Electronic Supplementary Information

Coupling solid-phase microextractions and surface-enhanced Raman scattering: Towards a point-of-need tool for hepatic cancer screening

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Table of Contents

1. Materials

60 nm gold nanoparticles (AuNPs) were purchased from Ted Pella. Sodium chloride, bovine serum albumin (BSA), and acetonitrile were obtained from Sigma Aldrich. StartingBlock, phosphate-buffered saline (10 mM) (PBS), and borate buffer (50 mM) (BB) packs were acquired from Fisher Scientific. 5, 5'-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB) was synthesized and purified as previously reported.[1] Human alphafetoprotein antibodies (α-AFP), both monoclonal mouse IgG_1 (MAB1369) and affinity purified polyclonal chicken IgY (AF1369), were purchased from R&D Systems. AFP from human cord serum (>99% pure by SDS-PAGE) was purchased from Lee Biosciences (#105-11). Pooled human serum (Accusera), was

purchased from Randox Laboratories. The level of AFP in the pooled serum was below that of the LoD for this methodology. The Miriad RVF toolkit, which was used for nitrocellulose (NC) membrane development, was obtained from N. Vats at MedMira.

2. Methods

Preparation of capture membranes. The nitrocellulose membranes in the Miriad cartridges were modified to capture AFP by spotting 1.0 μL of monoclonal α-AFP capture antibody at a concentration of 2 mg mL^{-1}. These membranes were placed in a desiccator and allowed to dry for ~12 h. Prior to use in an assay, the modified membranes were prewetted by the addition of 100 μL of elution buffer (Universal Buffer, MedMira). Serum sample calibrants containing AFP were then applied (10 μL), followed by 10 μL of ERLs. A final rinse with 100 μL of elution buffer was then performed prior to Raman readout.

Preparation of extrinsic Raman labels (ERLs). Prior to coating with the Raman reporter molecule DSNB, the pH of a 1.0 mL suspension of nanoparticles was adjusted to 8.5 by adding 50 μL of 50 mM borate buffer. This step was followed by adding 10 μL of 1.0 mM DSNB in acetonitrile to the colloidal suspension. This suspension was then mixed for 3 h on a rocker table. The next step added 20 μg of polyclonal α-AFP to the suspension, which was again mixed on a rocker table for 3 h. To stabilize the ERLs, the colloidal suspension was modified by the addition of 10% BSA in 2 mM BB (100 μL), mixed for 1 h, and then centrifuged at 2000*g* for 10 min to remove residual materials. The preparation of the ERLS was completed by carefully removing the colorless supernatant and re-suspending the pelleted nanoparticles in 1.0 mL of 2.0 mM BB containing 1% BSA. This procedure was repeated two more times, with the final resuspension using 250 μL of BB and 25 μL of 10% NaCl.

Preparation of calibration standards. As-received AFP was serially diluted into human serum to give calibrant concentrations of 500, 50, 10, 5, 0.1 μg mL^{-1}, and 10, 2.50, and 1.25 ng mL^{-1}. The serum used as a diluent for calibrant preparation was used as a blank.

Assay procedure. The nitrocellulose membranes in the Miriad cartridges were modified to capture AFP by spotting 1 μL of monoclonal α-AFP capture antibody at a concentration of 2 mg mL^{-1}. These membranes were placed in a desiccator and allowed to dry for ~12 h. Prior to use in an assay, the modified membranes were prewetted by the addition of 100 μL of elution buffer (Universal Buffer, Medmira, Inc.). Serum sample calibrants containing AFP were then applied (10 μL), followed by 10 μL of ERLs. A final rinse with 100 μL of elution buffer was then performed prior to Raman readout.

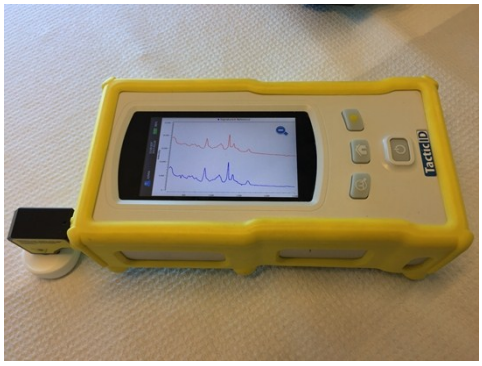

Figure SEI-1. Digital image for the Tactic ID with a 90° sampling accessory, which is placed to focus the laser light on the center of the membrane address.

Handheld spectrometer measurements. SERS spectra were collected using a TacticID handheld Raman spectrometer (B&W Tek, Inc.) equipped with a diode laser (785 nm) and a 90° sample adaptor accessory (**Figure SEI-1**). The unit is battery-operated, has a small footprint (7.5 x 3.9 x 2.0 in), weighs ~0.9 kg (< 2 lbs), and is WiFi enabled. The laser power at the sample was 90 mW. The average peak intensity (*i.e.,* height) of the symmetric nitro stretch [$\upsilon_s(NO_2)$] of DSNB at 1336 cm^{-1} was used for calibration plot construction.

3. Spectral band assignments of assay materials (Table SEI-1)

The band assignments and peak positions in Table SEI-1 were based on several recent studies,[2] including our earlier report on the Raman and infrared spectroscopy of DSNB.[3]

Table SEI-1. Band assignments and peak positions for the key components of the assay.

		Sample and Peak Position (cm^{-1})		
Mode Assignment[4]	Description	Nitrocellulose	DSNB	DSNB/AuNP/NC
$\nu_a(NO_2)$	asymmetric ONO stretch	1654		
ν(C=C)	aromatic ring stretch (8a*)		1567	1559 (DSNB)
δ(C-H)	C-H bending mode	1372		
$\nu_s(NO_2)$	symmetric ONO stretch	1285	1343	1336 (DSNB) 1289 (NC)
ν(C-O)	C-O stretches of pyranose group	~1190-1040		~1153 (NC)
ν(C-S)	C-S stretch (aromatic)		1077	1063 (DSNB)
$\delta(NO_2)$	ONO scissoring mode	850		
δ (C-H)	out-of-plane deformation mode		852	850 (DSNB)

*Wilson number notation.

4. Ligand binding curve analysis

The fit to the data in **Figure 3B** used the single-site binding isotherm model.[5] This model embodies two main assumptions. First, the binding between antigen and capture antibody obeys a 1:1 reaction stoichiometry and can be accurately represented by a single-valued equilibrium constant. Second, antigen is irreversibly bound to the capture antibody, meaning there is no desorptive loss of captured antigen during subsequent rinse and labelling steps. Our use of this model also implicitly asserts that the ERLs exhaustively tag the capture antigen at a 1:1 stoichiometry at all antigen concentrations, with the SERS response then being a quantitative measure of the captured antigen.

The formalization of the single-site binding isotherm develops by considering the equilibrium reaction between the antigen in solution [Ag(soln)] and surface-immobilized capture antibody [Ab(surf)], which can be written as:

$$Ag(soln) + Ab(surf) \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} Ag{:}Ab(surf) \qquad \textbf{Eqn. SEI-1}$$

where k_{on} and k_{off} are the respective reaction rate constants for the association and dissociation of the surface-bound antigen:antibody complex [Ag:Ab(surf)].

The dissociative equilibrium constant (K_d) for this reaction can be written as:

$$K_d = \frac{\Gamma_{Ag:Ab}}{[Ag]\Gamma_{Ab}} \qquad \textbf{Eqn. SEI -2}$$

where $\Gamma_{Ag:Ab}$ is the equilibrium concentration of the immobilized antigen:antibody complex, [Ag] is the equilibrium concentration of antigen in solution, and Γ_{Ab} is the equilibrium concentration of unbound immobilized antibody.

Next, the fraction of immobilized antibody bound to the antigen, θ, can be defined as:

$$\theta = \frac{\Gamma_{Ag:Ab}}{\Gamma_{Ab} + \Gamma_{Ag:Ab}} \qquad \textbf{Eqn. SEI-3}$$

This formulation can also be written in terms of the concentration of the immobilized antigen:antibody complex at saturation, $\Gamma_{Ag:Ab}^{satd}$, which equals $\Gamma_{Ab} + \Gamma_{Ag:Ab}$.

Eqn. SEI-3 can be rearranged in terms of K_d and [Ag] by multiplying through by [Ag]/[Ag] and substituting the expression for K_d (**Eqn. SEI-2**) into the result. Completing these steps gives **Eqn. SEI-4**.

$$\theta = \frac{[Ag]}{[Ag] + K_d} \qquad \textbf{Eqn. SEI-4}$$

Eqn. SEI-4 indicates that a plot of θ vs [Ag] will have a linear relationship when $[Ag] << K_d$, and a slope of K_d^{-1}.

The last two parts of the development for this formalization is to convert the *y*-axis from a measure of θ to that of SERS intensity and to account for the signal due to nonspecific adsorption. For the former, we can write:

$$\theta = \frac{I(C_i)}{B_{max}} \qquad \textbf{Eqn. SEI -5}$$

where $I(C_i)$ is the SERS signal at a given antigen concentration C_i, and B_{max} is the SERS signal at binding site saturation. Substitution of **Eqn. SEI-5** into **Eqn. SEI-4** and rearrangement yields,

$$I(C_i) = \frac{B_{max}[Ag]}{[Ag] + K_d} \qquad \textbf{Eqn. SEI-6}$$

For the latter, we will assume that the response from nonspecifically adsorbed ERLs for blank human serum (N_{blank}) is constant for all antigen concentrations. The total response for this system is therefore:

$$I(c_i) = \frac{B_{max}[Ag]}{[Ag] + K_d} + N_{blank} \qquad \textbf{Eqn. SEI-7}$$

This equation, listed as Eqn. 1 in the formal manuscript, can be readily solved for K_d, which is equal to the concentration of antigen at $0.5B_{max}$. Once K_d is known, the limit of detection (LoD) can be determined as the antigen concentration that has a signal equal to the blank measurement plus three times the standard deviation of the blank measurement (σ_{blank}). The data per **Eqn. SEI-7** was fit using a dynamic nonlinear regression per a Marquardt-Levenburg algorithm (SigmaPlot 11.0) and set a tolerance of 1×10^{-10}. From the fit, B_{max}= 17906 cts/s, $K_d = 1.67 \times 10^{-10}$ M ($\Delta G_{reaction}$ = -13.3 kcal mol^{-1} or 56.1 kJ mol^{-1} at 25 °C). The other components of the measurement related to the LoD estimate include N_{blank}= 180.3 cps and σ_{blank}= 25.9 cps.

5. Estimation of level of antigen:antibody dissociation from rinse/labeling steps

The single-site ligand binding analysis assumes that the equilibrium concentration for captured AFP is not perturbed by the rinse and labeling steps. An estimation of the off-rate kinetics for captured AFP can be calculated by recognizing that K_d (M) in **Eqn. SEI-2** can also be expressed as:

$$K_d = \frac{k_{off}}{k_{on}} \qquad \textbf{Eqn. SEI-8}$$

where k_{off} (s^{-1}) is the heterogeneous rate constant for the unimolecular dissociation of the antigen:antibody complex and k_{on} (M^{-1} s^{-1}) is the heterogeneous rate constant for the biomolecular formation of the antigen:antibody complex. This equation can be used to estimate a value for k_{off}. For our purposes, we will reasonably assume that the formation of the antigen:antibody complex proceeds at a diffusion-limited rate,[6, 7] which from collision theory is ~1 x 10^6 M^{-1}-s^{-1}.[8] Coupled with a value for K_d from the single-site ligand binding model (1.67 x 10^{-10} M), this analysis gives a k_{off} of ~2 x 10^{-4} s^{-1}.

The extent of dissociation (C_{rinse})/C_i) can then be projected by means of the expression for the first-order dissociation of the antigen:antibody complex given in **Eqn. SEI-9,**

$$C_{rinse} = C_i e^{-k_{off} t} \qquad \textbf{Eqn. SEI-9}$$

where C_i and C_{rinse} are the amounts of complex present before and after rinsing, respectively. Using the above value for k_{off} and the total time of exposure to solutions devoid of AFP (~40 s in total for the labeling step and three rinse steps), roughly 2% of the bond complex will dissociate. The validity of the nondissociation assumption can be further framed if the value for k_{off} is

increased by a factor of 5 to k_{off} = 1 x 10^{-3} s^{-1}. In this case, the percent of complex dissociation in 40 s increases to about 10%.

6. Literature Cited

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