Chapter 9

Ultrasensitive Detection of Metal Ions with DNA Nanostructure

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Abstract

In spite of its greatly scientific and technological importance, developing rapid, low cost and sensitive microarray sensors for onsite monitoring heavy metal contamination remains challenging. Here we develop a DNA nanostructured microarray (DNM) with a tubular three-dimensional sensing surface and an ordered nanotopography for rapid and sensitive multiplex detection of heavy metal ions. In our design, DNA tetrahedral-structured probes (TSPs) are used to engineer the sensing interface with spatially resolved and density-tunable sensing spots, improving the micro-confined molecular recognition. Meanwhile, a bubble-mediated shuttle reaction inside the DNM-functionalized microchannel improves the target-capturing efficiency. Thus, the sensitive and selective detection of multiple heavy metal ions (i.e., Hg^{2+}, Ag^{+}, and Pb^{2+}) with this novel DNM biosensor can be achieved within 5 min. Moreover, the detection limit is down to 10, 10, and 20 nM for Hg^{2+}, Ag^{+}, and Pb^{2+}, respectively. Therefore, the DNM biosensor capable of simultaneously detecting multiple heavy metal ions with sensitivity and selectivity shows great potential to be point-of-test devices.

Key words DNA nanostructures, Multiplex detection, Metal ions detection, Ultrasensitive detection, Microchannel

1 Introduction

The rapid and sensitive detection and monitoring of environmental toxins, including heavy metals is of great significance for the health of humans and the ecosystem [1–3]. Often, a contamination event results from multiple toxic ingredients and thus a multiplex detection strategy need to be adopted for the readout of all relevant chemicals [4–6]. The construction of advanced sensing interfaces and fluorescence imaging devices has enabled the use of high-throughput microarray-based platforms [7, 8]. Nevertheless, rationally building a fast, sensitive, and easy-to-use microarray sensor remains a challenge [9, 10], and engineering a heterogeneous sensing interface promoting the faster kinetics and advantageous thermodynamics for probe-target interactions is desired [11, 12].

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Sensor performance depends on the affinity between molecular targets and the surface-tethered probes [13] and by the interfacial properties including the orientation, ordering, and density of probes, as well as the diffusion rate of the target of interest to the probes [14]. The reduction of the sensors to the nanoscale can increase the mass transport rate and thus the detection sensitivity [15, 16]. However, a trade-off must be found, as too small a nanosensing area exposes a small number of molecular probes thus reducing the probability of collision and binding between the surface probes and the soluble analytes. Molecules can typically diffuse by 10–100 μm in solution [17], while binding events require nanometer proximity between binding partners: a trans-scale design of sensor integrating nanoscopic features onto macroscopic surfaces is a prerequisite. Probe-target recognition can be greatly improved once the dynamic targets are closely confined to the static surface-tethered nanoprobes arranged in the macroscale. Considering the radial-diffusion feature of analytes at the microscale or nanoscale interface, we assume that rolling up a traditional two-dimensional (2D) flat planar sensing surface into a three-dimensional (3D) tubular sensing surface in the inside face of a microfluidic channel is expected to improve the sensor performance [18].

Self-assembled DNA nanostructures have been used to a great advantage in engineering molecular recognition events and programmable sensing-interfaces [19–21]. Using these, we designed a spatially resolved 3D tubular DNA-nanostructured microarray (DNM) for the rapid and sensitive multiplex detection of heavy-metal ions (*see* Fig. 1). The microarray is organized inside a microchannel. To construct the DNM, target-specific DNA tetrahedral-structured probes (TSPs) (sequences of TSPs listed in Table 1, and sequences of TSPs probes and Reporter DNA listed in Table 2) were rapidly self-assembled and incorporated into a droplet array [22, 23]. This is then moved to the designated locations for immobilization and sensor engineering [24]. The DNM were evenly spaced along the microchannel, achieving probe surfaces with highly ordered probe orientations, controlled density and circular arrangement. This setup endowed the micro-confined microarray sensor with improved sensitivity and selectivity.

The method describes the preparation of the microchannel DNM biosensor with tetrahedral DNA nanostructures and its use with a low-cost bubble-based *shuttle reaction strategy*. This uses air bubbles to separate multiple reagent *plugs* (in segmented-flow microfluidics, discrete liquid droplets are encapsulated or spaced by a carrier fluid: these droplets are termed "plugs"). This bubble-mediated strategy avoided the designation and fabrication of complex microchannels as well as complex user procedures. With the method herein described, this DNM biosensor can detect multiple heavy metal ions (i.e., Hg^{2+}, Ag^{+}, and Pb^{2+}) within 5 min. The

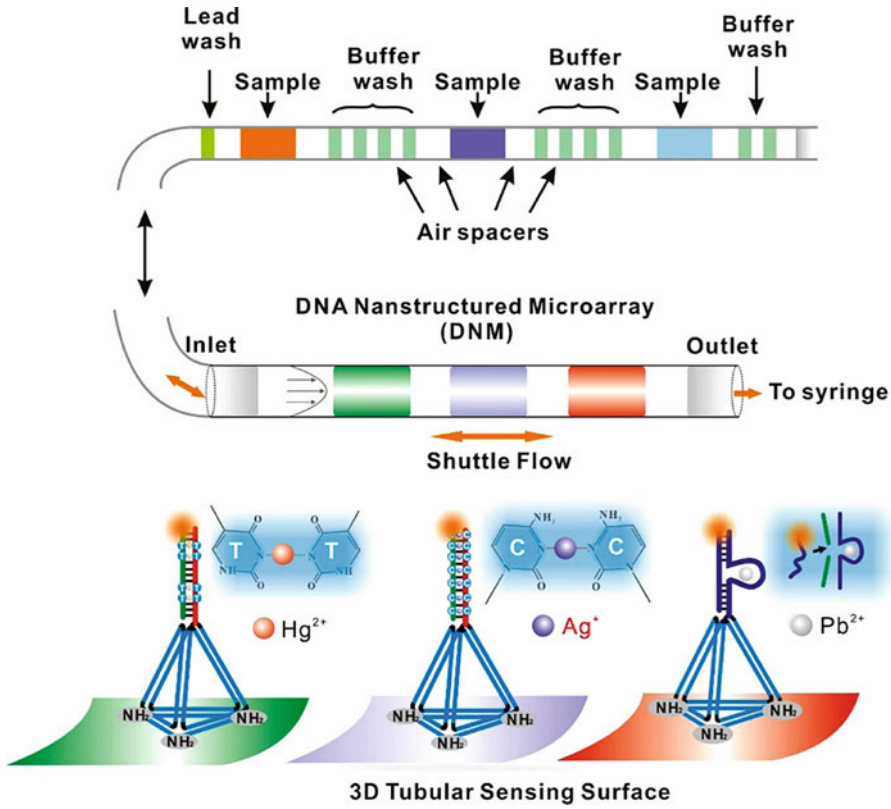

Fig. 1 Construction of 3D tubular DNM sensor inside microchannels for the detection of Hg^{2+}, Ag^{+}, and Pb^{2+} based on bubble-mediated shuttle reaction (reproduced from ref. [18] with permission from ACS)

Table 1
Sequences of TSPs

Oligo	Sequence (5′–3′)
TSP-A	Probe-ACATTCCTAAGTCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCC ATAGTA
TSP-B	NH_2-C_6-TATCACCAGGCAGTTGACAGTGTAGCAGCTGTAATAGATGCGAGGGT CCAATAC
TSP –C	NH_2-C_6-TCAACTGCCTGGTGATAAAACGACACTACGTGGGAATCTACTATGGC GGCTCTTC
TSP –D	NH_2-C_6-TTCAGACTTAGGAATGTGCTTCCCACGTAGTGTCGTTTGTATTGGA CCCTCGCAT

corresponding detection limit is down to 10, 10, and 20 nM for Hg^{2+}, Ag^{+}, and Pb^{2+}, respectively (*see* Figs. 2 and 3). In conclusion, this sensitive and selective DNM sensor has great potential developed as point-of-care test devices.

Table 2
Sequences of TSPs probes and reporter DNA

Description	Oligo DNA	Sequence (5′–3′)
Mercury ion assay	TSP-A1 (mercury ion specific sequence)	TTCGTTCCTGTTTGCGAAAAAAAAAAACATTCCTAAGTCTG AAACATTACAGCTTGCTACACGAGAAGAGCCGCCATAGTA
	Reporter sequence	CGCATTCAGGTTCGTA-Cy3
Silver ion assay	TSP-A2 (silver ion specific sequence)	CACACACACACACACACACACAAAAAAAAAAAACATTCCTAAG TCTGAAACATTACAGCTTGCTACACGAGAAGAGCCGCCAT AGTA
	Reporter sequence	CTCTCTCTCTCTCTCTCTCTC-Cy3
Pb ion assay	TSP-A3 (Pb ion specific sequence)	CATCTCTTCTCCGAGCCGGTCGAAATAGTGAGTCAGACATA TTTTTTTTTTACATTCCTAAGTCTGAAACATTACAGCTTG CTACACGAGAAGAGCCGCCATAGTA
	Substrate strand reporter sequence	TATGTCTGACTCACTTArAGGAAGAGATGTATGTCTGACTC ACTTAGGAAGAGATG-Cy3

2 Materials

Prepare all solutions using ultrapure water with a sensitivity of 18 MΩ cm at 25 °C and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 DNA TSPs Probe

1. TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0): dissolve 1.211 g of Tris and 0.372 g of Na_2EDTA in about 900 mL of ultrapure water (*see* **Note 1**). Mix and adjust the pH with HCl (*see* **Note 2**). Make up to 1 L with water. Store at 4 °C.
2. TM buffer (20 mM Tris, 50 mM $MgCl_2$, pH 8.0): dissolve 2.422 g of Tris and 2.975 g of $MgCl_2$ in about 900 mL of ultrapure water. Mix and adjust the pH with HCl (*see* **Note 2**). Make up to 1 L with water. Store at 4 °C.
3. Stock solutions of each DNA oligonucleotide at 100 μM in TE buffer (*see* **Note 3**).
4. 10× TAE buffer (400 mM Tris, 200 mM acetic acid, 20 mM EDTA): weigh 48.456 g of Tris and 7.444 g of Na_2EDTA, add 11.32 mL of concentrated acetic acid (*see* **Note 4**) and prepare a 1 L solution. Store at 4 °C.
5. PAGE running buffer (40 mM Tris, 2 mM EDTA, 20 mM acetic acid): weigh 4.846 g Tris and 0.744 g Na_2EDTA, add 1.132 mL acetic acid (*see* **Note 4**) and prepare a 1 L solution. Store at 4 °C. Alternatively, dilute from 10× TAE buffer with ultrapure water.

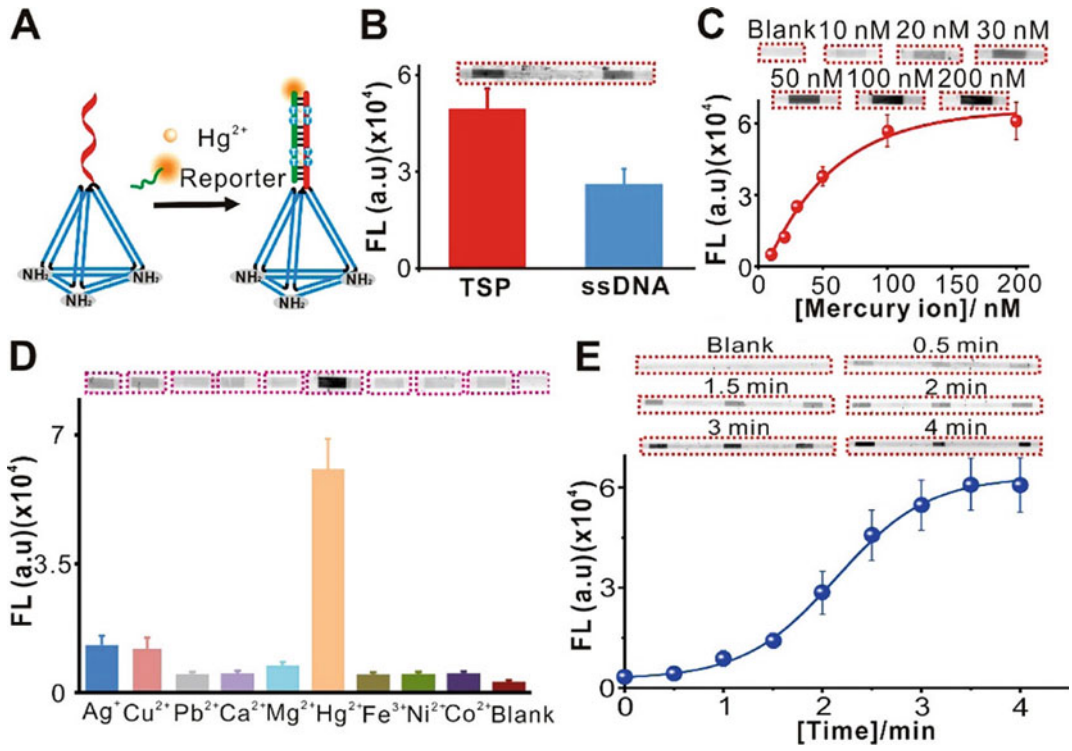

Fig. 2 Three-dimensional tubular DNM sensor for the Hg ion assay. (**a**) The presence of Hg ions is transduced to fluorescence signals through the formation of T-Hg^{2+}-T configuration. (**b**) Comparison of TSP- and ssDNA probe-based sensor performance (with 100 nM Hg^{2+}). The inset displays the corresponding fluorescent images. (**c**) Fluorescence intensities and corresponding fluorescent images (shown as inset) of DNM-sensing spots in the presence of Hg^{2+} at 10, 20, 30, 50, 100, or 200 nM. (**d**) Selectivity of DNM-based Hg^{2+} sensor over different metal ions. (**e**) Fluorescence intensity vs. shuttle hybridization time (with 200 nM Hg^{2+}). The inset displays the fluorescent images of three different DNM-sensing spots inside microchannels at corresponding hybridization times. (reproduced from ref. [18] with permission from ACS)

6. 30% acrylamide/Bis solution (29.2:0.8, acrylamide:bis): Add 29.2 g of acrylamide monomer and 0.8 g of bis-acrylamide (cross-linker) to about 40 mL of ultrapure water. Add a spatula of AG 501-X8 (D) mixed-resin beads (Bio-Rad) and stir for 30 min. Make up to 100 mL with water and filter through a 0.45 μm Corning filter (*see* **Note 5**). Store at 4 °C in a bottle wrapped with aluminum foil (*see* **Note 6**).
7. 10% Ammonium persulfate solution in water (*see* **Note 7**).
8. *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine. Store at 4 °C (*see* **Note 8**).
9. 6× gel loading solution for nondenaturing electrophoresis (Sangon Biotech, Shanghai or equivalent).
10. GelRed gel staining solution (formulate according to the manufacturer's instructions).

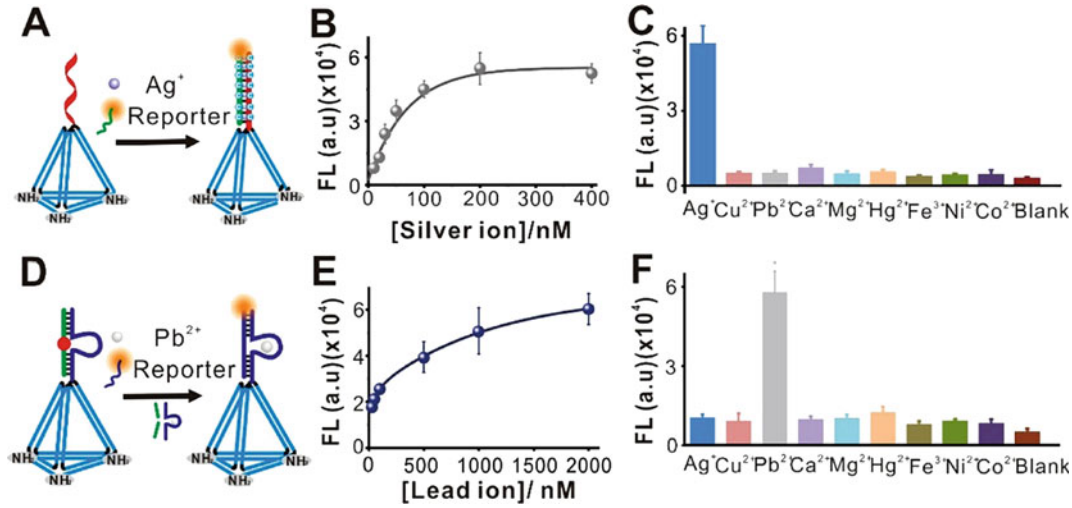

Fig. 3 Three-dimensional tubular DNM sensor for the silver and Pb ion assay. (**a**) The presence of silver ions was transduced to fluorescence signals through the formation of C-Ag^+-C configuration. (**b**) Fluorescence spectra of the DNM-based Ag^+ sensor upon addition of Ag^+ (10, 20, 30, 50, 100, 200, or 400 nM). (**c**) Selectivity of the DNM-based Ag^+ sensor over different metal ions. (**d**) The detection scheme for Pb ions. (**e**) Fluorescence intensities in the presence of Pb^{2+} (20, 50, 100, 500, 1000, or 2000 nM). (**f**) Selectivity of the DNM-based Pb^{2+} sensor to different metal ions. (reproduced from ref. [18] with permission from ACS)

11. DNA molecular weight size markers: 20 bp DNA ladder.
12. Dialysis tubing.

2.2 DNM Functionalized Microchannel

1. 10% NaOH: Weigh 10 g sodium hydroxide and bring to 100 mL with ultrapure water (*see* **Note 9**).
2. Capillary: Glass capillary with inner diameters of 200 μm.
3. Piranha solution (30:70 v/v sulfuric acid to 30% hydrogen peroxide): Add 70 mL 30% hydrogen peroxide to a 200 mL glass beaker. Make up to 100 mL with sulfuric acid (*see* **Note 10**). WARNING: piranha solution reacts violently with organic materials and should be handled with extreme caution. Wear safety goggles, appropriate gloves, and acid resistant apron over a lab coat. Always handle piranha solution under a fume hood. Prepare only as much as is needed; do NOT store any piranha solution or hold it in closed containers.
4. Mixture of ethanol/H_2O/3-Aminopropyltriethoxysilane (APTES) (95:3:2 by volume): Add 95 mL ethanol to a 100 mL graduated cylinder. Add 3 mL ultrapure water to the cylinder. Make up to 100 mL with APTES (*see* **Note 11**). Store at 4 °C.
5. 10× PBS buffer (100 mM phosphate buffer, pH 7.4): dissolve 11.51 g of Na_2HPO_4 and 2.28 g of NaH_2PO_4 in about of 900 mL of ultrapure water. Bring to 1 L with ultrapure water. Store at 4 °C.

6. 1× PBS buffer (10 mM phosphate buffer, pH 7.4). dissolve 1.151 g of Na_2HPO_4 and 0.228 g NaH_2PO_4 in about of 900 mL of ultrapure water. Bring to 1 L with ultrapure water. Store at 4 °C.
7. 5% glutaraldehyde: add 20 mL 25% glutaraldehyde to 80 mL of 10× PBS buffer (*see* **Note 12**). Store at 4 °C.
8. Aldehyde blocking solution (2.5 mg/mL $NaBH_4$ in PBS buffer with 25% (v/v) ethanol, pH 7.4): add 25 mL of ethanol to a 100 mL graduated cylinder. Add 0.25 g of $NaBH_4$ and 1× PBS buffer to 100 mL. Store at 4 °C (*see* **Note 13**).
9. Pentanol.
10. A slotted vial auto sample device: make slotted vials by cutting slots at the bottom of PCR tubes. Insert one side of the aldehyde modified capillary into the vial slot. Connect other side to the syringe pump.

2.3 Metal Ion Detection

1. A syringe pump (LSP02-1B; LongerPump).
2. 10 μL aliquots of various concentrations of metal ions to be tested.
3. 5 μL aliquots of 1.5 μM Cy3-labeled reporter oligos (*see* **Note 14**).
4. Washing buffer (20 mM Tris–HCl, pH 7.0): Add 24.227 g of Tris–HCl to about 900 mL of ultrapure water. Mix and adjust pH with HCl (*see* **Note 2**). Make up to 1 L with ultrapure water. Store at 4 °C.
5. A confocal laser fluorescence microscope (Typhoon 9210) and ImageQuant software (GE Healthcare) or equivalent.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Synthesis and Purification of DNA TSPs

1. Mix 1 μL of each of the four oligonucleotides stock solutions (TSP-A*n* for the specific ion, TSP-B, TSP-C and TSP-D, *see* Tables 1 and 2) with 96 μL of TM buffer (10 μM conc. of each oligo).
2. Heat the resulting mixture to 95 °C for 10 min and cool to 4 °C in 30 s using a Peltier model PTC-200 thermal cycler or analogous [25].
3. Mix 600 μL 10× TAE buffer, 2 mL acrylamide mixture, and 4 mL ultrapure water in a 20 mL centrifuge tube. Degas with helium for 10 min.

4. Add 60 μL of 10% ammonium persulfate solution to the acrylamide mix. Mix well.
5. Add 6 μL of TEMED, mix and immediately cast a gel within a 7.25 cm × 10 cm × 1.5 mm gel cassette. Allow space for stacking gel and gently overlay with isobutanol or water (*see* **Note 15**). Insert a 10-well gel comb immediately without introducing air bubbles. Allow the gel to polymerize for 30 min.
6. Take out the gel from the casting frame and clamp it in the gel apparatus. Fill the gel chambers with 1× TAE buffer. Remove the comb carefully (*see* **Note 16**).
7. Prepare the samples for the gel by mixing 5 μL of the DNA samples with 1 μL 6 × loading buffer.
8. Rinse the loading tip a few times with ultrapure water (*see* **Note 17**). Insert the loading tip to a few mm from the well bottom. Deliver the samples into the well. Repeat for all samples. Rinse the tip with ultrapure water after loading for a few times.
9. Attach the power supply to the electrophoresis apparatus (*see* **Note 18**) and run at 80 V for 1 h (*see* **Note 19**).
10. Following electrophoresis, switch off the power supply and take out the gel plates, remove the gel. Place the gel in the staining solution for 30 min. Stain the gel until the bands are properly seen. Determine the approximate molecular weight of the TSP bands by comparing them with the available DNA markers.
11. Cut out the TSP bands and obtain the gel slice. Place the gel slice in a small bag of dialysis tubing (*see* **Note 20**). Add a minimal volume of 1× TAE buffer just enough to surround the gel slice. Seal the bag (*see* **Note 21**). Fill the horizontal mini-gel apparatus with 1× TAE buffer. Place the dialysis bag in the apparatus, on the gel platform. Place the dialysis bag close to the negative electrode (*see* **Note 22**).
12. Apply 80 V to the apparatus, and allow the DNA to elute at this potential for 1 h. Recover the bag. Open carefully and recover the buffer with a pipette. Rinse the bag and gel slice once with 200 μL of 1× TAE buffer (*see* **Note 23**).
13. Measure the concentration of the recovered TSPs and, in case, set to 50 μM with the method of choice.

3.2 Fabrication of DNM Functionalized Microchannel

By this method, we commonly obtain DNM-functionalized capillaries with $3.72 \pm 0.27 \times 10^{12}$ TSPs/cm^2 [14].

1. Clean the capillary with 10% NaOH solution for 10 min.
2. Wash the capillary with ultrapure water and clean using piranha solution for 1 h at 80 °C.

3. Dry the capillary with nitrogen gas, and then treat the capillary with the ethanol/H_2O/3-Aminopropyltriethoxysilane (APTES) mix for 1 h.
4. Rinse the capillary successively with ethanol and ultrapure water.
5. Heat the capillary to 120 °C for 1 h.
6. Immerse the capillary in 5% glutaraldehyde overnight. Store at 4 °C, if needed.
7. Dry the aldehyde modified capillary under a flow of nitrogen gas.
8. Prepare a 50 μM solution of the TSPs in TM buffer. Generate a droplet array of amino-derivatized TSPs or amino-functionalized ssDNA droplet array using the slotted-vial auto sample device (using pentanol as the carrier). Incubate the capillary with the droplet array overnight at room temperature.
9. Rinse the capillary with TM buffer.
10. Reduce the functionalized capillary with the aldehyde blocking solution at room temperature. Store at 4 °C for further use.

3.3 Metal Ion Detection with DNM Functionalized Microchannel

1. Prepare a sample plugs array made by the desired amounts of aqueous solution plugs, an air bubble plug and a washing buffer plug (*see* Fig. 1b). Each aqueous solution plug is made by adding 10 μL of solution of the desired metal ion at the test concentration to 5 μL of Cy3-labeled reporter oligo solution (*see* Table 2).
2. Connect the DNM-functionalized capillary with the sample plug array via the inlet. Connect the DNM-functionalized capillary with a syringe pump via the outlet.
3. Drive the sample plug array from the inlet to the capillary with a flow rate of 5 μL/min. Move the sample plugs array successively through the DNM-sensing interface. Pause the syringe pump when the sample plugs array approaches the outlet of the capillary.
4. Flow the sample plugs array backward through the DNM-sensing interface with a flow rate of 5 μL/min. Repeat the process for multiple times.
5. Rinse the capillary passing the washing buffer plug over the DNM-sensing interface with a flow rate of 5 μL/min.
6. Utilize a confocal laser fluorescence microscope to evaluate the efficiency of the DNM sensor for the metal ion. Record the fluorescence signal at an excitation wavelength of 532 nm and an emission wavelength of 580 nm. Use a resolution of 10 μm.
7. Analyze the fluorescence image with ImageQuant software.

4 Notes

1. Warming up the water (to about 37 °C) helps in the dissolution of Tris. It is important to adjust the pH of the solution after equilibration at room temperature.
2. Concentrated HCl (12 N) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl (e.g., 6 and 1 N) with lower ionic strengths to avoid a sudden drop in pH below the required pH.
3. Centrifuge with 12,000 × *g* for 60 s before dissolving the DNA to avoid loss.
4. Operate in a fume hood and wear a mask and appropriate personnel protection equipment when using the concentrated acetic acid to avoid acid contacting with eyes and skin.
5. Wear a mask when weighing acrylamide. To avoid exposing acrylamide to coworkers, cover the weigh boat containing the weighed acrylamide with another weigh boat when transporting it to the fume hood. Transfer the weighed acrylamide to the cylinder inside the fume hood and mix on a stirrer placed inside the hood. Unpolymerized acrylamide is a neurotoxin and care should be exercised to avoid skin contact. Mixed resin AG 501-X8 (D) (anion- and cation-exchange resin) is used when acrylamide solution is made, since it removes charged ions (e.g., free radicals) and allows longer storage. Some investigators store the prepared acrylamide along with this resin in the refrigerator. However, we filter them out before storage. The used mixed resin should be disposed as hazardous waste. Manufacturer's warning states that this resin is explosive when mixed with oxidizing substances. The resin contains a dye that changes from blue-green to gold when the exchange capacity is exhausted.
6. The acrylamide solution can be stored at 4 °C for 1 month. Acrylamide hydrolyzes to acrylic acid and ammonia. The acrylamide mixture, buffer, and water can be prepared in large batches, frozen in aliquots, and used indefinitely. Remove the required amount, bring to room temperature, and add the other ingredients for casting our own gels.
7. We find that it is best to prepare this fresh each time.
8. We find that storing at 4 °C reduces its pungent smell.
9. Wear gloves and place a small beaker on the scale before weighing NaOH. Take out a certain amount of NaOH and quickly weigh the NaOH to avoid moisture. Add ultrapure water rapidly and stir gently with glass rod to avoid skin contact.

10. Wear a mask and use a glass rod for drainage when adding the sulfuric acid into 30% hydrogen peroxide. Do not change the order of addition. Stir gently in place for half hour before further use.
11. Wear a mask before using APTES to avoid breathing APTES. It would be better to operate in the fume hood.
12. Operate in a fume hood and wear a mask (besides appropriate gloves and other personal protective equipment) when using glutaraldehyde to avoid breathing vapors.
13. We find that it is best to prepare this fresh each time.
14. Wrap the Cy3-labeled reporter with aluminum foil to avoid light decomposition.
15. Seal the gel cassette with 1% agarose. Overlay the resolving gel with water for gels having acrylamide concentration lower than 8% and use isobutanol for gels of 10%. This overlay prevents contact with atmospheric oxygen in addition to helping to level the resolving gel solution.
16. Remove the comb carefully to avoid breaking the well.
17. Rinse the loading tip a few times with ultrapure water. Make sure that all the water in the loading tip is poured out before loading the samples.
18. Attach the power supply by putting the lid. Make sure that the connection is in correct way, ie., black to black and red to red. Make sure the migration in the right direction.
19. Centrifuging the samples prior to the run helps remove insoluble debris. Add a drop of 0.1% bromophenol blue to the upper chamber buffer and form a much stronger dye front during the electrophoretic run. Do not allow the dye front to go out of the gel.
20. The bag may be knotted to form the ends. Use dialysis clip for handling the bag easier and more convenient.
21. Seal the bag. Take care to exclude all air bubbles to avoid the separation effect.
22. Weigh the bag down with glass slides to prevent the dialysis clip from floating. Do not use metal as a weight, for it distorts the electric field. Tap the bag to the gel platform to keep it from floating if floating.
23. Rinse the bag and gel slice once with 200 μL of 1× TAE buffer. Add sodium acetate and ethanol if desired. Pellet the TSPs and wash one time with 70% ethanol.

Acknowledgments

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