Surface-Enhanced Raman Spectroscopy Detection of Ricin B Chain in Human Blood

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ABSTRACT: Over the past few years, ricin has been discussed frequently because of letters sent to high-ranking government officials containing the easily extracted protein native to castor beans. Ricin B chain, commercially available and not dangerous when separated from the A chain, enables development of ricin sensors while minimizing the hazards of working with a bioterror agent that does not have a known antidote. Recent events have increased the risk of ricin exposure for civilians, and there is a need for rapid, real-time detection of ricin. To this end, aptamers have been used recently as an affinity agent to enable the detection of ricin in food products via surface-enhanced Raman spectroscopy (SERS) on colloidal substrates. One goal of this work is to extend ricin sensing into human whole blood; this goal requires application of a commonly used plasmonic surface, the silver film-over-nanosphere (AgFON) substrate, which offers stable SERS enhancement factors of 10⁶ in human whole blood. Herein, this aptamer-conjugated AgFON platform enabled ricin B chain detection even after the aptamer-modified substrate had dwelled for up to 10 days in human whole blood. Principle component analysis (PCA) of the SERS data clearly identifies the presence or absence of ricin B chain in blood.

INTRODUCTION

Ricin is a naturally occurring toxin that is found in the castor bean to protect the plant from pests. The detailed protein structure and the mechanism of its toxicity have been extensively and thoroughly studied over the last two decades.1-3 This globular protein is comprised of two chains linked by a single disulfide bond.4 Ricin A chain is the ribosome-inactivating toxin that inhibits protein synthesis, while ricin B chain is a lectin that attaches to galactose residues on the cell surface, facilitating entry.5 Both chains are necessary for in vivo toxicity.6-10 Once the protein translocates into a cell’s cytosol, one molecule of ricin is sufficient to kill that cell. The median lethal dose (LD50) of crude ricin in mice is 8 µg/kg by injection and 10 µg/kg by inhalation.11,12 After exposure to a lethal dose of ricin, the time to death is typically 60-90 h.13 The relative ease of extraction and isolation of ricin, combined with the ubiquitous presence of the castor plant, make ricin an easily obtainable and frequently used bioterror agent. Ricin is considered the third-most toxic substance due to its toxicity and abundance according to the U.S. Environmental Protection Agency and the Center for Defense Information.14-17 Thus, rapid and easy-to-apply diagnosis of ricin presence in a biomatrix like human whole blood remains a priority. Many methods, including mass spectrometry, cell culture assays, and immunochemical assays, have been developed to detect ricin in various media;18,19 however, most of the methods to detect ricin can be confounded by complex biological environments and a lack of accuracy. Enzyme-linked immunosorbent assays (ELISAs) are the current commercial method for ricin detection. By using an antibody, ELISA is able to detect ricin selectively and sensitively. However, the antibody is expensive and sensitive to the environment, and nonspecific interactions with other proteins or molecules lead to large variance that also limits the application of this method in complex matrices.

Aptamers are short single DNA or RNA strands that, like antibodies, have specific affinity to analytes ranging from small inorganic molecules to proteins or even whole cells.20,21 Aptamers show many advantages in analytical detection applications, competing with traditional antibody-based methods. In contrast to antibodies, aptamers are easily synthesized in high purity and modified with desired functional groups. Unlike antibodies, aptamers are more stable in various conditions and can reversibly fold and unfold.

Sreevatsan and co-workers selected an aptamer specific to ricin B chain (RBC) from a combinatorial oligonucleotide library by systematic evolution of ligands by exponential enrichment (SELEX).22,23 This RBC aptamer is a 40-mer (5′-
ACACCCACCGCAAGAGCAGCAAGGCTCGGAGAC-CTAGCC-3′) that contains a bulge loop and a hairpin loop, and it is thermally stable from 4 to 63 °C. The thiolated RBC aptamer used in the experiments presented herein was heated to 95 °C for 5 min to fully unfold the hairpin structure and submerged in ice water to fold the aptamer into the most stable conformation prior to modification of the plasmonic substrate. Previous work with this aptamer showed that it enables detection down to 30 ng/mL of RBC within several liquid food matrices.22–24 The authors also demonstrated that the RBC aptamer was capable of detecting intact ricin (even though it was developed using only RBC) and demonstrated that this aptamer sequence had a binding constant better than other reported ricin aptamers and comparable to the current commercial ELISA antibodies.

Upon binding to the target analyte, the conformational change of aptamers can be detected by various optical analysis methods. The Labuza group reported the use of the RBC aptamer and surface-enhanced Raman spectroscopy (SERS) to detect ricin in food matrices.25 Because the Raman scattering cross-section of water is very small, SERS is a promising label-free detection method for analyte detection in aqueous environments, including both food matrices and blood.25,26 An additional advantage of using the aptamer affinity agent is that aptamers are small compared to antibodies, meaning that captured analytes are more likely to be detected by SERS, where the electromagnetic fields responsible for the enhancement decay exponentially with the distance between the analyte and the noble metal surface that tethers the aptamer.

To efficiently detect and monitor ricin in the human body, it is desirable to capture and detect ricin directly in whole human blood; however, this is likely to be challenging based on the biological complexity of blood. Several research groups have successfully used SERS to detect analytes in simpler body fluids. For example, Ridente et al. detected trace amount of amphotericin B, a drug for fungal infections, in dilute plasma via surface-enhanced resonance Raman spectroscopy.27 Also, Trachta et al. combined the techniques of SERS and HPLC to detect codeine and carbazepine in human blood and urine.28 In fact, several groups have reported SERS measurements in whole human blood. Liu et al. utilized a vancomycin-coated SERS substrate to capture and detect bacteria from human blood without interference from blood cells.29 Casella et al. were able to recognize and assign the fundamental features of β-carotene and the blood protein hemoglobin in human blood by SERS.30 Wang et al. and Sha et al. both reported the detection of circulating tumor cells in blood, relying on AuNPs conjugated with a Raman-active tag.31,32 Herein, RBC captured by the aptamer-modified AgFON is directly detected by SERS in human whole blood. This study also includes consideration of the reversibility of binding between ricin B chain and the aptamer-modified SERS substrates. While the detection of RBC is exciting, this approach is generalizable and could easily be adapted to detect other blood-borne protein targets with appropriate aptamer-modified SERS substrates.

**EXPERIMENTAL SECTION**

**SERS Substrates: Ag Film-over-Nanospheres.** Silver film-over-nanospheres (AgFON) substrates were fabricated as has been previously reported.33,34 Briefly, a silicon wafer was cleaned with a water:ammonium hydroxide: 30% hydrogen peroxide (10:1:1) mixture under sonication for 60 min. The wafers were thoroughly rinsed with water followed by silica nanosphere (Thermo Scientific, Waltham, MA) drop coating. Typically, 1–10 μL of the silica nanosphere solution was drop cast onto the wafer, followed by gentle agitation/mixing, and allowed to air-dry. Silver metal (200 nm thick, 99.999% Ag Kurt J. Lesker, Clairton, PA) was deposited with a vacuum evaporation chamber (Denton Vacuum, Moorrestown, NJ) equipped with a quartz crystal microbalance. Ahead of aptamer modification, freshly fabricated AgFONs were plasma cleaned using argon gas for 4–6 min in a plasma chamber.35 Then, 10–40 μM RBC aptamer solution (‘5’A’CCA ACC ACC GCA GCC AGA CGG ACC GCC TCG GAG ACT AGC C3’ dithiol spacer, Integrated DNA Technologies, Coralville, IA) (Figure S1) was added to the plasma-cleaned AgFON and incubated at 4 °C overnight, followed by rinsing with nuclease-free (Integrated DNA Technologies) water. The functionalized-AgFONs were then dried with a gentle stream of nitrogen gas.

**Ricin B Chain Incubation Experiments.** Individual AgFONs functionalized with the aptamer were incubated in either nuclease-free water, buffer, or human whole blood (the blood, containing EDTA anticoagulant, was donated from Memorial Blood Center and used on the same day as received). The solution was then spiked with a known concentration of ricin B chain (RBC, Vector Laboratories, Burlingame, CA) and gently mixed by swirling. Following the defined incubation time (1 h incubation in nuclease-free water, 6 h incubation in blood, overnight incubation with the substrates that had already been exposed to blood for 3 or 10 days), the AgFON was removed from the solution and rinsed with copious amounts of nuclease-free water. The AgFON was then dried with nitrogen gas ahead of SERS interrogation.

**Surface-Enhanced Raman Spectroscopy Measurements.** Raman spectra were acquired using a 632.8 nm HeNe excitation laser (Research Electro-Optics, Inc., Boulder, Colorado) that passed through an interference filter (Melles-Griot, Rochester, NY) ahead of direction to the sample using an aluminum-coated prism (final laser spot size on the sample was 1.26 mm²). The Raman scattered light was focused using a 50 mm-diameter achromatic lens (Nikon, Melville, NY), and the Rayleigh scattered light was rejected with a notch filter (Semrock, Rochester, NY). A 0.5 m SpectraPro 2500i single monochromator equipped with a Spec400B liquid nitrogen-cooled CCD (Princeton Instruments/Acton, Trenton, NJ) was used to collect and measure the Raman scattered light. The CCD was calibrated using the emission wavelengths of a neon lamp.

**Principal Component Analysis of SERS Data.** Principal component analysis (PCA) is a chemometric method capable of removing the fluorescence background from Raman spectra as well as discriminating different components within Raman spectra of complex mixtures such as pathogenic bacteria or paint pigments.36–40 PCA is a statistical technique that reduces the dimensionality of a spectral data set. It achieves this by reducing the data set to a linear combination of orthogonal vectors such that a majority of the variance between spectra is explained by the first vector. The dot product of the first vector with any spectrum gives the value of that spectrum’s first principal component. The projection of the second vector (which explains the second largest variance) gives the second principal component. Due to the variability in signal from SERS (due to subtle variations in laser power and substrate uniformity), PCA often does not discriminate well based on raw SERS spectra. Patel et al. have proposed the use of the magnitude of the second derivative of the spectra as input to
PCA.40 Here, we eliminate cosmic rays from our spectra, followed by removing the baseline by subtracting a wide (500 cm$^{-1}$) Hamming Window from each spectrum, followed by normalization, a spline interpolation of the data onto a 1 cm$^{-1}$ grid, and finally perform PCA analysis on the sign ($\pm$) of the second derivative of the data set.

Thermal and Ionic Strength Stability Assessment. All of the stability experiments were performed with both benzenethiol (a standard high scattering cross-section Raman scatterer) and RBC captured on the AgFONs. In the thermal stability tests, the AgFONs, following analyte incubation, were placed in 2 mL of nuclease-free water at various temperatures ranging from 70 to 95 °C for either 30 s or 5 min. The AgFONs were then rinsed with 1 mL of nuclease-free water to remove any free analyte after the thermal treatment. The SERS spectra were then measured from $N_2$ gas-dried AgFONs using the same experimental apparatus described above.

While elevated temperature is one possible way to compromise aptamer or protein stability, variations in ionic strength may have a similar effect by weakening electrostatic or H-bonding interactions. Herein, a 1 M NaCl solution was utilized as a high ionic strength solution. Similar to the thermal stability tests, the AgFONs with bound analyte were immersed in 2 mL of room temperature NaCl solution for either 30 s or 5 min. The resulting AgFONs were then rinsed with nuclease-free water, and SERS spectra were measured.

Possible destabilization due to pH variation was also assessed. Dilute aqueous HCl with pH of 4.96 and dilute aqueous NaOH with pH of 7.99 were prepared. Similar to the previous stability tests, AgFONs with bound analyte were immersed in 2 mL of either room temperature solution for either 30 s or 5 min. After rinsing, SERS spectra were measured.

Computational Modeling of Aptamer on a Ag Surface. Quantum chemistry calculations were carried out for geometry optimization of the aptamer on the SERS substrate by using density functional theory with the B3LYP functional.41-43 For C, H, N, and O atoms, the basis sets used were 6-311+G(df).44,45 The polarizable continuum model was used to consider the influence of water.26,46,47 On the basis of the optimized structures, the vibrational frequencies and Raman intensity were obtained. All calculations were performed with Q-chem 4.1.0.50

RESULTS AND DISCUSSION

With the aim of developing a sensor that could monitor blood continuously for at least a few days, stability of the sensor in blood is crucial. Stability of AgFONs in human whole blood has not previously been presented in the literature. To assess multiday stability of the AgFONs in human whole blood, a study was initiated with a standard SERS reporter molecule rather than the more expensive aptamer since both of the adenine and guanine, although differing in intensity compared to the natural aptamer backbone. Going forward, all experiments were done with the nuclease-resistant aptamer.

While single-stranded DNA has been used frequently on metal surfaces for sensing purposes,61,63-66 the use of ssDNA in complex matrices (such as food or body fluids) is not widely
Figure 1. Comparison of the theoretical SERS spectrum in the condition where (a) all bases beyond the 7th are considered to be the same distance from the surface or; (b) all bases beyond the 7th nucleotide are too far away from the surface to contribute significantly to the SERS intensity and (c) the experimental SERS spectrum for nuclease-resistant ricin B chain aptamer on a Ag surface.

reported, likely due to interference from proteins, native DNA, or possible degradation of the DNA by nucleases. Although it is hard to prove the orientation of the aptamer on the surface of the AgFON, there was a significant chance that it would not be possible to directly detect SERS of the protein target due to the exponential decay of the electromagnetic fields with distance from the noble metal. Figure 2 shows typical SERS spectra of aptamer on AgFONs (AgFON-apt), aptamer on AgFON in blood, and aptamer-modified AgFON exposed to ricin B chain (AgFON-apt-RBC) in blood. After AgFON-apt was exposed to human whole blood, the aptamer SERS spectral peaks broadened, and the signal-to-noise ratio decreased. The broadening may be attributable to a hypoxanthine scattering contribution near the observed 735 cm$^{-1}$ shift peak or nonspecific adsorption of albumin to the surface of the AgFON.$^{27,56}$ The decreased signal-to-noise ratio may be caused by native blood proteins nonspecifically adsorbing to the AgFON-aptamer conjugate, contributing either Raman scatter or background fluorescence. After exposure to 1 $\mu$g/mL RBC, new spectral features were apparent at 640 cm$^{-1}$ shift and 937 cm$^{-1}$ shift; these peaks may be due to either Raman scattering from the protein itself or possible conformational change of the aptamer after a binding event. The nature of the binding of aptamers to ricin has been explored in other publications, and it has been shown that these aptamers may bind to various sites on ricin.$^{2,3}$ Identifying the exact binding interaction of this aptamer is difficult and not critical to achieve the purpose of this study. In fact, the spectral changes of the aptamer-RBC interaction remain consistent over many measured spectra across multiple AgFON substrates, making these spectral changes potential predictors of RBC presence. As a secondary method, self-assembled monolayers and desorption-ionization (SAMDI) mass spectrometric measurements were performed on these substrates.$^{67}$ As can be seen in Figure S5, the measured $m/z$ ratios correspond well with the known mass of RBC. While this initial study proved promising, our overarching goal was to be able to detect RBC in whole human blood over multiple days without any decline in performance of the AgFONs or the aptamer affinity agent.

The aptamer-RBC binding experiments were repeated with AgFON-apt incubated in human whole blood for either 3 or 10 days followed by overnight RBC exposure (Figures 3 and S6); the goal of this work was to explore the stability of the AgFON-aptamer assembly during constant exposure to whole human blood. Principle component analysis (PCA) was applied to help differentiate small changes in the spectral patterns in these complex environments. Figure 3 shows the SERS spectra and PCA of the AgFON-apt, AgFON-apt after incubation in blood for 3 days, and AgFON-apt incubated in blood for 3 days then exposed to RBC (AgFON-apt-RBC). As can be seen in the spectra, the peaks that are normally very prominent for the aptamer after shorter blood exposure times are now shoulders of peaks due to scattering from blood components, and the spectrum changed significantly after exposure to RBC. The PCA in Figure 3 exhibits clear separation of the unexposed AgFON-apt, AgFON-apt after exposure to blood, and AgFON-apt-RBC. More importantly, there is no overlap between different spectral classes in the PCA. In future experiments, this may be applied to a training data set to identify the presence of RBC among unknown protein analytes. While the typical experimental protocol was to measure at seven different locations on each AgFON, the silver surface partially delaminated during 10 day blood exposure experiments, leaving only a few active areas on the substrate. During the RBC exposure, the AgFON further delaminated, leaving only three locations to be measured. However, the three different classes of experiments cluster separately following PCA analysis, indicating that, of the surfaces that remained intact, it was still possible to distinguish between the nuclease-resistant aptamer in blood and the nuclease-resistant aptamer exposed to RBC. Future work in substrate design will focus on increasing the mechanical robustness of these plasmonic surfaces while maintaining the enhancement factor and the ability to modify the structure with desired affinity agents.

Specificity is critical to the design of effective sensors: false positives may cause the dismissal of true positives. To evaluate
the specificity of the AgFON-apt for RBC, control experiments with BSA (bovine serum albumin) as a nonspecific binding agent were conducted. BSA was chosen to evaluate specificity because of the relatively high concentration of albumin in blood (3.5–5.0 g/dL) and its similarity in size to intact ricin.\textsuperscript{13,68}

AgFON-apt substrates were exposed to BSA, and acquired SERS spectra were compared to non-BSA exposed AgFON-apt substrates (Figure S7). PCA results in Figure S8 show the cluster due to AgFON-apt-BSA spectra overlap with those of AgFON-apt in blood. Moreover, the spectra of different concentrations of AgFON-apt-BSA clustered together, indicating that the AgFON-apt does not have affinity for BSA. In addition, the same substrates were exposed competitively to RBC, and the SERS spectra of those were compared to the AgFON-apt-BSA via PCA in Figure 4; the principal components due to the AgFON-apt-BSA clustered separately from the AgFON-apt-RBC. While there was some overlap between the AgFON-apt-BSA and the AgFON-apt, all of the AgFON-apt-RBC spectra were clearly separated from those of AgFON-apt. In addition, all the spectra for AgFON-apt exposed to high concentrations (10–40 \( \mu \)g/mL) of BSA were clustered together, likely due to saturation of the capture layer.

Ideally, the interaction between the aptamer and target analyte would be reversible so that the sensing platform could be reused. Ricin is known to be highly resistant to both heat and pH,\textsuperscript{16,69,70} and there are limited publications on the
inactivation of ricin.\textsuperscript{71–73} In an attempt to achieve reversibility, the interaction between the aptamer and RBC was studied under various conditions, including varied temperature, pH and ionic strength. For brevity, only the influence of elevated temperature on the sensor performance is discussed in detail; pH and ionic strength data are included in the SI.

Prior to direct study of the stability of the association between the protein and the aptamer, it was important to first characterize the physical conditions that might induce dissociation of the aptamer from the AgFON surface or cause damage to the AgFON itself. Again, benzenethiol was employed as a model molecule to study the stability of the thiolate-silver interaction as well as the stability of the AgFONs themselves.

Herdt et al. reported degradation of DNA–Au conjugation at 70 °C within hours.\textsuperscript{54,69} Therefore, to study the stability of S–Ag interaction at elevated temperature, the temperature range considered herein is 70–95 °C, where 95 °C is the temperature typically used to fully unfold the aptamer (RBC aptamer is known to maintain its conformation in the temperature range of 4–63 °C).\textsuperscript{22} The SERS intensity of BT reduced with higher temperature treatment (Figure S9). While these signal intensity losses may, in fact, be due to dissociation of the thiolate–Ag interaction, there are other potential contributing factors to keep in mind. First, silver is a highly oxidizable metal that can also undergo annealing processes and is known to be more vulnerable in nanoscale than bulk form.\textsuperscript{71} Second, Raman scattering efficiency is highly dependent on the molecular orientation of the scatterer on the SERS substrate; it is possible that the temperature elevation, even for short time periods, causes a reorientation of the benzenethiol, rendering it a less efficient scatterer. Thus, we assume that all three factors, dissociation of thiolate–Ag conjugate, damage to the AgFON surface, and reorientation of molecular species, contribute to the SERS signal decline. Figure 5 shows the SEM images of the AgFONs before and after the thermal treatment at 95 °C. The sharp features on the original AgFON anneal, decreasing the roughness of the surface and leading to a lower SERS enhancement. The visible black spots on the SEM image in the post-thermal treatment AgFON indicate areas devoid of Ag coverage. Based on these experiments, it is clear that high temperatures and long temperature excursion times will compromise the sensing system irreversibly.

Despite the apparent substrate damage, thermal treatment experiments were also performed on the RBC-exposed aptamer-functionalized AgFONs. The SERS signal from the aptamer at 731 cm\textsuperscript{-1} shift decreased 31.6% after thermal treatment at 80 °C, significantly (p value = 0.03) more than the benzenethiol signal decline under the same conditions (SERS signal of benzenethiol at 1071 cm\textsuperscript{-1} decreased 13.5% after thermal treatment at 80 °C), indicating that the conformational change of RBC aptamer caused by the thermal treatment contributes to the decline of the SERS signal. Even though there is a clear decline in the SERS signal intensity following thermal treatment, the 1274 cm\textsuperscript{-1} shift peak that indicates the presence of RBC was still measurable (Figure 6). Slight modifications of the detailed thermal treatment have been performed, including heating under N\textsubscript{2} gas and heating at a lower, physiologically relevant temperature for an extended time period (37 °C for 24 h). In all these variations, the 1274 cm\textsuperscript{-1} shift peak remains in the SERS spectrum.

Ionic strength and pH are known to influence the conformation of DNA, thus, variation in these conditions present another potential avenue for intentionally destabilizing the aptamer/target interaction. However, SERS spectra of the post-treatment AgFONs revealed no obvious changes when compared to the pretreatment conditions (Figure S10), indicating that the association of aptamer and RBC was intact during and after the treatments. In summary, 95 °C, 1 M NaCl, and pH 5–8 were not able to induce the dissociation of RBC from the capture aptamer without also irreversibly damaging the plasmonic substrate (Figure S11). If a reversible sensing platform is needed, either more robust substrates or more specific target denaturation strategies must be employed.

\section*{CONCLUSIONS}

The detection of ricin B chain (RBC) in human whole blood was performed on aptamer-functionalized AgFONs (AgFON-apt) via SERS. The AgFON-aptamer complex was shown to be stable in whole blood for 10 days and still able to detect RBC in blood. Principle component analysis (PCA) of the resultant SERS spectra shows a cluster of spectra from the AgFONs exposed to RBC clearly separated from the ones without RBC exposure as well as those exposed to other proteins, giving a straightforward yes/no answer regarding ricin exposure. Although our current detection limit (1 \mu g/mL) is higher than the LD50 upon injection into humans, with high enhancing plasmonic substrates (e.g., SERS enhancement factors of 10\textsuperscript{7})\textsuperscript{74} and/or an optimized aptamer with shorter overall length, the RBC limit of detection should improve significantly. In addition, this work makes it clear that the aptamer/RBC conjugate is stable even at elevated temperature as well as biologically relevant pH and ionic strengths; thus, the AgFON-apt could be used as a platform both to detect and effectively remove ricin from contaminated blood.
Ricin B chain aptamer structure, stability of AgFON-BT in blood for 3 or 10 days, computational modeling of aptamers with respect to the distance dependence of surface-enhanced Raman spectroscopy, characterization of ricin B chain on aptamer-modified AgFONs by SAMDI, stability of AgFON-apt in blood for 10 days and its ability to detect RBC after 10 day exposure to blood, nonspecific binding of BSA on ricin B chain aptamer-modified AgFONs, and effects of temperature, pH, and ionic strength on AgFONs with benzenethiol and RBC aptamer (PDF)

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