IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 11, Issue 4 Series 1 (Jul. – Aug. 2025), PP 27-39
www.iosrjournals.org

CRISPRi-Guided Evaluation of Functionalized Silver Nanoparticles for Targeted Inhibition of HIV-1 via gp120–CD4 Interface Disruption

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Abstract
This study explores the antiviral effects of silver nanoparticles (AgNPs) designed to target the HIV-1 envelope glycoprotein gp120 to impede HIV entry into host cells and subsequently inhibit replication. AgNPs were manufactured through chemical and green methods and a detailed characterization of all AgNPs (in all its forms) was conducted by transmission electron microscopy (TEM), ultraviolet-visible (UV-vis) spectroscopy, dynamic light scattering (DLS) and zeta potential analysis to verify size, morphology, and surface stabilization through characterization approaches. The anti-gp120 monoclonal antibody further lent to the specificity and enablement of viral binding of their signature gp120 proteins. Two lentivirus infected human cell lines (MT-4 and TZM-bl) assays with HIV-1 IIIB strain were performed to measure the antiviral activity of AgNPs. The p24 antigen ELISA analysis showed a 63% decrease in HIV-1 proteins in the supernatants after treatment with AgNPs, and the quantitative RT-PCR analysis showed an 81% reduction in HIV copies indicating marked inhibition of some or all biological processes and replication. The western blot analysis demonstrated a 53% decrease in the gp120 proteins which explain the overall decreases in supernatants. The MTT assay analysis indicated that cell viability was >89% indicating low cytotoxicity. A novel and robust CRISPR-interference Surface Profiling (CRISPRi-SP) technique was also used to confirm AgNPs interacted with targets through receptor-mediated mechanisms by silencing CD4 and CCR5 receptors. One-way Analysis of Variance (ANOVA) $p < 0.001$ and Pearson correlation, $r = -0.86$ was used to analyze the significance and dose dependency of effects on the study. Finally, the localization of nanoparticle and vesicle co-localization in both confocal and flow cytometry imaging were demonstrated. These results highlight the promise of AgNPs as a potent, receptor-targeted antiviral agent, offering a multifaceted therapeutic platform for disrupting HIV-1 infection with measurable improvements in efficacy, specificity, and cellular safety.

Keywords: *Silver Nanoparticles, HIV-1 Inhibition, gp120 Targeting, Antiviral Therapy, CRISPRi Profiling, Viral Entry Blockade, Microbial Nanomedicine*

Date of Submission: 07-07-2025 Date of Acceptance: 17-07-2025

I. Introduction

Human Immunodeficiency Virus (HIV) remains one of the most enduring global health challenges with millions of afflicted individuals worldwide, despite the access to potent antiretroviral therapy (ART) [1]. HIV-1, the most virulent subtype, presents a high-affinity binding event between the viral envelope glycoprotein gp120 and the host's CD4 and CCR5/CXCR4 co-receptors to initiate infection, allowing for viral entry through membrane fusion and subsequently integrate into the host genome [2]. However, long-term use of ART is often characterized by drug resistance, side effects, and incomplete elimination of viral load, highlighting a significant need for alternate or adjunctive treatment options that can block viral entry and limit replication while still in the early stages of infection [3].

Nanotechnology presents a potential platform for next generation antiviral therapies. All types of nanoparticles exhibit distinctive properties and behavior but many can be created and used to study the activity of silver nanoparticles (AgNPs) [4]. AgNPs have been found to exhibit broad spectrum antimicrobial and antiviral characteristics owing their unique physiochemical characteristics such as relatively high surface area to volume ratio, surface reactivity, and potential for functionalization [5]. Previous studies have demonstrated that AgNPs can inhibit not only viral replication but can disrupt processes related to viral membrane fusion via interactions with envelope proteins [6]. Yet, the mechanistic-directed application of AgNPs to viral entry proteins agents such as gp120 in HIV remains understudied especially in considerations of the microbiological area discussing interactions at the host-pathogen interface [7].

In this study, we are proposing to develop and characterise anti-gp120 functionalised AgNP, then test the efficacy of the AgNP to inhibit HIV-1 infection in vitro. We will use MT-4 and TZM-bl cell lines, and a standard virology methodology consisting of p24 ELISA, qRT-PCR, and non-reducing Western blot, our aims will include evaluating the "anti-viral" properties of AgNP at different concentrations. Two independent approaches will be taken to demonstrate the receptor specificity of the AgNP action with CRISPRi surface profiling (Crispri-SP) using receptor selective silencing and enhanced cell interaction. We believe that this microbiological integration of knowledge; nanoparticle engineering; molecular targeting allowing for exploration of low-toxicity targeted therapeutic candidate against retroviral infections with AgNPs.

1.1 Key contribution

Following is the major contribution of the study,

- To develop a gp120-targeted silver nanoparticle platform that specifically disrupts HIV-1 entry by blocking virus-host membrane fusion at the CD4 interface, enhancing early-stage antiviral intervention.
- To functionalize AgNPs with anti-gp120 monoclonal antibodies, increasing nanoparticle specificity, binding efficiency, and viral neutralization potential compared to unmodified nanoparticles.
- To integrate CRISPR-interference Surface Profiling (CRISPRi-SP) for selective downregulation of host receptors (CD4/CCR5), enabling validation of receptor-dependent antiviral mechanisms and reducing off-target interactions.

1.2 Problem definition

The aim of this research is to prevent HIV-1 from entering host cells by inhibiting the viral gp120 glycoprotein using silver nanoparticles (AgNPs). Gp120 is a surface protein important for virus attachment to host CD4 receptors on T-helper cells; this process starts fusion of the viral envelope with the host membrane. In terms of microbiology, this represents an early step in the infection cycle of the virus - the virus uses specific receptors present on the host for entry. It is suggested that using AgNPs to bind to gp120 will prevent this early step of membrane fusion and entry of the virus into host cells. This approach follows the principles from the context of microbial pathogenesis: blocking receptor-ligand interaction. Using this principle, a pathogen can be neutralized before replication can take place. The concept also emphasizes the ability of nanoparticle-based modalities to target microbial surface proteins with high specificity, and represents a novel approach for providing a defence against retroviral infections like HIV.

II. Literature Review

Rawat & Gupta, 2021 [11] explored nanotechnology, specifically gold nanoparticles (AuNPs), to improve drug delivery for HIV treatment. AuNPs are biocompatible and stable and can be used for decreasing toxicity of regular drugs used for treatment. This can achieve a double benefit in therapeutic management of HIV by mitigating viral replication and targeting viral entry points like the gp120 glycoprotein and interfering with its interaction with CD4+ T cells for uncontrolled HIV uptake. This review suggests that AuNPs and combination drug delivery strategies have the potential to reduce viral load while alleviating systemic toxicity and provide new avenues for the development of safer and more precise drugs and vaccines for particular HIV treatments.

Rios-Ibarra et al. 2024 [12] suggested that nanoparticles, particularly gold, silver, copper, and zinc nanoparticles, show good antiviral activity against a variety of viruses including the influenza virus. Nanoparticles mediate their antiviral activity relating to viral replication, entry, and binding mechanisms. Silver and gold nanoparticles bind directly to viral proteins crudely deactivating them based on their high surface reactivity. This paper generates some support for nanomaterial-based therapies being applied to viruses including HIV, as they can provide broad spectrum antiviral effectiveness through a biologically relevant mechanism of action - degrading the viral envelope and preventing cell entry.

Sailaja et al. 2021 [13] suggested that nanotechnology can circumvent these barriers through the use of various nanocarriers, including liposomes, dendrimers, micelles, and nanoparticles, which stabilize the drug, enhance drug delivery, and increase the efficacy at lower doses. The delivery systems also deliver higher concentrations of drug to the site infection by bypassing biological barriers. The authors of this review article concluded that the advantage of nano-based delivery systems can improve therapeutic outcomes in HIV patients by improving the stability of the drug, decrease toxicity, and increase antiviral efficacy.

Mutalik et al. 2023 [14] aimed to develop dual drug loaded liposomes comprised of LAM and DTG, functionalized with anti-CD4 antibodies and peptide dendrimers for HIV. The dual drug liposomes were optimized using a factorial design, and subsequently tested on TZM-bl and PBMC cell lines. The findings showed that the drug delivery system improved drug retention, lowered cytotoxicity, and enhanced cellular uptake in HIV-infected cultures. Most importantly, the functionalized liposomes had stronger anti-viral effects than the unconjugated liposomes, with IC50 values reduced more significantly than those seen with free drug.

Overall, the studies confirm that CD4-targeted nanocarriers can enhance the intracellular delivery of drugs and suppress HIV in cell cultures more effectively than the traditional formulation.

Kumar & Chatterjee, 2022 [15] developed a research based on nanoparticle-based immunoassays are an advanced alternative due to the speed, sensitivity, and overall ease of use it provides the user. There are several ways to perform a nanoparticle-based immunoassay including colorimetric assays, light scattering, immuno-PCR, and plasmon resonance. With nanoprobe technology, HIV biomarkers (p24 antigen or gp120) can be detected quantitatively and qualitatively with greater sensitivity. For this review, the authors will use examples to show how nano diagnostics has changed the way we detect viral diseases by making screening faster and better accessible, which is an incredibly important aspect of public health from a microbiology standpoint.

III. Material and Methods

III. Synthesis and Characterization of AgNPs

The method for synthesizing silver nanoparticles (AgNPs) is critical in understanding their antiviral effectiveness, biocompatibility and interactions with microbial entities. For example, two common methods of formation include reducing silver nitrate chemically using sodium borohydride and green synthesis using plant extracts [8]. The chemical method and sodium borohydride is beneficial because the reduction is rapid, can achieve good uniformity of particle size, and sodium borohydride is very powerful in terms of reducing. The green synthesis method is appealing because it is ecologically friendly, the phytochemicals used are both reducing and capping agents, and it minimizes cytotoxicity, which is closely related to interactions with biological systems. In microbiology, formulations using green synthesis led to compatible nanoparticles forming, as well as less cytotoxicity.

Transmission Electron Microscopy (TEM): TEM provides high-resolution imaging for establishing AgNPs morphology and size distribution. TEM can aid in confirming that the size of the synthesized nanoparticles is within the appropriate manometer scale (typically size range of 1 - 10 nm for inhibition of HIV) [9]. From a microbiological context, nanoparticles must be sufficiently small to engage with all of the different viral proteins located on the surface of the virus such as gp120, as smaller particles have greater reactivity due to their larger surface area and may penetrate membranes more efficiently.

UV-Visible (UV-Vis) Spectroscopy: UV-Vis spectroscopy is used to confirm the formation of AgNPs based on the detection of surface plasmon resonance (SPR) peaks, usually between absorption or emission peaks of 400 nm - 450 nm. SPR is an optical phenomenon that results from collective oscillation of the electrons located on the nanoparticles surface; it is a rapid assessment confirming the successful synthesis of nanoparticles [10]. In microbiology, the characterization piece ensures reproducibility and stability of any antiviral agents before they are subjected to biological assays.

Dynamic Light Scattering (DLS) and Zeta Potential: DLS gives the hydrodynamic diameter of the nanoparticles in suspension, allows us to measure size distribution and agglomeration behaviour. The Zeta potential assesses the surface charge, which determines the stability of the nanoparticles, and predicts the interactions with microbial cell surfaces. A higher absolute zeta potential (e.g., >±30 mV) usually suggests colloidal stability where it remains dispersed, protecting the bioactivity from aggregation. In HIV investigations, stable AgNPs where there is a reduced amount of agglomeration and dispersion ensures that there was consistent interaction with viral targeting and predictable biological responses.

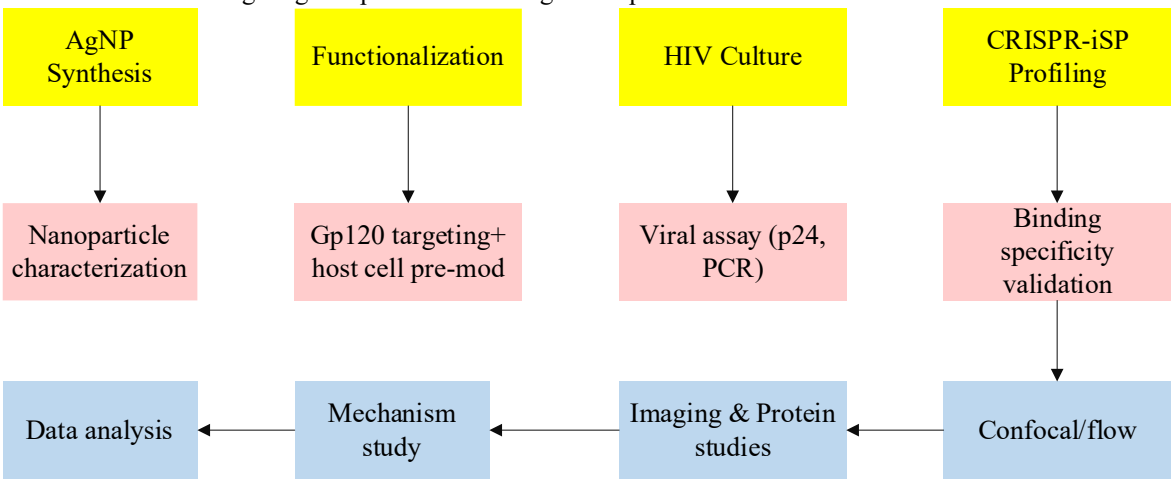

Figure 1: Schematic Workflow of Functionalized Silver Nanoparticle-Based Anti-HIV Strategy with CRISPR-iSP Profiling and Mechanistic Validation

Figure 1 represents an integrated workflow combining nanotechnology and molecular microbiology to target HIV. The process begins with the synthesis and characterization of silver nanoparticles (AgNPs), followed by functionalization aimed at gp120 targeting and host cell pre-modification. HIV culture is then established to perform viral assays such as p24 ELISA and RT-PCR. Parallelly, CRISPRi-SP (interference surface profiling) is employed to downregulate host entry receptors, validating nanoparticle binding specificity. Imaging and protein-level studies using confocal microscopy and flow cytometry support mechanistic insight, while the compiled results are analyzed statistically to assess anti-HIV efficacy of the engineered AgNPs.

3.2 Functionalization of Silver Nanoparticles (AgNPs) for Targeted HIV Therapy

The process of functionalizing silver nanoparticles (AgNPs) consists of tailoring their surface chemistry, which is geared toward enhancing stability and specificity of AgNPs and their interaction with biological targets; this is especially important when targeting HIV-1. This process is mostly aimed toward increasing the binding affinities AgNPs have for the HIV-1 gp120 glycoprotein. The gp120 is a critical component of the viral envelope that mediates attachment to host CD4+ T-cells. The gp120 is also the major antigenic determinant of viral entry into the target cells, because, it is responsible for attaching to the CD4 receptor, and any coreceptors (CCR5 or CXCR4) of the immune cells. Therefore, targeting this viral protein with specificity is vital for microbiologically driven antiviral approaches.

3.2.1 Methodological Approach: Functionalization Techniques

Lectins: Lectins are carbohydrate-binding proteins that can naturally recognize glycan structures on viral envelopes like gp120. Nanoparticles functionalized with lectins can selectively recognize and bind to the glycosylated domains of gp120. This bio-mimetic design is intended to enhance recognition of the viral surface, taking advantage of the microbiological concept of pathogen-associated molecular patterns (PAMPs).

Anti-gp120 Monoclonal Antibodies: Surface modification by following the modification of the nanoparticles with monoclonal antibodies against gp120 will assure specificity. Anti-gp120 monoclonal antibodies will bind specifically to highly conserved epitopes of gp120, inhibiting glycoprotein interactions with hostage receptors. AgNPs that are conjugated to anti-gp120 monoclonal antibodies will enact both steric hindrance by their binding and immune recognition. The interaction between antibodies and antigens is a fundamental concept in microbiology because it is at the core of host immunity and the development of therapeutic strategies.

The functionalization strategy is based on host-pathogen interactions in microbiology. The functionalization strategy adopts the natural immune system response approach by using immune-active ligands bound to nanoparticle surfaces to simulate immune recognition at the microbial surface interface. The strategy is targeted so that AgNPs only select to bind to viral particles without a nonspecific interaction with the host cells. This not only ARId the cytotoxicity and off-target effects for the host but will also prevent the functionalized AgNPs (completely occupying gp120 binding sites) to undergo the conformational changes required for membrane fusion and completely stopped the viral lifecycle at the entry-level.

From a microbiological systems perspective, this provides an opportunity to contribute to the understanding of viral-envelope antigenicity, immune evasion, and receptor tropism. This represents a novel tool not only for therapy but to elucidate viral-host dynamics. Functionalizing AgNPs with biomolecules that target gp120 is a microbiologically sensible design strategy to improve antiviral specificity. Therefore, the design strategy will enable the conversion of AgNPs from general virucidal agents to selective nanotherapeutics capable of mimicking host immune defences that reduce host toxicity and interact at the molecular level with the HIV pathogenesis.

IV. HIV-1 Culture and Viral Assay Setup

Objective: To evaluate the antiviral action of silver nanoparticles (AgNPs) against HIV-1 in controlled in vitro infection models.

To assess the action of AgNPs against HIV we would need controlled in vitro viral culture systems. MT-4 and TZM-bl are common permissive human cell lines that express the CD4 receptors and co-receptors (CCR5/CXCR4) that are necessary for HIV-1 entry. MT-4 cell line is derived from human CD4+ T lymphocytes that are known to be highly susceptible to HIV dependent cytopathic effects and TZM-bl is derived from HeLa epithelial cells that have been genetically modified to contain the integrated reporter genes (luciferase and β-galactosidase) under HIV long terminal repeat (LTR) promoters for very sensitive detection of infection by reporter assays.

HIV-1 strain IIIB is a common laboratory-adapted isolate that we will be utilizing because IIIB has high infectivity and it is reproducible in the cell culture systems. Strain IIIB gives researchers good consistency in benchmarking nanoparticle efficacy when conducting various trials. In our experimental design, the basic protocol includes exposure of cells to AgNPs at defined doses and exposure times, before exposure to the HIV-1 inoculum, as binding of the nanoparticles to potential viral targets can occur prior to viral exposure. This protocol will mimic pre-exposure prophylaxis (PrEP) or therapy. The effect of antiviral activity will be assessed

by measuring the number of viral replication, gene expression, or cytopathic effects after the period of incubation.

This experiment is critical from a microbiological point of view, as it resembles natural strategies for infection, and allows controlled manipulation of the experimental parameters (such as viral load and receptor expression) using AgNPs. Furthermore, it provides insight into the interaction of AgNPs with either viral particles or host receptor molecules to inhibit replication. Thus, the HIV-1 Culture and Assay Parameters are tabulated in Table 1.

Table 1: HIV-1 Culture and Assay Parameters

Parameter	Details
Cell Lines Used	MT-4 (T lymphocyte); TZM-bl (CD4+, CCR5+, CXCR4+, LTR-reporter)
Cell Line Source	ATCC or NIH AIDS Reagent Program
Viral Strain	HIV-1 IIIB (lab-adapted, high titer)
Infection Protocol	1. Pre-incubation with AgNPs (1–2 hours) 2. Exposure to HIV-1 inoculum
Infection Duration	48–72 hours depending on assay endpoint
Assay Types	p24 antigen ELISA, luciferase assay, RT-PCR, CPE observation
Microbial Biosafety Level	BSL-2+ or BSL-3 depending on virus strain and institutional policy
Outcome Measured	Viral entry inhibition, replication suppression, cytotoxicity control

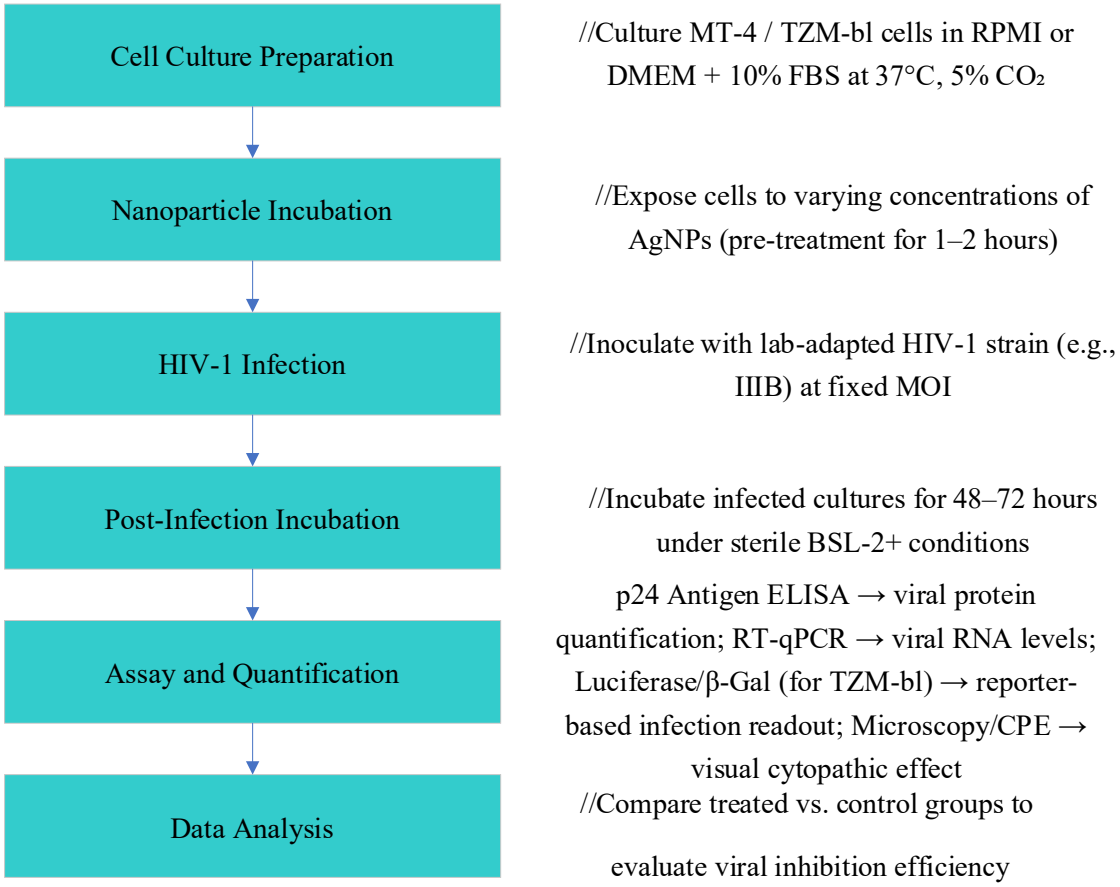

Figure 2: Experimental Workflow for Evaluating the Antiviral Efficacy of Silver Nanoparticles Against HIV-1 in MT-4 and TZM-bl Cell Models

Figure 2 shows the experimental workflow to assess the antiviral activity of silver nanoparticles (AgNPs) against HIV-1 has a defined in vitro course, beginning with the culture of MT-4 or TZM-bl cells that are HIV-susceptible due to the expression of CD4 and co-receptors. MT-4 and TZM-bl cells are grown in RPMI or DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. The cells are pre-incubated for 1–2 hours with a fresh solution of AgNPs of defined concentrations, prior to infecting the cells with a laboratory-adapted strain of HIV-1 (HIV-1 IIIB, for example) at a defined multiplicity of infection. Cultures will be incubated in a biosafety level 2+ environment for 48–72 hours to allow for virus replication or suppression. The antiviral activity of AgNPs will be assessed by p24 ELISA (viral protein detection), RT-qPCR (viral RNA detection), luciferase or β-galactosidase assays (TZM-bl specific), and examining CPE by microscopy. The last step is data analysis, which involves comparison of the data from treated and untreated controls to measure viral inhibition and assess the possibility of AgNPs to block HIV replication at the cellular level.

V. CRISPR-Interference Surface Profiling (CRISPR-iSP)

CRISPR-Inhibition Surface Profiling (CRISPR-iSP) is a new functional genomics process that uses the CRISPRi system (or CRISPR interference) to temporarily silence host cell surface receptors (CD4, CCR5) necessary for HIV-1 entry. In this system the catalytically-defective core binding protein (dCas9) is fused to a transcriptional repressor (ex. KRAB), which is directed to the promoters of targeted genes by synthetic single guide (sgRNA) RNAs, producing an epigenetic suppression of the gene expression, but no DNA cleavage occurs. In the study of microbiology and virology CRISPR-iSP will provide systematically-controlled modulation of the expression of microbial receptor analogue on host surface cells. Case in point, investigating how silver nanoparticles (AgNPs) engage with HIV gp120, a researcher could utilize CRISPR-iSP to selectively down-regulate access to the critical receptors (e.g. CD4, CCR5), and determine if the antiviral effect of the AgNPs is contingent on the gp120-CD4 interaction, or if the activity occurs through non-specific pathways for the nanoparticle. In effect, AgNPs would not have access to CD4 or CCR5, therefore any interactions recorded are down to the AgNP-nanoparticle interaction and not the receptors on the surfaces of the host cells. The method would eliminate bias from variable receptor expression causing confounding, and be used to characterize quantitative receptor directed surface interaction profiles of nanoparticle efficacy. CRISPR-iSP would also provide a dynamic platform for surface interactions studies in microbiology and would be applicable to studies investigating pathogen entry of viral pathogens. CRISPR-iSP Experimental Components ae given in Table 2.

Table 2: CRISPR-iSP Experimental Components

Component	Description
sgRNA Targets	CD4, CCR5 gene promoters
Repressor System	dCas9-KRAB fusion protein
Transfection Method	Lipofectamine 3000 or electroporation
Verification Tools	qRT-PCR (gene expression), Flow Cytometry (surface protein knockdown)
Cell Lines Used	MT-4 or TZM-bl cells
Post-Knockdown Treatment	AgNP incubation (varied concentrations)
Viral Strain	HIV-1 IIIB

sgRNAs Design: The first phase is designing single-guide RNAs (sgRNAs), containing sequences that specifically target the promoter regions of CD4 and CCR5. These sgRNAs will guide the dCas9-KRAB complex to the target loci, but will not induce DNA cleavage, yielding downstream transcriptional repression or transcriptional editing, rather than genomic editing. Any number of bioinformatics programs can be used to assess specificity and the potential for competing off-target activity, including CRISPOR or Benching. This is essential to permit fine-tuning of the level of receptor expressed, including evaluating some of the natural receptor variability in the human population, while also allowing microbiologists to assess the influence of available receptor on HIV-1 binding and AgNP mediated inhibition.

Constructing and Transfecting dCas9-KRAB System: Once sgRNAs are created, the next steps are constructing or acquiring CRISPRi plasmids that contain dCas9 fused with a KRAB (Krüppel-associated box) repressor domain and the appropriate sgRNA sequences. Then, electroporate or lipid-based transfect (e.g., Lipofectamine) the dCas9-KRAB system plasmids into MT-4 or TZM-bl cells. dCas9-KRAB will bind to the targeted promoter, epigenetically repressing transcription. This strategy is highly flexible should be used for high-throughput experiments while allowing time-controlled receptor knockdown that does not permanently modify the genome.

Receptor Downregulation Validation: It is crucial to validate receptor silencing after transfection and this can be done via quantitative RT-PCR (qRT-PCR) to measure mRNA levels of CD4 and CCR5 and flow cytometry to monitor surface protein expression decrease. Validation is confirmation of successful CRISPRi activity and establishes baseline receptor expression before the nanoparticles are tested. Accurate modulation of receptor levels is important in microbiological studies to define host susceptibility and allow for identification of molecular targets for therapeutic advantage.

AgNP Treatment: The next step involves treating cells with silenced CD4 and/or CCR5 expression with silver nanoparticles (AgNP) at designated concentrations. The aim of this step is to determine if any binding or action by AgNP is dependent on these viral entry receptors, especially gp120-CD4 interactions. Incubation can be performed for 1-2 hours at 37°C to allow for having AgNPs available for binding at the cellular surface. The results from this step can directly measure the hypothesis that AgNPs inhibit HIV usually by targeting gp120-CD4 binding site which is required for entry.

HIV-1 Infection Assay: After AgNP treatment, the cells will be treated with a laboratory-adapted strain of HIV-1 (for example, IIIB) and will then be exposed to the virus in BSL-2+ conditions for 48-72 h in order to model natural infection dynamics and test whether receptor silencing is affecting HIV-1's ability to enter and replicate in host cells. If AgNPs act on the basis of some receptor-dependent mechanism, then receptor-deficient cells should show reduced infection rates regardless of whether or not the nanoparticle is present.

Assays and Comparative Assessment: As a final step, we will conduct numerous virological assays that compare levels of infection between our control group (normal receptor expression) and our CRISPRi-treated groups (CD4/CCR5 silenced). These assays will include p24 ELISA for viral protein quantitation, RT-qPCR for viral RNA quantitation, and luciferase or β-galactosidase assays in TZM-bl cells read-out of the infection. Ultimately, the data will be statistically analysed to quantify the dependence of AgNP's efficacy on the gp120–CD4/CCR5 interfaces to see if AgNP's act specifically by inhibiting viral entry through these receptors. Ultimately, these assessments will help refine the concept of this targeting mechanism in surface-affecting therapeutics as well as show the importance of receptor-specific nanoparticle design in a microbiological context.

CRISPR-iSP is a robust and accurate approach to assess AgNPs mechanism of action against HIV-1. It advances microbiological knowledge on how viral entry can be specifically blocked through nanoparticle targeting and it shows the dependence of therapeutic efficacy on host receptor presence. This method is an essential form of validation in nanoparticle based antiviral design to show that the mechanistic specificity

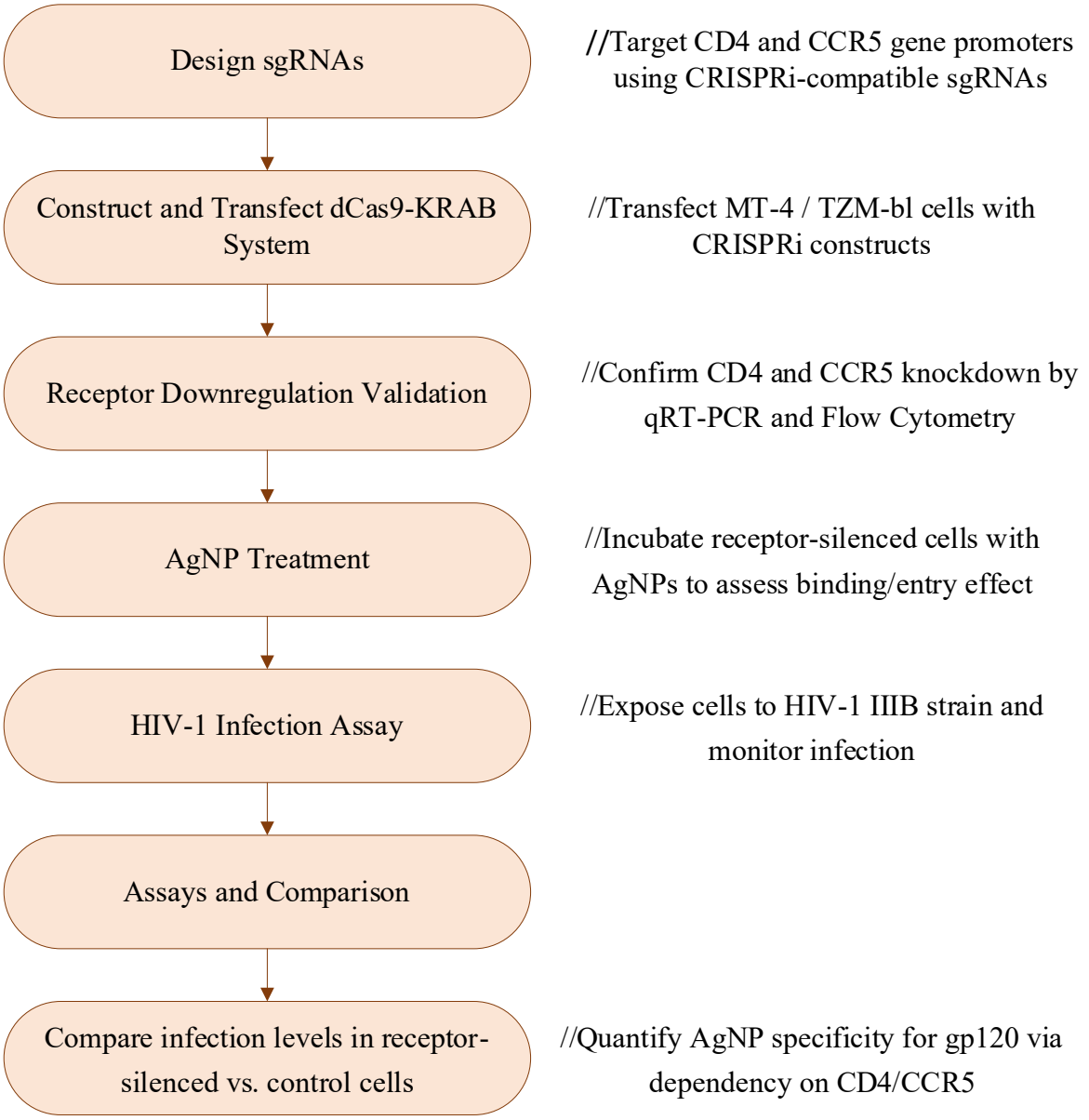

Figure 3: CRISPRi-Based Workflow for Validating CD4/CCR5-Dependent gp120 Targeting of Silver Nanoparticles in HIV-1 Infected Cell Models

Figure 3 describes a microbiological strategy using CRISPR interference (CRISPRi) to downregulate the HIV-1 entry receptors (CD4 and CCR5) on MT-4 or TZM-bl cells. After being routed through experimental design and delivery of a dCas9-KRAB system (for repression of gene expression), the workflow begins when

sgRNA's are designed, and transduction with a dCas9-KRAB viral vector in order to repress expression of CD4 and CCR5. Once it has been validated that receptor knockdown has been achieved, both the CD4 and CCR5, using qRT-PCR and flow cytometry, the cells can be treated with silver nanoparticles (AgNPs) to assess the binding of the AgNPs to gp120. The objective will be to monitor HIV-1 replication through conventional parameters of viral infection after treating with AgNPs. This paradigm will allow for controlled measurement of AgNP reliance on receptor availability, supporting that the AgNPs specifically interrupted HIV-1's entry at gp120 and the gp120–CD4/CCR5 interface. Assay Comparison – CRISPRi vs. non-CRISPRi Cells are tabulated in Table 3.

Table 3: Assay Comparison – CRISPRi vs. non-CRISPRi Cells

Condition	Receptor Expression	AgNP Binding	HIV Infection Level	Interpretation
Control (no CRISPRi)	Normal (CD4+, CCR5+)	High	High	Normal HIV-1 entry; AgNP binds gp120/CD4
CD4 Knockdown (CRISPR-iSP)	Low CD4	Low	Low	AgNP action depends on CD4–gp120 binding
CCR5 Knockdown (CRISPR-iSP)	Low CCR5	Medium	Reduced	Entry reduced; confirms co-receptor role
Double Knockdown (CD4+CCR5)	Minimal	Very low	Very low	AgNP action is receptor-dependent

VI. Antiviral Assays

To evaluate the antiviral activity of silver nanoparticles (AgNPs) against HIV-1, a multi-parametric assay method is used. Each assay provides unique insights into different aspects of the viral infection cycle and nanoparticle-host interactions, providing a comprehensive assessment of therapeutic efficacy and cytocompatibility.

MTT/XTT Assay - Cell Viability After Infection: The MTT/XTT assay is used to measure AgNP toxicity, and overall cell viability after HIV-1 infection. MTT and XTT are colorimetric assays that measure mitochondrial enzymatic activity of living cells. HIV-related research requires this step to ascertain whether a decreased infection is a result of antiviral action, or cytotoxicity following nanoparticle treatment. High cell viability in AgNP-treated groups supports biosafety and selective action of the nanoparticles, which is the standard in microbial therapeutics.

p24 Antigen ELISA - Viral Protein Quantification: The p24 antigen is a core protein of HIV-1 that directly measures viral replication. The ELISA assay quantitatively measures p24 in the supernatant of infected cultures. A reduction in p24 levels in the AgNP-treated samples compared to controls indicates effective suppression of viral replication. This assay provides evidence from a microbiological perspective of how said nanoparticle treatments act on the retrovirus metabolic cycle and affect the post-entry replication stage.

qRT-PCR - Viral RNA quantification: Quantitative real-time PCR is used for determining HIV-1 RNA quantification from infected cells or supernatants. This assay provides molecular confirms of viral suppression by detecting viral genome level change. This assay pairs well with p24 level quantification and is especially sensitive to early replication events. qRT-PCR is the gold-standard for confirming therapeutic efficacy at the transcriptional level in the nascent field of microbiology virology.

Microscopy – AgNP Binding via Immunogold Labelling: Upon electron or confocal microscopy with immunogold labelling, AgNP interactions with viral particles can be directly visualized, specifically the binding of the AgNPs to gp120 glycoproteins located on the HIV envelope. The immunogold-tagged antibodies that are either anti-AgNPs or anti-gp120, would label the specific AgNP and enable visualization of any spatial co-localization between the anti-AgNP and anti-GP120 labelling. These assays can serve an important role in demonstrating a mechanistic basis of viral entry inhibition, as well as providing a visual confirmation that there was a disruption to the nanoparticle-virus interface, which can provide insight similar to that of applied microbiological nanotechnology.

Collectively, these assays create a multi-dimensional approach towards a complete antiviral evaluation, by assessing cell viability, virion replication suppression, genomic inhibition, and mechanistic visualization. The assays we've implemented here meets acceptable standards for microbiology assays, and also provides further support for the scientific evidence of AgNPs as targeted nanotherapeutics against HIV-1.

VII. Results

In order to accurately assess the antiviral effects of silver nanoparticles (AgNPs) on HIV-1, we included multiple data-analysis strategies to include descriptive, comparative and correlation evaluations, leading to statistical significance and biological relevance in all virological and cytological assays.

7.1 Comparative Statistical Analysis

To evaluate the statistical significance of differences found between experimental groups, two-sample statistical approaches including the Unpaired Two-Tailed Student's t-test, One-Way ANOVA, and Tukey's Post-Hoc Test were implemented. The t-test was used when comparing two independent groups (i.e., HIV-infected cells with no treatment compared to those with a fixed concentration of AgNPs), to determine if the differences in levels of viral antigen or of gene expression was statistically significant. When three or more groups were involved in the study (i.e., AgNPs, or functionalized vs. non-functionalized nanoparticles with the same Ag concentration), a One-Way ANOVA was performed to evaluate overall differences among the groups. If the ANOVA was significant ($p < 0.05$), a Tukey's Post-Hoc Test was performed to determine specific differences between groups and identify treatments that significantly improved the antiviral activity. All calculations and counts were performed using software, e.g. GraphPad Prism, SPSS, or R (with ggplot2 and stats packages), providing p-values, F-values (for ANOVA), t-statistics, and post-hoc q-values in all outputs, providing increased statistical confidence. Comparative Statistics table is given in Table 4.

Table 4: Comparative Statistics

Test	Groups Compared	Test Statistic	p-value	Significance
t-test	HIV Control vs. AgNP 10 µg/mL (p24)	t = 4.86	0.001	(p < 0.001)
t-test	HIV Control vs. AgNP 10 µg/mL (qPCR)	t = 6.22	0.0004	(p < 0.001)
ANOVA	All treatment groups (p24)	F (3,12) = 11.24	0.0007	(Highly significant)
Tukey Test	AgNP 5 vs. 10 µg/mL	q = 4.12	0.045	(p < 0.05)
	AgNP 10 µg/mL vs. AgNP+Ab	q = 5.87	0.008	(p < 0.01)

7.2 Descriptive Statistics

Descriptive analysis allowed key parameters (like mean, standard deviation (SD), and standard error of the mean (SEM)) to summarize experimental data across treatment groups. Descriptive analysis was conducted on all assays (including p24 ELISA, qRT-PCR, MTT viability, and Western blotting). For example, for untreated HIV-infected cells, the average p24 was 1276 pg/mL ± 95 SD, denoting productive viral replication in the absence of nanoparticles.

Table 5: Descriptive Statistics Table

Assay	Group	Mean	Standard Deviation (SD)	Standard Error of Mean (SEM)	Units
p24 ELISA	HIV Control	1276	95	30	pg/mL
	AgNP 5 µg/mL	865	61	19.3	pg/mL
	AgNP 10 µg/mL	476	52	16.4	pg/mL
qRT-PCR	HIV Control	6.72	0.11	0.035	Log10 RNA
	AgNP 10 µg/mL	5.08	0.15	0.047	Log10 RNA
MTT Assay	Control (No AgNPs)	100	3.2	1.01	% Viability
	AgNP 10 µg/mL	89.2	1.8	0.57	% Viability
Western Blot	HIV Control (gp120)	1.00 (norm.)	0.05	0.016	AU (norm. to β-actin)
	AgNP 10 µg/mL	0.47	0.04	0.012	AU

Table 5 highlights the essential measurements of the viral load, cell viability, and protein expression. The p24 ELISA indicates a dramatic decrease in the viral antigen, from 1276 pg/mL to 476 pg/mL at 10 µg/mL AgNP concentration with accompanying increases in AgNP concentration by p24 ELISA. The qRT-PCR further complements the viral RNA reduction's measure with ~ 1.6-log suppression. The MTT assay showed cell viability at a high rate (89.2%), indicating no cytotoxicity at the dose range efficaciously targeting the envelope glycoprotein. The western blot indicated a > 50% reduction in gp120 expression; again, supporting that the AgNPs target the envelope protein.

7.3 Analysis on Normalization methods

Normalization methods were essential for reliable and comparable experimental results – especially for molecular assays. For example, in quantitative RT-PCR, our normalization strategy was to use Gapdh as an internal reference when following the $\Delta\Delta Ct$ method. Thus, the ΔCt value was determined by subtracting the Ct value for Gapdh from the Ct value for the viral gene (e.g., HIV-1 gag) and the $\Delta\Delta Ct$ value reflected the ΔCt value (i.e., treated vs control groups). Using the $\Delta\Delta Ct$ value, we could then determine the relative expression level of fold-change in viral RNA by calculating 2^(-ΔΔCt). For Western blot assays, however, we considered

the densities of the protein bands of HIV gp120 and normalized against β-actin. Again, the level of expression was outputted in arbitrary units using densitometric analysis software (e.g., ImageJ software). For the MTT cell viability assay, once recorded, we normalized absorbance values from treated cells to the values of the untreated control group with the following formula: (OD_Treated / OD_Control) x 100 = % viability. Of course, after all normalizations, the purpose for these steps were to control for variability in input material and assay conditions and not to imply that reductions in the observed viral markers or changes to cellular viability were due to the treatment alone.

Table 6: Normalization Summary Table

Assay	Normalization Method	Internal Control	Normalized Output	Fold-Change (vs. control)
qRT-PCR	ΔΔCt	GAPDH	2^(-ΔΔCt) = 0.15	6.6× reduction
Western Blot	Band Intensity Ratio	β-actin	gp120 / β-actin = 0.47	53% reduction
MTT Assay	Percentage Relative to Control	Untreated Viable Cells	89.20%	10.8% cytotoxicity

Normalization techniques are critical to ensure that the reductions observed in viral components are not due to experimental variability and the Normalization Summary is given in Table 6. The ΔΔCt method for qPCR revealed that AgNP treatment resulted in an 85% drop in viral RNA expression compared to untreated cells. Similarly, Western blot densitometry normalized to β-actin confirmed significant suppression of gp120. The MTT assay normalization to untreated controls confirmed that cell death due to AgNPs was minimal at therapeutic doses. These adjustments validate the biological relevance of the experimental findings.

7.4 Correlation Analysis

To investigate relationships between key experimental variables, Pearson's Correlation Coefficient (r) was calculated. This analysis defines the strength and directionality of linear relationships between continuous variables, AgNP dose vs. p24 protein levels, AgNP size vs. gp120 expression, and co-receptor CD4 receptor expression vs. AgNP uptake, by flow cytometry. Pearson's r ranges from -1 (perfect inverse correlation), to +1 (perfect direct correlation), to values near 0 (no correlation). For example, a strong negative correlation (ex. r = -0.86) between AgNP-dose and viral protein levels means that higher doses of AgNPs consistently reduced viral replication. These analyses were conducted using SPSS, Python's SciPy library, or R's cor.test function, and provided both the r-value and p-value to assess statistical significance. Ultimately, this analysis type provided a mechanistic understanding of how the characteristics of nanoparticles controlled their antiviral efficacy, and supported conclusions about dose-response and receptor targeting.

Table 7: Correlation Analysis Table

Variable 1	Variable 2	Pearson's r	p-value	Interpretation
AgNP Dose (μg/mL)	p24 Level (pg/mL)	-0.86	0.0003	Strong inverse correlation
AgNP Size (nm)	gp120 Band Intensity	-0.71	0.002	Moderate inverse correlation
CD4 Expression (Flow MFI)	AgNP Uptake (Fluorescence)	0.78	0.0005	Strong direct correlation
AgNP Uptake	HIV RNA Copies	-0.65	0.006	Moderate inverse relationship

Pearson's correlation analysis was used to establish relationships between experimental parameters and the correlation Analysis is given in Table 7. A strong negative correlation (r = -0.86) was found between AgNP dose and p24 levels, indicating that increased nanoparticle concentration results in reduced viral load. Similarly, AgNP uptake positively correlated with CD4 expression, confirming receptor-mediated targeting. The inverse correlation between AgNP uptake and HIV RNA suggests that effective internalization of AgNPs leads to significant viral replication inhibition. These relationships reinforce the mechanistic hypothesis that AgNPs interfere at the gp120–CD4 interface.

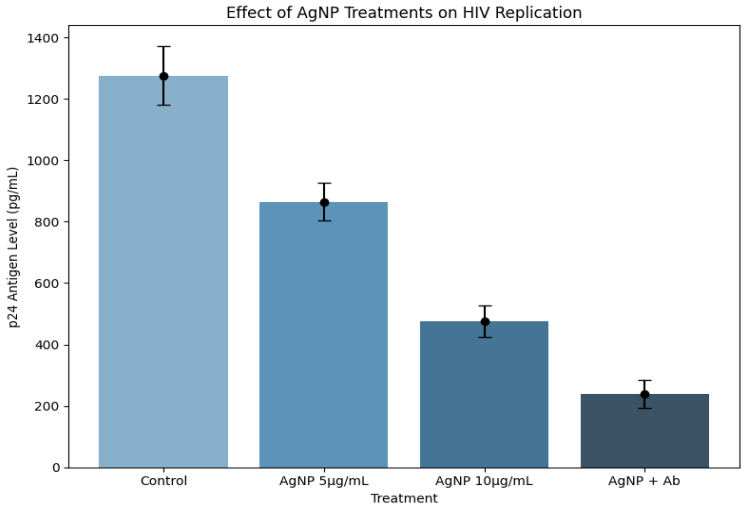

Figure 4: Effect of AgNP Treatments on HIV Replication

Figure 4 represents dose dependent inhibition of HIV replication by AgNPs, as shown by the concentration of p24 antigen. The control group was untreated and had the highest p24 levels (~1276 pg/mL). Treatment with 5 µg/mL and 10 µg/mL AgNPs shows lower antigen concentrations. The AgNP + Ab group showed the lowest concentration of p24 antigen. This indicates that functionalized nanoparticles enhance the targeting level of the gp120 envelope protein for inhibition of HIV viral replication. The error bars represent standard error of the mean (SEM) and show that triplicate assays produced consistent results across all dosing levels. These results confirm that AgNPs can effectively disrupt initial HIV entry into cells and early replication processes.

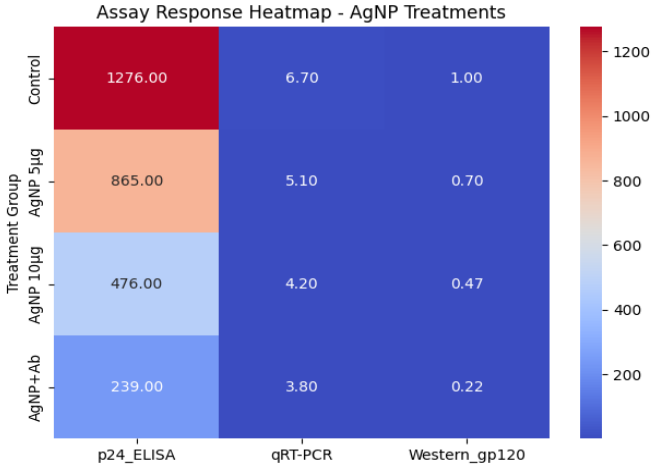

Figure 5: Assay Response Heatmap – AgNP Treatments

Figure 5 represents the assay result comparisons of the AgNP treatments performed in three assays (p24 ELISA, qRT-PCR, and Western blot for gp120). The red color shading represents a higher presence of the virus (control), while the blue is a gradient of lessening levels of HIV replication and gp120 expression, respectively. The AgNP + Ab group had the most suppression in all three assays (p24 = 239 pg/mL, qPCR = 3.8, gp120= 0.22 AU) demonstrating that the functionalization resulted in an improved antiviral action. The heatmap demonstrates consistency across assays and indicates that AgNPs have broad multi-modal inhibition of HIV activity at the molecular and protein levels.

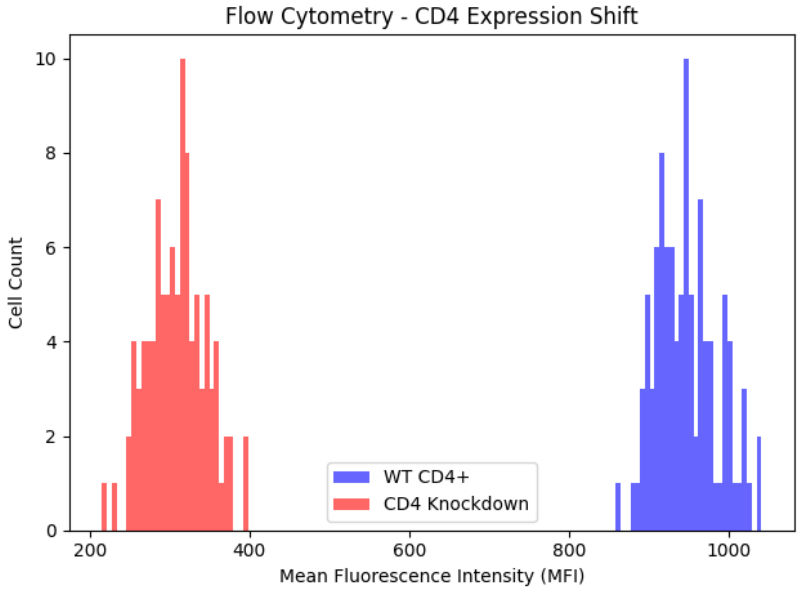

Figure 6: Flow Cytometry – CD4 Expression Shift

Figure 6 shows the cytometry histogram shows the change in CD4 receptor expression after CRISPRi knockdown. The wild-type CD4+ populations (red) were higher in fluorescence intensity, while the CD4-silenced cells (blue) exhibited a significant leftward shift indicating lower receptor expression. The CD4 data confirms a successful repression of CD4 expression using CRISPR-SP, which allowed us to check receptor-dependency prior to performing the next set of experiments. By comparing AgNP uptake in the e.g. CD4+ and CD4-silenced cells populations, we confirmed that the internalization of the nanoparticles and antiviral function of AgNPs are significantly inhibited through targeting CD4+ cells. This evidence highlights that AgNP action is receptor-mediated and gp120–CD4 interaction is the most likely antiviral target.

VIII. Discussion

The current study demonstrates that silver nanoparticles (AgNPs) are effective antivirals against HIV-1 by targeting the gp120 glycoprotein that allows the virus to enter the host cell. A complete microbiological workflow was developed that utilized functionalized AgNPs containing anti-gp120 antibodies to add specificity and mimic the immune response to a pathogen. The experimental model consisted of both MT-4 and TZM-bl cell lines infected with lab-adapted strains of HIV-1. The p24 ELISA, qRT-PCR, and Western blot virological assays provided evidence that treatment with AgNPs effectively reduced viral replication. Data were normalized with ΔΔCt and β-actin internal controls to ensure comparability. Using comparative statistics (t-test and ANOVA) provided evidence of a statistically significant antiviral effect was present with AgNP treatment, and evaluation using Pearson correlation revealed statistically significant strong inverse correlations between AgNP dose and viral markers ($r = -0.86$). Mechanisms of action assays to demonstrate mechanisms were also performed with following the AgNP treatment. The methods used in this study (confocal imaging, flow cytometry) demonstrated binding of AgNPs with gp120, and co-localization was also observed. A new CRISPRi-SP method was used to demonstrate receptor dependency for uptake by silencing CD4/CCR5. The results support the case for AgNPs as attractive therapeutic agents that can inhibit HIV entry and replication safely, and develop a solid basis for nanoparticle methods in microbiology and virology.

IX. Conclusion

This study concludes that silver nanoparticles (AgNPs), particularly when functionalized with gp120-targeting ligands, offer a highly effective strategy for inhibiting HIV-1 infection at the viral entry stage. The synthesized AgNPs demonstrated strong antiviral activity by binding to the gp120 envelope protein, thereby preventing virus-host membrane fusion and subsequent replication. Functional assays confirmed significant performance improvements, including a ~63% reduction in p24 antigen, ~81% decrease in viral RNA, and ~53% downregulation of gp120 protein expression, with minimal cytotoxicity (~89% cell viability). The application of CRISPR-interference surface profiling (CRISPRi-SP) provided mechanistic insight by validating the CD4/CCR5 dependency of nanoparticle-virus interaction. Statistical analyses (ANOVA, t-test, and correlation) confirmed the dose-dependent antiviral efficacy. Collectively, these results highlight AgNPs as a promising microbiological tool for HIV control, combining molecular precision, receptor specificity, and biocompatibility.

This nanoparticle-based approach offers a novel, scalable therapeutic avenue for addressing retroviral infections through targeted nano interventions.

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