Label-free optical biosensing as an alternative for HIV-1 drug-resistant mutation detection

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Abstract. Collectively, conventional assays for HIV-1 drug-resistant mutation detection have established the basis for understanding the mechanisms involved in drug-resistant mutations and have thus led to the development of antiretroviral therapy (ART) regimen that targets and suppress drug-resistant variants. Assays like Sanger sequencing, Next Generation Sequencing (NGS), and Polymerase Chain Reaction (PCR) are considered gold standards for HIV-1 drug-resistant mutation detection and have since been used to inform global guidelines, particularly the World Health Organization (WHO) resistance monitoring protocols. However, their significant impact has been hindered by high costs, turnaround time, speed, accessibility, complexity, and multiplexing. Hence, this study uses optical biosensors based on localized surface plasmon resonance spectroscopy as an alternative drug-resistant detection technique to counter some of the conventional assay shortfalls. Optical biosensors offer cost effectiveness, simple and potential for point-of-care development, which is vital particularly in resource-limited settings. In this study, localized surface plasmon resonance was successfully optimized for HIV-1 drug-resistant mutation detection.

1. Introduction

It has been more than four decades since the identification of human immunodeficiency virus (HIV-1), a causative agent of the acquired immunodeficiency syndrome (AIDS) and the first case was reported back in the year 1981 [1]. According to a report by UNAIDS/WHO, about 38.4 million individuals worldwide have HIV/AIDS (Ghosh, 2023). Also, about 40.1 million people have died because of HIV/AIDS since it started [2]. In the year 1991, the World Health Organization (WHO) predicted that 40 million people would be infected globally by year 2000 [3]. Since the 1980s, the fight against HIV has seen major progress with early efforts focused on community involvement through pleading for support, which resulted in making treatment affordable [4]. The introduction of highly active antiretroviral therapy (HAART) in the mid-90s revolutionized treatment by combining drugs to target the virus at multiple stages [5]. New drug classes emerged, and antiretroviral therapy (ART) proved highly effective in suppressing the virus [2]. By 2020, an estimated 87% of HIV-positive individuals aware of their status were receiving ART, with 90% of those on treatment achieving viral suppression [1]. However, challenges like limited access, stigma, and drug resistance remain counterproductive in the detection and subsequent mitigation of the spread of the virus. The emergence of drug-resistant mutations poses a significant and ongoing challenge to the efficacy of antiretroviral therapy (ART) [5]. Drug resistance occurs when the virus mutates, rendering antiretroviral drugs less effective in suppressing HIV replication. This can lead to virologic failure, where the virus remains detectable despite

treatment, increasing the risk of HIV-related illness, transmission, and the need for regimen adjustments [6]. Strategies such as regular monitoring of viral load and resistance testing are crucial for early detection and management of virologic failure. Combination therapies targeting multiple stages of the viral life cycle are being explored to prevent drug resistance and optimize treatment outcomes [6, 7]. Currently, drug-resistance mutations are detected through phenotypic assays and genotypic assays, including Sanger, next-generation sequencing, and Polymerase Chain Reaction (PCR), among other molecular assays. However, their significant impact has been hindered by high costs, turnaround time, speed, accessibility, complexity, and multiplexing.

This study proposes the use of label-free optical biosensors as an alternative for HIV-1 drug-resistant mutation detection. Optical biosensors are tools used to detect biological molecules. With advantages such as sensitivity, real-time monitoring, specificity, multiplexing capabilities, and not requiring biological labels for signal production. Optical biosensors can detect subtle concentrations of analytes, monitor interactions in real-time, provide specific detection of target molecules, and simultaneously detect multiple analytes in a single sample. Two key types of optical biosensors are Surface Plasmon Resonance (SPR) and Localized Surface Plasmon Resonance (LSPR). SPR occurs when light interacts with a metal surface, detecting shifts in resonance due to biomolecular interactions. LSPR involves the excitation of localized surface plasmons in metallic nanoparticles, providing enhanced sensitivity for detecting subtle biomolecular interactions [8]. This study employs LSPR for HIV-1 drug-resistant mutation detection and was optimized for this application.

2. Methods and Materials

2.1 Materials

The citrate stabilized gold nanoparticles (AuNPs) used in this study were purchased at Cytodiagnostics (Burlington, Canada). Phosphate buffered saline (PBS), nuclease-free water (H_2O), Biotin, Neutravidin, and Acetone were all purchased at Inqaba, South Africa. The (3-Aminopropyl) triethoxysilane (APTES), Polyethylene glycol (PEG), 1-Ethyl-3-diaminopropyl carbodiimide (EDC), N-hydroxysuccinimide (NHS) were purchased at Thermo-Fisher Scientific, South Africa. Isopropanol and absolute ethanol were used in this experimental approach.

2.2 Cleaning the slides and APTES incubation

The glass slides were cleaned via sonication in a glass Coplin staining jar of acetone, isopropanol, ethanol, and water for 5 minutes, respectively. The glass slides were dried with nitrogen (N2) gas, then they were dry baked on a hot plate (80 °C) for 10 minutes. To increase the surface energy and adhesion strength, the slides were exposed to O_2 plasma treatment. The slides were then incubated in a glass Coplin staining jar with APTES for 3 hours before washing them in glass Coplin staining jars containing toluene, methanol, ethanol, and water for 5 minutes, respectively. The glass slides were dried with N_2 gas and were ready to use immediately.

2.3 Conjugation of Gold Nanoparticles with PEG

To functionalize gold nanoparticles (AuNPs) with polyethylene glycol (PEG), prevent non-specific binding, and improve stability, 700 µl of AuNPs (60 nm) were centrifuged at 0.5 g for 30 minutes. After 30 minutes, the supernatant was decanted, and the absorbance of the AuNPs was screened through the UV-Vis spectrometer before diluting them with nuclease-free H_2O. Diluted AuNPs were conjugated with 0.2 mM of PEG to form a covalent (-SH-Au) bond and incubated for 3 hours. The unbound material were centrifuged at 0.5 g for 15 minutes to separate the PEGylated AuNPs. Then the supernatant was removed and AuNPs were resuspended in fresh PBS. To activate the PEGylated AuNPs with NHS and EDC for covalent immobilization, an NHS/EDC solution was prepared by dissolving EDC and NHS with concentrations of 5 mg/ml in dH_2O water. Thereafter, the NHS/EDC mixture was mixed well with PEGylated AuNPs at room temperature for 1 hour in an orbital shaker. Following 1 hour incubation, the solution was centrifuged at 0.5 g for 15 minutes to pellet AuNPs and the supernatant decanted, and the AuNPs were resuspended in fresh PBS.

2.4 Neutravidin Immobilization

To immobilize Neutravidin onto NHS/EDC-activated AuNPs, Neutravidin solution was added to the NHS/EDC-activated AuNPs. The solution was incubated at room temperature on an orbital shaker for 2 hours. The solution was centrifuged for 15 minutes to remove unbound Neutravidin, and the pellet was resuspended in fresh PBS. To conjugate the biotin-labelled probe to neutravidin-functionalized AuNPs, the biotinylated probe was diluted to 5 µg/mL in PBS. Then, the biotinylated capture probe solution was added to the Neutravidin-functionalized AuNPs in a 1:10 ratio. The mixture was incubated at room temperature on an orbital shaker for 2 hours. Post 2 hours of incubation, the mixture was centrifuged at 0.5 g for 15 minutes, and the pellet was resuspended in PBS and ready

for hybridization experiments.

3. Results and Discussion

Gold nanoparticles (AuNPs) of three different sizes, 50 nm, 60 nm, and 90 nm were characterized for their absorption peak wavelengths. The screening of the peak wavelengths for each size helped in selecting the most suitable size for this study. The chosen geometry would enable us to evaluate the LSPR peak wavelength shift sensitivity with the highest absorbance intensity for downstream experimental procedures. As shown in Figure 1, the UV-Vis spectra indicates that 60 nm AuNPs have the highest absorption, and the selection is further supported by Mayer & Hafner (2011) [9], who note that 60 nm AuNPs are considered ideal for balancing peak sharpness, as also shown in Figure 1. The 90 nm exhibits 0.372, the lowest absorbance intensity and a wavelength peak at 640 nm, 60 nm exhibits 0.659, the highest absorbance intensity at 580 nm, and finally the 50 nm exhibits 0.478 absorbance intensity at 577 nm.

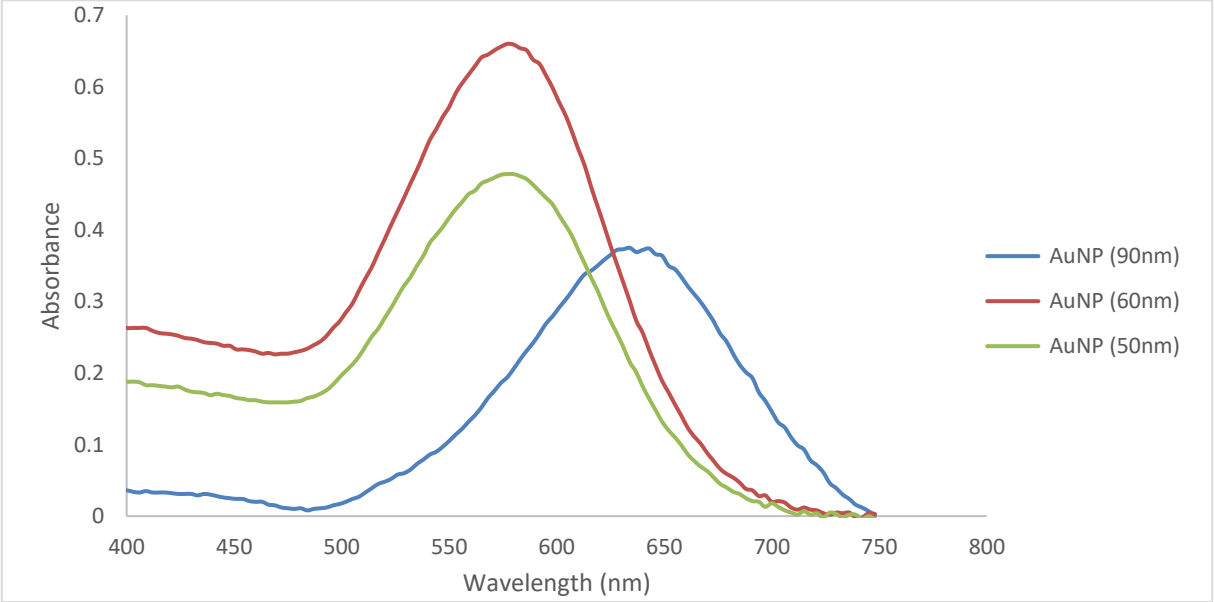

Figure 1: The UV-Vis spectra of gold nanoparticles of 50 nm, 60 nm, and 90 nm sizes screened on a UV-Vis Nanodrop 2000 spectrometer.

The above Figure 1 indicates a well-documented behavior of gold nanoparticles, as the AuNPs size increases, the LSPR wavelength peak shifts to longer wavelengths, described as a red shift [10].The red shift increase is due to the oscillation electron damping and the plasmon modes multipolarity in larger AuNPs [11]. The 60 nm AuNPs exhibit a distinct absorbance peak and thus were chosen for the downstream hybridization experiments for stable LSPR sensitivity. For hybridization experiments, 60 nm AuNPs were thus used and functionalized followed by immobilization of capture DNA probe and target DNA sequences. The capture DNA probe is made up of 24 bp and targets DNA in this study were a synthetic oligonucleotide of 90 bps. To mimic the clinical DNA, three oligonucleotides were designed which include sequences that do not possess the mutation of concern. In this study, the mutation of concern was M184V (-ATG to – GTG), and thus were looking at a single nucleotide polymorphism. A DNA capture probe included a CAT codon to directly hybridize with the wild-type codon, which acts as a positive control. The same capture probe would hybridize with the mutation oligonucleotide to form a partial duplex with a single mismatch. This allowed us to assess the sensitivity of our LSPR system in detecting small interactions and their alterations. The non-complementary DNA oligonucleotide included random codons (-TTT) which were acting as our negative control. The LSPR system has been previously optimized for the limit of detection, which was 0.003125 mg/ml of neutravidin and biotin capture probe binding [12].

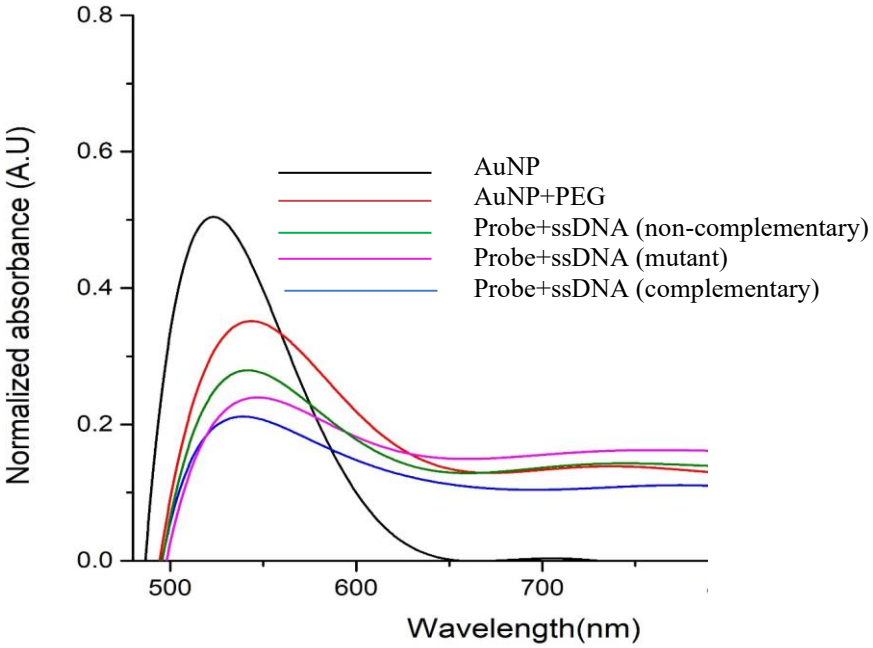

Figure 2: The normalized absorbance intensities of gold nanoparticles (AuNP) only, gold nanoparticles stabilized in PEG (AuNP+PEG), capture probe hybridized to a non-complementary single-stranded DNA (Probe+ssDNA) as a negative control, capture probe hybridized to a partial complementary ssDNA (mutant), and capture probe hybridized to a complementary ssDNA as a positive control.

Partial hybridization includes a single base change in the DNA sequence to mimic a mutation. The absorbance intensities decreased from those of AuNP alone, which has the highest absorbance, to the hybridization of the capture probe and complementary DNA, which has the lowest absorbance intensity. LSPR peak wavelength shifts, from AuNP alone and red shifts induced by refractive index changes through the hybridization of the capture probe and target analytes on the nanoparticle surface are observed in Figure 2.

The introduction of PEG sterically stabilizes gold nanoparticles, preventing aggregation and causing a slight decrease in absorbance intensity, as observed in Figure 2 above. This decrease can also be attributed to reduced particle-to-particle interactions as a result of the PEG molecule (5000 Da). DNA hybridization typically results in minimal refractive index changes, generally around 1.4 [13], which is slightly higher than water and close to glycerol[14]. Hybridization forms double-stranded DNA from two single strands, leading to a modest local refractive index change. The shifts in LSPR wavelength tend to follow, though not perfectly, a linear trend with the refractive index change of the local medium[8]. Therefore, an increase in the local refractive index on the nanoparticle surface will induce a corresponding increase in LSPR wavelength shifts. In Figure 2 above, the wavelength shifts are not distinctively defined. And thus, we further analyzed (MS Excel) our data to indicate that indeed the DNA hybridization events are causing an LSPR wavelength shift. These are presented in Figure 3 below.

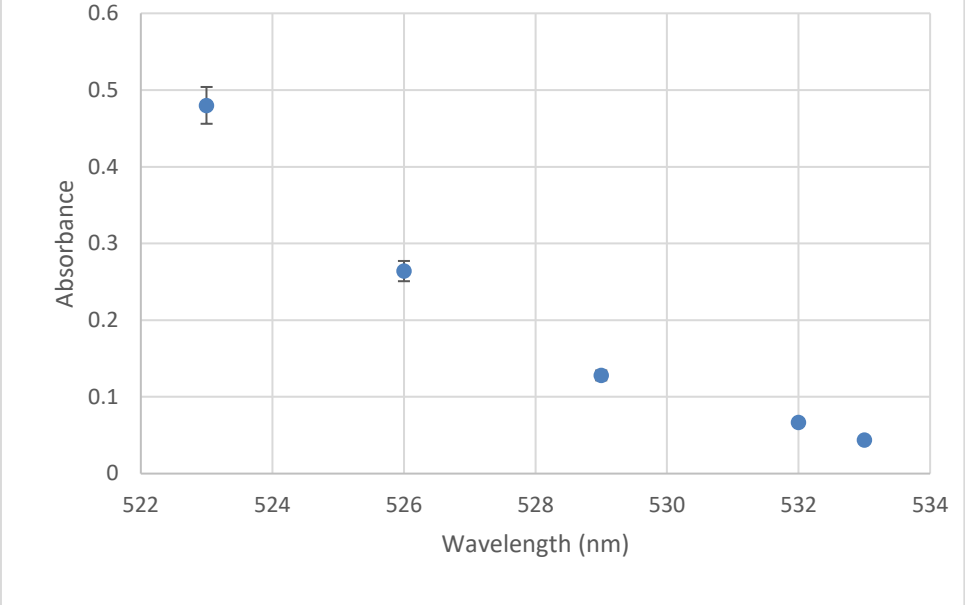

Figure 3: A curve quantifying the LSPR wavelength shifts and their absorbance intensity. The point with the highest absorbance intensity represents AuNP alone, and the point with the lowest absorbance intensity represents the hybridization between a capture probe and a complementary DNA.

This curve allows us to quantify LSPR wavelength shifts, which are sometimes subtle due to small refractive index changes on the nanoparticle surface. The trend suggests that PEG stabilization induces a larger LSPR wavelength peak shift than the hybridization of DNA sequences. Differences in hybridization of different DNA sequences, though small, are detected with this LSPR approach.

4. Conclusion

Early HIV-1 drug-resistant mutations detection can assist clinicians to advise on effective ART regimens, personalized therapy for targeted strains, and help in controlling the transmission and progression of the virus in communities. The detection turnaround time is thus critical to achieve these milestones, but this is not possible with the available laboratory-benchtop molecular techniques. LSPR can be developed into a point-of-care diagnostic assay for drug-resistant mutation detection, it is convenient and cost-effective. Our LSPR system was successful in detecting partial hybridization, which represents a single-nucleotide polymorphism pair mutation on our ssDNA target. With a distinctive peak wavelength shift of 3 (RIU) from uncomplimentary hybridization, our LSPR setup represents a sensitivity above the lower end (<1 nm) [15], thus indicating a successful mutation detection technique. This study thus forms the basis for further optimization and development into a point-of-care drug-resistant mutation detection assay.

5. Acknowledgments

This study was supported by the national research foundation (NRF), Council for Scientific and Industrial Research (CSIR), Department of Science, Technology, and Innovation (DSTI), and the South African Medical Research Council (SAMRC).

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