, or radiation therapy; (v) aged between 20 and 80 years; (vi) Eastern Cooperative Oncology Group performance status<sup>27</sup> of 0 or 1; (vii) sufficient organ function; and (viii) written informed consent.

The exclusion criteria were: (i) synchronous or metachronous malignancy; (ii) pregnant or breastfeeding women; (iii) active or

chronic viral hepatitis; (iv) active bacterial or fungal infection; (v) diabetes mellitus; (vi) systemic administration of corticosteroids; and (vii) unstable hypertension.

In all cases, the pathological stage of the disease was determined as per the 7th edition of the Union for International Cancer Control TNM Cancer Staging Manual.<sup>28</sup> Data on the clinicopathological features were collected from the patients' medical records and described using the abovementioned manual and Japanese gastric cancer treatment guidelines, 2010 (ver. 3).<sup>29</sup>

### Sample preparation

A 5.0-mL peripheral vein blood sample was obtained from each patient before surgery. The blood sample was drawn into tubes containing clot activator and polyolefin gel (Venoject II, VP-AS109K50, Terumo Corporation, Tokyo, Japan), and centrifuged at  $1600 \times g$  using a centrifuge separator (Model 5930, Kubota Corporation, Tokyo, Japan) for 7 min at room temperature. The serum extracted from each blood sample was stored at  $-20^\circ\text{C}$ .

### Preparation of NHCs on chip of test plate

We prepared examination plates with a round-shaped chip made with phosphor bronze (JIS H3110, C5191P) for sample analysis (Figure 1, A). Sodium thiosulfate pentahydrate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was dissolved in distilled water. Silver (I) chloride ( $\text{AgCl}$ , Wako Pure Chemical Industries, Ltd., Tokyo, Japan) was added to the solution and dissolved in a 3:1 molar ratio of  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  and  $\text{AgCl}$ . The final concentration of the resulting silver thiosulfate ( $\text{Ag}(\text{S}_2\text{O}_3)_2$ ) was adjusted to 0.1% with distilled water. We dropped 20  $\mu\text{L}$  of the  $\text{Ag}(\text{S}_2\text{O}_3)_2$  solution on the chip, and  $\text{Ag}(\text{S}_2\text{O}_3)_2$  complexes were directly converted to silver NHCs on the chip within a few minutes (Figure 1, B–D). Specificity of these silver NHCs has been previously reported by Yamamoto et al.<sup>30</sup> The NHCs gradually grow in size with time; no remarkable difference was observed in the intensity SERS spectra of silver NHCs obtained at 3 min and 30 min after dropping the  $\text{Ag}(\text{S}_2\text{O}_3)_2$  solution. Therefore, we used NHCs obtained after 4 min of dropping the  $\text{Ag}(\text{S}_2\text{O}_3)_2$  solution onto the chip. Excess  $\text{Ag}(\text{S}_2\text{O}_3)_2$  solution was removed by blow drying.

### Validation of measuring SERS spectra using silver NHCs chip

A laser Raman microscope (50 $\times$  objective lens, focal length 100 mm, and Andor CCD camera; RAM-300, Lambda Vision, Inc., Sagami, Kanagawa, Japan) with a helium–neon laser of 632.8-nm wavelength, at 2 mW was used to detect the Raman spectra of the serum samples. To validate the silver NHCs on the chip (silver NHC chip) and the Raman microscope, we measured the SERS spectra of control substances, viz., positively charged rhodamine 6G (R6G; Wako Pure Chemical Industries, Ltd., Tokyo, Japan), at a concentration of  $3 \times 10^{-6}$  M, and negatively charged Congo red (CR; Wako Pure Chemical Industries, Ltd., Tokyo, Japan), at a concentration of  $3 \times 10^{-6}$  M, after 20  $\mu\text{L}$  of the samples was dropped onto the prepared chips. Four minutes thereafter, the SERS spectra were measured, every second for 5 min. Because momentary atypical spectra frequently appeared, the spectra were recorded after the change in the peak intensity was within 20 arbitrary units for 3 s. The SERS spectra with

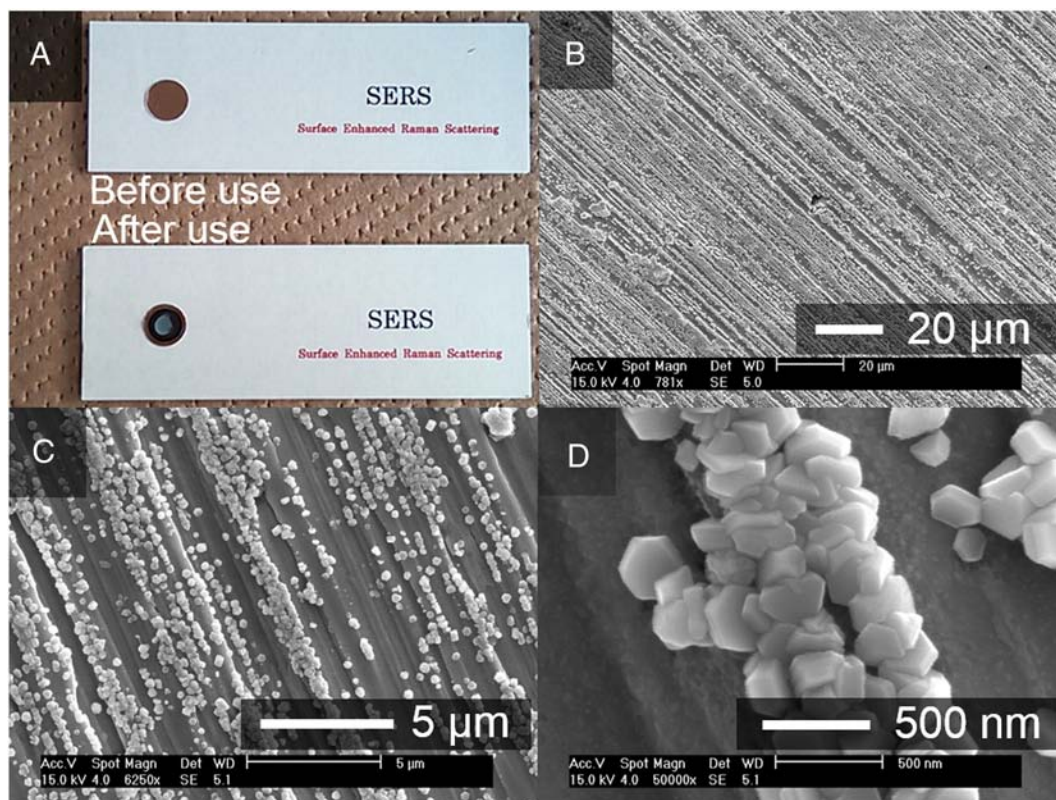


Figure 1. Silver thiosulfate complexes converted to nanoscale hexagonal columns (NHCs) on a chip made of phosphor bronze. (A) The examination plate for surface-enhanced Raman scattering (SERS) analysis of serum samples contains a small round-shaped chip made of phosphor bronze. (B–D) Scanning electron microscopy images of the nanoscale hexagonal columns (NHCs) on chip surface. After pipetting 20  $\mu\text{L}$  of silver thiosulfate solution on the chip, silver thiosulfate complexes were directly converted to NHCs within few minutes (B: scale bar, 20  $\mu\text{m}$ ; C: scale bar, 5  $\mu\text{m}$ ; D: scale bar, 500 nm). These images were recorded by Dr. Masafumi Fukuzumi (Hyogo Prefectural Institute of Technology, Hyogo, Japan) using a field emission type scanning electron microscope (Sirion, FEI JAPAN, Tokyo, Japan), 3 min after pipetting silver thiosulfate solution onto the chip.

individual peaks of R6G (Figure 2, A) and CR (Figure 2, B) were successfully measured.

Next, we dropped 20  $\mu\text{L}$  of 5% sodium hypochlorite solution onto the silver NHC chip to negatively ionize the NHCs before measuring the SERS spectra of the samples. Although the SERS spectra of R6G were not changed markedly, those of CR were attenuated on the negatively-charged silver NHC chip. In contrast, the SERS spectra of the clinical serum sample from one colon cancer patient were recognized only on the negatively-charged silver NHC chip (Supplementary Figure 1). Similarly, 20  $\mu\text{L}$  of control serum (Aalto control LIPID IIF, Aalto Scientific, Ltd., Carlsbad, CA, USA) showed no significant SERS peaks on the non-ionized silver NHC chip and a relatively small peak on the ionized chip compared to the clinical serum sample (Figure 2, C). Thus, we opted to ionize the silver NHC chip negatively by using sodium hypochlorite before measuring the SERS spectra of clinical serum samples. We found that silver chloride complexes combined with positively-charged small serum peptides (Figure 2, D). The procedures in this assay are shown in Figure 3.

#### Intensity of SERS spectra

The SERS spectra showed rapid increase until maximum peak intensity and gradual decrease thereafter. We measured the

average of 3 spectra, including the spectra with peak intensity, except for instantaneous flash spectra, spectra after 1 s, and those after 2 s. Two peaks repetitively appeared in the SERS spectra at approximately  $1350\text{ cm}^{-1}$  and  $1570\text{ cm}^{-1}$  in the Raman shift wavelength in all test serum samples, and the SERS spectra demonstrated a gently-sloping shape at shorter and longer wavelengths. Thus, we analyzed the SERS spectra using a polynomial expression. For this calculation, we used 2nd, 3rd, 4th, 5th, and 6th order polynomial expressions, and found that a 4th order polynomial expression fitted the SERS spectra (Figure 4, A). We defined the height from the bottom to the peak on the graph of the 4th order polynomial expression as the SERS peak height for each sample.

We confirmed the relationship between the SERS peak height and sample concentration using a clinical serum sample. Test samples were prepared by serially diluting the serum obtained from a colon cancer patient, 10, 100, 500, 1000, and 10000 times with distilled water; distilled water alone was used as the negative control. The 10-, 100-, 500-, and 1000-fold dilutions yielded 2 significant peaks in the SERS spectra; no peaks were obtained for the 10000-fold dilution or the negative control sample (Figure 4, B). Thus, the limiting dilution for detecting SERS spectra for serum was 1000-fold in this assay. Moreover, a significant correlation was obtained between concentration and SERS peak heights of the test 100-, 500-, 1000-, and 10000-fold

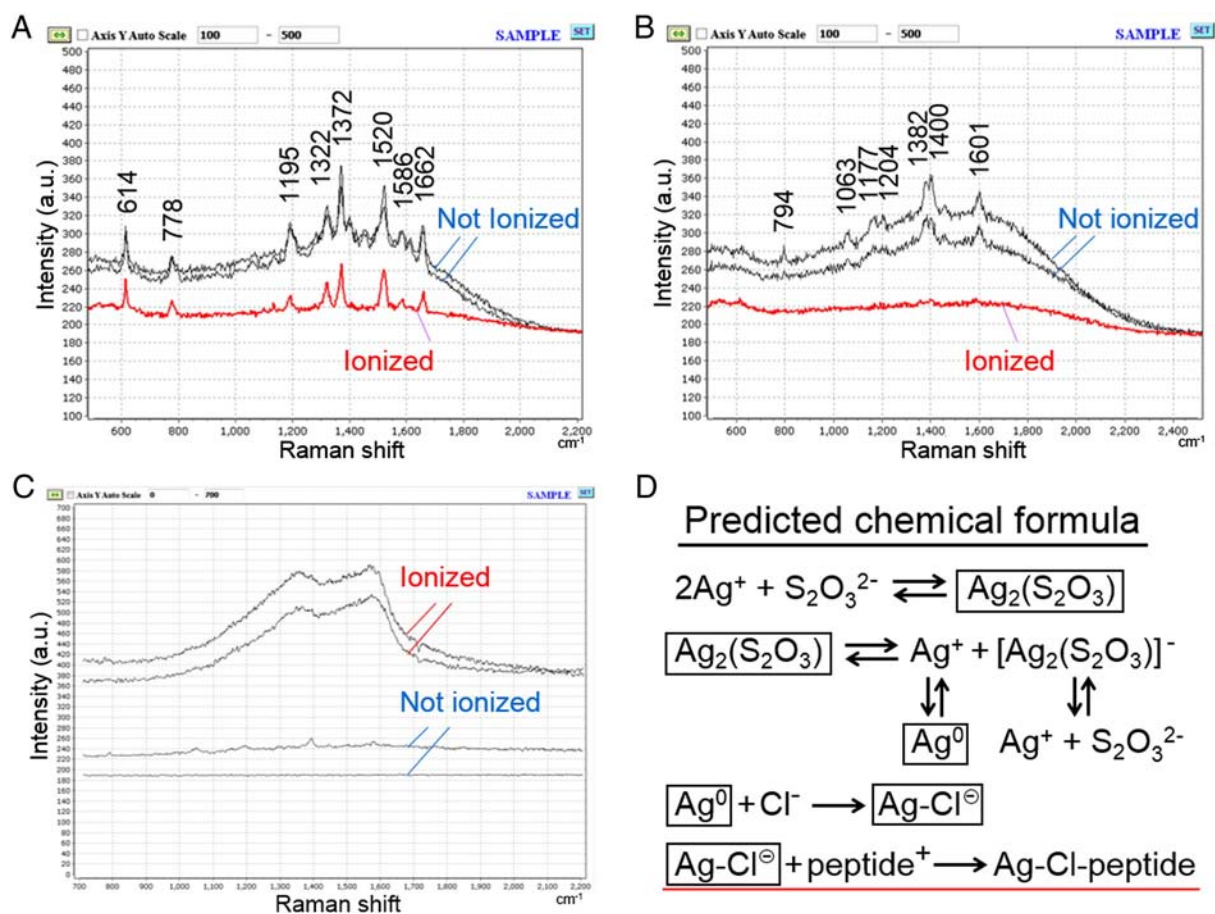


Figure 2. SERS spectra of standard samples. **(A)** SERS spectra of positively-charged rhodamine 6G. There was no marked change in the SERS spectra in the absence (black lines) and presence (red line) of negative ionization of nanoscale hexagonal columns on chip surface. **(B)** SERS spectra of negatively-charged Congo red chip. The SERS spectra were attenuated when using a negatively-charged chip (red line), as compared with spectra obtained without negative ionization. **(C)** SERS spectra of serum sample from a single patient with colon cancer. A peak of the SERS spectra was observed using the ionized chip, but not the non-ionized chip. **(D)** Predicted chemical formula. Silver nanoscale hexagonal columns on the chip were negatively charged.

diluted samples ( $y = 51.8$ ,  $R^2 = 0.796$ ; Figure 4, C). The SERS peak height for the 10-fold dilution did not fit the curve ( $y = 66.7$ ,  $R^2 = 0.481$ ); thus, the 10- and 100-fold dilutions were the saturating concentrations in this assay. Therefore, we used the 10-fold dilution for measuring SERS intensity.

#### Measure SERS spectra of clinical samples

We diluted each serum sample 10 times with distilled water prior to analysis. We added 20  $\mu\text{L}$  of the sample onto the chip, and used a distilled water sample as negative control. An examiner blinded to sample details manually focused the microscope with computer monitoring, and automatically measured the SERS spectra using a low intensity helium-neon red laser beam with a 632.8-nm wavelength. The SERS spectra were measured every second for 5 min. The 3 spectra, including the spectra at peak intensity, those after 1 s, and those after 2 s, were recorded for calculation of SERS peak intensity.

#### Statistical analysis

Statistical analysis was performed using JMP 9.0.3 (SAS Institute, Cary, USA). To compare the sex ratios, we used

Fisher's exact test. To calculate SERS peak height, we applied a 4th order polynomial expression. To evaluate the relationship among the SERS peak height, concentration of the samples, and total protein and albumin levels, we used the parametric Pearson product-moment correlation coefficient and the non-parametric Spearman's rank correlation coefficient. A value of  $P < 0.05$  was considered statistically significant.

## Results

### Patient characteristics

Patient characteristics are summarized in Table 1. The group of patients with benign disease included 7 patients with esophageal achalasia and 5 with cholecystolithiasis. Among the 12 gastric cancer patients, 7 (58.3%), 2 (16.7%), and 3 (25.0%) had pT1, 2, and 4 tumors, and 8 (66.7%) patients had lymph node metastases (pN1-3). Of these patients, 6 (50.0%), 2 (16.7%), 2 (16.7%), and 2 (16.7%) belonged to pathological stages I, II, III, and IV, respectively. In the patient group with colorectal cancer, 11 colon and 1 rectal cancer patients were

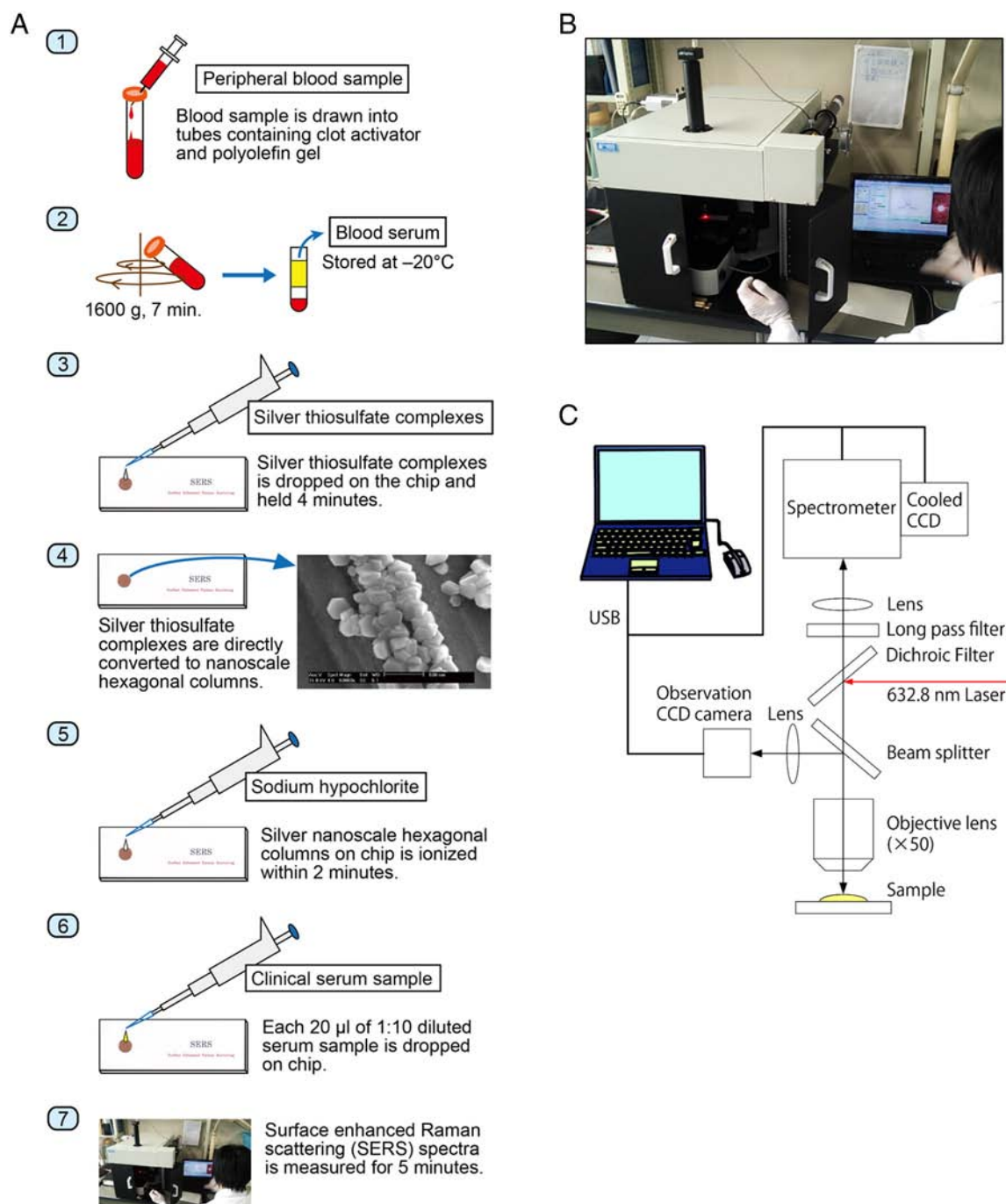


Figure 3. Graphical representation of SERS assay procedure. (A) Flowchart of SERS assay procedure. (B) Laser Raman microscope (RAM-300, Lambda Vision Inc, Sagami-hara, Kanagawa, Japan). (C) Schema of system for SERS spectra analysis.

included. pT1, T2, T3, and T4 tumors were noted in 0, 1 (8.3%), 8 (66.7%), and 3 (25.0%) of these patients, respectively. Furthermore, 9 (75.0%) of the 12 patients had lymph node metastases (pN1-3). The pathological stages I, II, III, and IV were noted in 0, 3 (25.0%), 7 (58.3%), and 2 (16.7%) patients, respectively.

The median (range) age in groups with gastric cancer, colorectal cancer, and benign disease was 72 (56-80), 69.5 (44-79), and 47 (28-75) years, respectively. The median age of

patients with benign disease was significantly lower than that of patients with gastric and colorectal cancer.

No significant differences in the values of total protein and albumin were observed among the 3 patients groups.

#### *SERS spectra of clinical serum samples from patients*

SERS spectra could be detected and recorded in all 36 patient samples. The distilled water sample showed no significant peak

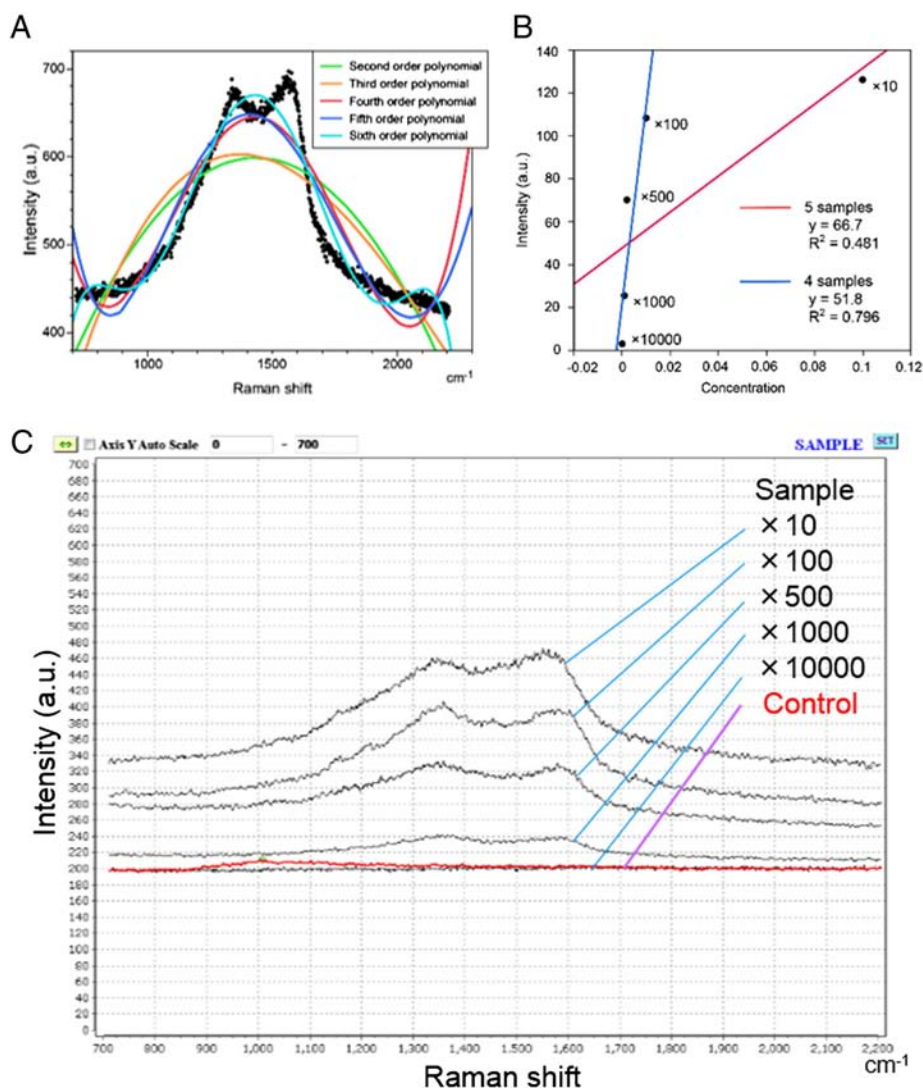


Figure 4. Definition of peak height of SERS spectra. **(A)** The peaks of SERS spectra of clinical samples appeared at 1350  $\text{cm}^{-1}$  and 1570  $\text{cm}^{-1}$  in the Raman shift, and the SERS spectra showed a gently-sloping shape at shorter and longer wavelengths. **(B)** Typical SERS spectra of the serum sample from a colon cancer patient, diluted 10, 100, 500, 1000, and 10000 times, are shown. The limiting dilution for detecting SERS in a serum sample was 1/1000. **(C)** Significant correlation between serum concentration and the SERS peak heights of 100-, 500-, 1000-, and 10000-fold dilutions (blue line,  $y = 51.8$ ,  $R^2 = 0.796$ ). No significant correlation was observed between concentration and SERS peak height for the 10-, 100-, 500-, 1000-, and 10000-fold dilutions (red line,  $y = 66.7$ ,  $R^2 = 0.481$ ). The SERS peak height of the 10-fold dilution did not fit the curve.

in SERS spectra. The typical SERS spectra of the serum samples from the 3 patient groups are shown in Figure 5, A-C.

Next, we compared the SERS peak heights of samples among the patient groups, and studied the relationship between SERS peak height and disease progression. SERS peak heights of samples from patients with benign disease were significantly lower than those of samples from patients with stage II-IV gastric ( $P < 0.001$ ), stage II-IIIa colorectal ( $P = 0.018$ ), and stage IIIb-IV colorectal cancer ( $P < 0.001$ ). The SERS peak heights of samples from patients with stage I gastric cancer were higher than those of samples from patients with benign disease ( $P = 0.068$ ). The SERS peak heights of samples from patients with stage II or more progressive disease were significantly higher than those of samples from patients with stage I gastric cancer

( $P = 0.045$ ). Although no statistically significant differences were observed, the SERS peak heights of samples from the patients with stage IIIb or more progressive disease were higher than those of samples from patients with stage II and IIIa colorectal cancer ( $P = 0.203$ ; Figure 5, D).

#### Relationship among SERS spectra and serum total protein and albumin levels

We evaluated the relationship among SERS spectra and the value of serum total protein and albumin as measured in a routine blood test. No significant correlation between SERS peak heights and total protein (Figure 6, A; parametric Pearson product-moment correlation coefficient,  $P = 0.691$ ; non-

Table 1  
Patient characteristics (n = 36).

Variables	Benign disease (n = 12)	Gastric cancer (n = 12)	Colon and rectal cancer (n = 12)	P value
Age (median, range)	47 (28-75)	72 (56-80)	69.5 (44-79)	B–G, 0.005; **, B–C, 0.030*; G–C, 0.505
Sex				B–G, 0.667; B–C, 0.500; C–G, 0.500
Male	8 (66.7%)	8 (66.7%)	7 (58.3%)	
Female	4 (33.3%)	4 (33.3%)	5 (41.7%)	
Disease	Achalasia: 7 (58.3%) Cholelithiasis: 5 (41.7%)	Gastric cancer: 12 (100%)	Colon cancer: 11 (91.7%) Rectal cancer: 1 (8.3%)	
Total protein (g/dL) (median, range)	6.8 (6.3-7.6)	6.65 (4.2-7.9)	6.4 (5.0-7.7)	B–G, 0.664; B–C, 0.093; G–C, 0.506
Albumin (g/dL) (median, range)	4.1 (3.4-4.6)	3.95 (1.8-4.4)	3.8 (3.1-4.3)	B–G, 0.283; B–C, 0.103; G–C, 0.431
Pathological depth of tumor invasion				
pT1	-	7 (58.3%)	0	
pT2	-	2 (16.7%)	1 (8.3%)	
pT3	-	0	8 (66.7%)	
pT4	-	3 (25.0%)	3 (25.0%)	
Lymph node metastasis				
pN0	-	4 (33.3%)	3 (25.0%)	
pN1	-	2 (16.7%)	6 (50.0%)	
pN2	-	2 (16.7%)	1 (8.3%)	
pN3	-	4 (33.3%)	2 (16.7%)	
Distant metastasis				
pM0	-	10 (83.3%)	10 (83.3%)	
pM1	-	2 (16.7%)	2 (16.7%)	
Pathological TNM stage				
Stage I	-	6 (50.0%)	0	
Stage II	-	2 (16.7%)	3 (25.0%)	
Stage III	-	2 (16.7%)	7 (58.3%)	
Stage IV	-	2 (16.7%)	2 (16.7%)	

parametric Spearman's rank correlation coefficient,  $P = 0.571$ ) or albumin (Figure 6, B; parametric Pearson product-moment correlation coefficient,  $P = 0.180$ ; non-parametric Spearman's rank correlation coefficient,  $P = 0.148$ ).

## Discussion

To improve survival of the cancer patients, it is important to have an early and precise diagnosis. Thus, the purpose of this study was to find a serum protein particular to cancer patients by using SERS. To date, there have been a few reports about cancer diagnosis using SERS,<sup>31,32</sup> but the small sample study presented here is the first report indicating that differences in the constituents of serum between cancer patient and those with benign disease can be detected using SERS analysis.

SERS makes use of the phenomenon that each substance disperses laser radiation at a particular wavelength;<sup>33</sup> moreover, this method can detect a single molecule of such material.<sup>34</sup> Additionally, the intensity of SERS correlates with the quantity of the material. Thus, the pattern of the SERS spectrum depends on the particular constituents and their quantities present in the sample, and different samples have dispersion spectral patterns. Because SERS spectra are affected by the laser wavelength and the surface properties of the base, the dispersion wavelength of

the dispersion may vary; however, the shapes of SERS spectra are maintained. Thus, the constituents of a substance can be deduced by inspection of the SERS pattern in an unknown sample, and unknown constituents can be distinguished as potential biomarkers. However, it is necessary to use methods such as liquid chromatography, electrophoresis, and mass spectrometry to determine the identity of such constituents. Furthermore, selective detection of the target molecule is possible by using a specific method such as antibody-based detection.

In this study, the targeted molecules were small serum peptides, including light chain, heavy chain, globulin, albumin, tumor-specific antigen (TSA), and tumor-associated antigen (TAA), and the molecular weights of these peptides were estimated to range from 25 to 20 kDa. Almost all peptides, including cationic peptides, include some positively-charged regions. The silver NHCs we developed can be prepared within a few minutes on the surface of a chip made with phosphor bronze, and the negatively-charged chip can then bind the small serum peptides; in fact, SERS spectra were not detected without a negatively-charged chip. Additionally, systems with a high signal/noise ratio that include stable low power and high brightness laser irradiation equipment and adequate software are necessary for detecting SERS of small serum peptides. We used a laser with a 632.8-nm wavelength

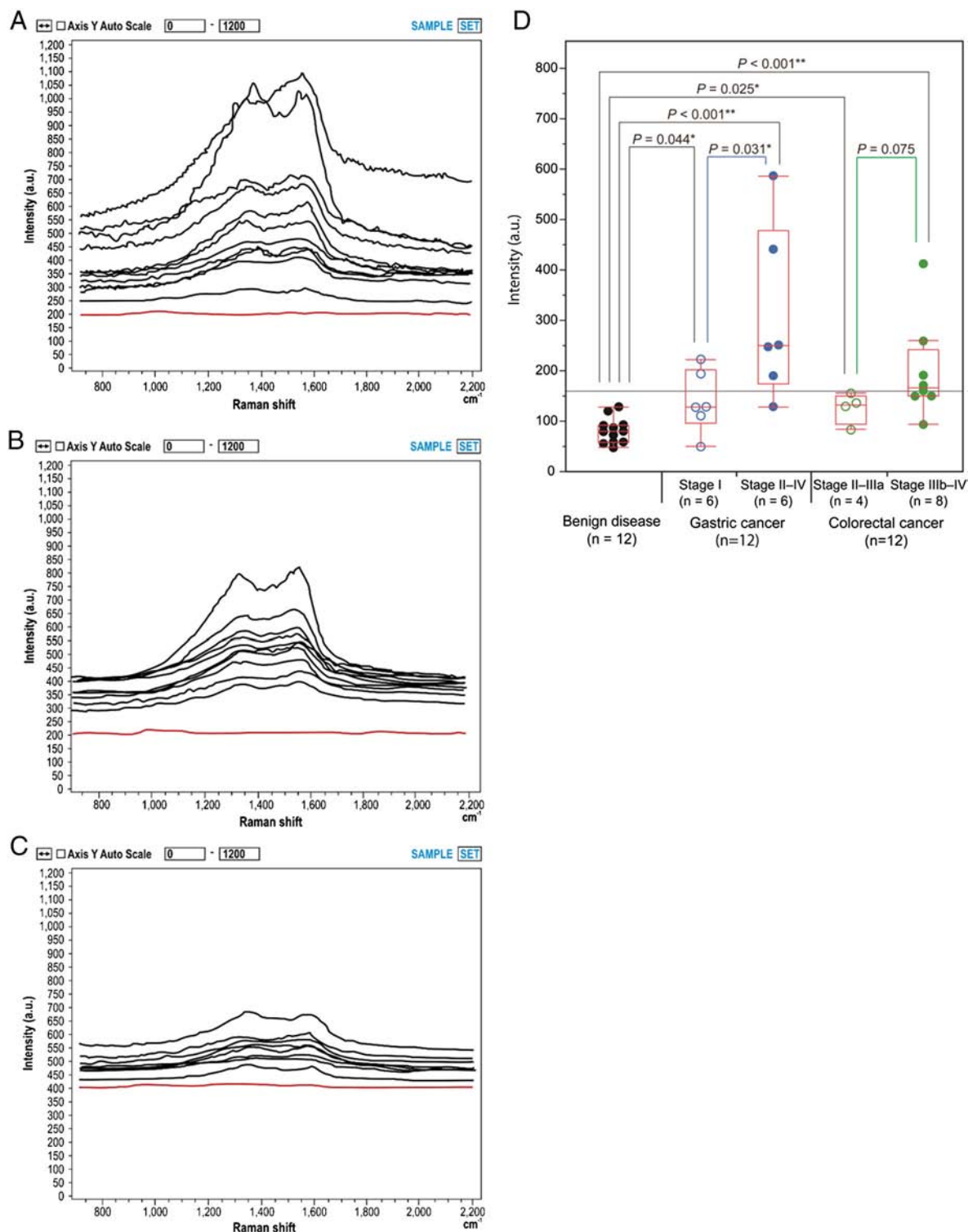


Figure 5. SERS spectra of serum samples from patients. SERS spectra as a function of peak intensity vs. wavelength for 12 samples from (A) 12 gastric cancer patients, (B) 12 colorectal cancer patients, and (C) 12 benign disease patients. (D) The dots represent the SERS peak heights of patient samples. The bottom and top of the red box represent the lower and upper quartiles, and the red band across the red box shows the median. The lower and upper red bars at the ends of the whiskers show the lowest data point within 1.5 interquartile ranges of the lower quartile, and the highest data point within 1.5 interquartile ranges of the upper quartile, respectively. The gray bar indicates mean value. The SERS peak heights of samples from patients with benign disease were significantly lower than those from the patients with stage II-IV gastric ( $P < 0.001$ ), stage II-IIIa colorectal ( $P = 0.018$ ), and stage IIIb-IV colorectal cancer ( $P < 0.001$ ).



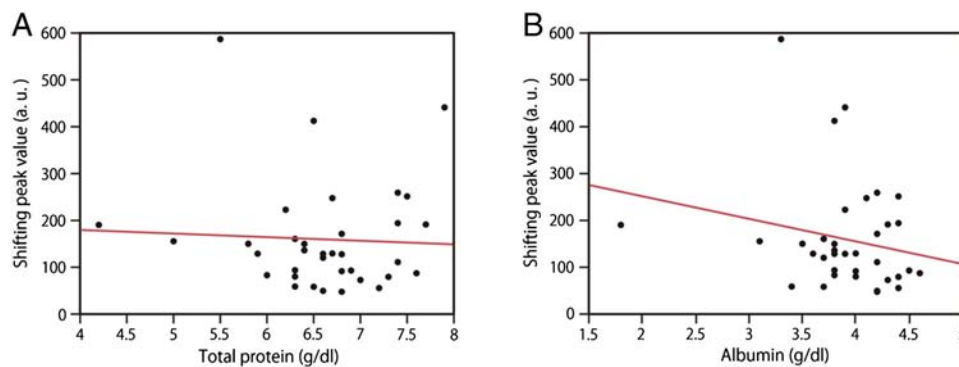


Figure 6. Relationship between SERS spectra and serum total protein or albumin. The relationships between SERS peak heights and total protein or albumin were determined by using linear regression analysis.

at a stable low-output power of 2 mW, to avoid evaporation and heat degeneration of the samples. However, when we used a laser with a 785-nm wavelength in a pilot study, a weaker signal was detected than that obtained using the 632.8-nm wavelength laser.

One of the most important points in the present study is the origin of the detected SERS spectra. The SERS peak height of samples from patients with cancer was significantly stronger than those with benign disease in this study, and the SERS peak height of samples from patients with progressive disease was stronger than those with early stage disease in gastric ( $P = 0.045$ ) and colorectal ( $P = 0.203$ ) cancer, indicating that cancer-related peptides in the serum increased and the SERS spectra intensified with progression of disease. Thus, it appears that our methodology could detect SERS spectra caused by cancer-related serum-constituents. We consider that TSA, TAA, and immunoglobulins with a positively-charged structure were the constituents detected in our assay.

There are certain limitations to this study. First, we could not confirm the structure of the cancer-related peptides and could not analyze the details of the Raman shift wavelength of the SERS spectra due to instrumental limitations. We intend to change the grating of the beam splitter to facilitate splitting of the laser spectra into narrow wavelengths. In the future, we will also analyze the serum samples by methods such as liquid chromatography, electrophoresis, and mass spectrometry to address this limitation. Another limitation is the variability in the measurement of the SERS spectra. To remove this variability and the necessity to record measurements at a particular point manually, we will integrate measurement of the intensity of the SERS spectra using a computer controlled measurement system and modified three-dimensional mapping software. One more limitation is the differences in patient demographics among the 3 surgical groups. The patients with benign diseases were relatively younger than the patients with cancer; this limitation can be addressed in a future large scale study.

In conclusion, we propose that the simple and rapid methodology using SERS with silver NHCs that we described here may be successfully implemented in blood tests for cancer screening and detection of unknown serum-constituents that are cancer-related biomarkers.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2013.09.006>.

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