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Multilayer gold nanoparticle-assisted protein tryptic digestion in solution and in gel under photothermal heating

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Abstract Elevating the reaction temperature is an effective method to accelerate protein enzymatic digestion because it can promote protein denaturation and enzyme activities. In this study, we demonstrated a new photothermal heating method to assist protein tryptic digestion on glass slides. A glass slide coated with layer-by-layer gold nanoparticles (Glass@AuNPs), combined with the use of a near infrared (NIR) diode laser, was used to raise reaction temperature during tryptic digestion in a short period of time. The modified glass slide is capable of absorbing NIR light arising from the dipole–dipole interactions between Au NPs immobilized on the slide. The temperature of Glass@AuNPs rapidly increased when irradiated by the NIR laser, accelerating protein enzymatic digestion conducted on the slide. Thus, when performing the tryptic digestion of proteins on the Glass@AuNPs slide under NIR irradiation, 3.5 min was sufficient to carry out the tryptic digestion of proteins in solution, while less than 5 min was adequate for in-gel tryptic digestion of proteins. Matrix-assisted laser desorption/ionization mass spectrometry was used for characterization of the tryptic digestion product. On the basis of the results, the time taken to analyze proteins could be greatly reduced using this current approach.

Keywords Gold NPs · Tryptic digestion · Photothermal · MALDI-MS

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Introduction

The development of biological mass spectrometry and bioinformatics promoted the progress of proteomic analysis. Nevertheless, time-consuming sample pretreatment steps are normally required. Among them, a critical pretreatment step is protein enzymatic digestion, which is generally performed prior to conducting mass spectrometric analysis. However, enzymatic digestion usually takes several hours. Thus, efforts have been devoted to accelerate the reaction [1–13]. Optimizing reaction temperature [1, 3–13], enzyme concentration [2, 7], and degree of protein denaturation [1, 5, 7] could greatly decrease the time required for enzymatic digestion. Elevating the reaction temperature could effectively promote protein denaturation and enzyme activities. On the other hand, if the temperature is elevated to over a certain degree, the activity of enzymes is affected. Thus, reaction temperatures should be adjusted to a proper level for remaining enzyme activity. Microwave- [3–8], thermocycler- [9], ultrasound- [10], and infrared-assisted [11, 12] enzymatic digestion have been successfully employed to reduce the time required for analysis.

Nanoparticles (NPs) have been extensively used in many research fields owing to their unique features. For example, on the basis of the visible color of spherical Au NPs, the NPs have been used as effective sensing probes to recognize specific biomolecules [14–17]. Furthermore, the color of spherical Au NPs can be adjusted from red to dark blue by increasing the particle size of the NPs or by adjusting the distance between the NPs. When spherical Au NPs are self-assembled layer-by-layer (LBL) on a substrate, that LBL Au NPs have an enhanced ability to absorb visible light, and a new absorption band occurs in the near infrared (NIR) region arising from the dipole–dipole interactions between Au NPs [18–20]. Additionally, the

λ_{max} in the NIR region can be adjusted by modifying the particle size of the spherical Au NPs.

Using an IR lamp as the light source, IR-assisted enzymatic digestion has been demonstrated in previous studies [11, 12]. The IR lamp has been directly used to irradiate enzymatic digestion samples mainly based on the absorption capacity of water at the far IR region. We believed that the time required for enzymatic digestion could be alternatively reduced by conducting IR-assisted protein enzymatic digestion on a substrate capable of absorbing IR light. Thus, we used LBL self-assembled Au NP-glass slides (Glass@AuNPs) capable of absorbing NIR light as the sample deposition substrate and light absorption medium. Proteins in solution and in gel were used as model samples to demonstrate the feasibility of this approach in accelerating protein enzymatic digestion on the Glass@AuNPs slide. A human serum sample separated by gel electrophoresis was also examined using the current approach.

Experimental

Reagents and materials Trifluoroacetic acid (TFA), acetonitrile, and hydrochloric acid were obtained from Merck (Seelze, Germany), while hydrogen peroxide, ammonium hydrogen carbonate (98%), sulfuric acid (95%), trisodium citrate (99.5%), and glycerol (99%) were obtained from Riedel–de Haën (Seelze, Germany). Acetic acid (99.8%), nitric acid (65%), and melittin were purchased from Fluka (Buchs, Switzerland). Methanol was purchased from Tedia (Fairfield, OH, USA). Acrylamide/bis (29:1, 40%), tris hydrochloride, and sodium dodecyl sulfate (SDS) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Tetrachloroacurate (99%) was obtained from Showa (Tokyo, Japan). *N*-[3-(trimethoxysilyl)propyl]-ethylenediamine (80%) (EDAS) and α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Aldrich (Milwaukee, WI, USA). Cytochrome C (horse), myoglobin, bovine serum albumin (BSA), human immunoglobulin G (IgG) trypsin (from bovine pancreas), tosyl phenylalanyl chloromethyl ketone (TPCK) treated, tris[hydroxymethyl]-aminomethane, brilliant blue R, brophenol bluse sodium salt, *N*,*N*,*N*,*N*-tetramethyl ethylenediamine (99%), and ammonium persulfate were obtained from Sigma (St. Louis, MO, USA). Glass slides were purchased from Matsunami (Osaka, Japan). Human serum samples were obtained from a healthy individual.

Preparation of Au NPs All glassware and stirring bars were rinsed with a HNO_3/HCl (1/3, *v*/*v*) solution, and then by deionized water. Au NPs were prepared using the Frens method [21]. An aqueous tetrachloroaurate solution (0.1 mg/mL, 50 mL) was heated to boiling point, to which trisodium citrate (0.1%, 0.3 mL) was added while stirring. The color change was observed, and the solution turned from pale yellow to gray black, and then to red violet, at which point monodisperse spherical particles were formed. After cooling to room temperature, the suspension of Au NPs was centrifuged at 2,500 rpm for 10 min. The supernatant was removed using a pipette. The remaining NPs (~51 nm) were re-suspended in a small amount of deionized water. The suspension (0.1 mL) was diluted to obtain optical density (OD) ~6 at a wavelength of 540 nm.

Fabrication of Glass@AuNPs slides Glass slides were pretreated by soaking in a piranha solution [H_2SO_4/H_2O_2, (3:1, *v*/*v*)] for 30 min to remove impurities. They were then washed with water and methanol under sonication. The glass slides were stored in methanol before use. The slides were dried using a hairdryer before surface modification. The surface of each slide was modified by depositing a thin film of 0.5 mL EDAS prepared in deionized water (0.25% *v*/*v*) for 10 min. The slide was rinsed with deionized water to remove unbound EDAS, dried with a hairdryer, and heated in an oven at 120 °C for 1 h. The surface of each slide was then coated with the Au NP suspension (0.2 mL) prepared via electrostatic interactions after the slide was cooled to room temperature. After standing at ambient temperature for 12 h, the slides were rinsed with deionized water to remove unbound Au NPs and were dried using a hairdryer. Afterwards, EDAS and Au NPs were coated twice onto the surface of the slides to generate an LBL Glass@AuNPs.

Protein tryptic digestion on slides Stock solutions (1 mg/mL) including cytochrome C, myoglobin, BSA, and IgG were prepared in deionized water. Protein samples were then diluted to given concentrations using deionized water. Trypsin was prepared in ammonium hydrogen carbonate (0.1 M, pH 8). The protein sample (0.2 mg/mL, 8 μL) was mixed with trypsin (0.08 mg/mL, 2 μL). The mixture (8 μL) was deposited on a Glass@AuNPs (or unmodified glass) slide, and an NIR laser (λ=808 nm) equipped with an optical fiber irradiated the slide from a distance of 2.5 or 3.0 cm with corresponding laser power outputs at ca. 390 and 356 mW, respectively. The power was measured by a PM-10 Laser power meter (Coherent). The temperatures on the Glass@AuNPs slide irradiated by the NIR laser from a distance of 2.5 and 3.0 cm for 1 min were raised to 53.8± 0.7 °C and 49.2±0.8 °C, respectively, measured by a Raytek MT4 thermometer (Santa Cruz, CA, USA). After a certain period of time, 0.5 μL of CHCA (10 mg/mL) prepared in the solution of acetonitrile/0.15% TFA (1/1, *v*/*v*) was mixed with the remaining solution (~0.5 μL) on the slide. The mixture was deposited onto a matrix-assisted laser desorption/ionization (MALDI) target. After solvent

evaporation, the sample was ready for MALDI-mass spectrometry (MS) analysis. The irradiation time was varied, mainly depending on the irradiation distance. In general, 3~5 min of light irradiation was required for protein tryptic digestion using this approach. Additionally, the Glass@AuNPs slide could be repeatedly used because the tryptic digestion was performed on the side that was free of Au NP coating. The side with sample deposition could be easily cleaned after the experiment using proper solvents without destroying the Au NP coating on the other side. Control experiments were conducted by digesting proteins using conventional overnight incubation at 37 °C. The protein concentration of the control sample was 160 ng/μL.

In-gel protein tryptic digestion on slides Protein samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [22]. After separating the proteins via SDS-PAGE, gels were stained with Coomassie brilliant blue R-250 to examine protein bands. The gel was then de-stained with a methanol/acetic acid/deionized water solution (4:1:5, *v*/*v*/*v*) under shaking at a speed of 50 rpm for 1 h. The protein band (ca. 1 mm^3) excised from the gel was placed into a tube followed by mixing with acetonitrile (0.1 mL). The mixture was gently vortex-mixed for 10 min, and the remaining solution was discarded. The excised gel was placed onto a Glass@AuNPs slide (or unmodified glass slide) under irradiation of the NIR laser with a distance of 1.5 cm for 5 min to eliminate the remaining acetonitrile from the gel. The dried gel was mixed with trypsin (10 μL, 0.05 mg/mL) prepared in aqueous ammonium hydrogen carbonate (100 mM) in an Eppendorf tube. The mixture was mixed by pipetting the solution within the pipette tip for 1 min under an ice bath followed by standing at ice bath for 5 min, which provided sufficient time for the gel to soak up the trypsin solution. The swollen gel was then placed onto a Glass@AuNPs slide, and aqueous ammonium hydrogen carbonate (100 mM, 4 μL) was added onto the gel to adjust the pH of the gel to an appropriate digestion condition. The gel was irradiated by the NIR laser for a given time from a distance of 2.5 or 3 cm. Subsequently, 5% TFA (10 μL) prepared in acetonitrile/deionized water (1:1, *v*/*v*) was added to the gel followed by ultrasonication for 15 min to extract the digestion product out of the gel. The extract (0.5 μL) was mixed with CHCA (10 mg/mL, 0.5 μL) prepared in acetonitrile/0.15% TFA (1/1, *v*/*v*) on a MALDI target. The sample was ready for MALDI-MS analysis after solvent evaporation.

Instrumentation All mass spectra were obtained using a Bruker Daltonics Biflex III time-of-flight (TOF) mass spectrometer (Bremen, Germany) equipped with a 337-nm nitrogen laser and a Bruker Daltonics Autoflex III TOF/TOF mass spectrometer (Bremen, Germany) equipped with a 355-nm laser. The mass spectral data was submitted via Biotools (v. 3.0) to MASCOT (www.matrixscience.com) using the following search parameters: the database searched was NCBI, the enzyme was trypsin, and MS tolerances were set at ±0.5 Da. Mass spectra were acquired from 100 laser shots. The absorption spectra were obtained using a Varian Cary 50 spectrophotometer (Melbourne, Australia).

Results and discussion

To fabricate a glass slide with the absorption capacity in the NIR region of the electromagnetic spectrum, the glass slide was coated with LBL Au NPs (~51 nm). Figure 1a displays the representative absorption spectrum of the glass slide coated with LBL Au NPs. In addition to the absorption band appearing at the wavelength of ~530 nm, another absorption band reveals at the NIR region. We then employed the glass slide as the sample holder for protein

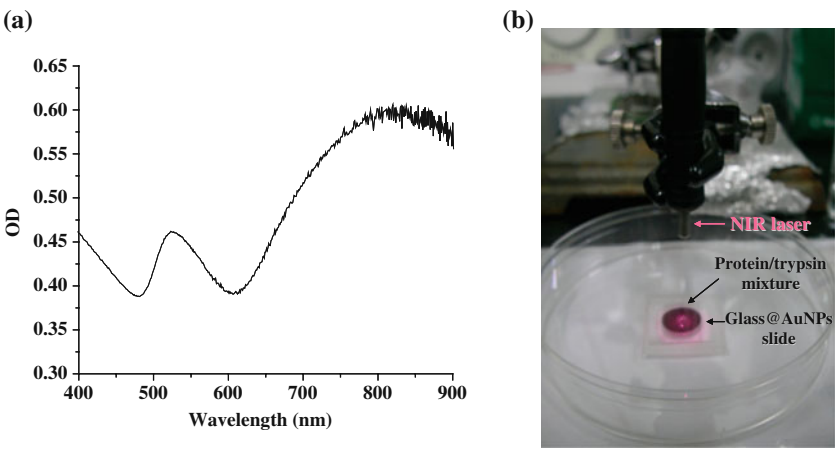

Fig. 1 **a** Representative absorption spectrum of the Glass@AuNPs slide. **b** Photograph of the setup of protein tryptic digestion conducted on Glass@AuNPs slide under illumination of an NIR laser (808 nm)

enzymatic digestion and the absorption medium for NIR light. The slide has a maximum absorption band at the wavelength of ~800 nm. Figure 1b presents the setup of the NIR-assisted protein tryptic digestion. Sample solution (8 μL) was deposited on the Glass@AuNPs slide, and an NIR laser (808 nm) was used as the light source. The laser power and heating rate depend on the distance between the glass slide and the NIR laser; thus, different irradiance distances were examined during on-slide enzymatic digestion. Figure 2a–d displays the MALDI mass spectra of the tryptic digest of cytochrome C on the Glass@AuNPs slide under irradiation of the NIR laser with the distance of 4, 3.5, 3, and 2.5 cm, respectively. The irradiation time varied with the irradiating distance. We stopped the light irradiation as the sample volume reduced to ~0.5 μL due to water evaporation, which occurred during heating. When the NIR laser was placed above the slide at a distance of 4 cm, a weak protein signal (MH^+) appeared in the mass spectrum (Fig. 2a) of the tryptic digest of cytochrome C conducted on the Glass@AuNPs slide under NIR light irradiation. Some peptide peaks appeared in the low mass region. However, the protein signal disappeared as the laser moved closer to the glass slide, suggesting that the tryptic digestion tended to be completed at the irradiation distance of 2.5~3.5 cm.

Generally, it took 4.5 and 3.5 min to reduce the sample volume to ~0.5 μL at the irradiance distance of 3 and 2.5 cm, respectively. Figure 2e, f presents the results obtained from control experiments. Figure 2e presents the MALDI mass spectrum of the tryptic digest of cytochrome C performed on an unmodified glass slide under illumination of NIR light for 3.5 min. The singly (MH^+) and doubly charged cytochrome C ions (MH_2^{+2}) appear in the mass spectrum. Few peptide peaks appear at m/z <3,000 in the mass spectrum. However, digestion efficiency is relatively poor compared with the result in Fig. 2d. The results demonstrate that the Glass@AuNPs slide could effectively assist the acceleration of protein tryptic digestion under illumination of NIR light. Figure 2f presents the MALDI mass spectrum of the tryptic digest of cytochrome C conducted on a Glass@AuNPs slide for 3.5 min without irradiating with NIR light. As can be seen, the singly, doubly, and triply charged cytochrome C ions dominate the mass spectrum. No peptide ions are observed in the low mass region. That is, without irradiating with NIR light, nearly no enzymatic digestion proceeded within 3.5 min. On the basis of the control and experimental results in Fig. 2, both NIR light and Glass@AuNPs are required components to assist the acceleration of protein tryptic digestion.

We then examined the digestion efficiency in terms of sequence coverage and MASCOT scores. When the distance between the glass slide and the NIR laser decreased to 2.5 cm, the sequence coverage obtained from on-slide tryptic digestion was 95% (see Table 1), and there were 20 peptides derived from cytochrome C observed in the MALDI mass spectrum (Electronic Supplementary Material Table S1). When conducting tryptic digestion of

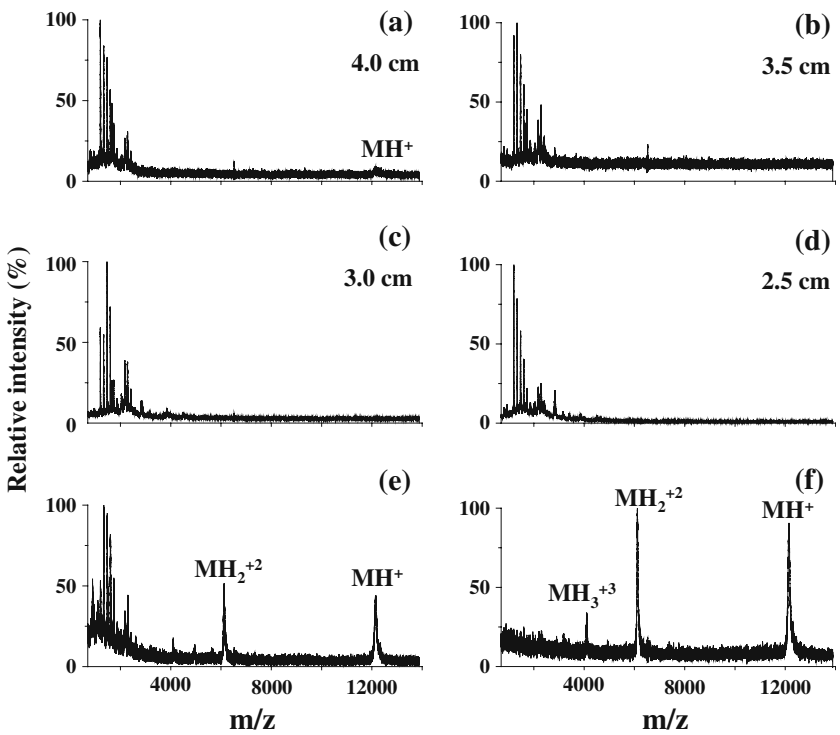

Fig. 2 MALDI mass spectra of the tryptic digest of cytochrome C performed on the Glass@AuNPs slide under irradiation of an NIR laser (808 nm) with the irradiance distances of (**a**) 4 cm (irradiance time, 6.5 min), (**b**) 3.5 cm (irradiance time, 5.5 min), (**c**) 3 cm (irradiance time, 4.5 min), and (**d**) 2.5 cm (irradiance time, 3.5 min). **e** MALDI mass spectrum of the tryptic digest of cytochrome C performed on an unmodified glass slide under illumination of NIR light for 3.5 min. **f** MALDI mass spectrum of the tryptic digest of cytochrome C performed on the Glass@AuNPs slide at room temperature for 3.5 min

Table 1 Peptide ion peaks observed in the MALDI mass spectra obtained from NIR-assisted tryptic digestion of cytochrome C and its control experiment

Observed $[M+H]^+$	Calculated $[M+H]^+$	Missed cleavage	Sequence	Control (160 ng/μL)	160 ng/μL	1.6 ng/μL
779.6	779.4	0	MIFAGIK (81–87)		[a]	
907.9	907.5	1	MIFAGIKK (81–88)		[a]	
1,168.6	1,168.6	0	TGPNLHGLFGR (29–39)	[a]	[a]	
1,296.5	1,296.7	1	TGPNLHGLFGRK (29–40)	[a]	[a]	[a]
1,306.5	1,306.7	1	GEREDLIAYLK (90–100)	[a]	[a]	
1,433.5	1,433.8	1	HKTGPNLHGLFGR (27–39)	[a]	[a]	[a]
1,434.6	1,434.8	2	GEREDLIAYLKK (90–101)	[a]	[a]	[a]
1,456.5	1,456.7	0	TGQAPGFSYTDANK (41–54)	[a]	[a]	
1,561.6	1,561.9	2	HKTGPNLHGLFGRK (27–40)		[a]	[a]
1,562.7	1,562.9	3	KGEREDLIAYLKK (89–101)	[a]		[a]
1,584.6	1,584.8	1	KTGQAPGFSYTDANK (40–54)	[a]	[a]	
1,606.6	1,606.9	3	MGDVEKGKKIFVQK (1–14)		[a]	
1,633.4	1,633.8	1	IFVQKCAQCHTVEK (10–23)	[a]	[a]	
1,675.7	1,675.9	2	GGKHKTGPNLHGLFGR (24–39)			[a]
1,698.6	1,698.8	1	TGQAPGFSYTDANKNK (41–56)		[a]	[a]
1,761.6	1,761.9	2	KIFVQKCAQCHTVEK (9–23)	[a]	[a]	
1,803.9	1,804.0	3	GGKHKTGPNLHGLFGRK (24–40)	[a]	[a]	[a]
1,826.8	1,826.9	2	KTGQAPGFSYTDANKNK (40–56)	[a]	[a]	[a]
1,849.8	1,850.0	3	GEREDLIAYLKKATNE (90–105)			[a]
1,875.7	1,875.9	2	IFVQKCAQCHTVEKGGK (10–26)		[a]	
2,009.9	2,009.9	0	GITWGEETLMEYLENPK (57–73)	[a]	[a]	
2,138.0	2,138.0	1	GITWGEETLMEYLENPKK (57–74)	[a]	[a]	[a]
2,797.8	2,797.4	2	GITWGEETLMEYLENPKKYIPGTK (57–80)		[a]	

[a] The peaks were observed in the mass spectra

cytochrome C at 37 °C for 12 h as a control experiment, few peaks appeared in the mass spectrum. The detailed sequences of the matched peaks are listed in Table 1. The sequence coverage is 75%, and 14 peaks matched the theoretical data (Electronic Supplementary Material Table S1). The corresponding MASCOT scores obtained from NIR-assisted and conventional tryptic digestion samples were 300 and 235, respectively (Electronic Supplementary Material Table S1). In addition, the reproducibility of this approach was also evaluated by performing NIR-assisted tryptic digestion on five different Glass@AuNPs slides (A, B, C, D, and E). Three sets of experiments (1~3) were performed on a same slide (Electronic Supplementary Material Table S2). Cytochrome C was used as the sample. The number of expected peptides is 83 when the number of missed cleavages is set at 3. Table S2 in the Electronic Supplementary Material lists the number of the peptides observed in the resulting MALDI mass spectra. Corresponding MASCOT scores are also listed in parentheses in the table. The results indicate that our current approach is comparable to the conventional one with acceptable reproducibility. Furthermore, the digestion time was dramatically reduced from 12 h to 3.5 min. We also examined the digestion efficiency of a low concentration of cytochrome C sample. When the concentration of cytochrome C was decreased to 1.6 ng/μL, tryptic digestion still proceeded quite well, and the sequence coverage of the resultant peptides observed in MALDI mass spectrum was 64% (see Table 1).

We further employed this approach to the tryptic digestion of myoglobin. Figure S1 in the Electronic Supplementary Material presents the MALDI mass spectrum of the tryptic digest of myoglobin conducted on the Glass@AuNPs slide under NIR light irradiation with an irradiation distance of 2.5 cm. The sequence coverage is 95% (see Electronic Supplementary Material Fig. S1a and Table S3), and there were 17 peptide peaks derived from myoglobin observed in the mass spectrum (Electronic Supplementary Material Fig. S1a and Table S1). Figure S1b in the Electronic Supplementary Material presents the MALDI mass spectrum of the tryptic digest of myoglobin at 37 °C for 12 h. As can be seen, fewer

peaks appear in the mass spectrum. The detailed sequences of the matched peaks are listed in Electronic Supplementary Material Table S3 and are marked as control. The sequence coverage is 70%, and only 13 peaks matched the theoretical data. The corresponding MASCOT scores obtained from experimental and control samples were 283 and 199, respectively (Electronic Supplementary Material Table S1). The results confirm that this current approach is effective in accelerating protein tryptic digestion. When the concentration of myoglobin decreased to 1.6 ng/μL, tryptic digestion still proceeded well, and the sequence coverage of the resulting peptides observed in MALDI mass spectrum was 75% (See Electronic Supplementary Material Table S3).

A larger protein, BSA, was also used as the sample to examine the feasibility of this approach. Figure S2a in the Electronic Supplementary Material presents the MALDI mass spectrum of the tryptic digest of BSA conducted on the Glass@AuNPs slide under NIR light irradiation with an irradiation distance of 2.5 cm. The sequence coverage (Electronic Supplementary Material Fig. S2a and Table S4) is 43%, and there were 27 peaks derived from BSA observed in the mass spectrum (Electronic Supplementary Material Fig. S2a and Table S1). Figure S2b in the Electronic Supplementary Material presents the MALDI mass spectrum of the tryptic digest of BSA at 37 °C for 12 h. The detailed sequences of the matched peaks are listed in Electronic Supplementary Material Table S4 and are marked as control. The sequence coverage is only 28%. Only 14 peaks matched to BSA appeared in the mass spectrum. The corresponding MASCOT scores obtained from the results in Electronic Supplementary Material Figs. S2a and S2b were 274 and 148, respectively (Electronic Supplementary Material Table S1). The experiment was also repeatedly performed to examine the reproducibility. Table S5 in the Electronic Supplementary Material summarized the results obtained from three replicates (1~3). Apparently, more peptides were identified when using the current approach for tryptic digestion than those obtained from conventional overnight digestion. Furthermore, the corresponding MASCOT scores and sequence coverage were higher than when NIR-assisted tryptic digestion was employed for tryptic digestion. Additionally, we also used human IgG as the sample. Although only a few peaks derived from IgG were observed in the resultant MALDI mass spectrum (Electronic Supplementary Material Fig. S3), those peaks were matched to the constant C region of Ig with a significant MASCOT score of 82 (Electronic Supplementary Material Table S6 and the last page in Electronic Supplementary Material). The results indicate that our current approach is suitable for the analysis of large proteins such as BSA and IgG. Furthermore, the results are comparable with those obtained using the conventional digestion method; the required digestion time is also shorter. Additionally, protein denaturation above 90 °C was required prior to IR-assisted digestion to improve the digestion efficiency previously [1, 12]. Because the heating efficiency obtained using this current approach was sufficient, the heating denaturation steps were therefore eliminated to further reduce the analysis time. Protein reductions by dithiothreitol (DTT) prior to tryptic digestion were necessary in previous work [23]; they are not required in this current approach. The protein samples used for NIR-assisted tryptic digestion experiments shown in Electronic Supplementary Material Figs. S1, S2, and S3 were all obtained without performing any protein denaturation and protein reduction steps beforehand. Nevertheless, our results were comparable with those obtained using denatured proteins as the samples. That is, shorter analysis time is one of the advantages of this current approach.

Gel electrophoresis (GE) is one of the most common methods used in proteomic analysis. To further examine this approach in practical analysis, in-gel tryptic digestion of proteins using our approach was performed. We used BSA in-gel as the sample to examine the effectiveness of this approach. Figure 3a presents the MALDI mass spectrum of in-gel tryptic digest of BSA performed on the Glass@AuNPs slide under NIR light illumination for 4.5 min. The peaks derived from BSA are marked with asterisks. Table 2 lists their corresponding sequences; the sequence coverage is as high as 45%. Figure 3b displays the MALDI mass spectrum of in-gel tryptic digest of BSA conducted at room temperature for 4.5 min. The mass spectral quality is poor, and few peaks derived from BSA (marked as asterisks) appear in the mass spectrum. Their corresponding sequences are listed in Table 2. The sequence coverage is only 12%. Generally, BSA in gel is relatively difficult to digest without conducting protein denaturation in advance. However, the in-gel tryptic digestion result using our approach is similar to that obtained from in-solution digestion (cf. Electronic Supplementary Material Fig. S2a). The results demonstrate that our approach is suitable in assisting in-gel protein tryptic digestion with a short analysis time.

To examine the feasibility of employing this approach in the analysis of real samples, we employed 100-fold diluted human serum as the model sample. The inset in Fig. 4a displays the portion of the resultant gel obtained from the human serum sample separated by GE. The band on the right-hand side is BSA and is used as a marker. The band on the left-hand side was obtained from the serum sample. Because the molecular weight of BSA is similar to that of human serum albumin (HSA), we excised the gel from the left band and conducted in-gel tryptic digestion on a Glass@AuNPs slide using the NIR-assisted approach to rapidly digest the protein in the gel. Figure 4a presents the

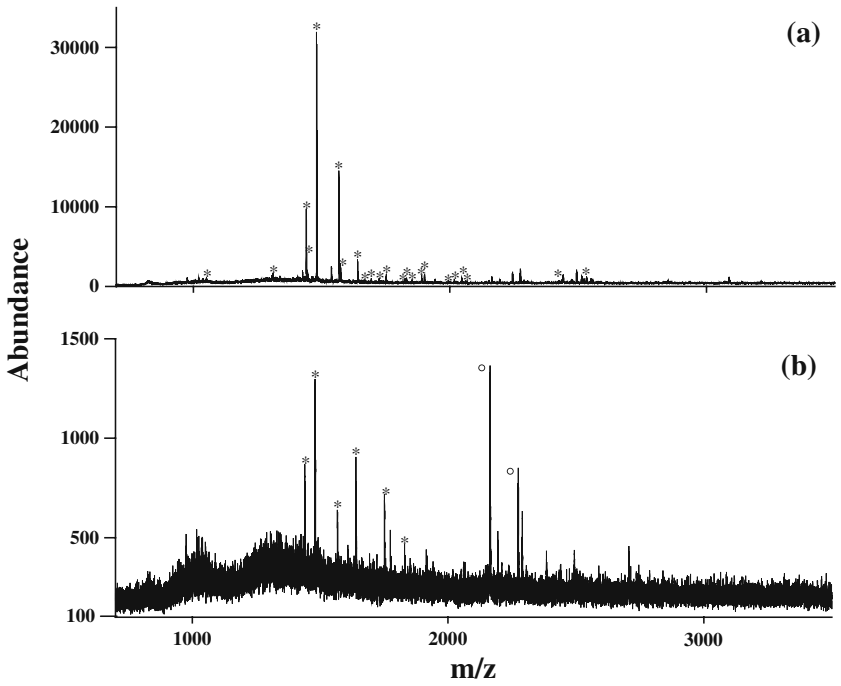

Fig. 3 **a** MALDI mass spectrum of NIR-assisted in-gel tryptic digest of BSA (2 μg) conducted on the Glass@AuNPs slide under illumination of NIR light for 4.5 min. **b** MALDI mass spectrum of in-gel tryptic digest of BSA performed at room temperature for 4.5 min. Peptides derived from BSA were marked with *asterisks*

Table 2 Peptide ion peaks observed in the MALDI mass spectra obtained from NIR-assisted tryptic digestion of the excised BSA gel conducted on Glass@AuNPs slide and its control experiment

Observed $[M+H]^+$	Calculated $[M+H]^+$	Missed cleavage	Sequence	BSA (2 μg)	Control (2 μg)
1,050.9	1,049.5	0	EACFAVEGPK (588–597)	[a]	
1,305.8	1,305.7	0	HLVDEPQNLIK (402–412)	[a]	
1,439.8	1,439.8	1	RHPEYAVSVLLR (360–371)	[a]	[a]
1,445.6	1,445.8	1	FWGKYLYEIAR (157–167)	[a]	
1,479.8	1,479.8	0	LGEYGFQNALIVR (421–433)	[a]	[a]
1,567.6	1,567.7	0	DAFLGSFLYEYSR (347–359)	[a]	[a]
1,573.9	1,574.8	2	KFWGKYLYEIAR (156–167)	[a]	
1,639.8	1,639.9	1	KVPQVSTPTLVEVSR (437–451)	[a]	[a]
1,667.7	1,667.8	0	MPCTEDYLSLILNR (469–482)	[a]	
1,692.7	1,692.9	1	AEFVEVTKLVTDLTK (249–263)	[a]	
1,723.7	1,723.8	1	DAFLGSFLYEYSRR (347–360)	[a]	
1,750.8	1,751.0	2	LSQKFPKAEFVEVTK (242–256)	[a]	[a]
1,823.9	1,823.9	0	RPCFSALTPDETYVPK (508–523)	[a]	
1,830.0	1,830.1	3	ALKAWSVARLSQKFPK (233–248)	[a]	[a]
1,850.8	1,850.9	0	LFTFHADICTLPDTEK (529–544)	[a]	
1,889.0	1,889.0	1	SLHTLFGDELCKVASLR (89–105)	[a]	
1,899.9	1,900.0	1	LGEYGFQNALIVRYTR (421–426)	[a]	
1,994.1	1,994.0	2	TPVSEKVTKCCTESLVNR (490–507)	[a]	
2,017.4	2,017.0	3	ADEKKFWGKYLYEIAR (152–167)	[a]	
2,045.1	2,045.0	1	RHPYFYAPELLYYANK (168–183)	[a]	
2,061.1	2,061.1	2	YTRKVPQVSTPTLVEVSR (434–451)	[a]	
2,424.5	2,424.2	3	DTHKSEIAHRFKDLGEEHFK (25–44)	[a]	
2,521.7	2,521.4	3	LSQKFPKAEFVEVTKLVTDLTK (242–263)	[a]	

[a] The peaks were observed in the mass spectra

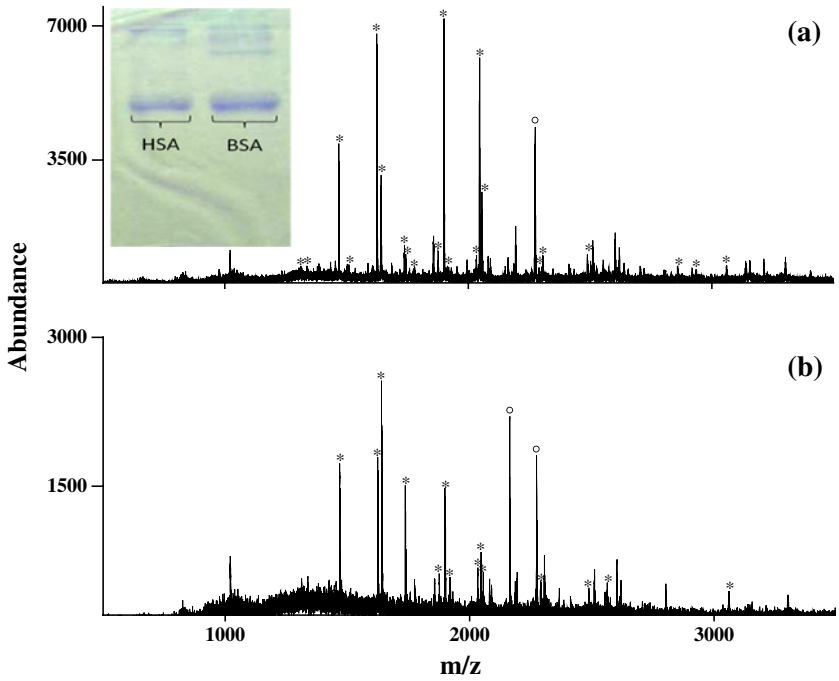

Fig. 4 **a** MALDI mass spectrum of the NIR-assisted in-gel tryptic digest of the excised gel obtained from the gel electrophoresis of a 100-fold diluted human serum sample, performed on the Glass@AuNPs slide under illumination of NIR light for 5 min. The *inset* is the resultant gel obtained from GE of the serum sample. **b** MALDI mass spectrum of in-gel tryptic digest of the excised gel obtained from the gel electrophoresis of a 100-fold diluted human serum sample, performed at room temperature for 5 min. Peptides derived from HSA were marked with *asterisks*

Table 3 Peak ions observed in the MALDI mass spectra obtained from the NIR-assisted tryptic digestion of the excised gel from serum sample conducted on the Glass@AuNPs slide and its control experiment

Observed $[M+H]^+$	Calculated $[M+H]^+$	Missed cleavage	Sequence	Serum sample	Control
1,379.9	1,379.7	2	LKCASLQKFGER (222–333)	[a]	
1,386.7	1,386.6	0	YICENQDSISSK (287–298)	[a]	
1,467.7	1,467.8	1	RHPDYSVVLLLR (361–372)	[a]	[a]
1,508.9	1,508.8	2	FGERAFKAWAVAR (230–242)	[a]	
1,623.7	1,623.8	0	DVFLGMFLYEYAR (348–360)	[a]	[a]
1,639.8	1,639.9	1	KVPQVSTPTLVEVSR (438–452)	[a]	[a]
1,736.9	1,736.9	2	LSQRFPKAEFAEVSK (243–257)	[a]	[a]
1,742.8	1,742.9	0	HPYFYAPELLFFAK (170–183)	[a]	
1,780.0	1,780.1	2	RHPDYSVVLLLRLAK (361–375)	[a]	
1,875.1	1,875.0	1	SLHTLFGDKLCTVATLR (89–105)	[a]	[a]
1,899.1	1,899.0	1	HPYFYAPELLFFAKR (170–184)	[a]	[a]
1,918.9	1,919.2	3	KQTALVELVKHKPKATK (549–565)	[a]	[a]
2,032.3	2,032.1	2	YTKKVPQVSTPTLVEVSR (435–452)	[a]	[a]
2,045.2	2,045.1	0	VFDEFKPLVEEPQNLIK (397–413)	[a]	[a]
2,055.3	2,055.1	2	RHPYFYAPELLFFAKR (169–184)	[a]	[a]
2,289.5	2,289.4	3	QTALVELVKHKPKATKEQLK (550–569)	[a]	
2,300.5	2,300.1	1	NYAEAKDVFLGMFLYEYAR (342–360)	[a]	[a]
2,488.6	2,488.1	1	EFNAETFTFHADICTLSEKER (525–545)	[a]	[a]
2,560.7	2,560.3	1	RMPCAEDYLSVVLNQLCVLHEK (469–490)		[a]
2,859.2	2,859.3	1	CCTESLVNRRPCFSALEVDETYVPK (500–524)	[a]	
2,934.5	2,934.5	2	QNCELFEQLGEYKFQNALLVRYTK (414–437)	[a]	
3,059.4	3,059.5	1	MPCAEDYLSVVLNQLCVLHEKTPVSDR (470–496)	[a]	[a]

[a] The peaks were observed in the mass spectra

MALDI mass spectrum of in-gel tryptic digestion of the band on the left-hand side conducted on a Glass@AuNPs slide under illumination of NIR light for 5 min. The peaks marked with asterisks were confirmed as the peptides derived from HSA. The corresponding sequences of these peaks are listed in Table 3. The sequence coverage is 35%. When the in-gel tryptic digestion was conducted at room temperature for 5 min, the sequence coverage was only 17% (see Fig. 4b and Table 3). The sequence coverage was 40% when in-gel tryptic digestion of HSA was performed at 37 °C for 16 h based on a previous report [12]. The results indicate that our result is slightly worse than that obtained from the conventional method. Nevertheless, the analysis time can be greatly reduced from 16 h to ≤5 min.

Conclusions

We have demonstrated a new NIR-assisted method combined with the use of Glass@AuNPs slide to accelerate enzymatic digestion of proteins in solution and in gel, reducing digestion time to less than 5 min. In addition, owing to the high heating efficiency, protein denaturation steps could be eliminated to further simplify the sample pretreatment steps. The sample volume and the gel required to successfully perform the experiment are only 8 μL and 1 mm^3, respectively. Furthermore, the sequence coverage obtained from our approach is better than existing rapid methods. The Glass@AuNPs slides could be repeatedly used because the digestion is performed on the side free of Au NP coating. Furthermore, the setup is very simple and straightforward. The advantages of this approach include good digestion efficiency, short analysis time, and ease-of-use. Additionally, we have demonstrated the feasibility of this approach to in-gel tryptic digestion of proteins derived from a human serum sample. The results suggest that this alternative photothermal heating method should be practical for being used in accelerating protein tryptic digestion either in solution or in gel. The disadvantage of this approach is that only one sample can be digested at one time. However, we believe that this approach can be employed to a large amount of samples simultaneously if the light source is changed to long-tube type of NIR lamps.

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