Nanomaterial-assisted laser desorption ionization for mass spectrometry-based biomedical analysis

Nanomaterials have been widely used to assist laser desorption ionization of biomolecules for mass spectrometry analysis. Compared with classical matrix-assisted laser desorption ionization, strategies based on nanomaterial-assisted ionization generate a clean background, which is of great benefit for the qualitative and quantitative analysis of small biomolecules, such as therapeutic molecules. As label-free platforms, they have successfully been used for high-throughput enzyme activity/inhibition monitoring and also for tissue imaging to map in situ the distribution of peptides, metabolites and drugs. In addition to widely used porous silicon nanomaterials, gold nanoparticles can be easily chemically modified by thiol-containing compounds, opening novel interesting perspectives. Such functionalized nanoparticles have been used both as probes to extract target molecules and as matrices to assist laser desorption ionization for developing new enzyme immunoassays or for studying DNA hybridization. More recently, semiconductor nanomaterials or quantum dots acting as photosensitive centers to induce source redox reactions for proteomics and to investigate biomolecule oxidation for metabolomics have been shown to offer new analytical strategies.

Matrix-assisted laser desorption/ionization mass spectrometry is an excellent method for the analysis of large biomolecules. However, mass ranges under 700 Da are usually seriously impeded by the presence of matrix cluster ions, therefore limiting the application of MALDI in the characterization of small molecules, such as therapeutic molecules. To solve this problem, inorganic materials have been developed to assist LDI of low-molecular-weight analytes. These materials should be able to absorb light energy in accordance with the laser wavelength and should not generate ions that can be detected by the MS. The first example of inorganic material-assisted LDI is indeed the initial work reported by Tanaka et al. in 1987, where glycercol and cobalt nanopowder were employed as absorbers of the laser energy [4]. In this article, we name this inorganic material-assisted LDI as matrix-free LDI. Specifically, when inorganic nanostructure material, such as nanoparticles, nanoporous materials or nanowires, is used to assist the LDI, the ionization method is named as nanomaterial-assisted LDI (NALDI).

In 1999, an important matrix-free LDI method was introduced by the Siuzdak group performed by depositing analytes directly on a porous silicon substrate. The technique was named desorption/ionization on porous silicon (DIOS), and is illustrated in Figure 1 [5]. Compared with...
that the capped nanoparticles could increase ion yields, decrease ion fragmentation and increase the useful analyte mass range (up to ~12,000 Da) when compared with bare AuNPs [20]. AuNPs were also modified with α-cyano-4-hydroxycinnamic acid, a typical organic matrix, for effective NALDI measurements [21]. It was found that the α-cyano-4-hydroxycinnamic acid-terminated AuNPs showed marked improvement on peptide ionization compared with citrate-capped or cysteamine-capped AuNPs. In addition, the α-cyano-4-hydroxycinnamic acid coating on AuNPs effectively suppressed formation of Au cluster ions and analyte fragment ions, leading to a cleaner mass spectra. Another special property of AuNPs is their ability to absorb visible light owing to localized surface plasmon resonance (SPR) and visible-LDI with the assistance of AuNPs was developed [22–24]. Generally, a 532-nm visible laser, corresponding to the SPR of approximately 15 nm diameter gold nanoparticles, is used instead of the typical UV laser of 337 and 355 nm. The visible laser NALDI may show advantages in the analysis of compounds sensitive to UV light.

Another interesting material for NALDI is nanotitania [25–28]. TiO$_2$ semiconducting nanoparticles are widely used in the field of photocatalysis [29] and for the design of dye-sensitized solar cells. Under UV light irradiation, electrons in the valence band of TiO$_2$ can be excited into the conduction band, and both holes and electrons can be trapped at the nanoparticle surface to form oxidative holes and reductive electrons able to react with surrounding molecules [30–32]. Using nano-TiO$_2$ to assist LDI, in-source redox reactions induced by these redox centers can be observed during the ionization processes [30–32].

Other inorganic materials are also used to assist LDI, such as graphite [33], carbon nanomaterials [34–36], nanodiamonds [37], porous alumina [38], nano-ZnO [39,40], EuF$_3$ hollow hexagonal nanodisks [41], silver nanoparticles [42], HgTe nanostructures [43], platinum nanoflowers [44], manganese oxide nanoparticles [45], Fe$_3$O$_4$ particles [46], fullerene-derivatized silica [47] and germanium nanodots [48], among others. Recently, we have shown that quantum dots (QDs) of cadmium selenide and cadmium selenide covered with cadmium sulfide can also be used to assist LDI [49]. Several of the most important publications regarding inorganic material-assisted LDI are listed in Table 1 with descriptions.

Generally, NALDI shows a cleaner background in the low mass range and more homogeneous surface geometry suitable for quantitative
NALDI in qualitative, quantitative & imaging analysis of small molecules

The most important application of NALDI is the characterization of small molecules, such as lipids, fatty acids, metabolites, drug molecules and enzymatic products, for the development of therapeutic and diagnostic strategies [53–61]. Furthermore, with experimental optimization, NALDI was proven to be capable of performing quantitative analysis [62], therefore opening new opportunities for studying enzyme activity and on-tissue medical imaging.

Siuzdak et al. demonstrated DIOS-MS as a quantitative analytical tool in the analysis of small peptides [63]. Electrospray deposition was used to improve sample homogeneity across the porous silicon surface, and therefore to improve the quantitative analysis [63]. A linear relationship between the mass spectra peak intensity and concentration ratios (analyte/internal-standard) in the logarithmic scale was observed. DIOS-MS was further employed for the quantification of codeine, where acceptable accuracy and precision were obtained by using DIOS-MS quantification compared with standard liquid chromatography-MS methods [64]. Similarly, other nanomaterials were also employed to perform quantitative analysis. Oxidized carbon nanotubes were tested as matrices for quantitative analysis of small molecules by MS [65]. Compared with nonoxidized carbon nanotubes, oxidized carbon nanotubes can facilitate sample preparation owing to their higher solubility in water. Moreover, the matrix layer of oxidized carbon nanotubes is much more homogeneous and compact than nonoxidized carbon nanotubes. Accordingly, the reproducibility of peak intensities within and between sample spots was greatly enhanced on the surface of oxidized carbon nanotubes. Quantitative analysis of jatrorrhizine and palmatine with a linear range of 1 to 100 ng/ml was achieved. Other typical examples of NALDI-MS quantitative analysis include the analysis of glucose from blood plasma using titania nanotubes as matrix [66] and the quantification of glucose in human urine by using AuNPs as matrix [67], which can be important for the diagnosis of diabetes.

Besides the direct characterization of metabolites and drug molecules, NALDI-MS was also used for the investigation of enzyme activity and inhibition. MS-based enzyme assay is a label-free method, therefore avoiding the labor-intensive tagging processes classically used in...
radioactivity- or fluorescence-based techniques. Siuzdak et al. employed DIOS chips (96 or 384 dots) to run high-throughput DIOS-MS to monitor enzyme activity and enzyme inhibition [68,69]. Inhibitors from a library were characterized and their activities against selected enzyme targets, including phenylalanine hydroxylase, glycosyltransferase and acetylcholinesterase, were monitored. For phenylalanine hydroxylase, phenylalanine was selected as substrate, and tyrosine was produced. For glycosyltransferase, β-(2,6)-sialylated trisaccharide was produced from the corresponding lactoside. For acetylcholinesterase, choline was generated from acetylcholine. The inhibition was determined by monitoring the substrate-to-product peaks ratio on the mass spectra. On two different commercially available instruments, a sampling rate of up to 38 inhibitors per min was accomplished, with thousands of inhibitors being monitored.

Reaction equilibrium can be monitored by NALDI-MS as well. Powell et al. used DIOS-MS to investigate the binding affinities between aldopentose isomers and boron [70]. 1,4-anhydroxyrititol was firstly used to compete with individual aldopentoses in forming borate complexes to determine the binding preference among the four aldopentoses. Peak intensities of different complexes were used to identify the quantity of each compound. Afterwards, 13C-labeled ribose was included in another set of competition experiments to further confirm the first competition results. The purpose of using 13C-labeled ribose is to eliminate the differences in ionization efficiency of various complexes induced by the differences in chemical structures. Ribose exhibited higher affinity to boron than other aldopentoses, and the binding preference was demonstrated to be ribose > xylose > arabinose > xylose. The result indicates that the favored binding between ribose and boron can be an important factor in RNA evolution. There are several reasons for using DIOS to perform such reaction equilibrium investigation. Besides the advantages, such as a clean background in the low mass range and a good reproducibility of signal intensity, DIOS-MS can avoid the use of organic matrices, which might change the pH condition of the reaction system and disturb the equilibria.

Mass spectrometry imaging has been used as a label-free method to investigate the in situ distribution of peptides, proteins, metabolites and medicines in tissues and cells [71]. The most popular imaging mass spectrometers involve MALDI-MS, LDI-MS, secondary ion MS and desorption electrospray ionization MS [71]. The theoretical and instrumental aspects, together with the applications of MS imaging, have been reviewed in recent publications [71–73]. A typical MS imaging process comprises the acquisition of mass spectra for a regular series of points across a section of tissue and then the plot of the relative intensities of individual m/z data across the tissue, thus visualizing the distribution of the individual molecules. NALDI-MS has also been employed to carry out MS imaging, especially for small molecules. Compared with MALDI-MS imaging, NALDI can avoid the decrease of spatial resolution that is induced by the matrix crystal size and the migration of analytes on the tissue surface during the matrix-depositing process. Since nanomaterials can usually generate a very homogeneous and regular surface, accurate quantification results can be obtained. He et al. used DIOS-MS to map small molecules on mouse liver tissues [74]. In addition, phosphatidylincholine and propidium iodide were used as cell membrane and nucleus markers, respectively, to ‘visualize’ the presence of HEK 293 cells. The tissue was cut into thin slices (50 nm) and then transferred onto a DIOS substrate for NALDI-MS imaging of the molecules of interest. As an alternative strategy, Setou et al. sprayed nanoparticles on the tissue surface to visualize peptides and lipids at a resolution of 15 µm in mammalian tissues [75,76]. Recently, Volny et al. reported an interesting MS imaging protocol using the commercial NALDI target plate, as shown in Figure 2 [55]. NALDI surfaces were used first as substrates for imprinting of tissue sections, and the transferred lipids were then washed and imaged by LDI-MS.

**Nanomaterials as both probes to extract biomolecules & matrices to assist LDI**

Functionalized nanomaterials can be used as probes to enrich molecules of interest, or to immobilize antibodies and DNA. The extracted molecules on the nanomaterials can then be directly ionized under laser irradiation and analyzed by MS. With this strategy, investigations, such as selective characterization of low-abundance molecules, MS-based enzyme immunoassay and MS-based DNA hybridization assay, were performed. As examples, TiO2, Al2O3 and ZrO2 were used to extract phosphorylated peptides from protein digests, and the enriched phosphopeptides were then analyzed directly by MALDI or NALDI-MS [66,77–80]. Titania nanoparticles were also used as selective probes and matrices for the determination of catechins in tea using NALDI-MS [82].
Among various nanomaterials, gold is indeed one of the most suitable for functionalization as it can easily bind thiol molecules. In a simple application, AuNPs nanoparticles were used to extract aminothiols, including glutathione, cysteine (Cys), and homocysteine \[83,84\]. The enriched aminothiols were directly ionized on the surface of AuNPs for qualitative and quantitative analysis. With this selective pre-concentration approach, the limits of detection at a MS signal-to-noise ratio of 3 were 25, 54, and 34 nM for glutathione, Cys and homocysteine, respectively, while limits of detection of 1.0, 2.0 and 1.3 µM were obtained without any pretreatment on the same mass spectrometer. Furthermore, the authors applied this method for the analyzes of glutathione in red blood cells and of Cys in plasma, which showed great potential for diagnosis.

By modifying AuNPs with special ligands that are enzymatic substrates, enzyme assay investigation by MS was realized. Kim developed a label-free assay of protein kinase on peptideconjugated AuNPs by using secondary-ion mass spectrometer, where the AuNPs acted as both signal enhancers and target concentrators \[85\]. Two Cys-terminated peptides with different lengths were conjugated to AuNPs based on the affinity between Au and thiol groups. The kinase reaction resulted in a
straightforward change in the mass of the peptide substrates, where a mass shift equivalent to that of HPO$_4$ (80 Da) could be observed. The phosphorylation efficiency was determined by the intensity ratio of the original unphosphorylated peptide peak and the phosphorylated peptide peak. This strategy holds advantages of label-free and reliable quantification results. Similarly, carbohydrate was modified with thiol and immobilized on the AuNPs for monitoring glycosyltransferase reactions using MS [86].

Modified AuNPs have also been used to amplify signals associated with DNA hybridization, allowing for sensitive detection of DNA, as shown in Figure 3 [87]. The AuNPs were modified with probe–DNA strand and small alkanethiol molecules. Target–DNA strands were immobilized on a glass or silicon wafer substrate through the capture strands. By incubating this substrate with the modified AuNPs, the probe strand could hybridize with the target strand. When these captured AuNPs were analyzed by NALDI-MS, alkanethiol molecules, which existed in large excess over the probe–DNA strands on the gold particle, were ionized and detected rather than the DNA strands. In this way, the DNA hybridization can be transduced to a highly amplified mass signal by observing a large number of small molecules instead of target DNA strands. As a result, detection limits as low as 100 pM were achieved. By using distinct alkanethiol molecules to modify the AuNPs that carried different probe strands, this DNA hybridization amplification method could also be applied to the analysis of several target DNA strands simultaneously. A similar strategy was also applied for the MS signal amplification of target proteins by changing the probe strands on AuNPs to ligands or antibody molecules and the target strands to proteins [88].

AuNPs are also promising candidates as drug delivery agents into cells. Rotello et al. demonstrated that MS can be used for the analysis of cellular uptake based on the functionalized AuNPs with cationic or neutral surface ligands (Figure 4) [89]. The surface ligands could be viewed as ‘mass barcodes’ that allow AuNPs with different modifications to be simultaneously identified and quantified at levels as low as 30 pmol. Specifically, AuNPs were firstly incubated with cells for 6 h for cellular uptake. Afterwards, the phosphate-buffered saline solution was used to wash the cells to remove extra AuNPs that were not taken up by the cells. Transmission electron microscopy was used to verify the cellular uptake of AuNPs. The cells were then lysed and the AuNPs taken up by the cells were collected as part of the precipitate after centrifugation of the lysate. By analyzing the collected AuNPs with NALDI-MS, the ‘mass barcodes’ ligands were ionized and detected to demonstrate and quantify the AuNPs. This study has shown that the cellular uptake of the functionalized AuNPs is dependent on the functionality of the nanoparticle surfaces, suggesting that

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![Figure 3](image.png)

**Figure 3.** Functionalized DNA hybridization assay with nanomaterial-assisted laser desorption ionization-time of flight mass spectrometry.

Au: Gold.

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differential cellular uptake and specific cell targeting might be possible if the appropriate surface functionalities are chosen.

**Nanomaterials to induce in-source reaction during the MALDI & NALDI processes**

Oxidation of biomolecules plays an important role in many human diseases. The most important oxidative species in a human body are reactive oxygen species (ROS) acting both as deleterious and beneficial species [90]. Common ROS include superoxide anion (O$_{2}^•$), hydroxyl radical (•OH) and peroxyl radicals (ROO$^•$) [90]. At low/moderate concentrations, ROS can be important messengers for signal transduction, while at high concentration, ROS can induce oxidative damage to DNA, lipids and proteins, and these processes are usually described as oxidative stress [90]. These oxidation reactions take place not only during diseases such as cancer, cardiovascular troubles, diabetes and neurological disorders, but also naturally during aging [90].

Nanosemiconductor materials or QDs, characterized by separated valence and conduction bands with a moderate band gap, have been widely used to investigate the oxidation of molecules. Under light irradiation, electrons are excited from the valence band to the conduction band, generating oxidative holes and reductive electrons that can induce redox reaction with target molecules. Combining this photoreaction with the LDI process, on-line redox reactions on biomolecules coupled with MS analysis [30–32]. The applications of this strategy involve on-line tagging of Cys residues by quinone compounds, in-source reduction of disulfide bonds in proteins and oxidation of peptides for α, χ-fragmentation, as shown in Figure 5 [30–32].

Commercial TiO$_2$ nanoparticles (Degussa P25) were separated in ethanol/H$_2$O and deposited on a stainless steel target plate or on an aluminium foil. After drying in ambient conditions, the modified substrate was heated at 400°C for on in-source redox reactions during NALDI include oxidation of ferrocene derivatives and reduction of organic dyes during DlOS [91–93]. Recently, we employed TiO$_2$ nanoparticle modified substrates to perform on-line redox reactions on biomolecules coupled with MS analysis [30–32]. The applications of this strategy involve on-line tagging of Cys residues by quinone compounds, in-source reduction of disulfide bonds in proteins and oxidation of peptides for α, χ-fragmentation, as shown in Figure 5 [30–32].

Commercial TiO$_2$ nanoparticles (Degussa P25) were separated in ethanol/H$_2$O and deposited on a stainless steel target plate or on an aluminium foil. After drying in ambient conditions, the modified substrate was heated at 400°C for...
2 h to sinter the TiO$_2$ nanoparticles and to form a stable photosensitive layer. For tagging Cys residues, Cys-containing peptides were deposited together with hydroquinone on the TiO$_2$ spot [32]. Under UV irradiation, photo-generated holes oxidized hydroquinone to produce benzoquinone, which could further react with the thiol group on Cys-containing peptides to tag them concomitantly with the sample ionization, as shown in Figure 5. As a result, the peaks for both untagged and tagged peptides were observed together on one mass spectrum. This on-line tagging method was proven to be sensitive and highly specific for the synthetic peptides, protein digests and even protein mixture digests. For example, for peptides containing three Cys residues, signals coming from untagged, single, double and triple tagged peptides could all be observed in one spectrum, therefore providing a convenient method for counting free Cys residues present in peptides. The number of Cys residues in a peptide is important supplementary information in the process of database interrogation as it is needed for MS-based protein identification, to distinguish isobaric peptides. For example, β-lactoglobulin A was identified with a database searching score of 196 by considering the information on Cys content based on peptide mass fingerprinting method by using Mascot as the searching engine and SWISS-PROT as the database, whereas a score of only 151 was obtained without this information. Higher score value indicates a more accurate identification. Similarly, the database searching score was enhanced from 261 to 424 for bovine serum albumin.

For the reduction of disulfide bonds in proteins, glucose was deposited on the TiO$_2$ layer to enhance the reductive ability of the semiconductor under light irradiation [30]. The hydroxyl group on glucose can extract the holes generated in the TiO$_2$ substrate and free electrons. These electrons could then react with the disulfide bond and break this bond, as shown in Figure 5. When using human insulin (a protein containing three disulfide bonds), only the peak corresponding to the intact protein was observed on the classical MALDI-MS spectrum, while the fragments of A- and B-chains, disulfide bond cleavage products, were observed by using the in-source reduction strategy.

During the investigation of photoinduced reductions, it was found that TiO$_2$ could also induce peptide regular fragmentation with the presence of glucose [31]. Specifically, glucose was oxidized by the holes, and the oxidized glucose could further oxidize the peptides and cleave the C$_a$–C$_a$ bond to generate $a, x$-fragments, as indicated in Figure 5. On the other hand, the electrons in the conduction band could directly react with peptides to induce the $c, z$-fragmentation on N–C$_a$ bond (Figure 5). However, because there was no effective electron conductor in the reaction system, reduction could only happen on the surface of TiO$_2$, while oxidation could also occur in the ionization plume due to the presence of glucose. As a result, stronger $a$-fragments were observed compared with the $c$-fragments. The $a, x$-fragmentation is usually relatively seldom observed when using common peptide dissociation strategies. The present TiO$_2$-induced in-source peptide fragmentation can be useful for peptide sequencing.

**Conclusion & future perspective**

This article shows how nanomaterials have been used for the ionization of molecules for MS detection, focusing on the applications of direct analysis of drug molecules, tissue imaging, enzyme assay and binding assays. This article also focuses on the major nanomaterials commonly used, namely silicon, gold and TiO$_2$. Silicon is the most widely used inorganic materials to assist LDI, where high sensitivity has been achieved especially for small biomolecules. AuNPs are specifically used as both probes to selectively extract molecules of interest and as matrices to assist LDI. By modifying these nanoparticles with specific ligands or functional groups, selective characterization of low abundant biomolecules, and sensitive detection of proteins, DNA and cellular uptake can be realized.

The limitation of all matrix-free LDI and NALDI techniques is that the ionization efficiency decreases for large molecules. As discussed above, matrix-free LDI and NALDI are normally used for the investigation of small molecules (MW: <2 kDa) of metabolites and medicine. In some research, the NALDI technology was used to ionize small proteins (MW: ~20 kDa) [28]. However, it is not interesting to develop NALDI-related methods just for the ionization of biomacromolecules that can be adequately detected with the typical MALDI-MS strategy. In our view, it is worthy to further develop LDI in-source reactions for protein/peptide tagging, oxidation and fragmentation by using TiO$_2$ or QDs. However, in order to enhance the oxidation or fragmentations efficiency of protein/peptide, different additive molecules containing special functions, new hole scavenger candidates and matrix composition should be characterized.
Semiconductor materials can also be coupled with electrospray ionization MS to achieve online oxidation of biomolecules, where ROS (94) of hydroxyl radical (‘OH) and peroxyl radicals ((ROO)·) can be generated, offering a novel strategy in the research of antioxidant filtration and disease mechanism study.

As another future perspective, novel functional mesoporous materials can be considered to facilitate biological reaction kinetics, owing to their major advantages of having large specific surface areas and uniform pore structure. Specifically, macroporous silica or titania can be used to immobilize enzymes for fast on-plate protein digestion [95–97], which can provide promising platforms for future tissue imaging of large biomolecules of interest.

Nanomaterial-assisted LDI has shown successful applications in metabolism investigation. The NALDI strategies were also combined with MS imaging techniques for the investigation of the distribution of medicine metabolites and proteins in tissues or cells. However, the study of NALD-MS imaging is still in the early stages. Further research in areas such as highly sensitive and selective imaging methods should be performed in the future.

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Bibliography
Papers of special note have been highlighted as:
* of interest
** of considerable interest


** The first laser desorption/ionization (LDI) publication.


The most widely used LDI method.


Peptide a.a-fragmentation during the LDI process, valuable for peptide sequencing.


Matrix-assisted LDI (MALDI)-based on-line cysteine tagging strategy, valuable for protein accurate identification.


NALDI for MS-based biomedical analysis

**Technology Report**

- **Tang HW, Ng KM, Lu W, Che CM:** Ion desorption efficiency and internal energy transfer in carbon-based surface-assisted laser desorption/ionization mass spectrometry: desorption mechanism(s) and the design of SALDI substrates. *Anal. Chem.* 81(12), 4720–4729 (2009).
- **A very interesting LDI-mass spectrometry (MS) imaging strategy, where the nanomaterial-assisted LDI (NALDI) plate was firstly used as a substrate for the imprinting of tissue section for MS imaging.**

**DIOS-MS as an ideal platform for the investigation of reaction equilibrium.**


88 MS-based signal amplification method for DNA hybridization investigation.


