CHAPTER – 3
MoS$_2$ NANOSHEETS BASED DESIGN OF PATHOGEN DETECTION SYSTEM

3.1 Overview

The monitoring of food and water for the infectious disease-causing organism is an important issue for ensuring its safe consumption for humans. Several infectious diseases occur through ingestion of contaminated water, and the cases of food and water borne illnesses are highest in developing nations. Water quality evaluation and the rapid diagnostics of pathogenic microorganisms remain a great challenge to ensure maintenance of public health and for prevention of bio-terrorism [1].

2-D inorganic nano transition metal dichalcogenides (TMDs) have attracted attention due to their widespread applications in the field of catalysis, energy storage and electronic devices [2]. Particularly, Molybdenum disulphide nanosheets (MoS$_2$-Ns) aggrandize TMD’s due to its exemplary electronic, optoelectronic and energy harvesting properties [3]–[6]. Recently, some new features of single layer MoS$_2$–Ns such as its fluorescence quenching properties have gained good popularity and opened new opportunities for exploiting these materials. Besides fluorescence quenching, MoS$_2$-Ns surface is also known to bind ssDNA molecules via van der waals forces of attraction between nucleobases and the basal planes of MoS$_2$-Ns [7]. In the case of ssDNA aptamer, its binding to target molecule induces rigid structure, effectively preventing interactions of nucleobases with MoS$_2$-Ns surface [8]. This makes aptamer-MoS$_2$-Ns interactions weak and thus results in restoration of quenched fluorescence.

3.2 Design of pathogen detection system

Herein in this chapter on the basis of above mentioned thought, the design of new pathogen detection system is reported. The system relies on fluorescence response of fluorescein tagged aptamer (Apt-FAM) probe, in which MoS$_2$-Ns are used for detecting the presence of a pathogen. We utilize Salmonella typhimurium as a model pathogen for sensing platform. The system workflow is very simple, just addition of Apt-FAM probe and water sample followed by MoS$_2$-Ns before answers can be read using a standard fluorescence detector. In the presence of target pathogen, the Apt-FAM probe selectively
binds with the pathogen, which fluoresces and then MoS$_2$-Ns is added. The Apt-FAM probe system weekly interacts with MoS$_2$-Ns and thus shows minimal quenching. However in the absence of a selective pathogen, the free nucleobases of Apt-FAM probe get stacked on MoS$_2$-Ns through van der waals interactions and fluorescence quenches. Figure 3.1 briefly illustrates the operational mechanism of biosensor.

**Figure 3.1** – Schematic illustration of MoS$_2$-Ns based bioassay for detection of Salmonella Typhimurium.

### 3.3 Experimental protocols

#### 3.3.1 Reagents used

Molybdenum disulphide, poly-D-lysine, Ammonium sulphate, EDTA, PBS buffer reagents (KCL, NaCl, KH$_2$PO$_4$, Na$_2$HPO$_4$) were purchased from Sigma Aldrich. Maxisorp flat and round bottom polystyrene microtiter black plates were products of Nunc, Thermo Scientific (Denmark). Goat anti-rabbit secondary antibody HRP labeled from Merck genei, Bangalore, India. Polyclonal antibodies against Salmonella typhimurium was produced in-house (details provided in Supplementary information). Water used for making buffers and MoS$_2$-Ns synthesis was purified with Milli-Q ultra pure system (Millipore, India) having a resistivity $\geq$ 18 M$\Omega$.cm. Biotek synergy h1 hybrid plate reader was used throughout for fluorescence studies.
3.3.2 Aptamer-FAM probe

Aptamer specific to target pathogen (Salmonella typhimurium cells) was identified from literature [9] and got synthesized from Sigma. The sequence of Apt-FAM probe is

\[(5^-\text{FAM-ATAGGAGTCACGACGACCAGAAA GTAATGCCCGGTAGTTATTCAAAGATGAGTAGGAAATGATATGTGCGTCTACCTCTTTGACTAAT-3^\prime})\].

The Apt-FAM probe was reconstituted in double distilled water and stock was prepared. Further dilution was prepared in 1X PBS using the stock.

3.3.3 Pathogenic strains and their preparation

Salmonella Typhimurium, Escherichia coli and Proteus vulgaris were procured from in house MTCC, IMTECH Chandigarh, India with strain number 3232, 6198, 744 respectively. Strains of pathogens were taken from MTCC in a lyophilized form. It was mixed with few microliters of nutrient broth and spread on a nutrient agar plate. Colony was taken and again streaked on another plate. From the streaked plate single colony was picked up and inoculated in 100 ml nutrient broth media and kept overnight at 37°C in incubator shaker. 0.1% of primary culture was inoculated in nutrient broth and kept overnight at 37°C in incubator shaker. Culture used for the experiment was of 1 O.D. (10^9 CFU/ml) and further dilution was made using the same

3.3.4 Synthesis of MoS₂-Ns

MoS₂-Ns for pathogen sensing were prepared according to mixed-solvent exfoliation method with little modifications [10]. Briefly, 35 mg of MoS₂ powder was added to 25 ml of ethanol-water (45% v/v) solution. The mixture was sonicated for 7 h, and then dispersion was centrifuged at 5000 rpm for 30 min. The colloidal supernatant was collected and was characterized by TEM and UV-Vis spectroscopy.

3.3.5 Concentration optimization and specificity check of Apt-FAM probe

For concentration optimization of Apt-FAM probe, different concentrations ranging from 20 nM–100 nM of Apt-FAM probe were used. Salmonella typhimurium (10^9 CFU/ml) was coated on a black well plate that was initially coated with 100 µg/ml poly-D-Lysine, overnight at 4 °C. The plate was then incubated for 2 h at 37 °C. After
washing with 1X PBS, different concentrations of Apt-FAM probe was added and incubated for 30 min. Fluorescence intensity was then recorded 494 nm (excitation) and 525 nm (emission) after multiple washings to remove unbounded Apt-FAM probe.

For specificity analysis, different pathogenic strains at the concentration of $10^5$ CFU/ml were incubated and optimized concentration of Apt-FAM probe was used for the checking the specificity. Pathogenic strains were coated on the black well microtitre plate, which was initially coated with 100 µg·ml$^{-1}$, poly-D-Lysine. Different pathogenic strains were then added and incubated for 2 h at 37 ºC. After washing with 1X PBS, Apt-FAM probe was added and incubated for 30 min.

3.3.6 Fluorescence quenching of APT-FAM probe and pathogen detection by MoS$_2$-Ns

In order to investigate fluorescence quenching behaviour of MoS$_2$-Ns, different concentration ranging from 3 µg/ml to 18 µg/ml of Ns (100 µl) were incubated with 50 µl of Apt-FAM probe for 5 min at 37 ºC. The optimum concentration of MoS$_2$-Ns which show maximum quenching was used for the detection of pathogens. Different concentrations of Salmonella typhimurium $10^8$ - $10^5$ CFU/ml (50 µl) prepared in De-Ionized water were incubated with 50 µl of the optimized concentration of Apt-FAM probe in PBS buffer pH 7.4 for 30 min at 37 ºC in a black well plate. Then, 100 µl of the optimized concentration of as prepared MoS$_2$-Ns was added followed by incubation for 5 min. The fluorescence readings were obtained using excitation wavelength of 494 nm and an Emission of 519 nm. The same protocol was used for different pathogenic strains in order to justify selectivity and specificity of biosensor.

3.4 Results and Discussions

3.4.1 Characterization of MoS$_2$-Ns

Figure 3.2 shows UV-Vis spectrum and TEM micrographs of as synthesized MoS$_2$-Ns. UV-Vis spectra obtained using Shimazdu UV-1800 spectrophotometer of colloidal suspension showed absorption peaks around 612 nm and 672 nm that can be attributed to characteristic A and B direct excitonic transitions, with energy split from valence band spin orbital coupling. These results are in agreement with previously reported results and indicate the presence of dispersed layered MoS$_2$-Ns [11]. High resolution transmission electron microscopy Hitachi (H-7500) was carried out to
characterize the surface morphology of the exfoliated MoS$_2$-Ns that clearly shows the formation of monolayer sheets during the process.

**Figure 3.2** – (a) UV-Vis spectroscopy of MoS$_2$-Ns depicts characteristic peaks at 614 and 674 nm. High resolution TEM image of MoS$_2$-Ns (inset showing photograph of as-prepared MoS$_2$-Ns).

To fabricate a method using MoS$_2$-Ns we first optimize the concentration of FAM labelled aptamer probe by coating a fixed concentration of Salmonella Typhimurium cells and varying the concentration of aptamer. An optimum 50 nM concentration of the aptamer was used in our experiments as it gives sufficient fluorescence window for pathogen detection as shown Figure 3.3 (a).

**Figure 3.3** – (a) Fluorescence intensity of different concentrations of Apt-FAM probe with $10^9$ CFU/ml Salmonella Typhimurium (b) Apt-FAM probe fluorescence quenches upon incubation with different concentration of MoS$_2$-Ns.
Initially, in order to prevent precipitation of aptamer molecules by ethanol present in colloidal MoS$_2$ supernatant, the solution was kept in hot air oven for 2-3 h at 60°C and afterwards volume makeup was carried out by using deionized water (resistivity $\approx 18$ M$\Omega$/cm$^2$).

Figure 3.3 (b) shows the fluorescence quenching of 50 nM Apt-FAM probe at different concentrations of MoS$_2$-Ns. An increase in concentration of MoS$_2$-Ns provides a decrease in fluorescence intensity, and quenching up to 82% was observed at 18 µg/ml. This quenching mechanism can be explained by weak van der Waals forces between nucleobases of Apt-FAM probe and surface of MoS$_2$-Ns, which dominates on increasing MoS$_2$-Ns concentration [12]. As the Apt-FAM probe approaches near the surface of MoS$_2$-Ns, hairpin structure of Apt-FAM probe tends to open, and nucleobases get stacked over the surface of MoS$_2$-Ns. As reported earlier [13], transition metal ions possess intrinsic fluorescent quenching properties for organic dye molecules. The Apt-FAM probe conformation brings organic fluorescent dye molecule and MoS$_2$-Ns more close to each other, and thus quenching the fluorescence. Thus, it is clear from above discussion that MoS$_2$-Ns act as an effective fluorescence quencher for Apt-FAM probe.

Figure 3.4 (a) shows the response of the biosensing system to the increasing concentration of pathogens in water samples. The fluorescence was found to restore by approximately 74% to 84% at a concentration of 10 CFU/ml to $10^8$ CFU/ml respectively. This fluorescence increase is due to the strong affinity of Apt-FAM probe with surface moieties of pathogen cell in comparison to weak interactions between MoS$_2$-Ns and Apt-FAM probe. As the Apt-FAM probe binds to the cell surface moieties the distance between organic dye molecule and MoS$_2$-Ns surface increases leading to increasing fluorescence. Interestingly, the biosensor does not show a clear linear response as the concentration of pathogen increases. On the other hand, it shows digital on/off response as it detects the presence of a pathogen.
3.4.2 Detection ability of developed technique

To demonstrate the specificity of biosensor for a single pathogen, we employed other commonly present pathogens in water as negative controls. Typically Escherichia coli, Proteus vulgaris were used as non-target pathogens at a concentration of $10^5$ and $10^7$ CFU/ml. As shown in Figure 3.4 (b) the biosensor does not show any response to the presence of these non-target pathogens and the response was similar to that of blank sample. The results clearly demonstrate that the assay detects Salmonella Typhimurium with high specificity and the system is not affected by the presence of any other pathogen.

We also observe that the addition of different levels of cells to bare Apt-FAM probe did not lead to any changes in the fluorescence intensity thus ruling out any influence of opacity (Figure 3.5 a). Moreover, in order to simulate real time conditions the experiment was also performed using tap water and skim milk. The assay shows a good response in both conditions as show in Figure 3.5 b. Thus, MoS$_2$-Ns based assay shows high selectivity and promising detection ability in the complex matrix also with a digital like response.

**Figure 3.4** – (a) Response of the system to different concentrations of Salmonella Typhimurium. The error bar correspond to three independent experiments ($n=3$). Line of proposed LOD is given by $(x + 3\sigma)$ rule. (b) Color coded pictures for the area spectral scans of the microwells showing visible change in fluorescence intensity at the surface. (c) Fluorescence intensity of Apt-FAM probe towards different pathogens.
Figure 3.5 – (a) Fluorescence intensity of Apt-FAM probe incubated with different pathogen concentrations. (b) Fluorescence intensity response of Apt-FAM probe with and without the presence of target pathogens in both skim milk and tap water.

Furthermore, to understand digital response experiments performed with MoS$_2$-Ns and Salmonella Typhimurium cells using a specific antibody probe that showed to us the interaction of MoS$_2$-Ns with pathogenic cells (Figure 3.6). This particular observation could explain the digital like response since the pathogen bound with Apt-FAM probe is free to interact with MoS$_2$-Ns leading to the quenching of fluorescence intensity that ideally should increase with an increase in pathogen concentration. At a first glance, this seems to be a weakness of these kinds of a system that does not exactly predict the concentration of pathogens but its ease of usage and simple workflow makes it fit as a platform for a qualitative analytical device with a possibility to detect the occurrence of harmful disease-causing microbes at a very low concentrations. Its sensitivity and time took to obtain results rivals many of the reported systems for Salmonella detection that are mainly based on calorimetric detection [14], electrochemical impedance immunoassay[15], chemiluminescent immunoassay [16] and quantum dot fluorescence detection [17]. Moreover, the reported system shows a much lower limit of detection for Salmonella typhimurium as compared to similar graphene oxide detection system [18].
Fig 3.6 – Graphical representation of MoS$_2$ Ns interaction with comparison to that of poly-D-lysine coated plate. Graph clearly shows binding of MoS$_2$ Ns with Salmonella cells.

3.5 Conclusion

A digital MoS$_2$-Ns based assay for the detection of harmful pathogen Salmonella Typhimurium at very low concentrations was developed. MoS$_2$-Ns were synthesized by an easy mix solvent method and then used for effective quenching of Apt-FAM probe. In the presence of a target pathogen, Apt-FAM probe induced a rigid conformation with pathogen and thus resulting in restoration of fluorescence intensity. The proposed technique shows a digital on/off response to the presence of target pathogen. The method also depicts an excellent sensitivity and selectivity to target pathogen up to a concentration of 10 CFU/ml. Main advantages of the assay include opportunities to develop rapid, simple, low cost technologies that can be conveniently executed using a handheld fluorescence detector for pathogen detection.
3.6 References


