Reviewer's report

Title: Label-free blood serum detection by using surface-enhanced Raman spectroscopy and support vector machine for the preoperative diagnosis of parotid gland tumors

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Reviewer: Alois Bonifacio

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The paper by Bing Yan et al. reports a classification method for parotid gland tumors based on SERS of serum samples, in combination with a SVM predictive model. In recent years, the use of SERS of serum and plasma in cancer diagnosis has already been described in several papers, as already pointed out by the authors in the “Background” section (page 6, lines 120-124, refs 14,15,17, but there are many others). Still, the use of SERS of serum to classify parotid gland tumors is new, and it is worth investigating.

The authors’ work clearly aims at answering a well-defined question, i.e. if SERS of serum can effectively be used for the classification of such kind of tumors. The method reported by the authors is interesting in itself, but its description is somewhat incomplete (vide infra). Without a complete, more detailed description of such methods it is impossible to estimate whether the data presented are sound or not. Thus, the conclusions drawn about the efficacy of the diagnostic method are only partially supported by data.

Moreover, the authors also did not clearly state the limitations of their work, such as the limited number of patients per class, the limitations and problems possibly related with their chemometric method (i.e. SVM), the quality of the age and gender matching for the different classes (vide infra), etc.

Unfortunately, the clarity of the manuscript, and thus the significance of the findings reported, is often compromised by the poor English. A thorough editorial revision of the manuscript is necessary before consideration for publication.

MAJOR COMPULSORY REVISIONS

1. Experimental methods should be detailed, enabling other researchers to repeat the experiments described (see also Minor Revisions). In particular, authors should:

   - (page 7, line 144) specify what they mean when they state that the subjects “were not treated prior to this study significantly”; in particular, they should explicitly state if any of the subjects underwent any kind of therapy specifically related to their cancer. This is a particularly important point, as treatments as chemotherapy of radiotherapy might induce effects in serum which are detectable with SERS.
- (page 9, lines 189-190) specify what they mean by “to extract pure Raman spectra from the raw spectra”

2. Data analysis

- the authors did not mention any normalization of spectral intensity prior to data analysis, and the use of “arbitrary units” (a.u.) in the figures is ambiguous, since the actual numbers on the y-axes suggest absolute photon counts. The use of un-normalized spectra would also explain the high intensity standard deviations depicted along with the average intensities in figure 2. Usually, intensity normalization is applied to all spectra, since only relative intensities (i.e. the spectral “shape”) and not the absolute intensities are relevant for this type of classification. In fact, absolute intensity might be related to transient experimental conditions (especially for SERS, considering the role of “hot-spots”) such as optical focusing, alignment or other conditions, and two spectra from the same sample (i.e. subject) might have different absolute intensities in spite of their identical spectral shape. The authors should comment on this aspect, explaining why they did not normalize their data, or, alternatively, they might repeat their calculations after normalization.

- according to the paper, 4 to 6 spectra were collected for each “drop” (i.e. subject), for a total of 454 spectra out of 91 samples (page 11, lines 232-233). In validating their model, the authors stated that they used the “leave-one-out” (LLO) validation method. However, they should specify if the method used was a “leave-one-SPECTRUM-out” (i.e. just one spectrum has been left out for testing for each validation step) or a “leave-one-PATIENT-out” (i.e. all the 4-6 spectra belonging to the same one patient must be left out for testing). In fact, validation should be carried out using a test set of “independent” samples, as indicated by several books on chemometrics, see for instance “Chemometrics: Data Analysis for the Laboratory and Chemical Plant” by Brereton (Wiley, 2003) or “Chemometrics with R” by Wehrens (Springer, 2011). Two samples belonging to the same patient are not independent, and cannot be used one for the training set and the other for the test set. If it turns out that the authors used a “leave-one-SPECTRUM-out” method, I recommend them to repeat the validation using the “leave-one-PATIENT-out” method.

3. Comparison of spectra reported with those available in literature

- The SERS spectra of serum presented in this paper (Fig.1b, Fig.2, Fig.3) are remarkably different from serum spectra obtained by other authors using similar conditions (see Refs. 14,15,17; but see also Li et al. Appl.Phys.Lett. 2014, 105, 91104; Laser Phys.Lett. 2014, 11, 65603; J.Biomed.Opt. 2013, 2, 27008; Lin et al. Opt.Exp. 2011, 2011, 19, 13565). The SERS spectra of serum reported by all these authors present several similarities, but all of them are remarkably different from the ones reported in this paper. The authors should comment this aspect, proposing some explanation.

- In the light of recent findings (see for instance Hu et al, J.Raman Spectrosc.
2014, 45, 565; Bonifacio et al. 2014, 406, 2355; Premasiri et al. J.Phys. Chem. B 2012, 116, 9376), the assignments proposed in table 2 are outdated (and partly unclear), and they should be revised. In particular, bands around 725 cm\(^{-1}\) were assigned to hypoxanthine, and not to adenine. Adenine also has an intense SERS band in that region, but its blood concentration is at least one order of magnitude lower than that of hypoxanthine (http://www.serummetabolome.ca/), and its contribution to the SERS spectrum of SERS has still to be ascertained. I leave to the authors the discussion on the reason why, in their spectra, two bands are observed in that region.

In some cases, the assignments were too vague: at page 12, line 256-257, they peaks in the broad region 1500-1600 cm\(^{-1}\) were assigned “to some special molecular bond or vibration”. The authors should also specify what they mean by “O-P-O symmetric stretching in proteins”, possibly adding a reference (table 2).

Moreover, one of the most intense band in the one around 1400 cm\(^{-1}\), as can be appreciated by looking at the intensity standard deviations in figure 2, and in the spectrum in Fig. 1b. As a suggestion, I would consider that band as possibly originating from the citrate ions adsorbed on the Au NPs, which are often present with interfering bands in SERS spectra (Sanches-Cortez and Garcia-Ramos, J. Raman Spectrosc. 1998, 29, 365).

4. Limits of this work

Despite the interesting work presented, the authors should explicitly mention its limitations. For instance:

- the limited number of patients per class (see Beleites et al. Anal.Chim. Acta 2013, 760, 25)
- the limitations and problems possibly related with their chemometric method (i.e.SVM): what are the typical problems associated with SVM if compared with other methods? Are there “overfitting” issues?
- the quality of the age and gender matching for the different classes: the different groups (see table 2) have remarkably differences in the median age (e.g. 29 years between the Normal and WT groups) and are relatively unbalanced as far as gender is concerned (e.g. Normal M/F ca. 2:1, WT M/F 4:1).

All these aspects should be mentioned, stressing that because of these limitations the results presented should be considered as “preliminary”, and further studies are needed, involving greater numbers of subjects.

MINOR ESSENTIAL REVISIONS

- (page 5, line 103) Water is a weak Raman scatterer; “scattering” is the process involved in Raman and SERS, “absorption” is the process involved in Infrared spectroscopy
- (page 6, lines 118-120) The sentence “But in our in vivo study, the laser of
Raman microscope cannot penetrate the skin covering the parotid gland in order to avoid the damage resulted by the high power of laser” is not clear, please rephrase.

- (page 8, lines 162-166) specify reagents concentrations as molarity (preferably), or as g/L

- (page 8, lines 173) specify the “g” value for the centrifugation step, instead of the rpm (centrifugal force depends on the rotor diameter)

- (page 8-9, lines 176-177) specify if the drop of serum-NPs mixture is left to dry (and if yes, at which temperature at for how much time), before collection of SERS spectra

- (page 9, lines 182) specify the numerical aperture (N.A.) of the objective used

- (page 3, lines 53) “Warthin’s tumor” instead of “warthin tumor” (see also lines 136, 411)

- (page 4, line 73) “specificity” instead of “specific icy”

- (page 4, line 88) “well-established” instead of “well established”

- (page 6, lines 117) “literature” or “studies” instead of “literatures”

- (page 6, line 114) “adsorbed” instead of “absorbed”

- (page 7, line 152) “Stomatology” instead of “Stomatoloy”

- (page 9, lines 191, 192) “OriginPro” and “OriginLab” instead of “Oiringin…”

- (page 9, line 192) “spectrum” instead of “spectral”

- (page 15, line 323) “Fleischmann” instead of “Fleischman”

- (page 20, line 434) “References” instead of “Reference”

- (table 1) “Tryptophan” instead of “Trytophan”

- (caption figure 1) “absorption” instead of “absorptiaon”

- (caption figure 2) “standard deviations” instead of “standard deciations”

DISCRETIONARY REVISIONS
None.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Not suitable for publication unless extensively edited

**Statistical review:** Yes, but I do not feel adequately qualified to assess the
statistics.

Declaration of competing interests:
I declare that I have no competing interests