Ultrathin Homogenous AuNP Monolayers as Tunable Functional Substrates for Surface-Assisted Laser Desorption/Ionization of Small Biomolecules

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ABSTRACT: A series of ultrathin, homogenous gold nanoparticle (AuNP) substrates for surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS) were prepared using a simple air/water interface approach. These SALDI substrates enabled soft ionization and provided significant improvements in terms of signal intensities and reduced background levels in comparison to other AuNP morphologies for different analytes such as fatty acids, peptides, amino acids, saccharides, and drugs. Through different microscopic and spectroscopic methods, we demonstrated that the packing homogeneity of the [AuNP]n substrates played a vital role in the efficiency of the SALDI process. We demonstrated that the signal intensities of the investigated analytes were readily optimized by manipulating the thickness of the [AuNP]n substrates. The desorption/ionization efficiency increased as a function of the number of layers and then reached a saturation point. The optimized [AuNP]n substrates not only exhibited high SALDI-MS desorption/ionization efficiencies but also showed excellent reproducibilities of the analyte signals.

KEYWORDS: gold nanoparticles (AuNP), surface-assisted laser desorption/ionization (SALDI), mass spectrometry (MS), biomolecules, soft ionization

INTRODUCTION

Matrix-assisted laser desorption/ionization (MALDI)-MS has been widely used in biological applications because of its soft desorption and ionization conditions and its broad application range. However, the requirement of a chemical matrix severely limits MALDI in the detection of small molecules at lower m/z ranges < 500, owing to matrix interferences. Surface-assisted laser desorption/ionization (SALDI) is frequently used as a matrix-free alternative to MALDI. Recent advances in the SALDI field are mainly due to the availability of efficient nanoparticulate substrates. A wide variety of different nanomaterials including carbon, silicon, metal (Au, Ag, Pa, etc.), metal oxides, and other nanomaterials have been successfully implemented for SALDI. Compared with MALDI, SALDI offers distinct advantages, namely its simple and flexible sample preparation, limited background signals in the low m/z range, and its ability to modify nanoparticles to selectively capture and ionize analytes. The desorption and ionization efficiency in SALDI is highly dependent on physical (size, surface roughness, electrical conductivity, light absorption, and melting point) and chemical properties (surface modification, binding energy to analytes) of the nanomaterials. Generally, rapid laser-induced heating of the substrates and slow energy dissipation, leading to desorption and ionization of analytes, is widely accepted as the primary mechanism of function.

Owing to the unique optical, thermal, and electrical properties, gold nanomaterials are promising candidates for SALDI-MS analysis of biomolecules. Mclean et al. used AuNP with different sizes (2–10 nm) to detect peptides. They found a size effect of AuNP on desorption and ionization efficiencies of peptides, where larger AuNP showed better performance. Amendlac et al. reported that chemical-free AuNP exhibited low background levels for m/z < 500 as.
compared to AuNP with surface-protected agents, providing picomolar level detection of small molecules. Gold nanorods were used to detect biomolecules after irradiation with an IR laser. Havel et al. used flowerlike gold nanoparticle as mediator to enhance ionization of peptides. Finally, fluorinated AuNP were synthesized for comprehensive analysis of metabolites in biological tissues with high sensitivity and minimal background noise levels. Lower laser energy was required during this process, which led to gentle desorption/ionization.

While the above inorganic nanomaterials were shown to be effective SALDI substrates for small molecules, signal fluctuations and poor reproducibility of analyte measurement remain a major problem. There are multiple reasons for this, including hot spot formation during sample deposition and solvent evaporation, leading to poor shot-to-shot and spot-to-spot reproducibility. Furthermore, the morphology of the substrate is often highly inhomogeneous, resulting in significant variations of ion signals. Recent studies showed that homogenous distribution of nanomaterials to form continuous thin films decreased sweet spot formation and increased signal intensity and reproducibility in SALDI-MS. Multilayered AuNP thin films were used to detect a bone biomarker (hydroxyproline) for assessment of osteoporosis with excellent reproducibility of 9.3 % RSD. Gold nanoporous films, modified by cysteine, were easily prepared to detect a wide range of compounds including amino acids, drugs, cyclodextrins, peptides, and polyethylene glycols with good reproducibility (<10% RSD). Kawasaki et al. used a layer-by-layer (LBL) method to obtain multilayer films of AuNP on a silicon wafer for SALDI, to detect angiotensin I at low concentration levels. The author showed that the sensitivity for angiotensin improved as the number of layers was increased; it reached a plateau for 5 layers. The same material was also used to extract and directly identify environmental pollutants at very low concentration levels (<100 pg/L). Choi et al. attached a homogenous layer of graphene foil on a target plate, and samples were deposited onto this foil. Sweet spots were effectively eliminated and reproducibility highly improved (<10% RSD). The method exhibited good linear response for small peptides (angiotensin II and Glu-angiotensin) and good linear response to angiotensin improved as the number of layers was increased; it reached a plateau for 5 layers. The same material was also used to prepare packed monolayer films, e.g., spin coating, physical deposition, controlled drying, or preassembly at air/water interfaces. The predominant air/water method is a Langmuir-based process, which enables the nanoparticles to preassemble in highly-ordered structures over large areas. This method is time-consuming, however, and requires specialized equipment. Nagel et al. introduced a much simpler method to produce high quality monolayers, where the colloidal nanoparticles were directly assembled at a water/air interface and a monolayer formed automatically without any additional compression. The monolayers of nanoparticles on the water surface are subjected to isothermal compression and undergo phase transition from liquid to solid as a function of the increase of surface pressure. Under optimal conditions, continuous layers of nanoparticles are formed and evenly arranged with sufficient density and space. These monolayer films can then be transferred to any substrate or surface.

In this study, a new method was developed to prepare homogeneous monolayers of small AuNP. Each monolayer was ultrathin (∼2.5 nm), and multilayer AuNP substrates ([AuNP]) were prepared by transferring individual monolayers of AuNP for n times on the substrate. In previous reports, LBL, controlled drying, and spin coating were used to prepare multilayer films for SALDI-MS. These methods were not as simple to use as our new method, however, and did not always provide the necessary homogeneity. For example, LBL nanomaterials can only be deposited onto specific substrates (e.g., silicon wafers or glass). Also, as electrostatic interactions are utilized to form the continuous layers, an equivalent counterpart is needed for the nanoparticles. Finally, the preparation process is very time-consuming, often requiring hours to complete. Compared to these methods, the approach presented here is very simple and fast (<10 min) and can be implemented on a variety of different substrates. In our experiments, [AuNP] substrates were used as functional substrates for SALDI-MS to detect small molecules, including fatty acids, amino acids, drugs, small peptides, and saccharides. These substrates offered several advantages: (1) the small AuNP provided a large surface area for improved contact with the analyte and increased LDI efficiency; (2) the [AuNP] substrates were homogeneous over extended areas, which avoided sweet spot formation and enhanced reliability; (3) there was almost exclusive formation of potassium adducts during ionization, which increased sensitivity for quantitative applications; and (4) the ionization efficiency for the analytes could be adjusted by changing the number of layers (n) of the [AuNP] substrates.

Experimental Section

Chemicals and Reagents. CHCA (α-cyano-4-hydroxycinnamic acid), palmitic acid, stearic acid, oleic acid, linoleic acid, erythromycin, A, cysteine, phenylalanine, and 4-amino-1-benzylpyridinium bromide (ABP) were purchased from Sigma-Aldrich (Steinheim, Germany). Flumequine, peometon, D-fructose, L-sorbose, D-maltose, and D-sucrose were from Merck (Darmstadt, Germany). Timolol maleate, gluthathione, and Leu-enkephalin were from Cayman Chemicals (Hamburg, Germany). Acetonitrile (ACN) and methanol were from VWR Chemicals (Darmstadt, Germany). Deionized water was generated by a Millipore (Bedford, MA, USA) water purification system.

Stock solutions of analytes were prepared as follows: fatty acids (palmitic acid, stearic acid, oleic acid, and linoleic acid) and drugs (erythromycin, flumequine, peometon, and timolol maleate) were dissolved in methanol at 1 mM; amino acids (cysteine and phenylalanine), small peptides (gluthathione and Leu-enkephalin), and saccharides (D-fructose, L-sorbose, D-maltose, and D-sucrose) were dissolved in water at 10 mM; ABP was dissolved in methanol at 10 mM. All stock solutions were stored at −20 °C and diluted to the required concentration prior to use. If not otherwise specified, fatty acids, drugs, amino acids, small peptides, saccharides, and ABP were diluted to 100 μM in methanol. Sodium and potassium salt were added to analytes solutions if needed.
Preparation of Small Gold Nanoparticles. Stock AuNP solutions were prepared according to a modified protocol, which was previously published. Ligand exchange from oleylamine to 11-mercaptoundecanoic acid was performed as follows: 10 mL of AuNP, dispersed in toluene, was heated to 80 °C at 200 rpm. 200 mg of 11-mercaptoundecanoic acid was dissolved in 5 mL of toluene and heated to 80 °C until the 11-mercaptoundecanoic acid was fully dissolved. Afterwards, the dissolved 11-mercaptoundecanoic acid was added to the AuNP solution. During ligand exchange, the AuNP agglomerate (11-mercaptoundecanoic acid capped AuNP do not disperse in toluene). Subsequently, the AuNP dispersion was centrifuged for 3 min at 3000 rpm. To remove excess ligand, the sediments AuNP were dispersed in 5 mL of toluene (at this state, the AuNP were still agglomerated). The suspension was sonicated for 5 min, followed by centrifugation (3 min at 3000 rpm) and removal of the supernatant. This was repeated 5 times. Finally, the AuNP were dispersed in 10 mL of methanol. The AuNP solution was stored at room temperature.

Preparation and Characterization of \([\text{AuNP}]_n\) Substrates. A homemade microtrough (130 × 80 × 30 mm, Figure 1) was used to prepare the AuNP substrates. Deionized water was first filled into the trough. Then 10 μL of the AuNP solution was deposited on the water using a microsyringe, by allowing the needle of the syringe to contact the water surface of water and gently pushing out the AuNP solution. The amphiphilic ligand of the AuNP (11-mercaptoundecanoic acid) automatically enabled the formation of an AuNP monolayer on the water surface. A clean steel target plate was then placed onto the water surface at an angle of ca. 45° and slowly “rolled” onto the water surface for full contact, held in that position for 10 s, then lifted up again to a 45° angle, and pulled out. The substrate was subsequently dried at ambient conditions. \([\text{AuNP}]_n\) substrates, where \(n\) denotes the number of layers, were prepared by placing additional layers on top of the previous layer.

UV/Vis experiments of films on glass were performed with a Lambda 750 spectrometer (Perkin Elmer, Shelton, CT, USA). The AuNP monolayer was carefully transferred to a carbon-coated copper TEM grid (Plano, Wetzlar, Germany), and TEM experiments were undertaken with a JEM-2010 microscope (Jeol GmbH, Munich, Germany) operated at an accelerating voltage of 200 kV. For X-ray scattering measurements, the AuNP films were carefully transferred to the thin rectangular glass substrates (borosilicate, 170 ± 5 μm thick, Thorlabs GmbH, Germany). X-ray scattering was performed with a Xeuss 2.0 setup (Xenocs, Sassenage, France) equipped with a GeniX Low Divergence Cu-Κα source. The X-ray wavelength was 1.54 Å. The scattering signal was collected with a Dectris Pilatus 1M detector, and the sample to detector distance was calibrated with silver behenate. A horizontal scanning of the sample was achieved with fixing its height position while moving the sample along the direction normal to the beam at a step of 5 mm. The conversion of 2D image to the 1D curve was done with Foxtrot software.

A scanning electron microscope (FEI Quanta 400F, FEI Europe, Eindhoven, The Netherlands) was used at an operating voltage of 10 kV to characterize the surface structures of the \([\text{AuNP}]_n\) thin films. The contact angle and its change with time was determined for aqueous dispersions (volume 2 μL) of the \([\text{AuNP}]_n\) films on cleaned silicon wafers with a OCA 20 contact angle measuring system (Data Physics Instruments, Filderstadt, Germany) at room temperature using the sessile drop method. The frequency for the contact angle data collection was 25 Hz. The video images were processed with the OCA 20 instrument software to obtain the contact angle values.

Preparation of MALDI and SALDI Samples. MALDI samples were prepared using the dried droplet method. CHCA solution (10 mg/mL in acetonitrile/water 70/30 v/v) was first mixed with the analytes, 1.0 μL of the mixture was deposited on the surface of the target plate using a micropipette, and the droplet was dried at ambient conditions. \([\text{AuNP}]_n\) films were transferred and dried on steel target plates, and 0.5 μL of the analyte solutions was deposited onto the steel surface and dried at ambient conditions. Two comparisons were made: (1) 0.5 μL of the original AuNP solution was pipetted onto the steel target. After the solvent evaporated, 0.5 μL of the analyte solution was placed onto the AuNP and dried; (2) the original AuNP solution was mixed with the analyte solution. Then, 1 μL of the mixture was pipetted onto a steel target and dried at ambient conditions.

Mass Spectrometry. MS experiments were performed using a Bruker Esquire HCT+ ion trap mass spectrometer (Bremen, Germany) coupled with a MassTech (Burlingtonville, MD, USA) atmospheric pressure (AP) MALDI source. An Nd:YAG laser emitting at 355 nm was used as the light source. Laser energy was set to 50%, and the repetition rate was set to 200 Hz. Mass spectra were acquired in positive ion mode from \(m/z\) 50 to 500 using a raster motion of the target plate. Ion currents were accumulated as follows: velocity, 40.0 mm/min; scan length, 2.0 mm; scan height, 2.0 mm; spacing, 0.5 mm; step size, 0.5 mm; raster direction, horizontal. A drying gas temperature of 50 °C at a flow rate of 5.0 L/min was used.

RESULTS AND DISCUSSION

In this study, small AuNP were used to prepare ultrathin films as functional substrates for SALDI-MS. By using a simple air/water interfacial approach, homogenous monolayer AuNP films were formed by adding AuNP colloidal onto the surface of water as shown in Figure 1. \([\text{AuNP}]_n\) substrates were prepared by repeatedly (n-fold) transferring the monolayer
AuNP films, followed by drying them under ambient conditions.

In all experiments described here, an atmospheric pressure (AP) MALDI source was implemented for SALDI-MS. AP-MALDI allows for much quicker and easier sample handling and often provides softer ionization conditions with reduced fragmentation levels,\textsuperscript{52,53} which is ideal for molecules that are unstable under vacuum conditions.\textsuperscript{54} While sometimes the mass range is not as wide as for vacuum MALDI and the ion transmission efficiency from ion source to analyzer is lower,\textsuperscript{55,56} the signal-to-noise ratios for analytes in AP-MALDI are usually comparable to vacuum MALDI.\textsuperscript{57} In our

Figure 2. (a) TEM image of a single layer of an AuNP film and (b) UV/Vis absorption of the [AuNP]₁ substrate. The mean diameter of the AuNP is ca. 2.5 nm; that is, the thickness of one layer of the AuNP film is also approx. 2.5 nm. The insert (left) shows the size distribution of the AuNP.

Figure 3. SALDI-MS spectra of (a) fatty acids, (b) drugs, (c) amino acids and small peptides, and (d) saccharides on [AuNP]₁ films at concentrations of 100 μM each.
exploratory SALDI-MS study, AP ionizations conditions were ideal for conducting the method optimization. AP conditions also avoided ablation with subsequent ionization of the AuNP substrate material and thus interference signals in the SALDI mass spectra, which will be further described below.

**Characterization of the [AuNP] Films.** The structures of the [AuNP] films were initially characterized by TEM and UV/Vis spectroscopy (Figure 2). The TEM images clearly showed single AuNP within the [AuNP] films. The AuNP exhibited uniform spherical shapes with narrow size distribution of diameters between 1.9 and 3.8 nm. We calculated the statistical mean diameter of the AuNP as 2.5 nm (see insert of Figure 2a). Considering that the [AuNP] film is a single layer, we inferred the thickness of the single [AuNP] film to be ca. 2.5 nm. In Figure 2b, the UV/Vis spectra showed several absorption peaks at 510, 395, 380, and 360 nm. The peak at 510 nm can be rationalized by the surface plasmon band of AuNP, while peaks below 400 nm were due to metal-centered (interband) transitions and/or ligand-metal charge-transfer transitions. Considering that these small AuNP exhibited strong absorption under 400 nm and that a laser wavelength of 355 nm was used for the MS experiments, we anticipated that photons would be readily absorbed by the AuNP for enhanced desorption/ionization efficiency of organic molecules. To further characterize the batch-to-batch reproducibility of the [AuNP] films, four further batches were prepared and

<table>
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<th>state</th>
<th>[M1 + H]+ (m/z 281)</th>
<th>[M1 + Na]+ (m/z 303)</th>
<th>[M1 + K]+ (m/z 319)</th>
<th>[M2 + H]+ (m/z 441)</th>
<th>[M2 + Na]+ (m/z 457)</th>
<th>[M2 + K]+ (m/z 473)</th>
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<td>1351</td>
<td>1076</td>
<td>4451</td>
<td>2628</td>
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<tr>
<td>deposited onto [AuNP] film</td>
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<td>1538</td>
<td>2597</td>
<td>4559</td>
<td>6683</td>
<td>14328</td>
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<tr>
<td>deposited onto [AuNP] film</td>
<td>29</td>
<td>201</td>
<td>10938</td>
<td>641</td>
<td>131</td>
<td>473</td>
</tr>
</tbody>
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"M1 and M2 represent linoleic acid and 11-mercaptoundecanoic acid, respectively.

**Figure 4.** SALDI-MS of (a) fatty acids, (b) drugs, (c) amino acids and small peptides, and (d) saccharides on [AuNP], for different layer numbers at concentrations of 100 μM each.
characterized by TEM and UV/Vis spectroscopy (Supporting Information, Figures S1 and S2), readily showing the excellent reproducibility of \([\text{AuNP}]_n\) film preparation.

**[AuNP]_n Substrate-Assisted LDI-MS of Small Molecules.** To demonstrate the ionizing abilities of the \([\text{AuNP}]_n\) substrates, they were evaluated for SALDI-MS analysis of different low molecular weight compounds. Different small molecules were investigated to demonstrate the proof-of-concept: fatty acids (palmitic acid, oleic acid, linoleic acid), synthetic drugs (erythromycin A, flumequine, pemetrexed, timolol maleate), amino acids (cysteine, phenylalanine), small peptides (glutathione, Leu-enkephalin), and saccharides (D-fructose, L-sorbose, D-maltose, and D-sucrose). As shown in Figure 3, all investigated analytes were clearly detected as protonated molecules, sodium and/or potassium adducts with very limited background signals from the \([\text{AuNP}]_n\) substrate. These initial results clearly revealed the potential of the \([\text{AuNP}]_n\) films as versatile substrates for SALDI-MS analysis. As SALDI-MS spectra are usually dominated by cation adducts, \(0.5 \text{ M} \text{ sodium or 0.5 } \text{ M} \) potassium ions were added to all analyte solutions, to further promote adduct formation. Except for some low intensity noise from the AuNP, no fragmentation of analytes was observed, suggesting that the \([\text{AuNP}]_n\) films provided soft ionization abilities during SALDI-MS.

**Comparison to Other Morphologies of AuNP.** In SALDI-MS, both structure and aggregate state of nanomaterials have a profound effect on the desorption/ionization process.\(^{26,28,29}\) Here, comparisons between different AuNP states were made using linoleic acid as the test analyte (Table 1). The SALDI spectra exhibited linoleic acid’s \([\text{M + Na}]^+\) and \([\text{M + K}]^+\) ions, as well as signals from the AuNP ligands (11-mercaptoundecanoic acid). Importantly, signal intensities of linoleic acid from \([\text{AuNP}]_n\) films were much higher than those generated from deposited AuNP or mixtures of AuNP and linoleic acid. This is likely the result of the improved contact of linoleic acid and AuNP in the films as well as the homogeneity of the \([\text{AuNP}]_1\) substrates.

\([\text{AuNP}]_n\) Films and Their Application in SALDI-MS. To optimize the desorption and ionization efficiency of the analytes, \([\text{AuNP}]_n\) \((n = 1–4)\) substrates of different thickness were prepared. When transferred onto a glass surface, \([\text{AuNP}]_n\) films visually appeared in purple color (Figure S3a). As the number of layers increased, the color of the \([\text{AuNP}]_n\) substrates deepened. \([\text{AuNP}]_n\) films were also characterized by UV/Vis spectroscopy as shown in Figure S3b. From this figure, it can be seen that the absorption of the \([\text{AuNP}]_n\) films at 355 nm increased with the number of layers (the quantitative increase is shown in the Supporting Information, Figure S4), allowing increasing energy absorption of the \([\text{AuNP}]_n\) substrates. The \([\text{AuNP}]_n\) films were then used to analyze different analytes by SALDI-MS (Figure 4). For some compounds, \(e.g.,\) fatty acids, signal intensities reached a plateau at two layers. For other analytes, such as drugs, three-layer substrates provided maximum ion currents. These results clearly showed that there was an optimal thickness of \([\text{AuNP}]_n\) substrates for different compounds, which are in agreement with previous reports on MoS\(_2\) thin films and graphene oxide films.\(^{39,41}\) There was no linear increase of signal intensity with the increased layer number, however, which might be related to the water contact angle changes of the AuNP substrates (Supporting Information, Figure S5). The contact angle on \([\text{AuNP}]_n\) substrates decreased and gradually leveled out as the layer number increased. From SEM experiments of the \([\text{AuNP}]_n\) substrates (Supporting Information, Figure S6), it can be seen that the surface of \([\text{AuNP}]_n\) films was already very smooth after two layers.

Considering the above results, we believe that the AuNP on the surface of the target plate reach approximate saturation after two (or three) layers; beyond this, oversaturation appears, with reduced energy uptake and less favorable desorption and ionization conditions, because of ligand restrictions of the AuNP. As the energy thresholds for desorption/ionization are compound dependent, it is possible to adjust and optimize the number of layers of the \([\text{AuNP}]_n\) substrates for different analytes to meet their individual energy requirements. We
hypothesize that the ability to control the energy uptake of the [AuNP]ₙ substrates would also allow the control of the internal energy and fragmentation behavior of labile ions and the possibility to reduce (or induce) fragmentations if needed.

**Soft Desorption/Ionization from [AuNP]ₙ Substrates.** Both MALDI and SALDI generally exhibit soft ionization characteristics, mostly producing intact molecule-related ions without fragments for most analytes. We chose erythromycin A as a “thermometer ion” to evaluate our new materials, as the ionized erythromycin A molecule is prone to losing a water molecule upon ion activation. Under MALDI conditions using CHCA as matrix, erythromycin A exhibited a protonated molecule at m/z 734, with strong accompanying dissociations leading to fragment ions at m/z 716, 698, 576, 558, 540, and 522 from water and sugar losses (Supporting Information, Figure S7a). This is very similar to the MS/MS spectrum after ion activation of m/z 734 (Figure S7b). Under SALDI conditions using [AuNP]₁ films, only molecule-related ions at m/z 734, 756, and 772 were generated, however, corresponding to [M + H]⁺, [M + Na]⁺, and [M + K]⁺ (Figure S5). As the layer number of the [AuNP]ₙ substrates increased from n = 1−4, the intensity of these molecule-related ions increased and gradually reached a plateau, without any fragmentation. Based on these observations, we conclude that the [AuNP]ₙ films provide soft ionization abilities during SALDI, much softer than MALDI using CHCA. The laser energy was adjusted to see whether higher laser energy induces fragmentation of erythromycin A in SALDI. The laser energy was varied from 50% to 99%. The ion intensities increased sharply with increasing laser energy as shown in Figure S5b, without any fragmentation at the highest laser energy available on the used instrument. Fragmentations under SALDI therefore appear to be induced by energy transfer to or from the substrate materials to the analyte rather than to the analyte directly.⁶²

**Desorption of Thermometer Benzylpyridinium Ions from [AuNP]ₙ Substrates.** To further explore the SALDI process on the [AuNP]ₙ substrates, benzylpyridinium thermometer ions were used to describe the desorption efficiency and survival yields.⁶³,⁶⁴ Precursor 4-amino-1-benzylpyridinium (ABP) ions were utilized here, and the desorption efficiency was described using the total intensity of the precursor ABP ions, [ABP]⁺ at m/z 185, and the dissociation product of ABP, [ABP-pyridine]⁺, at m/z 90. The survival yield of ABP was defined as the fraction of remaining precursor ABP ion intensity relative to the total ABP ion current. A plot of desorption efficiency of ABP ions against the layer number of the [AuNP]ₙ substrates is illustrated in Figure 6. The desorption efficiency of ABP ions from the [AuNP]₁ substrate was 32726 counts and significantly increased to 95374 for [AuNP]₂, 106058 for [AuNP]₃ substrates, and 113292 counts for [AuNP]₄ substrates. This increase was in accordance with the increased UV/Vis absorption, indicating that a thermally-driven desorption/ionization process might play an important role in the ion desorption process. In addition, this increase of desorption efficiency corresponded to a decreased water contact angle and a general increase of intensities with the layer number for small molecules on the [AuNP]ₙ substrates. From the mass spectrum of ABP, it can be seen that the intensity of the fragment ion at m/z 90 was very low (Supporting Information, Figure S8). Therefore, the survival yield of ABP from 4 different substrates was less sensitive to the layer number of [AuNP]ₙ substrates and generally very high (>99.9%). The reason for this is that ABP tended to be desorbed directly off the [AuNP]ₙ substrates as an intact precursor ion rather than as a dissociation product. As previously reported,⁶³,⁶⁴ high survival yields of ABP correspond to a small internal energy transfer from [AuNP]ₙ substrates under SALDI conditions, indicating that the higher survival yields of ABP are insensitive to internal energy transfers. This behavior cannot be explained by a thermal desorption mechanism, suggesting that some other nonthermal process occurs that dissipates the absorbed energy.

For the AuNP (diameter, 2.5 nm) used in our experiment, the melting point is only approx. 835 K.⁶⁵,⁶⁶ The nanoparticles therefore melt relatively easily. Gold ions are thus common in vacuum SALDI mass spectra.⁶⁰ This was confirmed for our
AuNP, by investigating linoleic acid on [AuNP]_2 substrates using vacuum MALDI (Supporting Information, Figure S9), clearly showing gold ions in both positive and negative modes (e.g., Au^+, Au^2+, Au^3+, Au^−, ...) under vacuum MALDI conditions, indicating ablation of AuNP during laser irradiation. However, under AP-MALDI conditions, no gold-related ions were observed (Supporting Information, Figure S9), suggesting insufficient energy for ablation and ionization of gold ions from the [AuNP]_2 substrates. We hypothesize that during the laser irradiation, the local temperature rise on the surface of the [AuNP]_2 substrate was not as pronounced under AP conditions as under vacuum conditions. Even the highest laser energy available on the AP-MALDI instrument provided no gold-related ions, while the vacuum MALDI instrument gave abundant gold ions, even at very low laser energies. This may be related to the effects of surface destruction, phase transitions, the air environment in the AP ion source, etc., that is, processes that may help to dissipate the energy. We therefore believe that the local temperature of the [AuNP]_2 substrates in AP-SALDI was not as high as the melting point of the AuNP but sufficient for desorption and ionization of the analytes.

**Structural Homogeneity of [AuNP]_n Films and Its Influence on Signal Reproducibilities and Detection Sensitivities.** The structural homogeneity of [AuNP]_n substrates was characterized by SAXS (Figure 7a). SAXS is a well-established protocol to evaluate the particles’ structure (size, shape, and assembly), and the scattering intensity is proportional to the particles’ number, form, and assembly. As seen from the TEM data, the AuNP have well-defined structures, i.e., narrow size distribution and uniform spherical shape. As the [AuNP]_n films were prepared by packing different numbers of the same single layers on top of each other, we used the intensity of this scattering peak for qualitative analysis of the structural homogeneity of the AuNP films. Moreover, we attributed the indicated peak in Figure 7a to the scattering signal of AuNP because a similar scattering peak was not observed for the neat glass substrate (Supporting Information, Figure S10a). As shown in Figure 7a, the peak intensities were virtually identical at different locations of the [AuNP]_2 substrates covering a horizontal space of 15 mm, indicating a homogenous structure in the [AuNP]_2 film. Similar homogeneity was found in the scattering curves of other [AuNP]_n substrates with different thickness (Supporting Information, Figure S10).

Next, we characterized the analytes’ homogeneity of dispersion on the [AuNP]_2 substrates (Supporting Information, Figure S11). From the SEM images, it can be seen that the analytes were almost homogeneously distributed on the surface of the [AuNP]_2 substrate, except for a minor coagulation effect.

We then assessed the signal reproducibility by evaluating the signal intensities for linoleic acid deposited onto an [AuNP]_2 substrate (Figure 7b), showing fluctuations of <8% RSD. These low numbers agreed well with the homogenous distribution of AuNP in the [AuNP]_2 films and also indirectly demonstrate the absence of major spots for deposited analytes. In addition, signal reproducibilities of linoleic acid from different batches of [AuNP]_2 films were also assessed (Supporting Information, Figure S12), showing comparable variations with RSD < 10% RSD.

Finally, we investigated fatty acids for [AuNP]_2 films at lower concentrations (Supporting Information, Figure S13). The approximate limits of detection for palmitic acid, stearic acid, oleic acid, and linoleic acid were 2.0 μM, 2.0 μM, 0.5 μM, and 0.5 μM, respectively. While these numbers are relatively high, they are mainly the result of the utilized quadrupole ion trap instrument, which was a less sensitive, older generation instrument used for the development work here. In addition, this instrument also did not allow high duty cycle data acquisition such as SIM or SRM, which could lower LOD 100–1000x further.

**CONCLUSIONS**

In this study, [AuNP]_n substrates were prepared by using a simple air/water approach. TEM and SAXS data showed that the resulting substrates were ultrathin and homogenous in terms of nanoparticle packing density. The [AuNP]_n substrates were successfully implemented for the SALDI-MS analysis of various small molecules, including fatty acids, drugs, amino acids, small peptides, and saccharides. The generated ion currents from the [AuNP]_2 films were much higher than those from other aggregate states of AuNP. The signal intensities depended on the number of layers of the [AuNP]_n films and gradually reached a plateau for [AuNP]_2 or [AuNP]_3. The [AuNP]_n substrates provide soft ionization characteristics with no observed fragmentations for the investigated analytes. We
utilized benzylpyridinium ions as thermometer ions to further describe the desorption and ionization process. The data showed that the desorption/ionization efficiency of ABP reached saturation for [AuNP]2, directly correlating with the saturation of AuNP on the surface of the substrates. The [AuNP]3 substrates exhibited no distinct sweet spots, and the generated signals under SALDI-MS conditions were very stable, readily allowing quantitative analyses.

**ASSOCIATED CONTENT**

1. Supporting Information
   The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.9b00038.

   Figure S1, TEM images; Figure S2, UV/vis spectra; Figure S3, visual image of [AuNP]6 substrates and corresponding UV/vis absorption spectra; Figure S4, UV/vis absorption of [AuNP]6 films; Figure S5, contact angle of multilayer AuNP films; Figure S6 and S11, SEM images; Figure S7, MALDI-MS and MS/MS spectra; Figure S8, SALDI mass spectrum; Figure S9, mass spectra; Figure S10, waterfall diagrams; Figure S12, stability of SALDI signal intensities; and Figure S13, signal-to-noise ratios (PDF)

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