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Peptide-Nanoparticle Hybrid SERS Probe for Dynamic Detection of Active Cancer Biomarker Enzymes

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Abstract— **Real-time *in situ* detection of protease enzymes is crucial for early-stage cancer screening and cell signaling pathway study; however it is difficult to be realized using fluorescence or radioactive probes. Here we devise a hybrid optical probe by incorporating nanocrescent particle and peptides with artificial tag molecules. The peptides have high specificity to PSA, one of the most prominent prostate cancer markers, and a serine protease present in patients' seminal fluid and serum. The extrinsic Raman spectral signal from the tag molecules is enhanced by the nanocrescent and the signal is monitored as the indicator for the peptide digestion in nanomolar PSA concentration and femtoliter reaction volume. Sensitive detection of cancer-related serine protease activity of PSA proteins in low concentrations and small volumes of biofluid is critical to early cancer diagnosis, clinical staging, and therapy. The high reaction specificity of the peptide and the monitored extrinsic Raman signal also minimizes the false detection of other serine proteases and intrinsic Raman signal, which results in a high-fidelity and high-signal-to-noise-ratio cancer nanoprobe. Peptide-conjugated nanocrescents should also be applicable for measuring the intercellular and intracellular activity of other cancer-related proteases and protease activity profiling-enabled cancer cell identification.**

I. Introduction

Prostate cancer incidence and mortality rates vary worldwide. It is the most common cancer in men in Europe and North America [1-3]. In the United States, prostate cancer is the second-leading cause of cancer death for men. One of the clinical diagnosis tools for prostate cancer is the measurement of plasma protein concentration of the prostate-specific antigen (PSA or hK3). PSA is a member of the large kallikrein (hK) protease family, which is normally secreted from prostate luminal epithelial cells. PSA activity is important in initiating the spermatozoa forward motility. In prostate cancer, PSA, and perhaps, other members of the hK family proteases, are involved in tissue remodeling aided by the proteolytic activities against the extracellular matrix, contributing critical control mechanisms to tumor invasion or progression.

The introduction of plasma PSA screening since the 1980s has greatly improved the diagnosis, staging, and management of prostate cancer [4]; however, many problems exist with this method. Measurement of plasma PSA concentration does not differentiate the prostate cancer patients from those with benign prostatic hyperplasia, leading to a high false positive rate, requirement for more expensive biopsies, and even unnecessary surgical procedures [4, 5]. Efforts to enhance the clinical value of the PSA for early detection of prostate cancer have included the characterization of various molecular variants of PSA, since PSA in prostate cancer exists in various isoforms and proteolytic activity [6-8]. Among those various isoforms, the proteolytically active subpopulation of PSA is a more useful tumor marker than the serum PSA concentration and is a better predictor for prostate cancer malignancy [9, 10]. Simple detection of the presence of PSA by a traditional immunostaining method can not reveal the proteolytic activity of the PSA; therefore, it is of great importance to develop new methods to discriminate the different PSA isoforms, especially the proteolytically active form. However, an assay that measures the proteolytic activity of PSA in seminal fluid is still not widely accepted, due to the quick decay of the proteolytic activity, the limited amount of seminal fluid available from old patients, and the difficulty in obtaining accurate results.

The sensitivity of current detection methods is limited to nanomolar concentrations, and relatively large sample volume (milliliter) is required. Here we introduce a new optical spectroscopic detection method for PSA proteolytic activity based on a peptide-conjugated crescent nanoparticle, which can be used on minute sample volumes (femtoliters), integrated into microfluidics, and multiplexed as high density nanoarrays or microarrays (with sub-microliter volume). The nanocrescent can serve as an individual surface enhanced Raman scattering (SERS) substrate [13], and after conjugation with a peptide specifically designed for PSA [5, 10-12, 14], it can be used to optically monitor PSA proteolytic reactions. Raman is a sensitive spectroscopic detection method for probing biochemical composition with abundant atomic level information without fluorophore labeling [15]. Various SERS substrates have been developed to enhance the weak Raman

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scattering signals in chemical and biomolecule detections on the substrate surface over several orders of magnitude [13, 16-21]. The nanoscale dimension and the high local electromagnetic field enhancement of our nanocrescent SERS substrate enable a high-sensitivity optical detection of biomolecular reactions on its surface.

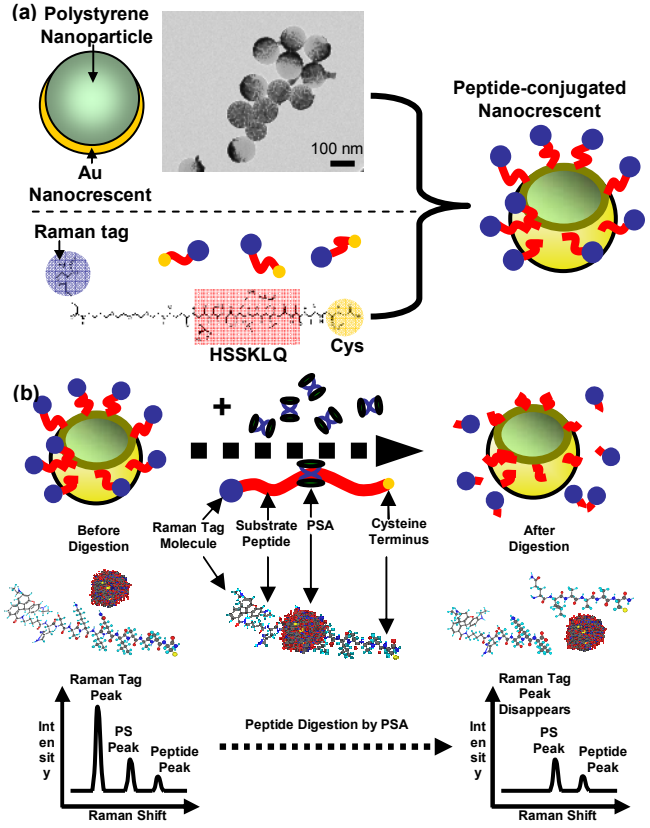

Figure 1. Peptide-conjugated nanocrescent for PSA detection. **(a)** Fabrication procedure. The nanoscale Au layer is evaporated on polystyrene nanoparticles to form the Au nanocrescent as shown in the TEM image. Peptides are synthesized with the specific PSA substrate sequence HSSKLQ and are terminated by a Raman tag molecule, biotin or R19 (not shown), respectively, and cysteine for both versions of tagged peptides. The peptides are conjugated to the Au surface of the nanocrescents through an Au-S bond. **(b)** PSA detection scheme. Before the proteolysis reaction, the SERS spectrum of the peptide-conjugated nanocrescent contains the characteristic peaks from the Raman tag molecules, polystyrene nanoparticle, and the peptides; after the digestion reaction by PSA, the peptide is cleaved between L and Q. The cleavage fragment containing the Raman tag molecules diffuses away from the nanocrescent surface, while the other fragment remains on the nanocrescent surface. The SERS spectrum of the peptide becomes different and the characteristic peaks from the Raman tag molecule disappear.

II. METHODS AND MATERIALS

Nanocrescents consist of a 100 nm polystyrene core and a 10~20 nm gold crescent shell. Fig. 1a shows the schematics and transmission electron micrograph of the nanocrescent. The nanocrescents are fabricated by angled Au deposition on the rotating polystyrene nanoparticle template [13]. The fabrication details were described previously [13]. In this paper, the polystyrene nanoparticle core is not removed and it serves as the internal control in the SERS detections. We then tether a specially designed peptide on the surface of the Au nanocrescent. The peptides contain the sequence of HSSKLQ which has been shown to have very high specificity to PSA [22]. A cysteine group at the carboxyl terminus of the peptide is used to attach the peptide to the Au surface, relying on the Au-thiol reaction to form a covalent bond. At the amino terminus of the peptide, Raman active molecules such as biotin (shown in fig. 1a) or Rhodamine 19 (R19) (not shown in the schematics) are grafted through a short polyethyleneglycol or aminovaleric acid linker. The detection scheme is shown in Fig. 1b. The SERS spectra of the artificial peptides change after digestion by PSA, and the characteristic SERS peaks of the molecular moieties with the biotin or R19 tags disappear due to the diffusive dislocation of the tag molecules from the nanocrescent surface into the solution after peptide digestion; therefore the existence and concentration of the proteolytically active PSA in solution can be probed by monitoring the SERS spectra of the peptide-conjugated nanocrescents. The Raman scattering signal of the peptide change is amplified by the nanocrescent. Our numerical simulation indicates the amplitude of the local electric field can be enhanced by close to 20 dB (100 fold) especially around the sharp edge. Due to the fourth power relation between the electric field amplitude and the Raman enhancement factor, the peptide Raman signal could be amplified 10^8 times by the nanocrescent.

The peptide-conjugated nanocrescents are incubated with PSA molecules in a closed transparent microchamber to minimize evaporation. The transparent microchamber is mounted on a 37 ºC thermal plate on an inverted Raman microscope with darkfield illumination for nanoparticle visualization. ~0.8 mW excitation laser spot is focused on single nanocrescents and the Raman scattering light is collected by the same objective lens.

III. RESULTS

The typical SERS spectra of the peptide-conjugated nanocrescents with biotin and R19 Raman tag molecules are shown in fig. 2a and 2b, respectively. By comparing the SERS spectra before and 2 hours after the peptide digestion experiments, the Raman peaks from polystyrene core, e.g. 1003 cm^{-1}, remains constant, which serves as an internal control signal as the indication of stably focused laser excitation. Some Raman peaks are from the partial amino acid chain remaining on the nanocrescent surface after digestion and they still appear in the spectra, although the peak positions have slight changes and the peak intensities decrease due to possible conformational changes upon peptide cleavage. Those peaks from the Raman tag molecules, such as 525 cm^{-1} from biotin in fig. 2a and 1183 cm^{-1} from R19 in fig. 2b, almost completely disappear after the digestion reaction is finished.

The digestion reaction dynamics can be monitored by time-resolved SERS spectra acquisitions. Because ~100 peptides are conjugated on each nanocrescent on average, the disappearance of the characteristic Raman peaks from the tag molecules is not abrupt. Since most of enhanced field

concentrated around the tip area which accounts for ~1/6 of total area of the nanocrescent, the actual molecule number contributing to the Raman scattering signal is less than 20. As shown in the time-lapse SERS spectra in Fig. 2a, the digestion of all of the peptides on each nanocrescent, as monitored by the disappearance of the biotin peak at 525 cm^{-1}, takes ~30 min at a PSA concentration of 420 nM. For the peptide with R19 as the Raman tag molecule, the disappearance of the R19 peak at 1183 cm^{-1} can be also observed after digestion by 420 nM PSA (Fig. 2b).

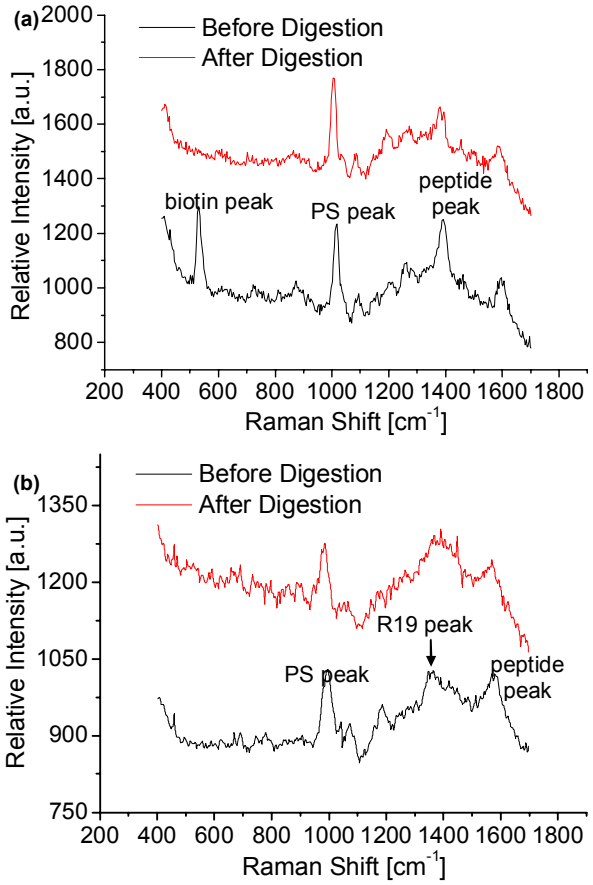

Figure 2. Typical SERS spectra of peptide-conjugated nanocrescents before and after PSA digestion reactions with **(a)** biotin and **(b)** R19 as the Raman tag molecules respectively.

In order to specifically inhibit the PSA-mediated proteolysis of the conjugated peptides, PSA inhibitors were introduced prior to the addition of 420 nM PSA. We also tested the specificity of the conjugated peptides to PSA using other serine proteases such as Granzyme B, which serves as a negative control here. Fig. 3c and 3d show the time-lapse SERS spectra of nanocrescents with R19 tag molecules in the above two control experiments with the PSA inhibitor and the serine protease Granzyme B, which has orthogonal substrate specificity to PSA, respectively. The peptide digestion by PSA is more than 90% suppressed after the addition of inhibitors given the same experimental conditions. In the control experiment of peptide digestion by 420 nM Granzyme B, the reaction rate showed no statistically significant difference from the inhibitor-treated reaction.

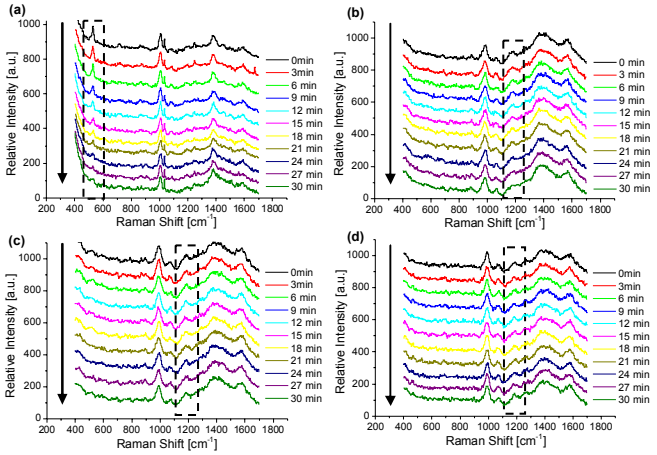

Figure 3. Time-resolved SERS spectra in PSA digestion reactions. **(a)** SERS spectra in the peptide digestion by 420 nM PSA with biotin as the Raman tag molecule. **(b)** SERS spectra in the peptide digestion by 420 nM PSA with R19 as the Raman tag molecule. **(c)** SERS spectra in the peptide digestion by 420 nM PSA in the presence of inhibitor with R19 as the Raman tag molecule. **(d)** SERS spectra in the peptide digestion by 420 nM Granzyme B with R19 as the Raman tag molecule.

The digestion rate is related to the PSA concentration and we found the minimal PSA concentration detectable in 30 min is around 1 nanomolar (with ~50% reduction in biotin signal intensity, data not shown). Since at most 100 peptide molecules are attached per nanocrescent, it is likely that the nanocrescent surface with the highest SERS signal is not fully taken advantage of, and only a small percentage of the peptides are attached to the region that provides the greatest enhancement in electromagnetic field. Fig. 4a shows the intensities of the biotin Raman peak at 525 cm^{-1} as a function of PSA digestion time for the PSA concentration of 0 M (buffer solution), 4.2 nM, 42 nM and 420 nM. Fig. 4b shows the time-lapse intensities of the R19 Raman peak at 1183 cm^{-1} in the digestion reaction with 420 nM PSA, 420 nM PSA with inhibitor, and 420 nM Granzyme B, respectively. All the peak intensity values are normalized to the internal control peak at 1003 cm^{-1} and the initial peak intensity at 525 or 1183 cm^{-1}. The results indicate that the peptides are efficiently and specifically cleaved by PSA even after the conjugation with Au nanocrescents; therefore this peptide-conjugated nanocrescent can be used as a specific screening tool to provide information on the concentration and activity of the cancer biomarker PSA.

IV. CONCLUSION

In conclusion, we have demonstrated the in vitro PSA detection using single peptide-conjugate nanocrescent SERS probes with nanomolar sensitivity. Compared to other cancer biomarker detection assays, our bioconjugated nanocrescent allows the detection of nanomolar concentrations of proteolytically active PSA molecules in femtoliter volumes, which is crucial especially for cancer screening at a single cancer cell level. In semen, the PSA concentration is

10-150M, with approximately two thirds of the PSA enzymatically active [14]. The sensitivity level achieved with the nanocrescent PSA probe (nanomolar range) is sufficient for a seminal fluid based assay, thus the nanocrescent SERS platform here could have potential clinical applications. We envision that, with additional spacer synthesized in between the substrate peptide sequence HSSKLQ and the Cys residue, we can improve the presentation of PSA substrate peptide HSSKLQ on the surface, and increase the detection sensitivity consequently. The real-time reaction monitoring also provides abundant information on the PSA activities, not only the presence of the protein. Two different Raman tag molecules are successfully utilized here indicating the potential of multiplexing the peptide-conjugated nanocrescents to detect two or more types of cancer-related proteases. Additional spatial multiplexing in a microarray or nanoarray format is under investigation in our laboratories. In addition, the fabrication scheme of the nanocrescent permits the creation of magnetically or laser maneuverable SERS nanoprobes for biosensing at desired locations [23], which would be useful for obtaining in situ measurements intracellularly.

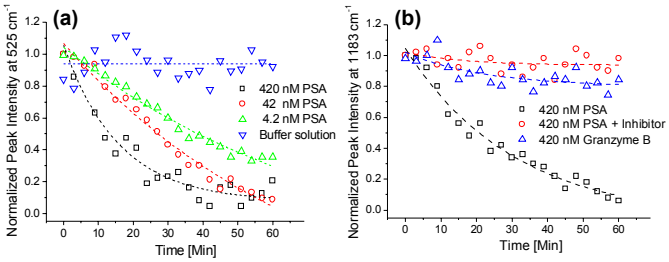

Figure 4. Time-dependent Raman peak intensities in PSA digestion reactions. **(a)** Raman peak intensities of biotin at 525 cm-1 in the digestion reactions with 0 M (buffer solution), 4.2 nM, 42 nM and 420 nM PSA, respectively. **(b)** Raman peak intensities of R19 at 1183 cm^{-1} in the digestion reactions with 420 nM PSA, 420 nM PSA with inhibitor, and 420 nM Granzyme B, respectively.

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