Cellular uptake of metal oxide-based nanocomposites and targeting of chikungunya virus replication protein nsP3

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ABSTRACT
BACKGROUND: Emergence of new pathogenic viruses along with adaptive potential of RNA viruses has become a major public health concern. Hence it becomes even more important to explore and evaluate the antiviral properties of nanocomposites which is an ever-evolving field of medical biology.

METHODS: In this study, series of metal/metal oxide (Ag/NiO : NiO, AN-5%, AN-10% and AN-15%) and ternary metal oxide nanocomposites (Ag2O/NiO/ZnO : N/Z, A/N/Z-1, A/N/Z-2 and A/N/Z-3) have been synthesized and characterized. Cellular uptake of nanocomposites was confirmed by ICP-MS.

RESULTS: Intriguingly, molecular docking of metal oxides in the active site of nsP3 validated the binding of nanocomposites to chikungunya virus replication protein nsP3. In-vitro antiviral potential of nanocomposites were tested by performing plaque reduction assay, cytopathic effect (CPE) analysis and qRT-PCR. The nanocomposites showed significant reduction in virus titre. Half-maximal inhibitory concentration (IC50) for A/N/Z-3 and AN-5% were determined to be 2.828 and 3.277 µg/mL, respectively. CPE observation and qRT-PCR results were consistent with the data obtained from plaque reduction assay for A/N/Z-3 and AN-5%. CONCLUSION: These results, have opened new avenues for development of nanocomposites based antiviral therapies.

Keywords: Nanocomposite, Antivirals, CHIKV, ternary metal oxides

1. Introduction
The global shock imposed by the COVID-19 pandemic is regarded as one of the most significant and widespread health crisis to hit humanity in recent history [1]. Several prominent recent cases of re-emerging infections are caused by RNA viruses like Chikungunya, Dengue, ZIKA, Mayaro, SARS-CoV-2, Ebola, Marburg, etc [2,3]. The high error rates of the viral polymerase enzymes allow viruses to easily adapt and exploit the varying conditions of the environment [4–6]. Among these viruses, CHIKV is an alphavirus which is transmitted in humans by a mosquito vector known as Aedes aegypti and Aedes albopictus. The first outbreak of CHIKV occurred in 1953 from Tanzania (East Africa) and then progressed towards Asia, Europe and America [7]. The first case of Chikungunya was reported in India in 1963 and re-emerged in the country in 2005. Due to the re-emergence of the disease, every region of the country has become endemic [8]. The major clinical symptoms of CHIKV infection are severe joint ache, fatigue, headache, muscle pain, joint swelling, rashes, nausea and high fever [7]. The rising temperatures due to climate change are expected to increase the number of people infected with the Chikungunya fever virus. Since, there are no effective vaccines or antiviral therapies available for
CHIKV, it is believed that the emerging viral disease may continue to cause outbreaks in the future and hence needs immediate medical interventions [9].

CHIKV is a small (70 nm), positive-sense, single-stranded RNA virus (11.8 kb) with 5’ cap and 3’ polyadenylated genome. It has two open reading frames (ORFs). The RNA replicate known as P1234, is encoded by 5’ ORF which is translated and cleaved at three positions to form four essential non-structural proteins (nsPs) namely nsP1, nsP2, nsP3 and nsP4 [10]. The 3’ ORF is a subgenomic positive-stranded mRNA that codes six proteins which includes three major structural proteins (capsid, E1, and E2) [11]. Like other alphaviruses, the CHIKV also exploits the nsPs to perform crucial activities for its infection and pathogenesis [12]. The non-structural protein nsP4 is the viral RNA polymerase that makes new viral RNA from a pre-existing viral RNA template [13,14]. After the replication of viral RNA, nsP1 and nsP2 are responsible for its capping and removal of terminal phosphate from the newly formed viral RNA [15–17]. The C-Terminal domain of nsP2 possesses cysteine protease activity which cleaves and processes non-structural polypeptide, and hence assists in formation of viral replication complex [18]. Structural and functional studies on nsP3 propose that it has three domains: the N-terminus macro domain, which exhibits both ADP hydrolase and RNA binding along with ADP-ribosylhydrolase capabilities. The second domain, is the alphavirus unique domain (AUD), is distinctly present only in the genus alphavirus and the third domain is called C-terminal hypervariable region which is intrinsically disordered [19]. While nsP3 is an essential component of the RNA replication process, its biochemical aspects in the virus life cycle are still under investigations.

Although, currently there are no drug in the market against alphaviruses, it might be useful to target nsP3 using nanomaterials with broad-spectrum antiviral activity [20,21]. The therapeutic potential of nanomaterials has been acknowledged well in literature. They have been shown to exhibit competitive antiviral properties and could potentially help alleviate the side effects of drugs [22–24]. It has been shown that metal and metal oxides nanoparticles have exhibited a significant antiviral effect on various pathogenic viruses. Metal nanoparticles such as Zn, Ag, Au, and Cu as well as metal oxide nanoparticles, such as ZnO,[25] Fe₂O₃, [26] CuO, [27] and TiO₂ are effective antiviral agents against a wide range of viruses such as herpes simplex virus type 1 (HSV-1), bovine herpesvirus-1 (BoHV-1), influenza virus A (H1N1) and human immunodeficiency virus (HIV-1) [28,29]. Nanomaterials have been explored intensively for therapeutic applications due to their unique physical properties such as morphology, dimension, high surface area, charge and high reactivity [30]. In metallic nanoparticles, silver has been widely utilised in biological and medical applications due to its antiseptic and anti-inflammatory properties, as well as its low toxicity. Silver nanoparticles have also shown great potential as an antimicrobial agent as they can work well against bacteria, fungi and viruses [31]. Metal oxide nanoparticles have also shown excellent results in the biomedical field, for example, Ag₂O [32], NiO [33], ZnO [34] and TiO₂[35] in drug delivery, as a skin protectant, coating material, antimicrobial and anticancer agent.

Recently, nanocomposites formed by mixing two or more components to obtain the best properties of each component are also being explored for biological applications. From the literature survey, it is found that noble metal/metal oxide and mixed metal oxide nanocomposites show excellent antimicrobial properties. For example, Ag@Fe₂O₃ [36], Ag/ZnO [37], Ag/Ag₂O/ZnO [38], NiO-ZnO [39], NiO-CdO [40] and NiO-CeO₂-ZnO [41] act against bacteria such as Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis and Pseudomonas aeruginosa. Further, no research on metal/metal oxide and mixed metal oxide-based nanocomposites as an antiviral agent have been reported so far, except PMMA/ZnO–Ag NFs used against coronavirus and the parainfluenza [42]. By reviewing the current properties of metal, metal oxides and their nanocomposites, this study has investigated the inhibitory potential of metal/metal oxide (Ag/NiO) and ternary metal oxide nanocomposites (Ag₂O/NiO/ZnO) with silver metal as one of the components using molecular docking to target the macrodomain of nsP3 and the antiviral potential of these nanocomposites has been
determined against CHIKV.

2. Materials and methods

2.1. Synthesis and characterisation of Ag/NiO and ternary Ag$_2$O/NiO/ZnO nanocomposites

Synthesis and characterization of Ag/NiO nanocomposites [AN-5% (5% Ag), AN-10% (10% Ag), AN-15% (15% Ag) and NiO], and Ag$_2$O/NiO/ZnO [A/N/Z-1 (10% Ag$_2$O), A/N/Z-2 (20% Ag$_2$O), A/N/Z-3 (30% Ag$_2$O) and N/Z] ternary nanocomposites [43,44] was done using the protocol of Bhatia and Nath, 2021 and Bhatia and Nath, 2022 respectively [43,44]. All of the chemicals, solvents and details of methodology used for the characterisation of the nanocomposites are as reported by Bhatia and Nath, 2021 and Bhatia and Nath, 2022 respectively [43,44]. The schematic routes for the synthesis of nanocomposites are illustrated in Fig. 1. Octahedron NiO was prepared by direct calcination of nickel nitrate hexahydrate at 300 °C for 3 h (at a heating rate of 2 °C min$^{-1}$) [43]. Further, three Ag/NiO composites [AN-5% (5% Ag), AN-10% (10% Ag) and AN-15% (15% Ag)] were synthesized as described below: 200 mg (26.77 mM) of octahedron NiO and different amounts of silver nitrate (Supplementary Table ST1) were mixed in 100 mL of water and sonicated for 1 h [43]. Further, trisodium citrate dihydrate (5 mL) and sodium borohydride (5 mL) were introduced under constant nitrogen purging and stirred for 30 min to reduce silver nitrate (in ice bath). After that, the reaction mixture was left at room temperature for 24 h with slow stirring before being centrifuged to collect the product. Finally, a grey powdered product was obtained by drying at 50 °C. The N/Z (NiO/ZnO = 1:1) nanocomposite was synthesized [44] using the sol-gel method with nickel nitrate hexahydrate (581.6 mg, 2 mmoL) and zinc nitrate hexahydrate (595.0 mg, 2 mmoL) as precursors, followed by calcination at 500 °C (at a heating rate of 2 °C min$^{-1}$) for 5 h. To synthesise ternary nanocomposites [44], N/Z nanocomposite (100 mg) and silver nitrate (amount as mentioned in ST2 of the supplementary file) were mixed together in the deionised water (150 mL) by sonication (1 h) followed by the addition of aqueous sodium hydroxide solution dropwise with continuous stirring. After 2 h, the reaction mixture turned brown, and the product was separated by centrifugation and washed with ethanol several times. A rotatory evaporator was used to dry the final product (Ag$_2$O/NiO/ZnO).

All of the synthesised composites were characterised using different spectroscopic techniques. The crystal phases of metal (Ag) and metal oxides (Ag$_2$O, NiO and ZnO) present in nanocomposites were confirmed by X-ray diffraction analysis (XRD). The morphology and particle size calculations of all the composites were performed using scanning electron microscopic (SEM) and transmission electron microscopic (TEM) images (Fig. 1). X-ray photoelectron spectroscopy (XPS) confirmed the constituent elements, their corresponding oxidation states and binding energies in nanocomposites. The specific surface area, pore size and pore volumes of all the composites were determined by Brunauer-Emmett-Teller (BET) analysis.

2.2. Cells and viruses

African green monkey kidney (Vero) cells (NCCS, Pune, India) were propagated at 37°C in 5% CO$_2$ in Dulbecco Modified Eagle's Medium (DMEM; HiMedia, India) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin sulphate (Gibco, USA). The clinical strain of CHIKV propagated in Vero cells monolayer was used for antiviral assay at a multiplicity of infection (MOI) of 1.0.

2.3. Cytotoxicity studies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the cytotoxicity of varying concentrations of nanocomposites on Vero cells.
Nanocomposites were dissolved in phosphate-buffered saline (PBS) to attain final working concentrations of 2.0 µg/mL, 4.0 µg/mL, 8.0 µg/mL, 10.0 µg/mL, 20.0 µg/mL, 40.0 µg/mL, 60.0 µg/mL and 80.0 µg/mL. Before adding nanocomposites to the cells, the diluted nanocomposites were briefly sonicated. Vero cells were treated with varying concentrations of nanocomposites for 48 h in a humidified incubator at 37 °C in 5% CO₂. 100 µL of MTT reagent (5 mg/mL, HiMedia) was added to the cell culture media and kept at 37 °C in 5% CO₂ for 4 h. Later, 100 µL of dimethylsulfoxide (DMSO) (HiMedia) was added to the wells to dissolve formazan crystals in each well. Absorbance at 570 nm was measured using a microplate reader (Cytation3, BioTek instruments). The percentage of metabolically active cells was compared to that of the control (non-treated) cells. Using the formula,
\[
\left( \frac{A_{\text{test}}}{A_{\text{control}}} \right) \times 100; \text{ where } A_{\text{test}} \text{ is the absorbance of the treated sample and } A_{\text{control}} \text{ is the absorbance of non-treated control sample), relative cell viability (\%) was calculated.}
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2.4. Cellular uptake studies using inductively coupled plasma mass spectroscopy (ICP-MS)

AN-5% and A/N/Z-3 nanocomposites uptake/association by Vero cells was measured on ICP-MS Triple Quad (Agilent). Vero cells were seeded onto a 24-well plate at a density of \(1 \times 10^5\) cells per well and incubated overnight at 37 °C with 5% CO\(_2\). Next day, to the Vero cell monolayer, 40 µg/mL of nanocomposites diluted in DMEM were added to the wells, and the plate was kept in the humidified incubator at 37 °C with 5% CO\(_2\) for 36 h. After 36 h, cell supernatant was removed, and cells were washed gently with sterile dH\(_2\)O twice. Next, 700 µL of 2:1 mixture of HNO\(_3\) (65%) and H\(_2\)O\(_2\) (30%) was added to each well of the Vero cell monolayer and digested for 12 h [45]. Finally, 7 mL of milli-Q water was added to dilute the samples, and the results were recorded using ICP-MS Triple Quad (Agilent).

2.5. Molecular docking

Crystal structure of CHIKV nsP3 macrodomain (nsP3MD) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (PDB ID: 6VUQ) [46]. The protein was sequentially optimised in terms of protein chain selection, removal of extra chain subunits, removal of crystallographic waters molecules, ligands, the addition of charges and so on. The AutoDock MGL tools 1.5.6 [47] were used for the complete optimisation of nsP3MD protein structure, and the optimised structure was saved in .pdbqt format for further analysis. The optimised three-dimensional structures of Ag\(_2\)O, NiO, and ZnO were constructed using VESTA 3 software by applying PXRD data, and the final structure file was saved as a .pdb format. After energy minimisation and optimisation of the metal oxides nanoparticles as ligands [48,49]. The .pdb file format was converted into a .pdbqt format using open Babel in PyRx 0.8 algorithm in macOS Mojave workstation (Ligand Preparation Wizard).

AutoDock Vina algorithm [50] was utilised for the ligand to interact with the nsP3MD to evaluate the receptor grid. The grid box for molecular docking aids the ligands to bind in various possible conformations in the specified target site in the nsP3MD protein. The grid box parameters were selected around the catalytic residues of nsP3MD of CHIKV, namely Asp 10, Ile 11, Asn 24, Asp 31, Gly 32, Val 33, Cys 34, Ser 110–Tyr 114, Val 133, and Arg 144 [15,51] (Fig. 5). Grid box was created with the defined centre point coordinates as X = 11.135, Y = 45.464 and Z = -7.041 and box dimensions as 68 Å × 60 Å × 78 Å with spacing 0.375. Other docking and analysis parameters were set as default.

After ligand docking to the nsP3MD of CHIKV, the .pdbqt output files for all three ligands were retrieved from the Vina algorithm. Further, PyMOL 2.3.4 [52] and LIGPLOT+ [53] tools were used to visualise and analyse the protein-ligand interactions.

2.6. Antiviral cell-based assays

For evaluation of antiviral effect of synthesised nanocomposites on CHIKV, nanocomposites were diluted in DMEM to obtain a final working concentration of 4.0 µg/mL, 10.0 µg/mL and 40.0 µg/mL for initial screening. To obtain IC\(_{50}\) value of nanocomposites showing the highest antiviral potential, the concentration was further lowered to 0.5 µg/mL, 2.0 µg/mL, 4.0 µg/mL, 10.0 µg/mL and 20.0 µg/mL. Vero cells were seeded onto a 24-well plate at a density of \(1 \times 10^5\) cells per well and incubated overnight at 37 °C with 5% CO\(_2\). Before adding nanocomposites to the cells, the diluted nanocomposites were briefly sonicated. Vero cells monolayer was infected with CHIKV at MOI of 1.0 for 1.15 h in the humidified incubator at 37 °C with 5% CO\(_2\). After infection, cells were washed twice with 1X PBS to ensure no chance of secondary infection. Later dilutions of nanocomposites prepared in 1X DMEM with 2% FBS were added to the cells infected with the virus and transferred to the humidified incubator.
at 37 °C with 5% CO₂ for 24 h. After 24 h post-infection, the cell supernatant was collected for plaque reduction assay. The cells were washed with 1X PBS twice and harvested to perform qRT-PCR to quantify viral RNA.

2.6.1 Cytopathic effect (CPE) analysis
Vero cells were seeded onto a 24-well plate at 1 × 10⁵ cells per well and incubated overnight at 37 °C with 5% CO₂. To observe the reduction in CPE induced by CHIKV after treatment with varying concentrations of different nanocomposites, monolayer of Vero cells was infected with CHIKV at MOI of 1.0 at for 1.15 h and plate was kept in the humidified incubator at 37 °C with 5% CO₂. Post-infection cells were washed twice with 1X PBS to remove the unbound virus. Later nanocomposites were diluted in DMEM with 2% FBS and added to the cells kept in the humidified incubator at 37 °C with 5% CO₂ for 48 h. After 48 h post-infection, cells were observed for CPE induced by CHIKV, where virus-infected cells without any treatment with nanocomposites were taken as control. The CPE images were obtained on a microscope (Leica, USA) at 10X in a bright field.

2.6.2. Plaque reduction assay
Plaque assay was performed on Vero cells to assess the inhibitory effects of nanocomposites on CHIKV infection and propagation. A 24-well plate was seeded with Vero cells in DMEM with 10% FBS and allowed to attach and grow at 37 °C with 5% CO₂ to reach 90% confluency. Later the cell supernatant was removed, and cells were washed with 1X PBS twice. Virus stock harvested after antiviral assay was serially diluted to 10 folds in DMEM and added to Vero cells monolayer. The plate was kept in the humidified incubator for 1.15 h with gentle mixing after every 15 min for efficient virus attachment onto the Vero cells. Post-infection, the media containing CHIKV was removed, and cells were washed with 1X PBS twice to remove any unbound virus. Next, the cell monolayer was overlaid with media containing 1% carboxymethyl cellulose (CMC) with minimal essential media (MEM) containing 5% FBS and incubated for 72 h at 37 °C with 5% CO₂. After 72 h post incubation, the overlay media was removed gently, and fixing solution (10% formaldehyde) was added in the wells for 1 h with gentle shaking. After 1 h, the fixing solution was discarded, and cells were stained with 1% crystal violet dye to visualise viral plaques. Each plaque assay was performed in triplicates to quantify the reduction in viral titre determined as plaque forming unit per millilitre (pfu/mL), and untreated virus stock was used as a control as described previously[54].

2.6.3 Quantitative real time polymerase chain reaction (qRT-PCR)
Total RNA was isolated from CHIKV-infected Vero cells using TRIzol (RNAiso, TakaraBio) reagent per manufacturer's instruction under RNAse free condition. cDNA synthesis was performed by AccuScript High fidelity 1⁰ stand cDNA kit (Agilent Technologies) using 1µg of RNA and random hexamer primers in a 20 µL reaction according to manufacturer's protocol. Experiments were performed in RNAse-free conditions to avoid any RNase contamination. qRT-PCR was performed to amplify the E1 gene of CHIKV by using KAPA SYBR fast universal qPCR kit and StepOnePlus real-time PCR system (Applied Biosystems, Carlsbad, CA). Fold-reduction in viral RNA in nanocomposite-treated cells relative to the control is plotted as described previously.[55] β-Actin: endogenous control; CHIKV E1: target gene. (***, p< 0.001.) Values are mean and error bars are Standard Deviation (n = 3).

3. Results and discussion
3.1. Synthesis and characterisation of Ag/NiO and ternary Ag₂O/NiO/ZnO nanocomposite
Two steps synthetic route was used for Ag/NiO composites. NiNO₃·6H₂O was directly calcined in a muffle furnace to obtain octahedron NiO with a particle size of 0.63±0.13 µm, as observed by SEM analysis. Synthesized octahedron NiO were anchored with Ag nanoparticles in different molar ratios to
obtain AN-5% (5% Ag), AN-10% (10% Ag) and AN-15% (15% Ag)). The anchored Ag nanoparticles in the AN-5% composite have a particle size of 16.54±1.88 nm [44]. Furthermore, a binary N/Z (NiO:ZnO = 1:1) nanocomposite was synthesised using the sol-gel method and reacted with different molar ratios of silver nitrate and sodium hydroxide to achieve ternary Ag2O/NiO/ZnO nanocomposites i.e., A/N/Z-1 (10% Ag2O), A/N/Z-2 (20% Ag2O), and A/N/Z-3 (30% Ag2O). The particle size of N/Z nanocomposite is 31.59±5.03 nm, and the size of Ag2O nanoparticles in A/N/Z-3 nanocomposite is 5.92±1.36 nm in accordance with the reported data reported by Bhatia and Nath, 2022 [43]. According to the BET model, the surface area of Ag/NiO nanocomposites and octahedron NiO is between 49 and 53 m² g⁻¹, with an average pore size between 2.26 to 2.43 nm [44]. Similarly, Ag2O/NiO/ZnO nanocomposites and N/Z possess surface areas between 17.96 and 20.54 m² g⁻¹ with an average pore diameter between 3.71 to 4.07 nm. XPS investigation confirms the oxidation states of elements in Ag/NiO nanocomposites i.e., Ag⁺, Ni²⁺ and O²⁻ [44]. Further, it is also confirmed Ag⁺, Ni²⁺, Zn²⁺, and O²⁻ states in nanocomposites Ag2O/NiO/ZnO [43]. XRD and XPS studies established the presence of Ag in Ag/NiO and Ag2O in A2O/NiO/ZnO nanocomposites. XRD, XPS, TEM with size and BET images of the most active nanocomposites, i.e., AN-5% (5% Ag) and A/N/Z-3 (30% Ag2O) are reproduced in the supplementary file [43,44].

3.2. Cellular uptake of nanocomposites and potential binding to nsP3

Absolute quantification of the cellular absorption of metal or metal oxide NPs is frequently achieved using ICP-MS. For elemental analysis, it provides great sensitivity and selectivity [56–58]. Cells must often be lysed prior to examination. After the measurement, the absolute uptake per cell can be estimated, taking into account the sample's total number of cells. In this study the uptake/association of AN-5% and A/N/Z-3 nanocomposites by Vero cells was measured by ICP-MS. In AN-5%, cellular uptake/association of Ni and Ag is 62.64% and 4.91%, respectively. The availability of silver is meagre, as silver is just 5% compared to NiO in the AN-5% composite. In the case of A/N/Z-3 nanocomposite, cellular uptake/association of Ni, Zn and Ag is 19.91%, 5.17% and 9.06%, respectively. The ICP-MS results indicate uptake of nanomaterial by cells, which indicates that the nanocomposites are available in the cytoplasm where the virus replication proteins including nsP3 replicate the viral genome. Therefore, these nanocomposites are available during viral genome synthesis to target virus specific enzymes and inhibit the replication of virus.

The ICP-MS cellular uptake results incited to undertake in-silico molecular binding of metal oxide based nanocomposites to the nsP3 protein of CHIKV as the macrodomain of alphavirus nsP3 protein has been reported and evaluated to be a potential molecular target for nanoparticles/nanocomposites [15]. In this study, molecular docking was performed to confirm the binding of A/N/Z-3 nanocomposite to the viral protein sites and identify the best nanocomposite candidates based on the docking scores. To obtain molecular details of A/N/Z-3 nanocomposite interaction with a viral protein, molecular docking analysis of Ag2O, NiO and ZnO targeting the macrodomain of nsP3 protein of CHIKV was performed. The details of the lattice parameter of cubic Ag2O (JCPDS no: 00-041-1104), cubic NiO (JCPDS no: 01-073-1523) and hexagonal ZnO (01-079-0207) are derived from PXRD data. The space group of cubic Ag2O is Pn3m, with a = b = c = 4.7263 Å, α = β = γ = 90° and structure parameter for Ag (¼,¼, ¼) and O (0, 0, 0) [59]. The cubic NiO has Fm3m space group, with a = b = c = 4.18 Å, α = β = γ = 90°, and structure parameter for Ni (0, 0, 0) and O (¼,¼, ¼) [60]. Similarly, P63mc space group of ZnO, with a = b = 3.2568 Å, c = 5.2125 Å, α = β = γ = 120° and structure parameter for Zn (1/3, 2/3, 0) and O (1/3, 2/3, 0.38261) [61]. The three-dimensional structures of metal oxide nanoparticles, i.e., Ag2O, NiO and ZnO, have been created using VESTA software with the coordinates mentioned above.
Fig. 5. Crystal structure of CHIKV nsP3MD (6VUQ) with catalytic residues generated using PyMOL. The protein surface is depicted in cyan, and the key catalytic residues used for docking nanomaterials are highlighted in yellow.

Using the AutoDock Vina algorithm, all three ligands were selected for molecular docking. The obtained binding energy of the three ligands against the catalytic residues of the nsP3MD of CHIKV from the docking is presented in Table 1. Furthermore, the LIGPLOT+ analysis of the three ligands revealed the hydrogen bond (H-bond) and hydrophobic interactions between the ligands and active site residues of nsP3MD (Fig. 6). Based on the interaction of the nsP3MD with different nanoparticles, nickel oxide nanoparticle exhibits the highest binding energy (kcal/mol) when compared to silver oxide and zinc oxide nanoparticles (Table 1). The comparative analysis shows that zinc oxide forms the highest number of hydrogen bonds (H-bond) where the nitrogen (N) atom of the macromolecule binds to the oxygen (O) atom of the ligand with the bond length ranging from -2.84 Å to -3.25 Å. In comparison, nickel oxide nanoparticle shows the highest hydrophobic bonds with the nsP3MD of CHIKV. Moreover, the complete analysis of the hydrogen bond and hydrophobic bond interaction of nanoparticle oxides with the functional groups of nsP3MD of CHIKV is presented in Table 1.

Table 1
Tabular presentation of docked ligands at the catalytic site of CHIKV nsP3MD of CHIKV displaying H-bond, hydrophobic interactions and binding energy.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Binding Energy (kcal/mol)</th>
<th>H-bond</th>
<th>Hydrophobic Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>NiO</td>
<td>-8.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residue No.</td>
<td>No. of Bonds</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gly32, Val33, Pro107, Thr111, Gly112, Cys143, Arg144, Asp145</td>
<td></td>
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</tbody>
</table>
An exhausted literature review survey revealed that metal oxides-based composites have not been tested against the CHIKV. Therefore, Table 2 is a compilation of metal and metal oxide (ZnO) nanoparticles tested for their antiviral activity against CHIKV. Compared to other reported noble metal nanoparticles, Table 2 clearly illustrates that the tested nanocomposites (Ag/NiO and Ag$_2$O/NiO/ZnO) have excellent antiviral activity against CHIKV. Ag$_2$O, ZnO, and NiO as a single metal oxide, have been used as antimicrobial agents in numerous reports. However, due to the synergistic effect, mixing these three metal oxides may play a crucial role in enhancing the properties of nanocomposites.

![Fig. 6](image)

**Fig. 6.** Schematic diagrammatic representation of docked NiO, Ag$_2$O and ZnO nanoparticles in nsP3MD of CHIKV using the LIGPLOT$^+$ and PyMOL analysis tool. In LIGPLOT$^+$, H-bonds are represented by green dotted lines (---) with a distance of Å and hydrophobic interactions are indicated with brown curvature (-----) around the macromolecule residues. In PyMOL, the protein surface is depicted in cyan, and the key interacting residues are highlighted in green sticks.

### 3.3. Evaluation of anti-CHIKV activity
One of the most critical factors that must be considered when developing effective nanomaterials is their non-toxicity. The cytotoxicity effect of varying nanocomposites concentrations was determined by performing an MTT assay on Vero cells. To exclude the likelihood of cell death due to toxicity caused by nanocomposites, cells were grown in 2.0 µg/mL, 4.0 µg/mL, 8.0 µg/mL, 10.0 µg/mL, 20.0 µg/mL, 40.0 µg/mL, 60.0 µg/mL and 80.0 µg/mL of all the nanocomposites for 48 h in the humidified incubator at 37 °C with 5% CO₂. Cell viability decreased to below 80% for concentrations above 60.0 µg/mL for all the nanocomposites. NiO showed >95% cell viability at the maximum dose of 80.0 µg/mL, as depicted in Fig. 2. Cytotoxicity was determined by measuring the absorbance values at 570 nm and the percentage viability of treated and control cells was calculated by using GraphPad Prism software. CC₅₀ for all the nanocomposites was >80.0 µg/mL. An increase in silver nanoparticles in Ag/NiO nanocomposites has not been associated with any discernible trend or major change in cellular toxicity. Similarly, in the Ag₂O/NiO/ZnO series, nanocomposites did not exhibit a significant change in toxicity with an increase in silver oxide concentration. Furthermore, ZnO and colloidal silver have been known to have relatively low toxicity and minimal adverse effects, as previously reported by Jennifer, M. et al [62]. Following the results of cytotoxicity assay, 4.0 µg/mL, 10.0 µg/mL and 40.0 µg/mL concentrations were selected for performing the antiviral assay for initial screening of antiviral potential of N/Z, A/N/Z-1, A/N/Z-2, A/N/Z-3, NiO, AN-5%, AN-10% and AN-15%.

![Graph 2](image.png)

**Fig. 2.** The effect of cytotoxicity of nanocomposites on Vero cells was analysed using MTT assay. The graph represents the percentage viability of Vero cells, treated with different concentrations of A) Ag₂O/NiO/ZnO series: N/Z, A/N/Z-1, A/N/Z-2 and A/N/Z-3, B) Ag/NiO series: NiO, AN-5%, AN-10% and AN-15%. Data shown are mean value ±SD (n = 3, t-test, p-value ≤ 0.01).

The antiviral activity of Ag₂O/NiO/ZnO: N/Z, A/N/Z-1, A/N/Z-2 and A/N/Z-3 and Ag/NiO: NiO, AN-5%, AN-10% and AN-15% are determined by plaque assay on Vero cells infected with CHIKV. Initial screening of the antiviral potential was performed at 4.0 µg/mL, 10.0 µg/mL and 40.0 µg/mL concentrations of nanocomposites. A comparison of CHIKV titre from untreated and treated cells was determined, and the percentage reduction in viral titre was calculated. Based on the results obtained from plaque assay, a significant reduction of viral titre is observed at the highest concentration of 40.0 µg/mL for both Ag₂O/NiO/ZnO and Ag/NiO nanocomposites (Fig. 3B). At 4.0 µg/mL concentration, A/N/Z-3 from Ag₂O/NiO/ZnO series showed 45.0% reduction and AN-5% from Ag/NiO series showed 57.1% in CHIKV titre; therefore, we proceeded ahead with the evaluation of antiviral activity at lower concentration of 0.5 µg/mL, 2.0 µg/mL, 4.0 µg/mL, 7.0 µg/mL, 10.0 µg/mL, 20.0 µg/mL for A/N/Z-3 and AN-5% nanocomposites. Even at the lowest concentration of 0.5 µg/mL, A/N/Z-3 and AN-5%
showed 3.8% and 5.1% reductions in CHIKV titre. IC$_{50}$ determined for A/N/Z-3 and AN-5% are 2.828 and 3.277 µg/ml, respectively (Fig. 4A).

The viral infections caused by alphaviruses are characterised by the rapid induction of cytopathic effect in host cells. CHIKV can infect various vertebrate cell lines, and most of these show an apparent cytopathic effect. Furthermore, studies have shown that CHIKV can induce more substantial apoptosis in Vero cells than in C6/36 cells [63–65]. Decreased CPE post-treatment with nanocomposites were recorded 48 h post-infection and compared to the non-treated Vero cells. Images of CPE were captured in the bright field at 10X using a microscope with an HD camera (Fig. 3A). Consistent with the plaque reduction assay results, qRT-PCR confirmed that the viral mRNA expression levels were reduced significantly after the treatment with 40.0 µg/mL of A/N/Z-3 and AN-5% nanocomposites. Furthermore, A/N/Z-3 and AN-5% showed the best antiviral activity at a lower concentration from a series of Ag$_2$O/NiO/ZnO and Ag/NiO, and respectively, thus they were selected for qRT-PCR analysis to delve deeper into further validation of the antiviral potential of selected nanocomposites (Fig. 4B).

**Fig. 3.** A) Microscopy observation of CHIKV-induced reduction in CPE in Vero cells. Vero cells infected with CHIKV (Virus control) and treated with 40.0 µg/mL of nanocomposites were observed for CPE appearance after 48 h post-infection. CPE Images were taken 48 h post-infection by inverted microscope in a bright field. Scale bar, 100 µm. B) and C) Graphical representation of pfu/mL determined by plaque assay. Inhibition of viral titre is represented as a percentage value compared to the control (non-treated, CHIKV infected) Vero cells. >90% reduction in viral titre is observed at the highest concentration of 40.0 µg/mL for all the nanocomposites. Data shown are mean value ± SD (n = 3, t-test, p-value ≤ 0.01).
Fig. 4. A) Antiviral effect of lower concentrations of AN-5% and A/N/Z-3 determined by plaque assay. Data is represented as a percentage reduction in viral titre compared to control (non-treated, CHIKV infected) Vero cells. B) Reduction in viral titre by AN-5% and A/N/Z-3 nanocomposites were confirmed by qRT-PCR analysis. A significant reduction in fold change is observed after treatment with 40.0 μg/mL of AN-5% and A/N/Z-3 obtained by ∆ct. Data shown are mean value ± SD (n=3, t-test, *** p-value ≤ 0.01, ** p-value ≤ 0.05).

As evident from Table 2 nanocomposites (Ag/NiO and Ag$_2$O/NiO/ZnO) under the present study perform better than other published metal or metal oxide against CHIKV because these nanocomposites have a multi-component system rather than a single-component system. The same rationale has also led to the excellent results of Ag/NiO and Ag$_2$O/NiO/ZnO nanocomposites as heterogeneous catalysts for reducing various pollutants, such as nitrophenols and organic dyes for several cycles [43,44]. Therefore, they can be utilised in many industries such as paper, textile, tanning, and petrochemical for pollutants’ removal in the wastewater treatment, as previously reported [43,44]. Globally, there is panic owing to SARS-CoV-2, and the fear prevails due to new and re-emerging pathogenic viruses such as monkey pox. Considering a clear need to prepare for a future pandemic, our tested nanocomposites can be explored as antiviral agents since several studies on using nanomaterials as effective antiviral agents have highlighted the growing interest in this field [66].

Table 2
Compilation of Various Nanomaterials with Potential Antiviral Activity against the Chikungunya Virus.

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Types of Nanomaterial</th>
<th>Effective concentration</th>
<th>Evaluation of antiviral activity</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ag, Fe and ZnO NPs</td>
<td>0.05 mg/mL, 0.1 mg/mL and 0.2 mg/mL</td>
<td>&gt;90% inhibition (Ag and Fe) at all concentrations evaluated through Plaque assay</td>
<td>[67]</td>
</tr>
<tr>
<td>2.</td>
<td>Ag nanoparticles treated with <em>Psidium guajava</em> extract</td>
<td>15.0 μg/mL (MNTD)</td>
<td>64.40% Vero cell viability observed through TCID$_{50}$ and CPE</td>
<td>[68]</td>
</tr>
</tbody>
</table>
3. **Andrographis paniculata** Ag NPs, **Phyllanthus niruri** Ag NPs, **Tinospora cordifolia** Ag NPs

| 3. | Andrographis paniculata Ag NPs, Phyllanthus niruri Ag NPs, Tinospora cordifolia Ag NPs | 31.25 μg/mL (MNTD) 125.0 μg/mL (MNTD) 250.0 μg/mL (MNTD) | 80.76% 30.56% 75.35% | CPE inhibition | [69] |

4. AN-5% and A/N/Z-3 nanocomposites

| 4. | AN-5% and A/N/Z-3 nanocomposites | 4.0 μg/mL, 10.0 μg/mL and 40.0 μg/mL | >95% antiviral activity at 40 μg/mL observed through Plaque assay and qRT-PCR | Present study |

*MNTD: Maximum Non-Toxic Dose, NPs: Nanoparticles, TCID\textsubscript{50}: Tissue Culture Infectious Dose*

### 4. Conclusion and future prospective:

The prevalence of Chikungunya is a significant public health concern in the country. Despite its relatively low mortality rate, the disease can still affect the quality of life for the individuals. Currently, there is no licensed vaccine or antivirals for treating Chikungunya disease. The alarming increase in the number of cases of Chikungunya in the last decade due to virus re-emergence has prompted us to focus on exploring the scope of nanocomposites as antivirals. ICP-MS and \textit{in silico} docking studies validated and confirmed the interaction of nanocomposites with the virus and viral replication protein nsp3. Although we have shown through \textit{in silico} docking studies that the nanocomposites are potentially interacting and inhibiting CHIKV nsp3 protein functionality, it should be emphasised that it does not limit the potential use of a single drug to treat multiple viral infections at different stages of its life cycle. This is advantageous in virology, where rapid and unforeseen infections can occur. The potential \textit{in vitro} antiviral activity of Ag/NiO and Ag\textsubscript{2}O/NiO/ZnO nanocomposites against the chikungunya virus has been investigated for the first time in this study. Cell viability was examined over a broad range, from 2.0 to 80.0 μg/mL, and good viability was seen up to 60.0 μg/mL for all the nanocomposites. The best antiviral outcomes among all the examined nanocomposites were demonstrated by AN-5% and A/N/Z-3, with >95% inhibition of the virus at a concentration of 40.0 μg/mL. This study is a significant step forward towards developing effective antiviral drugs from nanocomposites.

### Credit authorship contribution statement:

Pooja Bhatia: data curation, formal analysis, writing - original draft, validation. Vedita Anand Singh: data curation, formal analysis, writing - original draft, validation. Ruchi Rani: data curation. Mala Nath: methodology, writing - review & editing, supervision. Shailly Tomar: conceptualization, methodology, writing - review & editing, supervision.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### 5. References


