Supporting Information Available:

Simultaneous Detection of Attomolar Pathogen DNAs by Bio-MassCode Mass Spectrometry

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Table 1. Sequences of modified oligonucleotide strands and blockers.

No.	Name	Sequence (5' to 3')
DNA 1	HIV-NP	GCT GTC CCT GTA ATA AAC CCG AAA ATT TTT TTT TT–$(CH_2)_3$–SH
DNA 2	HBV-NP	CTC TGT GGT ATT GTG AGG ATT CTT GTC ATT TTT TTT TT– $(CH_2)_3$–SH
DNA 3	HCV-NP	CGC TTT CTG CGT GAA GAC AGT AGT TTT TTT TTT TT– $(CH_2)_3$–SH
DNA 4	TP-NP	GTG TAC TAG CCC TCC CTT CTA CCT GAT TTT TTT TTT– $(CH_2)_3$–SH
DNA 5	HIV-MP	SH–$(CH_2)_6$–TTT TTT TTT TTT GTA TGT CTG TTG CTA TTA TGT CTA TTA TTC TTT CCC CTG C
DNA 6	HBV-MP	SH–$(CH_2)_6$–TTT TTT TTT TCA AAC GGG CAA CAT ACC TTG GTA GTC CAG AA
DNA 7	HCV-MP	SH–$(CH_2)_6$–TTT TTT TTT TCG CAA GCA CCC TAT CAG GCA GTA CCA CAA
DNA 8	TP-MP	SH–$(CH_2)_6$–TTT TTT TTT TTT TGT AAT GTA TCG TTT GTT GCT TCT GTA TCT ATT TCT TGC
DNA 9	Passivation-T_{10}	SH–$(CH_2)_6$–TTT TTT TTT T
DNA 10	HIV-target	TTT TCG GGT TTA TTA CAG GGA CAG CGC AGG GGA AAG AAT AAT AGA CAT AAT AGC AAC AGA CAT ACA A
DNA 11	HBV-target	TGA CAA GAA TCC TCA CAA TAC CAC AGA GTT CTG GAC TAC CAA GGT ATG TTG CCC GTT TG
DNA 12	HCV-target	AAC TAC TGT CTT CAC GCA GAA AGC GTT GTG GTA CTG CCT GAT AGG GTG CTT GCG
DNA 13	TP-target	TCA GGT AGA AGG GAG GGC TAG TAC ACG CAA GAA ATA GAT ACA GAA GCA ACA AAC GAT ACA TTA CAA A
DNA 14	Random-target	CAG GGT TGG TAG GTC GTA AAT CCC CTA TTG GTC AAG AGA GAC AT
DNA 15	HBV-blocker1	GAA GAT TGA CGA TAT GGG TGA GGC AGT AGT CGG AAC A
DNA 16	HBV-blocker2	GAC AAG AGG TTG GTG AGT GAT TGG AGG TTG GGG AC
DNA 17	HBV-blocker3	CCT GGA AGT AGA GGA CAA ACG GGC AAC ATA CC

Chemicals

Amino-functionalized magnetic microparticle solution (Dynabeads M-270 Amine) was commercially available from Invitrogen Co. (Shanghai, China). $HAuCl_4.4H_2O$ (99.9%) was from Jiuyue Chemical Co., Ltd (Shanghai, China). Trisodium citrate dihydrate and other organic reagents (AR grade) were from Nanjing Chemical Reagent Co., Ltd (Nanjing, China). The chemicals dimethylsulfoxide (DMSO), succinimidyl 4-[p-maleimidophenyl] butyrate (SMPB), sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium dihydrogen phosphate anhydrous (NaH_2PO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich Chemical Co. (MO, USA). Thiolated oligonucleotide strands and blocking oligos were synthesized and purified by Invitrogen Co. (Shanghai, China). Nanopure water (18.2 MΩ•cm) purified by a Sartorius Arium 611 system was used

throughout the experiment. Organic molecules (namely disulfide homologs) were synthesized in-house by previously reported methods.[1] The MALDI TOF MS experiment was carried out on a Bruker Ultraflex III TOF/TOF mass spectrometry (Germany).

The molecular formulas of four disulfide homologs are shown below:

MI: $([S(CH_2)_{11}(OCH_2CH_2)_3OH]_2$, $[MI+Na]^+$ m/z 693

MII: $([S(CH_2)_{11}(OCH_2CH_2)_4OH]_2$, $[MII+Na]^+$ m/z 781

MIII: $([S(CH_2)_{11}(OCH_2CH_2)_5OH]_2$, $[MIII+Na]^+$ m/z 869

MIV: $([S(CH_2)_{11}(OCH_2CH_2)_6OH]_2$, $[MIV+Na]^+$ m/z 957

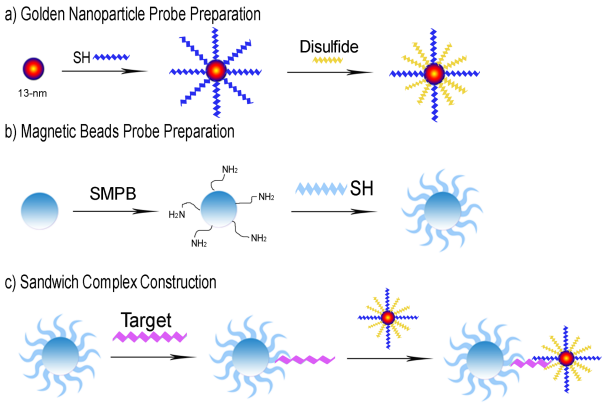

Fig. S1. Schematic Diagram of BMC probe-based on DNA hybridization assay with MALDI TOF MS. The BMC probe of 25~28 bp probing the target DNA-specific oligos (DNA1, DNA2, DNA3 or DNA4) is followed by modifying with the organic molecules MI (m/z 693), MII (m/z 781), MIII (m/z 869) or MIV (m/z 957), respectively. The target DNA-specific thiolated-oligos recognized the target of interest from 3' terminal and the disulfide acted as a reporter to decode the different sequence character of the targets. The reporter as surrogate targets was detected by MALDI TOF MS. The MPs of 29~44 bp probing another target DNA-specific oligos (DNA5, DNA6, DNA7 or DNA8) is covalently immobilized with magnetic beads. The MPs were utilized as capture probes for the target DNA from 5' terminal and also functionalized as a separation, purification and concentration tool for the target DNA in solution. (a). preparation of the NPs; (b). preparation of the MPs; (c). construction of the sandwich complete. SMPB: succinimidyl 4-[p-maleimidophenyl] butyrate.

Gold Nanoparticles Functionalization with DNA

Gold nanoparticles were prepared by citrate reduction of $HAuCl_4$[2]. The BMC probe, namely dual-modified golden nanoparticles (NP), was prepared according to literature procedures[3] shown in Fig. S1a. Briefly, about 35 μg of 3'-thiolated target DNA-specific oligos with T_{10}-spacer dissolved with 200 μL nanopure water was added to 2 mL of 13 nm gold nanoparticles[2a] and shaken gently for 24 h at room temperature. The sodium chloride concentration was brought to 0.1 M with buffer 1 (0.3 M NaCl, 10 mM phosphate, pH 7.0) in a stepwise manner. Over the course of 36 h for aging, 50 μL of 100 mM disulfide molecules was added and continued to be shaken gently overnight. These NPs should be preserved at 4°C. Prior to use, the particles were spun (12 000 rpm) for 30 min at low temperature (4°C) and rinsed three times with PB (0.1 M NaCl, 10 mM phosphate, pH 7.0) to remove any unbound specific-oligos. Following rinsing, the NPs were redispersed with buffer 1. The BMC probes should be stored in excess specific-oligos until needed, at which time they were purified as described above. As for HIV-NP, the recognized probe was 13 nm golden nanoparticles incubated sequencially in the HIV-specific oligonucleotide (5'-GCT GTC CCT GTA ATA AAC CCG AAA ATT TTT TTT TT -$(CH_2)_3$-SH-3') and the reporter MI of m/z 693. In the same manner, the NPs of HBV, HCV or TP were golden nanoparticle dual-functionalized with the 5'-thiolated target-specific oligos (Their sequences were shown in Table 1) and the different reporter (MII, MIII or MIV), respectively.

Magnetic Micropaticles Functionalization with DNA

Oligo-functionalized magnetic microparticles (MP) were prepared according to literature procedures[4] shown in Fig. S1b. Briefly, 2.8 μm amine-functionalized magnetic microparticles (30 mg/mL) were washed three times with DMSO and then coupled to 5'-thiolated oligonucleotide strands (35 μM) using the heterobifunctional cross-linker SMPB (1mg/100 μL). Over the course of 24 h, unreacted amine sites were passivated with passivation DNA (DNA 9, 72 μM) to minimize nonspecific binding. The MPs were stored at 4°C in PB (0.3 M NaCl, 10 mM phosphate, pH 7.2). The MPs were washed three times with PB (0.2 M NaCl, 10 mM phosphate, pH 7.2) prior to use in the assay. Taken HIV capture probe (HIV-MP) for example, magnetic microparticles were conjugated with 5'-thiolated oligos 5'-SH-$(CH_2)_6$-TTT TTT TTT TTT GTA TGT CTG TTG CTA TTA TGT CTA TTA TTC TTT CCC CTG C-3'. And for the other three magnetic capture probes (HBV-MP, HCV-MP or TP-MP), the preparation procedures were similar to that of HIV-MP. Their sequences linked with the magnetic microparticles were shown in Table 1.

Sandwich construction

The sandwich complex was formatted by the principles of base pairing as described before[4a] (Fig. S1c). The BMC-based assay was initiated by mixing MP solution (50 mL) and the appropriately artificial target ss-DNA solution (30 μL) in a tube (Axygen). The mixtures were heated at 45°C for 0.5 h by vortexing gently every 10 min, followed by incubation at 25°C for 2 h to allow hybridization as completely as possible between the MPs and the target DNAs of interest. The MP-target complexes were separated on a 6-Tube Magnetic Separation Rack (Invitrogen Co.) and washed three times with buffer 1; then the MP-target complexes were redispersed in 50 μL buffer 1. The NP solution (50 mL) was followed by adding to the tube. After hybridization for 2 h, the sandwich complex with three components, MP-target-NP, along with unreacted MPs was pulled to the wall of the reaction vessel by the magnetic separator. Unreacted NPs were washed away with buffer 1. This washing step was repeated several times to effect removal of the NPs that were not

specifically bound to the targets in hybridization reactions. Finally, the magnetic field was removed, 10 μL of nanopure water was added to the reaction vessel, and the tube was heated to 75°C for 5 min to dehybridizate the sandwich complex to release the BMC probes. Reintroduction of the magnetic field removed all of the MPs from solution, leaving dual-functionalized NPs for detection.

Sensitivity Improvement

We have optimized such parameters as NaCl concentration of hybrid reaction, NaCl concentration of washing buffer of Sandwich complex, proportion optimization of code molecules and identification molecules on recognition probe (AuNP), dispersed solution of sandwich complex, matrix, and plate of MALDI TOF MS to achieve the high sensitivity.

MALDI TOF MS Analysis of the Organic Molecules Served as the Reporters

The MALDI matrix in this study was selected from common ones, such as alpha-cyano-4-hydroxycinnamic acid (CHCA), 3,5-2-ethoxy-4-hydroxycinnamic acid (EHCA), 2,5-dihydroxybenzoic acid (DHB), 1-hydroxy-isoquinoline (1-HIQ), and self-assembled 13 nm golden nanoparticles with thiolated-oligos of ten units of thymine (Au-T_{10}).

The dehybridization products of the MPs-target-NP complexes, the BMC probes, were spotted onto 400 μm Anchor-Chips (Bruker Daltonics, Bremen, Germany). The Au-T_{10} was applied as the MALDI matrix onto MALDI "plate" (Anchor-Chips) presenting the BMC probes in duplicate and subsequently allowed to dry at ambient temperature, and 0.1% trifluoroacetic acid (TFA) was used for on-target washing if desired. All the samples were then analyzed on the mass spectrometry in the positive-ion reflectron mode. Mass spectra were acquired using 25 kV of ion source 1 and 21.55 kV of ion source 2 in accelerating voltage, linear operating mode, and the lens was 9.5 kV of the accelerating voltage, and the reflector 1 and reflector 2 were 26.3 kV and 13.85 kV of the accelerating voltage respectively. The detection of each spot was made under optimal conditions by adjusting laser energy.

Multiplexed Detection with the BMC Probes

Prior to the assay, the MP multiplexing solution composed of four MPs (HIV-MP, HBV-MP, HCV-MP and TP-MP) was prepared by diluting equal volumes of each capture probe in buffer 1 to a total concentration of 20 mg/mL. Similarly, the NP multiplexing solution constituted with four NPs (HIV-NP, HBV-NP, HCV-NP and TP-NP) was prepared by diluting equal volumes of each NP (10 nM) in buffer 1 to a total concentration of 500 pM. The sandwich complex was formatted by the principles of base pairing as described before[4a] (Fig. S1c). The BMC-based assay was initiated by mixing hybridization buffer (6 M NaCl, 10 mM phosphate, pH 7.0) (10 μL), MP multiplexing solution (50 mL) and the appropriately artificial target ss-DNA solution (30 μL) in a tube (Axygen). The mixtures were heated at 45°C for 0.5 h by vortexing gently every 10 min, followed by incubation at 25°C for 2 h to allow hybridization as completely as possible between the MPs and the target DNAs of interest. The MP-target complexes were separated on a 6-Tube Magnetic Separation Rack (Invitrogen Co.) and washed three times with washing buffer (0.6 M NaCl, 10 mM phosphate, pH 7.0); then the MP-target complexes were redispersed in 50 μL washing buffer. The NP multiplexing solution (50 mL) and hybridization buffer (10 μL) were followed by adding to the tube. After hybridization for 2 h, the sandwich complex with three components, MP-target-NP, along with unreacted MPs was pulled to the wall of the reaction vessel by the magnetic separator. Unreacted NPs were washed away with washing buffer. This washing step was repeated several times to effect removal of the NPs that were not specifically bound to the targets in hybridization reactions. Finally, the magnetic field was removed, 10 μL of nanopure water was added to the reaction vessel, and the tube was heated to 75°C for 5 min to dehybridizate the sandwich complex to release the BMC probes. Reintroduction of the magnetic field removed all of the MPs from solution, leaving dual-functionalized NPs for detection.

HBV Genomic ds-DNA Assay with the BMC Probe-based MS Detection

The real bio-sample of HBV genomic ds-DNA was extracted with boiling lysis by kit (Shanghai Kehua Bioengineering Co., Ltd.) from serum sample of the surface antigen positive individuals. 30 μL of the extracted HBV genomic DNA was then cut in 50 μL digestion system using the restriction endonuclease EcoN□(New England Biolabs) at 37°C for 1 h. A restriction digestion step was needed to prevent DNA super coiling during the heating and subsequent detection. Assays were assembled in nuclease-free tubes containing digested genomic DNA sample, 1 μL of each blocking oligonucleotides (200 μM), and 27 μL of 0.15 M NaCl, 10 mM phosphate buffer, pH 7.4 (buffer 6). The assays were mixed thoroughly and placed at 95°C for 5 min to denature the DNA fragments. After denaturation, the denatured target DNAs and 10 μL of hybridization buffer were added to the wet cake of 50 μL of MP multiplexing solutions in the tube. The reaction system were mixed well and incubated at ambient temperature for 2 h by mixing gently to facilitate target capture. The MP-target complexes were then washed 3 times with washing buffer. To wet cake of the washed MP-target complexes, 50 μL of buffer 6, 10 μL hybridization buffer and 50 μL of freshly cleaned NP multiplexing solution were added for sandwich complexes construction. The sandwich complexes were then washed 7 times using washing buffer to remove all unbound NPs. The supernatant was removed after the seventh wash, and the complexes were re-suspended in 10 μL of nanopure water. The BMC probes were released from the sandwich complexes by heating dehybridization and detected by MALDI TOF MS as described above.

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