

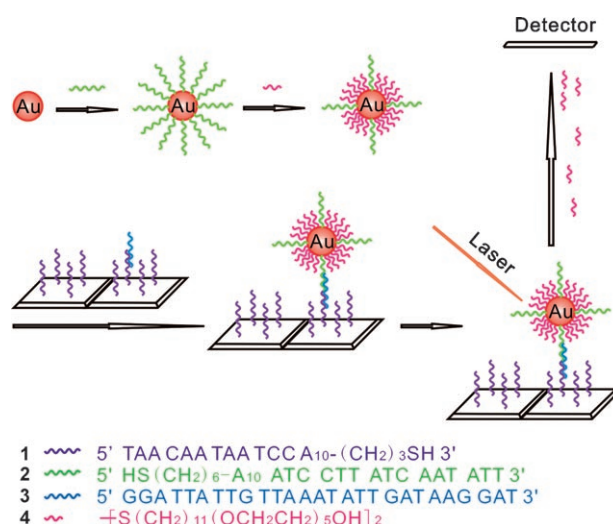
Monolayer-Barcoded Nanoparticles for On-Chip DNA Hybridization Assay**

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Molecular diagnostics lies at the heart of modern-day chemistry and biology. The identification of genetic and proteomic targets and patterns relies on the proper choice of detection format, a combination of biological recognition architecture and signal readout scheme. Biochip technology,^[1] by placing multiple probes on a single flat surface, allows for the storage of tremendous amount of information and massively parallel detection of target species. However, the convenience of use associated with this strategy usually comes at the cost of complexity in the fabrication of surface-bound recognition elements because of limitations in the signal readout.^[2] The serial generation of spatially resolved spot arrays, either photolithographically or through a microarrayer, is typically a labor-intensive procedure. Mass spectrometry (MS),^[3] a tool with inherent multiplexing capability, offers a potentially attractive alternative by providing mass-resolved signatures simultaneously for multiple analytes, thus obviating the necessity to use patterned arrays. However, for molecules with low ionization efficiency and facile fragmentation such as DNA,^[4] the implementation of such a technique reproducibly in chip-based biondiagnostics is extremely challenging. Thus far, only a couple of attempts have been documented and none have been elaborated into routine practice.^[5] Moreover, these methods suffer from restrictions on measurable DNA sizes, which pose significant obstacles for real clinical samples. Sequence-specific detection of DNA is crucial to the diagnosis of genetic and pathogenic diseases and central to the biological and medical research. A

diagnostic platform that takes advantage of massive parallelization offered by gene chips while at the same time eliminates the detection bottleneck associated with MS, which is broadly applicable for DNA of arbitrary length, can provide a powerful technology for numerous fields. Herein, we report on the utility of barcoded nanoparticles for on-chip DNA hybridization assay, by employing surrogate molecules as mass tags with amplified viable signals coupled with laser desorption/ionization time-of-flight (LDI-TOF) MS for readout. The research is part of our ongoing efforts to exploit nanoparticles functionalized with probe and MS tagging elements for multiplexed analysis of biomolecules.

A three-component sandwich assay, composed of surface-bound capture strands, target strands to be detected, and probe-strand-capped, monolayer-barcoded nanoparticles (MBNs), was designed for DNA hybridization (Scheme 1).



Scheme 1. MBN-based DNA hybridization assay with LDI-TOF MS (flat substrate is Si/SiO_x).

Key to the success of this method is the preparation of MBNs, which, by drawing on well-established monolayer chemistry,^[6] could be carried out with a one-pot, two-step strategy (Scheme 1). The first step involves functionalization of nanoparticles with appropriately modified oligonucleotide structures. Subsequent derivatization with mass-tag molecules for barcoding purposes, through ligand-exchange process, affords MBNs that simultaneously incorporate hybridization and readout elements. To be amenable to LDI-TOF MS analysis, the bonding interaction between the barcode molecule and nanoparticle surface must be carefully selected. Otherwise

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the barcode molecule cannot be easily desorbed by the application of laser pulses. In the initial proof-of-concept demonstration, we selected gold nanoparticles as the carrier for barcode and oligonucleotide, and a symmetric disulfide featuring ethylene glycol oligomeric unit (**4**) as barcode (Scheme 1). In this design, the well-studied chemisorption of **4** on gold nanoparticle surface^[7] ensures facile LDI-TOF MS identification of intact barcode molecules and, therefore, detection of DNA binding events. Indeed, surface-confined monolayers formed with a set of organic and biological molecules on flat gold substrates as well as nanoparticle surfaces have been investigated by matrix-assisted LDI-TOF MS (MALDI-TOF MS).^[8] In addition to the role of barcode, the ethylene glycol oligomeric unit could also act as a barrier to prevent nonspecific interactions between the biological molecules and substrate surfaces.^[9]

Compound **4** was synthesized by previously reported methods^[10] and characterized with ¹H and ¹³C NMR spectroscopy (see the Supporting Information), electrospray ionization (ESI) MS, and MALDI-TOF MS.^[11] In a typical experiment, gold nanoparticles (ca. 13 nm in diameter)^[12] were loaded with the oligonucleotide and organic molecule by the following protocol: First, citrate-stabilized particles (2 mL, ca. 17 nm) were incubated in thiol-terminated oligonucleotides (**2**; 200 μ L, 16 μ M). After 24 h, the solution was brought to 0.1 M phosphate-buffered saline (PBS) solution (0.1 M NaCl, 10 mM phosphate buffer, pH 7) and allowed to age for another 32 h. This solution was charged with a solution of **4** in ethanol (10 mM, 200 μ L) and allowed to stand for 12 h, during which time derivatization took place. Excess reagents were removed by centrifugation for 25 min at 15 000 rpm. The supernatant was removed, and the precipitate was washed three times by successive centrifugation and redispersion in PBS solution (0.1 M, 2 mL). The assay-ready MBN solutions were obtained by redispersing the red oily precipitate in PBS solution (0.3 M, 2 mL). The superior salt stability of MBNs over citrate-stabilized nanoparticles is consistent with successful surface modification.

Transmission electron microscopy (TEM) revealed virtually identical nanoparticle morphology before and after the surface modification (see the Supporting Information). A solution of MBNs exhibited a similarly shaped surface plasmon band (see the Supporting Information). The slight red-shift in the band position and apparent decrease in the band intensity have previously been observed in pure oligonucleotide-modified nanoparticles.^[13a] Derivatization of **4** onto the surface of the MBNs was validated by the observation of a peak in the LDI-TOF mass spectrum at m/z 869 ($[\mathbf{4}+\text{Na}]^+$) both with and without 2,4,6-trihydroxyacetophenone (THAP) as the matrix (Figure 1a).^[14] Additional control experiments indicate that the peak at m/z 869 is derived from the disulfide molecule **4**, rather than the oligonucleotides. In addition to the sodium adduct ions, fragmentation and potassium adduct ions could frequently be identified. The incorporation of probe oligonucleotide strands could be conveniently confirmed by a solution sandwich assay.^[15] This set of experiments are of prime importance because of the possibility of 1) complete exchange of **2** by **4** and therefore the loss of hybridization element in MBNs, and

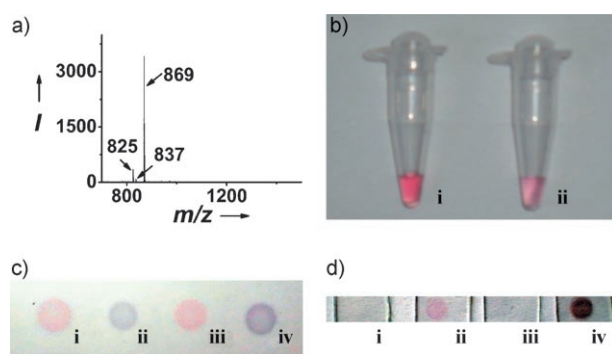


Figure 1. a) LDI-TOF MS of MBNs incorporating **2** and **4** (869, $[\mathbf{4}+\text{Na}]^+$; 837, $[\mathbf{4}+\text{Na}-\text{S}]^+$; 825, $[\mathbf{4}+\text{Na}-\text{CH}_2\text{CH}_2\text{O}]^+$). b) Solution assay i) without target **3** and ii) with target **3** at room temperature (image was taken 45 min after the addition of all the components). c) Spot test i) without target **3** and ii) with target **3** at room temperature (the solution was spotted on the thin layer chromatography plate 30 min after the addition of all the components), iii) with target **3** at 75 °C (the solution was spotted directly from the 75 °C mixture), iv) with target **3** by cooling a 75 °C solution back to room temperature (the solution was spotted after being kept at room temperature for 30 min). d) Images of **1**-functionalized glass slide incubated with MBNs (with **2** and **4**) in the i) absence and ii) presence of 1 μ M target DNA **3**. iii, iv) Slides (i) and (ii), respectively, after silver enhancement.

2) alteration of the oligonucleotide properties imposed by neighboring barcode molecules. Thus, when our MBN is mixed with pure oligonucleotide-modified nanoparticle in the presence of target strand (**3**), the solution color changes from red to reddish purple at the initial stage (Figure 1b, i and ii), indicating aggregation of the nanoparticles.^[13b] The nanoparticle aggregates would eventually precipitate out of the solution if left standing for an extended period of time. A spot test has been developed as an alternative way to provide a permanent record of the solution hybridization event.^[13b] In a fashion analogous to the earlier report, target strand-driven assembly of nanoparticles leads to the formation of blue spot, in contrast to the red color in the absence of target strand (Figure 1c, i and ii). Melting experiments further confirm that the nanoparticle assembly is directed by the DNA hybridization. If the sample is heated to 75 °C, a temperature at which nanoparticle assembly is unstable owing to DNA dehybridization, a red spot is observed (Figure 1c, iii). Upon cooling to room temperature, a blue spot is again developed (Figure 1c, iv).

Although the surface modification chemistry has not yet been optimized, the MBNs have already allowed us to carry out DNA hybridization assays in chip format. For this task, an oligonucleotide sequence (**3**) associated with the anthrax lethal factor was chosen as a test target because of its importance in the bioterrorism and biowarfare.^[13] In the first set of experiments, thiolated 22-nucleotide capture strands (**1**) were covalently spot-immobilized on the center area of a glass slide through succinimidyl(4-maleimidophenyl)-butyrate by a reported protocol.^[16] The covalent bond ensures the integrity of the surface-anchored oligonucleotides. The oligonucleotide-modified chip was allowed to hybridize with target DNA strands (**3**, 1 μ M) in 0.3 M NaCl, 10 mM phosphate buffer (pH 7) at room temperature for 4 h, followed by washing with

copious amount of 0.3 M PBS solution to remove unhybridized target. The slide was then incubated in a solution of MBNs for 4 h to effect the hybridization with the overhanging region of the target sequence. Following the incubation, nonspecifically bound nanoparticles were washed off with clean buffer (first with 0.01 % Tween 20, 0.3 M NaCl, 10 mM phosphate buffer, pH 7, then with 0.3 M PBS solution). The reddish spot that is selectively developed only in the oligonucleotide-functionalized slide area validates the efficacy of MBNs as the DNA hybridization probe (Figure 1 d, i and ii). In addition, silver enhancement could be employed for improved visualization of MBNs on the surface, where darkening of the spot could be clearly identified (Figure 1 d, iii and iv).

After successfully demonstrating surface hybridization, we next examined the feasibility of utilizing MBNs for LDI-TOF MS detection of DNA. To this end, a silicon wafer was treated with an identical protocol as described for the glass slide and mounted on a custom-made stainless steel sample plate (by matching the thickness of the wafers with the overall measurement requirement). Prior to the MS analysis, scanning electron microscopy (SEM) was used to provide further proof that MBNs are indeed capable of hybridizing onto the oxide surface (Figure 2 a and 2 b). Significantly, direct LDI-

deposition of silver on MBN surfaces (see the Supporting Information). After this treatment, enhanced LDI-TOF MS signal intensity could be achieved for the DNA hybridization detection (see the Supporting Information). Small silver nanoparticles generated on the MBN surfaces are anticipated to be conducive to laser-energy transfer^[18] and therefore the ionization process. Although not required for MS, spatially resolved spot arrays generated on biochips could be beneficial for information storage. In this context, the resolution of patterned biochip could benefit tremendously from matrix-free operation conditions, because of the limitations on the crystal size associated with the matrix (typically larger than 10 μm in size). The addition of matrix (THAP) could likewise provide a venue for improving the performance and increasing the sensitivity of our detection system. With this diagnostic format, we could detect target DNA at a concentration as low as 100 pM (equivalent to 3 fmol in a 30 μL solution of target DNA) under non-optimized conditions (Figure 2 d). Quantification of the number of barcode molecules and probe strands is a future endeavor that is necessary for maximizing the amplification factor. Optimization of the preparation conditions (e.g. conjugation chemistry) for MBNs is anticipated to lead to a further increase of the protocol sensitivity.

Several distinct features distinguish our detection platform from a previously developed solution masscode system:^[19] 1) the gold nanoparticle surface provides anchoring sites for multiple chemical and biological molecules, thus avoiding cumbersome conjugation methods that are required to link the masscode molecule to primer, 2) instead of relying on polymerase chain reaction, a time-consuming and expensive protocol, the amplification could be conveniently achieved by accommodating a high barcode-to-DNA ratio on the nanoparticles, 3) the bonding interaction between the barcode molecule and particle surface allows for direct on-chip LDI-TOF MS readout, rendering cleavage of small molecules from DNA unnecessary, 4) since our strategy is nanoparticle-based, it should offer intrinsically higher selectivity than conventional assays as a result of the unusually sharp DNA melting profiles.^[13]

Our barcode strategy could be easily adapted to high-throughput assays for screening DNA binding events. Multiplexing could be achieved by using distinct mass tags for each individual target. The high resolution of MS allows almost unlimited choice of mass tags, which offers an advantage over other multiplexing protocols. As a demonstration, we performed DNA hybridization assay with two target strands in one solution. To this end, a disulfide molecule ($[\text{S}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OH}]_2$, **8**) that differs from **4** in the mass signature was prepared by a similar procedure^[20] and used, together with **6** (5' HS(CH₂)₆-A₁₀TAAGTATTCCTATAT 3'), for the construction of a second solution of MBNs (see the Supporting Information). Target strand **7** (5' TATGTTAGTATGATATAGGAATAGTTA 3') is of an identical base composition to **3**, but with the sequence scrambled. The selection of this target strand is to illustrate that our barcode strategy could distinguish oligonucleotide sequences of the same molecular weight by appropriate choice of mass tags. Through sandwich hybridization, with capture strand **5** (5' CATA-

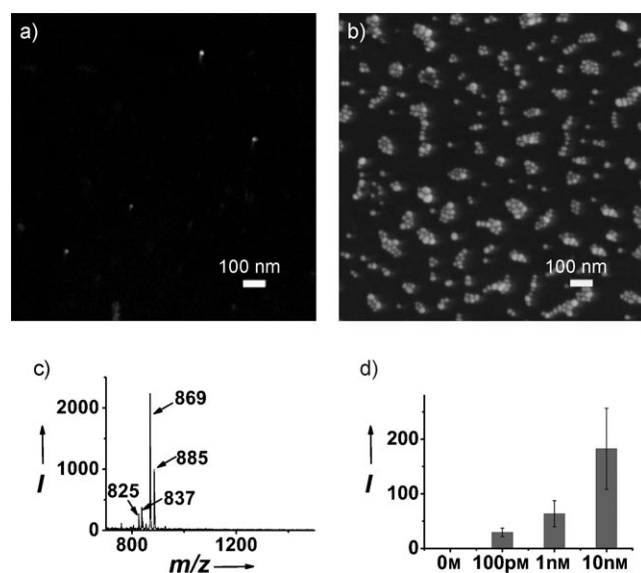


Figure 2. SEM images of 1-functionalized silicon wafer incubated with MBNs (with **2** and **4**) in the a) absence and b) presence of 1 μM target DNA **3**. c) LDI-TOF MS of surface b) (885, $[\text{4}+\text{K}]^+$; 869, $[\text{4}+\text{Na}]^+$; 837, $[\text{4}+\text{Na}-\text{S}]^+$; 825, $[\text{4}+\text{Na}-\text{CH}_2\text{CH}_2\text{O}]^+$). d) Signal intensity of $[\text{4}+\text{Na}]^+$ peak (MALDI-TOF MS) from the silicon wafer as a function of target DNA **3** concentration.

TOF MS characterization of the wafer is viable and shows a high-intensity peak at m/z 869 in the presence of 1 μM target DNA **3** (Figure 2 c).^[17] In comparison, no peak was observed at m/z 869 in the absence of target DNA **3**. The absence of oligonucleotide MS peaks under the experimental conditions employed herein manifests the advantage of using small molecules for MS identification purposes. In addition, silver enhancement for a short period of time leads to the

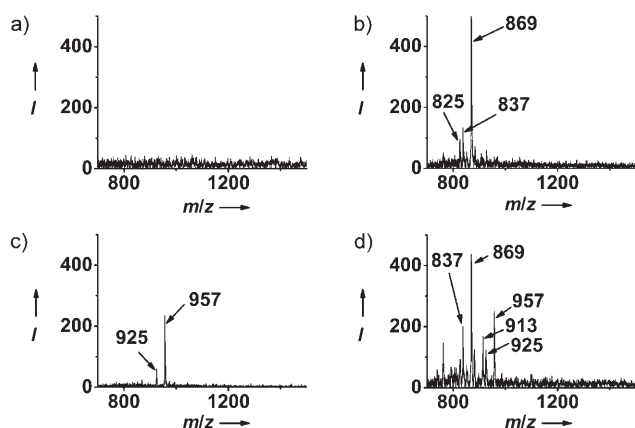


Figure 3. MALDI-TOF MS of 1- and 5-functionalized silicon wafer incubated with MBN mixture solution (two sets of MBNs: one with **2** and **4**, the other with **6** and **8**) a) with no target, and in the presence of b) 1 μM target DNA **3** (869, $[\text{4}+\text{Na}]^+$; 837, $[\text{4}+\text{Na}-\text{S}]^+$; 825, $[\text{4}+\text{Na}-\text{CH}_2\text{CH}_2\text{O}]^+$), c) 1 μM target DNA **7** (957, $[\text{8}+\text{Na}]^+$; 925, $[\text{8}+\text{Na}-\text{S}]^+$), d) 1 μM each of target DNA **3** and **7** (957, $[\text{8}+\text{Na}]^+$; 925, $[\text{8}+\text{Na}-\text{S}]^+$; 913, $[\text{4}+\text{8}+2\text{Na}]^{2+}$; 869, $[\text{4}+\text{Na}]^+$; 837, $[\text{4}+\text{Na}-\text{S}]^+$).

CTAACATA A₁₀-(CH₂)₃SH 3') immobilized on the chip, we were able to verify the effectiveness of this set of diagnostic components (see the Supporting Information). One of the most significant advantages of MS-based bioassay strategy is the ability to perform multiplexed analysis without resorting to patterned arrays. This capability is indeed a desired feature that is associated with our detection platform. By functionalizing the silicon wafer with capture strands **1** and **5** through the use of a thiolated DNA mixture solution, we could, with a sandwich assay, perfectly differentiate and detect target strands **3** and **7** simultaneously by virtue of the absence or presence of $[\text{4}+\text{Na}]^+$ and $[\text{8}+\text{Na}]^+$ indicator peaks (Figure 3) (the individual MS signature intensity for different target strands could differ owing to the possible variation in the oligonucleotide immobilization and target hybridization efficiencies^[16]). With the successful demonstration of the proposed diagnostic methodology, future research could be directed at evaluating more detailed yet important issues, such as selectivity (e.g. single-base mismatch differentiation), sensitivity (under optimized conditions), multiplexing capability (maximum number of simultaneously detectable targets), and target DNA quantification capability (based on MS signatures).

The MBN-based on-chip DNA assay strategy described herein offers a viable route for the characterization of DNA hybridization at the monolayer level. The approach could be readily extended to the identification of a vast range of biomolecular binding events that cannot otherwise be addressed by LDI-TOF MS in a chip format. Considering the diverse array of molecules that could act as mass tags, as well as nano-scaled materials that could act as carriers, the MBN-based LDI-TOF MS is likely to emerge as an important diagnostic tool, even in real biological settings.

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