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Diagnosis of breast cancer by analysis of sialic acid concentrations in human saliva by surface-enhanced Raman spectroscopy of silver nanoparticles

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ABSTRACT

Breast cancer is the most common type of malignant tumor among women and their second leading cause of cancer-related deaths. The most common method for screening and diagnosis is mammography. Nonetheless, two main problems have been identified. First, the dose of radiation received during the test prevents the method from the use on women who are < 40 years old. Second, there can be mammogram failure owing to the lack of tumor contrast with the fibrous tissue. Therefore, there is a need for screening methods that will help to identify high-risk cases. We developed a biological marker test that can help to identify them.

Increased levels of sialic acid (SA) in saliva are known to correlated with breast cancer. In this study, we evaluated the feasibility of Raman spectroscopy as a method for quantification of SA in saliva, using citrate-reduced silver nanoparticles (cit-Ag-NPs) as a surface-enhanced Raman spectroscopy (SERS) substrate. Quantification of SA was accomplished by measuring its intensity in saliva and comparing it with a calibration curve of SA standards. The mean SA concentration in saliva was found to be significantly higher among 100 breast cancer patients (18.3 ± 9.4 mg·dL^{-1}; mean ± SD) than among 106 healthy controls (3.5 ± 1.0 mg·dL^{-1}). The SERS test showed sensitivity of 94% and specificity 98% for detection of patients with breast cancer, assuming that SA concentration >7 mg·dL^{-1} is a cutoff for positive test results. Our findings prove the usefulness of this SERS technique as a simple, convenient, and highly sensitive method of quantitative analysis of SA in saliva. The simplicity of this nanotechnological test may help to substantially reduce the mortality among patients with breast cancer by providing women with a simple, noninvasive screening test that can be applied regardless of age or density of breast tissue.

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1 Introduction

Breast cancer is the most frequent malignant tumor among women in the world, being the cause of more than 520,000 deaths per year; it is the second leading cause of cancer deaths among women, being surpassed only by lung cancer [1, 2]. Early detection of breast cancer, combined with an aggressive and timely medical intervention, is necessary to substantially reduce the death rates among patients with this cancer.

The actual detection methods of breast cancer, such as X-ray mammography, ultrasonography, magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET), have considerable limitations, which affect their diagnostic effectiveness. For instance, CT and PET scans expose people to a relatively high dose of radiation in comparison with other types of diagnostic tests and are likely to cause new cancers in some patients [3, 4]. X-ray mammography, the most common and relatively cost-effective screening method, is limited by low sensitivity and specificity. Up to 75% of false positive results and 34% of false negative results have been reported in a published study on X-ray detection tests [3]. One of the main inconveniences of mammography is that it slightly increases the risk of radiation-induced breast cancer. This situation prevents mammograms from being universally applied to women younger than 40 years. In the case of women having dense breast tissue, a mammogram can fail because the tumor contrast is similar to that of a fibrous tissue. It has been found that almost 40% of women older than 40 years have dense tissue. Additionally, for younger women under 40, the dense adipose breast tissue masks most of the tumors at their initial stages, and X-ray mammography is normally inconclusive: when it is imperative to detect a tumor in order to provide proper treatment in a timely manner. As a consequence, breast cancer is often detected only at more advanced stages when the treatment options are significantly more limited. The 5-year relative survival rate for women with stage 0 or stage I breast cancer is close to 100%. Stage II and III breast cancers yield a survival rate of ~93% and 72%, respectively, and if the cancer has reached stage IV, prospects for survival among the patients are reduced to only 22% [2]. Therefore, there is an urgent need for a fast, accurate, relatively inexpensive, and noninvasive method for early detection of breast cancer regardless of age and breast tissue density.

Sialic acids are a family of nine-carbon acidic monosaccharides that occur at the end of oligosaccharide chains of mucins, glycoproteins, and glycolipids attached to the surface of cells and soluble proteins [5, 6]. N-acetylneuraminic acid (Neu5AC) is the predominant form of sialic acid (SA) and almost the only form found in human bodily fluids and tissues [7]. The chemical formula $C_{11}H_{19}NO_9$ and structure of sialic acid are presented in Fig. 1. The SA molecule is composed of a pyranose "chair" ring consisting of five carbon atoms and one oxygen atom, or "backbone structure", to which an N-acetyl (N–CH_3CO), a carboxyl group (CO–OH) and a glycerol "tail" are attached (–$C_3H_7O_3$).

Altered glycosylation is a universal feature of cancer cells, and certain glycan structures are well-known biomarkers of tumor progression [8, 9]. Recently, elevated levels of SA have been shown to be a characteristic feature of saliva of patients with breast cancer; therefore, this metric has been suggested as a noninvasive biomarker for diagnosis or theranosis of this type of cancer [10, 11]. The use of saliva as a diagnostic medium is advantageous because sample collection is simple, noninvasive, and safe. Vinogradova et al. have shown that small amounts of aqueous SA may be easily detected by surface-enhanced Raman spectroscopy (SERS) measurements with citrate-reduced silver nanoparticles (cit-Ag-NPs) [11]. These

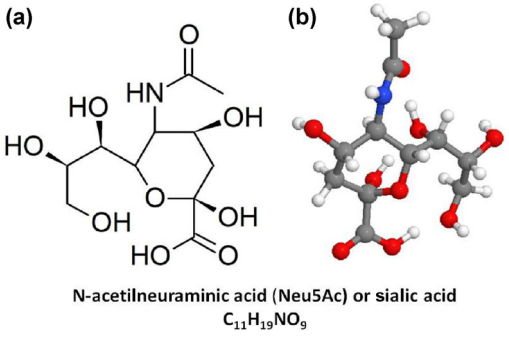

Figure 1 Chemical structure of Neu5Ac or sialic acid. (a) A schematic chemical model; (b) a stereographic model using color spheres; gray: carbon, red: oxygen, blue: nitrogen, white: hydrogen (mirror images).

results suggest the possibility of using SERS for the detection of SA in human saliva at the molecular level to diagnose breast cancer.

The most widely applied methods for quantification of SA in saliva are the acidic ninhydrin method described by Yao K. [12] and thiobarbituric acid assay modified by Skoza L. [13], which have passed the test of time. In studies published in recent years, quantification of SA in saliva continues to be performed by these methods [14, 15]. Nonetheless, quantification of SA by SERS is a novel technology that requires fewer reagents and could be useful for clinical diagnosis because it is highly sensitive, quick, and cheap, whereas the equipment may be portable, and results can be obtained in real time. There are some studies using SERS for the imaging of SAs on living cells [16, 17], but to our knowledge, there are no comparative reports on SERS in applications concerning its use for quantification of SA in human biological fluids or its systematic application to screening for them in human populations.

SERS is a Raman spectroscopic technique that has shown enhancement of the inelastic scattering of the outgoing radiation ranging from 10^6 to 10^{16} in amplification factors from molecules adsorbed on metals [18, 19]. Therefore, SERS has a great potential as a molecularly specific analytical tool for highly sensitive detection of weak Raman signals of proteins or other biological analytes with a small Raman scattering cross-section or at very low concentrations, as low as the single-molecule level [18–21]. Furthermore, colloidal suspensions of metallic nanoparticles, mainly silver, gold, or copper are the most common SERS substrates because of the ease of preparation, long shelf life, and high Raman signal enhancement factors [19–21]. One of the most widely employed silver colloids is prepared by the reduction of silver nitrate with trisodium citrate [22]. The chemical preparation process results in silver nanoparticles with a surface covered by a layer of negative citrate ions to compensate the positive charge of the NP surface. Cit-Ag-NPs have been demonstrated to be effective at SERS detection of not only positively charged analytes [23, 24] but also some negatively charged molecules, including SA [11, 25, 26].

The aim of this study was to examine the ability of Raman spectroscopy to measure concentrations of SA in human saliva, using a colloidal suspension of cit-Ag-NPs as a SERS substrate. A series of concentrations of SA in water showed a correlation with the total intensity of their SERS spectra indicating a good monotonically increasing relation. The obtained calibration curve was applied to determine SA levels in saliva of healthy women and patients with breast cancer. Salivary SA concentrations were found to be significantly higher in breast cancer patients than in controls. These results may lead to the development of a versatile and cost-effective diagnostic method for breast cancer on the basis of SERS.

2 Experimental

All the reagents were of analytical grade, purchased from Sigma-Aldrich, and were used without further purification.

Salivary samples were obtained from 106 healthy women and 100 patients with breast cancer receiving oncological treatment and follow-up supervision at the Civic Hospital of San Luis Potosí, S.L.P. México (Central Hospital or HCSLP). The age ranges were 18–60 and 25–78 years for the control and patient group, respectively. Inclusion criteria for the control and patient groups were the following: No systemic disease (except for pre-existing diagnosis of breast cancer for the patients) and no oral complaints. The exclusion criterion was unwillingness to participate in the study. The present study's protocol was approved by the HCSLP Ethics Committee. Written informed consent was obtained from all the participants.

Before saliva collection, each participant was required to perform two-step oral cleansing. The first step consists of vigorous teeth brushing, and the second one involves two subsequent oral rinses with commercial alcohol-free mouthwash.

After that, the subjects were instructed to refrain from swallowing for several minutes, before depositing 1.0–1.5 mL of the accumulated saliva into a sterile plastic vial. The saliva samples were centrifuged at 6,000 rpm (equivalent to 3,580 G) for 15 min, and the resulting supernatants were used to determine the concentration of SA. Reference solutions in water were prepared at SA concentrations of 1, 5, 10, 15, 20,

and 50 mg·dL^{-1}, and were measured in the same run, to preclude any unforeseen differences in performance of the Raman spectrometer or in cit-Ag-NP colloid inhomogeneities. The unused portions of the samples were stored at 4 ℃.

Cit-Ag-NPs were prepared by the standard Turkevich method [22]. High-resolution transmission electron microscopy images of the NPs were captured using a JEOL 2010-F transmission electron microscope operating at 200 kV. The transmission electron microscopy images indicated that the cit-Ag-NPs were polydisperse in size, varying in diameter from 5 to 50 nm with a predominant particle size range of 5–10 nm (Fig. 2).

The Raman measurements were performed on a Horiba Jobin Yvon XploRA ONE Raman spectrometer coupled to an Olympus BX41 optical microscope, using a green laser source at 532 nm with average power 20 mW at the sample. A green laser line was used instead of the more common 785 nm infrared (IR) line because the focused red laser tended to boil the liquid. The laser beam was focused on the surface of the liquid sample with a 10× objective. The diameter of the laser spot was approximately 8–10 μm. SERS were acquired across the 400 to 1,800 cm^{-1} spectral range as an average of four consecutive 10 s laser exposure periods. The fluorescence background was removed using the Vancouver algorithm.

To record the SERS spectra, 50 μL of a 2.5 × 10^{-3} M cit-Ag-NP suspension was placed in an aluminum container with a capacity of 100 μL and mixed with 25 μL of a saliva sample or with an equal volume of a reference SA solution.

The experimental data were fitted to the following logarithmic equation

$$Y = A - B \ln(x + C) \quad (1)$$

where A, B, and C are fitting parameters. Because Raman intensity of the three strongest peaks increases at different rates as the SA concentration increases, the calibration curve was constructed using the average values from peaks A, B, and C. The final margin of error associated with determination of the concentration of SA was ±0.5 mg·dL^{-1}. More information on the reproducibility of the calibration independently of the NP size is provided in the Electronic Supplementary Material (ESM).

Statistical analysis of the data was performed in the SPSS statistical software (IBM SPSS Statistics version 20 for Windows). Spearman's correlation was used to establish the degree of correlation between SA concentrations and the stage of breast cancer because the salivary SA values, particularly among the women with breast cancer, are not normally distributed. The results of the analysis are expressed in median values and the first and third quartiles: Q1 and Q3. In addition, as reference numbers, the mean and standard deviation of the numerical values of the concentrations that were measured were calculated and provided. As usual, data with P values < 0.05 were considered statistically significant.

3 Results and discussion

3.1 Experiments

Preliminary measurements were performed to establish optimal and reproducible conditions to obtain a

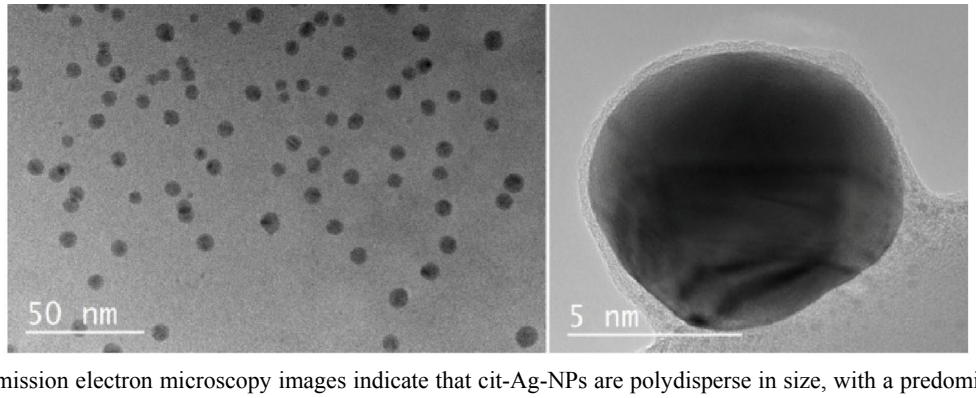

Figure 2 Transmission electron microscopy images indicate that cit-Ag-NPs are polydisperse in size, with a predominant particle size range of 5–10 nm.

calibration curve for the quantitative SERS analysis of SA adsorbed on cit-Ag-NP. Reference aqueous solutions were prepared at SA concentrations of 1, 5, 10, 20, 50, 100, 150, and 200 mg·dL^{-1}, and the SERS of each solution was acquired. SERS were also obtained for solutions with SA concentrations ranging from 1 to 15 mg·dL^{-1} (not shown), which showed features very similar to those in Fig. 3. The obtained SERS are in agreement with the spectra reported by Vinogradova et al. using a 785 nm laser [11]. The spectra are dominated by bands at 1,002, 1,237, and 1,391 cm^{-1} (labeled as A, B, and C), which correspond to the pyranose ring breathing mode, amide III (C–N stretching) mode, and stretching mode of the carboxyl group, respectively [11]. The figure indicates that the concentration of SA correlates well with the intensity of these peaks.

SERS intensity response of the A, B, and C bands as a function of SA concentration is shown in Fig. 4. The experimental points were fitted to the logarithmic function (red lines) discussed above, with a coefficient of correlation greater than 0.98, indicating the possibility of accurate determination of SA concentration by means of the calibration curve.

Figure 5 depicts a comparison of SERS of reference solutions of SA at concentrations 1, 5, and 10 mg·dL^{-1} with SA spectra acquired in saliva of eight healthy women. The close similarity of the SERS of SA measured in saliva to those obtained from a standard aqueous solution of SA validates the ability of SERS

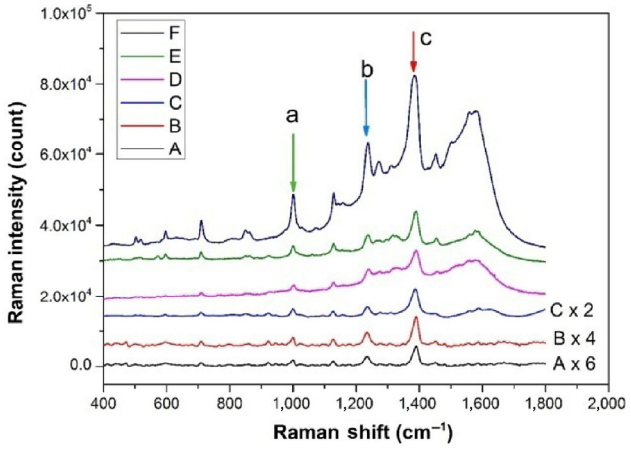

Figure 3 SERS of aqueous solutions of sialic acid at concentrations ranging from (a) 1 mg·dL^{-1}; (b) 5 mg·dL^{-1}, (c) 10 mg·dL^{-1}; (d) 20 mg·dL^{-1}, (E) 50 mg·dL^{-1}, to (F) 200 mg·dL^{-1}.

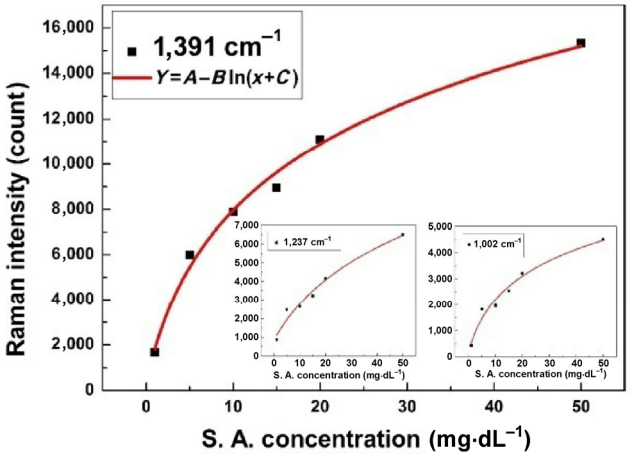

Figure 4 Plot of the 1,391 cm^{-1} peak intensity as a function of sialic acid concentration. The red line represents the logarithmic function of the best fit for the obtained data. Insets: Plots of the 1,237 and 1,002 cm^{-1} peak intensities as a function of sialic acid concentration.

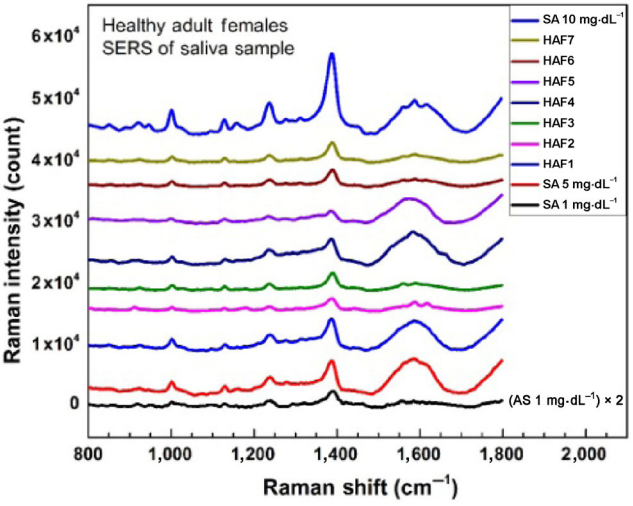

Figure 5 Comparison between the SERS data acquired from aqueous solutions of sialic acid at concentrations 1, 5, and 10 mg·dL^{-1} and sialic acid spectra recorded for saliva of eight healthy controls.

to measure SA in human saliva. As shown in the figure, the concentration of SA in saliva of these eight healthy women varied between 1 and 5 mg·dL^{-1}.

Figure 6 shows a comparison of the SERS of the reference solutions of SA at concentrations 5, 10, 15, and 20 mg·dL^{-1} with those of SA in the saliva of six patients with breast cancer. In contrast to the SERS obtained from healthy individuals, it can be concluded that the SA concentrations in the saliva of these breast cancer patients ranges between 10 and 20 mg·dL^{-1}.

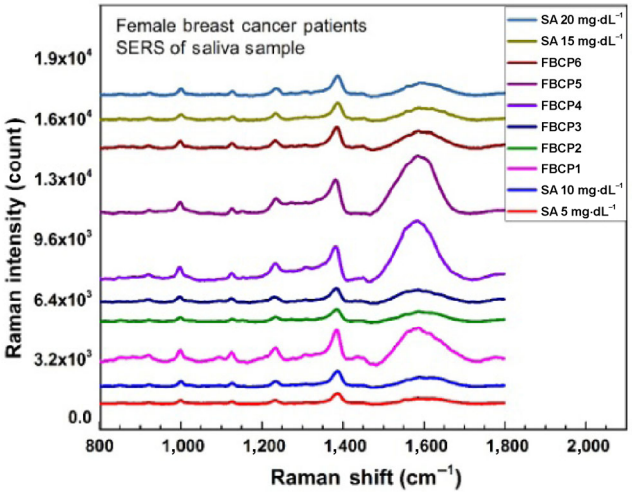

Figure 6 Comparison between SERS of sialic acid at concentrations 5, 10, 15, and 20 mg·dL^{-1} and sialic acid spectra recorded in saliva of six breast cancer patients.

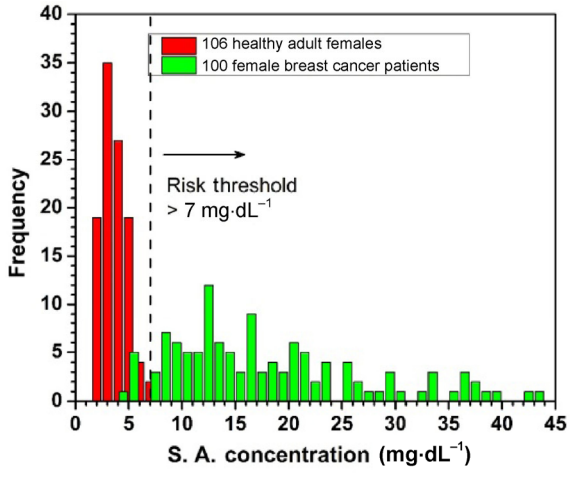

Figure 7 Plot of frequency of sialic acid concentrations with an interval width of 1 mg·dL^{-1} for both healthy controls (red bars) and patients with breast cancer, irrespective of cancer stage (green bars).

As a demonstration of the proposed application, Fig. 7 shows a plot of frequency of SA concentrations with an interval width of 1 mg·dL^{-1} for both healthy women (red bars) and breast cancer patients, irrespective of cancer stage (green bars). In this figure, one can easily see how the SA concentrations of the control group of healthy women are tightly clustered following a normal distribution with an estimated mean of 3.5 and standard deviation of 1 mg·dL^{-1}. In contrast, among the patients with breast cancer, there is broad variation of SA concentrations: from 5 to 40 mg·dL^{-1}, with a mean of 18.3 mg·dL^{-1} and standard deviation of 9.4 mg·dL^{-1}. This finding was used to decide that salivary concentrations of SA greater than 7 mg·dL^{-1} are indicative of the presence of a possible cancer condition.

3.2 Statistical analysis

One hundred patients, age ranging from 25 to 78 years old (51.8 ± 12.0 years, mean ± SD) and 106 healthy volunteers, ages ranging from 18 to 60 years (28.6 ± 12.7), all women in both groups, participated in this study. Figure 7 shows the SA concentration in each participant; the characteristics of the patients participating in the study are shown in Table 1. The statistical analysis was carried out in the statistical packages SPSS 20 and Origin Pro 8. The difference between mean concentrations of control subjects and

Table 1 Characteristics of the patients participating in the project. Data are presented as median (lower quartile to upper quartile). The Mann–Whitney *U* test was used for comparisons

	Control group	Positive group	*P*
Number of subjects	106	100	
Sialic acid concentration (mg·dL^{-1})	3.4(2.7 to 4.5)	15.4(11.3 to 22.5)	0.05

the patients with a positive diagnosis of breast cancer was assessed by the Mann–Whitney *U* test. A difference with $P \leqslant 0.05$ was considered statistically significant. The results of the Mann–Whitney *U* test supported the hypothesis that the concentrations of SA in the saliva of controls and patients are different. The assumption of nonparametric data was evaluated and further supported by a Kolmogorov–Smirnov test. Figure 8 shows the box plot of SA concentrations in both groups.

In Fig. 9, a box plot of the average concentration as a function of clinical stage is shown. Since only 3 patients were diagnosed with stage IV breast cancer, they were grouped together with stage III patients in the statistical analysis. The median numerical values, plus/minus the first and third quartile (Q1,Q3) are summarized in Table 2. Inspection of these data suggests that some correlation may exist between SA concentrations in the saliva of breast cancer patients and the clinical stage of the disease. A Kruskal–Wallis

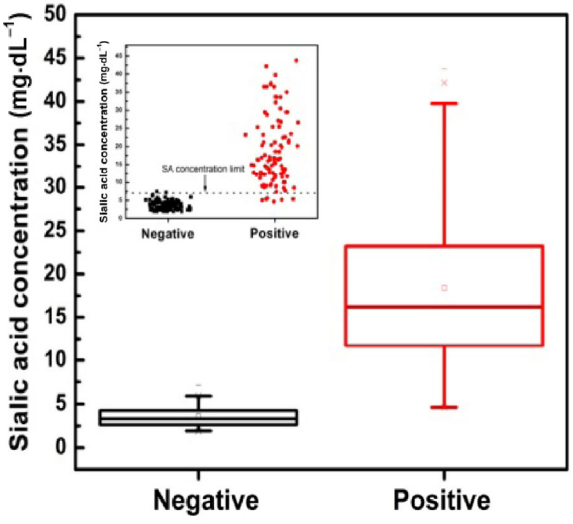

Figure 8 Box plot of sialic acid concentrations in both healthy women and patients with breast cancer. Inset: a cloud plot of sialic acid concentrations of each participant.

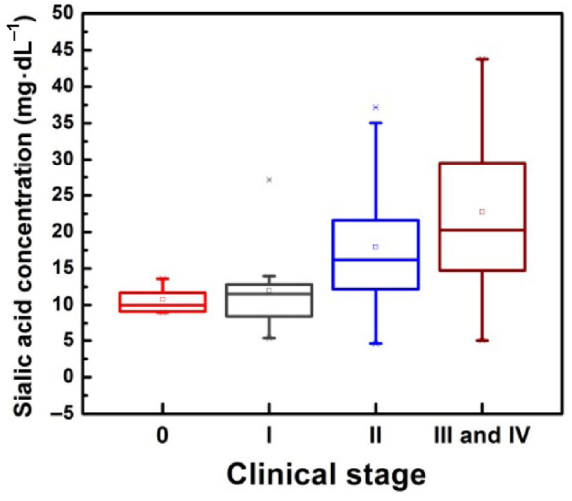

Figure 9 Boxplots of average and median concentrations as a function of clinical stage.

test was performed on all the SA concentration data and cancer clinical stages. A P value of 0.0007 was obtained, which indicates that there is a significant difference in the SA concentrations among different clinical stages. Subsequent Dunn's multiple-comparison test, or a Bonferroni test, both showed significance of differences between stages (0,I) and stages III&IV ($P < 0.05$). No significant differences were observed among the rest of the stages when compared in pairs, according to both tests. A simple Spearman correlation test indicates that r^2 is 0.16 for SA concentration vs. clinical stage.

Table 2 SA concentrations: Median, first quartile Q1, and third quartile Q3 of the data, for each patient with a diagnosis of a cancer stage

Cancer stage	Median	Q1	Q3	Frequency
Stage 0	11.2	10.1	12.7	8
Stage I	11.9	8.5	12.7	10
Stage II	16.3	12.2	22.0	48
Stage III & IV	20.7	15.1	29.5	34
				100

3.3 Sensitivity and specificity

Using the definition of diagnostic sensitivity of a test as the percentage of positives that are correctly identified, and the SA concentration cutoff of 7 mg·dL^{-1}, the sensitivity of the proposed method for breast cancer detection was ~94%. Defining diagnostic specificity as the probability that a healthy individual will have a negative result in the test under study and using the same SA concentration threshold of 7 mg·dL^{-1}, the calculated specificity of the method was found to be 98%. Accuracy of the test, defined as the proportion of true positives divided by the number of all positives, was 92% for the same SA concentration cutoff.

It has been noted that in salivary studies, oral diseases, such as gingivitis, should be taken into account to avoid false positive errors [10, 27]. Furthermore, Stefenelli et al. [28] reported that SA levels are increased in the serum of patients with uterus, lung, colon/rectum, stomach, or prostate cancer. In all these cases, the serum SA concentration was higher than the mean concentration that was detected in the serum of breast cancer patients. The same authors have demonstrated an increase in SA levels in rheumatoid arthritis, liver cirrhosis, and severe inflammatory diseases (such as pneumonia). As a result, increased salivary SA may be indicative of the presence not only of breast cancer but also of other types of cancer and/or cancer-unrelated severe inflammatory conditions.

It is important to note that in our study, the mean age of the control group is less than the mean age of the breast cancer group. It has been reported that the SA levels increase only by 4%–5% and 7% in age groups 50–59 and 60–69 years, compared to the group 20–49 years, in which the SA levels remain practically

the same [28]. Hence, the increment in the SA concentration expected for patients ⩾50 years old, is far below the 7 mg·dL^{-1} cutoff proposed for a negative result and is within one standard deviation of the mean: 3.5 ± 1.0 mg·dL^{-1}.

4 Conclusions

In view of its capacity for the detection of SA in human saliva, Raman spectroscopy may be a promising method for breast cancer diagnosis. Using the calibration curve obtained with reference aqueous solutions, this method was applied to quantification of SA in saliva of 106 healthy women and 100 patients with breast cancer. We found that the SA concentration in the saliva of the control group was 3.5 ± 1.0 mg·dL^{-1} (median, Q1, Q3: 3.4, 2.7, 4.5 mg·dL^{-1}) in contrast to 18.5 ± 9.7 mg·dL^{-1} in the breast cancer group (median, Q1, Q3: 16.3, 12.1, 23.3 mg·dL^{-1}). This method showed sensitivity of 94%, specificity of 98%, and accuracy of 92%. On the basis of these results, we suggest that salivary concentrations of SA greater than 7 mg·dL^{-1} may be indicative (with high probability) of breast cancer or some other cancer. We believe that this work has shown the possibility of developing a versatile SERS-based method that may be useful for the diagnosis of breast cancer. The adoption of the SERS test of human saliva by the medical community, taking advantage of the test's simplicity, its noninvasiveness, low cost, and applicability irrespective of age or breast tissue density, holds promise as a significant breakthrough in women's health care. This advance may help to substantially reduce the mortality caused by one of the leading causes of death among human females worldwide.

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Electronic Supplementary Material: Supplementary material (further details of the reproducibility of the SA concentration levels using different population size distributions of the citrate covered silver nanoparticles) is available in the online version of this article at https://doi.org/10.1007/s12274-017-1576-5.

References

[1] Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D. M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* **2015**, *136*, E359–E386.

[2] American Cancer Society. *Cancer Facts &Figures 2016* [Online]. Atlanta, GA: American Cancer Society, 2016. http://www.cancer.org/acs/groups/content/@research/documents/document/acspc-047079.pdf.

[3] Bird, R. E.; Wallace, T. W.; Yankaskas, B. C. Analysis of cancers missed at screening mammography. *Radiology* **1992**, *184*, 613–617.

[4] Berrington de Gonzalez, A.; Berg, C. D.; Visvanathan, K.; Robson, M. Estimated risk of radiation-induced breast cancer from mammographic screening for young BRCA mutation carriers. *J. Natl. Cancer Inst*. **2009**, *101*, 205–209.

[5] Pfaffe, T.; Cooper White, J.; Beyerlein, P.; Kostner, K.; Punyadeera, C. Diagnostic potential of saliva: Current state and future applications. *Clin. Chem.* **2011**, *57*, 675–687.

[6] Liu, J. Y.; Duan, Y. X. Saliva: A potential media for disease diagnostics and monitoring. *Oral Oncol.* **2012**, *48*, 569–577.

[7] Rosenberg, A. *Biology of the Sialic Acids;* Springer: US, 1995.

[8] Varki, A.; Kannagi, R.; Toole, B. P. Glycosylation changes in cancer. In *Essentials of Glycobiology*, 2nd ed. Varki, A.; Cummings, R. D.; Esko, J. D.; Freeze, H. H.; Stanley, P.; Bertozzi, C. R.; Hart, G. W.; Etzer, M. E., Eds.; Cold Spring Harbor Laboratory Press: New York, 2009; pp 617–632.

[9] Bates, S. E. Clinical applications of serum tumor markers. *Ann. Intern. Med*. **1991**, *115*, 623–638.

[10] Öztürk, L. K.; Emekli Alturfan, E.; Kaşikci, E.; Demir, G.; Yarat, A. Salivary total sialic acid levels increase in breast cancer patients: A preliminary study. *Med. Chem.* **2011**, *7*, 443–447.

[11] Vinogradova, E.; Tlahuice Flores, A.; Velázquez Salazar, J. J.; Larios Rodríguez, E.; José Yacaman, M. Surface-enhanced Raman scattering of *N*-acetylneuraminic acid on silver nanoparticle surface. *J. Raman Spect* .**2014**, *45*, 730–735.

[12] Yao, K.; Ubuka, T. Determination of sialic acids by acidic ninhydrin reaction. *Acta Med Okayama* **1988**, *41*, 237–241.

[13] Skoza, L.; Mohos, S. Stable thiobarbituric acid chromophore with dimethyl sulphoxide. *Biochem. J.* **1976**, *159*, 457–462.

[14] Dadhich, M.; Prabhu, V.; Pai, V. R.; D'Souza, J.; Harish, S.; Jose, M. Serum and salivary sialic acid as a biomarker in oral potentially malignant disorders and oral cancer. *Indian J. Cancer* **2014**, *51*, 214–218.

[15] Jacob, T. V.; Ramesh, M.; Murali, S.; Ramesh, K.; Sanjay, P. R.; Abraham, P. A non-invasive study to estimate and compare salivary sialic acid level as tumor marker in patients with pre-cancer and oral cancer. *J Cancer Res. Ther*. **2016**, *12*, 634–639.

[16] Song, W. Y.; Ding, L.; Chen, Y. L.; Ju, H. X. Plasmonic coupling of dual gold nanoprobes for SERS imaging of sialic acids on living cells. *Chem. Commun.* **2016**, *52*, 10640–16403.

[17] Gong, T. X.; Cui, Y.; Goh, D.; Voon, K. K.; Shum, P. P.; Humbert, G.; Auguste, J. L.; Dinh, X. Q.; Yong, H. T.; Olivo, M. Highly sensitive SERS detection and quantification of sialic acid on single cell using photonic-crystal fiber with gold nanoparticles. *Biosens. Bioelectron.* **2015**, *64*, 227–233.

[18] Haynes, C. L.; van Duyne, R. P. Plasmon-sampled surface-enhanced Raman excitation spectroscopy. *J. Phys. Chem. B***2003**, *107*, 7426–7433.

[19] Kneipp, K.; Wang, Y.; Kneipp, H.; Perelman, L. T.; Itzkan, I.; Dasari, R. R.; Feld, M. S. Single molecule detection using surface-enhanced Raman scattering (SERS). *Phys. Rev. Lett.* **1997**, *78*, 1667–1670.

[20] Nie, S. M.; Emory, S. R. Probing single molecules and single nanoparticles by surface-enhanced Raman scattering. *Science* **1997**, *275*, 1102–1106.

[21] Han, X. X.; Zhao, B.; Ozaki, Y. Surface-enhanced Raman scattering for protein detection. *Anal. Bioanal. Chem.* **2009**, *394*, 1719–1727.

[22] Turkevich, J.; Stevenson, P. C.; Hillier, J. A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discuss. Faraday Soc.* **1951**, *11*, 55–75.

[23] Meyer, M. W.; Smith, E. A. Optimization of silver nanoparticles for surface enhanced Raman spectroscopy of structurally diverse analytes using visible and near-infrared excitation. *Analyst* **2011**, *136*, 3542–3549.

[24] Cavalu, S.; Cîntă Pînzaru, S.; Leopold, N.; Kiefer, W. Raman and surface enhanced Raman spectroscopy of 2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy-3-carboxamide labeled proteins: Bovine serum albumin and cytochrome *c*. *Biopolymers* **2001**, *62*, 341–348.

[25] Munro, C. H.; Smith, W. E.; Garner, M.; Clarkson, J.; White, P. C. Characterization of the surface of a citrate-reduced colloid optimized for use as a substrate for surface-enhanced resonance Raman scattering. *Langmuir* **1995**, *11*, 3712–3720.

[26] Bell, S. E. J.; Sirimuthu, N. M. S. Surface-enhanced Raman spectroscopy as a probe of competitive binding by anions to citrate-reduced silver colloids. *J. Phys. Chem. A***2005**, *109*, 7405–7410.

[27] Shinohara, M.; Ohura, K.; Ogata, K.; Inoue, H.; Miyata, T.; Yoshioka, M. The relationship between the sialic acid concentrations in the serum and whole saliva in rats with naturally occurring gingivitis. *Jpn. J. Pharmacol*. **1994**, *64*, 61–63.

[28] Stefenelli, N.; Klotz, H.; Engel, A.; Bauer, P. Serum sialic acid in malignant tumors, bacterial infections, and chronic liver diseases. *J. Cancer Res. Clin. Oncol.* **1985**, *109*, 55–59.