Droplet Microarray Based on Nanosensing Probe Patterns for Simultaneous Detection of Multiple HIV Retroviral Nucleic Acids

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ABSTRACT: Multiplexed detection of viral nucleic acids is important for rapid screening of viral infection. In this study, we present a molybdenum disulfide (MoS2) nanosheet-modified dendrimer droplet microarray (DMA) for rapid and sensitive detection of retroviral nucleic acids of human immunodeficiency virus-1 (HIV-1) and human immunodeficiency virus-2 (HIV-2) simultaneously. The DMA platform was fabricated by omniphobic−omniphilic patterning on a surface-grafted dendrimer substrate. Functionalized MoS2 nanosheets modified with fluorescent dye-labeled oligomer probes were prepatterned on positively charged amino-modified omniphilic spots to form a fluorescence resonance energy transfer (FRET) sensing microarray. With the formation of separated microdroplets of sample on the hydrophobic−hydrophilic micropattern, prepatterned oligomer probes specifically hybridized with the target HIV genes and detached from the MoS2 nanosheet surface due to weakening of the adsorption force, leading to fluorescence signal recovery. As a proof of concept, we used this microarray with a small sample size (<150 nL) for simultaneous detection of HIV-1 and HIV-2 nucleic acids with a limit of detection (LOD) of 50 pM. The multiplex detection capability was further demonstrated for simultaneous detection of five viral genes (HIV-1, HIV-2, ORFlab, and N genes of SARS-CoV-2 and M gene of Influenza A). This work demonstrated the potential of this novel MoS2-DMA FRET sensing platform for high-throughput multiplexed viral nucleic acid screening.

KEYWORDS: droplet microarray (DMA), 2D MoS2 nanosheets, fluorescent resonance energy transfer (FRET), HIV nucleic acids, omniphobic

1. INTRODUCTION

Human immunodeficiency virus (HIV) is a lentivirus that causes HIV infection leading to acquired immunodeficiency syndrome (AIDS). Generally, the HIV family contains two major types of viruses, HIV type-1 (HIV-1) and HIV type-2 (HIV-2). The two types of HIV can infect a host through different pathological mechanisms and involve different clinical treatments. It is reported that coinfection of the two HIV viruses may complicate the management of HIV infections. An effective screening detection of the two HIV viruses in the early stage may provide important evidence for conducting clinical strategies and public health security in early time. In addition to HIV, there are many other emerging viral infections, including coronavirus disease 2019 (COVID-19) caused by the infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), requiring fast and efficient detection methods. The droplet microarray (DMA) sensing platform in this paper can be easily adapted to the detection of multiple viral genes for early diagnostics of other emerging viral infection diseases including COVID-19.

Nucleic acid testing and antibody testing are currently the dominating methods for HIV diagnostics. Since the host needs time to develop an immune response, antibody testing fails to detect early HIV infection. Retroviruses, such as HIV, infect patients by inserting their DNA within the genome of the host. Retroviral DNAs are important biomarkers existing in every infected host cells, which trigger pathological processes of HIV infection at an early stage. Thus, the detection of integrated retroviral DNAs is important for early estimation of the incidence and prevention of viral transmission. Polymerase chain reaction (PCR) is considered a standard genetic test for HIV nucleic acid detection. Besides, other biosensing methods are also developed for HIV nucleic acid detection, such as electrochemiluminescence, colorimetry, electro-
chemical, and fluorescence-based sensing. However, most of the current methods face the disadvantages of being time consuming, requiring large volumes of samples, and lacking multiplexed capability. Therefore, it is of high importance to develop a new sensing platform that is highly sensitive, fast, compatible with low sample/reagent consumption, and capable of multiplexed detection.

A droplet microarray (DMA)-based platform is a potential solution to meet these requirements. The DMA is a miniaturized high-throughput platform with individual droplet size down to nanoliters, which has been used in various biological applications such as cell screening, drug testing, lipidoids synthesis, and biofilm patterning. However, the application of DMA on parallel biosensing has not been explored yet. A small droplet microarray with a defined location of separated droplets is of high interest for high-throughput biosensing. Each droplet represents an ultrasmall liquid reservoir without solid walls, where liquid is confined by either hydrophobic or omniphobic surface properties. As a biosensing substrate, it could greatly reduce the cost of each sensing spot while enabling the formation of ultrasmall droplets. The DMA platform is usually based on a solid substrate, such as a glass surface coated with a nanoporous polymer layer functionalized to form either superhydrophobic/hydrophilic or omniphobic/omniphilic micropatterns via UV-induced thiol-yne or thio-e reactions.

The DMA platform will generate an interface that is easy to graft functional nanomaterials for biosensing purposes. In this paper, we further develop a droplet microarray platform based on dendrimer omniphobic–omniphilic coating with prepatterned nanosensing probes as a parallel biosensing platform. Dendrimers are monodispersed and multivalent molecules with highly branched structures, which have been used for many biomedical interfaces due to their polyelectrolytic self-assembling, electrostatic interactions, chemical stability, and low cytotoxicity. The dendrimer-based DMA platform will generate an interface that is easy to be grafted with functional nanomaterials for biosensing purposes.

Nanomaterial-based fluorescence resonance energy transfer (FRET) is a sensing mechanism based on energy transfer from a donor to an acceptor, which is used in many biosensing and bioanalysis applications. The sensing performance of the FRET assay is mainly determined by the design of donor and acceptor pairs. Recently, two-dimensional (2D) molybdenum disulfide (MoS2) nanosheets have aroused a lot of interest for biosensing due to their high surface-to-volume ratio, unique optical properties, good biocompatibility, and good quenching capability.

Compared with current organic quenchers, MoS2 nanosheets also showed advantages in protecting the loaded genes from enzymatic cleavage. Currently, nanomaterial-based FRET assays are mainly used in the bulk solution to detect various biomolecules. The application of nanomaterial-based FRET in the droplet microarray has not been explored yet.

In this paper, a droplet microarray with MoS2 FRET nanoprobe patterns (MoS2-DMA) was developed for multiplexed HIV gene biosensing, which provided a solid substrate-based FRET array sensing platform. In this design, dendrimer-based micropatterning was applied for DMA fabrication. MoS2 nanosheets were then adsorbed on dendritic spots as acceptors for FRET assays. The MoS2-DMA FRET sensing platform was established by further adsorption of fluorescent dye-labeled oligonucleotide probes on MoS2 nanosheet-coated hydrophilic spot surfaces as donors of FRET assays, which led to the “off” status of the fluorescence signal due to the energy transfer from dyes to MoS2 nanosheets. In the presence of HIV target nucleic acids, the specific hybridization between sensing probes and target nucleic acids would detach the probes from the MoS2 nanosheet surface, therefore triggering the “turn-on” of the fluorescence signal. As a proof of concept, we used this MoS2-DMA FRET sensing platform to realize simultaneous sensing of multiple samples of HIV genes with good sensitivity and specificity. The limits of detection (LODs) for HIV-1 and HIV-2 genes are 1.24 and 1.26 nM, respectively. Our MoS2-DMA FRET sensing platform combines a droplet microarray with nanomaterial-based FRET sensing and opens the possibility for high-throughput multiplexed viral gene screening and rapid diagnostics for emerging viral infection diseases such as COVID-19.

2. EXPERIMENTAL SECTION

2.1. Materials. The MoS2 material was purchased from Nanjing NKNANO Ltd., China. The oligonucleotides were synthesized and purified by Sangon Biotech Co., Ltd., Shanghai, China. The sequences of oligonucleotides applied are as follows: capture sequence HIV-1 (FAM-S′ CTGGGATTTAATAATAGAATGATATAGC3′), capture sequence HIV-2 (Cy5S′-AAAGACCAGCCGCACTAAATCCA-3′), target sequence HIV-1 (S′-GCTATACATTCTAATTTTATATATCCAG3′), target sequence HIV-2 (S′-TGAATTAGTTGCGCTGTCCTT-3′), four-base-mismatched sequence Probe-1 (S′-GCTAAAAACATCTTACTATTATAATATCCAG3′), four-base-mismatched sequence Probe-2 (S′-TGAAGTCTGCTGAACTGACCCCTT-3′), target sequence hepatitis B virus (HBV) (S′-CTGGATCCTGGCGCAGGTCCTT-3′), and target sequence hepatitis C virus (HCV) (S′-CACCACCAATCTCC-3′). All of the synthesized sequences were dissolved in DNAse/RNase free ultrapure distilled water as a stock solution and kept at −20 °C. Normal human serum samples were purchased from Thermo Fisher Scientific. The glass substrates were purchased from Marienfeld Superior Ltd., Germany. 4-(Dimethylaminophenyl)pyridine was purchased from Novabiochem, Germany. Ethanol/water (1:1, v/v) and acetone were purchased from Merck Millipore Ltd., Germany. N,N′-Diisopropylcarbodiimine was purchased from Alfa Aesar Ltd., Germany.

2.2. Characterization. The morphology of MoS2 nanosheets was observed by a JEOL-2100F transmission electron microscope installed on an Oxford Instruments EDS system (200 kV). The size distribution and zeta-potential of the MoS2 nanosheets were measured by a Zetasizer Nano Z system (Malvern Instruments Ltd.). The optical absorbance spectra of the nanosheets were obtained with a UV−vis spectrophotometer (Ultratop 2100 pro, Harvard BioScience, Inc.). The DMA surface and MoS2-modified DMA surface were characterized by a JEOL field-emission scanning electron microscopy system. Atomic force microscopy (AFM) characterization was performed on a Bruker Dimension ICON AFM machine with standard tapping mode. The Raman spectrum of dendritic spots was measured by a Renishaw Micro-Raman spectrometer system. A microscopy image of the droplet microarray was obtained by a Keyence BZ-9000 microscope. The fluorescence spectrum was characterized by an FLS920 photo-luminescence spectrometer (Edinburgh, UK.).

2.3. Dendrimer-Based DMA Fabrication. Dendrimers have been used to modify glass slides for DNA microarray fabrication. Here, a modified method was developed to fabricate dendrimer-based DMA. Vacuum plasma is a process of gas ionization in a vacuum chamber, which is often used for surface modification. The glass slides were first cleaned by vacuum oxygen plasma treatment for 10 min and then silanized with triethoxysilylane at 80 °C overnight. The slides were washed with ethanol and dried by compressed air. After silanization, the slides were modified with thioglycerol (3-mercaptopropylamine-1,2-diol). The cleaned slides were covered with 300 μL of thioglycerol solution and exposed to UV light (260 nm, 120 s) at an intensity of 3 mW/cm2 (OAI Model 30, San Jose). 4-Dimethylaminopypyridine (112 mg) and 250 μL of pentaenolic acid were mixed and dissolved in 90 mL of acetone.
as an esterification solution. Then, the slides were placed in the esterification solution for an overnight treatment at room temperature. The modification and esterification processes were repeated three times, resulting in a surface with abundant terminal alkenyl groups ready for patterning via the photoclick thiol-ene reaction.

The patterning was performed as follows. 1H1H2H2H2H-perfluorodecanethiol (PFDT) solution (300 μL, 10 vol %) was used to cover the slide. The surface was then exposed to UV light (260 nm, 60 s, 3 mW/cm²) through a quartz photomask (Rose Photomasken, Bergisch Gladbach, Germany). The hydrophobic boundary region was obtained after the fluorination process. Then, the slides were covered with 1.2 M cysteamine hydrochloride ethanol solution and irradiated with UV light (260 nm, 60 s, 3 mW/cm²) to functionalize the reactive spots with hydrophilic amino groups. The patterned slides were washed with ethanol and dried by compressed air for the following MoS₂ nanosheet coating.

2.4. Establishment of the MoS₂-DMA FRET Sensing Platform.

The MoS₂-DMA FRET sensing platform for a single type of viral nucleic acid detection is prepared as follows. Generally, MoS₂ nanosheets dispersed in ethanol solution were sonicated for 20 min for exfoliation. The well-dispersed nanosheets were then dialyzed with a filter membrane with a molecular weight cutoff of 3.5 kDa for at least 1 day to remove the lithium hydroxide. The dispersion solution was then sonicated for 15 min and then centrifuged at 2000 rpm for 5 min to eliminate the aggregated particles. Afterward, the nanosheet dispersion was collected and sonicated for an additional 10 min. The glass slides were cleaned by sonication in ethanol for 15 min and dried by compressed air. A suspension (100 nL) of MoS₂ nanosheets (200 μg/mL) in a mixture of ethanol and water (8:2) was printed on each hydrophilic spot by a noncontact liquid dispenser (I-DOT; Dispendix GmbH, Stuttgart, Germany), followed by a slight shaking for 5 min.

The droplets were then evaporated and kept at room temperature for 1–2 days. Then, 1.7 mL of water solution was used to clean the fabricated 14 × 14 array by gentle washing. To explore the optimal ratio of MoS₂ nanosheets and the fluorescence dye-labeled probe solution for optimal quenching efficiency, 150 nL of fluorescence dye-labeled probe solution with a series of concentration (0, 10, 20, 30, 40, 50, and 60 nM) was then printed on each spot by the I-DOT dispensing system and then incubated in a dark environment at controlled humidity for 1 h at room temperature. The DMA slide was raised and placed in a 100 mm filter membrane with a molecular weight cut-off of 100 kDa in a Petri dish with 4 mL of phosphate-buffered saline (PBS) and equipped with a wet humidifying pad in the lid to prevent evaporation during usage. The fluorescence images were then captured with excitation at 488 and 668 nm for fluorescein amidite (FAM)- and cyanine dye 5 (Cy5)-labeled probes, respectively, to check the quenching effect. Arrays for final testing of HIV-1 or HIV-2 nucleic acid detection with the maximum quenching efficiency were prepared with an optimal ratio of MoS₂ nanosheets (100 nL of suspension of 200 μg/mL) and the fluorescence dye-labeled probe solution (150 nL of 50 nM).

The MoS₂-DMA FRET sensing platform for simultaneous detection of multiple HIV nucleic acids was prepared as follows. A 4 × 4 droplet microarray is separated into an HIV-1 detection section, an HIV-2 detection section, an HIV-1 + HIV-2 detection section, and two negative control spots. The HIV-1 detection section included three testing spots and one positive control spot. The testing spot in the HIV-1 detection section was prepared by filling hydrophilic spots with 150 nL of PBS solution of FAM-labeled probes (50 nM) on the MoS₂-coated spot surfaces. The positive control spot was prepared by printing with 150 nL of PBS solution of FAM-labeled probes (50 nM) on the surface of a hydrophilic dendrimer spot without MoS₂ nanosheets. The HIV-2 detection section also included three testing spots and one positive control spot. The testing spot in the HIV-2 detection section was prepared by filling MoS₂-coated spots with 150 nL of PBS solution of Cy5-labeled probes (50 nM). The positive control spot was prepared by printing 150 nL of PBS solution of Cy5-labeled probes (50 nM) on a hydrophilic dendrimer spot without MoS₂ nanosheets. The HIV-1 + HIV-2 detection section included five testing spots and one positive control spot. The testing spot in the HIV-1 + HIV-2 detection section was prepared by printing 150 nL of PBS solution containing a mixture of FAM-labeled probes (25 nM) and Cy5-labeled probes (25 nM) onto the MoS₂-coated spot surfaces. The positive control spot was prepared by printing 150 nL of PBS solution of FAM-labeled probes (25 nM) and Cy5-labeled probes (25 nM) onto the surface of a hydrophilic dendrimer spot without MoS₂ nanosheets. The two negative spots were prepared by printing 150 nL of PBS solution on a MoS₂ coated spot. All of the printed spots were then dried for further usage.

2.5. MoS₂-DMA FRET Platform for Gene Detection.

For testing with the platform for a single type of viral nucleic acid detection, 150 nL of HIV-1 or HIV-2 target nucleic acid solutions with various concentrations of 0, 10, 20, 30, 40, 50, and 60 nM were printed on testing spots and kept at 37 °C for incubation in the dark environment for 1 h. The specificity testing was conducted using the four-base-mismatched probe sequences under the same conditions. To perform simultaneous detection of HIV-1 and HIV-2 target nucleic acids, 150 nL of sample solutions including target HIV-1 probe (50 nM), target HIV-2 probe (50 nM), four-base-mismatched HIV-1 probe (50 nM), four-base-mismatched HIV-2 probe (50 nM), HIV-1 + HIV-2 target probes (25 + 25 nM), and four-base-mismatched HIV-1 probe + four-base-mismatched HIV-2 probe (25 + 25 nM) were printed on testing spots and kept at 37 °C for incubation with 1 h in the dark environment. The fluorescence images were then captured with excitation at 488 and 668 nm for FAM- and Cy5-labeled probes, respectively. All of the
droplets were first focused to obtain the clear edges in bright-field images and then switched to the fluorescence excitation mode to take fluorescence images. For the concentration recovery test of the platform, complexed samples with different ratios of target Probe-1 to target Probe-2 (concentration ratios of 1:1, 1:2, 1:4, 4:1, and 2:1) were applied. All of the concentrations used were in the detectable range determined in this work. These complex HIV targets were added to 100-fold-diluted human serum samples and then detected with the sample protocol above. All of the concentrations used were in the detectable range determined in this work. These complex HIV targets were added to 100-fold-diluted human serum samples and then detected with the sample protocol above. All of the fluorescence images were measured and analyzed by ImageJ, and each spot average fluorescence intensity was obtained based on the defined square area.

3. RESULTS AND DISCUSSION

3.1. Mechanism of MoS2-DMA FRET Sensing Platform.

The MoS2 DMA platform fabrication process is shown in Figure 1a. Briefly, a glass slide is first silanized using triethoxyvinylsilane to modify the surface with alkene groups. Then, the surface is modified using the thiol–ene reaction with thioglycerol, followed by the esterification process with 4-pentenoic acid in a sequential two-step process to modify the surface with a layer of dendrons with a high density of terminal double bonds. After the dendrimer layer is generated, the surface is patterned by another thiol–ene reaction first with a fluorinated thiol (PFDT) to generate hydrophobic barriers and then with cysteamine to generate amino-functionalized hydrophobic spots at defined locations. Since cysteamine has a pK_{a2}(NH\textsubscript{3}) of 10.75, amino-functionalized hydrophobic spots will be protonated (NH\textsubscript{4}\textsuperscript{+}) at pH 7. The MoS\textsubscript{2} nanosheets show negative charges (Figure S1), which are then printed on the amino-functionalized hydrophilic spots to adsorb on the spots, forming the MoS\textsubscript{2}-DMA platform. The droplet microarray can be formed on the fabricated MoS\textsubscript{2}-DMA platform (Figure 1b). The platform is fabricated on a 7.5 cm × 2.5 cm glass slide with low intrinsic fluorescence. The microarray platform is divided into three arrays, and each array contains 14 × 14 hydrophilic square spots (1 mm × 1 mm), separated by 0.5 mm omniphobic barriers.

This portable DMA slide contained 588 individual micro-reservoirs filled with 150 nL of solutions for nucleic acid sensing. The fluorescence resonance energy transfer (FRET) sensing mechanism of the MoS\textsubscript{2}-DMA platform for viral nucleic acid detection is shown in Figure 1c. Fluorescein amidite (FAM)-labeled capture oligonucleotide probes of HIV-1 (capture Probe-1) and cyanine dye 5 (Cy5)-labeled capture oligonucleotide probes of HIV-2 (capture Probe-2) are absorbed onto MoS\textsubscript{2} nanosheets. MoS\textsubscript{2} nanosheets are able to adsorb nucleic acids via van der Waals force between nucleobases and the basal plane of MoS\textsubscript{2} nanosheets, which has been reported in both theoretical calculations and experiments.\textsuperscript{33,34} The fluorescence of the capture probes adsorbed on MoS\textsubscript{2} nanosheets was quenched via energy transfer from the fluorescent dye to MoS\textsubscript{2} nanosheets. When target HIV-1 nucleic acids (target Probe-1) or HIV-2 nucleic acids (target Probe-2) bind to the absorbed fluorescence-labeled capture probes on MoS\textsubscript{2} nanosheets, the hybridized double-stranded nucleic acid complex detaches from MoS\textsubscript{2} nanosheets due to weak affinity, leading to the recovery of a green fluorescence signal (FAM dye) for HIV-1 and a red fluorescence signal (Cy5 dye) for HIV-2. In hybridized nucleic acids, the nucleobases are surrounded by a dense phosphate layer, which weakens the interaction between MoS\textsubscript{2} and hybridized nucleic acids.\textsuperscript{35,36} It was shown that the adsorption energy between ssDNA and MoS\textsubscript{2} nanosheets was higher (>0.2 eV) than that for dsDNA (< 0.05 eV).\textsuperscript{37} With the addition of non-target nucleic acid, fluorescently labeled capture probes still attach to MoS\textsubscript{2} nanosheets without fluorescence signal recovery.

3.2. Establishment of the MoS\textsubscript{2}-DMA FRET Platform.

To establish the MoS\textsubscript{2}-DMA FRET platform, small MoS\textsubscript{2} nanosheets were first prepared by a sonication-assisted exfoliation approach from bulk MoS\textsubscript{2} powder. The average particle size of the MoS\textsubscript{2} powder was about 1 mm, and there was obvious deposition in the aqueous solution (Figure S2). Exfoliated MoS\textsubscript{2} showed a 2D layered structure with an average...
size of around 90 nm with good water dispersity (Figure 2a,b). MoS2 nanosheets showed a wide absorption spectral range from UV to near-infrared (NIR) in the UV–vis absorbance spectrum. The overlap of the absorption spectrum of MoS2 nanosheets with the emission spectra of FAM and Cy5 dyes could ensure the feasibility of the FRET process (Figure 2c). MoS2 nanosheets were then coated on the fabricated amino-functionalized hydrophilic spots to form a MoS2–dendrimer interface. Before coating, amino-functionalized hydrophilic spots show a smooth surface in the AFM image (Figure 2d). The cysteamine-modified hydrophilic spot surface was also characterized by Raman spectroscopy (Figure S3). The characteristic bands of cysteamine appeared at 1047, 1239, 1359, and 1420 cm⁻¹, which matched with the reference. The stability of adsorption of the nanosheets to the surface was proved by multiple washing cycles, followed by AFM imaging. The MoS2-coated spot surface showed abundant nanosheets with sizes ranging from 50 to 200 nm attached to the dendritic surfaces in the AFM image (Figure 2e), forming a stable MoS2 nanolayer with a thickness of 60–100 nm (Figure 2f).

In the case of adding aqueous solutions, small droplets were generated on the hydrophilic spots due to the difference in dewettability of the hydrophilic spots and omniphobic barriers. The geometry of the formed droplet array on cysteamine DMA and MoS2 nanosheet-coated DMA is shown in Figure 3a. The coating of MoS2 nanosheets did not change surface hydrophilicity and droplet geometry much. To test the quenching ability of MoS2-coated DMA, 150 nL of solutions of dye-labeled DNA probes was printed on the cysteamine DMA spots and MoS2 nanosheet-modified DMA spots by a noncontact dispenser and dried at 37 °C with slight shaking. The successful attachment of the fluorescence-labeled DNA probes on MoS2 nanosheets was confirmed by AFM analysis (Figure 3b). The droplets with fluorescence-labeled capture Probe-1 and capture Probe-2 (50 nM) showed strong fluorescence signals on cysteamine DMA spots but very weak fluorescence signals on MoS2 nanosheet (100 μg/mL)-coated DMA spots (Figure 3c). This demonstrated the quenching ability of MoS2 nanosheet-coated DMA on fluorescence-labeled DNA probes. However, the fluorescence signal on MoS2 nanosheet (100 μg/mL)-coated DMA spots was not fully quenched. The ratio between MoS2 nanosheets and fluorescence-labeled capture probes needs to be further adjusted to ensure a low fluorescence background before detection.

### 3.3. Optimal Quenching Efficiency of the MoS2–DMA FRET Platform

To obtain high quenching efficiency, a reasonable ratio between the FRET donor and acceptor was a prerequisite. Here, we first incubated 50 nM fluorescence-labeled capture probes in 150 nL droplets on hydrophilic spots modified with a series of MoS2 nanosheet concentrations (0–250 μg/mL) to explore the optimal ratio between fluorescence-labeled probes and MoS2 nanosheets (Figure 4a). Upon an increase of concentrations of MoS2 nanosheets printed on the spot surface, the fluorescence signals were gradually decreased for both capture Probe-1 and capture Probe-2 (Figure 4b,c). The quantitative analysis showed that the fluorescence intensity of spots decreased with an increase of concentration of MoS2 nanosheets and reached the lowest value at around 200 μg/mL MoS2 nanosheets for 50 nM concentration of fluorescence-labeled DNA probes (Figure S4). The quenching efficiency (Qₑ) was calculated by the equation $Qₑ = (F₀ − Fₚ)/F₀$, where the

![Figure 3](https://example.com/figure3.png)

**Figure 3.** (a) Geometries of droplets (150 nL) formed by the noncontact liquid dispenser on the cysteamine-modified (left) and MoS2 nanosheet-coated (right) spots. (b) Scheme of printing fluorescence-labeled capture probes on the MoS2 nanosheet-coated spot surface to form a FRET sensing system and AFM image of fluorescence-labeled capture probes adsorbed on the MoS2 nanosheet-coated spot surface. (c) Fluorescence images of labeled capture probes on cysteamine DMA and MoS2 nanosheet-coated DMA. The scale bar in the above images is 1 mm.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** (a) Scheme of the modulating ratio between fluorescence-labeled capture probes and MoS2 nanosheets to achieve an optimal quenching efficiency. Fluorescence-labeled probes (50 nM) incubated in 150 nL droplets on hydrophilic spots modified with MoS2 nanosheets with different concentrations (0–250 μg/mL) in 100 nL droplets. (b) Fluorescence images of 150 nL of FAM-labeled capture Probe-1 (50 nM) in various concentrations of MoS2 nanosheet-coated hydrophilic spots. (c) Fluorescence images of 150 nL of Cy5-labeled capture Probe-2 (50 nM) in various concentrations of MoS2 nanosheet-coated hydrophilic spots. (d) Quenching efficiency of the two fluorescence-labeled probes, capture Probe-1 and capture Probe-2, in spots modified with solutions of MoS2 nanosheets of variable concentrations.
original fluorescence intensity of the fluorescence-labeled capture probes on the hydrophilic spots without MoS2 was defined as \( F_0 \) and the fluorescence intensity of the dye-labeled probes on the MoS2-coated spots was defined as \( F_r \). It was observed that the optimal ratio of MoS2 nanosheets (100 nL of 200 \( \mu \)g/mL) and fluorescence-labeled capture probes (150 nL of 50 nM) could reach a high quenching efficiency of more than 95%, which was used for further sensing experiments (Figure 4d). The quenching capability of MoS2 nanosheets (100 nL of 200 \( \mu \)g/mL) against FAM-labeled capture Probe-1 and Cy5-labeled capture Probe-2 (150 nL of 50 nM) was also confirmed by fluorescence spectroscopy measurements in solution (Figure S5). This MoS2-DMA sensing platform showed good stability and the quenching efficiency for both capture Probe-1 and capture Probe-2 kept a high value (>90%) during 1 h, which covered the whole detection period (Figure S6).  

3.4. Detection of HIV-1 and HIV-2 Nucleic Acids via the MoS2-DMA FRET Platform. The established MoS2-DMA sensing platform was then tested for the detection of a single type of HIV-1 or HIV-2 single-stranded target nucleic acids. In practical applications, one more step is needed to separate the type of HIV-1 or HIV-2 single-stranded target nucleic acids. In this work, we developed a MoS2-DMA FRET sensing platform for the detection of HIV-1 and HIV-2 single-stranded target nucleic acids. The LODs for target Probe-1 and target Probe-2 with the fluorescence microscopy images analysis were calculated to be 1.24 and 1.26 nM, respectively. To improve the sensitivity of our platform, a miniaturized photodetector was put close to the backside of the glass substrate of our platform in a closed dark chamber to measure the fluorescence signals. It was observed that the LOD could be further improved to 50 pM for both HIV-1 and HIV-2 target probes (Figure S8). Our current platform used an amplification-free sensing approach. With the adoption of an amplification step in the droplet such as isothermal amplification, it is expected that the sensitivity could be further improved in the future.

Figure 5. (a) Fluorescence intensity of the MoS2-DMA FRET sensing platform in the presence of target Probe-1 with a concentration range from 10 to 50 nM. (b) Fluorescence intensity of the MoS2-DMA FRET sensing platform in the presence of target Probe-2 with a concentration range from 10 to 50 nM. (c) Quantitative analysis of the fluorescence intensity of the sensing spots and concentrations of target probes. (d) Diagram of the relative fluorescence recovery rate change with the logarithmic concentration of target Probe-1. (e) Diagram of the relative fluorescence recovery rate change with the logarithmic concentration of target Probe-2. (f) Specificity testing of the MoS2-DMA FRET sensing platform for HIV-1 and HIV-2 target probes using the four-base-mismatched probes, HBV target probes, and HCV target probes as controls.
The response time of this MoS2-DMA FRET sensing platform for HIV nucleic acid detection was around 60 min (Figure S9). To determine the specificity of the MoS2-DMA FRET sensing platform for the detection of HIV nucleic acids, different groups of nucleic acids, including target Probe-1, target Probe-2, four-base-mismatched HIV-1 probes, four-base-mismatched HIV-2 probes, target probes of hepatitis B virus (HBV), and target probes of hepatitis C virus genotype 1b (HCV), were used for sensing with the same condition (150 nL at 50 nM). The recovered fluorescence signals of target Probe-1 and target Probe-2 were 6.10 and 5.63 times that of the mismatched probes and 5.04 to 5.85 times those of HBV and HCV target probes, respectively (Figure 6a).

For simultaneous detection of dual HIV nucleic acids, a 4 × 4 MoS2-coated droplet microarray was prepared. This sensing platform is composed of an HIV-1 detection section, an HIV-2 detection section, an HIV-1 + HIV-2 detection section, and a negative control section (Figure 6a). In the HIV-1 detection section, 50 nM target Probe-1 led to the obvious green fluorescence signal recovery. In contrast, 50 nM four-base-mismatched HIV-1 probes and PBS solution did not have obvious recovery fluorescence signals (Figure 6b). Similar results were also observed for the HIV-2 detection section. Target Probe-2 (50 nM) led to obvious red fluorescence signal recovery. Four-base-mismatched HIV-2 probes and PBS solution did not lead to the recovery of the fluorescence signal. In the HIV-1 + HIV-2 detection section, the mixed sample of target Probe-1 (25 nM) and target Probe-2 (25 nM) led to the appearance of yellow fluorescence, indicating the capability of simultaneous detection of different genes in the same sample, in our example both HIV-1 and HIV-2 nucleic acids. In contrast, target Probe-1 (25 nM) only leads to green fluorescence signal change and target Probe-2 (25 nM) only leads to red fluorescence signal change, which indicated the capability of this sensing platform to differentiate HIV-1 and HIV-2 nucleic acids. Moreover, the mixed sample of base-mismatched HIV-1 probes (25 nM) and four-base-mismatched HIV-2 probes (25 nM) did not lead to either green or red fluorescence signal recovery, which demonstrated the specificity of this sensing platform. We also proved the repeatability of the test results in six independent experiments (Figure 6c).

In practical applications, it is very important to apply simple facility-free methods for rapid sample delivery. We developed a simple “brushing droplet” method to quickly generate hundreds of separated sample droplets by brushing bulk aqueous sample solution on our MoS2-DMA platform. The MoS2-DMA platform is composed of micropatterns of hydrophilic square spots separated by hydrophobic borders. MoS2 nanosheets with various capture oligos have been modified on the hydrophilic spots. When the bulk sample solution was brushed on the surface of the microarray surface, multiple and separated microdroplets of samples will be quickly generated due to the effect of discontinuous dewetting (refer to the Video S1 in the Supporting Information).

To explore the ability of the MoS2-DMA biosensing platform to quantitatively detect mixed HIVs’ target probes in complex samples, a variety of mixed molar ratios (1:1, 1:2, 1:4, 4:1, and 2:1; 1 represents 10 nM) of HIV-1 and HIV-2 target probes are spiked in human serum solution to simulate real clinical samples. First, calibration was performed with a series of concentrations of HIV-1 target probes and HIV-2 target probes to correlate the intensity of green and red recovered fluorescence signals with HIV-1 target probes and HIV-2 target probes, respectively (Figure S10a). Then, samples with various molar ratios of HIV-1 and HIV-2 target probes were then added to the HIV-1 + HIV-2 detection section of the platform for detection. It was observed that the ratio of detected green fluorescence signal intensity to red fluorescence signal intensity matched well with the molar ratio of HIV-1 target probes to HIV-2 target probes (Figure S10b). Based on the calibration curves, the detected concentrations of HIV-1 target probes and HIV-2 target probes in the complex samples could be calculated, which also matched well with the real concentrations of HIV-1 target probes and HIV-2 target probes (Table S1). The above results demonstrated that it was possible to detect concentrations of multiple HIVs in a complex sample by analyzing the fluorescence signal ratio. This MoS2-DMA biosensing platform has the potential for simultaneous detection of multiple HIVs quantitatively in a complex clinical sample.

The multiplex capability of the MoS2-DMA platform is based on the combination of two multiplex approaches including multicolor fluorescence probes for the detection of various viral genes of the same virus and spatially dividing the array into various sensing sections for different viruses. With the combination of these two approaches, the total number of genes of different viruses could be $N \times M$, where $N$ is the number of fluorescence colors and $M$ is the number of sensing sections. For example, if 4 color fluorescence probes and 20 sensing
sections are applied in the droplet array, the multiplex capacity of viral genes can reach 80. As a demonstration, we performed the experiments for five genes from three viruses including HIV-1 gene and HIV-2 gene for HIV, ORFlab and N genes of SARS-COV-2 for COVID-19 infection, and M gene of Influenza A with various concentrations (0.5, 1, 2, 5, and 10 nM) (Figure 7). The capture and target probes information are included in Table S2. Here, two fluorescence colors and three sensing sections were used. It was clearly observed that this MoS₂-DMA platform could detect all of the five genes with concentration-dependent fluorescence signals (Figure 7). As shown in Figure 1b, we have already fabricated the MoS₂-DMA platform with 588 droplets on a glass substrate. Using this combinational multiplex approach, our MoS₂-DMA platform is easy to be adapted for the detection of more genes.

4. CONCLUSIONS
In this work, a novel MoS₂-DMA FRET sensing platform was developed for the detection of multiple HIV retroviral nucleic acids based on the combination of the droplet microarray platform and MoS₂ nanosheet-based FRET nanoprobes. MoS₂ nanosheets were used to coat hydrophilic dendrimer-modified spots separated by a hydrophobic background as quenchers, and down to 150 nL droplets containing fluorescence-labeled capture probe DNAs were then deposited on each MoS₂-coated spot as donors. The Off status of the sensor array was formed due to energy transfer from absorbed fluorescence-labeled capture probe DNAs to MoS₂ nanosheets. In the presence of target viral (HIV-1 or HIV-2) nucleic acids, fluorescence-labeled sensing probes were detached from MoS₂ nanosheets coated on the hydrophilic spots, leading to a fluorescence signal recovery with the “on” status. As a proof of concept, this sensing platform has been successfully used for the detection of single type HIV-1 or HIV-2 nucleic acids with various concentrations, as well as simultaneous detection of both HIV-1 and HIV-2 nucleic acids with rapid response, good sensitivity, and good specificity. This MoS₂-DMA FRET sensing platform successfully transferred the solution-based FRET assay to a substrate-based microarray sensing platform with advantages of low sample consumption, parallel detection, as well as spatial separation between the sensor microspots, which provides the potential for parallel multiplexed detection of various viral and bacterial pathogens in the future.

Figure 7. MoS₂-DMA FRET sensing platform for the detection of five gene target probes of different viruses.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsami.0c16146.

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Notes
The authors declare no competing financial interest.

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**ABBREVIATIONS**

FRET, fluorescence resonance energy transfer  
MoS₂, molybdenum disulfide  
HIV, human immunodeficiency virus  
DNA, droplet microarray

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