Determining Estrogens Using Surface-Assisted Laser Desorption/Ionization Mass Spectrometry with Silver Nanoparticles as the Matrix

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We describe the application of silver nanoparticles (Ag NPs) as matrices for the determination of three estrogens using surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS). Because Ag NPs have extremely high absorption coefficients ($1.2 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$) at 337 nm, they are effective SALDI matrices when using a nitrogen laser. Three tested estrogens—estrone (E1), estradiol (E2), and estriol (E3)—adsorb weakly onto the surfaces of the Ag NPs, through van der Waals forces. After centrifugation, the concentrated analytes adsorbed on the Ag NPs were subjected directly to SALDI-MS analyses, with the limits of detection for E1, E2, and E3 being 2.23, 0.23, and 2.11 μM , respectively. The shot-to-shot and batch-to-batch variations for the three analytes were less than 9% and 13%, respectively. We validated the practicality of this present approach through the quantitation of E2 in human urine. Using this approach, we determined the concentration of E2 in a sample of a pregnant woman's urine to be $0.16 \pm 0.05 \mu\text{M}$ ($n = 10$). (J Am Soc Mass Spectrom 2008, 19, 1343–1346) © 2008 American Society for Mass Spectrometry

Estrogens are steroidal hormones. The most potent naturally occurring estrogen is estradiol (E2), which is interconvertible with estrone (E1). These hormones play important roles during the various stages of mammalian development, including prenatal development, growth, and reproduction, and influence sexual behavior, with E2 displaying the highest estrogenic activity. The concentrations of E1, E2, and estriol (E3) in body fluids are closely related to the stages of pregnancy [1].

Current techniques for the analysis of endogenous estrogens and their metabolites employ immunoassays [2], high-performance liquid chromatography [3], capillary electrophoresis [4], and mass spectrometry [5]. Of these techniques, only mass spectrometry provides structural information, but with higher cost and poorer sensitivity (pmol) relative to that of the immunoassay-based analyses (fmol) [6].

Recently, we developed surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) techniques using Au nanoparticles (NPs), aptamer-functionalized Au NPs, and TiO_2 NPs for the analyses of amino thiols, adenosine triphosphate, and catechins,

respectively [7–9]. SALDI-MS using NPs provides several advantages for the analysis of small molecules (<500 Da), including high reproducibility and a simpler sample preparation procedure [10].

We analyzed estrogens by SALDI-MS using Ag NPs as matrices. We carefully evaluated the effects of the pH of the sample solutions, the concentration of Ag NPs, and the laser fluence on the estrogens sensitivity of the present SALDI-MS system.

Experimental

Chemicals

Citric acid, E1, E2, E3, β -glucuronidase/sulfatase from *Helix pomatia* (Type H-2), silver nitrate, and trisodium citrate were purchased from Sigma (St. Louis, MO). Ammonia solution (28%) was purchased from Showa Chemical Industry (Tokyo, Japan). L-(+)-Ascorbic acid and dichloromethane were purchased from Acros Organics (Geel, Belgium).

Synthesis and Characterization of Ag NPs

Ag NPs was prepared through sodium citrate mediated reduction of silver nitrate, using a slight modification of a literature procedure [11]. An aqueous silver nitrate

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solution (1 mM, 100 mL) was heated to its boiling point and then trisodium citrate solution (20% wt/vol, 0.5 mL) was added while stirring. The solution was heated under reflux for an additional 10 min. A double-beam UV-vis spectrometer (Cintra 10e; GBC Scientific Equipment Pty Ltd., Victoria, Australia) was used to measure the absorbance of the as-prepared Ag NPs. The surface plasmon resonance wavelength of the Ag NPs appears at 410 nm. The size of the Ag NPs was further confirmed through analyses of transmission electron microscopy (TEM) images (H7100; Hitachi High-Technologies Corp., Tokyo, Japan). The concentration of the as-prepared Ag NPs was ca. 2.0×10^{15} NPs/L (3.3 nM) [12]. After being centrifuged at 12,000 rpm (Sigma 3K30 centrifuge; Sigma Laborzentrifugen GmbH, Postfach, Germany) for 10 min, the as-prepared Ag NPs were concentrated to the final concentration of 7.0×10^{16} NPs/L, which was estimated by comparing the absorbance values at 410 nm of the solutions after and before centrifugation.

Preparation of Samples

Stock solutions (10 mM) of E1, E2, and E3 were prepared separately in methanol. Ammonium citrate buffers at pH 4.0–11.0 were prepared by adjusting different amount of aqueous ammonia into 50 mM citric acid solutions to a final volume of 50 mL. Mixtures (1.0 mL) of ammonium citrate (50 mM, pH 10.0, 10 μ L), Ag NPs (1.7 nM, 5–500 μ L), and an estrogen (1–100 μ M, 10 μ L) were equilibrated at ambient temperature and pressure for 30 min before being centrifuged at 12,000 rpm for 10 min. For each mixture, ca. 970 μ L of the supernatant was discarded and the bottom portion (ca. 30 μ L) contained the Ag NPs and solution, which was further subjected to vortex to provide a homogeneous mixture. A sample (ca. 1 μ L) of each mixture was pipetted onto a stainless-steel 384-well MALDI target plate and dried in air at room temperature before SALDI-MS analysis.

Urine samples (ca. 250 mL) were collected from a pregnant woman (36 wk, age 35) in 500-mL bottles containing ascorbic acid (0.25 g) that was used for preservation and stored at -20°C until required for analysis. Because endogenous estrogens and their metabolites excreted in urine are present in glucuronidated (major) and sulfated (minor) forms [13], a hydrolysis step was conducted before SALDI-MS sample preparation. Hydrolysis and extraction were conducted according to the literature [14]. After extraction, the organic phase was collected, which was then subjected to evaporation under a vacuum pump. Solutions of ammonium citrate (0.5 mM, pH 10.0, 987.5 μ L) and Ag NPs (1.7 nM, 12.5 μ L) were added to the dried urine samples and equilibrated for 30 min. The samples were then centrifuged at 12,000 rpm for 10 min. For each sample, ca. 970 μ L of the supernatant was discarded. The bottom portion (30 μ L) contained the Ag NPs, the analytes, and solution, which was further subjected to vortex to provide a homogeneous mixture. Each solution (ca. 1 μ L) was pipetted onto a stainless-steel 384-well MALDI target plate and dried in air at room temperature before SALDI-MS analysis.

Estimation of Estrogens Adsorbed on Each Ag NP

A spectrofluorometer (Cary Eclipse, Varian, Palo Alto, CA) was used to measure the native fluorescence (λ_{ex} : 266 nm; λ_{em} : 312 nm) of the estrogens. Mixtures (1.0 mL) of ammonium citrate (50 mM, pH 10.0, 10 μ L), Ag NPs (1.7 nM, 12.5 μ L), and each estrogen (100 μ M, 10 μ L) were equilibrated at ambient temperature and pressure for 30 min before being centrifuged at 12,000 rpm for 10 min. For each mixture, ca. 970 μ L of the supernatant was collected for fluorescence measurements. By comparing the fluorescence intensity of the each estrogen in the absence and presence of the Ag NPs, the average number of estrogen molecules adsorbed to each Ag NP was estimated [8].

Figure 1. (a) Absorption spectra of Ag NP (0.02 nM) solutions in the absence (solid line) and presence (dotted line) of E2 (100 μ M). (b) TEM image of Ag NPs.

Table 1. Impacts of Ag NP concentration, buffer pH, and laser fluence on the S/N of estradiol

Ag NPs concentration (pM) ^a	S/N ratios of E2 peak				
	84.5	42.2	22.1	8.5	4.2
Buffer pH ^c	136 (33%) ^b	155 (25%)	130 (11%)	22 (11%)	10 (7%)
	4.0	6.0	8.0	10.0	11.0
Laser fluence (μJ) ^d	21 (15%)	8 (8%)	66 (28%)	130 (11%)	90 (31%)
	102.0	102.9	103.8	104.7	105.6
	25 (27%)	130 (11%)	145 (17%)	103 (24%)	21 (32%)

^a pH = 10.0 and laser fluence = 102.9 μJ.^b RSD, *n* = 10.^c [Ag NPs] = 0.02 nM and laser fluence = 102.9 μJ.^d [Ag NPs] = 0.02 nM and pH = 10.0.

SALDI-MS Measurement

MS experiments were performed in the negative-ion mode on a reflectron-type time-of-flight (TOF) mass spectrometer (Biflex III; Bruker Daltonics, Bremen, Germany) equipped with a 1.25 m flight tube [10]. To obtain good resolution and signal-to-noise (S/N) ratios, the laser fluence was adjusted to slightly higher than the threshold (102 μJ), and each mass spectrum was generated by averaging 250 laser shots.

Results and Discussion

Adsorption of Estrogens on Ag NPs

Figure 1a and b displays the UV-vis spectra (solid curve) and TEM image of the Ag NPs solutions, with a maximum absorption wavelength at 410 nm. From counting of 300 Ag NPs, we calculated the average size to be 34 (±3) nm. In the presence of E2 (100 μM), the SPR band shifted to 436 nm and the full width at half magnitude was broadened from 94 to 128 nm, suggesting their adsorption onto the surfaces of the Ag NPs. We note that the adsorption of the estrogens (neutral) onto the Ag NPs (hydrophobic surface) occurred through van der Waals interactions. After centrifugation, the mixture volume was reduced from 1.0 mL to 30

μL, leading to concentration of estrogens on the Ag NPs.

Optimization of SALDI-MS Analysis

We found that a high mass signal background noise was usually generated at high NP concentrations. From fluorescence data, we estimated that there were 4.5×10^4 molecules of E2 on each Ag NP, which corresponds to a saturation concentration of E2 of 0.90 μM when using 0.02 nM (1.2×10^{13} Ag NPs/L).

Table 1 shows that the optimal conditions for the concentration of Ag NPs, pH of ammonium citrate buffer, and laser fluence were 0.02 nM, 10.0, and 102.9 μJ, respectively. The signals and S/N ratios at pH 10.0 were greater than those at pH 4.0 and 6.0. Because the pK_a of E2 is 10.2 [16], ca. 0.0006%, 0.006%, and 59% of E2 exist in the deprotonated form at pH 4.0, 6.0, and 10.0, respectively. Similarly, the degrees of dissociation of E1 (pK_a 10.4) and E3 (pK_a 10.0) both increase upon increasing pH [15, 16]. The low S/N ratios obtained from the acidic buffers were due to the low desorption/ionization efficiency of E2 when the SALDI-MS system was operated in the negative ion mode.

Figure 2. SALDI mass spectrum of a mixture of E1 (100 μM), E2 (10 μM), and E3 (100 μM). Peaks from the background are marked as asterisks.

Figure 3. MS signal intensity of the $[E2 - H]^-$ ion plotted against the concentration of E2 (1–100 μM). The inset displays the linearity of the plot of the signal intensity for E2 against its concentration over the range from 1 to 20 μM.

Figure 4. SALDI mass spectra of a pregnant woman's urine: (a) original sample and (b) sample spiked with 5.0 μM E2.

Under optimum conditions, we detected the major ion peaks for E1, E2, and E3 at m/z 269.6, 271.5, and 287.5, respectively, which we assigned to the ions $[\text{E1} - \text{H}]^-$, $[\text{E2} - \text{H}]^-$, and $[\text{E3} - \text{H}]^-$, respectively. The mass spectrum presented in Figure 2 displays the simultaneous analysis of E1, E2, and E3. Each of the three mass spectra displays two high-intensity peaks at m/z 283.6 and 284.6, which we assign to $[\text{citrate} + 2\text{K} + \text{NH}_3]^-$ and its deuterated congener, respectively. On the basis of the peak heights, we estimated the LOD at values of S/N of 3 for E1, E2, and E3 to be 2.23, 0.23, and 2.11 μM , respectively. We note that SALDI-MS using Ag NPs provides advantages of small shot-to-shot (all less than 9%, $n = 10$), batch-to-batch (all less than 13%, $n = 7$) variations.

Determination of E2 in Human Urine

We obtained a calibration curve of the MS signals for E2 against its concentrations in the range from 1 to 100 μM (Figure 3). The inset in Figure 3 indicates that good linearity ($R^2 = 0.998$) existed in the range from 1 to 20 μM . Next, we tested the practicality of this present SALDI-MS approach through the determination of E2 in a pregnant woman's urine. Figure 4 displays a representative mass spectrum of the urine samples. The signals for the ions of E2 (m/z 271.5) and E3 (m/z 287.5) are readily identifiable. By comparing the peak high with the standard calibration curve, we estimated that the concentrations of E2 and E3 in the pregnant woman's urine were 0.16 (± 0.05) and 0.83 (± 0.13) μM ($n = 10$), respectively, which is in good agreement with literature values [4, 17].

Conclusions

In this study, we employed Ag NPs as SALDI-MS matrices for the analysis of estrogens. When using Ag NPs as matrices, the LOD of E2 was as low as 0.23 μM .

We used this approach, with its advantages of simplicity, repeatability, and sensitivity, to determine the level of E2 in a pregnant woman's urine. One drawback of using Ag NPs for the determination of estrogens, however, is that their interactions are weak and nonspecific, leading to an inefficient concentration effect. To further improve the sensitivity of the SALDI-MS approach when determining estrogens in a urine sample, we will focus on the preparation of Ag NPs bioconjugated with antibodies against estrogens in future studies.

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