Supporting Information

Realization of on-tissue protein identification by highly efficient in-situ digestion with graphene-immobilized trypsin for MALDI Imaging analysis

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Experimental Section

Materials and chemicals

Graphene oxide powder was purchased from Nanjing XFNANO Materials Tech Co., Ltd. α-Cyano-4-hydroxycinnamic acid (α-CHCA), 2,5-Dihydroxybenzoic acid (DHB), Tosylphenylalanine chloromethyl-ketone (TPCK)-treated trypsin, Myoglobin from equine heart (Myo), Bovine serum albumin (BSA), alpha-casein (α-casein) from bovine milk, ammonium bicarbonate (ABC), acetonitrile (ACN) were purchased from Sigma Chemical (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore, Molsheim, France). Trifluoroacetic acid (TFA) was purchased from Merck (Darmstadt, Germany). Ethanol of analytical grade and was purchased from Shanghai Chemical Reagent Company (Shanghai, China). Unless noted otherwise, other chemicals and analytically pure reagents were used as received. An aqueous solution of GO was prepared by transferring the powder to a 1.5 mL-Eppendorf tube and suspending in water. The stock solution was stored at room temperature and used for further experiments. The trypsin was suspended in 0.1% (v/v) TFA, reaching a concentration of 2 mg mL^{-1} and was stored at -20 ^{o}C.

Characterization of graphene oxide

Transmission electron microscopy (TEM) images were taken with a JEOL 2011 microscope (Japan) operating at 200 kV. The characterization data including SEM, TEM, AFM and UV-vis absorption spectra of commercial graphene oxide were provided by the manufacturer.

Enzyme immobilization

Before immobilization, stock solution of trypsin and yellow-brown aqueous suspension of GO was diluted in phosphate buffer to the desirable concentration. In a

typical immobilization experiment, GO solution was incubated with enzyme solution at certain concentrations for a period of time at room temperature. This solution was centrifuged at 16400 rpm for 5 min and the resulting supernatant was collected. Enzyme loading on GO was measured by the difference in the amount of total enzyme used and residual enzyme in the supernatant after immobilization. The bicinchoninic acid method was used to determine the enzyme concentration by measuring the UV absorbance at 562 nm. For accurate calculation of the amount of immobilized enzyme, a calibration curve was obtained at λ=562 nm by using a series of standard protein solution with different concentrations. To study the effect of pH, phosphate buffer with different pH was prepared and used as the incubation buffer during immobilization. The succeeding adsorption procedure was the same as described before.

Digestion of standard proteins

Three proteins, Myo, BSA and α-casein were used for digestion by immobilized enzyme and free enzyme. Before digestion, proteins were diluted in 25mM ABC buffer (pH 8.1), to a concentration of 10 ng μL^{-1}. For on-plate digestion, each protein solution (1 μL) was spotted on the MALDI plate. To simulate the on-tissue digestion, all the drops of protein were dried before digestion. Either the immobilized or the free enzyme was diluted with the 25 mM ABC buffer (pH 8.1) and deposited on the MALDI plate with several cycles of aspirating and dispensing. The MALDI plate was placed at room temperature. While the enzyme solution is slowly drying, the digestion can be performed directly on the plate. 0.5 μL CHCA matrix (in 50% ACN containing 0.1% TFA) for Myo and BSA or 0.5 μL DHB matrix (in 50% ACN containing 0.1% TFA) for α-casein was dropped and dried before mass analysis.

Tissue preparation

Normal human lenses were obtained from the eye bank of Eye and ENT Hospital of Fudan University and stored at -80 ^{o}C. The research followed the tenets of the

Declaration of Helsinki and was approved by the Ethics Committee of Eye and ENT Hospital of Fudan University.

Frozen human lenses were attached to specimen chucks with the aid of a small amount of optimal cutting temperature (OCT) embedding medium. When the tissue was cryosectioned into 14-μm-thick sections at -18 ^{o}C using a Leica cryostat (Leica CM3050S, Leica Microsystems Inc, Wetzlar, Germany), the lens were cut in parallel with the equatorial plane but the cryostat blade never contacted the OCT in order to avoid the background interference. Then the sections were mounted onto glass slides.

Several circles of ACN-water solution (50:50, v/v) spray were applied resulting in a tightly bound section.

On tissue digestion of crystallins

Crystallin tissue slices were mounted onto glass slides and washed successively for 60s each in 50%, 75%, and 100% ethanol. The washing step facilitated uniform matrix crystal formation across the entire lens section by removing the interfering species such as salts and lipids. Afterward, the crystallins tissue slice was immobilized on the MALDI plate by conductive adhesive. Immobilized trypsin solution (1.5 μL, diluted in 25 mM ABC) was deposited on the tissue slice. Several cycles of aspirating and dispensing were not desirable during on tissue digestion which would lead to the delocalization of the protein easily. The MALDI plate was then placed in a home-built humidity chamber, made from a plastic box with wet paper tissues in the bottom and a tight-fitting lid, and incubated at 37 ℃ for 10 min. CHCA matrix (in 50% ACN containing 0.1% TFA) was sprayed by a pneumatic TLC sprayer on the tissue before mass analysis.

MALDI-TOF MS Process

Matrix-assisted laser desorption/ionization (MALDI) mass spectra were acquired

in reflection mode between m/z 700 and 4000 on a 5800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) with a Nd:YAG laser at 355 nm, a repetition rate of 200 Hz, and an acceleration voltage of 20 kV. GPS Explorer software from Applied Biosystems, with Mascot as a search engine and SwissProt (Version 050303) as a database, was employed for protein identification. The peptide mass tolerance was set to 80 ppm.

MSI acquisition and data processing

After digestion and matrix coating, the plates were analyzed in 5800 Proteomics Analyzer (Applied Biosystems, Framingham, MA, USA) using 4800 imaging software. Mass conditions were set as described above. Images were performed in the positive reflection mode, and MALDI-MS spectra were acquired in the m/z range of 900-3500. Two-dimensional ion density maps were created using the image reconstruction software (BioMap, Novartis, Basel, Switzerland)

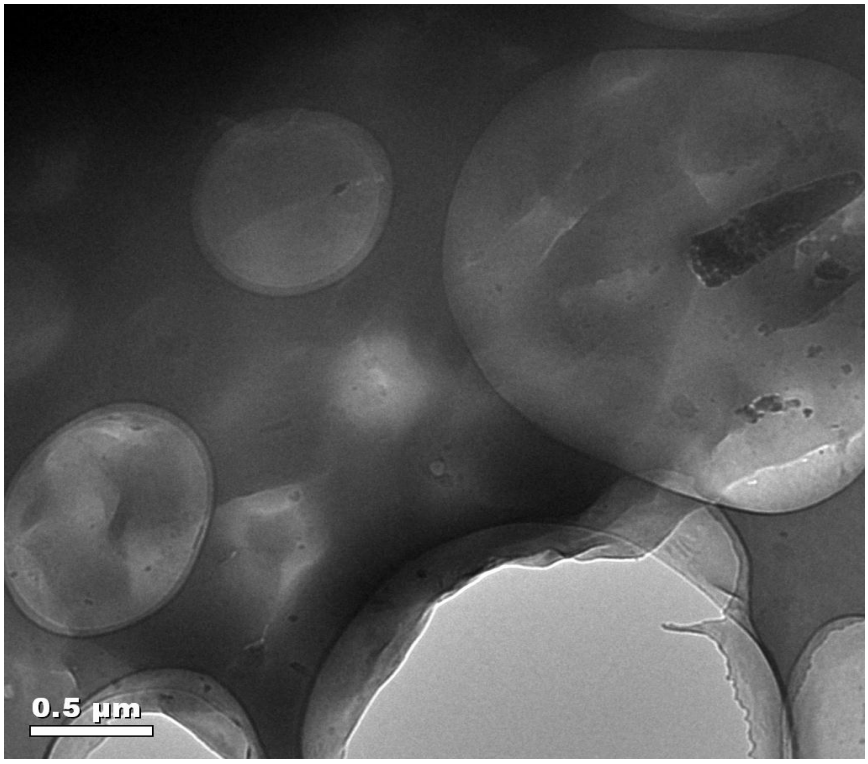

Fig. S1. TEM image of graphene oxide

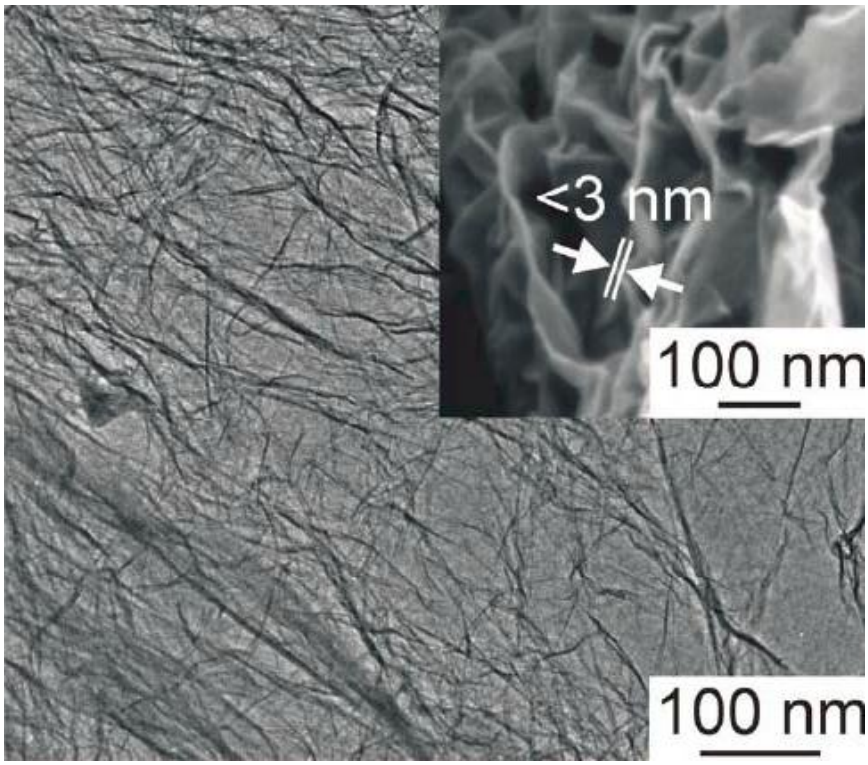

Fig. S2. TEM and SEM (inline) image of graphene oxide provided by manufacturer

The TEM and SEM show that the commercial graphene oxide was exfoliated to individual nanosheets. SEM image (inline) indicates that the graphene sheets were thinner than 3 nm

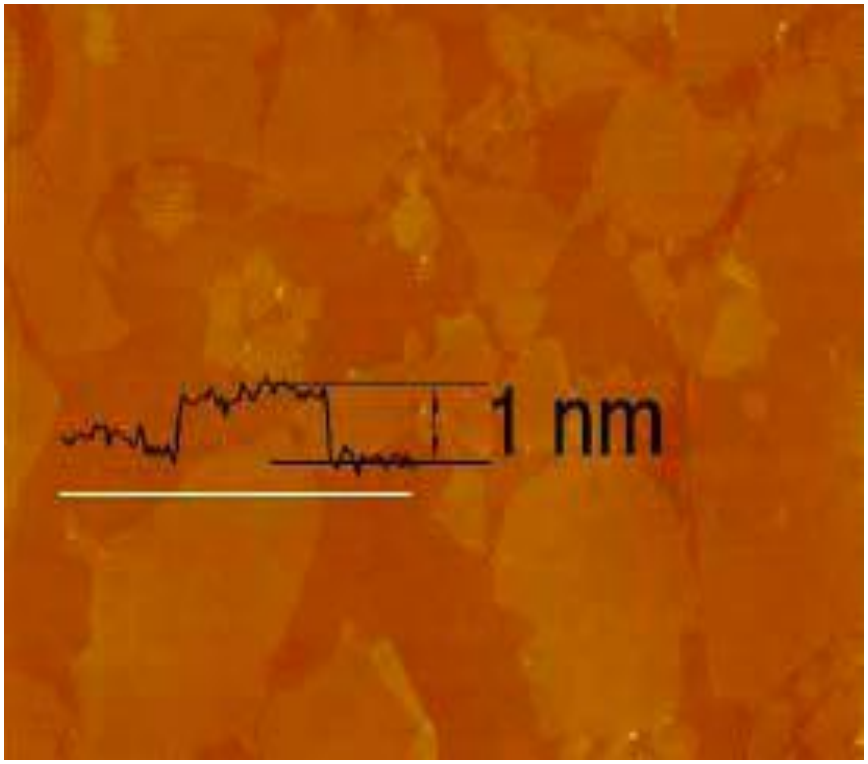

Fig. S3. AFM image of graphene oxide provided by manufacturer

As shown in Fig. S3, the height of the commercial graphene oxide sheets is about 1 nm, which confirms that they are monolayer graphene oxide, and in solution, they exist primarily as exfoliated single sheets.

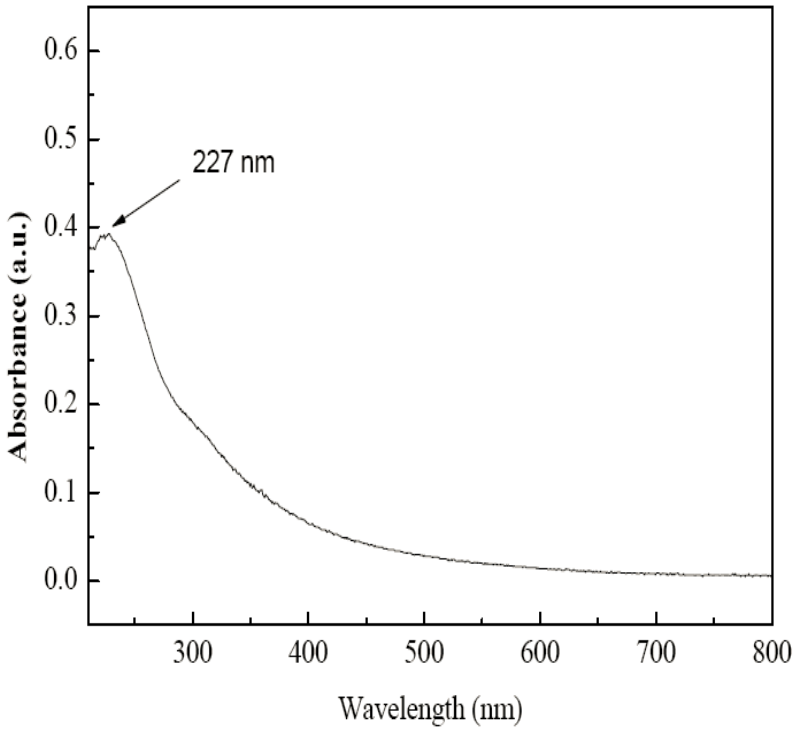

Fig. S4. UV-vis absorption spectra of graphene oxide provided by manufacturer

An absorption peak at 227 nm was observed at the commercial grapheme oxide, which was in accordance with the previous result reported.(J. Mater. Chem. 2012, 22, 8426; Electrochimica Acta. 2011, 56, 8168)

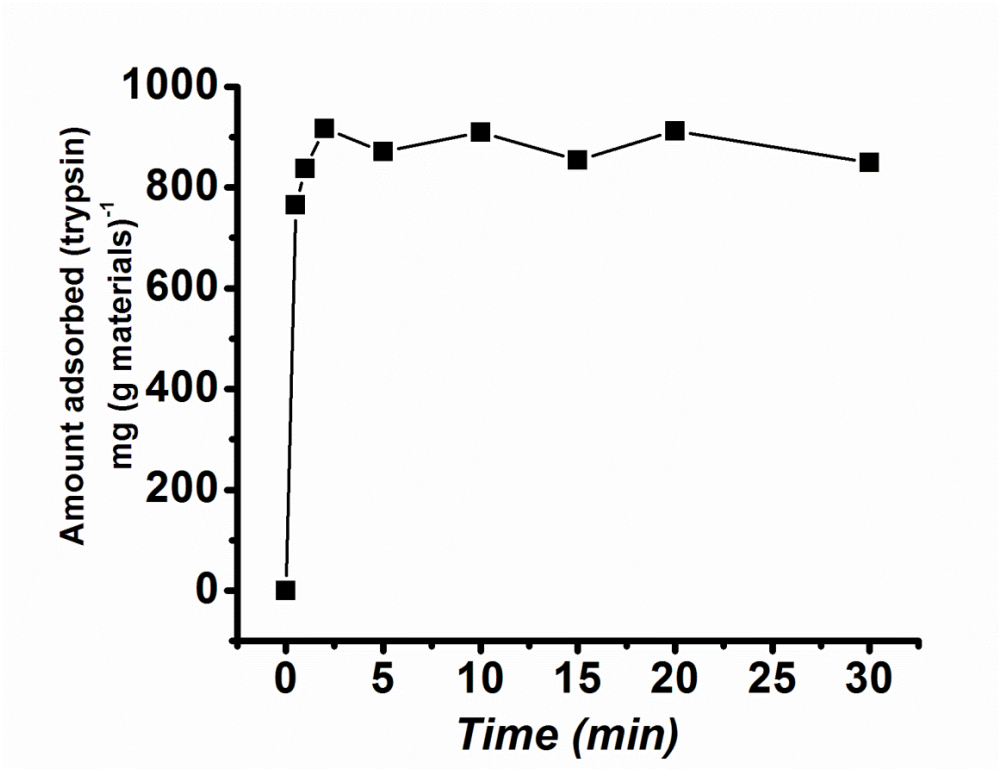

Fig. S5. Adsorption of trypsin on GO as a function of time.

As Fig. S5. showed, nearly 90% of the maximum adsorption amount can be reached in 1 min.

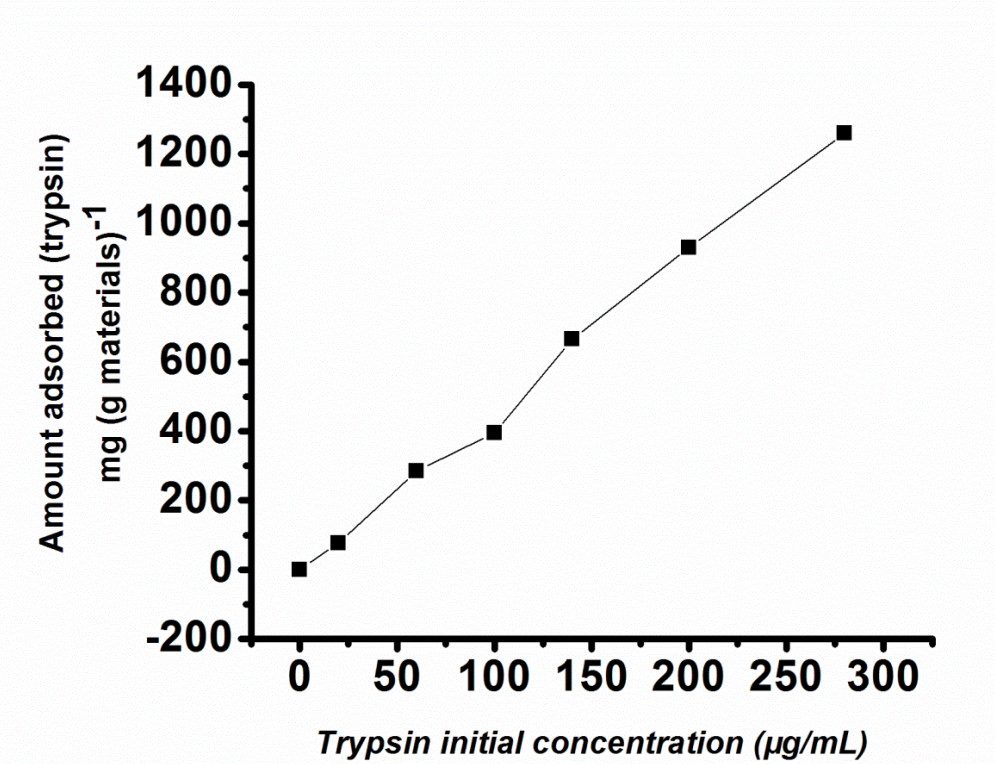

Fig. S6. Effect of the initial concentration of trypsin on the enzyme loading on GO.

When the initial concentration of the enzyme increased from 0.787 μM to 7.87 μM, the total amount of trypsin adsorbed increased from 72 μg mg^{-1} to 930 μg mg^{-1}.

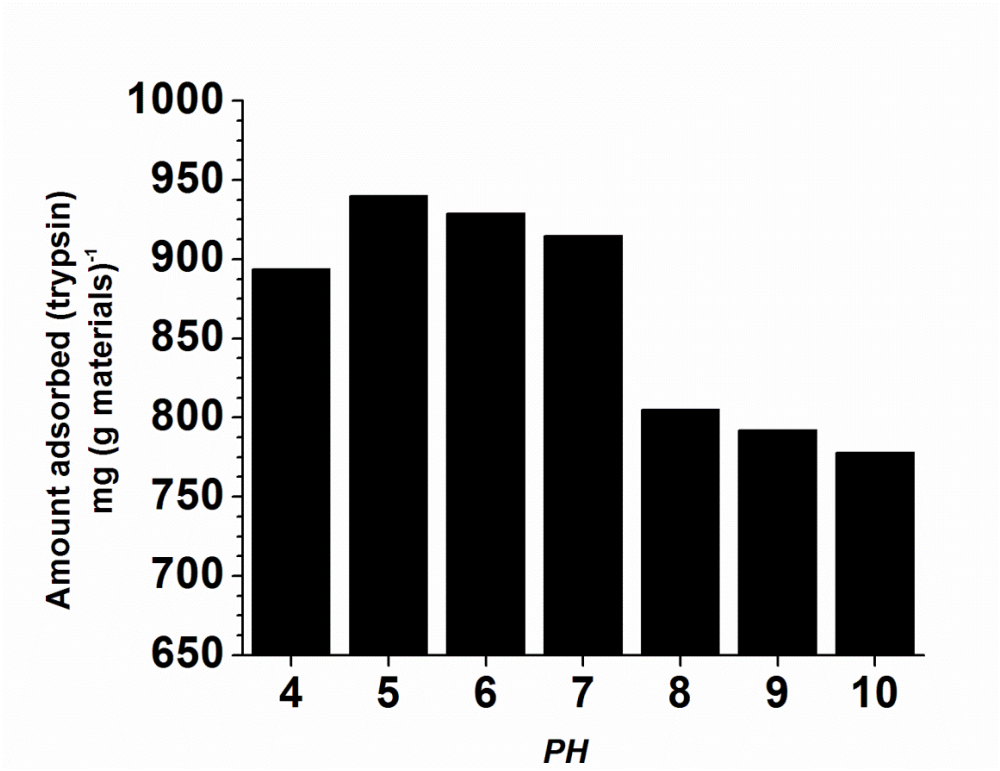

Fig. S7. Effect of pH on the adsorption of trypsin on GO

When the pH value of the buffer was higher than 7, the loading amount of trypsin decreased dramatically. It is well known that GO is negatively charged in aqueous solution with a pH value ranging from 4 to 11. Thus when the pH value of the incubation buffer was between 4 and 7, the positively charged surface of trypsin was favorable to an interaction with GO. However, when the pH value of the buffer was higher than 8, both the trypsin and GO had a negative charge. The loading capacity was limited as a result of repulsive interactions. Therefore, in the later immobilization operation, a buffer with a pH value between 5 and 6 will be chosen for the maximum enzyme loading amount.

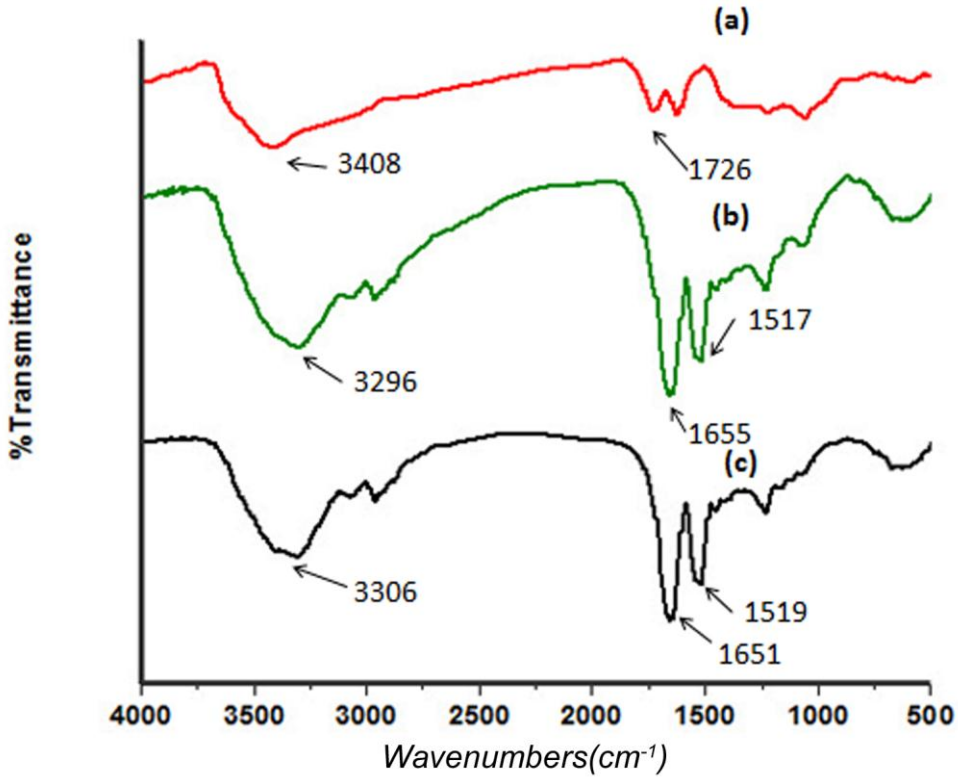

Fig. S8. FT-IR spectra of (a) GO, (b) trypsin and (c) GO-immobilized trypsin

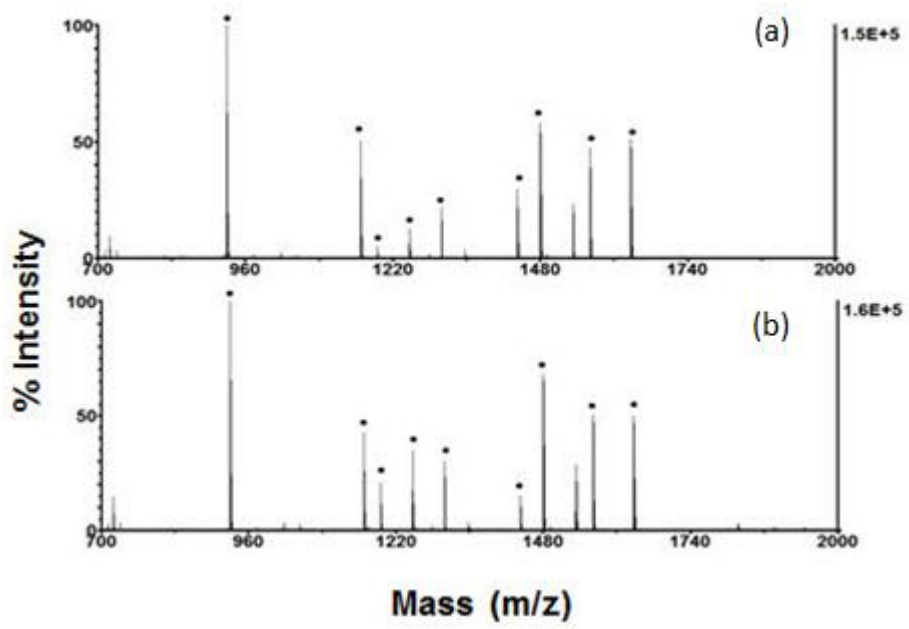

Fig. S9. MALDI-TOF mass spectra of 5 ng μL^{-1} BSA digests (a) with and (b) without GO

There were no obvious differences between the two spectra in signal intensity. The mass spectra illustrated that the existence of GO did not interfere with the MS analysis.

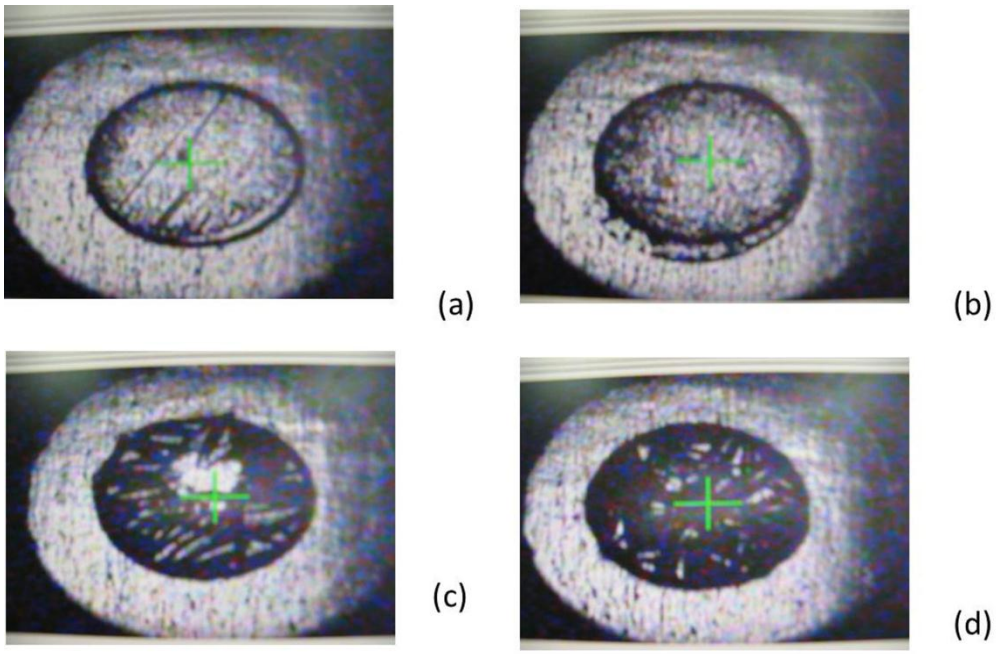

Fig S10. Digital photographs of prepared sample spots of Myo digests with (b) (d) and without (a) (c) GO. On each spot, 0.5 μL^{-1} Myo sample solution and 0.5 μL^{-1} matrix solution CHCA (a) (b) or DHB (c) (d) were spotted and mixed together on the spot with pipette.

Photographs showed that the crystal formations of (a) and (b) had no obvious differences. Similarly, the crystal formations of (c) and (d) were almost the same. Both illustrated that the existence of GO did not interfere with the formation of matrix and analyte crystals.

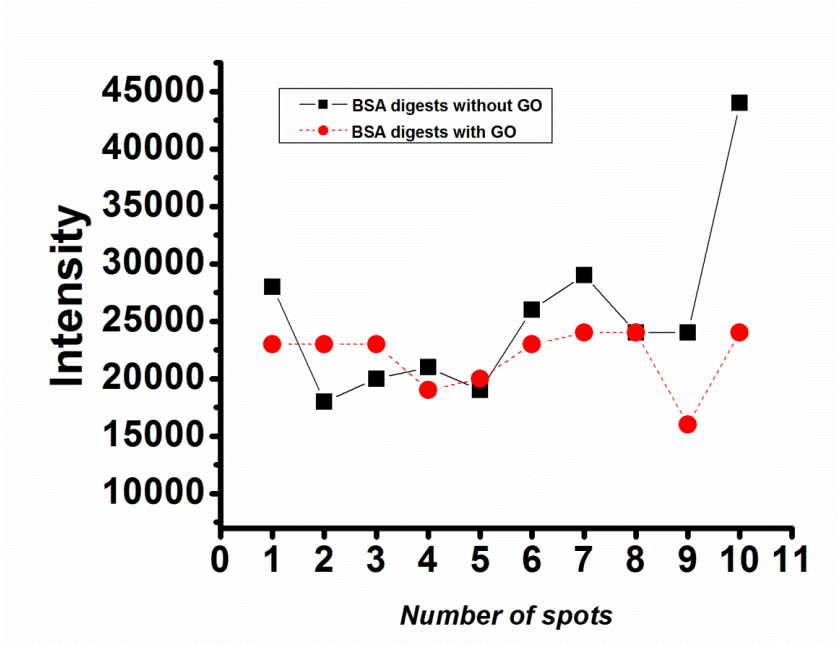

Fig. S11. Comparison of peak intensity stability of 10 ng μL^{-1} BSA digests with and without GO

Each sample spot were prepared by applying 1 µL mixture solution of bovine serum albumin peptides (red circle) with and (black square) without graphene oxide to each spot on the sample plate. The mass spectra were acquired from a discrete location in each spot by applying continuous stage motion of 200 laser shots for each mass spectrum and 50 sub-spectra pass acceptance. The red circle illustrated in Fig. S11, indicating that better shot-to-shot reproducibility of mass spectrometry signals of the bovine serum albumin peptides mixed with GO. Similarly, each black square represented mass spectrometry signal intensity of bovine serum albumin peptides without graphene oxide from individual spot. We can see that without the help of graphene oxide the signal intensity had a huge variance. Therefore ,GO contributed to better shot-to-shot reproducibility.

Table S1. Comparison of amino acid sequence coverage of On-Plate digestion with GO-IMER and free enzyme

Digestion method	Myoglobin %	BSA %	α-casein %
GO-IMER	77	30	57
Free enzyme	30	ND*	ND*

* The protein is not identified.

Table S2. Comparison of protein score and amino acid sequence coverage of on-Plate digestion with free enzyme and GO-IMER

Protein	concentration	Score		Protein sequence coverage	
		Free enzyme	GO-IMER	Free enzyme	GO-IMER
alpha-casein	100 ng μL^{-1}	56	102	53%	53%
	50 ng μL^{-1}	62	91	50%	69%
	25 ng μL^{-1}	44	71	53%	58%
bovine serum	100 ng μL^{-1}	60	79	33%	48%
albumin	50 ng μL^{-1}	83	107	35%	48%
	25 ng μL^{-1}	74	105	35%	52%

Table S3. Peptides detected in human lens sections after in-situ digestion.

Measured proteins in tissue	Observed MH^+	Calculated MH^+	Peptide source	Miss cleavage	Peptide
αA-crystallins	744.12	744.39	αA-(113-117)	1	EFHRR
	1599,26	1599.81	αA-(1-12)	1	MDVTIQHPWFKR
	1037.33	1037.54	αA-(13-21)	0	TLGPFYPSR
	1175.37	1175.63	αA-(55-65)	0	TVLDSGISEVR
	1655.51	1655.86	αA-(100-112)	1	AIPVSREEKPTSAPSS
αB-crystallins	700.30	700.44	αB- (158-163)	0	TIPITR
	900.43	900.49	αB- (150-157)	1	KQVSGPER
	1716.52	1716.90	αB- (93-107)	1	VLGDVIEVHGKHEER
	1821.65	1822.04	αB- (158-174)	1	TIPITREEKPAVTAAPK
	1944.55	1944.07	αB- (91-107)	2	VKVLGDVIEVHGKHEER
	2703.04	2703.52	αB-(150-174)	3	KQVSGPERTIPITREEKPAV TAAPK
	2831.14	2831.61	αB- (151-175)	3	QVSGPERTIPITREEKPAVT AAPKK
			αB- (150-175)	4	KQVSGPERTIPITREEKPAV TAAPKK
βA_2-crystallins	1037.33	1037.54	βA_2-(13-21)	0	TLGPFYPSR
	1457.70	1457.67	βA_2-(108-120)	0	VTLFEGDNFQGCK
	1481.43	1481.67	βA_2-(27-39)	1	CRLLSDCANVCER
	1655.51	1655.86	βA_2-(100-112)	1	AIPVSREEKPTSAPSS
βA_3-crystallins	1503.47	1503.76	βA3- (110-122)	0	LMSFRPICSANHK
	1584.50	1584.78	βA_3- (18-32)	0	MAQTNPTPGSLGPWK
	1727.46	1727.85	βA_3-(197-211)	0	EWGSHAQTSQIQSIR
	1857.51	1856.96	βA_3- (123-137)	2	ESKMTIFEKENFIGR

βB_1-crystallins	1087.34	1087.55	βB_1- (51-60)	0	AAELPPGNYR
	1568.26	1568.78	βB_1- (111-123)	1	GEMFILEKGEYPR
βB_2-crystallins	1269.17	1269.63	βB_2-(164-175)	1	EEKPAVTAAPKK
	1269.35	1269.63	βB_2- (190-198)	1	IRDMQWHQR
	1311.40	1311.67	βB_2- (146-157)	0	IQTGLDATHAER
	1426.42	1426.76	βB_2- (161-172)	1	GLQYLLEKGDYK
βB_3-crystallins	815.57	815.48	βB_3- (196-201)	2	RIRDQK
	1729.51	1729.83	βB_3- (153-167)	0	AINGTWVGYEFPGYR
γA -crystallins	2398.80	2399.26	γA-(81-99)	3	IIPHTSSHKLRLYERDDYR
γC -crystallins	1612.48	1612.84	γC-(78-91)	1	SCCLIPQTVSHRLR
γD -crystallins	909.42	909.47	γD- (4-10)	0	ITLYEDR
	1248.58	1248.71	γD- (164-174)	2	VGSLRRVIDFS
	1255.64	1255.61	γD- (143-152)	0	QYLLMPGDYR
	1454.51	1454.74	γD- (4-15)	1	ITLYEDRGFQGR
γS -crystallins	891.22	891.41	γS- (73-79)	0	WMGLNDR
	1120.50	1120.65	γS- (147-155)	2	GRQYLLDKK
	1355.64	1355,73	γS- (149-158)	2	QYLLDKKEYR
	1568.26	1568.85	γS- (147-158)	3	GRQYLLDKKEYR
	1729.31	1729.90	γS-(159-174)	0	KPIDWGAASPAVQSFR