Peptide-Nanoparticle Hybrid SERS Probes for Optical Detection of Protease Activity

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ABSTRACT

Real-time in situ detection of active proteases is crucial for early-stage cancer screening and cell signaling pathway study; however, it is difficult to achieve using fluorescence or radioactive probes at volumes below 1nL. Here we demonstrated a hybrid optical probe by incorporating nanocrescent particle and peptides with artificial tag molecules. We performed a proof-of-concept study using prostate specific antigen (PSA), one of the most prominent prostate cancer markers, and a serine protease present in patients’ seminal fluid and serum. The Raman spectral signal from the tag molecules is enhanced by the nanocrescent and the signal is monitored as the indicator for peptide cleavage in a femtoliter reaction volume, at levels close to a single proteolytically active PSA molecule. The high reaction specificity of the peptides on individual nanoparticles minimizes the false detection of other serine proteases and background Raman signal, which results in a high-fidelity and high-signal-to-noise-ratio cancer nanoprobe that can be easily incorporated into nano/microfluidic devices.

Keywords: Surface Enhanced Raman Scattering (SERS); Nanoprobe; Protease; Prostate Cancer, Prostate Specific Antigen (PSA), Proteolytically Active PSA
Prostate cancer is the most common cancer in men in Europe and North America \(^1\text{-}^3\). One of the clinical diagnosis tools for prostate cancer is the measurement of plasma protein concentration of the prostate-specific antigen (PSA or hK3), which is a member of the large kallikrein (hK) protease family (for reviews, see\(^4\text{-}^6\)) normally secreted from prostate luminal epithelial cells. Unlike other kallikrein family members, PSA is a chymotrypsin-like serine protease\(^7\). In prostate cancer, PSA, aided by the proteolytic activity, is involved in tissue remodeling 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\(^5\); however, 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\(^5\text{-}^6\). Efforts to enhance the clinical value of the PSA for early detection of prostate cancer have included the characterization of various molecular isoforms of PSA\(^8\text{-}^{10}\). Among those various isoforms, the proteolytically active subpopulation of PSA is accepted as a more useful tumor marker and malignancy predictor than the serum PSA concentration\(^11\text{-}^{12}\). Simple detection of the presence of PSA by a traditional immunostaining method can not reveal the proteolytic activity of PSA; therefore, it is of great importance to develop new methods to discriminate the proteolytically active isoform. Seminal fluid has been demonstrated to carry an abundance of proteolytically active PSA and is a biological source of PSA for protease activity assays\(^13\text{-}^{14}\). The concentration of proteolytically active PSA in seminal fluid is at 10-150 \(\mu\)M\(^14\), while its concentration in the plasma is much lower, from less than 0.1nM in healthy individuals to higher than 1nM in patients with prostate disease\(^15\). However, an assay that measures the proteolytic activity of PSA in seminal fluid or biopsy samples from fine needle aspiration is still not widely accepted, due to the quick decay of the proteolytic activity, and the limited amount of seminal fluid available from old patients or biopsy samples.

The sensitivity of current detection methods reaches subnanomolar concentrations for PSA protein\(^16\text{-}^{18}\).
(mostly determined by the binding affinity of the antibody to PSA), and relatively large sample volume (milliliter) is required. However, the enzymatic assays have not enjoyed the same sensitivity enhancement. Here we introduce a new optical spectroscopic detection method for PSA proteolytic activity based on a Raman tagged PSA specific substrate peptide\textsuperscript{7,12-14,19} conjugated on crescent-shaped nanoparticles, which can be used on minute sample volumes (femtoliters), integrated into microfluidics, or introduced intracellularly, and be used to optically monitor PSA proteolytic reactions in real time. Other proteases play similar roles in cancers as well, thus this proof-of-concept nanoprobe has the potential to be further refined and improved to be used as a general measurement tool for other proteases in cancer. The nanocrescent particles serve as an individual surface enhanced Raman scattering (SERS) substrate\textsuperscript{20}. Raman spectroscopy is a spectroscopic detection method for probing biochemical composition with abundant atomic level information without fluorophore labeling\textsuperscript{21}, however the Raman signal intensity (scattering cross-section) is much lower than fluorescence. Various SERS substrates have been developed to enhance the weak Raman scattering signals in chemical and biomolecule detections on the substrate surface over several orders of magnitude \textsuperscript{20,22-25}. 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 1c).

Materials and Methods

**PSA preparation**

PSA was purchased from CalBiochem (San Diego, CA). Cleavage of the substrate peptide immobilized on the Au nanocrescent was performed in buffer of 50mM Tris-HCl, pH 8.0, 100mM NaCl, and 0.1mM EDTA, and the reaction was monitored in real-time in 37°C. PSA inhibitor was obtained from CalBiochem and added to the reaction solution following the manufacturer’s instructions, so that the final reaction solution contained 5µM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 4.2nM Aprotinin, 200nM Elastatinal and 10nM 1,5-dansyl-L-glutamyl-L-glycyl-L-arginine.
chloromethylketone (GGACK).

**Peptide Synthesis**

Biotin-Ttds-HSSKLQLAAAC-NH₂ (1). 200 mg (0.140 mmol) of Rink Amide AM polystyrene resin (loading 0.69 mmol/g) was added to a 6 mL fritted syringe and swollen with dimethylformamide (DMF) (4 mL). The fluorenylmethoxycarbonyl (Fmoc) protecting group was removed [treatment with 20% piperidine in DMF (2 mL) for 25 min], and the resin was filtered and washed with DMF (3 x 3 mL). To load the α-amino acid residues, the resin was subjected to repeated cycles of coupling conditions, followed by washing (3 x 3 mL) of DMF, Fmoc deprotection [treatment with 20% piperidine in DMF (2 mL) for 25 min], and washing again (3 x 3 mL of DMF). The conditions used for coupling the α-amino acids to the resin were subjection of the resin to a 0.4 M solution of the suitably protected acid [Fmoc-Cys(Trt, trityl)-OH (375 mg), Fmoc-Ala-OH (199 mg), Fmoc-Leu-OH (226 mg), Fmoc-Gln(Trt)-OH (391 mg), Fmoc-Lys(Boc, tert-butoxycarbonyl)-OH (300 mg), Fmoc-Ser(O-t-Bu)-OH (245 mg), or Fmoc-His(Trt)-OH (397 mg)] (0.640 mmol) which had been pre-activated by incubation with DIC (100 µL, 0.640 mmol) and HOBt (98 mg, 0.64 mmol) in DMF (1.5 mL) for 10 min. Each coupling was allowed to proceed for 4 h. After coupling and deprotection of the final α-amino acid residue, the Ttds linker was added by subjection of the resin to a 0.4 M solution of N1-(9-fluorenylmethoxycarbonyl)-1,13-diamino-4,7,10-trioxatridecan-succinamic acid (Fmoc-Ttds-OH, 303 mg, 0.560 mmol) which had been pre-activated by incubation with DIC (88 µL, 0.56 mmol) and HOBt (86 mg, 0.56 mmol) in DMF (1.2 mL) for 10 min. The coupling was allowed to proceed overnight. The resin was washed with DMF (3 x 3 mL), the Fmoc protecting group was removed, and the resin was washed again with DMF (3 x 3 mL). The biotin group was incorporated by adding a slurry of biotin (137 mg, 0.560 mmol), PyBOP (281 mg, 0.540 mmol), and i-Pr₂NEt (94 µL, 0.54 mmol) in anhydrous DMF (1.5 mL) to the resin. After agitating the resin overnight, the resin was washed thoroughly with 20% piperidine in DMF (1 x 4mL), DMF (3 x 4 mL), THF (3 x 4 mL), MeOH (3 x 4 mL), THF (3 x 4 mL).
mL), and CH₂Cl₂ (3 x 4 mL). The substrate was cleaved from the resin by incubation with a solution of 94:2:2:2 TFA/triisopropylsilane/H₂O/ethanedithiol (3 mL) for 1 h, purified using preparatory C18 reverse-phase HPLC (CH₃CN/H₂O-0.1% TFA, 5-60% for 50 min, 8 mL/min, 210/220/254 nm detection for 100 min, tᵣ = 31.8 min), and lyophilized. The purity was checked by HPLC-MS analysis (CH₃CN/H₂O-0.1% TFA, 5-95% for 14 min, 0.4 mL/min, 220 nm detection for 22 min, tᵣ = 6.5 min). MS (ESI), m/z calcd for C₇₁H₁₂₁N₁₉O₂₂S₂: 1655.8. Found: m/z 828.2 (M + 2H)⁺.

R19-Ava-HSSKLQLAAAC-NH₂ (2). 401 mg (0.277 mmol) of Rink Amide AM polystyrene resin (loading 0.69 mmol/g) was added to a 12 mL fritted syringe and swollen with N-methylpyrrolidinone (NMP) (4 mL). The Fmoc protecting group was removed by treatment with 1:2:2 piperidine/NMP/CH₂Cl₂ solution (3 mL) for 30 min, and the resin was filtered and washed with NMP (3 x 3 mL) and CH₂Cl₂ (3 x 3 mL). To load the α-amino acid residues, the resin was subjected to repeated cycles of coupling conditions (method A or method B), followed by washing (5 x 3 mL NMP, 5 x 3 mL CH₂Cl₂), Fmoc deprotection [treatment with 1:2:2 piperidine/NMP/CH₂Cl₂ solution (3 mL) for 30 min], and washing again with NMP (5 x 3 mL) and CH₂Cl₂ (5 x 3 mL). The first α-amino acid residue was loaded by addition of a preformed solution of Fmoc-Cys(Trt)-OH (1.17 g, 2.00 mmol), PyBOP (1.04 g, 2.00 mmol), and HOBr (270 mg, 2.00 mmol) in 1:1 NMP/CH₂Cl₂ (2 mL) onto the resin and the resulting slurry was stirred for 5 min on a wrist-action shaker, followed by addition of i-Pr₂EtN (0.55 mL, 4.0 mmol). The reaction was allowed to proceed for 5 h. The resin was then filtered, washed (5 x 3 mL NMP, 5 x 3 mL CH₂Cl₂), and dried under high vacuum. The loading of Cys was determined to be 0.60 mmol/g (78% yield). Successive couplings were achieved either by method A or method B. Method A consists of addition of a preformed solution of Fmoc-protected amino acid [Fmoc-Cys(Trt)-OH (1.17 g, 2.00 mmol), Fmoc-Ala-OH (622 mg, 2.00 mmol), Fmoc-Leu-OH (707 mg, 2.00 mmol), Fmoc-Gln(Trt)-OH (1.22 g, 2.00 mmol), Fmoc-Ser(tBu)-OH (767 mg, 2.00 mmol), and Fmoc-His(Trt)-OH (1.24 g, 2.00 mmol)], PyBOP (1.04 g, 2.00 mmol), and HOBr (270 mg, 2.00 mmol) in NMP/CH₂Cl₂ (1:1, 2 mL), followed by addition of i-Pr₂EtN (0.55 mL, 4.0 mmol). The reactions were allowed to proceed for at
least 4 h. Method B consists of subjection of the resin to a 0.4 M solution of the suitably protected acid [Fmoc-Lys(Boc)-OH (375 mg)], which had been pre-activated by incubation with DIC (130 µL, 0.84 mmol) and HOBt (108 mg, 0.800 mmol) in DMF (2 mL) for 10 min. The coupling was allowed to proceed for 4 h. After each coupling the resin was filtered and washed (NMP: 5 x 3mL, CH₂Cl₂: 5 x 3 mL), followed by removal of the Fmoc protecting group. After coupling and deprotection of the final α-amino acid residue, the aminovaleric acid linker was added by subjection of the resin to a 0.4 M solution of Fmoc-S-Ava-OH (272 mg, 0.800 mmol) which had been pre-activated by incubation with DIC (120 µL, 0.80 mmol) and HOBt (108 mg, 0.800 mmol) in NMP (1 mL) for 10 min. The coupling was allowed to proceed overnight. The resin was filtered and washed (5 x 3mL NMP, 5 x 3 mL CH₂Cl₂), the Fmoc protecting group was removed, and the resin washed again. The rhodamine group was incorporated by adding a 0.4 M solution of rhodamine 19 (412 mg, 0.8 mmol), which had been pre-activated by incubation with DIC (130 µL, 0.84 mmol) and HOBt (108 mg, 0.800 mmol) in NMP (2 mL) for 10 min. The reaction was allowed to proceed for 6 h, the coupling procedure was repeated once more and the reaction was allowed to proceed overnight. The substrate was cleaved from the resin by incubation with a solution of 94:2:2:2 TFA/triisopropylsilane/H₂O/ethanedithiol (3 mL) for 2 h, purified using preparatory C18 reverse-phase HPLC (CH₃CN/H₂O-0.1% TFA, 5-95% for 50 min, 20 mL/min, 220/254/280 nm detection for 100 min, tᵣ = 24.3 min), and lyophilized. MS (MALDI), m/z calcd for C₇₈H₁₁₆N₁₉O₁₇S: 1622.85. Found: m/z 1623.90.

**SERS Spectroscopy**

A microscopy system with Raman spectrometer was used to acquire Raman scattering spectra from single nanocrescents. The system consisted of a Carl Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Germany) equipped with a digital camera and a 300 mm focal-length monochromator (Acton Research, MA) with a 1024 × 256-pixel cooled spectrograph CCD camera (Roper Scientific, NJ). A 785 nm semiconductor laser was used in our experiments as the excitation source of Raman scattering,
and the laser beam was focused by a 40X objective lens on the nanocrescent. The excitation power was measured by a photometer (Newport, CA) to be ~0.8 mW. The Raman scattering light was then collected through the same optical pathway through a long-pass filter and analyzed by the spectrometer.

**RESULTS**

The 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\textsuperscript{20}. The fabrication details were described previously\textsuperscript{20}. In this paper, the polystyrene nanoparticle core is not removed and it serves as the internal control in the SERS detections. We then tether on the surface of the Au nanocrescent a substrate peptide that can be specifically cleaved by proteolytically active PSA. The peptides contain the sequence of HSSKLQLAAAC which has been shown to have very high specificity for proteolytically active PSA\textsuperscript{26}. It has been shown that HSSKLQ-L is cleaved by PSA but not by any other proteases *in vivo* in a mouse model\textsuperscript{27}. 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 (Fig. 1a) or Rhodamine 6G (R19) (Fig. 1b) 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 cleavage 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 attached peptide is amplified by the nanocrescent. Our numerical simulation (Fig. 1c) 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.

Figure 2 shows the experimental system configuration. The peptide-conjugated nanocrescents are incubated with PSA molecules in a closed transparent microchamber. The microchamber is mounted on a 37°C thermal plate on an inverted Raman microscope with darkfield illumination for nanoparticle visualization. The inset pictures show the ~0.8 mW excitation laser spot focusing on a single nanocrescent.

The typical SERS spectra of the peptide-conjugated nanocrescents with biotin and R19 Raman tag molecules are shown in fig. 3a and 3b, respectively. By comparing the SERS spectra before and 2 hours after the peptide digestion experiments, the Raman peaks from the polystyrene core, e.g. 1003 cm$^{-1}$, remains constant, which serves as an internal control. 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. 3a and 1183 cm$^{-1}$ from R19 in fig. 3b, almost completely disappear after the digestion reaction is finished (Fig. 3).

The digestion reaction dynamics can be monitored by time-resolved SERS spectra acquisitions. Because ~100 peptides are used in conjugation reaction for each nanocrescent on average, the disappearance of the characteristic Raman peaks from the tag molecules is not abrupt. Since most of the enhanced field is 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 in this high enhancement area is less than 20, even if assuming the conjugation efficiency is 100% (Fig. 1d). As shown in the time-lapse SERS spectra in Fig. 3a, the digestion 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. 3b). These changes can be tracked with real-time monitoring of the SERS spectra, as depicted in Fig. 4.

In order to specifically inhibit the PSA-mediated proteolysis of the conjugated peptides, protease 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. 5b shows the time-lapse SERS spectra measurement 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. The inability for Granzyme B to cleave the peptide is also expected as PSA has been shown to be the only protease for the HSSKLQ sequence \textit{in vivo}\textsuperscript{27}.

The digestion rate is related to the PSA concentration (Fig. 5) and we observed PSA activity in 30 min for a concentration way below 1nM (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. 1c). Fig. 5a 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. 5b 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 the averages of 5 independent measurement results and 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 and the enzyme concentration, specificity and inhibition can be differentiated from the observed reaction rates with high statistical significance; therefore this peptide-conjugated nanocrescent can be used as a specific screening tool to provide information on the concentration and proteolytic activity of the cancer biomarker PSA.

**DISCUSSION**

In conclusion, we have demonstrated the *in vitro* detection of proteolytically active PSA using a single peptide-conjugate nanocrescent SERS probe with at least nanomolar sensitivity. We used a highly focused laser beam as our excitation source, and the detection volume is only about 10 femtoliter. The actual PSA molecule number for the nanomolar samples is close to the single molecule level. Higher sensitivity and signal level can be achieved with other SERS substrates with higher enhancement factors and the same detection scheme can be applied. The Raman signals would be significantly larger if we had used higher cross-section molecules such as crystal violet, malachite green, or anthracene and a green excitation laser. 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. Compared to fluorescence-based assays, the Au nanoparticle will not photobleach, and can withstand the continuous illumination during real-time measurement. The small volume requirement and sensitivity level makes it possible to detect PSA activity in captured circulating prostate cancer cells for indications of metastasis, which is not feasible with conventional techniques. In semen, the PSA concentration is 10-150µM, with approximately two thirds of the PSA enzymatically active. 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 can easily envision that semen can be diluted 1000 times before we take an accurate and quantitative protease measurement. Due to the lack of active PSA in serum, this assay will not be useful for detection of PSA protease activity in the plasma. However, as mentioned above, circulating prostate cancer cells, if captured using surface markers, will secrete active PSA, and the concentration range has been suggested to be in mid nanomolar range, as observed in xenograft mouse models\textsuperscript{26,27}. Our assay will be suitable for this situation. In the current generation design, the PSA digestion site is between and the Glutamine (Q) and Leucine (L) residues, and is very close to the Au surface, thus the PSA peptide could be sterically hindered from the PSA enzyme and not optimally accessible. We envision that, with an additional spacer synthesized in between the substrate peptide sequence HSSKLQ-L and the Cys residue, we can improve the presentation of PSA substrate peptide HSSKLQ-L on the surface and thereby increase the detection sensitivity. The real-time reaction monitoring also provides critical information on PSA activity rather than just measuring the presence of the protein. Given the spectroscopic detection system with higher sensitivity, the reaction dynamics can be tracked at higher temporal resolution and the single event of proteolysis event can be possibly visualized. 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. The core can be made of magnetic material to allow spatial addressing of individual nanoparticles\textsuperscript{28}. The nanocrescent can also be manipulated by laser to address at high accuracy spatially\textsuperscript{29}, so that it could be multiplexed as high density arrays (with sub-microliter volume). Additional spatial multiplexing for multiple proteases in a microarray or nanoarray format is under investigation in our laboratories. In addition, the magnetic or laser maneuverability allow biosensing at desired locations\textsuperscript{28}, which would be useful for obtaining \textit{in situ} measurements intracellularly. The sub-milliwatt laser excitation power and near infrared wavelength can prevent tissue damages in future living cell imaging experiments.
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Figure Captions

Fig. 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, with the crescent tip showing lighter density. 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 proteolytic 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 after 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. (c) (1) Simulated local electric field amplitude enhancement by nanocrescent. The tip region of the nanocrescent has an electromagnetic enhancement factor of 100 fold. (2) Polar electric field energy distribution on the nanocrescent. Almost 100% energy is concentrated near the tip area which accounts for ~1/6 of total area of the nanocrescent.

Fig. 2 SERS microspectroscopy system and nanocrescent visualization. The peptide-conjugated nanocrescents are suspended in the reaction buffer in an enclosed transparent microchamber. The nanocrescents can be visualized using the darkfield illumination from oblique angles as the bright dots shown in the inset pictures. The excitation laser is focused on the nanocrescents by a microscopy objective lens. The SERS signal is collected by the same objective lens and analyzed by a spectrometer.
**Fig. 3** Representative SERS spectra of peptide-conjugated nanocrescents before and after PSA digestion reactions with (a) biotin and (b) R19 as the Raman tag molecules respectively. The spectra are offset in the plot in order to displaying all the spectra clearly.

**Fig. 4** 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 spectra are offset in the plot in order to displaying all the spectra clearly.

**Fig. 5** 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. The error bars represent the 95% confidence interval value at each data point. The inset plot shows the peak intensity change in percentage after 30 min reactions for each concentration. (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. The error bars represent the 95% confidence interval value at each data point. The inset plot shows the initial reaction rate (slope of reaction curve) for the above three conditions in the unit of percentage change per minute.
Figure 1

(a) Polystyrene nanoparticle

(b) Peptide-conjugated Nanocrescent

(c) Enhancement [dB]

(1) Electric Field Energy Distribution [%]

(2) Degree
Figure 2
Figure 3

(a) Before Digestion
After Digestion

Relative Intensity [a.u.]
Raman Shift [cm$^{-1}$]

Before Digestion
After Digestion

(b) Before Digestion
After Digestion

Relative Intensity [a.u.]
Raman Shift [cm$^{-1}$]

Figure 3
Figure 4
Figure 5

(a) Normalized peak intensity at 525 cm\(^{-1}\) over time.

(b) Normalized peak intensity at 1183 cm\(^{-1}\) over time.

- 420 nM PSA
- 420 nM PSA + Inhibitor
- 420 nM Granzyme B