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Detection of Tacrolimus in Saliva using a Lateral Flow Assay and Surface-Enhanced Resonance Raman Scattering

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Abstract

The lives of ~900,000 US citizens have been extended by organ, and more recently, limb transplants from 1988 to 2021. Currently, there are over 250,000 patients in the US with a functioning kidney transplant. Unfortunately, the success of these methods requires lifelong drug treatment that includes immunosuppression drugs, such as tacrolimus. Furthermore, the dosage and regiment must be monitored to ensure that concentrations are su f cient to avoid transplant rejection, yet not lead to toxicity. Patients must constantly visit a hospital or clinic to have the drugs in their blood measured. Unfortunately, this burden has led to non-adherence and consequently, transplant failure in over 20% of patients, most often resulting in death. In an e f ort to reduce this burden, we have developed a simple to use assay

enhanced Resonance Raman spectroscopy (SERRS), which has been used to detect single molecules in water [32,33]. Here we describe the development of a SERRS LFA. In use, a saliva sample is added to the LFA, the saliva ows through the conjugate pad of SERRS probes, that bind tacrolimus, which if present, bind to a second antibody at the Test Line, where a Raman spectrometer measures the SERRS probe dye intensity representative of the tacrolimus concentration.

Materials and Methods

Tacrolimus and all reagents were obtained from Sigma-Aldrich (Allentown, PA), while tacrolimus antibodies were obtained from Creative Diagnostics (Shirley, NY), and de-identi ed, pooled saliva was obtained from Lee Biosolutions (Maryland Heights, MO). e SERRS probes consisted of synthesized gold nanoparticles [21], coated with a dye, as a reporter molecule, and functionalized with antibodies speci c to tacrolimus [25]. e LFA cassettes were of standard construction (nanoComposix, San Diego, CA) consisting of

1) A conjugate pad containing the SERRS probes,

2) A Test Line functionalized with the tacrolimus antibodies,

3) A Control Line functionalized with goat anti-mouse IgG,

4) A wicking pad, all on

5) A nitrocellulose support,

6) Enclosed in a plastic cassette containing a sample addition port and a viewing section.

e LFA cassettes were measured using a compact, 5-lb Raman spectrometer of in-house design. It employed a 785 nm diode laser and a room temperature 512 channel Si array detector. e cassette Control Line was visually examined a er 10 minutes to con rm sample ow. e cassettes were inserted into a simple enclosure attached to the spectrometer that xed the distance of the laser focal point on the cassettes. e cassettes were positioned manually on the Test Line for measurements. All measurements used 40 mW laser power and a 1-sec acquisition time. All sample preparations and measurements were performed in a Biosafety Level 2 cabinet following standard safety procedures.

Results

e capabilities of the LFAs were initially tested using tacrolimus in phosphate bu ered saline (PBS) at 0 to 25 ng/mL. For each concentration, 0.1 mL of sample was added by pipette to a cassette and allowed to ow for 15 min, a er which the Test Line was measured by the Raman spectrometer. e measured height of the dye SERRS peak at 595 cm^{-1} was used to develop a concentration plot (Figure 1). e peak height intensity is a function of the tacrolimus concentration, which is limited by the concentration of the available antibodies on the Test Line, and follows a standard Langmuir equation [34] (Equation 1).

$$[Tacro] = (kxISERRS/(1+kxISERRS))xS$$

Where [Tacro] is the tacrolimus concentration, k is a constant, ISERRS is the measured 595 cm^{-1} peak height, and S is a constant representing the available antibodies on the Test Line, which is equivalent to the maximum peak height.

Next measurements of tacrolimus added to de-identi ed, pooled saliva were performed. A stock solution of tacrolimus was prepared at 0.1 mg/mL in PBS, and serially diluted in saliva to obtain concentrations from 0 to 25 ng/mL. For measurements, 0.1 mL of each sample concentration was added to three cassettes (Figure 2). Each cassette was measured by the Raman spectrometer, and the spectra were averaged for each concentration (Figures 3A). e averaged measured height of the dye SERRS peak at 595 cm-1 for each concentration was used to develop a concentration plot, which was t by a Langmuir equation (Figure 3B). For these saliva samples, the peak heights were lower in intensity by 13-17% compared to the PBS samples (Figure 2A). is intensity decrease was expected as the saliva mucins block some of the antibody binding sites, as re ected by the constant S (Figures 1-3).

e standard deviation generally increased with concentration, and ranged from 0.3 to 2.7 ng/mL, ignoring the sample with no tacrolimus added (Table 1).

e averaged peaks heights were also used to predict the concentrations using the Langmuir equation that was t to the data, as would be performed by so ware in a product. e error for these predictions ranged from -1.9 to 0.5 ng/mL, ignoring the 25 ng/mL sample, which had a large error due to the imprecision of the equation at this higher concentration.

e lowest measured saliva sample, 2.5 ng/mL, was used to calculate a limit-of-detection (LOD) based on the signal-to noise ratio (S/N) of

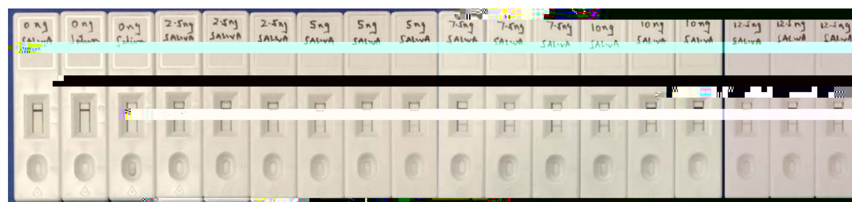

Figure 2: Photograph of LFAs used to measure 0 to 10 ng/mL tacrolimus spiked in saliva (12.5 and 25 ng/mL cassettes not shown).

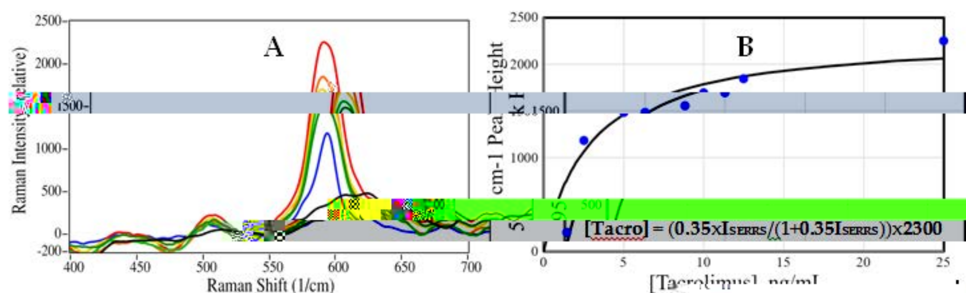

Figure 3: A) SERRS of 25, 12.5, 10, 7.5, 5, 2.5 and 0.0 ng/mL in pooled saliva, and B) corresponding plot of the SERRS 595 cm^{-1} peak height as a function of sample concentration with the expected Langmuir dependence fit to the data. Spectral conditions: 40 mW at 785 nm, 1 sec acquisition, baseline set to 0 at 535 cm^{-1}.

Table 1: Measured SERRS peak heights at Test Lines for 3 cassettes each at 25, 12.5, 10.0, 7.5, 5.0, 2.5, and 0 ng/mL Tacrolimus with average and standard deviation, as well as concentration standard deviation, and Langmuir calculated concentrations and error.

SERRS Peak Height 9595 (cm^{-1})					Tacrolimus (ng/ml)		
Cassette 1	Cassette 2	Cassette 3	Average	Stan Dev	Prepared ± Stan Dev	Calculated	Cllc Error
2534	2136	2085	2251.7	245.8	2.5 ± 2.7	60	35
1723	2054	1763	1846.7	180.7	12.5 ± 1.2	11.5	-1
1620	1522	1951	1698	224.6	10 ± 1.3	8.1	-1.9
1659	1456	1559	1558	101.3	7.5 ± 0.5	6	-1.5
1831	1263	1357	1483.4	133.4	5 ± 1.0	3	0.5
1167	1326	1061	1184.4	133.4	2.5 ± 0.3	3	0.5
125	189	296	203.3	86.4	0 ± 0	0.28	0.28

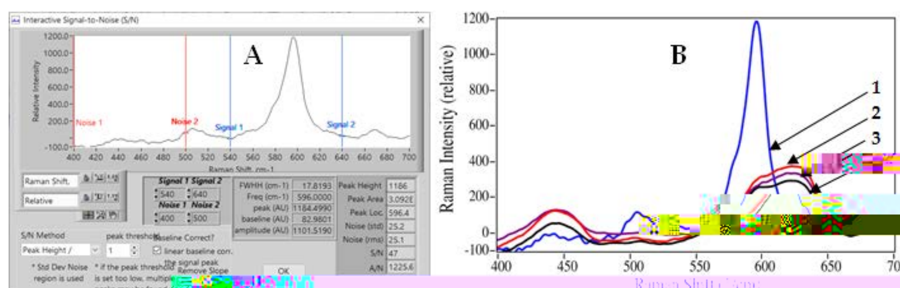

Figure 4: A) Image of RTA software used to calculate the LOD and LOQ using the 2.5 ng/mL Tacrolimus in saliva SERRS peak height. B) SERRS of 1) 2.5 ng/mL tacrolimus, 2) sirolimus, 3) cyclosporin, and 4) saliva as a blank (same scale, but offset for clarity). Spectral conditions as in Figure 1.

prepared at 500 ng/mL, 50 times the normal tacrolimus dose, yielded no spectra of the SERRS Probes demonstrating excellent antibody specificity, even at this high concentration. In fact, the spectra were identical to that of a blank sample of PBS (Figure 4B).

Conclusions

A lateral flow assay cassette for Tacrolimus in saliva was developed that employed novel SERRS probes that bind tacrolimus, which were quantitatively measured using a Raman spectrometer. Successful measurements of tacrolimus in phosphate buffered saline were followed by measurements of tacrolimus in purchased de-identified, pooled saliva covering the treatment range of 5 to 15 ng/mL. The SERRS intensity as a function of tacrolimus concentration followed a standard Langmuir equation, which was fit to the data and used to predict the concentrations based on the SERRS peak heights. The average error for the samples from 2.5 to 12.5 ng/mL was ±1 ng/mL tacrolimus. In addition, measurements of sirolimus, another immunosuppressant drug with a very similar chemical structure, and cyclosporin, an often used co-drug, showed no antibody cross reactivity at concentrations 50 times those used during treatment. While these measurements represent a limited set of ideal samples, these results are in-line with the requirements for monitoring dosage. Future research will test the ability of the LFA cassettes and SERRS measurements to detect tacrolimus in actual human samples with the goal of developing a simple-to-use, at-home immunoassay drug monitor. We believe that the elimination of the 1-4 monthly visits to hospitals or clinics for painful blood draws, will greatly reduce non-adherence, and thereby reduce organ rejection, and in many cases extend lives.

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