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Synthesis, characterization and bifunctions of bidentate silver nanoparticle assisted single drop microextraction as a highly sensitive preconcentrating probe for protein analysis

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Abstract

Synthesis, characterization and bifunction applications of silver nanoparticles with two different surface capping reagents were reported. The surface engineering of Ag NPs with 1-Octadecanethiol (1-ODT)/4-Aminothiophenol (4-AMP) 'Ag@ODT/AMP' and 1-Octadecanethiol (1-ODT)/1-thioglycerol (1-TG) 'Ag@ODT/TG' were synthesized, characterized and applied for microextraction. Presence of two functional groups on the surface of Ag NPs produce multidendate that can interact with proteins and peptides such as insulin, heart cytochrome C, ubiquitin, lysozyme, cysteine, and homocysteine. Thus, they were applied for single drop microextraction (SDME) and were termed as silver nanoparticles assisted dingle drop microextraction (SASDME). The proteins after separation were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The effects of different parameters were optimized, such as sample pH, stirring rate, salt concentration, extraction time, matrix type, and the least amount of eluents for

elution of the lead using the Ag NPs. The present methodology has been successfully applied for detection of insulin and cytochrome c of real samples (urine & milk) and for cysteine and homocysteine in urine sample. SASDME is a simple and effective microextraction technique for real sample analysis with high sensitivity, selectivity and rapidity.

Key words: Ag nanoparticle, single drop microextraction, proteomics, mass spectrometry,

Introduction

Among the different nanoparticles, silver nanoparticles (AgNPs) received fervent efforts for synthesis, characterization and applications [1-2]. Thus, it becomes star for various applications in many fields. It was used intensively for bioanalytical chemistry because of their unique properties such as surface-plasmon resonance (SPR), larger surface area, catalytic properties, and quantum size effects, which can contribute to the signal amplification of bioassays [3-4]. It has been applied for colorimetric assay for protein detection [5], separation [6], fluorescence [7], electrochemical immunosensor [8], antibacterials or others [1-2]. Recent developments of synthesis and functionalization methods for Ag NPs were reviewed and highlight in Ref. [9].

Sample-pretreatment methods such as separation or preconcentration prior to matrix assisted laser desorption/desorption mass spectrometry (MALDI-MS) analysis is highly demanded [10-15]. It could increase the sensitivity and improve analysis of extremely low levels of different analytes in diverse matrices [10]. Among the different preconcentration techniques, liquid-liquid extraction (LLE), in which the target analyte will be separated using two immiscible liquid phases with or without NPs, is highly used as preconcentration method[11].. In order to increase separation efficiency, selectivity, reduces extraction time and for environmental

concerns, nanoparticles were integrated for LLE. Thus, silver nanoparticles (AgNPs) received tremendous attention for separation, surface for ionization and preconcentration for mass spectrometry [12-15]. These techniques are very important for the analysis of protein biomarkers in order to get faster and effective disease diagnosis [10].

Herein, we reported the chemical engineering modification of AgNPs surface with two different capping agents using the 1-Octadecanethiol (1-ODT)/4-Aminothiophenol (4-AMP) 'Ag@ODT/AMP' and 1-Octadecanethiol (1-ODT)/1-thioglycerol (1-TG) 'Ag@ODT/TG'. The nanomaterials were synthesized and characterized using TEM, SEM, FTIR, and UV-vis absorption. The materials were applied for SDME of proteins such as bovine heart cytochrome c (MW 12.3 k Da, pI 9.5), bovine pancreas insulin (MW 5.7 kDa, pI 5.3) in real samples. Data revealed that SASDME is simple, sensitive, selective and fast for protein separation in real samples such as urine and milk. The Ag NPs have bifunctional capability so it can be uses as effective concentrating probes for separation and also for surface assisted laser desorption/ionization mass spectrometry (SALDI-MS). Separation and identification of cysteine and homocysteine from urine sample was also reported.

Experimental section

Chemicals and materials

All chemicals used were of analytical reagent grade. Bovine heart cytochrome c (MW 12.3 k Da, pI 9.5), bovine pancreas insulin (MW 5.7 kDa, pI 5.3), α-cyano-hydroxycinnamic acid (CHCA) and trifluoroacetic acid (TFA) were purchased from Sigma chemical Co. (St. Louis, MO, USA). Toluene and $AgNO_3$ were obtained from Mallinchrodt chemicals (Phillipsburg, NJ, USA). 1-Thioglycerol (99%, GC) was obtained from Fluka chemicals (Steinheime, Germany). 1-

Octadecanethiol (96%) and 4-Aminothiophenol (97%) was purchased from Alfa Aesar (Johnson Matthey Company, Karlsruhe, Germany). For SDME, the microsyringe (1-10 μL) was purchased from Hamilton Company (Reno, NV, USA). The microsyringe tip has an angel cut needle. All chemicals and reagent was used in deionized water using Milli-Q reagent water system.

Synthesis of the Ag Nanoparticles modified with binary functional groups in toluene

0.9 mmol $AgNO_3$ was dissolved in 200 mL water at 0ºC. 0.45 mmol of a mixture of thiols were then added and stirred for 10 min. Typically, ligand used were 1-Octadecanethiol (1-ODT): 4-Aminothiophenol (4-AMP)/1-thioglycerol (1-TG) in 2:1 ratio in 20 mL of toluene and saturated solution of $NaBH_4$ was then slowly added drop wise. After complete addition, the solution was stirred 20 mins and 2 mL HCl (1M) was further added and was stirred for another 2 hours and placed in a refrigerator overnight to complete the reaction. The layer with dark brown color was collected using separation funnel and washed with deionized water to remove the impurities and clean it up.

Instrumentation for characterization of AgNPs

The UV-vis absorption spectra of bifunctionalized AgNPs were measured on a double-beam UV-350 spectrophotometer at room temperature (Hitachi, Tokyo, Japan). The morphology and size of the AgNPs were evaluated using scanning electron microscopy (SEM, JEOL-6700) and high resolution transmission electron microscope (HRTEM, JEOL TEM-3010, Tokyo, Japan). FTIR was recorded using Bruker IFS 66 v/s vacuum (Bruker, Germany) to confirm the surface modification. The spectra were collected over 500 scans at resolution of 8 cm^{-1}.

MALDI- MS analysis

All mass spectra were obtained in positive ion mode using matrix-assisted laser desorption/ionization–Time of flight- mass spectrometry (Microflex , Bruker Daltonics, Bremen, Germany) with nitrogen laser (337 nm). Ions were produced with delayed extraction period of 200 ns and accelerating voltage was set at 20 kV. All experiments were carried out in linear mode (> 5000 Da) and reflectron mode (< 5000 Da) using a 96 wells of target plate with 200 laser shots and the laser power was attenuated properly to provide the ideal resolution of mass spectra.

Procedure for milk preparation

Standard stock solutions of 1 mg/mL for proteins (Cytochrome c and insulin) were prepared separately in deionized water and were further diluted to 0.7 nM for carrying out the experiments using SDME. Analysis of lysozyme from real milk sample (purchased from the local market, protein content of 3.1 g/100 mL) was performed. The sample treatment was performed according to the described scheme [16]. Milk sample (5 mL of milk sample and 15 mL of NaCl (1 mol/L)) were added into a 50 mL breaker. The pH of the sample solution was adjusted to 6.0 by NaOH of 1 mol/L. After stirring the solution for 1h at the temperature of 40 °C, the pH of the solution was changed to 2.2 by addition of hydrochloric acid solution (1 mol/L). Using Whatman filter paper the solution was filtered out to finally use for the analysis of the lysozyme and other protein present in the milk samples.

Procedure for urine preparation

For the identification of cysteine and homocysteine in an urine sample, the morning urine of healthy human donor (male) was taken and treated according to the procedure describe elsewhere [17]. Briefly, a polyethylene vial containing 50 mL of the urine sample was reduced using a solution $NaBH_4$ (1 mol/L) and NaOH (0.1 mol/L) prepared in 90% CH_3OH. The solution was kept for 10 minute incubation at 45°C to break the disulphide bond and then solution of 5 μL of hydrochloric (0.1 mol/L) was added to break the excess of borohydride in the solution. The final solution obtained was centrifuges at 2500 rpm (5 mins) and the supernatant was used for the analysis of cysteine and homocysteine using our AgNPs

Procedures of silver assisted single drop microextraction (SASDME)

The SDME was performed by taking 3 μL of AgNPs solution prepared in toluene phase in a microsyringe (10 μL). All the protein solutions with desired concentration were taken in 1 mL of microreaction vessels (Supelco, INC, USA) and one fifth of needle height of the microsyringe was dipped inside the solution (5-6 mm below the sample solution) as shown in Figure S1 (supporting information file, ESI). Slowly the head of the syringe was pressed to allow 1 μL organic phase containing the NPs to form a droplet at the tip of the micro-needle syringe. It is important to take care in order to avoid the drop collapse during the experiments. The pH of the sample solution was adjusted by suing NaOH or HCl solution (0.1 mol/L). After keeping for 10 mins as extraction time with 200 rpm of stirring rate by using magnetic stirrers (for proper mixing the solution and to attain equilibrium in the sample solution) the microdroplet was with draw back into the microsyringe. Because of the perfect match of the extraction volume and the volume required for MALDI-MS analysis, MALDI-MS is the best analytical tools. The

microdroplet of the AgNPs of the microsyringe was dropped onto the MALDI-TOF target plate and leaved it few mins for drying at room temperature. The matrix solution (1 μL of CHCA or SA) was deposited on the spot and kept for drying before MS analysis. Matrix solutions were prepared (SA or CHCA, 5 mg/mL) in 2:1 of acetonitrile/H_2O containing 0.1% TFA.

Results and Discussion

Characterization of the AgNPs

Silver nanoparticles (AgNPs) modified two different capping agents, called 1-octadecanethiol (1-ODT)/4-aminothiophenol (4-AMP) 'Ag@ODT/AMP' and 1-octadecanethiol (1-ODT)/1-thioglycerol (1-TG) 'Ag@ODT/TG' (as shown in Figure 1A) were synthesis by reduction of $AgNO_3$ by $NaBH_4$. It can be chemically written as '$AgNO_3 + NaBH_4 = Ag + NaNO_3 + BH_3$'. The morphology and size of Ag@ODT/AMP and Ag@ODT/TG were evaluated by SEM (Figure 1B(a,c)) and high resolution TEM (Figure 1B(b,d)). SEM (Figure 1B (a,c)) reveal that the morphology of the nanoparticle is spherical. TEM images (Figure 1B(b,d)) indicate that the size of Ag@ODT/AMP and Ag@ODT/TG are narrow distribution with average size 25 and 30 nm, respectively. The images of electron microscopy (SEM or HRTEM) reveal the successful synthesis of the AgNPs with the desired size. The presence of the surface modified molecules with ODT/AMP and ODT/TG were further confirmed by FTIR spectroscopy. Figure 2(A) shows the broad signal at 3385 cm^{-1} ($-NH_2$) and 1516, 1487, 1423 cm^{-1}, (all for aromatic CH_2) shows the presence of 4-aminothiophenol on the surface of the AgNPs. On the other hand the signals at 2952 ($-CH_3$) and 2816 ($-CH_2$) shows the presence of 1-octadecanethiol group. Thus, the IR signals confirm that both the groups are attached to the Ag nanoparticle. Similarly in case of Ag@ODT/TG, the signal at 3376 cm^{-1} confirm the presence of OH groups of 1-thioglycerol and the signal at 2904 cm^{-1} confirm the presence of $-CH_3$ group of 1-octadecanethiol as shown

in Figure 2(B). UV-vis absorption (Figure S2) of different prepared materials shows the surface plasmonic resonance (SPR) of AgNPs. The absorption prove also that the AgNPs could assist desorption/ionization process because the absorption wavelength is matched with the wavelength of the N_2 laser (337 nm). Thus, it can be called as surface assisted desorption/ionization mass spectrometry (SALDI-MS). It is also can called as surface enhanced desorption/ionization mass spectrometry (SELDI-MS). It is mainly relay on the large surface area of AgNPs, not only the absorption. These features not only provide high sensitivity, but also offer high S/N. Furthermore, AgNPs decrease the amount of conventional matrix. Thus, it decreases the probability of detector saturation that can be caused by the presence of the matrix ions. It is very important for biological detection where biomarkers are present in complicated system with trivial concentration.

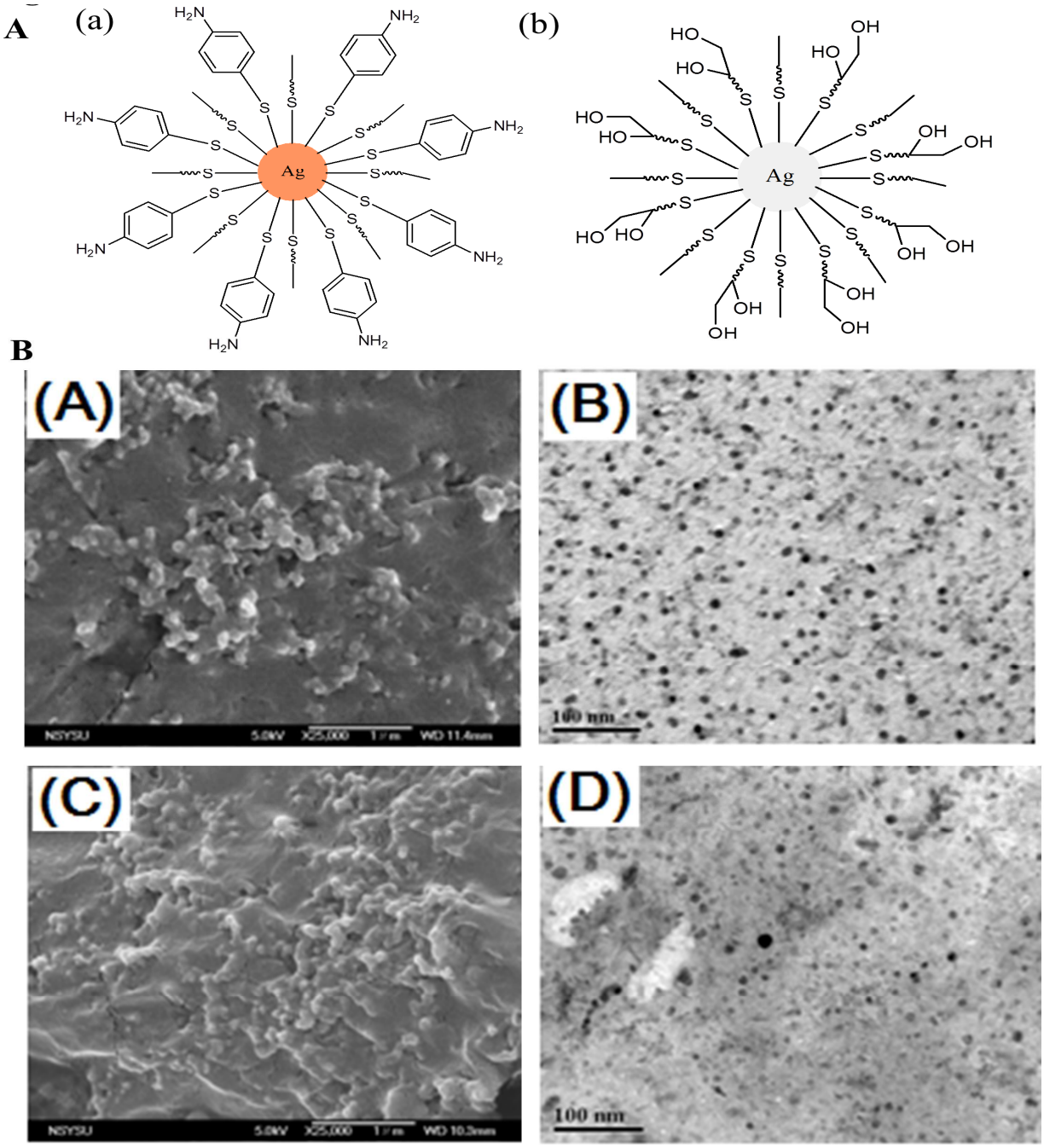

Figure 1. (A) Structure of Ag@ODT/AMP and Ag@ODT/TG and (B) their characterization using SEM for (a) Ag@ODT/AMP and (c) Ag@ODT/TG and TEM (b) Ag@ODT/AMP and (d) Ag@ODT/TG

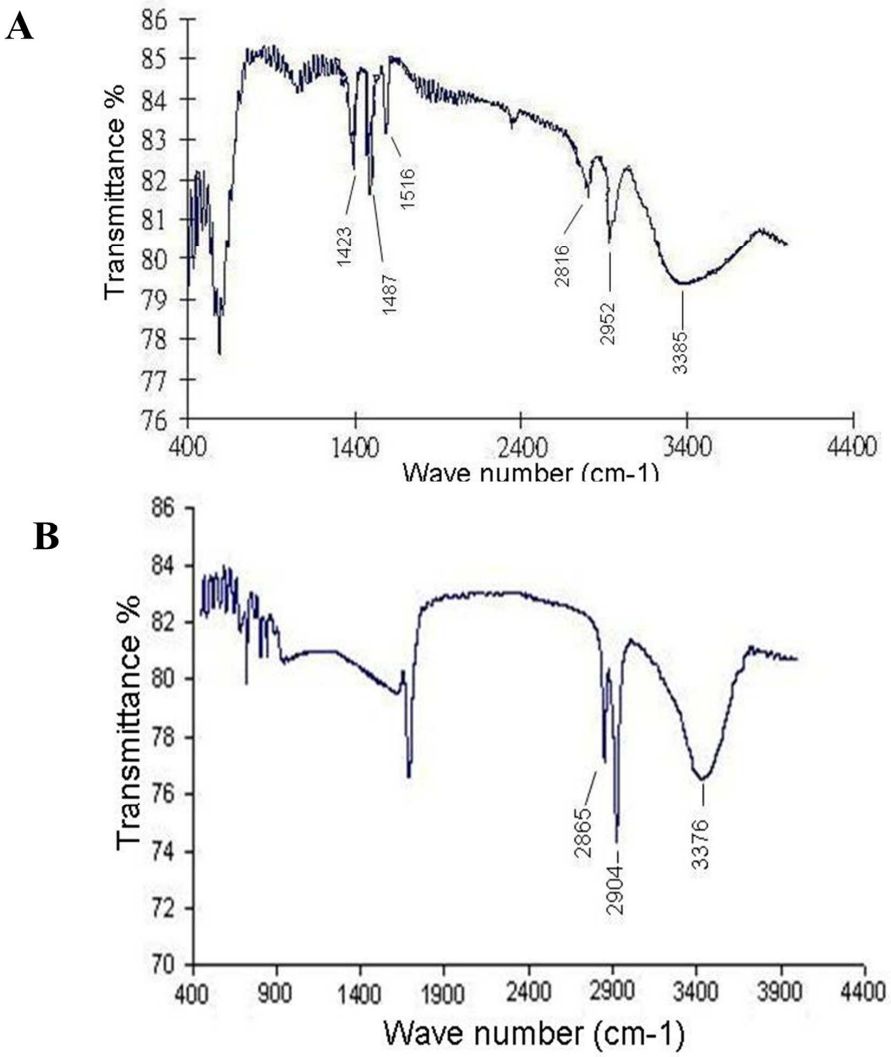

Fig 2. FTIR spectra of (a) Ag@ODT/AMP and (c) Ag@ODT/TG

Protein biomarker analysis using silver nanoparticles assisted single drop microextraction

The syntheses materials i.e silver nanoparticles (Ag@ODT/AMP and Ag@ODT/TG) can be used as a preconcentration probe for proteins biomarkers analysis. Various proteins including Lysozyme, Ubiquitin, Insulin, Heart Cytochrome C, Cysteine, and Homocysteine were

investigated. Lysozyme is part of the innate immune system. For instance, when the level of lysozyme decreases, it causes bronchopulmonary dysplasia in newborns [10]. Ubiquitin protein is associated with endocytic trafficking, inflammation, translation and DNA repair. Cytochrome *c* is an intermediate in apoptosis (process of programmed cell death, PCD) or in response to infection or DNA damage. Probe cysteine and homocysteine from urine or plasma is related to several human diseases, in particular metabolic disorders including cystinuria and homocystinuria. Unfortunately, biomarker present in biological samples typically is a tiny (or trace) amount and they exist in a complicated environment such as blood, milk, urine, ..etc. Thus, preconcentration or sample pretreatment become the *sine qua non* for protein biomarker analysis.

The schematic representation of single drop liquid-liquid microextraction (SDME-MALDI-MS) is shown in Figure S1. In order to achieve high effective extraction/detection of proteins by SDME-MALDI-MS, a number of key instrumental factors should be taken into account, namely: (1) ionization laser power, (2) pH of the solutions, (3) sample stirring rate, (4) salt concentration extraction time, (5) least amount of proteins, and (6) the appropriated matrix. Because the large number of the required parameters, we specified the suitable parameters that achieve higher sensitivity and fast extraction.

In general, laser ionization achieve by adjust the laser intensity 10% above the threshold ionization energy of the protein [18]. At this condition, protein was ionized softly without any observable fragmentation. It was also reported that matrix selection effect the spectra quality. In general, 3,5-dimethoxy-4-hydroxycinnamic acid 'sinapinic acid, SA', α-Cyano-4-hydroxycinnamic acid 'CHCA' [20] are the common matrix for protein and peptides. However, the former is suitable for proteins while the latter is suitable for peptides.

Conventional MALDI-MS of tiny concentration is crucial. The low concentration is difficult to detection mainly due to saturation of the detector by the matrix ions that is 100-1000 folds higher than the target analysis. Thus, preconcentration or sample pretreatment is highly demanded in order to improve the detection sensitivity and achieve higher spectrum quality (higher signal to noise ratio, S/N). Combination of the nanomaterials with high surface area and conventional matrix could improve the detection sensitivity and increase S/N ratio [21]. Comparison among the detection of insulin without and with separation using (A) Ag@ODT/AMP and (B) Ag@ODT/TG were investigated as shown in Figure 3. It is obvious that Ag@ODT/AMP is better for matrix hybrid and also for SDME. The main reason is due to the multifunction that Ag@ODT/AMP has. It contains aromatic benzene and NH_2 groups that facilitate π-π, hydrophobic and hydrogen bonds. However, Ag@ODT/TG has two hydroxyl groups; intrahydrogen bonds decrease interhydrogen bonds with the proteins. Thus, it shows lower resolution at low concentration of Insulin (80 nM). Thus, Ag@ODT/AMP is selected for as promising material for preconcentration the proteins. Different targets called Cytochrome c (80 nM, Figure 4A) and ubiquitin; ubiquitin-like proteins from oyster mushroom sample (50 µL, Figure 4B) were also investigated. Data (Figure 4A) show that Ag@ODT/AMP assisted SDME provide higher S/N and low limit of detection as tabulated in Table 1.

Table 1. Validation parameters for the proposed prior to Ag assisted SDME method.

			Insulin	**Cytochrome c**	**Ubiquitin**	**Cystein**	**Hcystein**
Analytical limits	LOD[a] (nM)	A	80	80	50	1	10
		B	100	100	120	10	20
Extraction parameters	Time (mins)	A	10	10	10	10	10

	pH	A	7	3	7	7	7
	Stirring rate	A	5000	5000	5000	5000	5000
MALDI parameters	Matrix		CHCA	SA	SA	CHCA	CHCA

[a] LOD: limits of detection calculated for a signal to noise ratio of 3.

A with Ag@ODT/AMP assisted SDME

B with Ag@ODT/GT assisted SDME

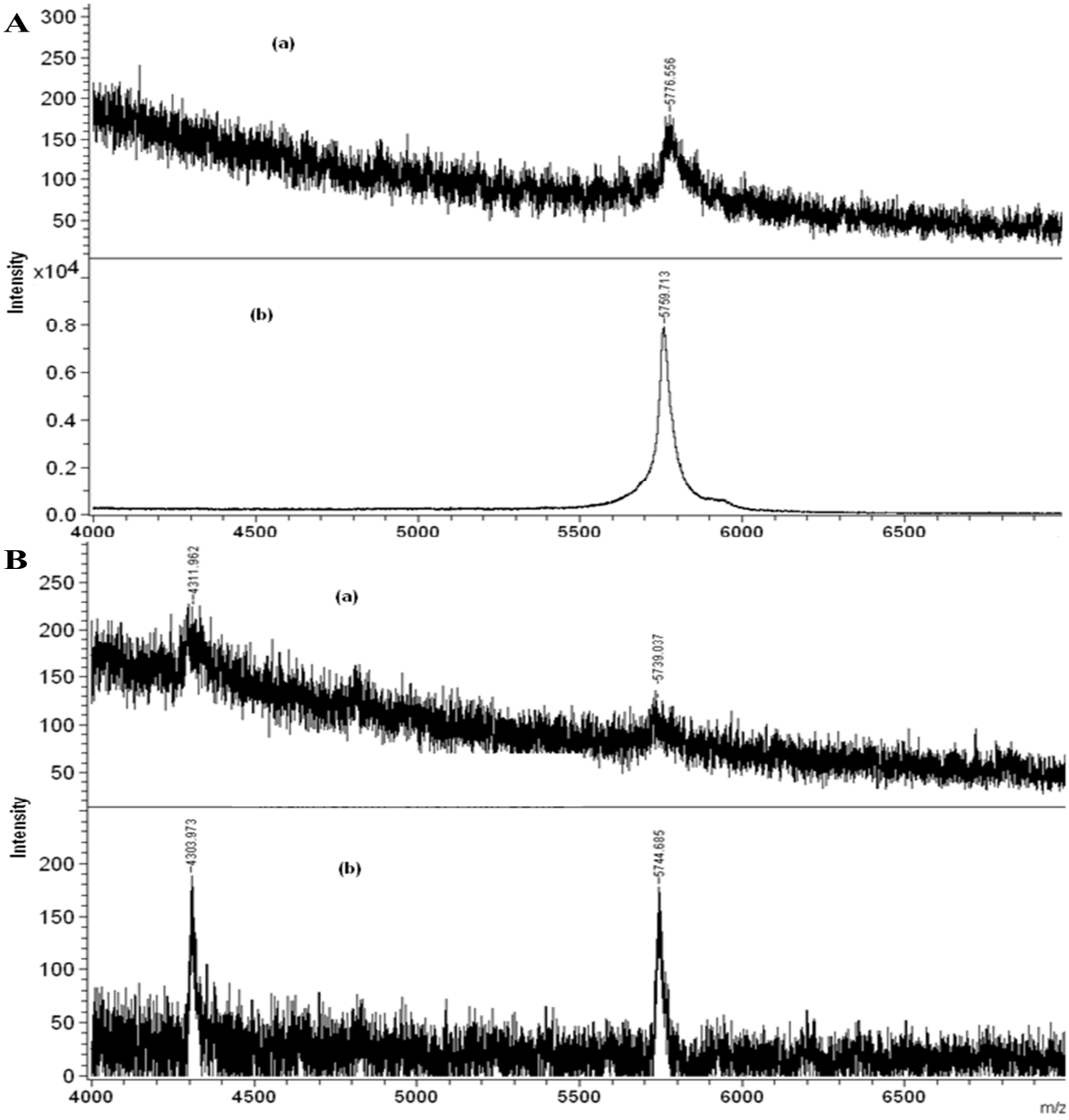

Figure 3. MALDI-TOF mass spectra of Insulin (80 nM) using (A) Ag@ODT/AMP and (B) Ag@ODT/TG (a) without AgNPs-SDME, direct analysis of aqueous sample with CHCA (5000 ppm) (b) with AgNPs- SDME and CHCA by using AgNPs- SDME at room temperature for 5 min extraction and stirring at 200 rpm and CHCA (5000 ppm) at pH 4, laser energy 60 %

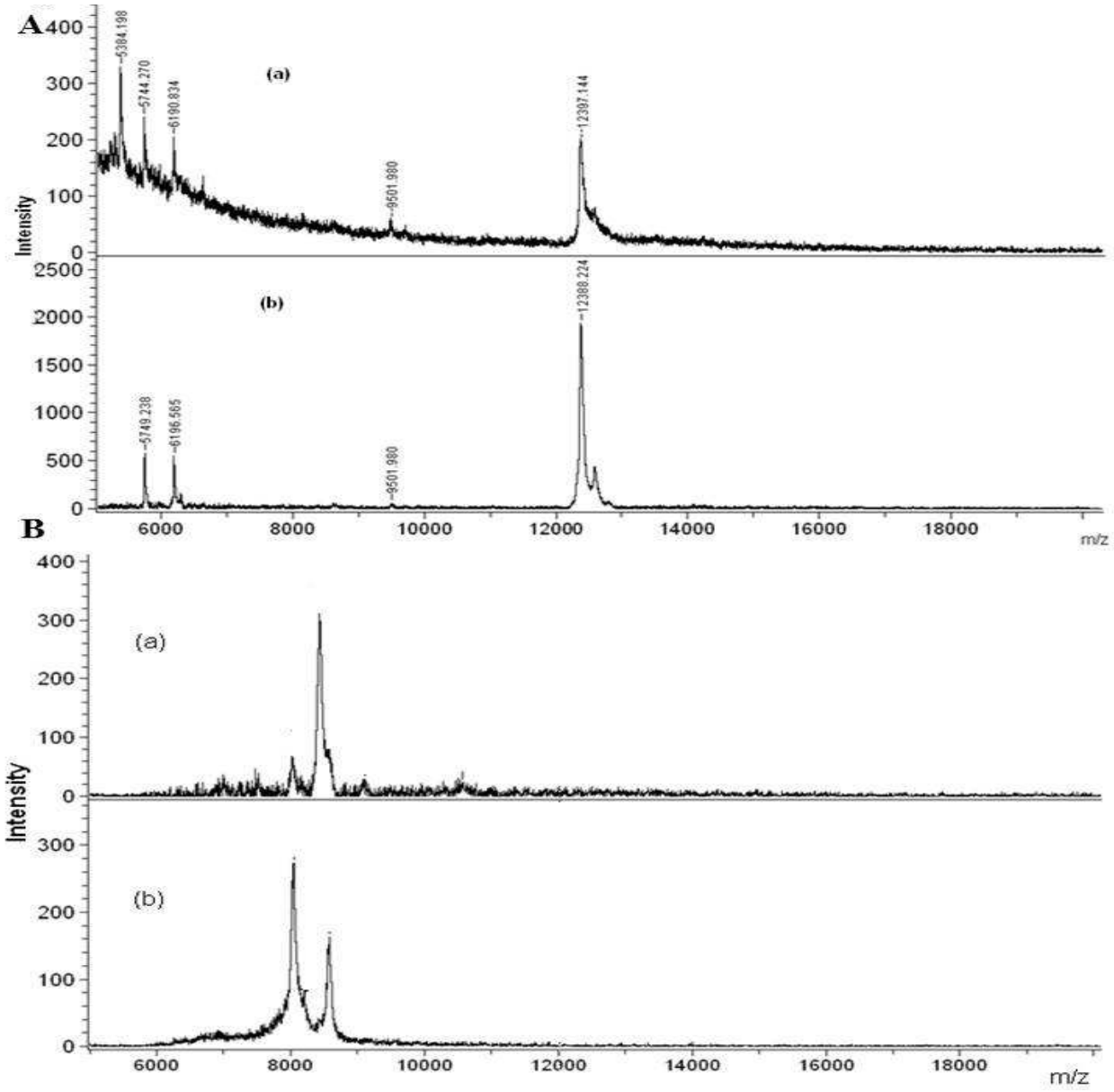

Figure 4: MALDI-TOF mass spectra shows analysis of (A) Cytochrome c (80 nM) and (B) ubiquitin, ubiquitin-like proteins from oyster mushroom samples (50µL) (a) without using AgNPs- SDME, and direct analysis on aqueous samples with SA (5000ppm) (b) with AgNPs-SDME (2.85 mM) and SA (5000ppm), using AgNPs- SDME at room temperature for 10 min of extraction at pH 7.0 and laser energy 65 %

In order to minimize the time of extraction, different intervals of time periods were investigated. We found that more amounts of analytes could be extracted into the organic solvent if the extraction phase is exposed to the sample solution for a longer time [21]. However, long equilibration times may result in the loss of analytes in the extraction process. Figure 5 shows the extraction at different extraction time (a) at 0 min (b) at 2 mins (c) at 10 mins (d) at 15 mins (d) at 20 mins at two different pH values. It is obvious that the sample pH has no observable effect on the separation. However, it effects on the MALDI-MS detection. The optimized pH was selected at pH 7 which is near the biological pH of humans (pH 7.2-7.4) and the extraction time was fixed at 10 mins. Under this condition, high extraction efficiency with higher S/N and minimal fragmentation from protein samples could be obtained.

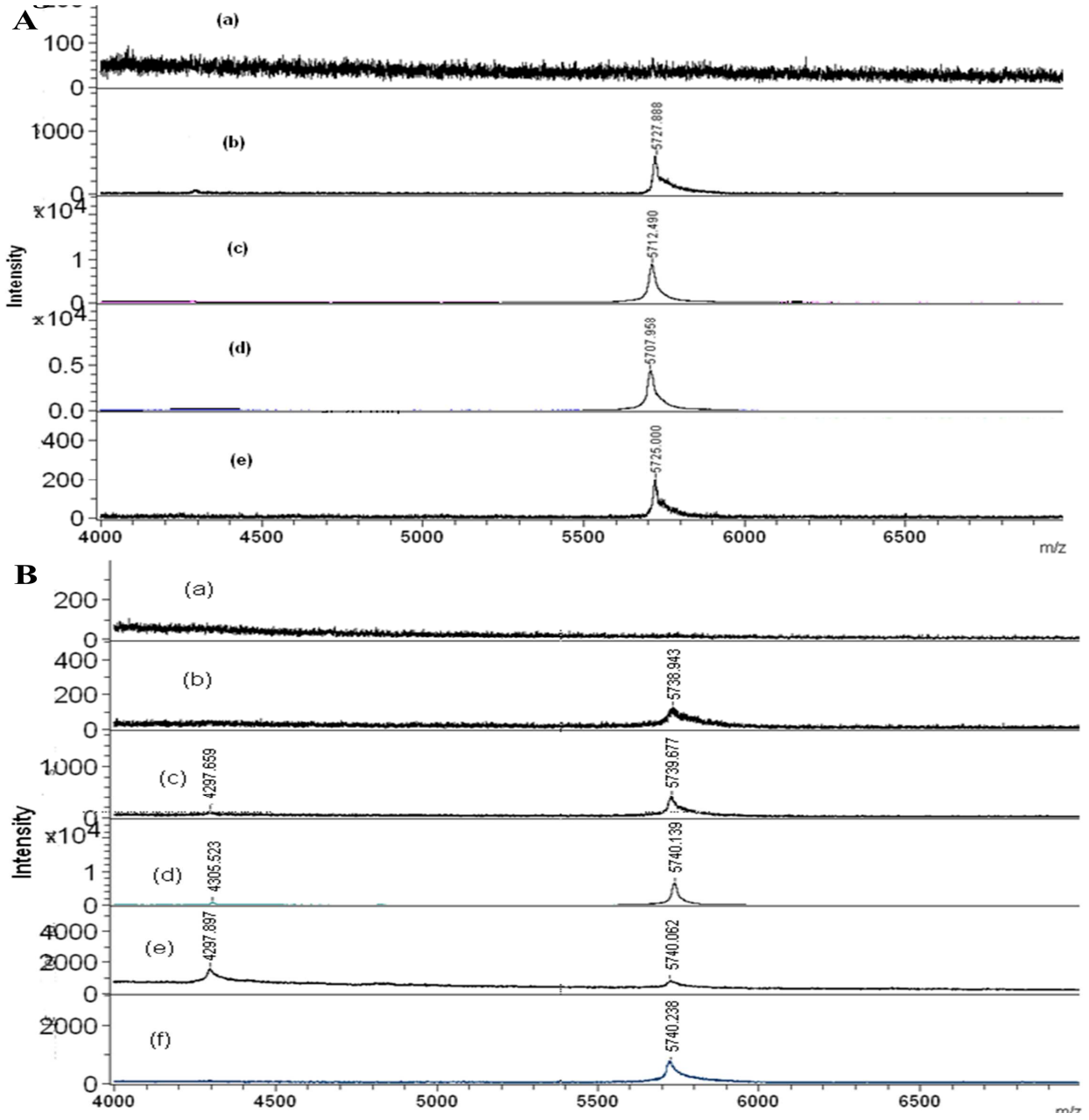

Figure 5: MALDI-TOF mass spectra show the results of various extraction time of Insulin (80 nM) (a) at 0 mins (b) at 2 mins (c) at 10 mins (d) at 15 mins (e) at 20 mins by using Ag@ODT/AMP- SDME at room temperature and CHCA (5000ppm) at (A) pH 3.0 and (B) pH 7.0.

Analysis of Real samples

The main purpose of the present study is to apply Ag@ODT/AMP assisted SMDE for the extraction and detection of proteins of milk (Figure 6) and Cysteine and Homocystiene (Figure 7) obtained from urine samples. Because real world sample is complicated and contain many interferences that will not to be easily detected in the MALDI MS spectra, and they can suppress the ionization [22]. In addition, the presence of salts and high concentration of small matrix molecules that have higher ionization ability would decrease the ionization efficiency of analytes. However, these disadvantages of MALDI mass spectrometry are still sufficient to detect small molecules, peptides, and protein in real sample such as milk or urine [23-24]. It is because the compatibility of separation techniques such as solid phase microextraction (SPME) and, liquid-liquid microextraction (LLME), ...etc are well suitable to couple with mass spectrometry for high sensitivity detection and it also can provide the fingerprint of target analytes. Ag@ODT/AMP assisted SMDE offer higher S/N over the direct detection of milk proteins as shown in Figure 6.

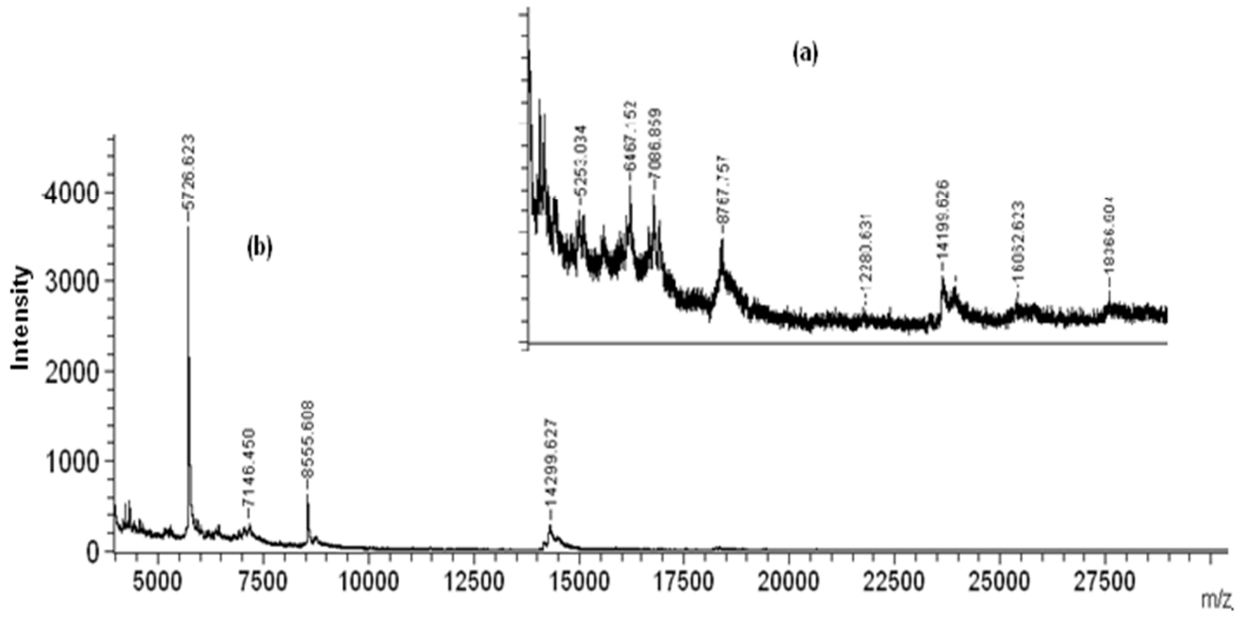

Figure 6: MALDI-TOF mass spectra Milk sample (100µL) analysis after addition of NaCl (5M) (a) without AgNPs-SDME, direct analysis of aqueous sample with SA (5000 ppm) (b) with AgNPs-SDME and SA (5000ppm) by using AgNPs-SDME at room temperature for 15 min extraction and stirring at 200 rpm, at pH 4.5 and laser energy 50 %.

Detection of small molecules for amino acids such as 'Cysteine and Homocystiene' is a crucial challenge in conventional MALDI-MS because the small organic matrix could be easily ionized to generate fragmentation or cluster ions. Thus, ionization/detection for small target analytes is difficult. The detection of cysteine and homocystiene after extraction from urine is reported in Figure 7. The peaks at *m/z* 121.782, 136.595, 145.746 and 171.790 marked with star represents [Cys], [HCys+ Na], [HCys+H] and [HCys+K] ions, respectively. Pokhrel et.al [25] reported that local Na^+ pairing with carboxylates on AgNPs surfaces is kinetically faster and remarkably favored over K^+. This interaction could cause sometimes aggregation. We did not observe this phenomenon here as Ag@ODT/AMP is dispersed in organic solvent 'toluene'. They claimed

also that AgNPs transform into micron-size aggregates upon release into aqueous environments [25].

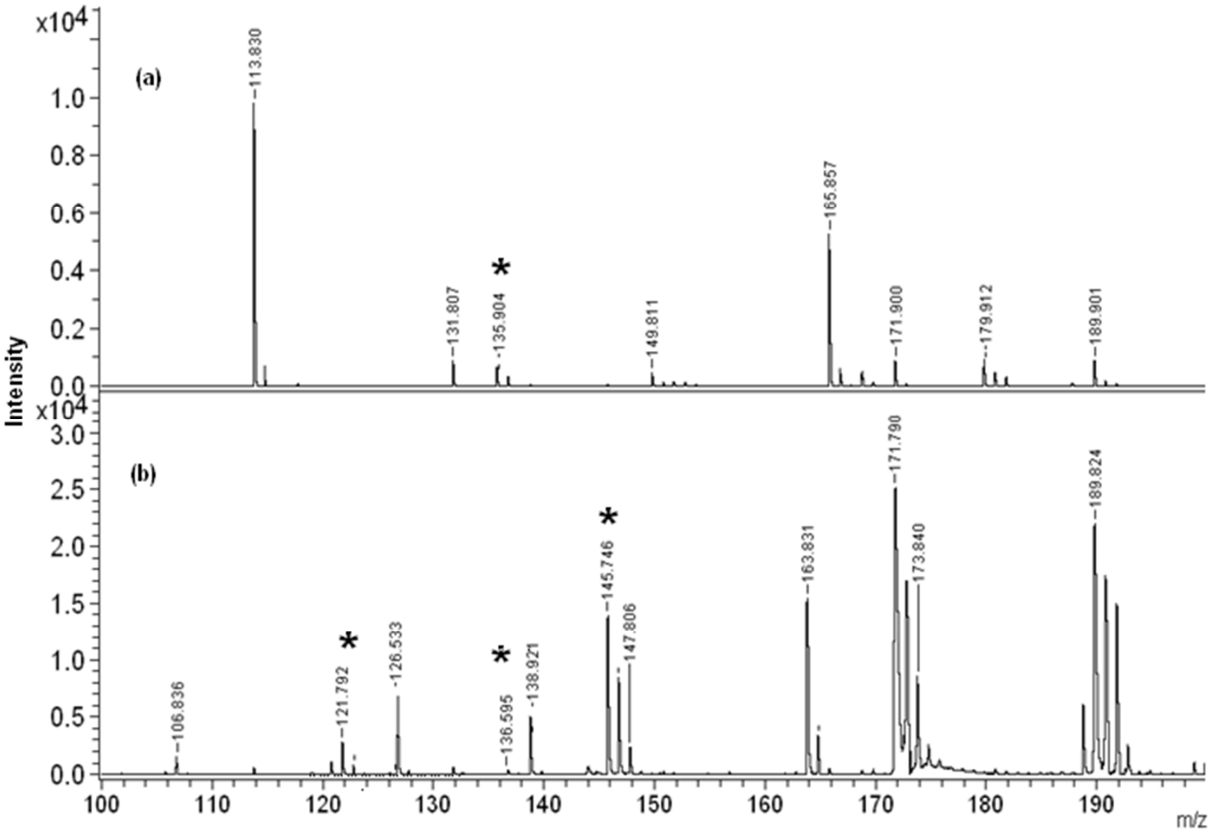

Figure 7: MALDI-TOF mass spectra of Cysteine and Homocystiene obtained from Urine sample using AgNPs-SDME at room temperature for 10 min extraction, stirring at 200 rpm and CHCA (5000ppm) at pH 7.5 and Laser energy 36%.

Generally, AgNPs were widely used as sorbents due to their intrinsic properties, such as high surface area, high adsorption capacity, functionality, and wide ability for surface chemistry modification to obtain a new selective solid-phase extractant as tabulated in Table 2 [26]. Silver nanoparticles loaded on NH_4-type zeolite (AgNPs–NH_4 ZSM5) was reported as an inorganic matrix for laser desorption/ionization mass -spectrometry (LDI-MS) of low molecular weight compounds [12]. The Ag_2Se nanoparticles were reported as extracting probes for NPs-based liquid-phase microextraction (NPs-LPME) to extract and detect hydrophobic peptides and proteins [13]. AgNPs provide matrix-free LDI-MS detection [14] and selective ionization probes [15]. The plasmonic nanoparticles such as Ag are typical inert in LDI MS. Sherrod et al. [26] utilized AgNPs in a laser desorption/ionization (LDI) procedure to show the selectively ionization of the olefinic compounds [e.g., cholesterol, and 1-palmitoyl-2-oleoyl-sn-glycero-3hosphocholine (POPC)] and carotenoids, and finally to analyze them by mass spectrometry (MS). Sudhir et.al reported the application of AgNPs nanoparticles as electrostatic probes for peptide analysis in atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry and comparison with the gold electrostatic probes and silver hydrophobic probes [27]. It requires few apparatus and simple [28]. AgNPs provide inert sorbent, high surface area, multifunctionality with different chemical modification and higher sensitivity.

Conclusion

The bidentate capped silver nanoparticles were successfully synthesized and coupled with single drop microextraction as highly affinity probes for the analysis of protein biomarkers using MALDI-TOF-MS. At the optimal condition (extraction time 10 mins, stirring rate 200 rpm, and 5M salt (NaCl)), high S/N and excellent sensitivity could be obtained. Compared to the convectional MALDI-TOF-MS, 10-15 folds of improvement in the signal intensity were

observed at the optimized parameters with Ag@ODT/AMP whereas the improvement in signal intensity is less by using Ag@ODT/TG. We have successfully tested our protocols for biomolecules identification from real sample i.e milk (proteins and peptides), and urine (cystein and homocystein). Therefore, we can conclude that our bifunctionalized/capped AgNPs - SDME can effectively enhance the signal of proteins in MALDI-MS and thus paves a new research platform for quick, simple, low cost and rapid methods for high throughput analysis of proteins and biomolecules in the MALDI-TOF-MS. The AgNPs used in SDME and coupled to MALDI MS is a powerful tool and they may be popular to be used as both pseudostationary phase and SALDI-MS due to their extremely high surface area and high sensitivity to match the laser energy used in MALDI-MS which enables high speed and highly sensitive detection and separation for various biomolecules.

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Reference:

1. N. G. Bastús, F. Merkoçi, J. Piella, V. Puntes, *Chem. Mater.*2014, **26**, 2836–2846
2. (a) K. Loza, J. Diendorf, C. Sengstock, L. Ruiz-Gonzalez, J. M. Gonzalez-Calbet, M. Vallet-Regi, M. Köller and M. Epple, J. Mater. Chem. B, 2014, **2**, 1634-1643; (b) R. Foldbjerg, X. Jiang, T. Miclăuş, C. Chen, H. Autrup, C. Beer, Toxicol. Res., 2015, DOI: 10.1039/C4TX00110A;
3. H. Li, D.Xu, *Trend. Anal. Chem*. 2014,**61**, 67–73

4. A. Liang, Q. Liu, G. Wen, Z. Jiang, *Trends Anal. Chem*. 2012, **37**, 32–47.
5. J. Li, W. Li, W. Qiang, X.Wang, H. Li, D. Xu, *Anal. Chim.Acta* 2014, **807**, 120– 125
6. H. N. Abdelhamid, H.F. Wu, *Mater. Sci. Engineering C* 2014, **45**, 438–445
7. D. Ghosh, N. Chattopadhyay, *J. Luminescence* 2015, **160,** 223–232
8. N. Li, Y. Wang, Y. Li, W. Cao, H. Ma, D. Wu, B. Du, Q. Wei, *Sensors and Actuators B* 2014, **202**, 67–73
9. K. Dastafkan, M. Khajeh, M. Ghaffari-Moghaddam, M. Bohlooli, *Trends. Anal. Chem.* 2015, **64**, 118–126
10. (a) H. N. Abdelhamid, H.F. Wu, *Colloids and Surfaces B: Biointerfaces*, 2014, **115**, 51–60; (b) H. N. Abdelhamid, H.F. Wu, *Trends. Anal. Chem*., 65, 2015, **65**, 30–46; (c) H. N.Abdelhamid, H.F. Wu, J. *Amer. Soc. Mass. Spectrom*. 2014, **25**, 861-868; (d) H. N. Abdelhamid, B.S. Wu, H.F. Wu. *Talanta*, 2014, **126**, 27–37; (e) H. N. Abdelhamid, M. Shawan Khan, H.F. Wu, *Anal. Chim. Acta*, 2014, **823**, 51–60; (f) M L. Bhaisare, H. N. Abdelhamid, B.S.Wu, H.F. Wu, *J. Mater. Chem. B*, 2014, **2**, 4671-468; (g) P.Y. Hua, M. Manikandan, H. N. Abdelhamid, H.F. Wu, J.Mater. Chem. B, 2014,**2**, 7334-7343; (i) H. N. Abdelhamid, J. Gopal, H.F. Wu, Anal.Chim.Acta. 2013, 767, 104–111; (j) J. Gopal, H. N. Abdelhamid, P. Y. Hua, H.F. Wu. J.Mater. Chem. B, **2013**,**1**, 2463-2475; (k) H. N. Abdelhamid, H.F. Wu, *J. Mater. Chem. B*, 2013,**1**, 3950 - 3961
11. S. K. Kailasa, H.F.Wu, *Trends.Anal. Chem*. 2015, **65**, 54–72
12. M.Yang, T. Fujino, Chemical Physics Letters 2013, **576**, 61–64
13. S. K.Kailasa, H.F. Wu, *Talanta* 2010, **83**, 527–534
14. J. Niziol, W. Rode, Z. Zieli´nski, T. Ruman, Inter. J. Mass Spectrom. 2013, **335**, 22–32

15. S.D. Sherrod, A.J. Diaz, W.K. Russell, P.S. Cremer, D.H. Russell, Anal. Chem. 2008, **80**, 6796–6799.

16. A. Verma1, U. Uzun, Y.Hu, Y. hu, H.S. Han, N. Watson, S. Chen, D.J. Irvine, F. Stellacci . *Nat. materials* 2008, **7**, 588-595.

17. K. Agrawal, H.F. Wu. *Rapid Commun. Mass Spectrom*. 2008, **22**, 283–290.

18. HN Abdelhamid, HF Wu, *Talanta* 2013, **115**, 442-450

19. R.C. Beavis, T. Chaudhary, B.T. Chait, Organic Mass Spectrometry 1992, **27**, 156–8.

20. HN Abdelhamid, HF Wu, *Analyst*, 2015, **140**, 1555-1565 Y. He, H.K. Lee, Anal. Chem., 1997, **69**, 4634

21. G Gedda, HN Abdelhamid, M Shahnawaz Khan, HF Wu, *RSC Advances* 2014, **4**, 45973-45983

22. (a) M. Busman, J. R. Bobell, C. M. Maragos, *Food Control* 2015 , **47**, 592-598; (b) M.Mateos-Vivas, E. Rodríguez-Gonzalo , J. Domínguez-Álvarez, D. García-Gómez, R. Ramírez-Bernabé, R. Carabias-Martínez, Food Chemistry, 2015, **174**, 348–355

23. (a) Z. Liu, P. Moate, B. Cocks, S. Rochfort, J.Chromatography B, 2015, 978, 95-102; (b) Y. Yang, N.Zheng, X. Zhao, Y.Zhang, R.Han, L. Ma, S. Zhao, S. Li, T. Guo, J.Wang, Journal of Proteomics 2015, **116**, 34– 43

24. L.R. Pokhrel, C.P. Andersen, P.T. Rygiewicz, M. G. Johnson, Science of the Total Environment 2014, **490**, 11–18

25. (a) M. Khajeh, E. Sanchooli, *Appl. Nanosci*. 2011, **1**, 205–209; (b) M. Khajeh, *Biol. Trace Elem. Res*. **2010**, **138**, 337–345;

26. S.D. Sherrod, A.J. Diaz, W.K. Russell, P.S. Cremer, D.H. Russell, Anal. Chem. 2008, **80**, 6796–6799.

27. P.R. Sudhir, K. Shrivas, Z.C. Zhou, H.F. Wu, Rapid Commun. Mass Spectrom. 2008, **22**, 3076–3086.

28. H.N. Abdelhamid, M.L. Bhaisare, H.F. Wu, Talanta 2014, **120**, 208-217

Figure captions

Figure 1(A) Strcutre of Ag@ODT/AMP and Ag@ODT/TG and (B) their characterization using SEM for (a) Ag@ODT/AMP and (c) Ag@ODT/TG and TEM (b) Ag@ODT/AMP and (d) Ag@ODT/TG

Figure 2. FTIR spectra of (a) Ag@ODT/AMP and (c) Ag@ODT/TG

Figure 3. MALDI-TOF mass spectra of Insulin (80 nM) using (A) Ag@ODT/AMP and (B) Ag@ODT/TG (a) without AgNPs- SDME, direct analysis of aqueous sample with CHCA (5000 ppm) (b) with AgNPs- SDME and CHCA by using AgNPs- SDME at room temperature for 5 min extraction and stirring at 200 rpm and CHCA (5000 ppm) at pH 4, laser energy 60 %

Figure 4: MALDI-TOF mass spectra shows analysis of (A) Cytochrome c (80 nM) and (B) ubiquitin, ubiquitin-like proteins from oyster mushroom sample (50μL) (a) without AgNPs-SDME, direct analysis aqueous sample with SA (5000ppm) (b) with AgNPs- SDME (2.85mM) and SA (5000ppm), using AgNPs- SDME at room temperature for 10 min extraction at pH 7.0 and laser energy 65 %

Figure 5: MALDI-TOF mass spectra shows optimization of extraction time of Insulin (80 nM) (a) at 0 min (b) at 2 min (c) at 10 min (d) at 15 min (d) at 20 min by using Ag@ODT/AMP-SDME at room temperature and CHCA (5000ppm) at (A) pH 3.0, and (B) pH 7.0.

Figure 6: MALDI-TOF mass spectra Milk sample (100μL) analysis after addition of NaCl (5M) (a) without AgNPs- SDME, direct analysis of aqueous sample with SA (5000ppm) (b) with AgNPs- SDME and SA (5000ppm) by using AgNPs- SDME at room temperature for 15 min extraction and stirring at 200 rpm, at pH 4.5 and laser energy 50 %,

Figure 7: MALDI-TOF mass spectra of Cysteine and Homocystiene obtained from Urine sample using AgNPs- SDME at room temperature for 10 min extraction, stirring at 200 rpm and CHCA (5000ppm) at pH 7.5 Laser energy 36%.

Table 2: Comparison among techniques involved nanoparticles for protein separation

Techniques	**Nanoprobes**	**Detection Technique**	**Analytes**	**samples**	**LOD**	**Ref.**
NPs-LPME	Ag_2Se NPs@MUA	MALDI-MS	valinomycin and 100–180 nM for gramicidin D	hydrophobic peptides and proteins from biological samples (urine and plasma)	20–68 nM for valinomycin and 100–180 nM for gramicidin D	13
SDME	Tetraalkylammonium bromide coated silver nanoparticles	AP-MALDI-MS	Met- and Leu-enkephalins (Met-enk and Leu-		160 and 210 nM	27

			enk)			
SDME	Ag@ODT/AMP	MALDI-MS	Insulin, cytochrome c, ubiquitin, cyctein, homocystein	Milk, urine	20-80 nM	Here
UESA-DLLME	CeO_2@CTAB	MALDI-MS	Pathogenic bacteria	Blood	3.8×10^4 - 1.5×10^4 cfu/mL	28

Notes: atmospheric pressure matrix-assisted laser desorption/ionization ion trap mass spectrometry (AP-MALDI-MS); NPs-based liquid-phase microextraction (NPs-LPME); octadecanethiol (ODT) and 11-mercaptoundecanoic acid (MUA) on Ag_2Se NPs; ultrasound enhanced surfactant-assisted dispersive liquid–liquid microextraction (UESA-DLLME)