

Hard Nanomaterials in Time of Viral Pandemics

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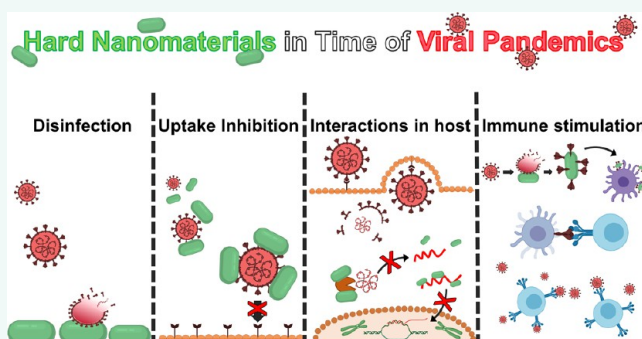
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ABSTRACT: The SARS-Cov-2 pandemic has spread worldwide during 2020, setting up an uncertain start of this decade. The measures to contain infection taken by many governments have been extremely severe by imposing home lockdown and industrial production shutdown, making this the biggest crisis since the second world war. Additionally, the continuous colonization of wild natural lands may touch unknown virus reservoirs, causing the spread of epidemics. Apart from SARS-Cov-2, the recent history has seen the spread of several viral pandemics such as H2N2 and H3N3 flu, HIV, and SARS, while MERS and Ebola viruses are considered still in a prepandemic phase. Hard nanomaterials (HNMs) have been recently used as antimicrobial agents, potentially being next-generation drugs to fight viral infections. HNMs can block infection at early (disinfection, entrance inhibition) and middle (inside the host cells) stages and are also able to mitigate the immune response. This review is focused on the application of HNMs as antiviral agents. In particular, mechanisms of actions, biological outputs, and limitations for each HNM will be systematically presented and analyzed from a material chemistry point-of-view. The antiviral activity will be discussed in the context of the different pandemic viruses. We acknowledge that HNM antiviral research is still at its early stage, however, we believe that this field will rapidly blossom in the next period.

KEYWORDS: nanoparticles, biomaterials, antiviral, immune system, vaccine, virucidal, surface chemistry, phototherapy



The current emergence caused by SARS-Cov-2 is dramatically changing the everyday life of all of us. Due to the high globalization, new viruses can spread all over the world much faster than ever, infecting the communities worldwide. The current technologies and measurements are able to sensibly slow down the infection spread. However, their cost is tremendously high, impacting the healthcare systems and causing the shutdown of industries and the lockdown of the population. The impact of SARS-Cov-2 on the worldwide economy is estimated to be a 2–3% decline, making this the biggest crisis since the world wars.¹ A possible vaccine is hopefully expected to come in 1–2 years, originating a buffer period pretty much uncertain for many people. Vaccination is the only way known to accelerate the flock immunity without causing further death by this pandemic. Contemporary history has seen the spread of other viral pandemics such as H2N2 flu (1956–1958), H3N3 flu (1968), HIV (peak reached between 2005 and 2012), SARS (2009), while MERS (2012 to now) and Ebola (1975 to now) viruses are in a prepandemic phase. The continuous colonization of wild nature lands may touch unknown virus

reservoirs causing the spread of contagious epidemics. Due to these facts, there is a clear urgency in the development of viral treatments to avoid the risk of new pandemics.² In particular, the possibility to have smart antiviral tools able to efficiently disinfect surfaces, block the viral spreading, enhance the survival of infected people, and boost immunization are highly desirable. In particular, more investments in the next years are expected in the antiviral research.

Hard nanomaterials (HNMs) have been extensively studied for many types of applications including drug delivery, bioimaging, and biosensing.^{3–5} The use of nanomaterials in biomedical research is highly developed, reaching in some cases clinical approval.⁶ Despite that, nanomaterials have been mainly developed for cancer therapy, while scarce attention has

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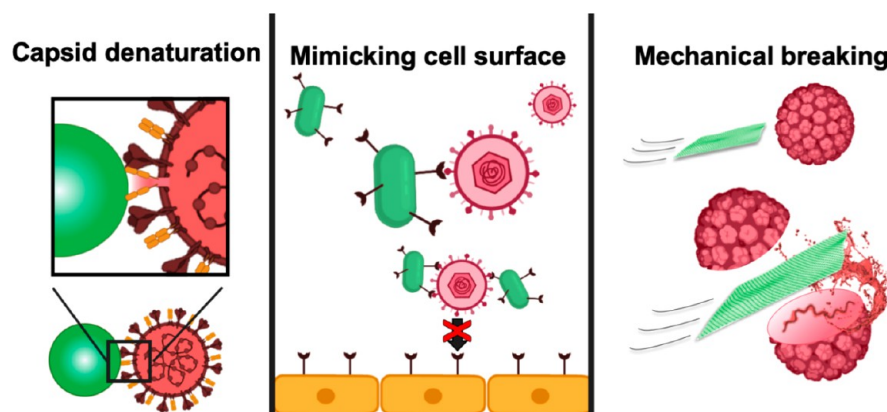


Figure 1. Illustration of the main mechanisms of blocking virus entry into host cells: capsid denaturation, mimicking cell surface, and mechanical breaking of the virus.

been spent on their application in viral infections.⁷ The continuous virology research has more and more increased into viral replication machinery, allowing the preparation and rationalization of more sophisticated vaccine formulations and viral inhibitors. The use of HNMs may be one of the keys to provide more effective biomedical agents with a wide spectrum of activity in viral pandemics.⁸

An increasing number of reports describe how HNMs can be successfully applied to block viral spread. HNMs can be used at different stages of viral infection: blocking viral entry, hampering interaction with infected host cells, and modulating immune responses. Due to their core composition (e.g., metal oxides, noble metals), HNMs can have an important antiviral activity inactivating some specific proteins of the capsid or dysregulating radical homeostasis in the virus particles. Additionally, surface functionalization can sensibly increase HNM antiviral activity, enabling the mimicking of host cells or enhancing the targeting efficiency.⁹

In this review, we will critically analyze different strategies for the application of HNMs as antiviral agents. The rational and the synthetic strategies will be highlighted and correlated to different relevant examples. Particular attention will be paid on the mechanisms of antiviral action and on HNM applicability and efficacy. For the sake of clarity, this review is divided into three parts, namely: blocking viral entry, antiviral activity in host cells, and stimulation of immune system. We have focused on the different stages of infection. In the section dedicated to blocking viral entry, the application of HNMs for surface disinfection and inactivation of the virus prior to interaction with host cells will be described. Subsequently, the interaction of HNMs after internalization into host cells will be addressed, stressing their antiviral delivery features and their activity in viral replication blockage, leading to host cell survival. Then, activation of immune response induced by HNMs, triggering the innate and the adaptive (e.g., nanovaccines) immunity, will be presented. The different adopted strategies will be correlated to the nanomaterial core (e.g., composition, size, shape) and surface (e.g., chemistry, surface charge) properties. Finally, limits (e.g., unknown long-term toxicity), advantages (e.g., high and wide spectrum virucidal activity), and perspectives will be discussed with particular attention to the applications in viral pandemics (e.g., HIV, SARS, and influenza viruses). This review is addressed to material and biomaterials scientists who are interested in antiviral research. We acknowledge that

application of HNMs as antiviral agents is still in the early stages; however, we believe that the research on this topic is going to grow soon. Thus, with this contribution we genuinely hope to inspire researchers in the preparation of smart and efficient HNM antiviral agents.

BLOCKING VIRAL ENTRY

The last decades have been characterized by increasing investigation on viruses. In particular, their surface charge, protein composition, and host cell entry mechanism have been elucidated, allowing to formulate the first-generation wide spectrum antivirals. Blocking the viral entry is one of the most common known antimicrobial procedure to stop infections at the early stage. In this context, antiviral materials have been used for surface disinfection or for epidemic limitation in humans and animals. Due to their high surface to volume ratio, composition, and tunable surface chemistry, HNMs are now more and more studied as powerful agents in blocking viral entry. As for other biological interactions, the attachment and entry of viruses into host cells are mediated by multivalent interactions between the surface of the virus and cell surface receptors.¹⁰ Nanomaterials can display multivalency that makes them able to compete with the host cells on virus attachment, limiting their infectivity. The mechanism of antiviral actions relies on the inactivation of the capsid proteins. As a matter of fact, HNMs have been used for: (1) blocking target proteins for viral entry, (2) capsid protein oxidation, (3) mimicking cell surface, and (4) mechanical rupture of viruses (Figure 1). These strategies target proteins and mechanisms of entry common in most of the viruses, thus allowing the preparation of wide spectrum antiviral agents. In this section, the application of different HNMs as powerful inhibitors of viral entry will be discussed.

Noble Nanoparticles. Noble nanoparticles (NPs), made of gold and silver, are attractive as antiviral agents for their surface functionalization versatility and their capacity to cleave disulfide bonds. Their use in disinfection has been extensively studied for different types of viruses. The morphology and the size of the NPs play a crucial role in their ability to efficiently interact with the capsids and in their toxicity for the organism. These nanomaterials are characterized by a very large specific surface area (inversely proportional to the particle diameter). As the particle size becomes smaller and smaller, the percentage of surface atoms increases, creating many unsaturated bonds due to lack of neighboring atoms. As a

consequence, AgNPs and AuNPs have unstable atoms with high surface energy. This kind of structure provides a lot of contact adsorption sites and reaction points for further modifications. These chemical features allow to easily combine surface NP atoms with other atoms through chemical bonds.

Besides the composition of the metal core, several studies have pointed out the importance of the control of the surface chemistry. The surface groups can: (1) stabilize NPs in the biological media, (2) insert targeting agents, and (3) enhance the circulation time inside the body. Antiviral efficiency can be also enhanced by the multivalency effect, where highly branched ligands are used to locally augment the local concentration of the targeting molecules. In this section the main strategies and results for silver (AgNPs) and gold (AuNPs) nanoparticles in blocking viral entry will be critically discussed.

Silver Nanoparticles. Many studies have shown that naked AgNPs have a good effect on the control and prevention of a variety of viral diseases (Table 1). However, the antiviral mechanism of nanosilver is still unclear. The antiviral action is associated with the following mechanisms: Nanosilver can prevent the virus from entering the host cells and inhibit the virus from binding to the cell receptor, thereby stopping the virus from infecting the targeted cells. AgNPs may be able to bind the viral surface protein and inhibit the interaction between the virus and the cell membrane receptors (Figure 2, left). However, it has been also reported that AgNPs can inactivate the virus through denaturation of surface proteins containing cysteine and methionine residues present on the viral capsid, in a similar way reported for bacteria. For example, AgNPs smaller than 10 nm were shown to interact with the sulfur-bearing residues of gp120 glycoprotein knobs distributed on the lipid membrane of HIV-1 virus, preventing the virus from binding to CD4 receptor site on the host cells, thus inhibiting the viral infection.¹¹ By means of a viral adsorption assay, it was shown that the AgNP mechanism of anti-HIV action is based on the inhibition of the initial stages of the HIV-1 cycle. To demonstrate that the antiviral effect of AgNPs is due to the particle structure rather than to silver ions present in solution, the antiviral activity of silver sulfadiazine (AgSD) and silver nitrate (known antibacterial silver salts) was evaluated. Both salts showed a much lower therapeutic index than AgNPs *in vitro*, indicating that silver ions themselves are less efficient.¹² These results point out that the antiviral efficacy is not only related to the dose of Ag⁺ ions present in solution but is also regulated by different other parameters (*e.g.*, size, charge, and surface functionalization) associated with the nanosize dimension. For instance, in the case of Herpesviridae and Paramyxoviridae viruses (both enveloped viruses with embedded viral-encoded glycoproteins), AgNPs can effectively reduce their infectivity, by blocking the interaction between the viral particles and the host cells with an antiviral activity strictly dependent on the size and ζ potential of the AgNPs. As a general observation, it was reported that smaller nanoparticles have better antiviral effect. This effect was associated with the increase of the surface area, where smaller-sized AgNPs could bind more efficiently to the viral particles exerting a higher antiviral activity.¹³ Another study reported the impairment of Peste des petits ruminants virus (PPRV) replication after incubating infectious viral particles with AgNPs, which did not exhibit any virucidal effect even up to 900 $\mu\text{g/mL}$. This result suggested that the anti-PPRV activity of the AgNPs is due to the inhibitory effect

Table 1. Antiviral AgNPs and Their Possible Mechanisms of Action

virus	shape	size (nm)	active concentration	mechanism of action	ref
HIV-1	spherical	1–10	25 $\mu\text{g/mL}$	interaction with gp120	11
HIV-1 III _B	—	30–50	440 $\mu\text{g/mL}$	interaction with gp120	19
HSV-1, HSV-2, and HPIV-3	—	20–50	not available	possible interaction directly with the viral envelope or its protein	20
Adenovirus type 3	spherical	5–18	25 $\mu\text{g/mL}$	direct destruction of virus particles and DNA structure	15
H1N1 influenza A virus	spherical	5–20	12.5 $\mu\text{g/mL}$	inhibition of respiratory enzymes and electron transport components and interference with DNA function	21
HBV	spherical	10–50	5 μM	interaction with double-stranded DNA and/or binding with viral particles	16
PPRV	spherical	5–30	11.1 $\mu\text{g/mL}$	interaction with virus surface and core	12
Vaccinia virus	spherical	25	not available	preventing viral entry into host cells	13
Monkey pox virus (MPV)	—	10–80	12.5 $\mu\text{g/mL}$	blocking virus-host cell binding and penetration	22
Tacaribe virus (TCRV)	—	5–10	25 $\mu\text{g/mL}$	inactivation of virus particles before entry	23
Poliovirus	spherical	4–9	3.1 ppm	preventing viral particles from binding to the receptors of RD cells	24
TGEV	spherical	<20	12.5 $\mu\text{g/mL}$	direct interaction with TGEV surface protein, such as TGEV S glycoprotein	18
	linear	60000–80000			
	linear	20000–30000			

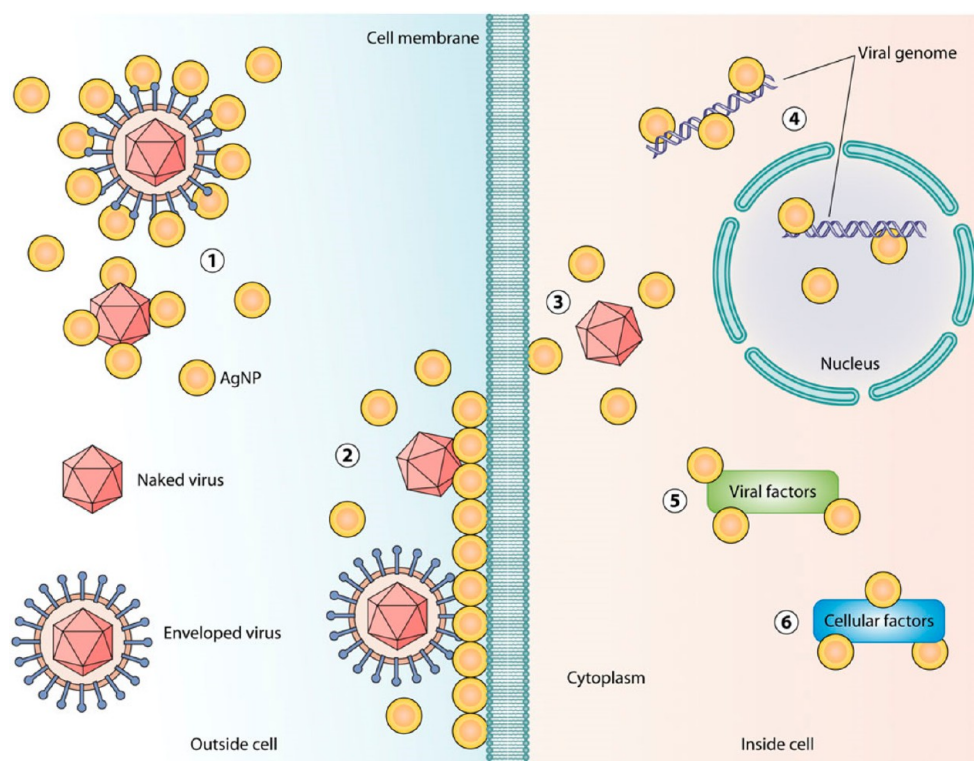


Figure 2. Potential antiviral mechanism of AgNPs. (1) AgNPs interact with viral envelope and/or viral surface proteins; (2) AgNPs interact with cell membranes and block viral penetration; (3) AgNPs block cellular pathways of viral entry; (4) AgNPs interact with viral genome; (5) AgNPs interact with viral factors necessary for viral replication; and (6) AgNPs interact with cellular factors necessary for productive viral replication. Reproduced with permission from ref 14. Copyright 2016 Taylor and Francis.

Table 2. Surface-Modified Antiviral AgNPs and Possible Mechanisms of Action

virus	shape	size (nm)	coating	mechanism of action	ref
HIV-1	spherical	1–10	foamy carbon, PVP and BSA	interaction with gp120	11
RSV	spherical	–	PVP, BSA, and recombinant F protein (RF 412)	interaction with the G-protein on the virus surface	26
H1N1 influenza virus	spherical	2–5	Oseltamivir Amantadine Zanamivir inhibition of accumulation of reactive oxygen species (ROS)	inhibition of the activity of neuraminidase and hemagglutinin	34 35 36
HAV, NoV and CoxB ₄	spherical	–	polyphosphonium-oligochitosans	preventing viral attachment and penetration	29
MPV	spherical	10–80	polysaccharide	blocking virus-host cell binding and penetration	22
TCRV	spherical	10	polysaccharide	inactivation of virus particles prior to entry	23
HSV-1	spherical	4	mercaptoethanesulfonate	competition for the binding of the virus to the cell	30
Enterovirus 71 (EV71)	spherical	2–5	PEI and antiviral siRNA	inhibition of the accumulation of ROS and activation of AKT and p53	37
HSV-2	spherical	13, 33, 46	tannic acid	direct interaction and blocking of virus attachment, penetration and spread	28

on viral replication in the target cells. AgNPs do not prevent the binding of PPRV to host cells, but inhibit the entry of viruses into these cells. AgNPs can also interact with the surface and core of PPRV, but this interaction cannot kill the virus directly.¹² The same results were then confirmed on other viruses. AgNPs with a diameter of 25 nm inhibited Vaccinia virus replication by preventing viral entry into host cells. However, AgNPs cannot prevent the virus from adsorbing onto the cells, and this virus is still infectious, indicating that AgNPs lack a direct virus-killing effect.¹³

Alternatively, nanosilver can be combined with viral nucleic acids to change the capsid structure, affect the replication of

viral genetic material, and make the virus inactive. For example, TEM analyses have shown that NPs can cause a change of the structure of the Ad3 virus from a hexahedral shape to an irregular shape, destroying its fibers and capsid proteins, leading to inhibition of the virus from binding to the host cells and destroying the DNA structure, preventing adenoviral infection.¹⁵ Nanosilver can also bind directly to the double-stranded DNA of hepatitis B virus to inhibit its replication.¹⁶

In other studies, it has been demonstrated that silver ions released from nanosilver can directly damage the viruses. Based in this property, an interesting application has been proposed. AgNPs were used as a coating on polyurethane condoms,

effectively inhibiting the activity of HIV and herpes simplex virus (HSV). The hypothesized mechanism is that silver ions are transferred directly from oxidized NPs to biological targets, such as viral membrane proteins gp120 and gp41. In addition, a small amount of silver ion is also released from the coated contraceptives to improve the antiviral level.¹⁷

Although the studies on naked AgNPs to reduce viral infectivity have shown their potential as broad-spectrum antiviral agents, the understanding of the specific antiviral action mechanism still needs to be elucidated in depth. Many studies have shown that the antiviral performance of naked AgNPs is related to their size, and smaller nanoparticles have better antiviral activities.¹⁶ In addition to particle size, the antiviral action of AgNP morphology has also attracted interest to fight against coronavirus. AgNPs and two types of silver nanowires were able to significantly cause an inhibitory effect on coronavirus transmissible gastroenteritis (TGEV)-induced host cell infection and TGEV replication. The mechanism is likely based on a direct interaction of AgNPs with TGEV surface proteins (e.g., TGEV glycoproteins) to inhibit the beginning of viral infection. It is possible that AgNPs and Ag nanowires alter the structure of some surface proteins of TGEV and then inhibit their recognition and adhesion to the cellular receptor PAPN.¹⁸

Although the potential of AgNPs as antiviral agents has been commonly recognized, unfortunately, their wide biological applications are limited by the risks of self-aggregation and environmental pollution. Silver ions can be released from the surface of AgNPs and potentially pollute the environment, and their agglomeration into bulkier particles or fibers may change their biological characteristics, diminishing the antiviral effect. In several cases, it has been reported that naked AgNPs may affect human health.²⁵ Therefore, research and development of AgNPs whose surface is modified or stabilized by protecting molecular layers is an urgent need to overcome these problems (Table 2). Poly(*N*-vinyl-2-pyrrolidone) (PVP) is the most commonly used stabilizer of AgNPs. The PVP-coated AgNPs are able to inhibit the activities of HIV-1, herpes simplex 2 virus (HSV-2), and respiratory syncytial virus (RSV).^{11,26,27} But compared to foamy carbon, small-sized PVP and BSA-coated AgNPs showed poor antiviral activity to the HIV-1 virus.¹¹ For RSV, PVP-coated AgNPs have a specific binding capacity to the viral surface, evidencing a regular spatial arrangement and a clear interaction with G-protein.²⁶ In addition, to improve the stability of AgNPs, their surface modification with antiviral drugs was proved to reduce the drug resistance caused by the drugs administered alone. Tannic acid-modified AgNPs showed good antiviral effects on HSV-2 infection *in vitro* and *in vivo*. The viral infection was inhibited only when these NPs directly interacted with HSV-2 virions. Indeed, the pretreatment of host cells with such AgNPs did inhibit the entry of HSV-2. Due to the high affinity of tannins to proteins and sugars, tannic acid can bind glycoproteins on the surface of viruses to make them inert, impairing glycoprotein function and preventing viruses from attaching and entering host cells.²⁸

The surface modification can also exert a synergistic antiviral effect. AgNPs decorated with polyphosphonium-oligochitosan (PQPOC) exhibited moderate to excellent antiviral activity against HAV, NoV, and CoxB₄. In addition, AgNPs could interact with the virion glycoproteins and prevent viral attachment and penetration. PQPOC can also serve as an effective virus inhibitor by blocking the interaction of the

targeted virus with the host through the electrostatic interaction between the cationic polymers and the negatively charged binding sites of the virus.²⁹

Surface-modified AgNPs can also prevent viral infection by competitive adsorption on host cells. The process of infection of cells by herpes simplex virus type 1 (HSV-1) involves the interaction between viral envelope glycoproteins and heparan sulfate (HS) on cell surface. Therefore, researchers designed AgNPs capped with mercaptoethanesulfonate (Ag-MES) to compete with the cellular HS through the sulfonate end groups, thereby blocking the virus from entering the cells.³⁰

A few years ago, it was shown that curcumin could prevent the replication and the budding of RSV,³¹ but the disadvantage of poor solubility and low bioavailability limited its clinical application.³² Curcumin was used as a reducing and capping agent to prepare stable curcumin AgNPs (cAgNPs) under physiological conditions. cAgNPs could reduce cytopathic effects induced by RSV and showed efficient antiviral activity against infection by directly inactivating the virus prior to entry into the host cells. Its antiviral effect was higher than curcumin alone or unmodified AgNPs (Figure 3).³³

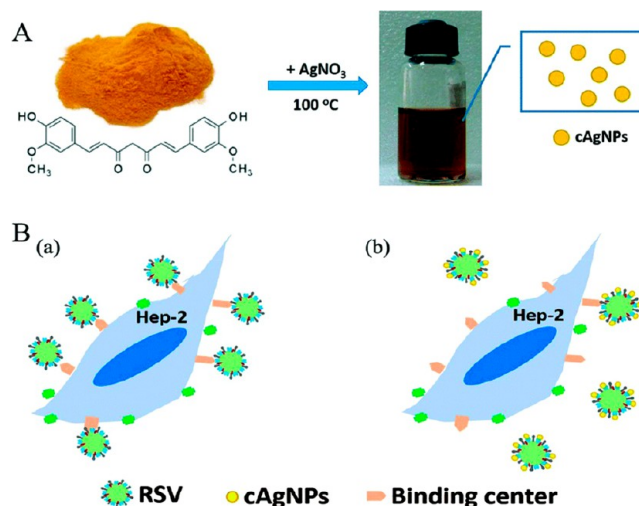


Figure 3. Schematic representation of the synthesis of cAgNPs (A) and a proposed inhibition mode of cAgNPs against RSV infection (B). The inhibition mode of (B) shows that cAgNPs can reduce the binding ability of virus with the binding centers on the surface of cells (b) as compared to those without cAgNPs (a). Reproduced with permission from ref 33. Copyright 2016 Royal Chemistry Society.

Alternatively, Zhu *et al.* prepared AgNPs surface-modified with oseltamivir, amantadine, and zanamivir (Ag@OTV,³⁴ Ag@AM,³⁵ and Ag@ZNV³⁶), by chemical methods. The results showed that these nanoparticles can directly interact with the virions, resulting in viral function damages.

Overall different studies have reported the capacity of AgNPs to block viral entry. However, there is not a concerted antiviral mechanism, but their activity differs from case to case, based on viral particle adsorption, capsid structure alteration, or surface protein denaturation. For AgNPs, the antiviral activity can be associated with different parameters including size, shape, surface charge, and functionalization but also to the topical release of silver ions able to disturb the viral cycle replication. As described before, bare AgNPs can be used as disinfectant agents, however their use in biological media is

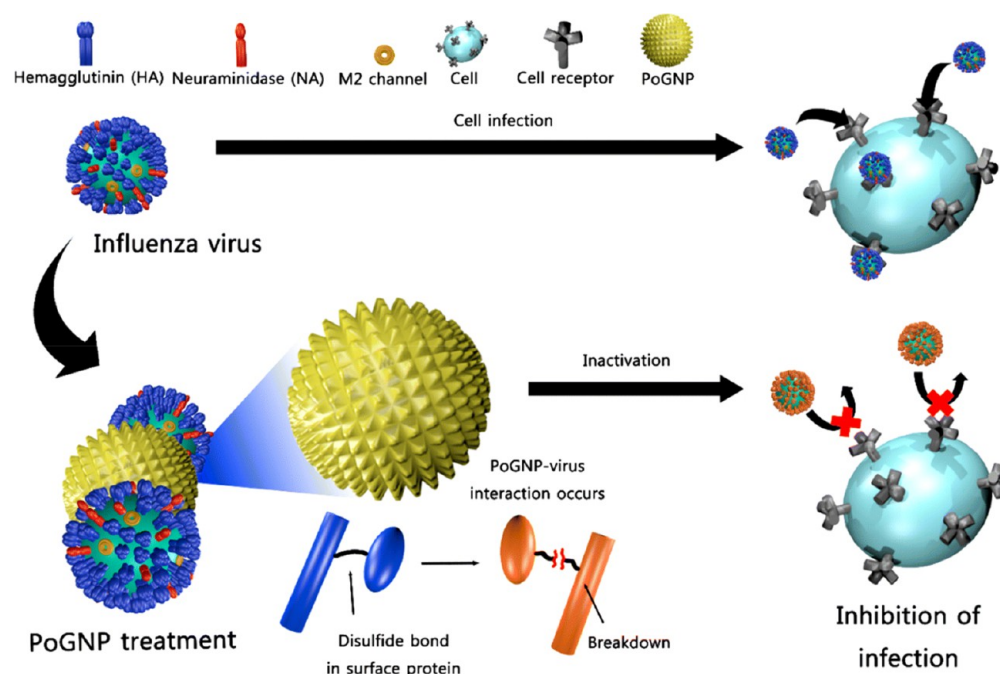


Figure 4. Schematic illustration of inactivation of influenza A virus (IAV) treated with porous AuNP (PoGNP). PoGNP interacts with IAV surface proteins and cleaves their disulfide bonds. Inactivated viruses exhibit lower infectivity to cells. Reproduced with permission under a Creative Commons CC-BY license from ref 39. Copyright 2020 BioMed Central Ltd., Springer Nature.

limited by their low colloidal stability and potential cytotoxicity. Surface functionalization can alleviate cytotoxicity, but it can also mask the nanoparticle surface, reducing their affinity for viral particles, thus reducing AgNP antiviral activity. For these reasons, AgNPs at the moment could find application mainly for surface disinfection and for topical administration. Further studies are needed to prepare safer AgNP formulations for systemic administration. In particular, the clarification of the antiviral mechanisms and the use of surface functional groups able to stabilize AgNPs in biological fluids without affecting their prominent antiviral activity are probably the most important challenges to tackle.

Gold Nanoparticles. Compared to AgNPs, AuNPs exhibit reduced toxicity on healthy cells, making them more attractive for *in vivo* and clinical applications.³⁸ Indeed, AuNPs have been successfully tested as inhibitors of viral entry into the host cells. AuNPs interact with hemagglutinin (HA), where Au is able to oxidize the disulfide bond of this glycoprotein causing its inactivation, thus impeding the membrane fusion of the virus with host cells. Targeting HA has emerged as an alternative strategy to the actual therapies (e.g., matrix protein 2 and neuraminidase), especially to pandemic viruses that show an accelerated mutation speed of their surface proteins, hence a resistance to conventional treatments increasing their infectivity and mortality.³⁸ This strategy has been applied to influenza (e.g., H1N1, HCV) and herpes viruses.^{39–44} The activity of AuNPs is proportional to the surface area exposed. As a consequence, the size and the morphology of these metal NPs play a substantial role in their antiviral activity. Recently, Kim *et al.* have reported that porous AuNPs are able to inhibit influenza A infection more efficiently than nonporous AuNPs.³⁹ This effect has been associated with the higher surface area of the porous material that favors their interaction with capsids and thus increases their antiviral activity (Figure 4).

Besides the *per se* antiviral activity, AuNP surface modifications have been developed in order to enhance their overall therapeutic benefits. The engineering of tailored AuNPs with selected ligands has allowed the preparation of efficient antiviral nanoagents. The target ligands can be introduced directly during the particle synthesis *via* ligand exchange reactions or ligand modifications. For instance, direct reduction of gold ions in the presence of gallic acid produced homogeneous AuNPs able to sensibly reduce herpes simplex virus infection *in vitro*.⁴⁰ Compared to free ligand NPs, functionalized AuNPs benefit from the multivalency effect and higher circulation times, decreasing the needed therapeutic concentrations.³⁹ Functionalized AuNPs can present organic groups that mimic host cell surfaces or other specific molecular patterns that selectively target the virus. Normally, negative charges are used to mimic cell surfaces and favor the interaction between the particles and the capsid. In particular, sulfonates and organic sulfates have been used for their capacity to attract the virus *via* capsid protein interaction and block the HA activity.⁴¹ AuNPs functionalized with sulfonates showed an increasing inhibition of influenza A compared to the nanoparticles capped with succinic acid.⁴² This study also demonstrated that there is not a correlation between the negative charge and the antiviral activity, but instead the inhibition depends mainly on the organic groups used. Thiol-capped AuNPs also displayed powerful inactivation of bovine viral diarrhea virus *in vitro*.⁴³ Multivalency has been exploited in more complex systems using dendrons as capping agents. This strategy allows to generate higher concentrations of the target ligand in close proximity to the AuNPs and to increase the binding efficiency of the nanoparticles to the capsid. The driving force of the antiviral efficiency relies on the concentration of the targeting agent onto the particles. Sulfonated dendrons were grafted to AuNPs *via* a sulfide bond and tested for HIV inhibition.⁴⁴ The results showed that

the decorated AuNPs exerted a higher affinity to the virus. Additionally, comparing AuNPs functionalized with different generation dendrons, those with a third generation displayed the highest inhibition performance with an IC_{50} below 0.1 $\mu\text{mol/mL}$, thus making them attractive for *in vivo* translation. It is worth noting that the inhibition efficiency is strictly dependent on the available sulfonate groups present on the surface of the NPs, making crucial a thorough characterization of the material.⁴⁴ The size of the AuNPs clearly plays an important role in the concentration of targeting ligands exposed per particle.⁴⁵ Indeed, too big NPs have a limited surface area, while too small would not allow an efficient grafting of the dendrons due to steric hindrance. For instance, it has been shown that dendron-functionalized AuNPs showed a size-dependent antiviral activity for influenza virus, where 14 nm particles exhibited a higher efficiency than 2 nm AuNPs. This has been associated with the low functionalization grade of the small nanoparticles and to the inappropriate spatial distribution of the interacting ligand/receptor pairs.

The development of viral proteomics has profoundly transformed the antiviral and disinfection strategies. In particular, small molecules and peptides able to target and block the viral biochemical machinery have been developed. However, despite these efforts into the drug design, many of these molecules suffer from poor biological effect, low concentration in the diseased areas, and undesired side effects. In this context, AuNPs have been coupled to biologically inactive small molecules to create biologically active multi-valent AuNP therapeutics. A bright example has been reported by Bowman *et al.*, where the authors functionalized AuNPs with SDC-1721, a small membrane fusion inhibitor of HIV.⁴⁶ The results demonstrated that, while pure SDC-1721 has low activity, functionalized AuNPs are able to inhibit HIV replication at μM concentrations. Similar results have been reported using targeting peptides. In particular, it was evidenced that the functionalized AuNPs can sensibly reduce the IC_{50} up to 2 orders of magnitude compared to pure peptides.⁴⁷ Preliminary results *in vivo* confirmed the biosafety of the AuNPs.⁴⁸

Nanoparticles Generating Reactive Oxygen Species.

One of the main advantages of using NPs compared to oxidized metals relies on the slow release of ions and clusters from these particles, leading to an enhancement of the antiviral activity. Additionally, the use of metal NPs containing Cu or Fe in ionic form catalyzes the generation of radicals *via* Fenton and Fenton-like reactions oxidizing the capsid proteins and consequently blocking the viral infection at early stage. For instance, copper ions (derived from sulfates or iodide salts) have been widely used as antiviral agents because of their activity on several kinds of enveloped and non-enveloped viruses including influenza virus,^{49–51} herpes simplex virus^{52–54} and hepatitis A virus.⁵⁵ Their mechanism of action relies on the formation of Cu^+ ions (from soluble salts or nanoparticles) that generate hydroxyl radicals.⁵⁶ The use of metallic copper nanostructures in the form of particles or sheets has shown only a moderate efficiency due to the low concentration and low release of Cu^+ .⁵⁶ For these reasons, Cu^+ salts, where the copper ions are readily present in their active monocationic form, have been favored. In particular, CuI nanoparticles (stable at room temperature) have been extensively studied for deactivation of feline calicivirus⁵⁶ and H1N1 pandemic influenza virus.⁵⁷ However, the use of copper salts at high concentrations can irreversibly alter reactive

oxygen species (ROS) homeostasis of healthy cells, provoking a general toxicity for the organism, limiting their applications to disinfection.⁵⁶ Nanostructured cuprous and cupric oxides have been also extensively employed as antiviral agents for *in vitro* applications. For instance, cuprous oxide nanoparticles (CuONPs) were successfully employed against hepatitis C.⁵⁸ In particular, it was found that these NPs exerted a favorable antiviral activity with no cytotoxic effects. CuONPs target the binding and entry step of viral infection to hepatic cells (Figure 5). Similar results were reported on the use of CuONPs against HSV-1, however without any profound investigation on the antiviral mechanism.⁵⁹

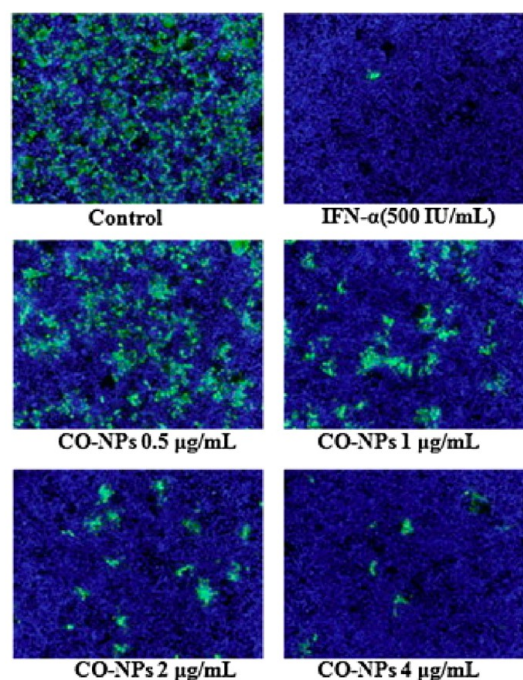


Figure 5. Huh7.5.1 cells at 72 h post-infection were stained with HCV-positive serum from patients (green signal) and with DAPI (blue signal). Cuprous oxide NPs (CuONPs) are able to reduce viral infection *in vitro*. Reproduced with permission from ref 58. Copyright 2015 Elsevier B.V.

Alternatively, zinc salts have been successfully used as antimicrobial agents from research up to clinical trials for viral warts.^{60,61} More recently, ZnO nanoparticles (ZnONPs) were developed for the treatment of HSV-2. ZnONPs were prepared with a tetrapod morphology.⁶² The results showed that they can mimic cell surface interacting with the HS present on the viral capsid. Additionally, these particles have been used for photocatalysis showing to efficiently destroy the viral proteins upon UV irradiation.⁶² Besides all these interesting examples, *in vivo* applications are still needed to validate this therapeutic modality. Due to the generation of high levels of ROS, the toxicity of copper nanoparticles has been widely debated. The antiviral activity of copper nanoparticles is generally associated with the release of Cu^+ ions in solution, thus the leakage of cytotoxic cationic species can be modulated by surface functionalization before *in vitro* and *in vivo* applications. On the other side, the use of nanomaterials generating ROS can find applications in textile and surface coating. The general broad virucidal efficiency of copper oxide nanoparticles shown for H1N1 pandemic influenza⁵⁷ should be tested on SARS-

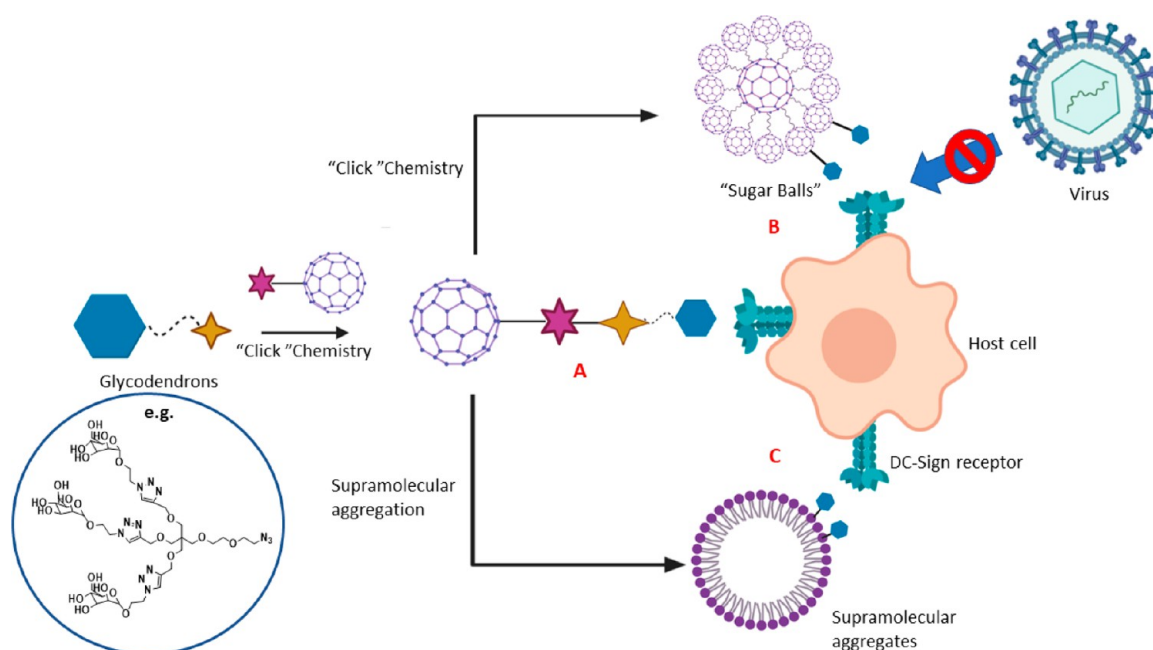


Figure 6. Chemical design and general scheme to block the viral entry by glycofullerenes. Three different shapes of glycofullerenes can act as inhibitor of viral infection: (A) shape composed of monodisperse fullerene bearing mannose, (B) assembly as "sugar balls" of tridecafullerenes exhibiting mannose on the edges, and (C) supramolecular micellar aggregates of fullerenes bearing mannose.

Table 3. Antiviral Activity of Different Fullerenes Functionalized with Mannose

material	functional groups	core function	number of mannose moieties	virus	number of atoms between fullerene and mannose	IC ₅₀	ref
glycofullerene	monosaccharide	malonate	12	Ebola	8	2 μ M	66
glycofullerene	monosaccharide	trialkynyl pentaerythritol	36	Ebola	22	68 μ M	
glycofullerene	monosaccharide	trialkynyl pentaerythritol	36	Ebola	28	0.3 μ M	
tridecafullerenes ^a	monosaccharide	malonate	120	Ebola	8	20.375 nM	69
tridecafullerenes ^b	monosaccharide	malonate	120	Ebola	8	0.667 nM	
glycofullerene	disaccharide	trialkynyl pentaerythritol	36	Zika	29	8.35 nM	70
				Dengue		7.71 nM	
tridecafullerenes	disaccharide	trialkynyl pentaerythritol	120	Zika	29	0.52 nM	
				Dengue		0.098 nM	
tridecafullerenes	disaccharide	trialkynyl pentaerythritol	360	Zika	48	0.067 nM	72
				Dengue		0.035 nM	
micellar glycofullerenes	monosaccharide	trialkynyl pentaerythritol	6	Ebola	23	424 nM	
micellar glycofullerenes	monosaccharide	trialkynyl pentaerythritol	12	Ebola	25	196 nM	

^aSmall spacer between core C₆₀ and surrounding fullerenes. ^bLarge spacer between core C₆₀ and surrounding fullerenes.

Cov-2 and might be used for improving mask protection efficiency.

Carbon Nanomaterials. Due to their diversity, versatility, and tunable surface chemistry, carbon nanomaterials have been attractive for several types of applications. In particular, the past decade has seen a tremendous raise in the preparation of performant carbon-based nanomaterials in the antiviral field. Fullerene and its derivatives are the most studied carbon nanomaterials for their virucidal activity. Due to the lack of solubility of pristine fullerene, functionalization strategies have been developed to prepare water-soluble drugs. Investigations in the biomedical field evidenced the membranotropic capacity of fullerene derivatives.⁶³ By modulating shape and functions,

fullerene derivatives have been shown to possess antiviral properties through inhibition of viral entry and blockage of viral replication. From these results, the attention has been directed also to other carbon nanomaterials. In particular, functional carbon dots (CDs) and graphene oxide (GO) have been investigated for their ability to block viral entry into host cells.

Glycofullerenes. The emerging of mortal viruses, like Ebola or Zika, and the lack of suitable treatments led the academic and the industrial communities to look for alternative therapeutic routes. Most of these pathogens are RNA enveloped viruses, and they share common infection mechanisms that can be targeted for the preparation of wide

spectrum antivirals. The external surface of the envelope of these viruses is covered by glycans that tightly interact with lectin receptors on host cells.⁶⁴ This strong interaction allows the attachment of the virions to the cells, followed by internalization and infection. Blocking lectin receptors is a general strategy used to stop viral infection at an early stage. Fullerenes have been widely investigated as antiviral molecules, drug carriers, or tissue scaffolds.⁶⁵ Fullerene applications have been recently extended to the design of mannosylated derivatives to block the entry of viral particles into host cells. Mannose, due to the high affinity with lectin receptors, competes with the virus in the interaction with the host cells. For example, one of the targets is the inhibition of viral particles through the interaction of mannose with the dendritic cell-specific ICAM-grabbing non-integrin (DC-sign). DC-sign receptors mediate the interactions between DCs and T cells.^{66,67} To exploit these characteristics, mannose was combined with fullerene in the design of the so-called glycofullerenes to study their capacity to inhibit Ebola, Dengue, and other pathogens.

For this purpose, different glycofullerenes were synthesized by changing the number of mannose units (from 12 to 36) and the spacers between the fullerene moieties and by varying steric hindrance in order to obtain a library of molecules.⁶⁸ The synthetic route is composed of three steps based on “click chemistry”: (1) assembly of glycodendrons by Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), (2) synthesis of alkyne-substituted Bingel-Hirsch hexakis-adducts, and (3) the coupling between the last two products again by CuAAC. To increase the number of mannose moieties up to 36, the glycodendron core was changed from malonate to trialkynyl pentaerythritol. In order to compare the different derivatives, *in vitro* studies were performed. Jurkat cells (lymphocyte T CD4 immortalized cells) expressing DC-sign were used to prove the inhibition capacity of the glycofullerenes on viral infection of Ebola (Figure 6, route A). The study revealed an IC_{50} in the μM range for the 12 mannose fullerene, a lower efficiency with the 36 mannose fullerene with a short spacer (PEG, with 2 ethylene oxide units), while a nanomolar IC_{50} was achieved with 36 mannose fullerenes with a longer spacer (PEG, with 3 ethylene oxide units) (Table 3). This first proof-of-concept study was then expanded, aiming to obtain a better antiviral activity by increasing the valence and inserting longer and flexible spacers.⁶⁸

Based on these studies, another class of multivalent fullerene dendrimers was then designed. A fast and controlled synthetic route was developed to achieve giant globular multivalent fullerenes, containing hundreds of functional groups. The first study was performed with tridecafullerenes containing 120 mannoses.⁶⁹ The molecular structure is composed of 12 hexakis C_{60} surrounding a C_{60} core (Figure 6, route B). Compared to the previous study, an IC_{50} 3 orders of magnitude lower was measured on the inhibition of Ebola virus (Table 3).^{66,68} In order to present more carbohydrates at the periphery of the dendrimer, a trialkynyl pentaerythritol derivative allowed to afford a tridecafullerene with 360 carbohydrates. In this case, the molecule was synthesized with a C_{60} tridecafullerene bearing $\alpha(1,2)$ mannobioside.⁷⁰ The use of this disaccharide was already investigated, showing an increase of affinity with DC-sign receptors by a factor of 3–4.⁷¹ The synthetic strategy exploited also the use of strain-promoted copper-free cycloaddition of azides to alkynes (SPAAC) for the coupling of the core fullerene to the

surrounding fullerenes. SPAAC allows an easier purification avoiding the removal of cytotoxic copper ions. The inhibition performance of this molecule was studied *in vitro* with viral pseudoparticles of Dengue and Zika. The comparison was made between 360 and 120 disaccharides tridecafullerenes and 36 disaccharide monofullerene. The results highlighted a picomolar IC_{50} inhibition on both Zika and Dengue models for the 360 disaccharide glycofullerene (Table 3). The ability to inhibit other types of viruses allows the use of glycofullerenes as broad spectrum antiviral drugs. Moreover, the negligible toxicity to other cells proved the biocompatibility of these molecules.

Following an alternative strategy, a supramolecular assembly of monodisperse glycofullerenes, leading to the formation of micelles, was achieved and tested.⁷² These micelles present a uniform and spherical shape (Figure 6, route C). The aggregation synthetic route is faster compared to a controlled synthesis of giant glycofullerenes, but it might suffer from low reproducibility and batch-to-batch differences between each formulation. This self-assembled C_{60} functionalized with 6 or 12 mannoses exposes a large amount of carbohydrate at the surface, leading to an inhibition of Ebola virus in the nanomolar range (IC_{50} of 424 nM for six mannoses and 196 nM for 12 mannoses, respectively, Table 3). Further *in vitro* studies evidenced again a good biocompatibility of these glycofullerenes. While there are no *in vivo* studies yet, these promising results enlarge the panel of molecules in the fight against new emerging viruses.

Functionalized fullerenes have been used for their ability to compete with viral particles through lectin receptors in host cells. There has been a tremendous advancement in the functionalization of fullerenes leading to the preparation of derivatives with a high amount of mannose, capable to enhance the multivalency effect and thus to increase the therapeutic outcome. First, glycofullerenes do not have an intrinsic virucidal activity. They can reduce the infectivity, but they are not able to completely inactivate the virus. Second, the mechanism of action of glycofullerenes relies on their interaction with host cells and not with viral particles. Thus, for a therapeutic application, they should be injected at different time points, ensuring that the local concentration is therapeutically relevant to prevent the virus from invading the host cells. In addition, glycofullerenes can be internalized into host cells losing their viral “shield” activity. On the other hand, the well-developed surface chemistry of glycofullerenes can be used for other key receptors involved in viral entry. For instance, in the case of the current SARS-Cov-2 pandemic, a similar click chemistry strategy can be used to anchor ligands recognized by human lung ACE2 receptors and so inhibiting viral entry.⁷³

Other Carbon Nanomaterials. Alongside fullerenes, other carbon nanomaterials (NMs) have been scrutinized for their ability to block viral entry. CDs and GO are the most known and studied carbon NMs with marked antiviral properties. CDs are zero-dimensional carbon nanoparticles. They are generally produced *via* hydrothermal decomposition of carbon containing “low-cost” precursors. The use of CDs in the biomedical field has been encouraged by their easy preparation, low toxicity, fluorescence properties, and easy surface functionalization. Pristine CDs have shown moderate viral blocking activity for HIV infection *in vitro*.⁷⁴ This has been associated with the surface of the material rich in carboxylic and hydroxyl groups prone to form noncovalent

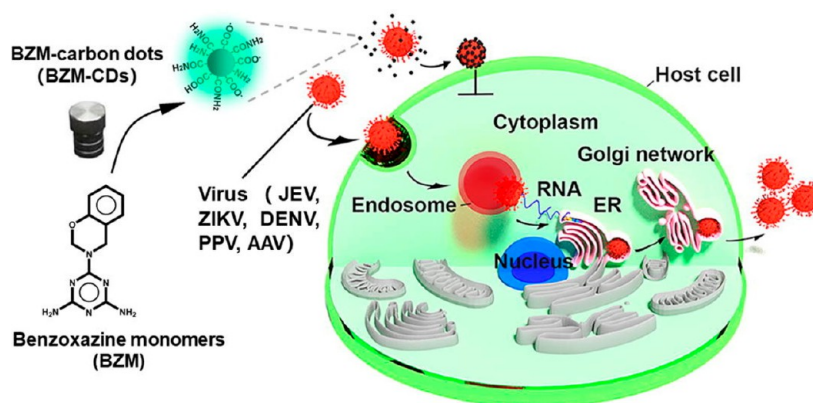


Figure 7. Illustration of benzoxazine-functionalized CDs and their broad antiviral entry activity. Reproduced with permission from ref 76. Copyright 2019 Elsevier B.V.

interaction with viral membranes. Moreover, due to the complexity of the biological systems these nonspecific interactions could not be so effective *in vivo*, likely reducing the antiviral efficacy. Therapeutic targeting molecules can be grafted onto a CD surface to enhance their antiviral activity. In this context, the design of multifunctional CD platforms can be obtained through two different strategies. The first consists in a single-step reaction that foresees the insertion of the therapeutic molecule directly into the step of preparation. Target molecules are decomposed with the other precursors, generating the desired functional CDs. This protocol is fast and efficient, however the drug loading as well as its activity are hard to estimate. Indeed, the hydrothermal treatment can alter the chemical structure of the active molecule, thus vanishing its therapeutic effect. For these reasons, the reaction conditions must be carefully controlled.⁷⁵ The second method is a two-step reaction and implies the postfunctionalization *via* amide formation on the surface of the CDs rich in carboxylic groups. This strategy offers a better chemical control, but the yield and the drug loading may not be quantitative and high, respectively. Different functionalized CDs were prepared to hamper host cell viral entry. For instance, benzoxazine (a low water-soluble antiviral agent) was incorporated into the CD structure during their preparation (Figure 7). The as-prepared CDs showed a broad spectrum viral blocking capacity *in vitro* for enveloped (e.g., Japanese encephalitis virus, Dengue virus, and Zika virus) and non-enveloped viruses (e.g., porcine parvovirus and adenovirus-associated virus).⁷⁶ These positive results were explained by the efficient binding and deactivation induced by the multivalent effect of the CDs to the viral particles.

Amino-functionalized CDs were also tested for the treatment of human norovirus. In this study, CDs were functionalized with 2,2'-(ethylenedioxy)bis(ethylamine) (EDA) and 3-ethoxypropylamine (EPA) *via* amide bond formation.⁷⁷ These NMs exerted a good viral blockage. In particular, EPA-functionalized CDs were able to inhibit 100% of viral infection at concentration of 2 $\mu\text{g/mL}$, while in the case of CDs prepared with the other amines, 80% of inhibition was reported. These effects have been associated with the higher positive charge of CD-EDA compared to CD-EPA. Another surface group used for viral targeting is boronic acid (BA), which can bind glycosylated surfaces forming boronic esters. This strategy was successfully adopted to treat HIV where the boronic groups, linked to different nanoparticles (e.g., silica

nanoparticles and nanodiamonds) can target gp120 receptors on the viral envelope inhibiting the infection.⁷⁸ Another recent study proposed the use of CD functionalized with phenylboronic acid for prevention of HIV infection.⁷⁴ The functional materials showed good inhibition properties compared to nonfunctionalized CDs by preventing the binding to the target cell *in vitro*. Overall, the use of CDs for stopping host cell viral entrance has shown good results *in vitro*. However, there is a lack of proofs *in vivo* limiting their applications to surface disinfection or masks. In addition, most of the *in vitro* studies foresee first the contact of the CDs with the viral particles and then their incubation with host cells. Deeper investigations should be performed adding the NMs at other time points (for instance in infected cells) to understand if the antiviral activity is maintained.

In addition, CDs have been successfully used for photodynamic therapy (generating radicals upon light irradiation) in cancer treatment. The same approach may be used to combat viral infections, where the antiviral activity induced by the surface modification can be sensibly enhanced by ROS generation under irradiation.

Graphene materials, and in particular GO and reduced GO (rGO), have been used for different biomedical applications including drug delivery, biosensing, and tissue engineering.⁵ GO platforms have shown also interesting antimicrobial activity.⁷⁹ Regarding viral infection, GO was used to block the virus entrance in host cells. GO and rGO can be considered as two-dimensional materials that contain hydrophilic and hydrophobic domains allowing to adsorb many biological molecules including nucleic acids and proteins. GO showed low interaction with viruses, however its surface functionalization with target molecules can sensibly enhance its affinity for the viral particles. Additionally, GO can be used as photothermal agent (generation of heat by NIR irradiation) or photodynamic therapy (using visible light irradiation) inactivating the capsids by local thermal shock or by radical formation during irradiation, respectively. The use of phototherapies may significantly augment the antiviral properties of the materials. However, we must keep in mind that these therapeutic modalities can be applied only to disinfection, since the radical/heat production may be harmful for the healthy tissues *in vivo*. Photodynamic therapy has been successfully exploited using bacteriophage MS2 as a model virus.⁸⁰ In this study, GO was functionalized with an aptamer recognized by the viral surface. The results showed that this

functionalization is able to enhance the binding efficiency of the MS2 capsids onto the GO surface compared to nonfunctionalized GO. Subsequently, irradiation in the visible light was able to disinfect the solution, while nonfunctionalized GO showed much less activity due to the lack of adsorption. Despite these interesting results, this pioneer work remains at an early research stage since the use of high light dose (300 W for 10–140 min) and the lack of material recovery and reuse make its application for surface disinfection difficult.

GO can be also used as a platform to link antiviral agents. Encouraging results were reported using GO with hypericin for the treatment of a recently appeared duck reovirus.⁸¹ More recently, Deokar *et al.* reported an original rGO-based multifunctional platform for HSV-1 treatment.⁸² In this work, the authors functionalized the material with organic sulfate groups and iron oxide magnetic nanoparticles (FeNPs). The rGO functionalized with the sulfate is able to mimic the host cell surface and to bind HSV-1. Subsequently, the viral particles captured onto the rGO-FeNP surface can be concentrated *via* magnetic precipitation and destroyed *via* photothermal therapy. This approach is highly efficient for disinfection with low energy (1.6 W/cm² for 7 min) and cost effectiveness. HS is a common entry receptor in various types of viruses (*e.g.*, herpes viruses, human papillomavirus, Dengue virus).⁸³ The use of organic sulfate-functionalized graphene sheets mimicking HS, like GO and rGO, has been already explored. However, it is worth noting that these NMs are prone to strongly adsorb proteins in culture environments (coronation), likely inhibiting their antiviral efficacy.⁸⁴ High loadings of sulfate groups were introduced onto rGO using polyglycerol sulfate.^{85,86} This approach has been used for inhibition of orthopoxvirus, pseudorabies virus, and African swine fever virus *in vitro*.^{85,86}

Graphene has also been used as antiviral material. Polysulfates and fatty amines were grafted onto graphene surface *via* triazine chemistry for the treatment of herpes simplex virus.⁸⁷ This strategy promotes the synergy between the electrostatic and hydrophobic interactions, showing incredibly high inhibition efficacy. Overall, graphene materials have shown a good capacity to block host cell viral entry. Disinfection with graphene family materials is also promising, offering the possibility to couple high viral binding with phototreatments. Regarding the GO and rGO activity in cellular environments, different parameters must be considered such as protein coronation, blood circulation time, and activity *in vivo*. So far, the use of sulfonic groups introduced *via* diazonium salt decomposition has been largely privileged. We take this opportunity to encourage future studies using other targeting groups (*e.g.*, boronic acids) and grafting methods (*e.g.*, epoxide ring opening or hydroxyl esterification reactions).

Mechanical Disruption of the Capsid. The most direct way to suppress viruses and stop the spreading of viral infection is to inactivate them before the attachment to the host cells, by binding to the acceptor proteins.⁸⁸ One of the most conserved targets of viral attachment ligands is the heparan sulfate proteoglycan (HSPG), previously mentioned. HSPGs are expressed on the surface of almost all eukaryotic cell types, and many viruses like HIV-1, HSV, human papilloma virus (HPV) exploit HSPGs as the target of their viral attachment ligands. Bearing in mind this behavior, different studies have used HSPG-mimicking materials to target this type of virus–cell interaction and to achieve broad spectrum efficacy. For example, in one key study, AuNPs were

functionalized with mercaptoethanesulfonate (MES) based on its mimicry of HS (Au-MES NPs). Au-MES NPs were shown to interfere with viral attachment, viral entry, and cell-to-cell spreading.⁸⁹ The importance of the polyvalent interactions with the virus makes these NPs a good candidate for antiviral therapy. However, Au-MES NPs presented virustatic activity, meaning that upon dilution of the NPs, the virus recovers its infectivity due to the reversibility of the cell-virion interaction. This problem was solved by Stellacci and colleagues who developed NPs coated with mercapto-1-undecanesulfonate (MUS) ligands (Au-MUS NPs). The long aliphatic and flexible linkers provide stronger associations with the viral particles compared to Au-MES NPs, leading to local distortions and eventually inducing a global deformation and breaking of the capsid that inactivates its contagion irreversibly (Figure 8).⁹⁰

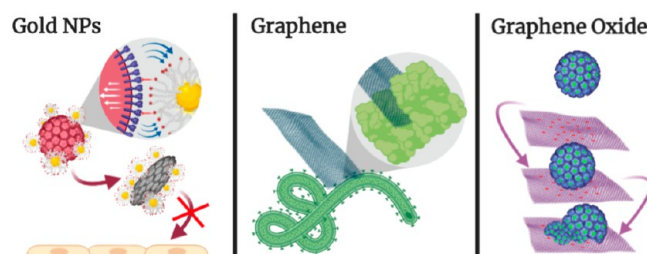


Figure 8. Mechanical disruption mechanisms of different NMs. Left: Au-MUS NPs inducing mechanical forces in the virus capsid leading to inactivation.⁹⁰ Center: Graphene NSs disrupting VP40 hydrophobic interactions in Ebola.⁹³ Right: Interaction between negatively charged surface of GO and positively charged capsid.⁹⁴

These Au-MUS NPs were tested against different HSPG-dependent viruses, showing a high viricidal activity over HSV, HPV, and RSV (Figure 9A). Furthermore, the activity of the Au-MUS NPs was studied *in vivo* using mice infected with RSV, indicating that the material can prevent pulmonary

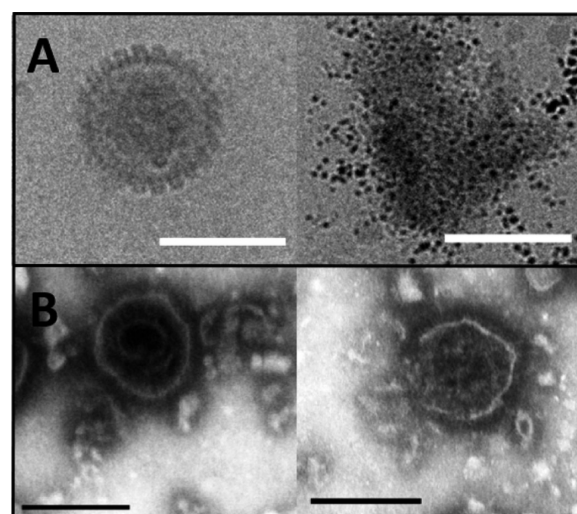


Figure 9. Interaction of NMs with viral capsid. (A) Gold NPs acting on HSV-2 virus. After 90 min, the percentage of destroyed virus was significantly increased. Scale bars: 100 nm. Reproduced with permission from ref 90. Copyright 2018 Springer Nature Limited. (B) GO acting on Pseudorabies virus. After incubation with GO for 1 h, part of the virus envelope and spikes were destroyed. Scale bars: 200 nm. Reproduced with permission from ref 94. Copyright 2015, American Chemical Society.

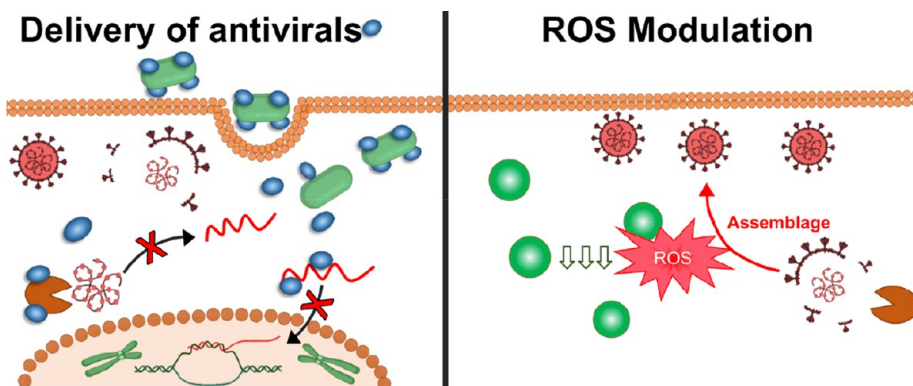


Figure 10. Illustration of NMs interacting with host cell to prevent viral spreading. Left: NMs are used for delivery of antiviral agents. Right: NMs can change the ROS homeostasis slowing down the infection and helping cell survival.

dissemination of the infection and showing potential use as medically relevant virucidal drugs to fight viral infections. More recently, the concept was further extended to cyclodextrins modified with mercapto-1-undecanesulfonate, proposing this system as a broad spectrum virucidal macromolecule.⁹¹ Besides Au-NPs, a similar mechanism of disruption was studied with other materials like graphene or GO. In a study, in which the toxicity of graphene was evaluated theoretically, it was also shown that graphene nanosheets can interrupt the hydrophobic protein–protein interaction, which is essential to biological functions.⁹² This feature was attributed to the hydrophobic nature of graphene. Thus, it seems energetically favorable for graphene to slide between the interface of two proteins in contact, due to hydrophobic interactions. In another study inspired by this behavior, the authors performed molecular dynamics simulations of graphene nanosheets in the proximity of the surface of the Ebola viral matrix protein VP40 showing that the nanosheets can break the hydrophobic interactions in VP40, a key protein for the replication and stability of Ebola virus (Figure 8).⁹³ These findings suggest that graphene nanosheets might have potential antiviral activity against Ebola; however, there is a lack of experimental evidence that corroborates this mechanism of disruption. On the other hand, GO was tested experimentally against Pseudorabies virus and Porcine epidemic diarrhea virus (PEDV), showing significant decrease in the infectivity.⁹⁴ It was found that the negatively charged surface of GO is important for the adsorption of the virus, whose surface is positively charged, and that GO could directly interact with the viral particles and destroy their structures due to the sharp edges of the material (Figures 8 and 9B).

The mechanical disruption of the capsid is a peculiar antiviral mechanism associated with some NMs. In particular, the use of specific sulfonates able to mimic heparan sulfate can be also used to target SARS-Cov-2 infection.⁹⁵

ANTIVIRAL ACTIVITY INSIDE HOST CELLS

Blocking the viral entry, *via* liquid/surface disinfection or once in the body, is a powerful strategy to hamper early stage viral contagions. On the other hand, when infections have already spread and reached middle and late stages, alternative pharmacological strategies are required. The study of the viral pathogenesis and machinery inside host cells has allowed the preparation of different drugs. Due to the present pandemic, such antiviral drugs are now of top interest in the scientific and medical communities. So far, few antiviral drugs

are clinically available, and their mechanisms of action consist on the inhibition of reverse transcriptase (HIV, hepatitis), DNA inhibition of polymerase (herpes, HIV), inhibition of protease (HIV), blockage of ion channels (influenza), and inhibition of neuramidase (HIV, influenza, hepatitis). However, these drugs suffer from moderate to severe side effects. Additionally, rapid mutations in the viral machinery make them resistant to the treatments, making the control and the stop of the infection challenging. The use of HNMs in drug delivery has shown several advantages. First, HNMs can increase drug solubility and its circulation time. Additionally, they can be functionalized with targeting molecules able to direct the drug to the desired organs and so avoiding side effects and reducing the dosage. More recently, new antiviral mechanisms were discovered. In particular, it was found that different types of HNMs are able to change the ROS homeostasis in infected host cells, stopping the viral replication and preserving the cell survival. In this section both drug delivery materials and HNMs with ROS modulation properties will be critically presented (Figure 10).

Nanomaterials for Drug Delivery. Different HNMs have been widely tested for drug delivery applications. The advantages of this strategy are several including enhance drug solubility and the possibility of targeting and multivalent effects. As a potential broad spectral antiviral agent, AgNPs can prevent the virus from adsorbing to the host cell in the early stage of infection and thus show strong antiviral activity. After the cells are infected by the virus, there are ways to inhibit cell apoptosis (Figure 2, right). For example, Lv *et al.* studied the anti-TGEV activity of AgNPs in swine testicle cells and explored the possible mechanism of AgNP inhibition of TGEV infection-induced apoptosis.¹⁸ The results showed that these AgNPs are able to decrease cell apoptosis through the activation of p38/mitochondria-caspase-3 signaling.¹⁸

Although the use of AgNPs has shown good antiviral action to further improve the therapeutic effects and reduce both side effects and drug resistance, the way of binding drugs or genes and other therapeutic agents to AgNPs has received particular attention. It has been observed that AgNPs inhibit the activities of neuraminidase and hemagglutinin, preventing the H1N1 influenza virus from attaching to host cells. At the same time, the potential molecular mechanisms revealed that caspase-3-mediated apoptosis was inhibited by ROS generation. AgNPs modified with polyethylenimine (PEI) can bind siRNA. Ag@PEI@siRNA exhibited superior abilities for enhanced cellular uptake and blocking EV71 virus infection

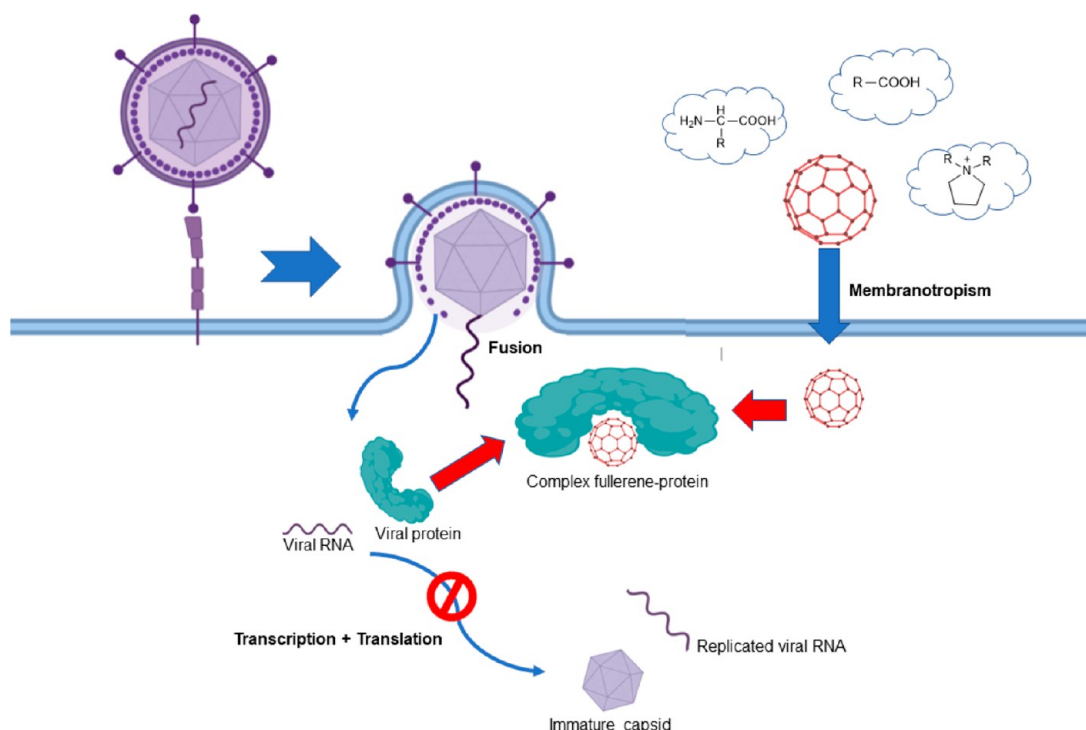


Figure 11. Illustration of fullerene affecting the viral replication mechanism. Interaction between fullerene and viral protein blocking either the transcription or the translation in the viral replication.

and significantly decreased the apoptotic cell population, which prevented the spread of EV71 virus.³⁷ In addition to drugs and siRNA, neutralizing antibodies in combination with AgNPs were developed. The results demonstrated that there is an additive effect between the antibody and AgNPs when combined against cell-associated HIV-1 infection *in vitro*.⁹⁶

The membranotropic properties of fullerenes were widely exploited. For example, pristine C₆₀, after accumulation at the cell membrane, can translocate into the cytoplasm by crossing the membrane through multiple energy-dependent pathways despite its hydrophobic character.⁹⁷ Fullerenes can be made water dispersible using surfactants, sonication or first dissolving them in appropriate organic solvents (DMSO). The use of fullerenes as inhibitors of viruses started in 1993 with a study focused on HIV infection.⁹⁸ This work revealed the interaction between C₆₀ derivatives and HIV protease through molecular modeling and experimental verification. HIV protease (HIV-PR) is involved in the mechanism of replication in the maturation of HIV virion, cleaving newly synthesized proteins. The active site of HIV protease has a cavity of 10 Å closed to the diameter of C₆₀ cage.⁹⁹ Molecular docking and experiments on HIV-PR catalytic activity revealed a blockage of the active site of the enzyme by van der Waals interactions leading to an antiviral effect. The following studies were based on a structure–activity relationship between functionalized fullerenes and HIV-PR with the aim to increase the antiviral activity. The introduction of pyrrolidinium salts onto C₆₀ was tested against the activity of HIV-1 strain.¹⁰⁰ In a second study, C₆₀ bearing two ammonium groups was applied against the activity of HIV-1 and HIV-2 strains.¹⁰¹ The results showed the importance of having two moieties in a precise position on the fullerene cage and the influence of the charge of the different salts sensibly increasing its antiviral activity. Further investigations using different functional groups (*e.g.*, amino acid

derivatives) were explored. C₆₀ functionalized with amino-butyric acid and aminocaproic acid was able to inhibit HIV viral replication at subnanomolar concentrations.⁶³ In another work, anionic and cationic pyrrolidinium salts and amino acid functionalized fullerene derivatives were used for the inhibition of HIV reverse transcriptase (HIV-RT). Fullerene compounds were compared to nevirapine, an available drug against HIV-RT, revealing a better inhibition compared to the pure drug.¹⁰² The antiviral property of carboxylated fullerenes was confirmed by another study.¹⁰³ Results obtained *in vitro* on CEM cell line showed low toxicity and a submicromolar EC₅₀ against HIV-1 and HIV-2 strain viral replication.

Several mechanisms of HIV protease inhibitors have been hypothesized and simulated. Some studies investigated the action of fullerene derivatives on viral replication cycle and the virus maturation (Figure 11).^{98,104} For the latter, it was suggested an impairment due to a strong interaction between fullerene and the immature capsid.¹⁰⁵

Other RNA viruses share ways of viral replication similar to HIV.¹⁰⁶ C₆₀ functionalized with an amino acid derivative was investigated against Hepatitis C RNA polymerase (HCV-RP).¹⁰² This essential enzyme for viral replication was inhibited in a submicromolar range, similarly to benzo-1,2,4-thiadiazine, a potent specific inhibitor of HCV-RP. Another publication highlighted the effect of a C₇₀ poly(carboxylic acid) derivative on different strains of influenza virus (*e.g.*, A, H1N1, H3N2, and B).¹⁰⁷ The inhibition was comparable to Tamiflu, rimantadine, ribavirin, and amantadine, but the mechanism of action remains still unclear. These data emphasize that fullerenes C₆₀ or C₇₀ can be potent universal antiviral drugs for RNA enveloped viruses. However, clear elucidations of the mechanisms of action and *in vivo* studies are still a missing point.

Due to their high surface/weight ratio and capacity to pass through cell membranes, carbon nanotubes (CNTs) have been extensively explored for drug and gene delivery applications.¹⁰⁸ CNTs have been also successfully used for the delivery of antiviral agents. Compared to pristine materials, oxidized CNTs (ox-CNTs) showed an inhibitory activity for HIV viruses *per se*.¹⁰⁹ This effect has been associated with the oxygenated groups that increase the hydrophilicity and the colloidal stability of the material. The antiviral efficiency has been correlated to the ox-CNT interaction with host cells.¹⁰⁹ However, it is not clear how and what kind of mechanism blocks the viral machinery in host cells. Different anchoring strategies have been explored to link antiviral drugs onto CNT surface. For instance, ox-CNTs were covalently linked to 2-amino-3-nitro-1-(3,5-dimethylbenzyl)-aniline (CHI360) and *N*-(2-aminophenyl-3-nitro-)-3,5-dimethylbenzenesulfonamide (CHI415), two active non-nucleoside reverse transcriptase inhibitors for HIV treatment.¹⁰⁹ Following the covalent conjugation, only a moderate antiviral effect was observed compared to pure drugs, indicating that most probably the NM cell trafficking played a key role on the virucidal activity.¹⁰⁹ In another study, ox-CNTs functionalized with cyclodextrin were used for the delivery of acyclovir (a prodrug inhibitor of the viral DNA polymerases) for the treatment of HSV-1. Preliminary results showed that when acyclovir was delivered *via* the nanotubes, the viral antireplicative effect was higher than the free drug.¹¹⁰ More recently, a similar approach was applied to herpes virus using cyclodextrin and PEI-functionalized CNTs for co-delivery of cidofovir and plasmid DNA. However, the antiviral effect of the materials was not explained, and the transfection effect was not satisfactory.¹¹¹ Functionalized CNTs have been also used for delivery of ribavirin *in vivo* using grass carp as an animal model for the study of grass carp reovirus. ox-CNTs were first functionalized *via* amidation with BSA, and then ribavirin was covalently bound to the protein *via* esterification (Figure 12).¹¹²

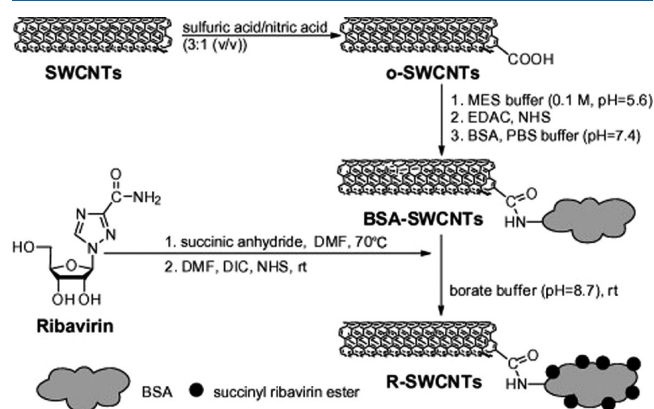


Figure 12. Schematic procedure of the functionalization of single-walled CNTs (SWCNTs) and ribavirin. Reproduced with permission from ref 112. Copyright 2015, Elsevier B.V.

In vivo tests demonstrated that, when ribovirin was shuttled by ox-CNTs, the antiviral efficiency was significantly increased without any evident toxicity and no significant changes in ROS-generating enzymatic activities. The use of CNTs in drug delivery is however still controversial.¹¹³

Graphene-based materials have been limitedly studied as antiviral drug delivery carriers. Only a few examples can be found in the literature. Graphene quantum dots (GQDs) were

used for drug delivery of CHI360 and CHI415 and tested *in vitro* against HIV similarly to CNTs.¹⁰⁹ Both the prepared GQD-CHI360 and GQD-CHI415 showed a high antiviral activity once into host cells, with low toxicity. GO has been also used for the delivery of DNAzyme into hepatic cells allowing to block the hepatitis C infection. This specific DNA single strand is able to recognize the viral mRNA and to silence its expression.¹¹⁴

Overall, carbon NMs proved to be interesting carriers for antiviral drugs. However, several questions need to be answered before their safe application as antiviral materials. Different reports have demonstrated that pristine CNTs display relevant toxicity for healthy cells, but by oxidation of the tubes, the side effects can be sensibly reduced.¹⁰⁹ In addition, more investigations should be performed on CNT toxicity. These nanocarriers indeed are not “innocent delivery agents”, but they play a key role in drug internalization pathway and in host cell machinery that might be averse to the expected therapeutic effects.¹⁰⁹ So far, CNT application in biological systems has been studied for 20 years. However, due to their possible toxicity, their real application in clinics seems to be steeper and difficult to achieve.¹¹²

Materials Tuning Reactive Oxygen Species. ROS homeostasis in infected cells has been studied for both RNA and DNA viruses. For instance, it was shown that infection of mice with influenza A decreased the concentration of lung glutathione and the antioxidant vitamin C, providing evidence that the viral infection was associated with oxidative stress *in vitro* as well as *in vivo*.¹¹⁵ Similarly, in HIV infection, induced oxidative stress in host T cells and high concentrations of antioxidants are able to slow down the cell-to-cell viral spreading.¹¹⁵ The increase of ROS concentration is a common process in most of the viral infections. However, the mechanism of radical generation is different from case to case. Several proofs suggest that modulating ROS homeostasis in infected cells can slow down or block the infection.¹¹⁶

HNMs have been shown to be powerful allies against viral infections. In particular, metal or metal oxide NMs, once internalized, can regulate the radical production into infected host cells. In this scenario, the NMs can work following two different mechanisms: (1) enhancing radical activity or (2) quenching ROS inside cell compartments. In the first case, metal oxide NPs are able to convert superoxide ions into more reactive hydroxyl radical species *via* Fenton or Fenton-like reactions. The excess of superoxide ions is able to oxidize the viral proteins and the genetic material and therefore efficiently block the infection. This approach has been reported using ZnO NPs.^{117,118} In these studies, ZnO NPs have been successfully applied for the treatment of H1N1 influenza virus and Herpes simplex virus. The preliminary results showed that PEGylated ZnO particles were able to efficiently reduce the viral infection with IC₅₀ similar to acyclovir. More importantly, toxicity of ZnO was modulated by functionalization with PEG, which allowed a higher colloidal stability and a more controlled release of Zn²⁺ ions to catalyze ROS formation. Indeed, ROS (e.g., superoxide and hydroxyl radicals) produced by the NPs should be highly reactive and should not only damage the exogenous biological molecules but also attack different cell compartments. Overproduction of ROS may reduce virus spread but also induce cell death. Interestingly, this approach is applicable only at the early infection stage (after 1 h incubation of the host cells with a virus), but it loses its activity at later time points. These

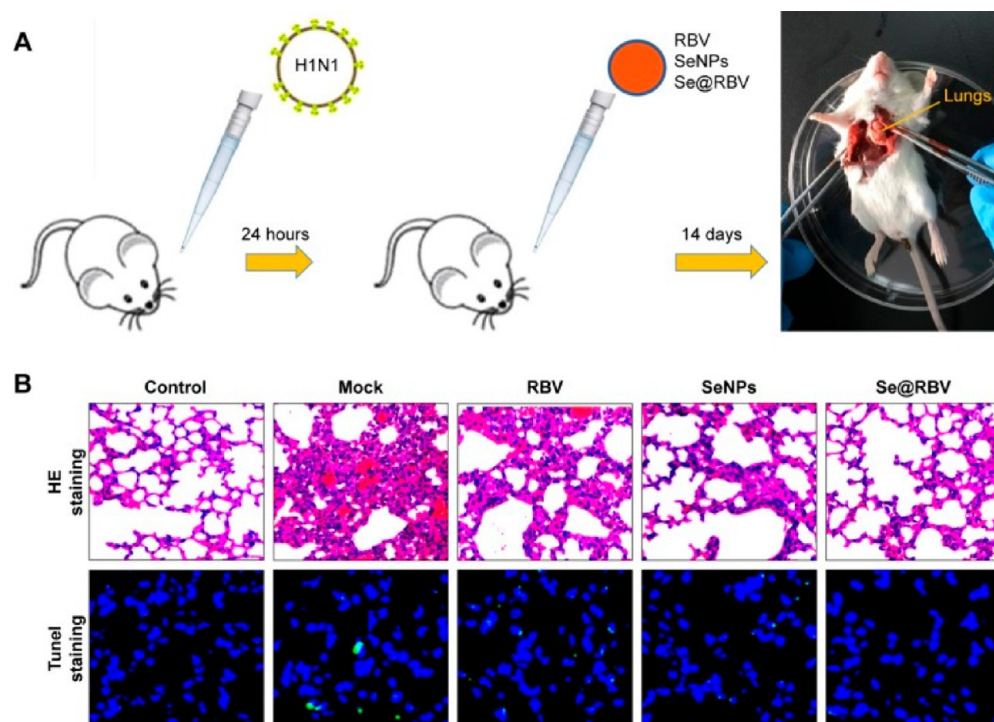


Figure 13. *In vivo* antiviral efficiency of SeNPs functionalized with ribavirin (Se@RBV). (a) Mice infected by H1N1 virus were treated with physiological saline (Mock), RBV, SeNPs, or Se@RBV. (b) H&E and tunnel staining showing that Se@RBV-treated mice displayed reduced lung damages compared to Mock. Reproduced with permission from ref 119. Copyright 2018 Dove Press Ltd.

experimental evidences suggest that materials capable of triggering the formation of ROS are essential in the first phase of the viral replication, most probably inducing the arrest of the viral DNA polymerases, which is active in the first 1–3 h of viral contamination.^{117,118} This specific mechanism of action restricts ZnO NPs to an application only at the early stage of infections. However, the same approach with other metals able to induce Fenton or Fenton-like reactions (*e.g.*, Fe, Cu, and Mn) has not been reported yet. The choice of proper capping agents may allow to control the metal ion release and thus tune the ROS-mediated antiviral activity. We would like to take an advantage here to suggest the growth of the antiviral research in this direction.

Another successful approach relies on the reduction of ROS concentration in host cells. ROS scavenging is able to alleviate the toxicity of the infection enhancing cell viability, giving time to start its endogenous antiviral mechanisms. So, this approach may both block infection and ensure host cell survival. In this context, selenium NPs (SeNPs) have been extensively studied for their antiviral activity. The mechanism of action of these NPs relies on the quenching of the radicals into host cells due to the infection, stopping the mitochondria depolarization and the consequent apoptotic cascade.¹¹⁹ Additionally, SeNPs can also adsorb onto the viral capsid sensibly reducing their infectivity. SeNPs can be prepared *via* classical mixing of selenium salt precursors in the presence of a reducing agent. More recently, SeNPs have been instead biosynthesized from *Actinobacteria* showing good stability and capacity to inhibit Dengue virus *in vitro*.¹²⁰ Moreover, SeNPs were used to carry different antiviral drugs including zanamivir,¹²¹ oseltamivir,¹²² amantadine,¹²³ and ribavirin.¹¹⁹ Their functionalization with the desired drug can be easily achieved adding the molecule during their synthesis through the Se ion controlled reduction. These NMs have been applied for the treatment of H1N1

virus. Notably, SeNPs with ribavirin (administered *via* intranasal absorption every 24 h for 3 days) showed that infected mice had much less alveolar collapse and perivascular and peribronchiolar edema, compared to the group challenged with the virus (Figure 13).¹¹⁹

Due to their efficacy and low toxicity, SeNPs can be considered a useful material for the treatment of other viral diseases including SARS-Cov-2. As a matter of fact, oxidative stress as well as chronic inflammation may contribute to the aggravation of the Covid-19 symptoms and to the general spread of the infection.¹²⁴ The use of SeNPs could eventually alleviate the toxicity of infected patients, giving time for the immune system to react against the contagion. However, the lack of preclinical studies on SeNPs, together the scarce knowledge of their biosafety and long-term toxicity, still remain the main challenges to tackle for clinical translation.

INTERACTION WITH THE IMMUNE SYSTEM

The vast majority of the studies show that human survival to viral attack is based on the stimulation and response of our immune system. When a virus enters and starts infecting tissues, the body reacts and triggers a strong immune response to overcome the pathogen invasion and spread. Normally, two different immunological reactions take place. The oxidative stress induced by infection causes the activation of the inflammasome through upregulation of pro-inflammatory cytokines such as IL-1 β , pro-IL-18, and NLRP3.¹²² Excessive upregulation of this mechanism leads to cell damage and eventually to pyroptosis activated by caspases.¹²² It was also shown that generation of radicals is able to depolarize mitochondria, hence affecting host cell respiration and inducing ROS-mediated apoptosis at the late stage of infection.¹²³ Besides, activation of pro-inflammatory cytokines can alert the immune system blocking the infection (the innate

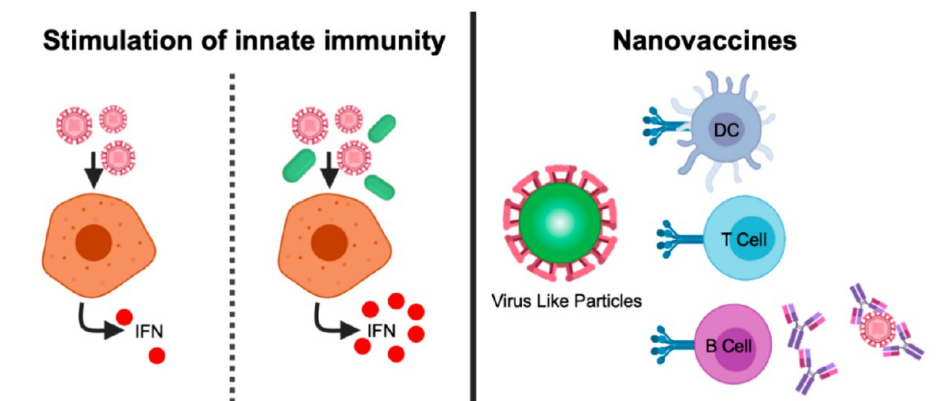


Figure 14. Illustration of HNM interaction with the immune system. Left: HNMs can enhance the production of interferon (IFN) alerting the immune system of the infection. Right: Virus-mimicking particles composed of viral proteins and HNMs can induce a strong immunological response activating dendritic cells, T cells, and stimulating B cells to produce specific antibodies.

immune response).¹²² The second immune response is specific (the adaptive immune response). Immune cells are trained to attack the virus (cellular response), while specific antibodies are produced by B cells (humoral response). In the last years, HNMs were proved to tune the immune responses, demonstrating to be a possible alternative against viral infection. In this section the most relevant strategies for the activation of innate and adaptive immune responses using NMs will be described (Figure 14).

Innate Immune Response. Innate immune response is the first response that takes place in the presence of any type of infection. During this early stage, interferon stimulating genes (ISGs) are upregulated in the infected cells.¹²⁵ This self-defense mechanism slows down the viral replication and alerts sentinel immune cells that start producing proinflammatory cytokines and trigger inflammation.¹²⁵ However, many viruses are able to escape this complex mechanism, retarding the immune response and spreading the infection. The interaction of HNMs with the immune system has been more and more studied. In the case of antiviral HNMs, many examples can be found in the literature where the NMs not only slow down the infection but also tune the innate immune response. Certain HNMs can display an intrinsic immune stimulation. We have already mentioned above that in the early stage of infection, AgNPs mainly prevent the virus from entering the host cell through the interaction with the external capsid, but *in vitro* cellular experiments lack to understand the complex interaction with primary immune cells. Recent studies have shown that AgNPs can potentially induce the expression of genes involved in innate and adaptive immunity-associated pathways, which are known to play crucial role in immune regulation. For example, Toll-like receptor 7 can be upregulated by AgNPs after 24 h, by recognizing the single-stranded RNA of the viruses and regulating the antiviral immune response.¹²⁶ Another study showed that in RSV-infected mice treated with AgNPs, the particles reduced the production of pro-inflammatory TNF- α and IL-6 cytokines, but potentiated the anti-RSV activity of neutrophils in an experimental mouse model.¹²⁷ However, activation of AgNPs in the reduction of RSV has been noted only when the NM was intranasally inoculated together with the virus, and no results have been reported on the use of AgNPs administered on infected mice. In the case of influenza virus infection of lung epithelial cells, it was found that AgNPs targeted infected lung epithelial cells and reduced viral replication, by preventing

autophagy. However, the blockage of the autophagic flux by AgNPs does not inhibit viral replication in already infected cells. Therefore, AgNPs are more suitable as viral preventive agents due to their pro-inflammatory response rather than drugs.¹²⁸ More recently, AgNPs were combined with graphene materials and exploited as antiviral material for the treatment of Porcine reproductive and respiratory syndrome virus (PRRSV).¹²⁹ GO-AgNPs were able to clump the virus diminishing its fusion with cell membrane. Additionally, once GO-AgNPs were internalized in host cells, they stimulated the ISGs that blocked viral budding and its diffusion to other cells *in vitro* (Figure 15).¹²⁹

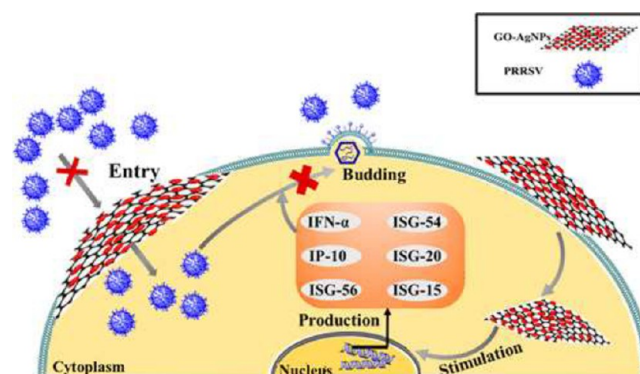


Figure 15. Scheme of the mechanism of action of GO-AgNPs on activation of innate immunity. Reproduced with permission from ref 129. Copyright 2018 American Chemistry Society.

Similarly, CDs used for the treatment of RSV and PRRSV were able to activate the innate immune response *via* an upregulation of ISGs *in vitro*.¹³⁰ Gold nanorods were applied to boost the innate immune response against RSV *in vivo*.¹³¹ Interestingly, it was shown that these nanorods (when intranasally administered with RSV) were not only able to activate the ISGs but also to tune the production of pro- and anti-inflammatory cytokines, resulting in the blocking of the infection with a reduced pulmonary inflammation.¹³¹ As drug carriers, HNMs can also affect the internalization pathways, modulate drug efficacy, and the immune cell responses. For instance, it was found that the isoprinosine immunomodulatory antiviral drug displays a much higher antiviral efficacy *in vivo* when delivered with CNTs than as a pure drug (in

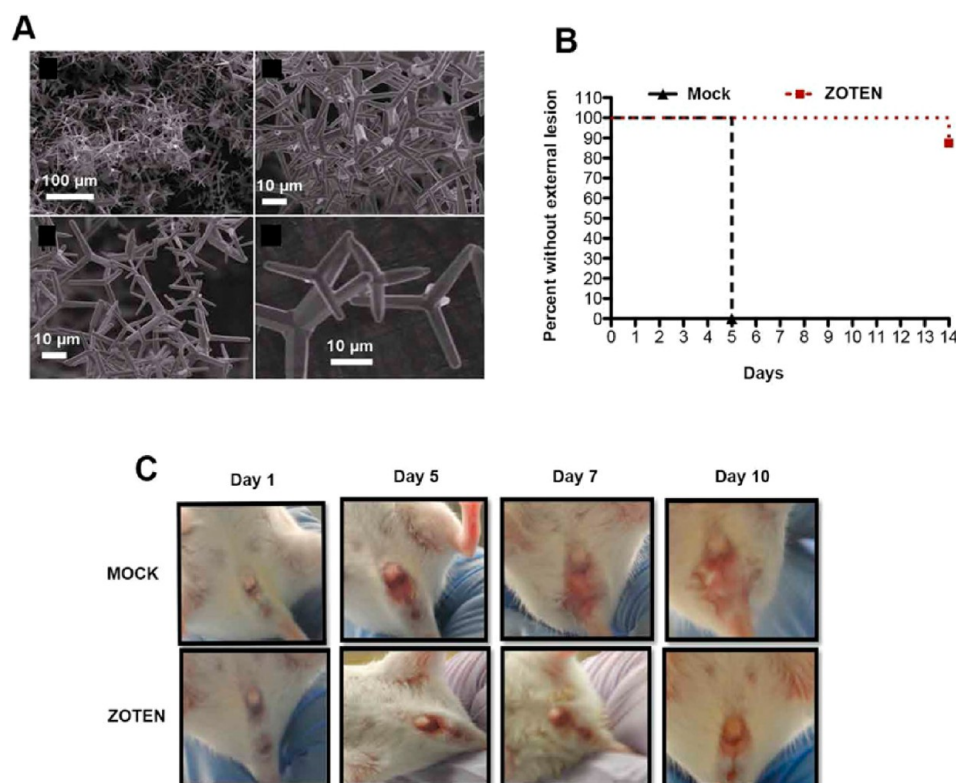


Figure 16. (A) Scanning electron microscopy images of ZnO tetrapod nanoparticles (ZOTEN) synthesized by flame transport synthesis. (B) Mice were challenged intravaginally with HSV-2 333 with or without ZOTEN. (C) To monitor progression of infection, mice were observed daily for the development of lesions around the vaginal opening, and base of the tail. Representative images from three independent experiments are shown. Reproduced with permission from ref 137. Copyright 2016 the American Association of Immunologists, Inc.

zebrafish larvae food administration), probably due to a better cellular uptake and the anti-inflammatory properties of the nanotubes.¹³²

Fullerenes also exhibit immunomodulation properties through the release of cytokines by bovine alveolar macrophages.¹³³ A study explored the influence of functionalization of C₆₀ with molecules presenting different surface charges like hydroxyl groups and amino acids.¹³⁴ The study concluded that negative charges upregulated TNF- α up-secretion in RAW 264.7 macrophages, but highly positively or negatively charged surfaces increased cell toxicity. The upregulation of the cytokine TNF- α is a proof that fullerene can promote cellular immune response.

Despite these preliminary results, the actual mechanisms of interaction between HNMs and the immune system are still at early stage of understanding. We must consider that the immune response needs to be proportional to the infection grade. If on one side nanosized immuno-boosters can alert more efficiently the sentinel cells, the use of HNMs that trigger an exaggerated immune response can promote excessive inflammation, damaging healthy cells and promoting uncontrolled side effects. In the particular context of SARS-Cov-2, the use of AgNPs, fullerenes, or other pro-inflammatory HNMs at the middle and late infection stage may cause an aggravation of the symptoms due to already diffused inflammation. In particular, most of the studies showed the ability to reduce infection when HNMs were first incubated with the pathogenic virus, thus limiting their potential use in the early stage infection.¹²⁴ The formulation of HNMs able to both alert the immune system (e.g., upregulating cytokine) and control lung inflammation (e.g., ROS scavenger) even after the

early stage infection may be a possible strategy for treatments against SARS viruses.

Adaptive Immune Response. Adaptive immune response is the specific response that the immune system exerts against pathogens. This mechanism is particularly active toward viral infections where the immune system produces specialized lymphocytes (to fight the virus), called memory B cells (to be effective in case of new infections) and antibodies (corresponding to the humoral response).¹³⁵ The stimulation of adaptive responses in case of specific infections can be induced artificially through the introduction of attenuated pathogens, stimulating the production of specific antibodies. This is the principle of vaccination, which is the most common procedure for immunization of large areas of population against many kinds of lethal viruses.¹³⁵ Besides, the use of viral proteins as antigens in the vaccine formulation leads to neutralizing antibodies, but, due to the low immunogenicity of isolated proteins, does not always stimulate sufficiently the immune system to reach total protection. More recently, nanotechnology has been applied to develop more efficient vaccines (e.g., nanovaccines). The use of nanostructures with a size similar to virus (virus-like nanoparticles) sensibly enhances the response helping to reach immunity.¹³⁵ HNMs can adsorb viral particles and present them to the immune system.^{136,137} This method of vaccination has been successfully applied *in vivo* for the challenge of herpes simplex 2 virus. HSV-2 starts its spreading in vaginal tissues and then diffuses to the neurons causing death in mice. ZnO NPs (teardrop morphology), after vaginal inoculation with HSV-2, are able not only to prevent viral cell adhesion but also to expose viral antigens to T cells and DCs, leading to immunization. The preclinical trials

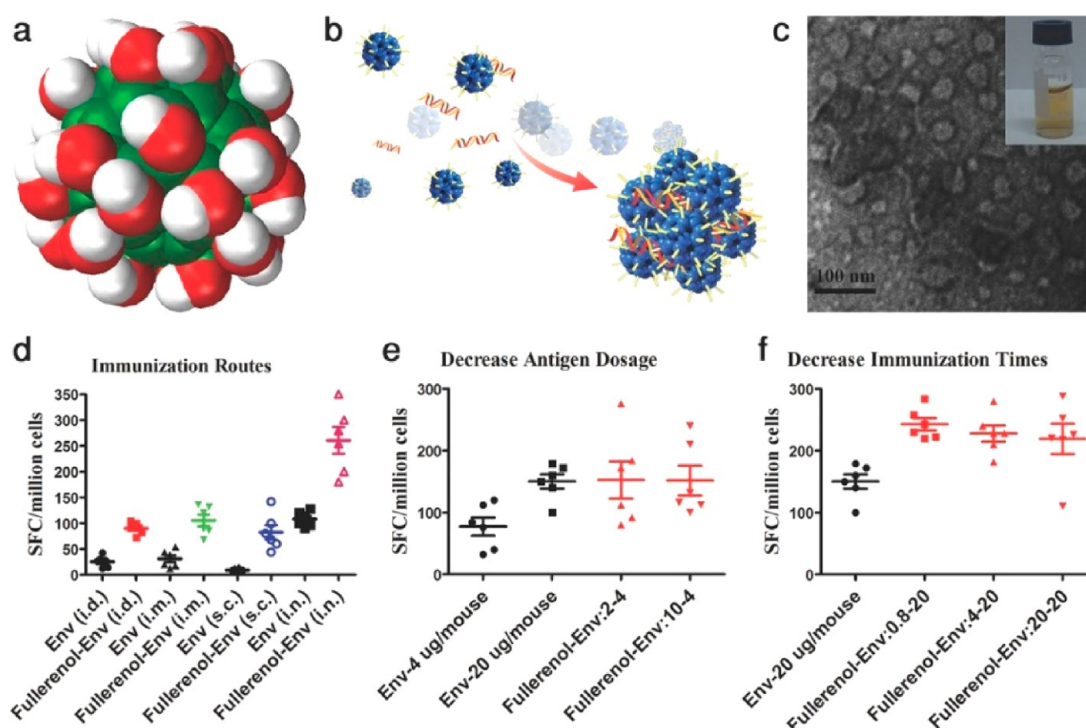


Figure 17. Use of fullerene as co-adjuvant for vaccination. (a) The structure of fullerene, red balls represent O and white for H on the fullerene surface, and green balls represent C atoms. (b) The schematic diagram of HIV Env plasmid DNA encapsulated during the self-assembly of fullerene. (c) TEM image of Env entrapped by fullerene. (d) Compared to naked Env immunization group, IFN- γ production (immunospot) was significantly enhanced when mice were immunized with the formulation via various immunization routes, including intradermal (i.d.), intramuscular (i.m.), subcutaneous (s.c.), and intranasal (i.n.) injections. Fullerene could decrease the antigen dosage (e) and immunization times (f). Reproduced with permission from ref 141. Copyright 2013 John Wiley & Sons, Inc.

against HSV-2 showed a survival to infection higher than 90%. This approach highlights the possibility to couple cell mimicking NMs to other co-adjuvants for the formulation of large spectrum nanovaccines (Figure 16).^{136,137}

HNMs have been also applied to the delivery of antigens, exposing them to the immune system. Fullerenes were found as suitable carriers for the delivery of drugs or nucleic acids.¹³⁸ Functionalized fullerene can also self-assemble into virus-sized NPs.¹³⁹ Investigated as vaccines in cancer immune therapy,¹⁴⁰ polyhydroxy fullerenes (called fullerenols) display interesting properties for antiviral therapy, based on their capacity to self-assemble into virus-like particles (VLPs) and so to enhance the immunogenicity of the antigens.¹⁴¹ The great advantage of this strategy relies on the easy encapsulation process during the self-assembly making fullerenols versatile for the formulation of different kinds of vaccines. These VLPs were investigated against HIV-1¹⁴¹ and hepatitis C viruses.¹⁴² Compared to conventional protein- or peptide-based vaccines intended to induce antigen-specific adaptive immune responses, DNA vaccines are more stable, cost-effective, easy to manufacture, and safe in handling.¹⁴³ However, DNA vaccines have the disadvantage of being poorly immunogenic.¹⁴⁴ Fullerene VLPs allow to avoid the use of other adjuvants. In the case of a vaccine against HIV-1, fullerene VLPs penetrated easily into the cells resulting in an enhancement of DNA transfection. This was proved in a study using fullerene encapsulating DNA encoding the HIV-1 envelope protein gp145 (Figure 17).¹⁴¹ *In vitro* assays were performed in human embryonic kidney cells line (HEK293) showing good transfection ability. Following various immunization routes (e.g., activation of Toll-like receptor signaling or effector memory T cell immune

response), fullerene VLPs can induce an innate and a cellular immunity. A similar study was performed for hepatitis C using the HCV recombinant protein as antigen,¹⁴² confirming the potential efficacy of using fullerenols as antiviral vaccines. Nevertheless, these results require further mechanistic investigations. Indeed, *in vitro* studies also evidenced a suppressive effect of acquired immune response of C₆₀ pyrrolidine tris-acid and fullerene C₆₀(OH)₃₆.¹⁴⁵ The fullerene had a dose-dependent effect on T cell receptor-mediated activation and antibody production by B cells under anti-CD40/IL-4 stimulation. However, the molecular mechanism is still unknown.

Other HNMs including AuNPs (two subcutaneous injections in guinea pigs) and nanodiamonds (three subcutaneous injections in Balb/C mice) were explored as carriers of viral proteins for immunization of swine transmissible gastroenteritis virus and H7N9 influenza, respectively, with good preliminary results.^{146,147} In these cases, the vaccine formulation relies on the adsorption of the viral antigen onto the surface of the nanoparticles. However, an effective vaccination depends on several factors. First of all, the size of the NPs plays a key role on the immune system. For instance, size-dependent vaccination efficacy has been reported in mice immunization against the foot-and-mouth disease virus (intraperitoneal and subcutaneous injection, every 7 days for 7 weeks) using AuNPs as antigen carriers. In this study, the most effective activity to stimulate the immune system was exerted by particles with a diameter in the range of 8 nm.¹⁴⁸ Both smaller or bigger particles evidenced a drop-off of the immunization effect. This aspect cannot be ascribed to the antigen concentration, but must be associated only to the

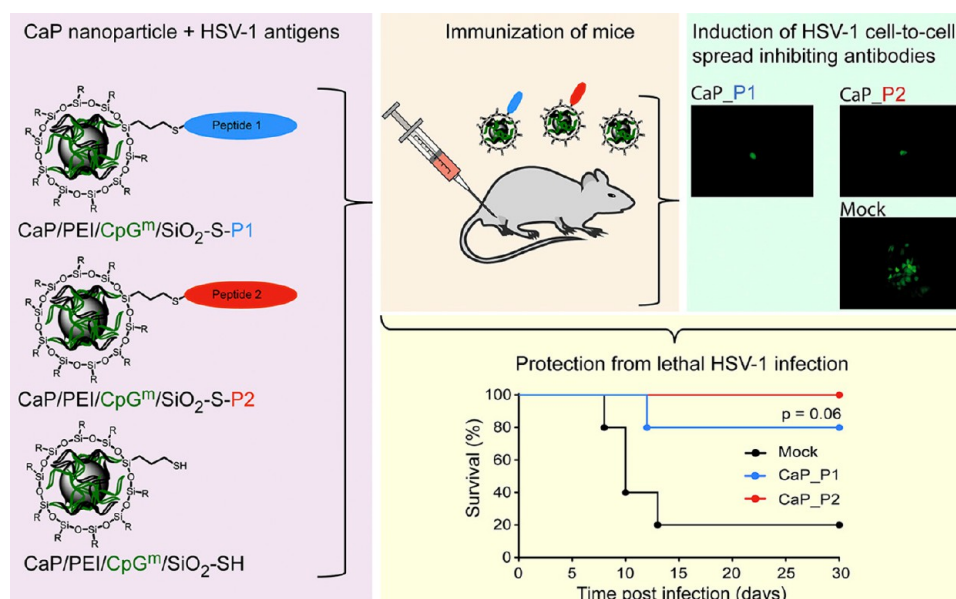


Figure 18. Scheme of CaPNPs used for vaccine. Left: CaPNPs functionalized with CpG^m adjuvant and two different peptides as antigens. Top right: CaPNPs were able to reduce cell-to-cell virus spread *in vitro*. Bottom right: CaP nanovaccines were able to immunize mice against HSV-1. Reproduced with permission from ref 153. Copyright 2019 Elsevier B.V.

nanoparticle size; however, the mechanism of interaction remains unknown. The selected antigen plays also a crucial role in the preparation of wide spectrum vaccines. For instance, in the case of influenza, two major membrane glycoproteins, hemagglutinin and neuraminidase, are generally used as antigens. However, the antibodies produced by this vaccination strategy are selective to the dominant epitope which has a low effectiveness or is totally ineffective against other epitopes or other kinds of influenza viruses. M2 (a viral protein responsible for the budding and scission of the influenza virus) is commonly expressed in different types of influenza viruses with a high rate of conservation but with low antigenicity. It has been shown that AuNPs functionalized with M2e protein have a high immunization capacity in comparison to the antigen alone. Mice immunized with AuNPs (two intranasal injections), and then challenged, showed a survival rate higher than 90% to California-H1N1pdm, Victoria-H3N2, and Vietnam-H5N1 infections.¹⁴⁹ This strategy shows that HNMs can be used to boost the immune response of low immunogenic molecules, providing a wide spectrum vaccination potential. Unfortunately, it has not been determined if the budding process in SARS-Cov-2 is mediated by viral proteins or *via* the host cell's endosomal sorting complex, thus more research on the SARS-Cov-2 viral machinery is highly desirable.

All these approaches are based on the capacity of the nanoparticles to adsorb the antigens and expose them to the immune system in the appropriate conformation to produce the neutralizing and protective antibodies. However, the adsorption is not an easy process to control. For instance, AuNPs have been ineffective in the immunization against SARS-Cov, when S viral proteins were used as antigens.¹⁵⁰ In particular, the immunization with the protein alone was more efficient than when it was adsorbed onto the AuNP surface. This failure was associated with a conformational change or denaturation of the antigen, which did not lead to the production of the specific antibody.¹⁵⁰

Covalent chemistry strategies offer a higher control on the antigen quantification with higher reproducibility and possibility to bind different groups onto the surface of HNMs. For example, calcium phosphate nanoparticles (CaPNPs) were successfully covalently functionalized with Hen Egg lysozyme as model antigen showing an immunization 100 times higher *in vivo* compared to antigens alone using (one subdermal injection in mice).¹⁵¹ A similar approach was used with iron oxide NPs using mannose (to target DCs) and hepatitis B antigen showing good immunological activity *in vitro* (two subdermal injections in mice at 14 days distance).¹⁵² More recently, other types of vaccination strategies have been applied using HNMs. A smart example has been reported using multifunctional CaPNPs on herpes virus. In this study, CaPNPs have been covalently functionalized with alum/MPL as the adjuvant and two peptides as antigens selected *via* reverse vaccination. The NM stimulated the immune system generating highly efficient antibodies able to block cell-to-cell infection of herpes virus *in vivo* (three intramuscular injections in mice every 14 days), increasing the survival rate of immunized mice to 100% against the controls (20% survival, Figure 18).¹⁵³

SUMMARY AND PERSPECTIVES

The recent history has shown the spread of different viral pandemics such as H1N1 flu, HIV, and SARS. Nowadays, the SARS-Cov-2 pandemic global lockdown has profoundly changed the daily life of most humans, causing uncertainty in short and middle time perspectives. In this context, the scientific community has responded to protect the population by studying new vaccines and disinfection methods to be applied in the near future. Despite this tough work, a SARS-Cov-2 vaccination will hopefully be available in 1–2 years, making this epidemic transient period gloomy with increased instability. The study of more effective vaccines and the production of a wide range antiviral agents is nowadays an extremely hot topic.

HNMs comprise a family of materials that share a nanostructured hard core and a tunable surface chemistry. In this contribution, we have methodically reviewed different HNMs for antiviral properties. HNMs can have antiviral properties *per se*, blocking the viral replication and diffusion, or their antiviral properties can be tailored, playing with surface chemistry. HNMs can be used to block viral entry and arrest infection at the early stage. The mechanisms rely on different actions including breaking of capsid disulfide bonds (e.g., noble metal nanoparticles), capsid oxidation (e.g., CuONPs), mimicking of cell surface (e.g., carbon NMs), or mechanical disruption (e.g., AuNPs or graphene). Surface functionalization additionally confers a higher specificity and pharmacological activity toward the targeted virus. In particular, the high local concentration of ligands on functionalized HNM surface imparts a high multivalent effect, enhancing the viral trapping efficiency of NMs. Interestingly, HNMs that show an intrinsic antiviral activity can further enhance their antiviral efficacy *via* surface functionalization. Some antiviral HNMs are good photosensitizers (e.g., CuONPs) or exert photothermal activity (e.g., carbon NMs), thus their antiviral activity can be triggered by light stimulation. More importantly, most of the settled strategies target common viral entry mechanisms and can be adopted to fight a wide viral spectrum. HNMs have been also applied as antiviral agents by their interaction with host cells. HNMs are able to block viral replication machinery in host cells (e.g., CNTs and fullerenes), inhibiting endogenous enzyme activity. Additionally, HNMs can be explored for the delivery of antiviral molecules, showing a better antiviral activity and reducing side effects *in vitro* and *in vivo*. Some HNMs can also regulate the ROS homeostasis of host cells, reduce apoptosis, and enhance host cell survival during the infection. Finally, we have reviewed the role of HNMs on immunity. In particular, HNMs can stimulate the innate immune response, mainly inducing overexpression of interferon and cytokines. This effect can alert sentinel cells and generally warn the immune system of the infection. HNMs can be also used for the activation of the adaptive immune response, foreseeing vaccination. Due to the similar size of a virus, functionalized HNMs with antiviral molecules (virus-like particles) can enhance their immunogenicity.

Certainly a huge effort should be done for translation of the research into clinics. Indeed, HNM applications as antivirals are still in the early phase of research. Several challenges still need to be tackled before their safe use. At the current stage, some HNMs have been approved only for surface disinfection. For instance, CuNPs have been used in filters for the preparation of highly efficient broad spectrum antiviral masks.⁵⁰ Some HNMs have been already clinically approved. For example, FeNPs were approved for imaging and as a drug to treat iron deficiency anemia in adult patients with chronic kidney disease, while AuNPs are in clinical trials for the treatment of prostate cancer (photothermal therapy).^{154,155} However, at the moment no clinical trials are running for the use of HNMs as antiviral agents. In fact, there are still several concerns on the applications of HNMs in drug formulations. Compared to molecules, where mainly concentration and exposure routes are concerned, solving HNM toxicity issues is much more complicated. Composition, size, shape, and surface functionalization must be considered to respond to the requirement for safety regulations. Additionally, interaction on HNMs with the immune system must be better elucidated. In particular, the activation of the immune system and

complement activation-related pseudoallergy must be taken into account.¹⁵⁶ For instance, ferumoxylol (a FeNP-based drug) has been reported to generate severe anaphylactic reactions in humans, 18 of which were fatal.¹⁵⁶ This is due to the possible interaction of HNMs with mast cells, provoking their degranulation/activation and release of histamine even at the first exposure (pseudoallergic-mediated hypersensitivity). Besides, the mechanism of activation of these cells is still unknown, although it was found that it depends on the HNM composition, size, and surface chemistry and on the corona formation.¹⁵⁶ On the other hand, it has been demonstrated that HNMs can travel to the draining lymph nodes, targeting resident dendritic cells and macrophages. Therefore, they are able to interact with antigen presenting cells to stimulate innate and adaptive immune responses.¹⁵⁶ We believe that a joint venture of different chemists, materials scientists, virologists, toxicologists, and medical doctors can push forward the preparation and safe application of HNMs in this field, hoping to prevent and eventually block the rise of new viral pandemics.

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Notes

The authors declare no competing financial interest.

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VOCABULARY

Hard nanomaterial, nonpolymeric organic or inorganic materials with sizes comprised between 1 and 100 nm; antiviral, medical term used for any agent or drug altering virus integrity or process involved in viral infection disease; viral infection, process by which viruses invade the body through multiple pathways and multiply in susceptible host cells; viral pathogenesis, approach in biomedical research to understand the process by which a viral infection leads to disease, including mechanism of infection into the host (e.g., viral entry, viral replication) and factors that affect this mechanism (e.g., virus susceptibility to host defenses); membranotropism, the ability of an organism or an agent to interact with biological barriers; immunogenicity, capacity of an exogenous substance or material to trigger an immune response in humans and other animals or to induce a humoral and/or cellular immune responses; multifunctional platform, material modified with different functionalities to achieve a variety of combined treatments

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