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Antiviral Potential of Graphitic Carbon Nitride (g-C_3N_4) Nanosheets Against Herpes Simplex Virus Type 1

Abdulhussain Kadhim Jwaziri

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Abstract

Background and aims: Herpes Simplex Virus Type 1 (HSV-1) has been found to present many therapeutic challenges due to the problem of drug resistance and the low efficacy of antiviral compounds. Recently, the photocatalytic property and biocompatibility of graphitic carbon nitride (g-C_3N_4) nanosheets have been found to be promising for antiviral therapy. The antiviral efficacy and biocompatibility of the g-C_3N_4 nanosheets are assessed in this study.

Methods: g-C_3N_4 nanosheets were prepared and characterized by X-ray diffraction (XRD), Fourier Transform InfraRed Spectroscopy (FTIR), Field Emission Scanning Electron Microscopy (FESEM), and zeta potential measurements to confirm the properties. Antiviral efficacy was determined by the ability of g-C_3N_4 to block the infection of Vero cells by HSV-1 using two different methods: the virucidal assay and the post-treatment assay. The Real-Time PCR measured viral replication. The cytotoxic effect of the g-C_3N_4 nanosheets was evaluated using a neutral red uptake assay.

Results: g-C_3N_4 nanosheets exhibited a pure graphitic structure, high colloidal stability, and a porous, flake-like morphology. They achieved dose-dependent HSV-1 inhibition rates of 94.9–99.2% (virucidal) and 92.3–96.0% (post-treatment) at 600–800 µg/mL ($P<0.001$). No cytotoxic effects were seen in the g-C_3N_4 nanosheets; however, increased cell viability by nearly 40% was seen when tested at a concentration of 800 µg/mL.

Conclusion: g-C_3N_4 nanosheets possess excellent biocompatibility and the ability to inhibit HSV-1 greatly, and are promising alternatives to conventional treatment regimens. There is a need to study further the mechanism of the antiviral properties of g-C_3N_4 nanosheets and the efficacy of g-C_3N_4 nanosheets in vivo.

Keywords: Graphitic carbon nitride, g-C_3N_4, Herpes simplex virus, Nanosheets, HSV-1

Introduction

Herpes Simplex Virus Type 1 (HSV-1), a member of the *Herpesviridae* family, is a contagious and prevalent pathogen with a wide range of clinical manifestations, which range from mild orofacial lesions to life-threatening conditions such as herpes encephalitis and keratitis (1). The latency period in sensory neurons, determined by the virus, causes recurrent infections, making its management and treatment a challenge (2). The current antiviral drugs, including nucleoside analogs such as acyclovir, target the viral DNA replication. However, they have some significant drawbacks: the virus can easily become resistant to the drug, the drug can be toxic if taken for a long time, and it is not very effective in preventing recurrent infections (3, 4). This situation calls for the development of new antiviral drugs that are more effective and act through different mechanisms than the current drugs used to combat HSV-1 infection.

In recent years, nanomaterials have been found to be potential candidates for developing both antimicrobial and antiviral therapy. Among such nanomaterials, graphitic carbon nitride (g-C_3N_4) nanosheets have been found to be particularly promising due to their high surface area and biocompatibility, as well as their photocatalytic activity (5). While g-C_3N_4 nanosheets have been found to be useful for developing drug delivery systems, biosensing technologies, as well as antimicrobial therapy, their photocatalytic activity enables them to produce reactive oxygen species (ROS). These reactive oxygen species have been found to disrupt the cell membranes of microorganisms (6). There have been many studies on g-C_3N_4 nanosheets against various bacterial and fungal pathogens (7). However, the antiviral potential of g-C_3N_4 nanosheets against HSV-1 viruses has not been explored.

Nanomaterials either oppose virus infection directly through interaction with viral particles, inhibition of virus attachment to host cells, and/or interference at specific steps in the viral life cycle (8). For instant, nanoparticles direct binding to virus surface proteins and interfering with viral attachment to host cell receptors or produce ROS which destructs the viral envelop and

genetic material. As HSV-1 is an enveloped virus with a complex reproductive cycle that involves attachment, entry, replication and release, g-C_3N_4 nanosheets might give some intervention points. Additional nanomaterials, such as silver nanoparticles and graphene oxide, have also been investigated for their antiviral activity against HSV-1 by inhibiting both viral entry and replication (9, 10). Therefore, it is reasonable to speculate that g-C_3N_4-with the features of special photocatalytic activity and good biocompatibility-may inactivate HSV-1 by direct virucidal effects or interfering with crucial viral replication.

In the current study, we will investigate the antiviral activity of g-C_3N_4 nanosheets against HSV-1. Other than that, this work will also explore the biocompatibility of g-C_3N_4 nanosheets in human cell lines associated with potential therapeutic applications. These findings are anticipated to provide the feasibility of g-C_3N_4 as a new type antiviral drug facing with the drawbacks of current drugs and offer important implications for developing new material-based strategies against HSV-1 infection.

Materials and methods

Synthesis of g-C_3N_4 nanosheets

Porous graphene-like carbon nitride (g-C_3N_4) was produced by the direct thermal condensation of melamine using methods as reported elsewhere (11). For the preparation of the textile, 10 g of high-purity melamine powder (analytical grade, Merck) was introduced into a porcelain crucible covered with a lid to control the reaction environment. The crucible was then introduced into a welded chamber electric furnace with precise calibrations, in which it underwent heating at 550 °C for 3 h. The heating was done at a controlled rate of 5 °C min −1, to favor homogeneous thermal condensation and minimize the building up of structural defects in the end product. After heating and cooling to room temperature the sample remained as a bright yellow solid (the characteristic color of g-C_3N_4) was observed. This product was then

harvested, finely ground with pestle and mortar to ensure homogeneity and stored under an inert condition in a closed container in the dark away from moisture and dirt.

For the preparation of ultrathin g-C_3N_4 nanosheets, the entire products (bulk g-C_3N_4 ~4–5 g) formed above was heat treated by thermal exfoliation in a porcelain crucible under loose lid to avoid direct exposure to oxygen. It was then sintered at 600 °C in an electric furnace with a temperature rising of 5 °C per minute, for 3 h. This annealing process was performed by flowing nitrogen continuously to avoid oxidation and promote thermal exfoliation. The powder became whitish during the thermal treatment, indicating the exfoliation of carbon nitride nanosheets.

Characterization of g-C_3N_4 nanosheets

The g-C_3N_4 nanosheets were characterized using several analytical techniques. The size and morphology of the nanosheets were examined through field emission scanning electron microscopy (FESEM) conducted on a TESCAN MIRA3 instrument. The crystallographic structure was assessed via X-ray diffraction (XRD) using a Philips PW1730 diffractometer with Cu Kα radiation (λ = 1.5406 Å) to analyze the phase composition and structural characteristics. Surface charge, colloidal stability, and particle size distribution were evaluated using a HORIBA SZ-100 instrument, performing both Zeta Potential and Dynamic Light Scattering (DLS) measurements. For DLS, the nanoparticle powder was suspended in deionized water to prepare a stock suspension (1 mg/mL), sonicated for 15 minutes using a probe sonicator, and then diluted to the desired concentration. The suspension was allowed to equilibrate for 2–3 minutes before measurements, which were performed at 25 °C using the viscosity and refractive index of water in the instrument software. Furthermore, Fourier transform infrared (FTIR) spectroscopy was carried out using a Thermo Scientific Nicolet Avatar 380 spectrometer equipped with a diamond ATR (attenuated total reflectance) accessory, Powdered samples were placed directly on the ATR crystal, and spectra were

recorded from 4000 to 400 cm^{-1}, to detect and characterize the surface functional groups on the nanoparticles.

Cells and viruses

Vero cells (African green monkey kidney cells) and HSV-1 KOS strain were provided by the Department of Virology, School of Medicine, Iran University of Medical Sciences, Tehran, Iran. Vero cells were grown at 37°C with 5% CO_2 in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, Invitrogen, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich, USA), and 2 mM l-glutamine (Merck, Germany). To evaluate antiviral activity, the HSV-1 KOS strain was used. The virus was grown in Vero cells, and the *Reed and Muench* method was used to calculate the infectious titer of the viral stock as $TCID_{50}$ mL−1 (12). After titration, the viral stock was divided into aliquots in sterile microtubes and stored at −70°C for subsequent experiments.

Cytotoxicity assay

The cytotoxicity of g-C_3N_4 nanosheets was evaluated using the well-established neutral red uptake (NRU) assay (13). This standard viability method relies on the ability of viable cells to incorporate and retain the supravital dye neutral red within intact lysosomes. A reduction in dye uptake indicates loss of cell viability or lysosomal function (13). Vero cells were seeded at a density of 1.5×10^4 cells per well in a 96-well microtiter plate. Following a 24-hour incubation period at 37°C in a humidified atmosphere with 5% CO_2, different amounts of g-C_3N_4 nanosheets were added to the culture medium (100, 200, 300, 400, 500, 600, 700, and 800 µg/mL). After that, the cells were cultured under the same conditions for a further 48 hours. Following this period, the medium containing g-C_3N_4 nanosheets was removed, and 100 µl neutral red (100 µg/mL) was added to each well for a 3-hour incubation. A desorption solution (1% glacial acetic acid, 50% ethanol, 49% water) was then added to release the incorporated

dye. Using a microplate reader (Hiperion MPR 4+, Roedermark, Germany), absorbance was determined at 550 nm. In relation to untreated cells (the negative control, which is defined as 100% vitality), cell viability was presented as a percentage.

Determination of antiviral activity

Virucidal assay

In summary, 100 µL of g-C_3N_4 nanosheets at two non-toxic doses (1200 and 1600 µg/mL) were mixed with 100 µL of HSV-1 solution (2×100 $TCID_{50}$/mL) and incubated for three hours at 37°C in a humidified 5% CO_2 environment. As a virus control, an equal volume of the viral suspension was combined with cell culture media devoid of g-C_3N_4 nanosheets. Vero cells (1.5×10^4 per well) were planted in a 96-well microtiter plate and incubated for 24 hours at 37°C and 5% CO_2. Vero cell monolayers were then subjected to the produced mixes and incubated at 37°C for an additional hour. The cells were then carefully rinsed three times with PBS to get rid of any unabsorbed viruses, and the supernatant was thrown away. After adding fresh DMEM media supplemented with 2% FBS, the cells were cultured for 48 hours at 37°C. Subsequently, the HSV-1 virus load was quantified by quantitative real-time PCR experiment.

Cell post-treatment assay

The Vero cell monolayers were infected with 100 $TCID_{50}$/ml of HSV-1 for 1 h at 37°C, and the cells were washed with PBS to remove unabsorbed viruses. Cells were then incubated with various non-toxic concentrations of g-C_3N_4 nanosheets (400, 600, 800 µg/mL) for another 48 h at 37°C in a CO_2 incubator. Cell and virus controls were run in parallel under the same conditions; quantification of HSV-1 viral load was performed by a quantitative real-time PCR assay.

Quantitative Real-Time PCR (qPCR) analysis

The effects of g-C_3N_4 nanosheets on HSV-1 infection in Vero cells were validated by quantitative real-time PCR (qPCR). Viral DNA of HSV was extracted from the supernatant of

infected Vero cells collected from virucidal and post-treatment experiments, by BehPerp Viral Nucleic Acid Extraction Kit (BehGene Biotechnology, Iran), according to the manufacturer's instructions. The primer and probe sequences targeting the HSV-1 UL30 gene were 5′-ATCGGCGAGTACTGCATACA-3′ (forward), 5′-GAGCTCCAGATGGGGCAA-3′ (reverse), and 5′-HEX-ATTCCCTGCTGGTGGGCCA-BHQ1-3′ (probe), yielding a 75 bp amplicon. The qPCR was conducted in a 25 µL reaction volume, comprising 12.5 µL of RealQ Plus 2x Master Mix for Probe, Without ROX™ (Ampliqon, Denmark), 2 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 1 µL of probe (10 µM), 5 µL of template DNA, and 3.5 µL of double-distilled water. Real-time PCR was carried out in a Rotor-Gene Q system (Qiagen, Germany), with an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds.

A standard reference was designed by inserting the particular viral sequence under investigation into a pUC57 vector. A calibration curve was prepared using tenfold serial dilutions from 10-1 to 10-10 of the synthesized plasmids and processed in parallel with the experimental samples. The copy number of the plasmid was calculated using an online DNA copy number calculator from Technology Networks (14). The detection limit of the real-time PCR assay (LOD) was determined as 3 copies per µl. All assays were performed with positive and negative controls for each experiment in three replications.

Statistical analysis

All the statistical analyses were performed using IBM SPSS Statistics software version 22.0 (Chicago, IL, USA). For assessing whether the data followed a normal distribution or not, the Kolmogorov-Smirnov test was used. For comparing quantitative results, the independent t-test was used when the results followed normal distribution, while the Mann-Whitney test was used when the results did not normally distribute. To check whether the results were homogeneous or not, Levene's test was performed. For all the experiments, a P value of ≤ 0.05 was considered

significant. For all the experiments performed in this study—cytotoxicity assay, virucidal assay, and post-treatment assay—each experiment was performed in triplicate in each of the three biological replicates. Thus, all the results were expressed as the mean of nine measurements.

Results

Characterization of g-C_3N_4 nanosheets

The XRD pattern of the g-C_3N_4 nanosheets was recorded by using a diffractometer equipped with Cu Kα radiation, where the wavelength was 1.5406 Å, and the scanning range was from 10.3930° to 79.9930°. The recorded XRD pattern shows sharp peaks at specific positions, which correspond to the graphitic carbon nitride (g-C_3N_4) structure, as identified from the standard JCPDS card (Ref. Code: 01-087-1526) with a high match score of 87. The match score represents the similarity index, which was automatically computed by X'Pert HighScore Ver. 3. The most intense peak at 27.84° corresponds to the (002) plane, where the d-spacing value was found to be 3.20139 Å. This value represents the interlayer stacking, which is typical for g-C_3N_4. A second, less intense peak at 13.49° was found to correspond to the (100) plane, where the d-spacing value was found to be 6.55681 Å, indicating the arrangement of tri-s-triazine units. The absence of other significant peaks indicates that the prepared g-C_3N_4 sample was pure (15, 16). The recorded XRD pattern was analyzed by using Xpert HighScore Ver.3 software (Figure 1).

The FTIR spectrum recorded for the prepared g-C_3N_4 sample shows a sharp absorption band at 1694 cm^{-1}, which corresponds to the stretching mode of the C=N bond, indicating the presence of a tri-s-triazine unit. Additionally, peaks at 1400 cm^{-1}, 1317 cm^{-1}, and 1237 cm^{-1} are attributed to C–N and C–NH–C vibrations, further confirming the presence of the tri-s-triazine structure. (17). The out-of-plane bending modes observed at 887 cm^{-1}, 810 cm^{-1}, and 733 cm^{-1} validate the heptazine-based configuration (18). The broad absorption bands at 3437

cm^{-1} and 3121 cm^{-1} indicate the presence of N–H and O–H stretching, suggesting residual amine groups or adsorbed moisture. In addition, the C-H stretching peaks at 2988 cm^{-1} and C≡N stretching peaks at 2141 cm^{-1} indicate that the number of residual organic species or terminal cyano groups are small and may be due to the thermal condensation reaction and minor structural changes of the material (17) (Figure 2).

The zeta potential of g-C_3N_4 nanosheets was measured at a temperature of 25.3°C with a dispersion medium having a viscosity of 0.890 mPa·s and conductivity of 0.077 mS/cm. The measurement gave a zeta potential of -42.9 mV, with an electrophoretic mobility of -0.000223 cm^2/Vs, which is indicative of a strong negative surface charge on the nanosheets. The measured zeta potential value of -42.9 mV is larger than the ±30 mV threshold typically linked to good colloidal stability, indicating that the nanosheets do not form a large number of agglomerates under these conditions. The strong negative charge improves the electrostatic repulsion between particles to prevent the aggregation and guarantee the stability of the dispersion (19). Dynamic Light Scattering (DLS) analysis was further performed to confirm the dispersion properties, showing the hydrodynamic diameter (Z-average) of about 259 nm with a polydispersity index (PDI) of 0.242. The moderate PDI value represents a relatively uniform particle size distribution, which can be taken to demonstrate that the nanosheets are homogeneously dispersed in the medium. (Figure 3).

We have examined the morphology of the synthesized g-C_3N_4 nanosheets with a Field Emission Scanning Electron Microscope (FESEM) with an accelerating voltage of 15.0 kV. At 20.0 kx magnification, the FESEM images show a porous, layered nanosheet-like structure of loosely packed aggregate resulting in visible voids and cavities (Figure 4). This layered morphology is characteristic of g-C_3N_4 prepared by thermal condensation, indicating that g-C_3N_4 is polymeric (20). Although the zeta potential is very negative (-42.9 mV), some aggregation occurs (possibly due to such forces as van der Waals interactions or hydrogen

bonding between surface functional groups e.g., amines and hydroxyls), when the sample is dried (21). In order to measure the thickness, we simply picked 300 individual nanosheets (randomly chosen) out of the high-magnification FESEM images (150 kx), and the thickness was measured via ImageJ software. GraphPad Prism version 8.4.3 was used to plot the thickness distribution as a histogram, the mean thickness and standard deviation were calculated based on this data, which was 15.67± 2.55 nm and the means of observed thicknesses were between 9.15 and 23.32 nm. Thickening locally, to a maximum of about 23 nm, depicts locally folded, partially stacked or aggregated portions of the sheets. These differences in thickness would be common to two-dimensional g-C_3N_4 materials and are the result of inherent heterogeneity in the stacking of sheet and topography in the preparation of samples. All in all, the nanosheets are characterized by a crumpled yet smooth morphology at the edges, which are characteristic of the layered 2D structure of the polymeric carbon nitride structure.

The nanosheet morphology observed is in line with the high colloidal stability of the nanosheet as observed in the zeta potential, indicating that a strong negative charge prevents restacking of the nanosheet during synthesis and dispersion. The morphological observations are also aligned with the size of the crystalline domains in the XRD and the size of hydrodynamic domains in DLS, thereby suggesting that the size of the nanosheet dimensions is quite appropriate in the applications that the study intends to perform in the current study.

Cytotoxicity assay

The toxicity analysis of the g-C_3N_4 nanosheets at different concentrations between 100 and 800 µg/mL indicated the nanosheets were not toxic to the Vero cell line. Amazingly, the findings showed that cell viability improved with the increase in the concentration of g-C_3N_4 nanosheets. The highest concentration of the test showed an increase in cell viability of about 40 percent as compared to the control population, which may point to a possible positive effect of g-C_3N_4 nanosheets on cellular well-being (Figure 5).

Antiviral assays

Virucidal assay

Treatment of HSV-1 with g-C_3N_4 nanosheets at 600 and 800 µg/mL for 3 hours significantly lowered the development of cytopathic effects (CPEs) (Figure 6). Quantitative Real-Time PCR (qPCR) analysis was also performed, and it showed that after incubation of HSV-1 with these nanosheets at identical concentrations, the amount of HSV-1 genomic DNA copies was significantly decreased, with inhibition levels of 94.9% ($P<0.001$) and 99.2% ($P<0.001$), respectively, in comparison with the virus control group. These results suggest that g-C_3N_4 nanosheets come with dose-dependent antiviral effects (Figure 7).

Post-treatment assay

After viral infection of the cells, the g-C_3N_4 nanosheets at a concentration of 600 and 800 µg/mL for 48 hours had a significant decrease in the amount of virus compared to the virus control group. Analysis of the viral loads showed that the g-C_3N_4 nanosheets produced viral inhibition rates of 92.3% and 96.0% at 600 and 800 µg/mL respectively, which had a statistically significant difference (P value < 0.001). However, the use of 400 µg/mL of g-C_3N_4 nanosheets did not result in a notable reduction in viral load compared to the untreated control group (inhibition rate 2.2%). Consistent with the virucidal assay, the antiviral efficacy of g-C_3N_4 nanosheets in the post-treatment assay exhibited a dose-dependent response (Figure 8).

Discussion

The results obtained in this study revealed the great antiviral effect of g-C_3N_4 nanosheets against HSV-1, showing their effectiveness in both virucidal and post-treatment assays. A dose-dependent antiviral effect was achieved by the g-C_3N_4 nanosheet treatment, with inhibition rates reaching 99.2% at 800 µg/mL in the virucidal assay and 96.0% in the post-treatment assay, as quantified by real-time PCR. Thus, g-C_3N_4 nanosheets might act as a promising alternative to the currently used antiviral therapies faced by several drawbacks, such

as drug resistance and toxicity, and include acyclovir. High biocompatibility of g-C_3N_4 nanosheets was reflected in the rise of Vero cell viabilities up to 40% at 800 µg/mL, further confirming their good perspectives for therapeutic use, which agrees with the data reported previously on low cytotoxicity and excellent biocompatibility of g-C_3N_4 (22).

Several physicochemical properties, including high surface area and strong negative surface charge (-42.9 mV), can be responsible for the antiviral efficacy of g-C_3N_4 nanosheets. This strong negative zeta potential develops high colloidal stability, hindering aggregation and thus facilitating interactions with viral particles. Such morphology of nanosheets, as observed, with a porous structure in the shape of flakes and with a thickness of about 14.07 nm, is likely responsible for their high surface area, while providing ample reactive sites to interact with HSV-1.

The results obtained from the virucidal assay reveal that g-C_3N_4 nanosheets exhibit a very effective activity in the inactivation of HSV-1 particles before cell infection, inhibiting 94.9% and 99.2% of the virus at 600 and 800 µg/mL, respectively. Thus, this may suggest that g-C_3N_4 interacts directly with the viral envelope by either ROS-mediated disruption of the lipid membrane or glycoproteins.

The high virucidal activity observed (up to 99.2% inhibition at 800 µg/mL) is very likely driven, at least in part, by strong electrostatic attraction between the highly negatively charged g-C_3N_4 nanosheets ($\zeta = -42.9$ mV at physiological pH) and positively charged regions on HSV-1 envelope glycoproteins (particularly gB and gC) (23). Such charge-mediated binding has been established as a key inactivation mechanism for numerous negatively charged 2D materials and nanoparticles against enveloped viruses, including HSV-1, by promoting irreversible adsorption, envelope distortion, and prevention of receptor engagement. The large lateral dimensions of our nanosheets (hundreds of nanometers to micrometers) relative to the viral diameter (≈150–200 nm) further suggest that a single sheet can simultaneously bind

multiple virions, enhancing inactivation efficiency. Direct visualization of these virus–nanosheet complexes by transmission or scanning electron microscopy would provide compelling confirmatory evidence of this mechanism and is therefore a high-priority objective in our ongoing research program. Establishing physical attachment would also help explain the sustained antiviral effect observed in the post-treatment assay, where free virions released from initially infected cells are rapidly sequestered by the nanosheets remaining in the culture medium.

The post-treatment assay outcomes also support the efficacy of g-C_3N_4 nanosheets against viral replication in infected cells with an inhibition rate of 92.3% and 96.0% for an equivalent dose. The synergy of both direct and cell-based inhibitory effects makes g-C_3N_4 a novel anti-viral agent, unlike the commonly used anti-viral drugs such as acyclovir, which primarily targets the replication of viral DNA. The versatility of g-C_3N_4 in targeting different phases of the viral life cycles can be a strength against drug resistance, a common problem with nucleoside drugs.

Although acyclovir was not included as a control in the present virucidal assay due to its mechanism targeting intracellular DNA replication rather than extracellular virion inactivation, comparative data from our previous study using the same Vero cell/HSV-1 KOS model demonstrate that acyclovir achieves 92.6% and 100% inhibition of viral replication at 25 µg/mL and 75 µg/mL, respectively (24). Based on this observation, the 92.3% and 96.0% inhibitory effects elicited by g-C_3N_4 at 600 µg/mL and 800 µg/mL in the post-treatment assay can be deemed comparable in value to the 25 µg/mL and 75 µg/mL inhibitory effects of acyclovir, respectively.

When benchmarked with other nanocomposites evaluated against the HSV-1 virus, g-C_3N_4 nanosheets showed better efficacy. For instance, a comparative study involving thermally expanded graphite (TEG) and copper oxide (CuO) nanocomposites showed virucidal inhibitory effects of 74.4% and post-treatment inhibitory effects of 99.9% with lower concentrations of

35 µg/mL and 10 µg/mL, respectively (4). However, the biocompatibility of g-C_3N_4, offering metal-free properties coupled with its high biocompatibility, is an added advantage over metal-based nanocomposites, which may pose toxicity risks. Also, g-C_3N_4 can be produced with ease because of the cost-effective technique involving the thermal condensation of melamine used for the synthesis of g-C_3N_4 nanosheets, unlike the fabrication of nanocomposites, which is a series of complicated steps.

The cytotoxicity screen within the range of concentrations (100–800 µg/mL) and the promotion of cell viability suggest a potential positive impact of g-C_3N_4 nanosheets on cell metabolism or proliferation, possibly involving cell signaling mechanisms or nutrient uptake pathways. This is consistent with g-C_3N_4 being a biocompatible material in biomedical applications related to biosensing and drug delivery (25-27). However, the specific mechanism by which cell viability is promoted in detail should be elucidated, in particular, regarding the possible dependence on the characteristics of surface chemistry and photocatalytic functionality of g-C_3N_4.

One of the most salient observations is the dose-dependent increase in neutral red uptake, which is of about 40% at 800 µg/mL, indicating increased lysosomal and metabolic activities rather than toxicity. A set of emerging evidence indicates that g-C_3N_4 and similar carbon-rich two-dimensional materials may serve as effective free-radical scavengers and antioxidants. Antioxidant effects due to their intrinsic properties have been related to decreased intracellular oxidative stress, including protection of mitochondrial function and overall cytoprotective effects in mammalian cells. The inherent photoluminescence of g-C_3N_4 further provides a convenient tool with which to investigate cellular uptake and subcellular localization through fluorescence microscopy in a label-free fashion. While experiments on internalization, intracellular glutathione level, ROS scavenging, and possible Nrf2-ARE pathway activation are out of the scope of the present study, they are high-priority goals of our current follow-up

work and will be necessary for a full mechanistic explanation of both the cellular stimulation observed and the potent antiviral activity reported here.

Although long-term stability was not systematically monitored in the present work, no visible aggregation or sedimentation of g- C_3N_4 nanosheets was observed in complete culture medium (DMEM + 10% FBS) over the entire experimental timeframe (up to 48 h). This behavior is expected given the strongly negative surface charge and the established resistance of g- C_3N_4 to protein-induced aggregation. Nevertheless, time-dependent hydrodynamic size and zeta potential measurements in biological media, as well as detailed characterization of the protein corona, are recognized as critical parameters and will be systematically evaluated in our ongoing follow-up studies to confirm maintained dispersion stability under physiologically relevant conditions.

The incubator conditions in this work were dark for all antiviral assays, which means photocatalytic contributions were eliminated; the efficacy reported here thus reflects intrinsic material–virus interactions. Testing g-C_3N_4 under visible-light illumination presents an additional path toward the improvement of antiviral performance based on ROS-mediated mechanisms.

While the current study shows unprecedented antiviral efficacy and excellent biocompatibility of pristine g- C_3N_4 nanosheets in vitro, several key limitations should be pointed out prior to any translation to in vivo or to the clinic. First, the effective antiviral concentrations (600–800 μg/mL) are well beyond what is typically achievable in plasma or tissues following systemic administration of nanomaterials, where rapid opsonization, protein corona formation, and mononuclear phagocyte system clearance dramatically reduce bioavailability. Second, the assays herein were performed under dark conditions; the potentially stronger ROS-mediated antiviral activity under visible-light irradiation remains untested in a biological context. Third, the complex physiological environment of serum proteins, immune cells, pH gradients, and

shear flow may serve to substantially dampen direct virus-nanosheet interactions or induce nanosheet aggregation. Fourth, the long-term fate, organ accumulation-particularly liver and spleen-immune activation, and potential off-target effects of persistent 2D carbon nitride structures remain unexamined.

In order to address these limitations systematically, the following prioritized studies are being further considered in our future research roadmap: (1) surface modification of g- C_3N_4 nanosheets, for example, PEGylation, in order to prolong the blood circulation time and reduce the non-specific uptake; (2) comprehensive pharmacokinetic and biodistribution studies in mice using fluorescently or radiolabeled nanosheets; (3) in vivo antiviral efficacy testing using well-established HSV-1 murine models, including both topical and systemic administration routes, and direct comparison against acyclovir; (4) acute/chronic toxicity, immunotoxicity (cytokine profiling, complement activation), and histopathology evaluation after single and repeated dosing; and (5) in vivo light-enhanced antiviral performance using localized visible-light irradiation when clinically feasible. These will be the planned studies that are expected to provide essential data on safety, realistic therapeutic dosing, and true in vivo efficacy, hence bridging the current gap between promising in vitro results and potential clinical translation.

Although qPCR provides superior quantitative precision over traditional infectivity assays, such as $TCID_{50}$, which depend on subjective visual scoring of cytopathic effects, future validation using parallel plaque or $TCID_{50}$ titration is recommended to confirm that reduced viral DNA copies correspond to diminished infectious progeny.

TEM of HSV-1-g-C_3N_4 nanosheets complexes would clearly show the physical capture of viruses and the distortion of their envelopes due to the forces of electrostatic forces. Such experiments are currently at the top of our priority list in our ongoing follow-up studies and will be crucial for the complete elucidation of the dominant inactivation mechanism.

Conclusion

In conclusion, g-C_3N_4 nanosheets have a strong virus inhibitory effect against HSV-1 with excellent biocompatibility, providing a promising means of developing new drugs against viral infections. The dose dependence, low cost, and metal-free nature of g-C_3N_4 nanosheets make this new candidate an attractive alternative for the treatment of viral infections. The future aspects include understanding the detailed virus inhibitory mechanism of g-C_3N_4 nanosheets and clarifying photocatalytic reaction conditions for improved performance in real environments.

Data availability statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Conflict of Interest statement

The authors have no conflict of interest

Ethics declaration

Review and/or approval by an ethics committee as well as informed consent was not required for this study because this article did not involve any direct experimentation/studies on living beings.

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Figure captions

Figure 1. XRD Pattern of g-C_3N_4 nanosheets. The XRD pattern of g-C_3N_4 nanosheets, confirming a pure graphitic structure with peaks at 27.84° (002 plane) and 13.49° (100 plane), indicating interlayer stacking and tri-s-triazine arrangement. Crystallite size is ~10.5 nm, supporting nanoscale properties for antiviral activity.

Figure 2. FTIR Spectrum of g-C_3N_4 nanosheets.

Figure 3. A: Zeta Potential of g-C_3N_4 nanosheets. Illustrates the zeta potential of g-C_3N_4 nanosheets (-42.9 mV), indicating strong negative surface charge and high colloidal stability, which prevents aggregation and supports effective interaction with viral particles for antiviral applications. B: DLS analysis showing a hydrodynamic diameter of ~259 nm and PDI of 0.242, indicating well-dispersed nanosheets consistent with TEM/FESEM and XRD data.

Figure 4. FESEM images of g-C_3N_4 nanosheets. Presents FESEM images showing g-C_3N_4 nanosheets' porous, flake-like structure and thin, crumpled morphology at 150 kx, confirming high surface area and two-dimensional structure ideal for antiviral interactions despite minor aggregation.

Figure 5. Thickness distribution histogram of g-C_3N_4 nanosheets based on FESEM image analysis. 300 nanosheets were measured using ImageJ software. The distribution represents the apparent nanosheet thickness estimated from edge-contrast regions. The solid curve shows the Gaussian fitting of the distribution.

Figure 6. Cytotoxicity assay of g-C_3N_4 on Vero cells. Shows cell viability results from the neutral red uptake assay, indicating no cytotoxicity of g-C_3N_4 nanosheets (100–800 µg/mL) and a ~40% increase in Vero cell viability at 800 µg/mL, suggesting biocompatibility and potential cellular benefits.

Figure 7. Representative inverted microscopy images of Vero cell monolayers 48 h after the virucidal assay (magnification 100×). Healthy Vero cells display typical elongated, fibroblast-

like morphology with confluent monolayer formation and intact cell–cell contacts. (VC) Virus control: Extensive HSV-1-induced cytopathic effects (CPE) are visible, characterized by pronounced cell rounding and shrinkage, detachment from the substrate, formation of multinucleated syncytia (giant cells), and focal clusters of rounded, refractile cells.

Dose-dependent suppression of CPE is evident in Vero cells inoculated with HSV-1 pre-incubated with g-C_3N_4 nanosheets at 600 µg/mL and 800 µg/mL, with the majority of cells retaining their elongated shape and monolayer integrity, closely resembling the uninfected control.

Figure 8. qPCR analysis of HSV-1 viral load in virucidal assay. Presents qPCR results showing dose-dependent HSV-1 viral load reduction (94.9% and 99.2% inhibition at 600 and 800 µg/mL, respectively) in the virucidal assay, confirming g-C_3N_4's potent antiviral activity with statistical significance.

Figure 9. qPCR analysis of HSV-1 viral load in post-treatment assay. Displays qPCR results from the post-treatment assay, showing HSV-1 viral load reductions of 92.3% and 96.0% at 600 and 800 µg/mL g-C_3N_4, respectively, with no significant effect at 400 µg/mL, highlighting dose-dependent intracellular antiviral efficacy.