SERS-based biosensors for the detection of human Thyroglobulin in liquid biopsies

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Abstract. Here we have developed an advanced surface-enhanced Raman scattering (SERS) platform that enables the ultrasensitive, rapid and highly specific identification of tumour biomarkers in liquid biopsies. Our focus is on the detection of Thyroglobulin (Tg), the most important tumour biomarker for the diagnosis and prognosis of thyroid cancer. Specifically, SERS-active substrates fabricated by nanosphere lithography on chip or on tips of optical fiber (OF) were functionalised with Tg Capture antibodies. Gold nanoparticles were functionalized with Detection antibodies and conjugated with a Raman reporter. The sandwich assay platform was validated in the planar configuration and a detection limit of only 7 pg/ml was successfully achieved. A careful morphological characterisation of the SERS substrates allowed us to strictly correlate the coverage area with the Tg concentration. The same approach was successfully demonstrated in the washout of fine needle aspiration biopsies from cancer patients. The strategy was transferred to the Lab On Fiber (LOF) SERS platform and successfully used to detect Tg concentration. The proposed SERS-enhanced immunoassay platform has proven to be highly versatile and can be used with both microfluidic chip POC devices and SERS-OF-based optrodes to perform sensitive, specific and rapid *ex vivo* assays for Tg detection in liquid intraoperative biopsies.

1 Introduction

Thyroid cancer, the most common endocrine malignancy, has an increasing incidence rate and ranks ninth worldwide in 2021 [1]. The disease generally has a good prognosis, but recurrence can occur in up to 30% of patients within 10 years of initial diagnosis [2]. Total thyroidectomy with "therapeutic" cervical lymph node dissection for affected lymph nodes is the standard of care. However, several retrospective studies indicate that regional lymph node metastases are associated with tumour recurrence and an unfavourable survival rate. Fine needle aspiration biopsy (FNAB) cytology is the gold standard for the detection of metastases in the cervical lymph nodes [3]. However, cytological analysis of the FNAB is not performed immediately.

We present the development of a biosensor based on surface-enhanced Raman spectroscopy (SERS) for Tg measurement in humans [4], which can be easily integrated into smart needle systems or point-of-care devices with microfluidic chips [5]. The sensor technology is based on a self-assembled SERS-active substrate functionalized with Capture antibodies specific for the Tg biomarker. To selectively capture the target biomarker, we also use gold nanoparticles (AuNps) functionalized with the Detection antibody and 4-mercaptobenzoic acid (4-MBA), which acts as a Raman reporter (RR). The sensing platform was initially developed as an on-chip architecture. The same functionalized SERS-active substrates were then fabricated directly on the OF tip. We relied on our previous research [5-8] to fabricate highly ordered, reproducible and cost-effective SERS-active substrates on the fiber tip (OF) using nanosphere lithography. For the Tg sandwich test, we used a periodic pattern called "Close Packed Array-Sphere Removal" (CPA-SR), which consists of triangular nano-islands with hexagonal symmetry, as the SERS substrate. The CPA-SR pattern is characterised by its excellent repeatability, its accessibility to hotspots and its good gain [6].

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2 Materials and Methods

For SERS Tg detection, the sandwich structure comprises a first layer of Capture antibodies immobilized on SERS substrates [7, 8]. After exposure to the molecular target, the captured antigen is selectively labeled by functionalized nanoparticles (f-AuNps). f-AuNps are developed by sequential functionalization of AuNps with the Raman reporter, followed by Detection antibodies for the selective detection of Tg. The resulting biosensor platform can generate a strong and detectable SERS signal, which depends on the interaction of plasmonic forces between the f-AuNps and the SERS substrate. The sandwich assay platform was validated in the chip configuration with different Tg concentrations and clinical samples from patients with suspicious thyroid lymph nodes (Federico II University Ethics Committee approval no. 790/2014). Finally, SERS optrodes were fabricated [6] and used to measure Tg concentration.

3 Results

The sensing capability of the proposed sandwich immunoassay platform is demonstrated by exposing the SERS on-chip platform to different Tg concentrations: 0.1, 1, 10 and 100 ng/mL. To quantitatively evaluate the SERS response of the chip to different Tg concentrations, we introduced an indicator that takes into account the number of spectra with the characteristic RR peaks that exceed the noise threshold. Figure 1A (blue curve) shows the calibration curve, which indicates the average pixel number as a function of the Tg concentration and 7 pg/mL as the detection limit. The correlation between the Tg concentration and the average pixel number, shows that the f-AuNps bound on the SERS chip increases with the Tg concentration. The red dots in Figure 1A show the Tg detection in the clinical sample. As with the *in vitro* samples, the pixel number increases with Tg concentration, indicating that the number of f-AuNps bound to the sample surface increases. Figure 1B shows the average pixel number of SERS spectra as a function of coverage area for different Tg concentrations. The increase in coverage area is consistent with the increment of the Tg concentrations and shows the strong correlation between the results of SERS and AFM analysis. This confirms the hypothesis that the SERS response is due to the detected hotspots associated with the presence of f-AuNps on the SERS-active substrate through the specific interaction of the Capture and Detection antibodies with Tg. We investigated the possibility of applying the proposed SERS–assisted immunoassay strategy to the optical fiber tip. For this purpose, Tg was added to the optical fiber tip at concentrations of 0.1 µg/mL (1.5 nM), 1 µg/mL (15 nM) and 10 µg/mL (150 nM). The SERS spectra for the different Tg concentrations are shown in Figure 1C. The SERS signal increases in intensity with increasing Tg concentration, indicating the expected dose-response effect.

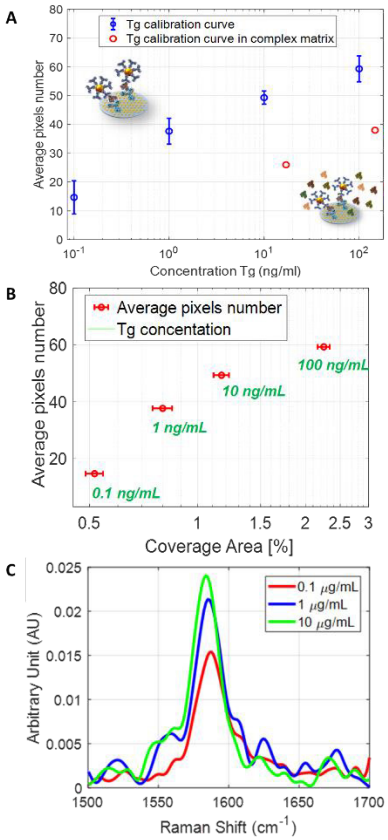

Fig. 1. (A) Dose-response curve showing the average pixel number of SERS on-chip for the detection of Tg at different concentrations (0.1, 1, 10, 100 ng/mL) in PBS (blue dots) and in the complex matrix (red dots). (B) Average pixel number of biofunctionalized CPA-SR as a function of the coverage area for the Tg concentrations. (C) SERS spectra highlighting the peak intensity 1580 cm^{-1} for different Tg concentrations.

4 Conclusions

The proposed SERS-assisted immunoassay platform has proven to be highly versatile and easy-to-use and can be applied to both on-chip devices and OF SERS-based optrodes to perform sensitive, specific and rapid *ex-vivo* assays for Tg detection in liquid intraoperative biopsies.

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