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Surface modified silver selinide nanoparticles as extracting probes to improve peptide/protein detection via nanoparticles-based liquid phase microextraction coupled with MALDI mass spectrometry

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ABSTRACT

We report the first use of functionalized Ag_2Se nanoparticles (NPs) as effective extracting probes for NPs-based liquid-phase microextraction (NPs-LPME) to analyze hydrophobic peptides and proteins from biological samples (urine and plasma) and soybean in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Surface modified functional groups such as octadecanethiol (ODT) and 11-mercaptoundecanoic acid (MUA) on Ag_2Se NPs were found to play an important role for efficient extraction of peptides and proteins from test samples through hydrophobic interactions. The peptides can be efficiently extracted using functionalized Ag_2Se NPs as extracting probes in the presence of high concentration of matrix interferences such as 4 M urea, 0.5% Triton X-100 and 3% NaCl. Ag_2Se@ODT NPs have shown better extraction efficiency and detection sensitivity for peptides than Ag_2Se@MUA NPs, bare Ag_2Se NPs and conventional MALDI-MS. The LODs are 20–68 nM for valinomycin and 100–180 nM for gramicidin D using Ag_2Se@ODT NPs-LPME in the MALDI-MS. The current approach is highly sensitive and the target analytes can be effectively isolated without sample loss and efficiently analyzed in MALDI-MS.

1. Introduction

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is one of the most powerful analytical instrument for the rapid analysis of a variety of biomolecules with the advantages of rapidity, simplicity and sensitivity [1]. Recently, nanoparticles (NPs) have been extensively applied as matrices or affinity probes for the analysis of biomolecules in MALDI-MS [2–5]. The functionalized NPs exhibited high surface area-to-volume ratio, excellent chemical and physical properties that make them promising materials for efficient extraction of biomolecules at low concentration in the MALDI-MS [6–8]. Chen et al. employed magnetic NPs coated with alumina and zirconia as concentrating probes for phosphopeptide analysis [9,10]. Kirk and Bohn utilized colloidal gold NPs for analysis of organomercaptans [11]. Silica NPs were used as affinity probes for the identification of peptides [12]. Zeolite NPs and magnetic particles immobilized with metal ions [13,14] and C_{18} functionalized magnetic NPs [15] were applied for phosphopetide enrichment. We have also investigated the effects of functional groups on ZnS nanoparticles to serve as affinity probes for biomolecule analysis [16]. Turney et al. applied silica-C_{18} functionalized NPs as sensitive probes for peptide extraction and their analysis in atmospheric pressure (AP) MALDI-MS [17]. Iron oxide capped TiO_2 NPs were used as affinity probes for the peptide/protein analysis [18]. However, all these approaches can be classified into two different approaches: (1) solid phase extraction (SPE) which is performed by using ion oxides capped with various NPs to extract oligonucleotides [4] or phosphopeptides [9,10,14,15,18]. These methods typically required multistep synthesis processes which involve tedious washing/separation procedures. (2) Using water soluble NPs (NPs prepared in aqueous phase) [5–7,11,12,16] as affinity probes can enhance detection sensitivity in MALDI-MS. However, this approach is challenged when applied to complex biological samples since it is not easy to perform the extraction because the NPs are in aqueous solution. To overcome these problems, in 2005, we first introduced nanoparticle based-single drop microextraction (NPs-SDME) technique using functionalized gold NPs prepared in organic solvent (toluene) to serve as effective preconcentrating probes for peptide extraction in complex samples in AP-MALDI-MS [19]. This method success-

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fully extracted peptides by modified gold NPs via electrostatic interactions. This is a novel type of LPME method which can be applied for the extraction of biomolecules using NPs prepared in organic phase. Meanwhile, using traditional LPME methods small molecules such as drugs or toxic compounds were successfully extracted but it has limitations for the extraction of peptides or proteins.

Liquid phase microextraction (LPME) coupled with MALDI-MS (LPME/MALDI-MS) has been proven to be a feasible tool for efficient extraction and detection of small target analytes from complex or biological samples [20–24]. However, for biomolecule analysis such as peptides/proteins, the LPME/MALDI-MS technique has limitations because of the inability of organic solvents for the extraction of peptides/proteins [19]. Moreover, the solubility of hydrophobic biomolecules (peptides and proteins) is very low in aqueous solution so it is difficult to extract the hydrophobic peptide/protein by aqueous phase nanoparticles, especially for the detection of biomolecules at low concentrations or from biological matrices. Therefore, we proposed a novel approach of NPs-based liquid phase microextraction (NPs-LPME) using functionalized silver selinide NPs prepared in organic solvent (toluene) for improved detection sensitivity of hydrophobic peptides and proteins in the MALDI-MS. The extraction efficiency of NPs-LPME/MALDI-MS technique was optimized with respect to (i) the effect of peptide/NPs ratio, (ii) extraction time, (iii) sample pH and (iv) salt concentration. We also investigated surface modified effect of Ag_2Se NPs on extraction efficiency of hydrophobic peptides in NPs-LPME/MALDI-MS by comparing three different types of Ag_2Se NPs including the bare Ag_2Se NPs, Ag_2Se@ODT (octadecanethiol) and Ag_2Se@MUA (11-mercaptoundecanoic acid) as extracting probes. Furthermore, the applicability of the current proposed method was investigated with respect to the analysis of peptides in the presence of high concentration of matrix interferences such as urea, Triton X-100 and salts (NaCl). In order to demonstrate the feasibility of the current approach toward real sample analysis with MALDI-MS. Furthermore, the proposed method was used with human urine and plasma as well as soybean samples.

2. Experimental

2.1. Reagents

Silver nitrate and toluene were purchased from Mallinckrodt Chemicals, Paris, KY, USA. Octadecylamine (ODA) was supplied by Lancaster Chemicals, Ward Hill, MA, USA. Selenium powder, octadecanethiol (ODT), 11-mercaptoundecanoic acid (MUA), acetonitrile (ACN), valinomycin, gramicidin D, α-cyano-4-hydroxycinnamic acid (CHCA) and trifluoroacetic acid (TFA) were obtained from Sigma Chemicals (St. Louis, MO, USA). Methanol was supplied by Echo chemicals, Toufen, Taiwan. Ultrapure deionized water was used in all of the experiments by a Milli-Q water purification system (Millipore, Milford, MA, USA). The samples were agitated on a vortex agitator (VM 2000, Digisystem Laboratory, Taipei, Taiwan).

2.2. Solution preparation

Stock solutions of valinomycin and gramicidin D (1 mg/mL) were prepared in methanol. The stock solutions of two peptides were further diluted to appropriate concentration with deionized water. The matrix solution (CHCA, 14 mM) was prepared in acetonitrile and water (2:1, v/v) containing 1% TFA. The structures of valinomycin and gramicidin D are shown in Figure S1 of Supporting Information.

2.3. Synthesis of functionalized Ag_2Se NPs

Silver nitrate ($AgNO_3$), selenium powder (Se) and octadecylamine (ODA), octadecanethiol (ODT) and 11-mercaptoundecanoic acid (MUA) were used for synthesis of functionalized silver selinide (Ag_2Se) nanoparticles (NPs). Briefly, 0.5 g of silver nitrate and 0.12 g of selenium powder were transferred into a conical flask (100 mL) containing 10 mL of ODA solvent and the mixture was stirred at 180 °C for 10 min. The formed Ag_2Se NPs were collected at the bottom of beakers and washed several times with ethanol and then dispersed in toluene using an ultrasonicator. The functionalized Ag_2Se@ODT and Ag_2Se@MUA NPs were prepared by the same procedures with the addition of 0.125 g of ODT and MUA to prepare the Ag_2Se@ODT and Ag_2Se@MUA NPs. The formed NPs were washed with ethanol and then dispersed in toluene using an ultrasonicator for further use.

2.4. Procedure for NPs-LPME/MALDI-MS analysis of hydrophobic peptides

900 μL of standard peptide mixture (valinomycin (0.40 μM) and gramicidin D (0.80 μM)) was taken into a 1.0 mL polyethylene vial. The pH of sample solution was carefully adjusted by addition of 1.0 M HCl or NaOH. To this, 100 μL of toluene containing functionalized Ag_2Se NPs was added and vortexed for appropriate extraction time at 900 rpm in room temperature. The sample vials were allowed to stand for 3 min to separate organic and aqueous layers. After that, 2 μL of organic solvent containing Ag_2Se NPs and peptides was mixed with equal volume of matrix solution and then directly deposited onto the MALDI plate (standard steel substrate). This was air-dried and then analyzed by MALDI-TOF-MS. Fig. 1 shows a schematic depleting of the proposed method.

2.5. Procedure for pretreatment of soybean

Prior to the NPs-LPME/MALDI-MS analysis, the soybean was pretreated according to [25]. Briefly, 150 g of finely ground soybean was taken into a 500 mL beaker containing 60% ethanol. The above solution was left standing at room temperature for 15 h, after which the soybean flour was removed by filtration. The filtrate was used for the extraction of hydrophobic proteins using the functionalized Ag_2Se NPs and then analyzed by MALDI-TOF-MS.

2.6. MALDI-TOF-MS

All experiments were carried out in a Bruker Daltonics Microflex MALDI-TOF mass spectrometer (Bremen, Germany). The mass spectra were obtained in the positive ion mode with a 337 nm of nitrogen laser for desorption/ionization of analytes and with an accelerating voltage of 20 kV for accelerating ions into the mass analyzer. A 96 plates well was used for sample acquisition with average of about 200 laser pulses. For analytes <5000 Da, a reflectron mode was applied for detection of ions in order to improve the resolution while for analytes >5000 Da, a linear mode was applied for detection of ions. To obtain highest signal-to-noise (S/N) ratios and best resolution, the laser energy was carefully adjusted to slightly above the threshold of analytes for each spectrum and other parameters were as described earlier [16].

2.7. UV–vis, FT-IR, SEM and TEM

UV–vis absorption spectra of Ag_2Se NPs were measured on a double-beam spectrophotometer, Hitachi U3501 (Tokyo, Japan). FT-IR spectra were recorded with a FT-IR spectrometer, Bruker IFS-66 v/s (Bruker, Germany). Scanning electron microscopy, JOEL SEM

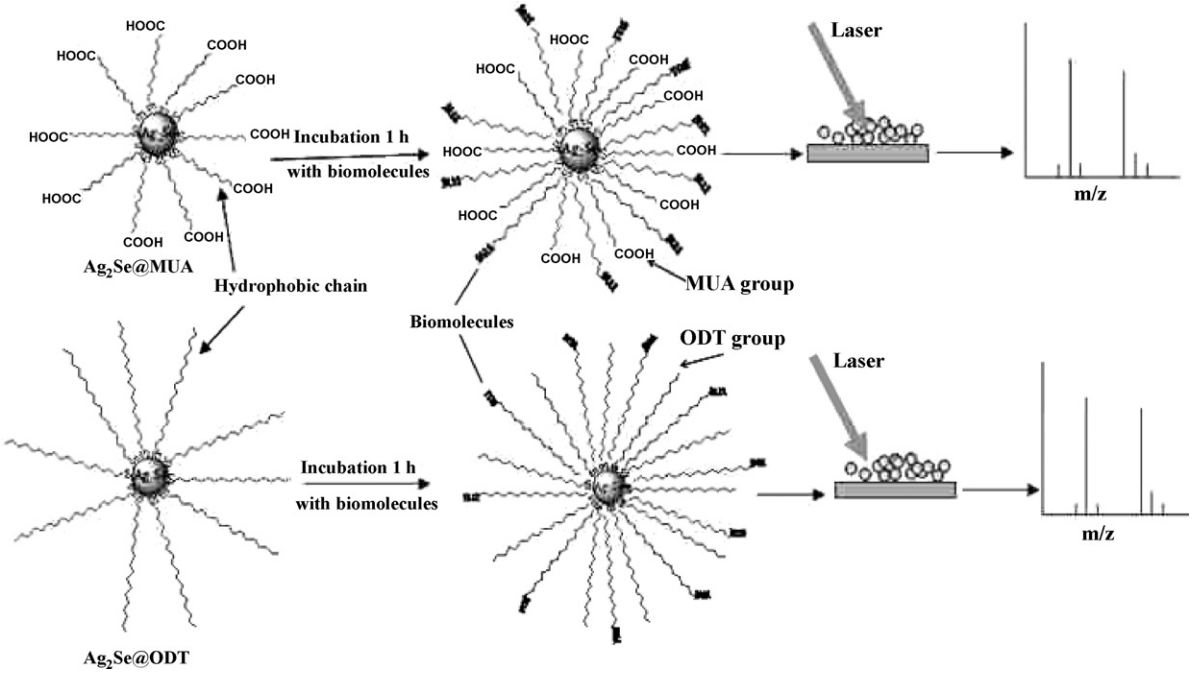

Fig. 1. Schematic procedure for NPs-LPME for hydrophobic peptide/protein analysis using functionalized Ag_2Se NPs as extracting probes and followed by MALDI-TOF-MS.

6700F (Tokyo, Japan) and high resolution transmission electron microscopy, JOEL HRTEM 3010 (Tokyo, Japan) were used to collect the SEM and TEM images of Ag_2Se NPs.

3. Results and discussion

3.1. Characterization of functionalized Ag_2Se nanoparticles

UV–vis, FT-IR, SEM and TEM have been used for characterization of Ag_2Se NPs. Fig. 2a–c displays UV–vis spectra of bare Ag_2Se NPs, Ag_2Se@MUA NPs and Ag_2Se@ODT NPs, respectively. The maximum absorption wavelength of three different Ag_2Se NPs shifted from 408 nm to 428 nm (408 nm for bare Ag_2Se, 420 nm for Ag_2Se@MUA and 428 nm for Ag_2Se@ODT NPs). This is because the UV–vis absorption spectra of Ag_2Se NPs are affected by surface modification of NPs, resulting in variation on the absorption wavelengths. Also, it is well known that the absorbance of functionalized nanoparticles mainly depends on carbon chain lengths and functional groups. Hence, the modified Ag_2Se NPs absorption wavelengths are different from the bare Ag_2Se NPs. Successful surface modified Ag_2Se@MUA and Ag_2Se@ODT NPs were confirmed by FT-IR spectra as shown in Fig. 2d and e. The FT-IR spectra confirm the characteristic features of hydrocarbon chains on the functionalized Ag_2NPs. The C–H stretching bands of MUA and ODT observed at 2920 and 2850 cm^{-1} are corresponding to the asymmetric and symmetric modes of $–CH_2$ groups. The stretching vibration of methyl group ($–CH_3$) was observed at 2938 cm^{-1} for Ag_2Se@ODT NPs. In addition, symmetric carboxylate stretching ($\nu_s(COO^-)$) was found at 1425 cm^{-1}, and the stretching bands of C=O and OH in carboxylic acid group (COOH) were observed at 1705 cm^{-1} and 3427 cm^{-1} (a broadband peak) for Ag_2Se@MUA NPs, respectively. Note that the stretching vibration band of –SH group was not observed in the region of 2545–2600 cm^{-1}. Also, thiol vibrational stretching band was observed at 2549 cm^{-1} for both free MUA and free ODT. This confirms that the formation of chemical bonds between Ag_2Se NPs and thiol (–SH) group of MUA and ODT molecules to form Ag_2Se-S-$(CH_2)_{10}$-COOH and Ag_2Se-S-$(CH_2)_{17}$-CH_3, respectively. The size and morphology of the Ag_2Se NPs were further confirmed by SEM and TEM (Fig. 3). Fig. 3a–c shows that the SEM images of functionalized Ag_2Se NPs. TEM images of bare Ag_2Se, Ag_2Se@MUA and Ag_2Se@ODT NPs are shown in Fig. 3d–e. From the above TEM images, we confirmed that these three types of Ag_2Se NPs were well-dispersed in toluene and exhibited spherical shapes and the sizes were ranging from 7 to 10 nm.

3.2. Effect of peptide/NPs ratio and NPs concentration for NPs-LPME/MALDI-MS analysis of peptides

To optimize the extraction efficiency for the NPs-LPME/MALDI-MS analysis of peptides from aqueous solution, various extraction parameters such as extraction time, sample pH, salt (NaCl) concentration and NPs concentration (peptide/NPs ratio) were carefully investigated. The extraction efficiency of peptides was monitored based on the signal intensity (absolute abundance) of the most abundant ions (potassium adduct ions for both valinomycin and gramicidin D) from the mass spectra for each peptide solution at five successive analyses. The number of laser shots for each spectrum was collected at a constant number (200 laser shots) in order to fairly evaluate the detection sensitivity of peptides in the MALDI-MS. All experiments were performed at optimal conditions for peptide/protein analysis. The extraction efficiency and signal intensities of the peptides were strongly depending on peptide/NPs mole ratio. Therefore, we examined the effect of peptide/NPs molar ratio for NPs-LPME of hydrophobic peptides. Hydrophobic peptides solutions of valinomycin (0.40 μM) and gramicidin D (0.80 μM) were extracted by various concentrations of Ag_2Se@ODT and Ag_2Se@MUA NPs (10–50 μM) and then detected by MALDI-MS. Figure S2a of Supporting Information displays the plot of signal intensities verses peptide/NPs mole ratio. The results reveal that the maximum signal intensities of peptides were observed at 1:20 of peptide/NPs ratio. Higher than this ratio, the signal intensities of peptides decreased as the NPs concentration increased. This indicates that the NPs concentration plays a significant role on the extraction efficiency and detection sensitivity of peptides using

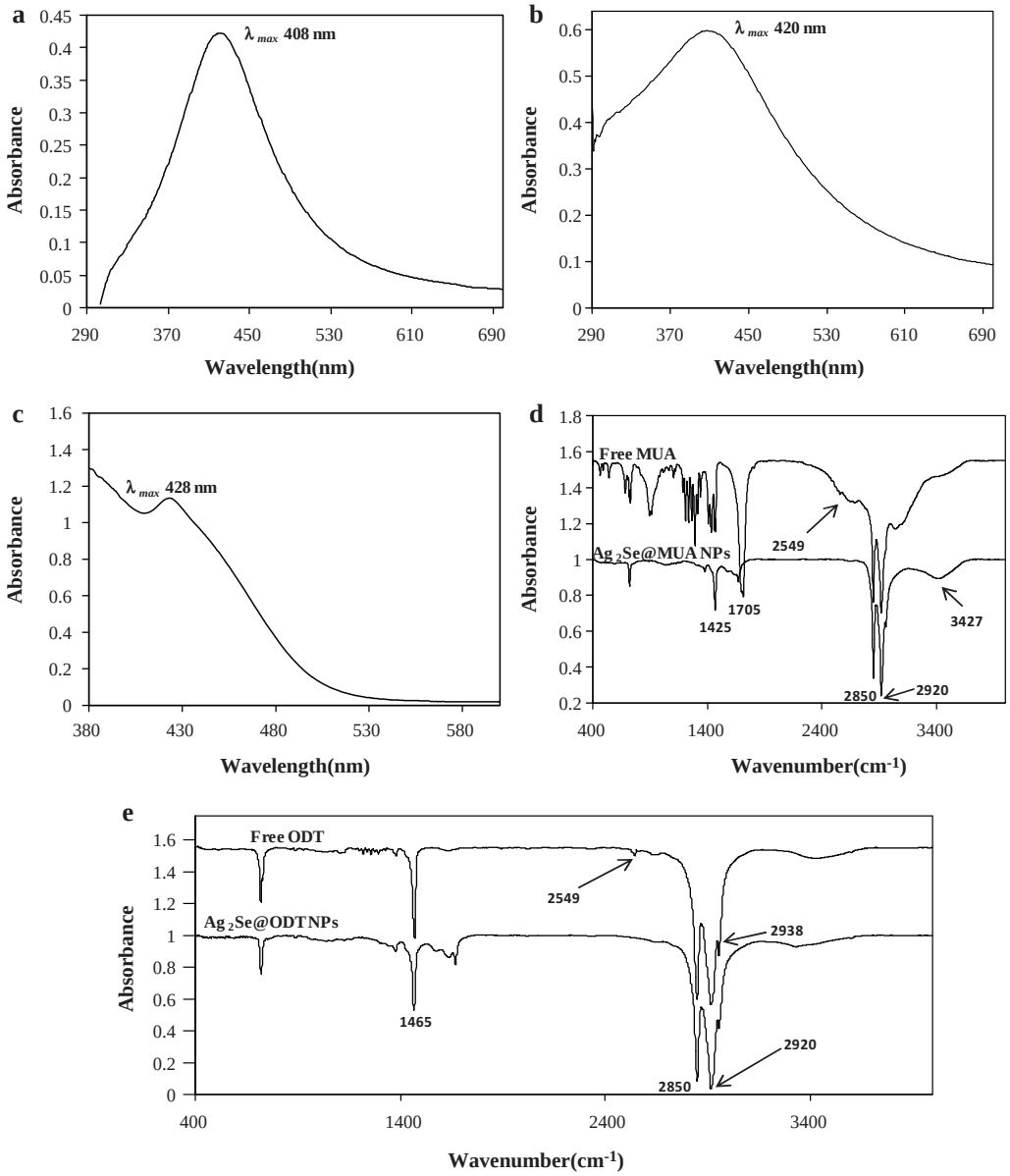

Fig. 2. UV–vis spectra of (a) bare Ag_2Se NPs, (b) Ag_2Se@MUA NPs and (c) Ag_2Se@ODT NPs. FT-IR spectra of (d) Ag_2Se@MUA NPs and (e) Ag_2Se@ODT NPs.

NPs-LPME/MALDI-MS. Therefore, 20 μM of Ag_2Se NPs was selected for extraction of target peptides. The expression and calculation details for the concentration of the Ag_2Se NPs were provided in the text of Supporting Information.

3.3. Effect of extraction time for NPs-LPME/MALDI-MS analysis of peptides

Mass transfer of analyte in LPME is a time-dependent process and equilibrium is reached only after a certain period of time in the extraction [20–24]. Since the nanoparticles-based LPME is mainly depends on the interactions of NPs with biomolecules via electrostatic or hydrophobic interactions [19]. It is necessary to optimize the extraction time in order to obtain the maximum extraction efficiency. Various extraction time intervals ranging from 0.3 to 2 h were investigated for extraction of peptide mixtures (valinomycin and gramicidin D) from aqueous solution by the NPs-LPME/MALDI-MS with agitation rate of 900 rpm at room temperature. We observed that the extraction efficiency increased with extraction time from 0.3 to 1 h and then decreased since the NPs-LMPE break through was reached to equilibrium at 1 h between peptides and NPs. Therefore, the optimal extraction time was carried out at 1 h for efficient extraction of peptides/proteins in this study. Note that the extraction time (1 h) was much longer than that of Au NP-SDME approach [19]. This is because the current approach is primarily based on the hydrophobic interaction of NPs with peptides which was much weaker than the electrostatic interactions of Au NPs with peptides [19]. Thus, longer extraction time was required to achieve an efficient extraction.

3.4. Sample pH effect on the NPs-LPME/MALDI-MS

The effect of sample pH on the Au NP-SDME extraction [19] for peptide analysis was very important because the surface of Au NPs modified with tetraoctylammonium bromide (TOAB) carried positive charges and could attract peptides (with net negative

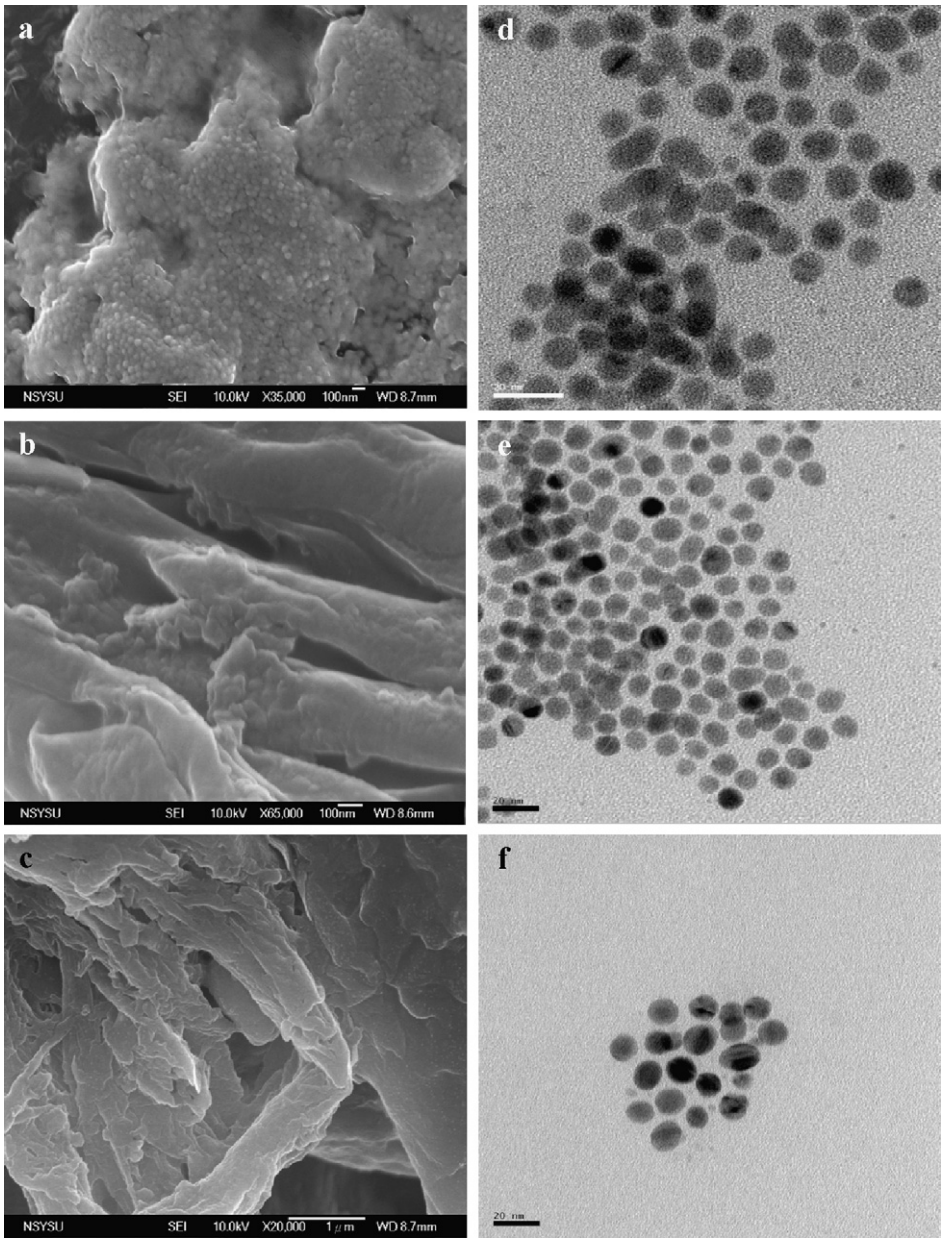

Fig. 3. SEM images of (a) bare Ag_2Se NPs, (b) Ag_2Se@MUA NPs and (c) Ag_2Se@ODT NPs. TEM images of (d) bare Ag_2Se NPs, (e) Ag_2Se@MUA NPs and (f) Ag_2Se@ODT NPs.

charges) in pI < pH of peptide solution [19]. The Au NP-SDME served as an electrostatic probe. In this study, we applied two types of Ag_2Se NPs capped with MUA (Ag_2Se-S-$(CH_2)_{10}$-COOH) and ODT (Ag_2Se-S-$(CH_2)_{17}$-CH_3) for peptide extraction via hydrophobic interactions. The sample pH effect toward the hydrophobic interactions was investigated by using extraction efficiencies of two peptide solutions of valinomycin and gramicidin D at pH 2–12 (Figure S2b of Supporting Information). The solution pH was found to have a significant impact on extraction efficiency of peptides in NPs-LPME/MALDI-MS. The peptide signals increased from sample pH 2–7, and then decreased to pH 12. The maximum intensities of peptides were observed at pH 7. Hence, pH 7 was applied for all further extraction. Comparison of extractions of peptides using Ag_2Se@ODT NPs and Ag_2Se@MUA NPs showed an increase in the amount of peptides extracted by Ag_2Se@ODT NPs. There are several reasons that could be responsible for the observation. These include: (1) hydrophobic interaction increasing with the length of alkyl chains on the surfaces of Ag_2Se NPs and (2) the terminal of Ag_2Se@MUA NPs containing a carboxylic group (–COOH) which reduces the hydrophobic nature.

3.5. *Effect of sodium chloride addition*

We previously demonstrated the extraction efficiency of monensin can be significantly enhanced by the presence of sodium chloride in SDME/MALDI-MS [21]. We therefore investigated the effect of NaCl addition on extraction efficiency of the peptides detected by Ag_2Se NPs-LPME/MALDI-MS and the results are shown in Figure S2c of Supporting Information. The extraction efficiency of peptide solutions was monitored as a function of NaCl addition from 1 to 7%. The signal intensities of these peptides were significantly enhanced; when the concentration of NaCl was increased from 1 to 3%; beyond 3% there was no change in the signal. This was probably due to the fact that salt (NaCl) leads to reduction of water molecules attached to the peptides. Therefore, the salt is used as an adsorber to concentrate and to enhance the hydrophobic interac-

tions of peptides toward NPs [26,27]. The 3% NaCl concentration as earlier indicated was an optimal parameter in the enhancement of valinomycin and gramicidin D extraction from aqueous solution. Figure S3 of Supporting Information displays the mass spectra of valinomycin and gramicidin D using Ag_2Se NPs as extracting probes at 3% of NaCl. The results show high abundance of potassium and sodium adduct ions at high S/N ratios for these NPs under high salt concentration. The order of detection sensitivity for these three types of NPs was Ag_2Se@ODT NPs > Ag_2Se@MUA NPs > bare Ag_2Se NPs under high salt conditions.

3.6. Effect of interferences from Triton X-100 and urea

The effects of Triton X-100 and urea matrices on biological samples were investigated using the proposed method. It is established that the presence of biological matrices in biological samples result in ion suppression in MALDI-MS [28]. Therefore, the concentration levels of urea and Triton X-100 were varied. Figure S4 of Supporting Information shows the results of the variation of urea and Triton X-100 of the proposed method. The optimum interferences concentration was up to 4 M and 0.5% urea and Triton X-100, respectively.

3.7. Effect of solvent selection

The selection of solvent used was based on previous publication [19,23] for isolation of analyte by LPME. Only water immiscible solvents were considered for efficient extraction of peptides. Therefore, common solvents methanol and acetonitrile were unsuitable due to their hydrophilic nature. An array of water immiscible solvents that included toluene, octanol, chloroform and hexane were used for LPME extraction by Au NP-SDME. The results showed that toluene was the best choice [19]. In our previous publication on LPME/MALDI-MS we demonstrated that toluene was the most suitable solvent for LPME [23] because it exhibited many attractive properties that included less evaporation during the extraction process, high viscosity for easily handling the organic microdrop, excellent reproducibility, and solubility for analytes, matrices and NPs [19]. In addition, TEM images of bare Ag_2Se NPs, Ag_2Se@MUA NPs and Ag_2Se@ODT NPs (Fig. 3d–f) show that these NPs can be well dispersed in toluene and this phenomenon plays a significant role for efficient extraction of peptides in NPs-LPME/MALDI-MS. Therefore, we selected toluene as the extraction solvent for Ag_2Se NPs-LPME experiments in this study.

3.8. Surface modification effect on Ag_2Se NPs-LPME for hydrophobic peptides analysis in MALDI-TOF-MS

To investigate the surface modification effect of Ag_2Se NPs for hydrophobic peptides analysis in NPs-LPME/MALDI-TOF-MS technique, Fig. 4 shows MALDI-TOF mass spectra of hydrophobic peptide mixture (valinomycin and gramicidin D) detected from aqueous solution using three types of Ag_2Se NPs. Fig. 4a–c shows mass spectra of hydrophobic peptides obtained from three different Ag_2Se NPs as extracting probes through NPs-LPME. Compared with the results of bare Ag_2Se NPs and direct analysis (Fig. 4c and d) we found that the extraction efficiency (signal intensities) of the peptides were enhanced about 3–4-fold using Ag_2Se@ODT NPs and Ag_2Se@MUA NPs (Fig. 4a and b). The detection sensitivity for peptides was highest using Ag_2Se@ODT NPs as extracting probes. The reason is probably due to the Ag_2Se@ODT NPs contain highest hydrophobic nature in comparison with modification of [$-(CH_2)_{17}CH_3$ group] than that of Ag_2Se@MUA NPs [$-(CH_2)_{10}COOH$ group] and bare Ag_2Se NPs (no $-CH_2$ group). Although the signal intensity of unmodified Ag_2Se NPs is appropriately equal to that of direct analysis, it is still superior than the direct analysis since we can observe intense interferences produced from the organic matrix (Fig. 4d). The use of Ag_2Se NPs as extracting probes is therefore responsible for suppression of background interferences. These results confirmed the role played by hydrophobic interaction in the efficient extraction of hydrophobic peptides using functionalized Ag_2Se NPs-LPME from aqueous solution.

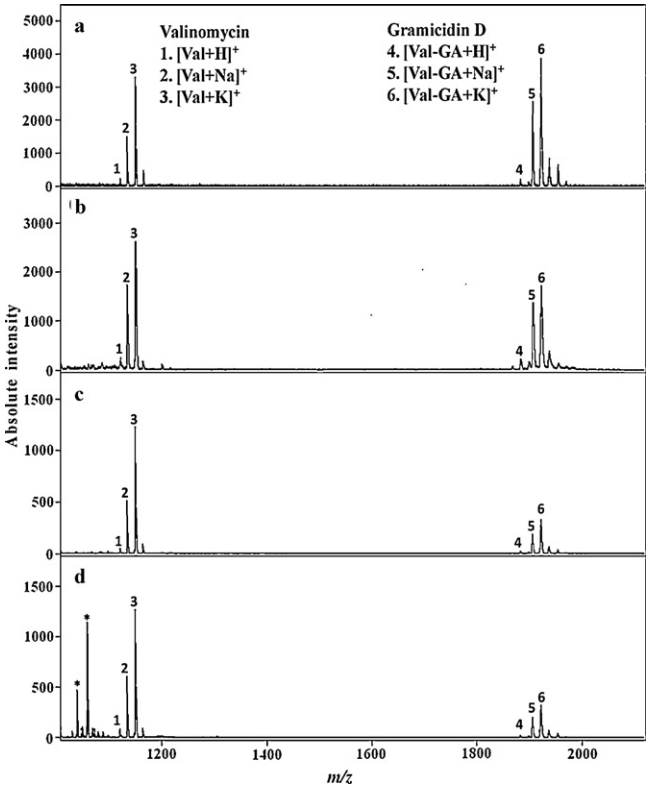

Fig. 4. MALDI-TOF mass spectra of valinomycin (0.40 μM) and gramicidin D (0.80 μM) with (a) Ag_2Se@ODT NPs-LPME, (b) Ag_2Se@MUA NPs-LPME, (c) bare Ag_2Se NPs-LPME and (d) direct analysis from aqueous solution. The peptide/NPs ratio was 1:20 and the mass peaks obtained at *m/z* 1112.31, 1133.82, 1150.19 corresponded to $[Val+H]^+$, $[Val+Na]^+$ and $[Val+K]^+$ ions of valinomycin; 1883.24, 1904.56, 1921.32 are assigned as $[Val\text{-}GA+H]^+$, $[Val\text{-}GA+Na]^+$ and $[Val\text{-}GA+K]^+$ for gramicidin D. The concentration of CHCA was 14 mM; sample pH of solution was 7. Mass spectra were generated by applying 49 μJ of laser fluence at 200 laser shots. The peaks marked with asterisks are interferences produced from the organic matrix.

3.9. Quantitative analysis of peptides using Ag_2Se NPs-LPME/MALDI-TOF-MS

Quantitative analysis of peptides using Ag_2Se NPs-LPME/MALDI-MS was carried out using the proposed method. The calibration graphs were constructed between peptides concentrations (0.2–1.0 μM for valinomycin and gramicidin D) and their signal intensities. The correlation coefficients (R^2) were found to be 0.998 (Val) and 0.997 (Val-GA) for Ag_2Se@ODT NPs, 0.996 (Val) and 0.998 (Val-GA) for Ag_2Se@MUA NPs and 0.992 (Val) and 0.995 (Val-GA) for unmodified Ag_2Se NPs. The limit of detections (LODs) was found to be 20 nM (Val) and 100 nM (Val-GA) for Ag_2Se@ODT NPs, 37 nM (Val) and 126 nM (Val-GA) for Ag_2Se@MUA and 68 nM (Val) and 180 nM (Val-GA) for unmodified Ag_2Se NPs. The above results confirm that the current approach is highly sensitive and can be applied for quantitative analysis of hydrophobic peptides at trace levels.

Reproducibility and repeatability are essential for the assay of potentiality of the developed method. Therefore, we studied intra- and inter-day precision and accuracy of Ag_2Se NPs-LPME coupled with MALDI-TOF-MS for the analysis of valinomycin and gramicidin

Table 1
Precision and accuracy for the analysis of hydrophobic peptides using Ag_2Se NPs-LPME coupled with MALDI-TOF-MS.

Name of NPs	Name of the analytes	Known concentration (μM)	Intra-day			Inter-day		
			Found concentration (μM)[a]	R.S.D. (%)[b]	Accuracy (%)[c]	Found concentration (μM)[a]	R.S.D. (%)[b]	Accuracy (%)[c]
Ag_2Se@ODT	Valinomycin	0.40	0.38 ± 0.012	3.1	−5.0	0.39 ± 0.007	1.7	−2.5
NPs	Gramicidin D	0.80	0.76 ± 0.027	3.5	−5.0	0.77 ± 0.029	3.7	−3.7
Ag_2Se@MUA	Valinomycin	0.40	0.37 ± 0.015	4.0	−7.5	0.38 ± 0.008	2.1	−5.0
NPs	Gramicidin D	0.80	0.75 ± 0.013	1.7	−6.2	0.76 ± 0.034	4.4	−5.0
Bare Ag_2Se	Valinomycin	0.40	0.35 ± 0.031	8.8	−1.2	0.36 ± 0.037	10.2	−10.0
NPs	Gramicidin D	0.80	0.74 ± 0.020	2.7	−7.5	0.75 ± 0.030	4.0	−6.2

[a] Mean ± standard deviation ($n = 5$).
[b] Relative standard deviation.
[c] Accuracy was calculated from (found concentration − known concentration)/known concentration.

D and results are shown in Table 1. The results indicate that relative standard deviation values are less than 10.2% and accuracy values are observed from −1.2 to −10.0%. These results confirmed that the Ag_2Se NPs-LPME coupled with MALDI-TOF-MS have good precision and accuracy for analysis of hydrophobic peptides. Spot-to-spot reproducibility was determined by replicate spotting ($n = 5$) of extracted valinomycin (0.40 μM) and gramicidin D (0.80 μM) using Ag_2Se@ODT NPs as extracting probes. Spot-to-spot reproducibility MALDI-TOF mass spectra of valinomycin and gramicidin D using Ag_2Se@ODT NPs-LPME are shown in Figure S5 of Supporting Information. Meanwhile, we also determined the sample-to-sample reproducibility using five different samples of soybean hydrophobic proteins extracted by Ag_2Se@ODT NPs-LPME. Five MALDI-TOF mass spectra of hydrophobic proteins extracted soybean are taken from five different samples using Ag_2Se@ODT NPs as extracting probes (Figure S6 of Supporting Information). These results suggest that the Ag_2Se NPs provided good spot-to-spot and sample-to-sample reproducibility in MALDI-TOF mass spectra for the analysis of hydrophobic peptides (valinomycin and gramicidin D) in spiked water and hydrophobic proteins from soybean.

3.10. Application of Ag_2Se NPs-LPME/MALDI-MS on detection of biological samples

To further demonstrate the feasibility of the present method for analysis of biological samples, the Ag_2Se@ODT NPs-LPME technique was applied for the extraction of peptide mixture (valinomycin and gramicidin D) from biological samples mainly urine and plasma. Fig. 5 shows results of clean mass spectra with high abundances of sodium and potassium ion adducts of both peptides. These results indicate that the current approach is a promising tool for interference free detection of hydrophobic peptides from biological samples.

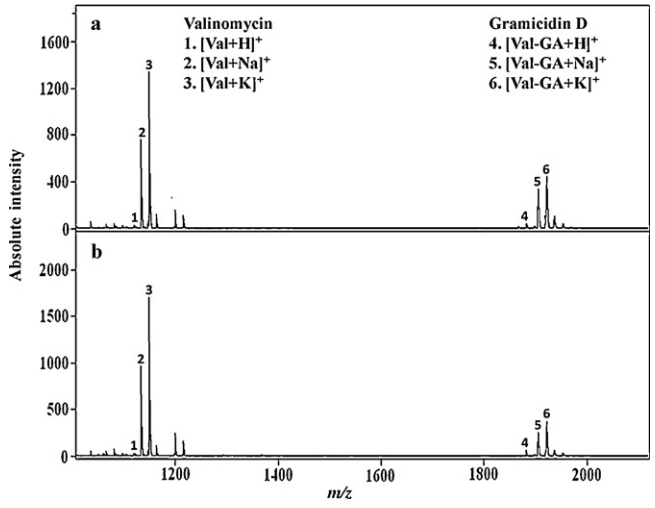

Fig. 5. MALDI-TOF mass spectra of valinomycin and gramicidin D obtained with Ag_2Se@ODT NP-LPME from (a) urine (4-fold dilution) and (b) plasma (5-fold dilution) samples. The other conditions are the same which was shown in Fig. 4.

3.11. Applications of functionalized Ag_2Se NPs for LPME/MALDI-MS analysis of hydrophobic proteins from soybean

Fig. 6 shows the results of functionalized Ag_2Se NPs-LPME method, a strong demonstration that the proposed method can be utilized for real samples. Comparing the MALDI mass spectra for the analysis of hydrophobic proteins from soybean by direct analysis

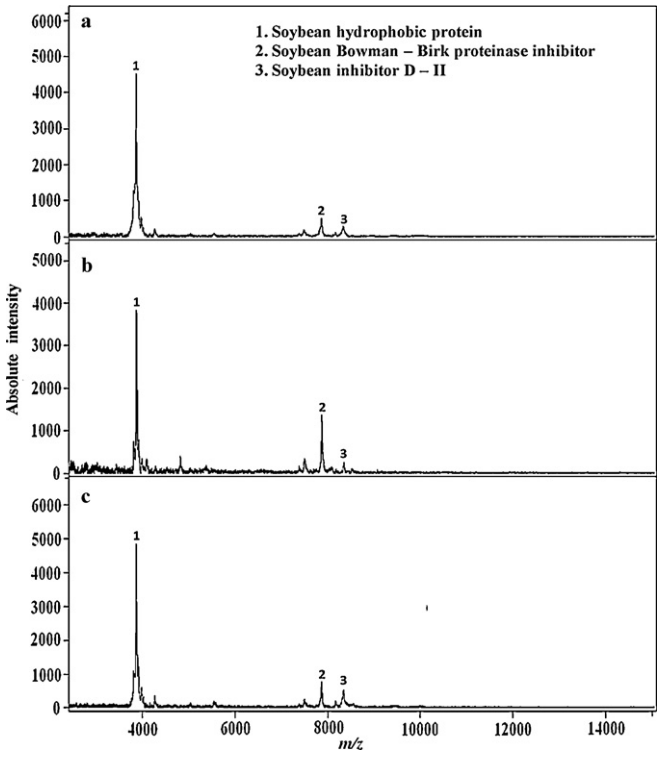

Fig. 6. MALDI-TOF mass spectra of hydrophobic proteins in soybean seeds using (a) direct analysis, (b) Ag_2Se@ODT NPs-LPME and (c) Ag_2Se@MUA NPs-LPME. The mass peaks of soybean proteins obtained at m/z 3879.21, 7855.32 and 8570.21 corresponded to soybean protein, soybean Bowman–Birk proteinase inhibitor and soybean inhibitor D–II, respectively. The concentration of CHCA is 14 mM; sample pH is 7. Mass spectra were generated by applying 67 μJ of laser fluence at 200 laser shots.

(Fig. 6a), Ag_2Se@ODT NPs (Fig. 6b), and Ag_2Se@MUA NPs (Fig. 6c) as extracting probes, although these three methods can detect the three peptides from soybean, the Ag_2Se@ODT NPs showed the best sensitivity for peptides at m/z 7855. The mass peaks generated at m/z 3879, 7855 and 8570 corresponding to soybean hydrophobic protein, soybean Bowman–Birk proteinase inhibitor and soybean inhibitor D–II, respectively.

4. Conclusions

We have successfully developed a novel liquid-phase microextraction method using functionalized Ag_2Se NPs as efficient extracting probes for analysis of hydrophobic peptides/proteins in aqueous solutions, biological samples and soybean based on hydrophobic interactions between the Ag_2Se NPs and hydrophobic biomolecules. The Ag_2Se@ODT NPs served as the best hydrophobic nanoprobe. Additionally, the mass peaks of peptides can be successfully generated in the presence of matrix interferences such as urea, Triton X-100 and salt (NaCl). The functionalized Ag_2Se NPs-LPME/MALDI-MS method is a potential tool for efficient extraction of hydrophobic peptides/proteins from biological and complex samples.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.09.040.

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