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Sensitive surface enhanced Raman scattering multiplexed detection of matrix metalloproteinase 2 and 7 cancer markers

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Abstract: A surface enhanced Raman spectroscopy (SERS) based platform was developed for sensitive multiplexed detection of matrix metalloproteinases (MMP) (MMP-2 and MMP-7) with low limit of detection and high specificity. Detection is based on the virtue of enzymatic reaction where a peptide can be cleaved only by its corresponding enzyme. The platform comprises two components, a specialized SERS-based bimetallic-film-over-nanosphere (BMFON) substrate and gold nanoparticles (AuNPs). The two components were functionalized such that binding between the two would occur through biotin-avidin-biotin complexation. Binding is hindered by MMP peptide chains conjugated onto the surfaces of the substrate and AuNPs, and can be removed only by cleaving the peptide chains with corresponding enzymes. Since AuNP binding sites become free after the peptides are cleaved, the number of binding sites for AuNPs onto the substrate would increase. By tagging the AuNPs, concentrations of MMP-specific enzymes can be quantified through examining intensities of signature SERS peaks of the tags. This cleave-and-bind mechanism was first validated by individual detection and quantification of MMP-2 and MMP-7. The platform was demonstrated to be able to sensitively detect concentrations of specific enzymes ranging from 1 ng/mL to 40 µg/mL, with close correlation between SERS intensity and concentrations. Finally, the multiplexed detection of MMP-2 and MMP-7 was demonstrated. The multiplexity of this platform provides a robust way to analyze diseases associated with MMP-2 and MMP-7 enzymes. Our work can be further developed as a clinical diagnostic tool to detect other MMP proteinase in bio-fluids samples, widening the number of biomarkers needed to characterize diseases better.

References and links


Many approaches have been developed to detect MMPs, including gel zymography [24, 25], enzyme-linked immunosorbsent assay (ELISA) [26], fluorescence resonance energy transfer (FRET) [27], and colorimetric method [28]. Although sensitive, gel zymography and
ELISA typically require long and tedious preparations, and are restricted in their applications due to low-throughput assaying. FRET detects MMPs based on tagging a fluorophore and quenching pair on the opposite ends of the peptide. The two ends separate upon MMP enzyme cleavage of the peptide, resulting in intensified fluorescence intensity. However, FRET possesses the inherent problem of photo-bleaching in molecular probes and high toxicity associated with the use of quantum dots. In colorimetry, Peng Chen et al. demonstrated its use for MMP-7 detection with a detection limit of 0.1µg/mL [28]. However, as MMP physiological concentrations in respective carcinomas are between a few ng/ml to µg/ml [8, 9, 29, 30], this method is not sensitive enough, especially for early stage cancer diagnosis. Furthermore, as there are several types of carcinomas that correlate with multiple MMPs, achieving accurate analysis and diagnosis require monitoring of the concentrations of multiple MMPs level simultaneously.

To address these issues, we proposed a SERS based sensing platform to achieve sensitive multiplexed detection of MMP-2 and MMP-7 proteases. This platform adopts a SERS-active planar substrate known as BMFON [31]. It comprises multiple nanostructures formed by depositing polystyrene nanospheres onto a glass slide, followed by systematically sputtering a silver (Ag) underlayer and gold (Au) overlayer to impart a gold-silver bimetallic coat. Classical film over nanosphere (FONs) which are single-layered in nature are typically made of silver for its strongly enhancing property. However, Ag is susceptible to atmospheric oxidation. The merit of BMFON lies in retaining this property while insulating it from oxidation by means of gold over-coating. More importantly, the outer Au layer is advantageous to biosensing because of its binding affinity to thiol groups that assist in bioconjugation. The BMFON substrate was first functionalized with biotin-PEG-thiol through thiol-gold bonding. NeutrAvidin was then conjugated to the substrate through strong biotin-avidin binding. In order to detect MMP-2 and MMP-7 enzyme, enzyme-specific MMP-2 (G-K(TAMRA)-G-P-L-G-V-R-G-C-CONH2) and MMP-7 (G-K(TAMRA)-G-V-P-L-S-L-T-M-G-C-CONH2) peptide were adopted. As both peptides have thiol-containing amino acids cysteine (C), BMFON substrate’s active surface can be conjugated with MMP-2 and MMP-7 peptides through thiol-gold interaction [32]. Two types of biotinylated AuNPs were prepared, of which one was tagged with 4-aminothiophenol (ATP), and the other with 2-naphthalenethiol (NT). ATP and NT are commonly used SERS reporter molecules which can provide strong unique SERS peaks at 1584cm\(^{-1}\) and 1377cm\(^{-1}\), respectively, from their ν(CC) ring stretch [33, 34]. The ATP-tagged and NT-tagged AuNPs were then conjugated with MMP-2 and MMP-7 peptide, respectively. The detection is based on the virtue of enzymatic reaction where a peptide can be cleaved only by its corresponding enzyme. To perform detection, these AuNPs were loaded onto the BMFON substrate, and the binding between AuNPs and BMFON via biotin-avidin interaction is initially blocked by long chains of the MMP peptide present on AuNPs and substrate that hinder such binding. The binding of AuNPs on BMFON can only been observed after peptide chain cleavage by presence of corresponding MMP enzyme (the scheme of detection mechanism were shown in Fig. 1). As the number of AuNPs binding on BMFON is proportional to the amount of peptide cleavage, the concentration of the MMP enzyme can be quantified by monitoring the SERS intensity of Raman tag on AuNPs. Since the type of AuNP that binds on BMFON is enzyme-specific, multiplexed detection of MMP-2 and MMP-7 enzymes can be achieved by identifying the signature peaks of the Raman tags and noting their intensities.
2. Materials and methods

2.1 BMFON substrate fabrication

Clean microscope glass slides were cut into 10mm x 10mm squares. They were then sonicated in a bath of ethanol for 20 min and dried before using. 400 µL of monodispersed polystyrene (PS) colloidal suspension (Ø=400 nm, 2.5 wt%, Kisker) was centrifuged at 7,000 rpm for 10 mins. After removing the clear supernatant, 50 µL of water and 50 µL of surfactant sodium dodecyl sulfate (SDS) (15 wt%, Sigma Aldrich) were added to form a composite solution. To form a monolayer of closely packed PS spheres on the glass slides, 5 µL of the prepared colloidal solution was loaded onto the center of each glass slide. Each glass slide was spin coated at 2,000 rpm for 20 s and placed in a desiccator overnight at 0.6 Pa. Each glass slide was then sputter-coated with 80 nm sublayer of Ag and 40nm of Au overlayer using JEOL JFC-1600 auto fine coater.

2.2 Conjugation of BMFON

Substrates were first incubated in 50 µM biotin PEG thiol (Nanocs) solution for 30 mins and rinsed with water. 15µL of NeutrAvidin (5mg/mL in PBS solution, Life technologies) was subsequently dropped onto the SERS-active surface and incubated for 3 hrs. 1mM of MMP-2 peptide (G-K(TAMRA)-G-V-P-L-G-V-R-G-C-CONH₂) and 1mM of MMP-7 peptide (G-K(TAMRA)-G-V-P-L-S-L-T-M-G-C-CONH₂) were dissolved in 1x phospho-buffered saline and 0.005M EDTA (pH 7.4) separately. As both peptides have thiol-containing amino acids cysteine (C), these peptides will therefore bind to BMFON through thiol-gold interaction. For initial verification, MMP-2- and MMP-7-conjugated BMFON substrates were prepared by incubating biotin-avidin conjugated BMFON substrates in MMP-2 and MMP-7 peptide solution, respectively. In order to perform multiplexed detection, BMFON substrate conjugated with a mixture of MMP-2 and MMP-7 were prepared by incubating biotin-avidin conjugated BMFON in solution containing 50% of MMP-2 and 50% of MMP-7 peptides. After 3hrs incubation, these BMFON substrates were rinsed with water to remove unbound peptide before use.

2.3 AuNPs preparation and characterization
For initial verification, two types of AuNPs were prepared.

ATP tagged AuNPs: 1.9mL of 60 nm diameter AuNPs (OD=1.19 @ 520nm, 2.60×10^{10} particles/mL, BBI Solutions) were mixed with biotin PEG thiol (50µL, 20µM) and ATP (50µl, 10µM) for 15 mins and centrifuged twice at 8,500 rpm for 1min. After removing the supernatant, water was added to make a final volume of 200 µL.

NT tagged AuNPs: The AuNPs were prepared in the same process as described above but with NT (50µl, 30µM) instead of ATP and centrifuged at 9,000 rpm.

For multiplexed detection of MMP-2 and MMP-7 peptides, two types of AuNPs were prepared.

MMP-2 conjugated AuNPs: NT tagged AuNPs were prepared as described above, but with a single round of centrifugation was applied. After removal of the supernatant, 1.9 mL of water and 0.25 µL of 1mM MMP-2 peptide were mixed with the AuNPs for 2 hrs. Following centrifugation, excess MMP-2 peptides were washed away and water was added to make a final volume of 200 µL.

MMP-7 conjugated AuNPs: MMP-7 conjugated AuNPs was prepared in the same process as described above but with ATP tagged AuNPs and MMP-7 peptide.

Hydrodynamic diameters of these AuNP were studied by using a dynamic light scattering (DLS) system (Zetasizer Nano series, Malvern).

2.4 Detection of individual MMP enzyme

MMP-2 and MMP-7 enzymes (0.1mg/mL, Sigma Aldrich) were diluted in 0.001 µg/mL, 0.01 µg/mL, 0.1 µg/mL, 0.5 µg/mL, 1 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL and 40 µg/mL using buffered solutions containing 50 mM Tris-Cl, 5 mM CaCl2 and 0.005% Brij-35 at pH 7.4. A buffered solution containing no enzyme was used as control.

MMP-2 enzyme detection: MMP-2 conjugated BMFON substrate was incubated with 100 µL of MMP-2 enzyme solution at 37°C for 3 hrs. The substrates were then rinsed with water and 20 µL of NT tagged AuNPs was loaded. After incubation for 2 hrs, the substrates were rinsed again to remove unbound AuNPs, SERS measurement was then performed.

MMP-7 enzyme detection: The same process as described above was carried out using MMP-7 conjugated BMFON substrate and ATP tagged AuNPs.

2.5 SEM Sample preparation

Three BMFON substrate samples were prepared.

Clean BMFON substrate: newly prepared BMFON substrate without any conjugation.

MMP-7 conjugated BMFON substrate: as described above

AuNPs bound BMFON: prepared as same process as described in MMP-7 enzyme detection, were 20µg/ml of MMP-7 enzyme was used.

2.6 Multiplexed detection of MMP-2 and MMP-7 enzymes

Four types of enzymes were prepared: 0.5µL of MMP-2 (0.1mg/mL), 0.5µL of MMP-7 (0.1mg/mL), 0.5µL solution contain 0.25µL of MMP-2 (0.1mg/mL) and 0.25µL of MMP-7 (0.1mg/mL), 0.5µL of buffer solution as control.

Each of MMP-2- and MMP-7-conjugated BMFON substrate were place in solution containing 50 µL of MMP-2 conjugated AuNPs, 50 µL of MMP-7 conjugated AuNPs and 0.5µL of enzyme solution. After 2 hrs of incubation at 37°C, the substrates were rinsed with water and performed with SERS measurement.
2.7 SERS measurement

SERS spectra were obtained using a Raman microscope system (Renishaw InVia) with a Peltier cooled CCD detector and a laser excitation wavelength at 785 nm. The laser was coupled through a 50× objective lens, which was used to collect the Stokes-shifted Raman signal. Rayleigh scattering was blocked with a notch filter. In this study, the samples were excited with a laser power of ~1.09 mW with 10 s exposure time throughout the measurements. The instrument was calibrated with a silicon standard at a Raman peak of 520 cm\(^{-1}\).

3. Results and discussion

3.1 Characterization of the BMFON

To evaluate the cleave-and-bind mechanism of the enzyme detection platform, SEM imaging was performed on the unfunctionalized BMFON substrate, MMP-7 conjugated BMFON substrate, and AuNPs-bound BMFON. As shown in Figs. 2(a) and 2(d), a large area of monolayer of hexagonally and closely packed spheres was formed on the glass slide due to the self-assembly mechanism. These metal-coated nano-structures are capable of generating plasmonic hot spots in the crevices between spheres upon laser excitation, thus strongly enhancing the Raman signal from molecules on the substrate. The far-filed property of BMFON is investigated by UV-vis diffuse reflectance experiment. The measured spectrum indicates a maximum LSPR absorption at 566 nm. The SEM images of peptide conjugated BMFON substrate were shown in Figs. 2(b) and 2(e). It is clearly observed that there is a thin layer of peptide coated on the substrate, resulting in a fuzzy image of the nano-structures. This layer of peptide serves to shield the substrate from binding of AuNPs. To understand the relative distribution of peptide on substrates, we carried out SERS mapping after incorporating peptide to substrates. In order to generate Raman signal from peptide, we embedded a Carboxytetramethylrhodamine (TAMRA) segment in MMP-2 and MMP-7 peptide, allowing the distribution of peptide to be examinable by SERS mapping of the strong Raman peak originated from TAMRA at 1354 cm\(^{-1}\). SERS mapping is a method for generating detailed chemical images based on a sample’s Raman spectrum. A complete spectrum is acquired at each and every pixel of the image in the selected area, and then interrogated to generate false color images based on intensity of a signature Raman peak. The SERS mapping of the distribution of TAMRA peak intensity at 1354 cm\(^{-1}\) over a 20 µm x 20 µm selected area on peptide conjugated substrate was shown in red color in Fig. 2(g). This mapping result confirms that peptide is distributed uniformly on the SERS substrate. In contrast, the TAMRA peak intensity is much weaker after incubating the substrate with MMP enzyme (Fig. 2(h)), indicating that a majority of the peptide has been cleaved by the enzyme. This can be also indicated from SEM imaging in Figs. 2(c) and 2(f), in which peptide layer disappears, indicating successful cleaving of peptide layer by enzyme. As a result, large amount of bound AuNPs on the nano-structures could be observed.
3.2 Validation of the mechanism

To further validate the mechanism for enzyme detection, we perform a quantitative study by detecting individual MMP-2 or MMP-7 enzyme. The binding of AuNPs onto BMFON substrate occurs when the peptide on the substrate is cleaved by the corresponding enzyme.
thus allowing binding on the biotin-avidin bond. By notion of the proportionality between the amount of AuNPs bound on BMFON substrate and peptide cleavage, the concentration of enzyme can be quantified by the intensity of target peaks of Raman tags. In this experiment, MMP-2 peptide- and MMP-7 peptide-conjugated BMFON were incubated with different concentrations of enzymes of MMP-2 and MMP-7, respectively. After peptide cleavage, NT- and ATP-tagged AuNPs were then loaded on their corresponding substrates. The AuNPs bound substrates were then measured using Raman spectroscopy. Peaks at 1584 cm\(^{-1}\) and 1377 cm\(^{-1}\) were selected for intensity analysis as they are the most prominent and unhindered Raman peaks for ATP and NT, respectively (Figs. 3(a) and 3(b)). We first investigated the SERS spectra of BMFON after bound with biotin-PEG-SH and biotin-avidin complex. As shown in Figs. 3(c) and 3(d), the SERS intensities are weak and show no significant difference between BMFON with biotin and biotin-avidin complex. More importantly, the major peak at 1631 cm\(^{-1}\) is not interference with ATP or NT peaks. The SERS spectra after peptide cleavage and AuNPs binding were then investigated. Here, intensities of ATP and NT peaks increase with increasing enzyme concentrations, as shown in Figs. 3(e) and 3(f). For BMFON incubated with control buffer containing no enzyme, the ATP or NT peak are negligible, indicating that the peptides have fully covered the substrates, thus preventing the binding of the AuNPs. The intensity curves of ATP and NT with enzyme concentrations are plotted in Figs. 3(g) and 3(h). As tagged AuNPs bind onto substrates in a sandwich manner, thus greatly enhancing Raman intensities of ATP and NT. Therefore, the signal is detectable even in extremely low enzyme concentration. In the case of high enzyme concentration, a large majority of peptide is cleaved and AuNPs are fully bound to the substrate, causing SERS intensity of tagged AuNPs to saturate. The observations made above indicate that this platform is highly sensitive to MMP-2 and MMP-7 enzymes and can provide a wide detection range from 0.001 µg/mL to 40 µg/mL.
Fig. 3. Detection of individual MMP enzymes. Chemical structure and Raman spectra of (a) ATP and (b) NT. SERS spectra of (c) biotin-PEG–SH bound BMFON and (d) NeutrAvidin-biotin-PEG–SH bound BMFON. (e) SERS spectra of ATP tagged AuNPs bound MMP-7 conjugated BMFON after incubation with different concentrations of MMP-7 enzyme. (f) SERS spectra of NT tagged AuNPs bound MMP-2 conjugated BMFON after incubation with different concentrations of MMP-2 enzyme. (g) Intensity curve of the ATP Raman peak at 1584 cm⁻¹ (x-axis is log10 scaled). (h) Intensity curve of the NT Raman peak at 1377cm⁻¹ (x-axis is log10 scaled).
3.3 Multiplexed detection of MMP-2 and MMP-7 enzymes

To extend this platform for multiplexed detection of MMP-2 and MMP-7 enzymes, the BMFON substrates were conjugated with both MMP-2 and MMP-7 peptide whereas the NT- and ATP-tagged AuNPs were conjugated with MMP-2 peptide and MMP-7 peptide, respectively. In order to prove that the MMP peptides are able to fully shield the AuNPs, hydrodynamic diameters of the untagged AuNPs, tagged AuNPs and MMP shielded AuNPs were studied using DLS. The mean hydrodynamic diameter of untagged AuNPs was found to be 65.35nm, while the size increased to 69.00nm after biotin conjugation and tagging with Raman reporter. After peptide shielding, the mean hydrodynamic diameter of MMP-7 conjugated AuNPs and MMP-2 conjugated AuNPs further increased to 71.47nm and 73.14nm, indicating that a covering outer shell of MMP peptide has formed on AuNPs. In demonstrating the specificity in AuNP binding, four samples were prepared for detection: MMP-2 enzyme, MMP-7 enzyme, a mixture of MMP-2 and MMP-7 enzymes, and a control buffer containing no enzymes. As MMP enzyme cleaves only its designated peptide conjugated on the BMFON substrate and on its corresponding AuNP, allowing for specific labeling, multiplexed detection can be achieved from the unique Raman peaks and their intensities. As shown in Fig. 4 (a), in the presence of control buffer, neither MMP-2 nor MMP-7 peptide is cleaved from BMFON substrate and AuNPs, resulting in no binding of AuNPs and, in turn, leading to negligible peak intensity at 1377cm$^{-1}$ and 1584cm$^{-1}$. In Fig. 4(b), the MMP-2 enzyme cleaves MMP-2 peptides from BMFON and the NT-tagged AuNPs, allowing for sandwich formation between the substrate and AuNP, yielding a sharp 1,377 cm$^{-1}$ peak characteristic of NT. It is worthy to note that the 1584 cm$^{-1}$ peak of ATP is negligible because no MMP-7 enzyme was present to cleave the MMP-7 peptide from BMFON and ATP-tagged AuNP for binding. Conversely in Fig. 4(c), ATP peak at 1584 cm$^{-1}$ could be observed while NT peak is negligible in the presence of MMP-7 enzyme. As in the case of the enzyme mixture, both NT and ATP peaks could be observed (Fig. 4(d)). Intuitively, due to the presence of the two types of enzymes, both types of peptides were cleaved and AuNP binding to substrate could occur. In order to understand the Raman peaks, we tentatively assigned all major peaks observed in our study, as shown in Table 1.

![Fig.4. Multiplexed detection of MMP-2 and MMP-7 enzymes. Raman spectrum of MMP-2 and MMP-7 shielded BMFON substrate samples in the presence of (a) control solution contain no enzyme (b) MMP-2 enzyme (c) MMP-7 enzyme (d) both MMP-2 and MMP-7 enzyme.](image-url)
Table 1. Peak Assignment of Raman Spectra

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<tr>
<td>1354</td>
<td>CH$_3$ bend, TAMRA</td>
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<tr>
<td>1377</td>
<td>C=C ring stretching, NT</td>
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<tr>
<td>1534</td>
<td>amide II, TAMRA</td>
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<tr>
<td>1584</td>
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<tr>
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4. Conclusion
We successfully demonstrated a SERS based platform for sensitive multiplexed detection of MMP-2 and MMP-7 proteinase using cleave-and-bind mechanism. To the best of our knowledge, this is the first reported SERS based MMP enzyme detection. This platform demonstrated a wide detection range from 1ng/mL to 40µg/mL for individual enzyme detection, thus it is able to detect the enzyme concentration from various clinical samples with different stages of cancers. In comparison to previously reported methods, our detection platform is simple and fast, providing readings from a much wider detection range with higher sensitivity. As a proof-of-concept, we successfully demonstrated the multiplexed detection for MMP-2 and MMP-7 enzymes as many cancer types have a close correlation to both enzyme expression levels, the multiplexed detection capability of this platform therefore provides a robust way to analysis these diseases. More importantly, this platform can be developed to clinical diagnostic tool to detect other MMP proteinase using the cleave-and-bind mechanism in bio-fluids samples.

Acknowledgments
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