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Raman Based Hepatocellular Carcinoma Biomarker Detection

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Abstract— **Highly sensitive and selective biomarker detection is required for early detection of hepatocellular carcinoma (HCC). Disease progression has been shown to correlate with specific fucosylation of a validated HCC serum glycoprotein biomarker, alpha-fetoprotein (AFP) Carbohydrate binding proteins, such as lectins, can be used as diagnostic indicators for monitoring glycosylation changes during disease progression in hepatitis B virus (HBV) or hepatitis C virus (HCV) infected patients. We prepared surface-enhanced Raman spectroscopy (SERS) substrates, which provide controllable, well-organized nanoparticles on the surface, for the analysis of a fucose binding lectin AAL. The SERS based assay provides fast (<10 s), and reproducible (<5% variation) detection.**

I. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide.[1, 2] It has been estimated that the number of cases of HCC will continue to increase by 81% by the year 2020, primarily due to the hepatitis C (HCV) epidemic.[2] A particularly disturbing feature has been the inability to substantially lower mortality rates in HCC, likely due to the fact that the majority of patients with HCC are diagnosed at advanced stages leading to an overall 1-year survival rate of 25% in the United States.[3] The poor prognosis for survival is related to the lack of sensitive assays for early diagnosis.

Biodetection methods for early biomarker diagnostics can be grouped into 3 major categories (i) electrochemical, (ii) mechanical, and (iii) optical. Raman spectroscopy is a non-destructive photon-scattering technique, which provides information regarding vibrational energy levels of analyte molecules. Conventional Raman spectroscopy is limited by the very low intensity of Raman scattering, which leads to low sensitivity, often requiring powerful excitation sources and long integration times for measurements. However, in its surface enhanced mode (i.e. SERS), this technique offers great limits of detection sensitivity and selectivity as an optical method.[4] Advances in nanotechnology have initiated an interest in integrating Raman spectroscopy with metallic nanoparticles (e.g. SERS based detection) in order to enhance the sensitivity of biomarker detection.[4]-[8] The ability to detect biomarkers with label-free (no fluorescent dye), minimal sample preparation efforts, low reagent costs, ease of operation, and rapid and reliable detection offer a substantial advantage of Raman technology.[9], [10] The vibration spectrum displays fingerprinting information of the chemical composition of the biomarker.[7] Additionally, when SERS is coupled with resonant-Raman, the detection limit becomes 1000 times better than conventional fluorescent assays due to the coupling of laser frequency with the electronic transition of the fluorescent dye.[11], [12] Thus SERS techniques can be applied to a broad variety of applications, which includes single molecule detection,[13] rapid DNA sequencing,[4] pathogen detection,[7], [8] and biomarker analysis.[14]

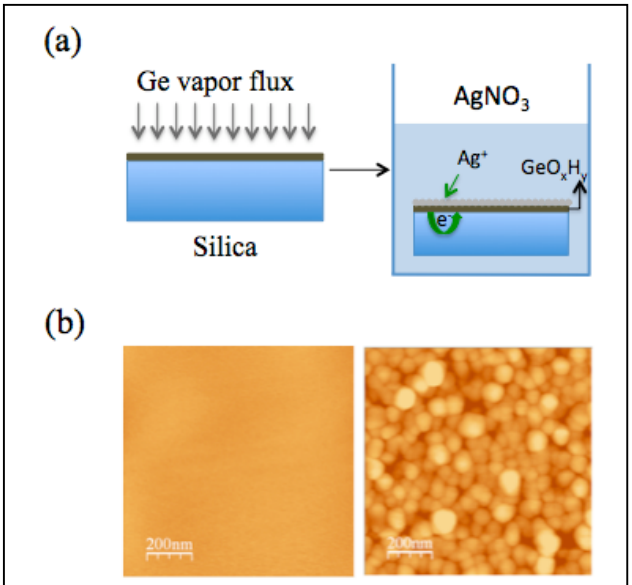

Figure 1. (a) Schematic depiction illustrating the electroless deposition of Ag^+ onto a Ge layer. (b) Left: Tapping mode atomic force micrograph (AFM) of Ge deposited on a quartz substrate via physical vapor deposition. Right: Tapping mode AFM micrograph of a Ge layer immersed in a 2 mM $AgNO_3$ solution during 30 s at room temperature.

While, human proteins could be measured by Raman spectroscopy, the signal is too weak to be measured over a short time interval. Surface enhanced Raman spectroscopy (SERS) provides enhanced detection at physiological concentration due to the adsorption of human proteins onto the surface of nanoparticles.[9], [10] In this study, we focused on detection of recombinant lectins

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(carbohydrate binding proteins) using SERS assays. We demonstrated the measurement of lectin with fucose via Raman spectroscopy on solid substrates. The recombinant lectin has higher affinity and specificity for biomarker glycoforms (i.e. fucose in our study) predictive of HCC and liver disease than the natural lectin products.

II. Experimental

A. Materials

Silver nitrate ($AgNO_3$) 99%, Germanium chips ≥99.9%, anhydrous ethanol ≥99.5% and L-fucose ≥99% were purchased from Sigma-Aldrich. Glass slides were purchased from Corning Glass Works.

B. AAL preparation

AAL is a mushroom derived lectin that preferentially binds to fucose linked (α -1,6) to N-acetylglucosamine but also binds to fucose linked (α -1,3, α-1,2, α-1,4) to N-acetyllactosamine related structures. Crystal structural data shows that AAL has 5 (genetically distinct) fucose binding sites with each site potentially having unique binding affinities for different fucopyranosyl linkages.[15] The lack of fucopyranosyl linkage specificity in native AAL is problematic for a specific, high affinity detection reagent. We have produced and purified a recombinant form of AAL, which has higher fucose binding affinity than the natural product. Briefly, we synthesized a DNA encoding the AAL gene sequence modified to include a C-terminal histidine tag followed by two cysteine residues. Using standard molecular biology cloning and expression techniques the sequence was expressed from a T7 inducible pET vector in a BL21 (D3) bacterial strain. Expression of rAAL was induced using 0.5 mM IPTG. Cells were harvested, lysed and the rAAL was purified using FPLC immobilized metal affinity chromatography (IMAC). Purity of rAAL was verified by SDS-PAGE analysis followed by coomassie staining.

C. Nanofilm preparation

Silica slides were coated with 5 nm of Ge via physical vapor deposition.[16] The thickness of the Ge sacrificial layer was monitored by a quartz crystal microbalance sensor (QCM) positioned perpendicular to the vapor flux. Then, Ag nanoparticles were deposited through galvanic displacement by immersing the Ge-coated slides in a 2mM $AgNO_3$ solution for 30s at room temperature. Subsequently the Ag/Ge films were rinsed with anhydrous ethanol to stop the galvanic displacement process and dried with a stream of N_2. A cartoon illustrating the galvanic displacement process is shown in figure 1(a).

D. SERS measurements

Detection studies were performed by adding 1 µL of 1µM AAL onto the Ag/Ge nanostructured films and SERS spectra was recorded 1 minute after addition. Also contrast studies were performed using L-fucose as a target sugar. Previously the signature spectra for the L-fucose sugar were recorded. Then, 10 µL of 100 µM of sugar in a phosphate buffer solution (PBS, pH 7.25) was mixed with 10µL of AAL and incubated at 3ºC under constant stirring during 3 h. SERS spectra were recorded using a Renishaw inVia micro-Raman instrument equipped with a HeNe laser source (633 nm), a motorized microscope stage, and a CCD detector. The acquisition parameters were 20 X objective 10 s acquisition time at 250 µW, and plotted using Renishaw software.

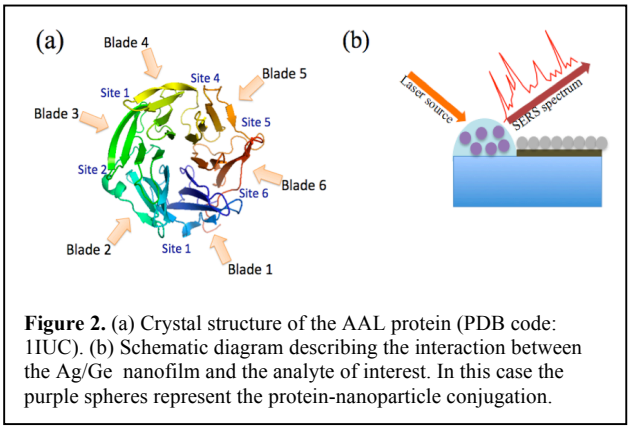

Figure 2. (a) Crystal structure of the AAL protein (PDB code: 1IUC). (b) Schematic diagram describing the interaction between the Ag/Ge nanofilm and the analyte of interest. In this case the purple spheres represent the protein-nanoparticle conjugation.

III. Results and Discussion

The scheme in figure 1A illustrates the galvanic displacement of Ag^+ onto the Ge surface.[13] Galvanic displacement is a type of electroless deposition in which a semiconductor surface (e.g. Ge) serves as an electron source for the reduction of a metal ion (e.g. Ag^+). Nanoparticles are formed as a consequence of aggregation during the reduction of the metal ion.[17] As depicted in figure 1(b), a smooth film of Ag nanoparticles with average particle size of ~40 nm is obtained upon immersion of a Ge-coated silica slide in $AgNO_3$.

Lectins are carbohydrate binding proteins that may be found on plants, animal, and fungi.[18] AAL is a fucose binding lectin (depicted in figure 2(a)) derived from the orange peel mushroom *Aleuria aurantia*. In this study a recombinant form of AAL, with higher fucose binding affinity than the natural product was used as biotarget. The structure of the AAL protein has been elucidated by Olausson and coworkers.[15] The protein is organized as a six-fold β-propeller protein, with 6 blade-shaped β-sheets arranged toroidally around the central axis of the protein. Although AAL has 6 blades, the 6th blade does not contain the conserved amino acids required for fucose binding. Therefore, AAL has only five binding sites for L-fucose molecules.

A 1 µl sample of AAL was applied to a silver nanoparticle substrate. SERS spectra were collected on the substrate in aqueous solution via direct detection method without using label molecules. When the analyte interacts with the Ag/Ge nanofilm, the buffer immediately dissolves the GeO_2 and Ge, releasing the nanoparticles as illustrated in figure 2(b). Then, the nanoparticles interact with AAL through a thiol linker termination. Conformational changes of the sample when it gets dry may affect the vibrational modes observed in the SERS spectra due to denaturalization of the protein

samples as well as structural changes induced during the drying event. Therefore, for all the samples, the SERS measurements were recorded in solution, in order to get uniform spectra with a small spot-to-spot signal variation.

Table I. SERS peaks assignment for L-Fucose and AAL.

L-Fucose Peak (cm^{-1})	L-Fucose Peak Assignment	AAL Peak (cm^{-1})	AAL Peak Assignment
1391	ν_{C-O}	1324	ν_{C-H}
1223	ν_{C-O-C}	1262	$\nu_{Amine\ III}$
963	ν_{C-H}	1170	ν_{Phe}
		1003	ν_{C-N}
		942	ν_{Phe}
		923	ν_{Tyr}

Figure 3 shows the SERS spectra recorded at 633 nm for the, AAL, and AAL-L-fucose. L-fucose sugar has characteristic resonance bands (as presented in Table I) at ν_{C-O} 1393 cm^{-1} and ν_{C-H} 963 cm^{-1}, and the ν_{C-O-C} antisymmetric stretch at 1223 cm^{-1}.[19] SERS spectrum of AAL shows vibration bands at ν_{C-H} 1324 cm^{-1}, $\nu_{Amide\ III}$ 1262 cm^{-1}, ν_{Phe} (1170 and 923 cm^{-1}), ν_{C-N} 1003 cm^{-1}, ν_{Tyr} 942 cm^{-1}.[20] For AAL with L-fucose, a distinguishing peak is observed at 1198 cm^{-1}, which is attributed to the C-O-C antisymmetric stretch of the fucose molecule. A slight shift of the Raman peak, ν_{C-O-C} , between the bare fucose (1223 cm^{-1}) and lectin bound fucose (1198 cm^{-1}) could be attributed to binding between the fucose and lectin. This peak is not observed for bare AAL sample.

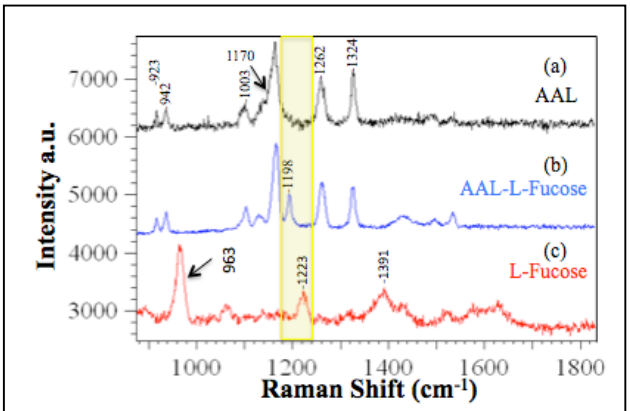

Figure 3. SERS spectra recorded at 633 nm of (a) AAL, (b) AAL with L-fucose, and (c) L-fucose.

IV. Conclusions

In the present study a novel approach for SERS detection was described. The high density of nanoparticles allows for real-time detection of proteins in solution. The SERS spectra of AAL, and AAL with L-fucose imply that Raman substrates can be utilized for the direct detection of proteins and proteins binding carbohydrates, which show potential for the elaboration of human-derive biomarkers involved in HCC.

The advantage of these new types of substrates is that direct binding of lectin can be measured, thus providing a simple, inexpensive method to achieve highly sensitive and spatially uniform signals. We demonstrate a highly sensitive rapid data analyses (i.e.,10s) at low laser power (i.e., 250-mW laser at 632 nm). Ongoing work will focus on the utilization of SERS to quantitatively measure the fucose binding lectin using our nanoparticle-based assay. These advantages open the feasibility of developing an early diagnosis sensor for hepatitis and HCC looking at fucosylation changes of biomarker proteins such as AFP. Future research will focus on the feasibility of selective lectin detection from blood samples of HCC patients.

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