

Aptamer-Based SERS Detection of Lysozyme on a Food-Handling Surface

Victoria Boushell, Shintaro Pang, and Lili He

Abstract: Undeclared food allergens due to cross contamination of processing equipment is a leading cause for food product recalls. Therefore, there is a great need for developing rapid and sensitive methods to detect food allergens. In this paper, an aptamer highly specific to egg white lysozyme was coupled to dendritic silver nanoparticles in order to perform surface enhanced Raman spectroscopy (SERS). The procedure was successfully tested in water and on a stainless steel food-handling surface. The lowest detectable concentration for lysozyme was 0.5 $\mu\text{g/mL}$ in water and 5 $\mu\text{g/mL}$ on a stainless steel food-handling surface. Principal component analysis shows a significant change in SERS spectra when lysozyme was present, suggesting the successful capture of lysozyme by the aptamer. Quantification of lysozyme target was also shown from 0 to 6 $\mu\text{g/mL}$, that is, 0, 0.5, 2, 6 $\mu\text{g/mL}$. Overall method took less than 40 min. The developed method can be extended to detect other food allergens using specific aptamers.

Keywords: aptamer, Detection, LYSOZYME, SERS

Practical Application: We developed a simple, rapid, and sensitive method to detect an egg allergen on a food-handling surface by using aptamers (that is, artificially engineered single stranded DNA) as the capturer and surface enhanced Raman spectroscopy (SERS) as the detector. Our tests showed low concentration detection capability in the parts per million (ppm) level with minimal analysis time (that is, <40 min). This technique can potentially be used for rapid screening of food-handling surfaces to detect undeclared allergens.

Introduction

Food allergy is an immunogenic response to food that can cause adverse health effects, and has been associated with a significant reduction in the quality of life of affected individuals and their family members (Primeau and others 2000; Gupta and others 2004). Recent reports indicate nearly 5% of adults and 8% of children have some form of food allergy, with growing evidence that its prevalence is increasing (Gupta and others 2011; Sicherer and Sampson 2014; van Ree and others 2015). As no curative treatments are available, these patients are often advised to strictly avoid contact with the allergens (Otsu and Fleischer 2012). Unfortunately, cross contamination of food products and mislabeling of food packages are not uncommon. In fact, undeclared food allergens have been reported as the leading cause for food product recalls (FDA 2014), with cross contamination of processing equipment being a significant problem. These issues pose a need for rapid and sensitive detection techniques to monitor the presence of food allergens on food-handling surfaces.

Currently, the most common methods for detecting food allergens are immunological (that is, antibody) based and uses colorimetric readouts to identify or quantify its presence (for example, ELISA; van Hengel 2007). However, these methods carry several limitations such as high cost and high false-positive/negative results (for example, cross-reactivities). Hence, this has attracted attention to develop alternative food allergen detection methods. Aptamers are single-stranded DNA or RNA oligonucleotides that can bind specifically to a target analyte through intermolecular recognition

(for example, electrostatic interactions, hydrogen bonding, van der Waals interaction, and so on) and an induced fit mechanism (Osborne and Ellington 1997; Patel and others 1997; Hermann 2000). The dissociation constants, K_d , of aptamers typically falls in the picomolar to micromolar range, which is comparable to antibodies. However, since they are selected *in vitro* using a process called SELEX, no animals are required and it can be reproduced at low cost with minimal batch-to-batch variation using standard phosphoramidite chemistry (Ellington and Szostak 1990; Tuerk and Gold 1990). Furthermore, aptamers are thermostable (that is, regenerate easily after denaturation) and can be easily modified for immobilizing onto a surface or labelling (Dong and others 2013; Kim and Gu 2014). These factors make aptamers a great alternative to antibodies as a capture agent for food allergens.

Surface enhanced Raman spectroscopy (SERS) is a relatively new and emerging technological tool that is gaining popularity for the detection of food contaminants (Craig and others 2013; Zheng and He 2014; He and others 2015). This is because SERS is able to provide strong and distinct molecular fingerprints while avoiding interference from water signals and suppressing fluorescence (Haynes and others 2005), thus facilitating identification, characterization and quantification of samples. It works on the principle that noble metal nanosubstrates greatly enhance weak Raman signals through electromagnetic (Gersten and Nitzan 1980; Moskovits 1985) and chemical (Burststein and others 1979; Gersten and others 1979) means, making it possible to detect many compounds at parts per million (ppm) or even parts per billion (ppb) levels (Doering and Nie 2002; Kneipp and Kneipp 2006). Because of its high sensitivity and unique Raman profile for each molecule, SERS methods can be label-free, thus simplifying and reducing detection time as well (He and others 2011a, 2011b). Furthermore, when compared to commonly used methodology, such as the change of color, as the basis of detection, SERS has a

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relatively lower possibility for false results as we can verify target capture based on its peak position. These properties make SERS a promising signaling tool for bio-sensing detection.

In this study, we immobilized an aptamer that was highly specific to lysozyme, a major egg allergen, onto dendritic silver nanoparticles for SERS detection. Our previous studies showed dendritic silver nanoparticles were able to provide sensitive and consistent SERS signals (He and others 2010; Zheng and others 2013; Pang and others 2014; Hou and others 2015). In addition, we validated our method by capturing and detecting lysozyme spiked onto a stainless steel that simulates a food-handling surface. To the best of our knowledge, this is the 1st paper that utilizes an aptamer-based SERS method for the detection of food allergens.

Materials and Methods

Materials

The lysozyme aptamer (that is, specific to egg white lysozyme) was synthesized by Eurofins Genomics (Ala., U.S.A.), and contained the following sequence: 5'-GCAGCTAAG-CAGGCGGCTCACAAAACCATTCGCATGCGGC-3' as described by previous literature (Tran and others 2010) with disulfide (S-S) modification at the 5' end. This aptamer sequence was chosen because it has been validated by several labs (Tran and others 2010; Wang and others 2011; Truong and others 2013) and has high binding affinity to egg white lysozyme ($K_d = 2.8 \pm 0.3 \text{ nM}$). The random ssDNA sequence used as a negative control had the following sequence: 5'- CTC CTC TGA CTG TAA CCA CGC ACA AAG GCT CGC GCA TGG TGT GTA CGT TCT TAC AGA GGT -3'. This sequence was randomly selected from our pool of available aptamers used to capture a different target. Egg white lysozyme, silver nitrate, zinc and stainless steel coupons were purchased from Fisher Scientific (Pittsburg, Pa., U.S.A.). Double distilled water was used for all aqueous solutions.

Preparation of dendritic silver nanoparticles for SERS analysis

Silver dendrites were synthesized according to a previously published method suitable for SERS analysis (He and others 2010; Zheng and others 2013; Pang and others 2014; Wijaya and others 2014). Briefly, a zinc metal plate was immersed in 200 mM AgNO_3 (aq) solution for exactly 1 min to produce silver dendritic

nanoparticles with $\approx 50 \text{ nm}$ size diameter. After incubation, the silver dendrites were washed copiously with water and then stored in a sealed, glass vial. This nanoparticle has been previously shown to be stable for at least 6 mo at room temperature (He and others 2010).

Preparation of aptamer-modified silver dendrites

The optimized conditions for preparing aptamer-modified silver dendrites were as follows: 1st, $10 \mu\text{L}$ ($\approx 40 \mu\text{g}$) silver dendrites (Ag) were deposited into 1 mL of $8 \mu\text{g/mL}$ thiol-modified lysozyme aptamer (Ap) and incubated overnight (room temperature) on a Nutating mixer (Fisher Scientific) at 24 rpm. After incubation, the mixture was centrifuged at $6,000 \times g$ for 1 min to separate the silver-aptamer (Ag-Ap) complex from the supernatant. The supernatant was removed and the Ag-Ap complex was rinsed 3 times with 1 mL double distilled water. One microliter of Ag-Ap complex was then deposited onto a clean, glass slide, dried, and then subjected to Raman analysis to validate the formation of Ag-S bond between Ag and the thiol-modified aptamer.

Capture and detection of lysozymes using traditional and aptamer-based SERS

The Raman spectrum of lysozymes was initially characterized so that it could be used as a reference to validate its capture by the aptamer. Then, 1 mL of varying concentrations of lysozymes (0, 0.5, 2, 6, 10, and $14 \mu\text{g/mL}$) was incubated with $5 \mu\text{L}$ Ag-Ap complex in double distilled water for 15 min under rotation at 24 rpm (Nutating mixer, Fisher Scientific). After incubation, the mixture was rinsed 3 times with double distilled water by centrifuging and replacing the supernatant. Lastly, $1 \mu\text{L}$ of sample was deposited onto a clean, glass slide and dried before being subjected to Raman analysis.

Detection of lysozymes on a food-handling surface

To verify the detection of lysozyme on a food-handling surface, 1 mL of known concentrations of lysozyme solutions (0, 5, 10, 20, 25, 50, and $75 \mu\text{g/mL}$) were deposited onto clean, stainless steel coupons (1 inch by 1 inch, Fisher Scientific) and dried quickly using low heat (Dial 3, Thermolyne hot plate, Waltham, Mass., U.S.A.). A surface swab method (He and others 2014; Wijaya and others 2014) was used to recover the lysozyme from the surface. Briefly, swab sticks (diameter 0.25 inch, ITW Texwipe, Kernersville, N.C., U.S.A.) were used to swab the spiked area for 30 s,

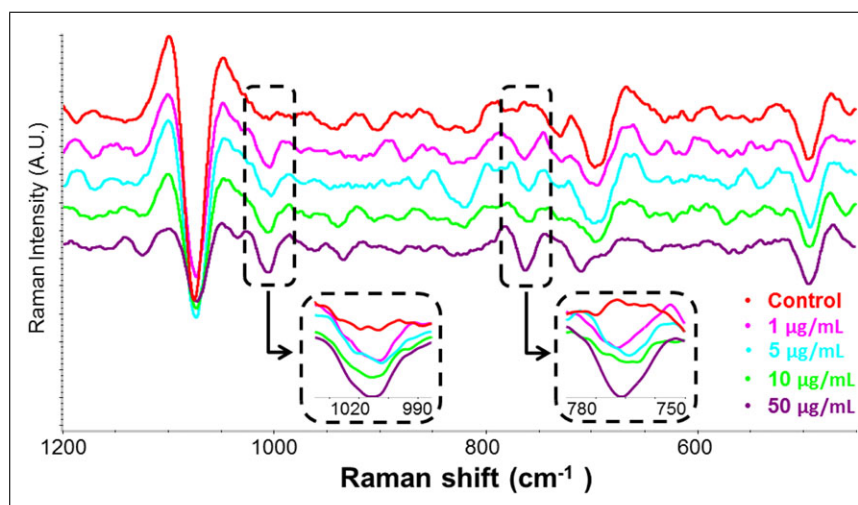


Figure 1—Second derivative SERS spectra of control (Ag) and varying concentrations of lysozymes (1, 5, 10, 50 $\mu\text{g/mL}$) on Ag between 720 and 1060 cm^{-1} .

which were then vortexed for 30 s with 1 mL deionized water in a microcentrifuge tube. The same procedure as the capture and detection of lysozymes using the above-described aptamer-based SERS method was performed with the collected sample.

Raman instrumentation and data analysis

Raman analysis was performed using a DXR Raman microscope (Thermo Scientific, Waltham, Mass., U.S.A.). All parameters were optimized and remained consistent throughout this study as follows: Excitation wavelength = 780 nm; Laser power = 4 mW; Slit width = 25 μm ; Spot diameter $\approx 3 \mu\text{m}$; Spectral resolution = 5 cm^{-1} ; Integration time = 2 s; Objective lens = 10x. Quadruplicate SERS measurements were collected for each sample using the Thermo Fisher Scientific OMNICTM Software (version 9.1) with Array Automation function. TQ Analyst Software (version 8.0) was then used to perform statistical analysis of the spectra. Second derivative transformation and smoothing was applied throughout to separate overlapping bands and reduce background noise. The

SERS spectra of each sample from multiple spots were then averaged and compared against other samples.

Results and Discussion

Determination of lysozyme Raman indicator

In order to verify that lysozyme was being captured by the aptamer, lysozyme was initially analyzed directly on Ag dendrite to obtain its Raman spectra. Figure 1 shows the characteristic SERS signals of the protein lysozyme, at various concentrations. Two prominent Raman peaks were identified at 1008 and 765 cm^{-1} . Separate studies have also identified these peaks to be attributed to lysozyme (Hédoux and others 2011; Vergara and others 2013). Hence, for the purpose of this study, the peak at 1008 cm^{-1} was selected as an indicator for the capture of lysozyme.

Optimization of aptamer-modified silver dendrites

The maximum binding capacity of lysozyme aptamer (Ap) onto silver dendrites (Ag) was investigated. This is a crucial step

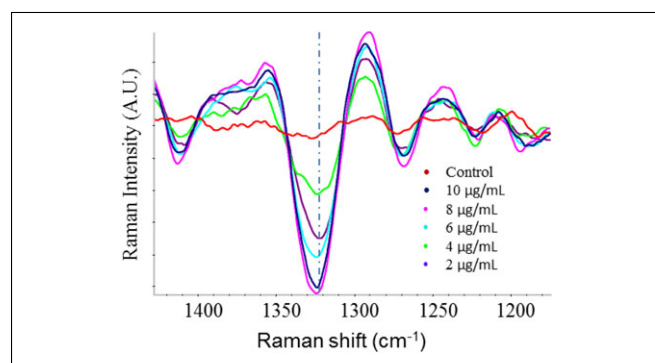


Figure 2—Second derivative SERS spectra of different concentrations of aptamer (2, 4, 6, 8, 10 $\mu\text{g/mL}$) bound on Ag (Control) between 1160 and 1430 cm^{-1} .

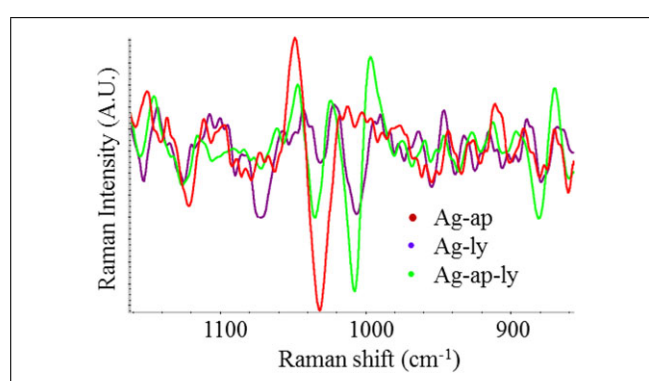


Figure 4—Second derivative SERS spectra of Ag-Ap, Ag-Ly and Ag-Ap-Ly between 860 and 1160 cm^{-1} . Ag: silver dendrite, Ap: aptamer, Ly: lysozyme.

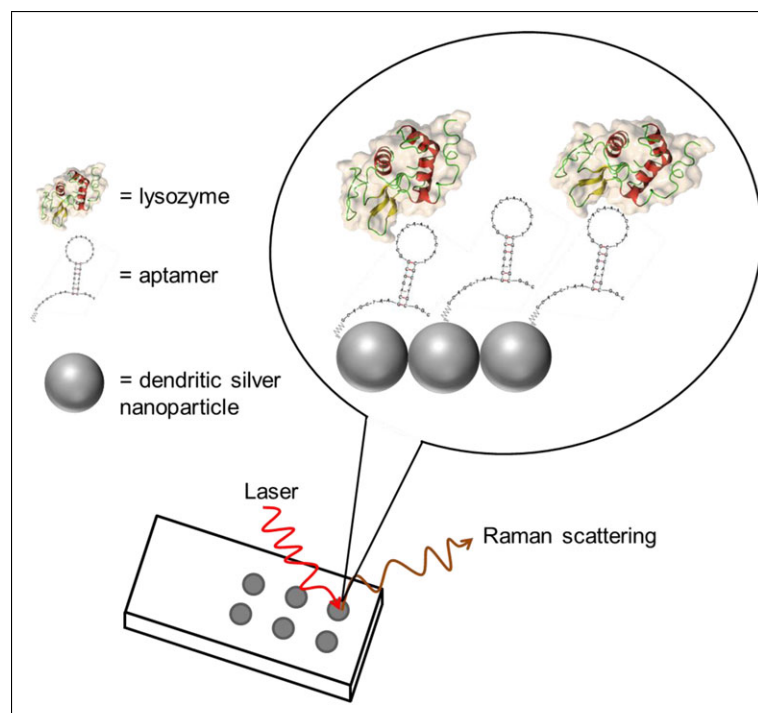


Figure 3—Schematic illustration of the capture of lysozyme Raman signals using aptamer-based SERS (aptamer secondary structure obtained from Tran and others 2010).

because a higher concentration of aptamer would allow more target molecules to bind and consequently improve sensitivity. In addition, by fully covering the Ag dendrite with aptamer molecules, binding of proteins and other molecules on Ag becomes unlikely. Thus, nonspecific binding from SH and $-\text{NH}_2$ available in many proteins are prevented (Niemeyer 2001; Chen and Schluesener 2008; Sanghi and Verma 2009). Figure 2 shows a characteristic Raman peak of the lysozyme aptamer around 1320 cm^{-1} with different aptamer concentrations. The peak intensity maximized at $8\text{ }\mu\text{g/mL}$, and was determined to be the maximum binding capacity based on the following equation (Kneipp and others 1999):

$$I_{\text{SERS}} \propto \sigma_{\text{ads}}^{\text{R}} \times I(\nu_{\text{L}}) \times N'$$

where I_{SERS} = Total Stokes Raman signal, $\sigma_{\text{ads}}^{\text{R}}$ = Raman cross section of the adsorbed molecule, $I(\nu_{\text{L}})$ = excitation laser intensity and N' = number of molecules. In other words, the height of the aptamer peak is directly proportional to the number of aptamer molecules bound to Ag.

Capture and detection of lysozymes using aptamer-based SERS

Figure 3 shows a schematic illustration of the capture of lysozyme Raman signals using aptamer-based SERS. The change in SERS spectra before and after capturing lysozyme is the basis of this sensor.

The specific changes in spectra such as new peaks, Raman shifts and peak height/width can be further analyzed to deduce the presence of new compounds, change in chemical structure and/or

target–nanosubstrate interactions (Lamhert 1987; Barhoumi and others 2008; He and others 2011a).

Figure 4 shows the change in Raman peaks when lysozyme was introduced to Ag–Ap. The lysozyme peak indicator around 1008 cm^{-1} increased significantly using the aptamer (that is, peak intensity was twice that of Ag–Ly), which demonstrated the successful capture and concentration of lysozyme by the aptamer, as well as improved detection sensitivity. This is most likely due to the strong target–aptamer binding interactions promoted through unique three-dimensional (3-D), folded structures, hydrogen bonds, electrostatic interactions, and so on (Hermann 2000; Li and others 2002) and validates the aptamer's high binding affinity, K_d , reported in literature. Furthermore, there was a slight Raman shift, which could be attributed to conformational changes in the 3-D structure or due to the modified aptamer surface.

Concurrently, the aptamer peak around 1032 cm^{-1} , which represents the DNA phosphate backbone functional groups (Barhoumi and others 2008), decreased by half when lysozyme was captured, which again suggests a conformation change in the aptamer. A new peak was also seen at 880 cm^{-1} when lysozyme was captured, suggesting a new chemical bond between the target and aptamer. All these changes in SERS spectra indicate the successful capture and detection of lysozyme. Another peak to note is at 1075 cm^{-1} , which indicates NO_3^- ions left from the synthesis of silver dendrites (He and others 2010). The results obtained from these SERS spectra agrees with previous findings that aptamers may be more suitable than antibodies for SERS analysis when capturing proteins because the distinction between antibodies and their target proteins are not as clear as the peak changes between nucleic acids and proteins. (He and others 2011a, 2011b)

Discriminant analysis was conducted to evaluate statistical differences in SERS spectra, and is reported using a principal component analysis (PCA) plot shown in Figure 5. The results clearly show a statistical difference between classes (that is, Ag–Ap, Ag–Ly and Ag–Ap–Ly). Seven spectral readings were analyzed for each class, and the PCA plot shows no overlap in PC1 and PC2 scores between the data clusters of each class. This result proves that aptamer-based SERS is a suitable method to capture and detect lysozyme.

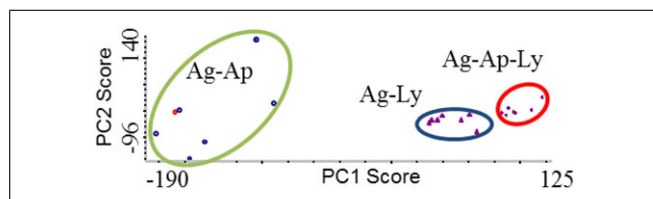


Figure 5—Principal component analysis (PCA) plot of Ag–Ap, Ag–Ly, and Ag–Ap–Ly.

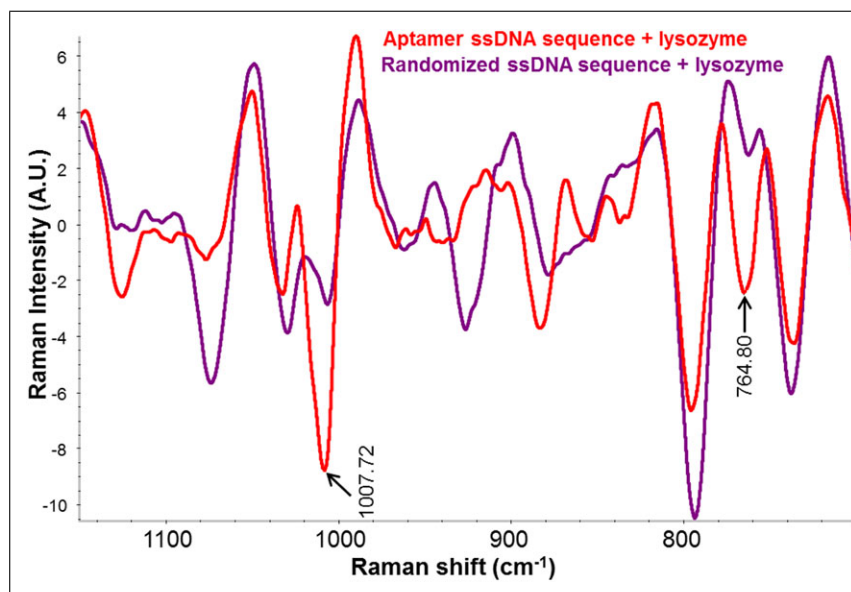


Figure 6—Second derivative SERS spectra of a lysozyme aptamer sequence and randomized ssDNA sequence exposed to 10 ppm lysozyme, respectively.

One interesting observation is the relatively high PC1 score of Ag-Ap-Ly (that is, $\text{Ag-Ap} < \text{Ag-Ly} < \text{Ag-Ap-Ly}$), which again suggest the improved sensitivity of using aptamer-modified Ag as compared to Ag itself. When compared to previous aptamer-based SERS using similar methods (He and others 2011a; He and others 2013), this result had higher PC1 scores, and therefore, further analysis might help us elucidate the mechanism behind the improved sensitivity of this method. To further prove the specificity of this aptamer for capturing the lysozyme target, a randomized ssDNA sequence was evaluated for the capture of lysozyme as a comparison. It is clear to see that the lysozyme peaks around 1008 and 765 cm^{-1} were clearly shown only when the specific aptamer was used (Figure 6). Although, the 2 small peaks appeared at similar locations in the randomized ssDNA negative control could be resulted from the background signals of the ssDNA or nonspecific binding of the lysozyme. Further improvement on the washing process may reduce the nonspecific binding. In practical application, the whole spectrum rather than just these 2 peaks would be concerned for accurate identification.

Figure 7(A) shows the effect of lysozyme concentration on the relative Raman peak intensity at 1008 cm^{-1} . The graph shows a sharp increase in peak intensity up to 6 $\mu\text{g/mL}$ and starts to plateau at higher concentrations. This suggests the potential for

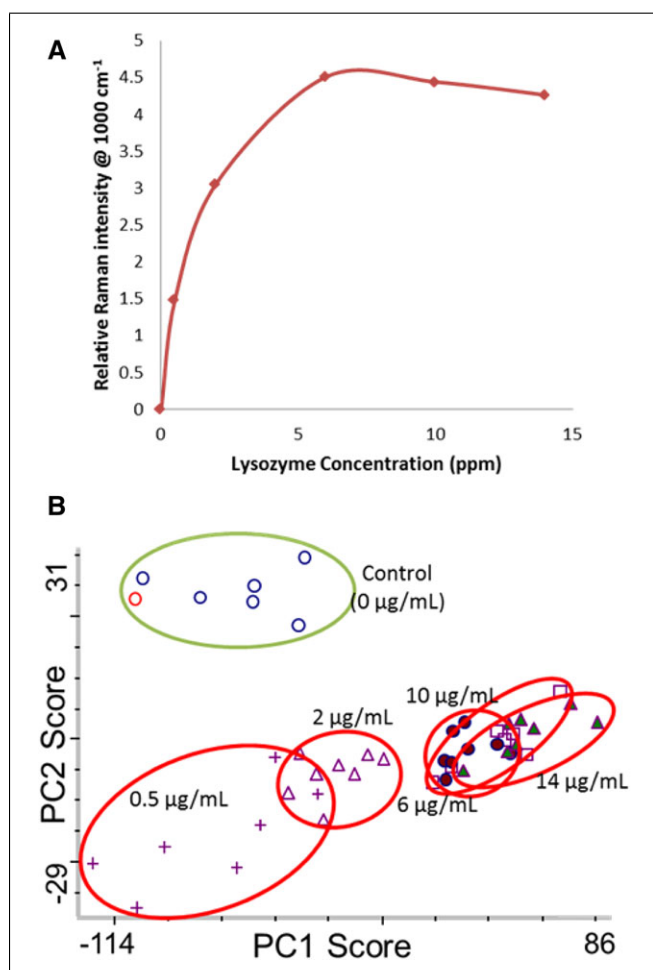


Figure 7—(A) Relative SERS peak intensity at 1000 cm^{-1} plotted against various lysozyme concentrations mixed with Ag-Ap; (B) principal component analysis (PCA) plot of Ag-Ap incubated with lysozyme at 0, 0.5, 2, 6, 10 and 14 ppm ($\mu\text{g/mL}$).

quantitation of lysozyme using aptamer-based SERS. The PCA plot in Figure 7, which is based on the SERS spectra used to obtain the graph in Figure 7(A), shows a significant difference between control and 0.5 $\mu\text{g/mL}$ lysozyme as the sample classes are not overlapping each other, suggesting that the lowest detectable concentration of lysozyme using aptamer-based SERS was 0.5 $\mu\text{g/mL}$. However, there were some overlaps between different lysozyme concentrations, suggesting that the method might require further optimization (for example, incubation time, nanoparticle amount) if an accurate quantification method is desired. Despite this limitation, a linear concentration standard curve was obtained between 0.5 and 6 $\mu\text{g/mL}$ for this method ($R^2 = 0.8834$).

Currently, there is no established threshold concentration to regulate egg white lysozymes as a food allergen. However, Poms and others (2004) indicated that a reasonable lowest detectable concentration for allergens is between 1 and 100 $\mu\text{g/g}$. Our method is able to achieve a comparable lowest detectable concentration as well as acceptable concentration ranges, although the linearity (that is, correlation coefficient) of the detectable concentration range is not as good as some other lysozyme detection methods. For example, an ELISA-based method was able to quantitatively detect lysozyme at concentrations between 0.38 and 4.8 $\mu\text{g/mL}$ ($R^2 = 0.991$; Vidal and others 2005). In another study, HPLC was used to quantitatively detect egg white lysozyme from 0.2 to 1 $\mu\text{g/mL}$ ($R^2 = 0.990$) and from 2 to 10 $\mu\text{g/mL}$ ($R^2 = 0.996$; Kerkaert and others 2010). The specificity of this detection method is also comparable to other detection methods commonly used to detect lysozyme. For instance, while Kerkaert and others (2010) used HPLC and relied on elaborated extraction procedures, specific fluorescence detection and good chromatographic separation without interfering peaks to show the specificity of the assay, this aptamer-based SERS method relied on the molecular fingerprint specificity of lysozyme and the functionalization of SERS substrates with a highly specific lysozyme aptamer. Hence, based on the sensitivity and specificity of the aptamer-based SERS method for lysozyme detection, it has great potential to be an alternative rapid detection method.

Detection of lysozymes on a food-handling surface

In order to verify if the developed aptamer-based SERS method was feasible for industrial application, detection of lysozyme was

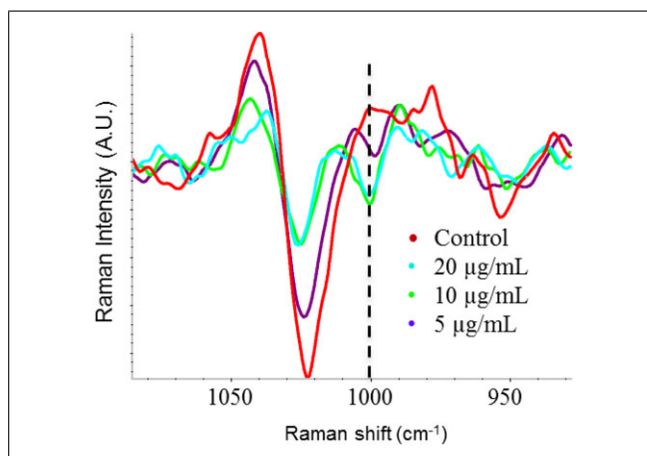


Figure 8—Second derivative SERS spectra of lysozyme recovered from a stainless steel food-handling surface at various lysozyme concentrations (0, 5, 10, 20 $\mu\text{g/mL}$).

tested on a stainless steel surface that simulates a contact surface of food processing equipment.

A clean swab was used to swab a one in by one in surface previously spiked with a known amount of lysozyme. The lysozyme was then recovered using a simple recovery method described in the method section. Figure 8 shows the 2nd derivative SERS spectra of lysozyme conjugated onto aptamer at various lysozyme concentrations. Once again, we see that the lysozyme peak at 1008 cm^{-1} was evident even at $5\text{ }\mu\text{g/mL}$ and the peak intensity maximized at $10\text{ }\mu\text{g/mL}$. The capability of this method to capture and detect lysozymes at such low concentrations makes it a potential detection tool for the food industry. When combined with other currently used technique, it can also provide more reliable information such as validation and characterization of the captured analyte.

Conclusion

In summary, an aptamer-based SERS method was successfully developed to detect lysozymes, a major egg allergen on a stainless steel food-handling surface. Minimal sample preparation was required and the overall detection time was only 40 min. The sensitivity of this detection method was also comparable to other lysozyme detection methods (Vidal and others 2005; Kerkaert and others 2010), based on its fingerprint peak at around 1008 cm^{-1} . This method can also give us further insights into the molecular interaction between aptamer and lysozymes. Other major egg allergens (such as ovomucoid, ovalbumin, and conalbumin) have their own unique molecular characteristics, and as such, may provide distinct Raman peak information when captured by their specific aptamer, which can then be exploited to perform simultaneous detection. This will be possible once aptamers targeting the other egg allergens are developed. A portable Raman spectrometer could even be used for onsite detection, thus reducing the need to send samples for laboratory work.

Acknowledgments

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

Author Contributions

V. Boushell came up with the experimental design, conducted the experiments and analyzed the results. S. Pang also analyzed the results and drafted the manuscript. L. He supervised the whole project and interpreted results obtained.

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