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Targeting Biological Sensing with Commercial SERS Substrates

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ARL-RP-0398

September 2012

A reprint from the *Proc. of SPIE*, Vol. 8366, 836602-1.

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A reprint from the *Proc. of SPIE*, Vol. 8366, 836602-1.

REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE (DD-MM-YYYY) September 2012		2. REPORT TYPE Reprint		3. DATES COVERED (From - To)	
4. TITLE AND SUBTITLE Targeting Biological Sensing with Commercial SERS Substrates				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Mikella E. Farrell, Srikanth Singamaneni, and Paul M. Pellegrino				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) U.S. Army Research Laboratory ATTN: RDRL-SEE-E 2800 Powder Mill Road Adelphi, MD 20783-1197				8. PERFORMING ORGANIZATION REPORT NUMBER ARL-RP-0398	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution unlimited.					
13. SUPPLEMENTARY NOTES A reprint from the <i>Proc. of SPIE</i> , Vol. 8366, 836602-1.					
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15. SUBJECT TERMS SERS, sensor, chemical, biological, Raman, detection, peptide, biomimetic					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 18	19a. NAME OF RESPONSIBLE PERSON Mikella Farrell
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (Include area code) (301) 394-0948

Targeting Biological Sensing with commercial SERS substrates

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SUMMARY

There is an increasing need for rapid and accurate detection, identification, and quantification of chemical, biological, and energetic hazards in many fields of interest. To meet these challenges, researchers are combining spectroscopy with nanoscale platforms to create technologies that offer viable and novel solutions for today's sensing needs. One technology that has gained increasing popularity to meet these needs is surface enhanced Raman scattering (SERS). For ideal SERS sensing, commercially available uniform and reproducible nanoscale surface demonstrating high sensitivity are desirable. If these surfaces can be modified for the selective sensing of hazard materials, an ideal sensor platform for dynamic in field measurements can be imagined. In this proceedings paper, preliminary efforts towards the characterization and application of commercially available next generation Klarite substrates will be demonstrated and efforts towards selective sensing will be discussed.

ABSTRACT

There is an increasing need and challenge for early rapid and accurate detection, identification, and quantification of chemical, biological, and energetic hazards in many fields of interest (e.g., medical, environmental, industrial, and defense applications). Increasingly to meet these challenges, researchers are turning interdisciplinary approaches combining spectroscopy with nanoscale platforms to create technologies that offer viable and novel solutions for today's sensing needs. One technology that has gained increasing popularity to meet these needs is surface enhanced Raman scattering (SERS). SERS is particularly advantageous as it does not suffer from interferences from water, requires little to no sample preparation is robust and can be used in numerous environments, is relatively insensitive to the wavelength of excitation employed and produces a narrow-band spectral signature unique to the molecular vibrations of the analyte.

SERS enhancements (chemical and electromagnetic) are typically observed on metalized nanoscale roughened surfaces. For ideal SERS sensing, commercially available uniform and reproducible nanoscale surface demonstrating high sensitivity are desirable. Additionally, if these surfaces can be modified for the selective sensing of hazard materials, an ideal sensor platform for dynamic in field measurements can be imagined. In this proceedings paper, preliminary efforts towards the characterization and application of commercially available next generation Klarite substrates will be demonstrated. Additionally, efforts toward chemical modification of these substrates, through peptide recognition elements can be used for the targeting sensing of hazardous materials will be explored.

KEYWORDS: SERS, sensor, chemical, biological, Raman, detection, peptide, biomimetic

I. INTRODUCTION

There are many unique and exquisitely organized solutions for sensitive and selective sensing in biological systems. This innate biological ability to guide and assemble nanoscale components into precisely controlled and sophisticated structures from very basic components has motivated intense defense, industrial and academic research efforts to develop and harness these directed artificial methods that mimic or exploit the selective recognition capabilities and sensitive interactions inherently found in biological systems. Many of these approaches have resulted in the successful

merging of more traditional sensing techniques (Fluorescence⁽¹⁾, Raman⁽²⁾, Raman-based spectroscopic techniques⁽³⁻⁹⁾) in novel nano-scale synthetic platforms combined with biomolecular recognition capabilities, thus demonstrating viable and transformative solutions for today's sensing needs. In this proceedings paper, we discuss the development of a novel selective and sensitive sensor platform.

One of the main challenges in the design and fabrication of a sensor platform is analyte selectivity and sensitivity. A high degree of sensor selectivity can be achieved by using biological recognition elements (e.g. antibody), and thus fabricating a⁽¹⁰⁻¹⁴⁾ sensor. Biomimetic sensing entails the adaptation of biological principles, designs, selective sensitive materials and signal processing schemes merged with artificial sensors. One type of biomimetic sensing recognition element demonstrating increased utility employs peptides as the biological recognition element.⁽¹⁵⁻¹⁹⁾ A peptide is a short (less than 50) chain of amino acids. Generally, in sensing platforms peptides are advantageous to use as they 1) are stable and robust in a myriad of environments, 2) can be easily synthesized, 3) are cost and time efficient, 4) can be easily modified to recognize a target, 5) are well characterized and 6) have been shown to easily immobilized to various surface platform materials (metals, plastics, fabrics, tissue sample). The ability to immobilize peptides to various platforms and targets has resulted in an upsurge in the development of⁽²⁰⁻²⁴⁾ peptides constructed from a fusion of functional peptides. There are many examples in the literature of fusion peptides being used in biomimetic sensors in many areas of research (defense, medical, hazard detection, and environmental studies).

Fusion peptides have been identified following many different experimental methods. Historically, methods for peptide selection include identification from natural resources and most commonly using combinatorial peptide-phage display libraries selected via a technique called biopanning. The power of the phage display library is in its ability to efficiently and rapidly identify ligands with a desired target property from a large population of colonies displaying highly diverse surface peptides. Biopanning can generally be described as an affinity selection technique for choosing a peptide that can bind to a given target (demonstrating desired properties). The biopanning process typically involves four main steps: preparation of the phage displayed peptide libraries, capture of specific phage that binds to the target, washing of low affinity or non specific phages from the cell surface, and then finally recovery through elution of the enriched target binders for the next round of selection.⁽²⁵⁻²⁸⁾ Using these types of selection techniques, it has been possible to identify and select several different material specific binding peptide sequences (examples include metal compounds, semiconductor materials, minerals, and polymers)⁽²³⁾ as well as particular targets (chemical⁽²⁹⁻³²⁾, biological^(16, 33, 34), or energetic hazards⁽³⁵⁻³⁹⁾). The screening and selection of such phage displayed target binding peptides has attracted particular interest in the research areas of nanotechnology and sensor design. Thus the power of these high affinity target peptide binders are increasingly being utilized as the recognition element in new sensor platform designs.

With a new strategy for sensor design, there is a renewed effort to address the outstanding need and challenge for rapid, sensitive, accurate detection, identification, and quantification of chemical, biological, and energetic hazards in many fields of interest (e.g., medical, environmental, industrial, and defense applications).⁽⁶⁾ One spectroscopic detection method that is gaining significant popularity for meeting these sensitivity needs is surface enhanced Raman scattering (SERS). SERS-based techniques and platforms combine traditional spectroscopy with nanotechnology. SERS is particularly advantageous and an appropriate detection technique to utilize in biomimetic sensors as it does not suffer from interferences from water, requires little to no sample preparation, is robust and can be used in numerous environments, is relatively insensitive to the wavelength of excitation employed and produces a narrow-band spectral signature unique to the molecular vibrations of the analyte.

Since the discovery of SERS in the 1970's, it has been experimentally shown that the SERS signal enhancements are typically observed on metalized (typically silver or gold) nanoscale roughened surfaces.⁽⁴⁰⁻⁴²⁾ Although several open debates regarding the exact magnitude of contributions are still ongoing, the basis of the SERS mechanism is fairly well understood. In the SERS literature, the two mechanisms that control the enhancement of the Raman scattering (SERS) are considered to be the electromagnetic fields generated at or near nanoparticle surfaces and the physical (chemical) adsorption of a target analyte onto a surface.⁽⁴³⁻⁴⁶⁾ The electromagnetic enhancement (EM) is typically deemed to be the stronger contribution, with an enhancement factor (as compared to spontaneous Raman) ranging from approximately 10^4 to 10^{14} ,⁽⁴⁷⁾ while the chemical enhancement (CE) has been suggested to contribute at most 10^2 .⁽⁴⁸⁾ Because of the many sensing advantages of SERS-based techniques, significant research efforts (defense, industrial and academic) have been directed toward fabricating "better" SERS substrates for SERS-based sensor platforms.⁽⁴⁸⁻⁵⁷⁾ Some of these SERS platforms are fabricated from colloids⁽⁵⁸⁾, film over nanospheres⁽⁵⁹⁻⁶²⁾, fiber optic bundles⁽³⁾, nanoparticles,^(55, 63-66) and

lithographically⁽⁵⁾ produced structures. The more sensitive substrate platforms generally have a 15% relative standard deviation (RSD; the measure of the reproducibility of an analysis) from substrate-to-substrate and SERS signal enhancements of 7 to 8 orders of magnitude.^(60, 61) Consequently, many researchers in academia, industry, and government have focused concerted efforts toward increasing the signal enhancement ability, reproducibility, and mass production manufacturing of substrates to increase the utility of this technique.^(5, 67) For the Army and first responders, such a substrate platform with increased sensitivity and reliability would be very advantageous for the detection and identification of unknowns.^(68, 69)

Significant research efforts have also been concentrated on better directing the optimization of the substrate surface from which the SERS enhancement occurs.^(5, 70-73) Based in part on experimental and as well as theoretical efforts, the directed fabrication of SERS platforms has focused on modifying the feature size⁽⁷⁴⁻⁷⁶⁾, spacing between objects, geometry and shape of structures⁽⁷⁷⁻⁷⁹⁾, identity and incorporation of metals on the surface⁽⁸⁰⁾, feature height, and the character of the foundation layer^(59, 81-83) on which the architecture is fabricated.^(69, 84, 85) There are numerous examples in the literature detailing how variation in some of these parameters in some cases can result in very dramatic changes to the overall SERS enhancing capabilities of the substrate surface. Rigorous efforts continue to focus on developing an understanding about how these parameters can synergistically work together to result in a highly reproducible and sensitive SERS substrate. As focus continues on improving the overall sensing capabilities of the SERS surface, congruently research continues to push towards the development of a uniform reproducible mass produced platform necessary to facilitate widespread incorporation of SERS in viable biomimetic sensing platforms.

Some success fabricating both spectrally and physically reproducible SERS substrates has been demonstrated with commercially available standard Klarite™ substrates (Renishaw Diagnostics).^(4, 71, 86-88) These substrates were developed using Si-based semiconductor fabrication techniques.⁽⁴⁾ Klarite substrates are fabricated using a well defined silicon fabrication technique in which a silicon diode mask is defined by optical lithography, and then potassium hydroxide (KOH) surface etched. The process results in an array of highly reproducible inverted pyramid features.⁽⁴⁾ These array pyramids are reported to have “hot spots” or “trapped plasmons” located inside the wells.⁽⁴⁾ These substrates have been previously characterized.⁽⁸⁹⁾ Atomic force microscopy (AFM) images have demonstrated that the inverted pyramids features are about 1.47 μm in width and 1 μm in depth. These substrates have plasmon absorbance bands located at 577 nm and 749 nm, thus demonstrating the usefulness of this substrate for a range of excitation sources. Additionally, due to the fabrication process used, under ideal conditions these substrates have demonstrated typical RSDs ranging from 10-15% under drop and dry conditions. While these standard Klarite substrates do demonstrate a high degree of substrate reproducibility and very low substrate background (SERS signal and surface morphology), for many applications to real-world situations increased analyte sensitivity is still necessary. Recently new prototype Klarite based substrates have been fabricated by Renishaw Diagnostics with the intent to expand substrate sensing capabilities. The morphologies of these substrates dramatically differ in overall shape, pitch and spacing as compared to the standard Klarite substrate resulting in very interesting sensing capabilities.^(90, 91)

In this proceedings paper, preliminary efforts functionalizing commercially available SERS substrates for directed biomimetic hazard sensing will be discussed and demonstrated. These chemical modification methods are achieved using fusion peptide recognition elements for the targeting sensing of hazardous materials such as the energetic sample TNT. By demonstrating these preliminary results, the potential of these SERS substrates for future incorporation in fielded Raman systems for the selective sensing of hazard materials can be imagined.

II. EXPERIMENTAL

Peptide Samples.

Potential peptide recognition elements were selected following a comprehensive literature search. Peptide materials were commercially purchased from RS Synthesis (Louisville, KY 40270), Genscript (Piscataway, NJ 08854) or Peptide 2.0 (Chantilly, VA 20153) and used without additional modification (> 85% purity). Fusion peptides were created with a glycine-glycine-glycine (GGG) spacer between the material binder and the target recognition element. The material binder, target recognition sequence, and naming systems used are listed in Table 1. Upon receipt, all samples were stored at -20°C or below and kept dry. Utilizing the GenScript Peptide Property Calculator, peptides were determined to have a neutral charge, and were suspended in a water only solution. Concentrations of peptide used are listed in the text.

After suspension in water solution, peptide solutions were stored refrigerated while being used for a maximum of one week, and then fresh solutions were made.

Table 1. Example potential peptide recognition elements with material target, sequences investigated, and naming systems listed.

Material of Interest	Peptide Sequence	Literature Reference	Name
Gold Binder	VSGSSPDS	Science, 2006, 312, 885-888	
Gold Binder	LKAHLPPSRLLPS	Science, 2006, 312, 885-888	
Gold Binder	TGTSVLIATPYV	Acta Biomater., 2010, 6, 2681-2689	
Gold Binder	WAGAKRILVLRRE	J. of Coll. and Interf. Sci., 2012, 365, 97-102	
Gold Binder	AYSSGAPPMPFF	Nat. Mater. 2002, 22, 201-204	A3 Binding domain
Gold Binder	AYSSGAPPMPFF	Small 2005, 1, No. 1, 1048-1052	A3 Binding domain
Gold Binder	AYSSGAPPMPFF	Adv. Mater, 2006, 18, 1988-1992	A3 Binding domain
Gold Binder	VSGSSPDS	Nanolett., 2005, 15 (7), 1429-1434	Au bind
Gold Binder	CGPWALRRSIRRQSY GPC	Langmuir, 2008, 24 (21), 12440-12445	cAuBind2
TNT Binder	WFVI	J. of Neurosci., 1999, 19 (17), 4768-7475	ASP1
TNT Binder	WHRTPSTLWGVI	Analytica Chim, 2002, 457, 13-19	
TNT Binder	WHRTPSTLWFVI	ARL Stock	
TNT Binder	WHWQRPLMPVSI	Langmuir, 2008, 24 (9), 4938-4943	TNT-BP
DNT Binder	HPNFSKYLHQR	Langmuir, 2008, 24 (9), 4938-4943	DNT-BP
PA Binder	STLKILPCTFCIWPS	ARL stock	YZ72
PA Binder	VYNGPRYVCTFCTWN	ARL stock	YZ91
PA Binder	VSHILCTFCLWSIP	ARL stock	YZ128

Energetic Materials

Certified energetics material TNT was purchased from Cerrilant. Samples were received in an acetonitrile solution, and were resuspended at a required concentration in a water solution for biological applications.

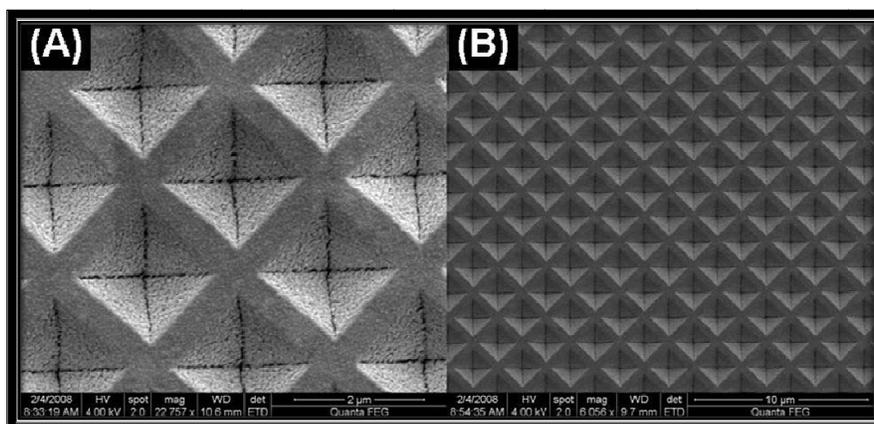


Figure 1. Example SEM images of commercial SERS substrate shown at different magnifications (A) and (B).

Commercially available SERS substrates.

Commercially available slide mounted standard Klarite™ 302 SERS substrates were purchased from Renishaw Diagnostics, see Figure 1 for an example SEM image of a typical substrate. Slides were individually wrapped and vacuum sealed. The SERS active area on these slides is a small 4 mm x 4 mm wafer with a gold surface. The standard Klarite slides were only used once and opened just prior to measurement to reduce any possible surface fouling. Additionally, the substrate was submerged in ethanol to remove any possible contamination that may have accumulated on the surface. Typically, in SERS data collection on the standard substrates, five measurements across the substrate surface were collected. Most data in this proceedings paper will be presented as an average of a collected data set and the standard deviation error shown, unless otherwise indicated.

Instrumentation and Data Analysis.

Scanning electron microscope (SEM) images was obtained using a FEI environmental SEM (Quanta 200 FEG).

A Renishaw inVia Reflex Raman microscope was used for SERS and Raman spectra collection. Spectra were collected using the NIR 785 nm laser. The laser light was focused onto the sample using a 20X objective, over a range of $100\text{--}3200\text{ cm}^{-1}$, exposures were 10 seconds in length, and 3 accumulations were collected per spot. Approximately 7 mW of power irradiated the surface of the substrate. A total of 10 spectra were collected from each spot on the substrate. Samples were positioned using a motorized XYZ translational stage internal to the microscope. Spectra were collected, and the instrument was run using Wire 3.2 software operating on a dedicated computer.

Data analysis was achieved using IgorPro 6.0 software (Wavemetrics).⁽⁹⁰⁾

III. RESULTS AND DISCUSSION

Gold binding capabilities of peptide

To assess the utility of the fusion peptides, it is critical to first evaluate the gold binding capabilities of the peptide recognition elements. To do this, the dry peptide was resuspended in a water and a solution of 0.8mg/mL of peptide was made. The solution was then vortexed for 2 minutes to assure complete mixing, and then a 4 μ L aliquot of analyte solution was deposited both on the active and non active areas of the Klarite substrate. The sample was then allowed to incubate covered in the refrigerator for 20 minutes to assure ample time for binding. The SERS substrate surface was then repeatably washed with a 3mL aliquot of water 4 times to remove any incompletely bound peptide, and then suspended in a water solution to prevent the peptide from drying. Using the Renishaw InVia microscope system both the nonactive and active areas of the SERS substrate were interrogated.

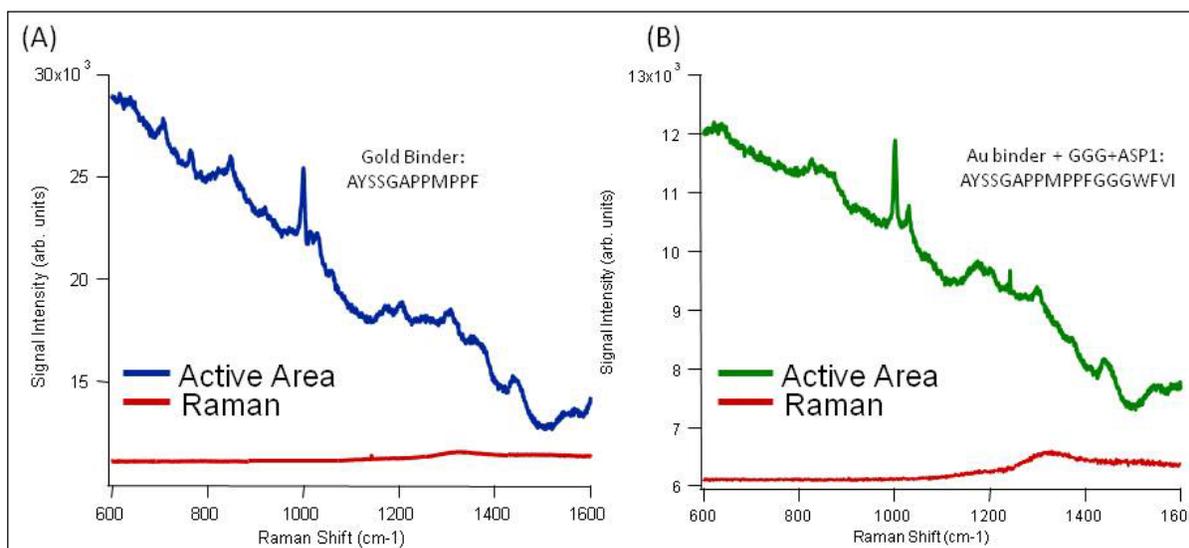


Figure 2. Example spectra from Au binder (A) and Au binder + TNT binder (B). Spectra are shown on active and non active areas of SERS substrate and after multiple washes to remove any unbound sample.

An example of a gold binding peptide commonly encountered in the literature has the sequence AYSSGAPMPPF. Since this peptide has been well characterized and demonstrated to bind effectively to gold, we wanted to complete a proof of principle study to assure binding and detection of a SERS. In Figure 2A, the SERS and Raman response are shown. In Figure 2A, SERS bands are located at 641 cm^{-1} , 721 cm^{-1} , 1001.8 cm^{-1} , 1031 cm^{-1} , 1204 cm^{-1} and 1443 cm^{-1} . For analysis, the 1001.8 cm^{-1} band was analyzed to determine a signal-to-noise (SNR) ratio. For Figure 2A, the SERS SNR is 30.54 and the Raman SNR is 0. Thus indicating that the peptide has successfully bound to the substrate surface

and does exhibit a distinguishable SERS spectrum. Repeating these studies, it was possible to detect the Au binder on the surface after multiple washings deposited at a concentration as low as millimolar levels.

Recognition elements- TNT binder

For the past decade, significant defense, industry and academic resources and research efforts have focused on the detection and recognition of hazardous materials. Efforts have been centered on such tasks as securing public locations and transportation, maintaining the integrity of checkpoint stations at military sites, and assuring public safety in food chain supplies and the environment. In many of these scenarios, the hazardous material of interest can be energetic in nature. In particular, the detection of high explosives such as 2,4,6-trinitrotoluene (TNT) and their byproducts like dinitrotoluene (DNT) have garnered significant attention because of its proliferations mainly due to availability and ease of use. Increasingly to detect and identify this threat, research agencies are focusing on new innovative bio-inspired technologies for possible solutions.

There are several examples of bio-inspired technologies being used for the detection of explosive materials. In the past decade there have been several example publications that have discussed using various peptides as a capture mechanism for TNT and TNT byproducts. Some examples in the literature that have been highlighted, include an article published in 1999 by Danty et.al.⁽⁹²⁾, in which a binding protein found in an olfactory mechanism of the honeybee was determined to be specific for TNT. The olfactory peptide sequence is WVFI. In an article published in 2002 by Goldman et. al.⁽⁹³⁾, phage displayed peptides were selected for the detection of TNT. From this paper, the sequence for TNT peptide detection was determined to be WHRTPSTLWGVI. In a 2008 article by Jaworski et al.⁽⁹⁴⁾, evolutionary screening of peptides for the selective detection of explosives was conducted. From this screening process, the following peptide sequences specific for TNT and DNT selectivity were determined, WHWQRLMPVSI and HPNFYSKYLHQR.

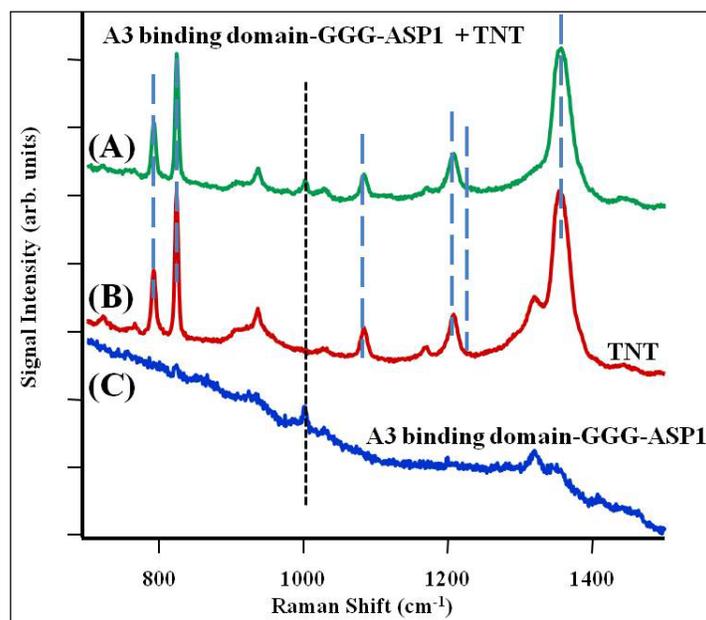


Figure3. Example spectra from (A) A3 binding domain +GGG- ASP1 + TNT, (B) TNT, and (C) A3binding domain –GGG-ASP1 (no TNT). Note several common bands found in (A) are clearly in (B) and (C).

To determine the effectiveness of these peptides for capturing TNT on the commercial SERS substrate, peptide sequences consisting of a gold binding motif, a spacer sequence and the previously mentioned TNT binding peptides were prepared. In this proceedings paper, we will present results from one combination of this fusion peptide. These fusion peptides were immobilized on the Klarite substrate surface and washed several times to insure monolayer coverage. The peptide containing SERS substrate was then exposed to a high concentration (3.67×10^{-2} M) TNT in a water/alcohol/some residual acetonitrile solution. The TNT solution was in a mostly water solution to prevent any

potential damage to the peptide sequence from acetonitrile. A high concentration of TNT was originally selected for these proof-of-principle demonstrations. The TNT solution sat on the SERS substrate surface for 20 minutes to ensure complete binding between the peptide and TNT. The surface was next washed with 2 mL of deionized water to remove any excess and unbound TNT from the surface. See Figure 3 for example SERS spectra from the peptides bound to the surface, and with the addition of TNT. In Figure 3, (C) has the characteristic peptide band located at 1001.8cm^{-1} , while (B) TNT exhibits SERS bands located at 793 cm^{-1} , 823 cm^{-1} , and 1356 cm^{-1} . In Figure 3A, the A3 binding domain-GGG-ASP1 sequence with TNT added demonstrates SERS bands located at 793 cm^{-1} , 823 cm^{-1} , 1001.8cm^{-1} and 1356 cm^{-1} , thus demonstrating that the peptide remained bound and captured TNT onto the sensing surface.

After a demonstration of preliminary sensing with the targeted peptide sequence, it was necessary to determine if the gold binding motif was necessary for immobilizing the peptide on the surface. For these experiments, the Au binding capabilities of the A3 binding domain as compared to a non material specific binding domain were assessed. Peptides of the sequence A3 binding domain-GGG-ASP1 and Graphene binding -GGG-ASP1 were immobilized onto different Klarite substrates overnight. After immobilization, the surface was washed with 2 mL of water, and 5ul of TNT was added to the surface for 1 hour. Following TNT exposure, the surface was washed with 2 mL of water and SERS measurements were collected. In Figure 4, examples of results collected are shown. In Figure 4A, bands common to TNT are clearly shown. In Figure 4B, there are some minor TNT bands shown, however the overall signal analyzing the TNT 1356 cm^{-1} band is determined to be almost 20X greater. From this Figure, it can be clearly concluded that the A3 binding domain is necessary for peptide immobilization onto the Klarite surface.

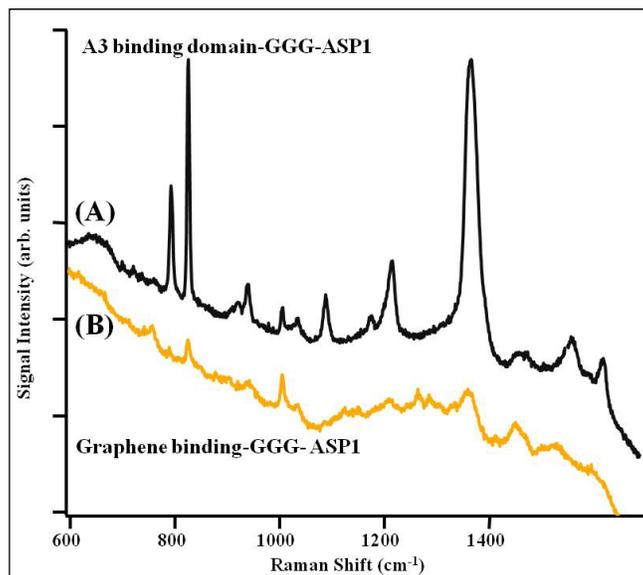


Figure 4. Example spectra TNT exposed to (A) A3 binding domain +GGG- ASP1 and the (B) Graphene binder-GGG-ASP1. From these results it can be concluded that A3 binding domain is necessary for immobilizing the peptide onto the gold Klarite surface.

After preliminary efforts to bind the A3-GGG-ASP1 binding motif to the Klarite surface and detect TNT were demonstrated, and it was conclusively shown that the A3 binding domain aids in peptide immobilization the specificity of the peptide for TNT detection was preliminarily assessed. A proof-of-principle experiment was conducted to determine if the peptide binder caused preferential binding of TNT to the surface as compared to non-specific binding. For these experiments, the peptides A3 binding domain-GGG-ASP1 (TNT binder) and A3 binding domain-GGG-Graphene binder (non specific to TNT) were immobilized on the Klarite substrate surface overnight, and washed with 2 mL of water to remove any unbound peptide. The SERS surface was then exposed to 5 uL of TNT for one hour. Following TNT exposure, the surface was washed with 2 mL of water and SERS measurements were collected. In Figure 5, the results for this binding study are shown. In Figure 5A the A3 binding domain-GGG-ASP1 peptide exposed to TNT is shown. In this figure the peaks observed are located at 793 cm^{-1} , 823 cm^{-1} , 1001.8cm^{-1} and 1356 cm^{-1} , thus demonstrating that the peptide remained bound and captured TNT onto the sensing surface. In Figure 5B, an example spectrum from the non specific peptide exposed to TNT is shown. In this figure, it can be clearly seen that the peptide

band early discussed at 1001.8cm^{-1} is clearly shown, however no SERS bands for TNT are observed. Thus it can be seen that the ASP1 binding domain is necessary for TNT binding to the peptide and being detected.

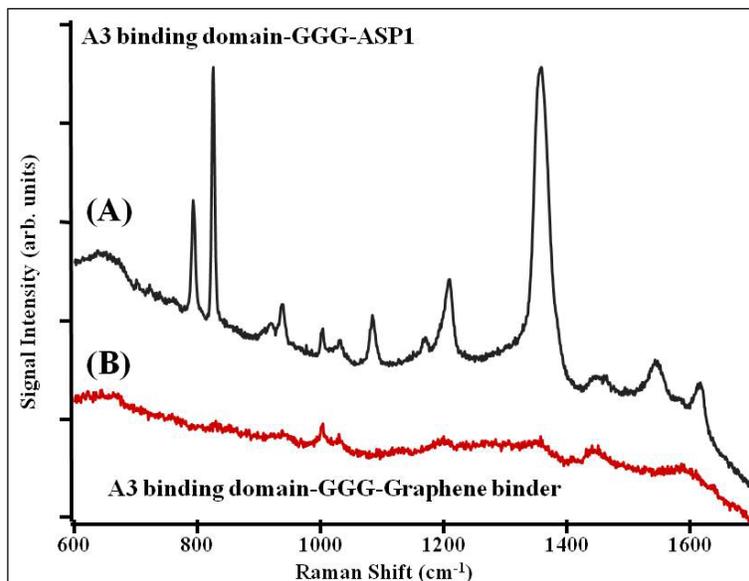


Figure 5. Example spectra TNT exposed to (A) A3 binding domain +GGG- ASP1 and the (B) A3binding domain –GGG-Graphene binder. From these results it can be concluded that TNT is preferentially bound to the ASP1 containing peptide.

IV. CONCLUSIONS

In conclusion, we have shown a preliminary proof-of-principle demonstration that using specially targeted fusion peptides it is possible to selectively bind hazardous materials (like the energetic TNT) to a SERS Klarite sensing surface. Additional studies need to be conducted to determine the optimized conditions needed for higher sensitivity detection of energetic and other Army relevant hazardous materials. We also plan to transition these biological sensing schematics onto next generation Klarite sensing.

ACKNOWLEDGEMENTS

Thank you to team members Dimitra Stratis-Cullum, Josh Kogot and Deborah Sarkes for initial assistance with peptide experiments.

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