Nanomedicine formulations for the delivery of antiviral drugs: A promising solution for the treatment of viral infections

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Abstract

I**ntroduction.** Viral infections represent a public health problem and one of the leading causes of global mortality. Nanomedicine strategies can be considered a powerful tool to enhance the effectiveness of antiviral drugs, often associated with solubility and bioavailability issues. Consequently, high doses and frequent administrations are required, resulting in adverse side effects. To overcome these limitations, various nanomedicine platforms have been designed.

Area covered. This review focuses on the state of the art of organic-based nanoparticles for the delivery of approved antivirals. A brief description of the main characteristics of nanocarriers is followed by an overview of the most promising research addressing the treatment of most important viral infections.

Expert Opinion. The activity of antiviral drugs could be improved with nanomedicine formulations. Indeed, nanoparticles can affect the fate of the encapsulated drugs, allowing controlled release kinetics, enhanced bioavailability, modified pharmacokinetics, and reduced side effects. In addition, the physicochemical properties of nanocarriers can enable their capability to target specific sites and to interact with virus structures. In this regard, nanomedicines can be considered an opportunity to enhance the therapeutic index of antivirals. Efficacy, safety, and manufacturing issues need to be carefully assessed to bring this promising approach to the clinic.

Keywords: antiviral drugs, nanomedicines, viral infections, nanoparticles, nanotherapeutics, targeted delivery.

Article highlight box

- Nanocarrier incorporation of antiviral drugs can modify their pharmacokinetics and pharmacodynamics properties and reduce the side effects.
- Long-acting nanoformulations can be applied in the treatment of infectious diseases to promote compliance and patient adherence.
- Nanomedicines formulations can overcome biological barriers and achieve effective drug concentrations in viral reservoirs.
- The peculiar physico-chemical properties of nanocarriers can enable their capability to interact with virus structures.
- Nanomedicine can provide an effective platform for treating viral diseases by small interfering RNA (siRNA) delivery.
- Nanomedicine strategies can be considered a powerful tool to enhance the potential of currently approved antiviral drugs.

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1. Introduction

Viral infections pose significant global health challenges, particularly because resistant viral strains and adverse side effects associated with the prolonged use of antiviral drugs can reduce the effectiveness of antiviral therapies. In the past several decades, a new paradigm for enhancing the efficacy of bioactive molecules – the use of nanotechnology in medicine (i.e., nanotherapeutics and nanodelivery systems) – has attracted increasing attention and has been largely exploited in many medical fields, both in basic research and in the drug development pipeline [1].

The use of nanodelivery systems offers some advantages that can improve therapeutic treatments. Indeed, the biopharmaceutical properties, and those of absorption, distribution, metabolism and excretion (ADME), of conventional drugs depend upon their physicochemical characteristics.

Nanomaterials can affect and govern the fate of the encapsulated drug [2]. Interestingly, nanocarrier formulations can modify the physico-chemical properties of the incorporated molecules, thereby allowing sustained/controlled release, modified pharmacokinetics, and targeting specific sites of action. This can lead to an increase in the effectiveness of the drug and a decrease in the associated side effects [3].

In this context, the nanomedicine rationale can be exploited to enhance the therapy of viral infections, taking into consideration the narrow therapeutic indices, high doses, and frequent administration needed for a number of antivirals drugs due to their limited aqueous solubility, short half-life, and/or slow uptake by the body tissues [4]. In addition to the physico-chemical limitations of drugs, prolonged anti-viral therapy can result in the development of drug-resistant virus strains necessitating daily repeated administrations, which can lead to a demanding drug-regimen. This is especially the case of chronic diseases, such as the acquired human immunodeficiency syndrome (AIDS) due to human immunodeficiency virus (HIV) infection, where no suspension of treatment is allowed in order to avoid viral resistance [5]. Thus, it is clear that the treatment of viral diseases is a challenging problem.

However, nanomedicine-based strategies may be useful for both the prevention and therapy of these diseases. The design of a nanodelivery systems can be approached from different points of view, although it generally takes into account the type of nanocarrier/nanomaterial used and the type of viral disease, leading to approaches that are respectively technologically and biologically driven.

Viruses act as intracellular parasites that use the replication system of the infected cells because they lack the genetic information necessary for the generation of metabolic energy and macromolecular synthesis. This behavior leads to an intricate relationship between viruses and infected cells that should be considered in the development of efficient, safe and targeted therapies. Current antiviral drugs interfere with specific phases of the virus life cycle by targeting viral structures and enzymes that are essential for virus replication. The major mechanisms of antivirals actions against DNA and RNA viruses are reported in Figure 1. Most RNA viruses replicate in the cytoplasm, whereas DNA viruses, Influenza virus and retroviruses (HIV) enter into the nucleus for genome replication. Notably, HIV reverse transcriptase acts before the entry of the genome into the cellular nucleus; by contrast, HBV reverse transcriptase acts on a viral RNA genome of new viral progeny.

Intriguingly, nanomedicine formulations could represent a new avenue for controlling the amount, dosage frequency, and delivery site of antivirals, as well as for targeting the virus life cycle. Indeed, the features of nanoparticles, such as sizes, morphology and surface charge, can be modulated to promote the targeting of the drugs. Moreover, nanocarriers can be engineered to enhance their ability to reach specific extracellular or intracellular targets and to compete with viruses for attachment to cell surface receptors, both of which are key factors for controlling viral diseases and overcoming drug resistance. Consequently, passive and active drug targeting are possible with nanomedicine approaches, as previously described for anticancer drugs [6]. Passive targeting depends on the composition and physicochemical characteristics of the nanocarriers; active targeting is based on the presence of a specific ligand on the nanocarrier surface, which acts as a recognition device for viral nanostructures.

These drug-targeting strategies might facilitate the local and specific release of antiviral drugs while minimizing damage to healthy cells and tissues, thereby preventing side effects.

In addition, these approaches can be exploited for the design of broad-spectrum antiviral-based nanodelivery systems that target the viral membrane or cell receptor to compete with attachment of the virus to the target cell, thereby preventing virus internalization [7]. Recently, suitable engineered nanoparticles (NPs) were developed that interact with cell receptors (i.e., glycan receptors) and competitively bind to cells to prevent viral infection [8]. Interestingly, this rationale has been proven by using liposomal decoys to capture influenza virus and delay the disease [9]. Alternatively, nanoassemblies have been generated to mimic glycan receptors, thereby neutralizing heparin sulfate dependent viruses [10]. In addition, the site-specific drug release potential, as well as the ability to overcome biological barriers of nanomedicine, can offer the advantage of decreasing the administered dose regimen of antivirals. To date, a number of nanocarriers, either organic (e.g., liposomes, NPs, micelles) or inorganic (e.g., silver and gold NPs), have been studied to improve the delivery and therapeutic efficacy of antiviral medicines. Consequently, various potential nanostructures might be fine-tuned and employed to meet the different needs of viral disease therapy [11].

This review highlights the state of the art of organic NPs used for the delivery of approved antiviral drugs. A brief classification of the main types of nanomedicines is given, and a detailed overview of the most common viral infections is provided. Given the large number of papers and reviews on this topic, it has not been possible to reference all the literature in the field, but some examples are reported which indicate how the formulation of antivirals as nanomedicines could represent a promising solution for the prevention and treatment of viral infections.

2. Nanodelivery systems for antiviral drugs

Various organic-based nanodelivery systems for antiviral agents, including liposomes, polymeric NPs, solid lipid NPs, hybrid NPs, dendrimers, nanoemulsions, micellar systems and self-assembled nanostructures, have been proposed [12]. To develop efficient nanocarrier platforms, various criteria should be considered, including safety, biocompatibility, biodegradability and compatibility with the drug. It is worth noting that the main parameters that determine NP functionality are particle size, size distribution, shape and surface characteristics (e.g., chemistry, charge) [13]. Additionally, NP features should be fine-tuned to optimize the pharmacokinetics profile, *in vivo* biodistribution and viral interactions. Figure 2 reports an overview of the main drawbacks of antivirals and nanotechnological strategies to overcome the limits of antiviral therapy. For example, nanocarriers for oral nanoformulations should be resistant to the acidic pH of stomach and intestinal enzymes, and be able to penetrate the mucus secretion that limits the intestinal presence of these drugs. Drug release kinetics is another important parameter that influences the therapeutic effectiveness of nanodelivery systems. An ideal system should incorporate a good amount of drug and release its payload once it has arrived at the site of action. Smart nanocarriers that are able to control and self-regulate drug delivery have been investigated, particularly for cancer therapy and also for infectious diseases [14]. Finally, to enhance the effectiveness of the drug, it is necessary to take the route of administration into account. The majority of antiviral drugs are administered orally, although some are delivered via intravenous or subcutaneous routes (Fig. 3). With regard to the toxicity of organic NPs based on biocompatible components, many papers have reported no or slight toxicity related to the surface charge or solvent residues [15]. The general characteristics of current nanoformulations for antivirals are described below.

2.1 Liposomes

Liposomes are nanocarriers comprised of phospholipid bilayers encapsulating an aqueous core [12]. These spherical lipid vesicles improve the solubility of compounds by compartmentalizing, solubilizing, and delivering a wide range of hydrophilic and hydrophobic molecules. These unique capabilities, coupled with their biocompatibility, biodegradability and low toxicity make liposomes very attractive as drug delivery vehicles. They have consequently been widely investigated as one of the most extensively used nanocarriers, and in 1995 the first liposomal product, consisting of doxorubicin-loaded pegylated liposomes was approved by the US Food and Drug Administration (FDA) for cancer treatment. Liposomes range in size from 20 nm in the case of small unilamellar vesicles, to more than 100 nm for large and multilamellar vesicles. In basic research, the most common preparation method is the dehydration-rehydration technique, which has relatively high loading efficiency compared with other methods, such as reverse-phase evaporation or detergent depletion, all of which have lower encapsulation efficiencies. Liposomes have a natural tendency for hepatic accumulation, and thus represent an optimal drug delivery system for the treatment of liver diseases, including chronic hepatitis C virus infections [16]. However, as drug carriers, liposomes have certain drawbacks. Besides their low *in vitro–in vivo* stability, they have low encapsulation efficiency and have a high production cost. In addition, their rapid removal from the blood and capture by cells of the reticuloendothelial system restrict their therapeutic applications.

2.2 Niosomes and ethosomes

Niosomes are vesicular delivery systems that result from the self-assembly of hydrated surfactant monomers. They may have some advantages over liposomes with respect to chemical stability, lower cost of chemicals, and the large number of surfactant classes available for the design of this vesicular system [17]. Niosomes can encapsulate both hydrophilic and lipophilic molecules, by entrapping

hydrophilic ones in vesicular aqueous core or adsorbed on the bilayer surfaces, while lipophilic drugs are encapsulated by their partitioning into the lipophilic domain of the bilayers. Because of their nonionic nature and high biodegradability, niosomes have shown excellent biocompatibility and low toxicity. However, segregation of non-ionic surfactants may cause toxicity [18].

Ethosomes are lipid multilamellar vesicles composed of phospholipids, ethanol and water. Because of their ability to fuse with skin lipids, ethosomes deliver topical agents to the skin more efficiently than liposomes. On the basis of their composition, they can be classified into classical ethosomes, binary ethosomes, and transethosomes. Classical ethosomes are composed of phospholipids, a high concentration of ethanol up to 45% w/w, and water. They are a modification of classical liposomes showing better skin permeation and stability profiles. Binary ethosomes were developed by adding another type of alcohol (i.e. propylene glycol and isopropyl alcohol) to the classical ethosomes. Transethosomes are the new generation of ethosomal systems developed in order to combine the advantages of classical ethosomes and deformable liposomes (transfersomes). They contain the basic components of classical ethosomes and an additional compound, such as a penetration enhancer or an edge activator (surfactant) in their formula [19].

2.3 Polymeric nanoparticles

Polymeric nanoparticles (NPs) were developed later than liposomes to improve their stability and drug payload. They are solid colloidal particles, generally below 500 nm in size, and are composed of a biocompatible polymeric matrix that can be made of synthetic or natural polymers [12]. The therapeutic molecule is either entrapped, adsorbed or covalently attached. Because of their polymeric composition, polymeric NPs may have greater stability than liposomes in biological fluids and under storage conditions. Polymeric NPs are prepared by several methods, including solvent evaporation, spontaneous emulsification, solvent diffusion and polymerization. The preparation conditions have

important effects on the characteristics of the NPs obtained, such as size and release rate. NPs can be loaded with lipophilic and hydrophilic drugs, and different chemical approaches have been proposed, including covalent chemistry, hydrophobic interactions and entrapment.

The synthetic polymers most widely used are polyesters, such as polylactides (PLAs), and polylactide–polyglycolide copolymers, poly(lactic-co-glycolic acid) (PLGAs), polycaprolactones (PCLs) and polyacrylates (PCAs). The most common polymer used is PLGA, and several PLGA drug delivery systems have been approved by the FDA. Natural polymers, such as alginate and chitosan, have also been used, and they generally exhibit immunogenicity lower than that of synthetic ones.

2.4 Polymeric micelles

Polymeric micelles are colloidal structures of block copolymers, such as poly(butyl methacrylate) (PBMA), polystyrene (PLS), PLA or poly(ethyleneoxide)–poly(propylene oxide) (PEO–PPO). The polymers spontaneously form micellar structures above the critical micelle concentration and temperature, causing the polymer chains to become hydrophobic and resulting in aggregation [12]. On the other hand, the polymers become insoluble in aqueous solution, and they act like inert materials below the aforementioned critical conditions. The hydrophobic fragments form the spherical inner core, in which poorly-water soluble-drugs can be encapsulated, and hydrophilic fragments form the outer shell. The outer hydrophilic shell can be functionalized with different moieties, such as folate, monoclonal antibodies and monosaccharides (i.e. mannose, glucose, fructose), to achieve active targeting and/or pH/temperature responsive nanocarriers. They have raised particular interest as nano-sized drug delivery systems, not only because they provide increased solubility and stability of hydrophobic drugs, but also due to their *in vivo* advantages versus the free drug, such as increased circulation half-life, enhanced bioavailability and improved cellular uptake [20]. Polymeric micelles suitable for drug delivery have the appropriate charge density and polymer chain length, and the

particle diameter is usually within 5–100 nm. Size control depends on the chemical structure of the polymers and is not dependent on the preparation process, which is an advantage that other types of NPs lack. Similar to other organic NPs, polymeric micelles have low toxicity because they disassemble into single polymer chains that can be easily excreted and exhibit no toxicity. Micellar stability mainly depends on the copolymer self-aggregation tendency. Since polymeric micelles are dynamic systems, they are liable to dissociate, especially upon administration when they are diluted to a concentration below the CMC. Moreover, blood components can alter the kinetic stability of micelles and cause dissociation [21].

2.5 Solid lipid nanoparticles

Solid lipid nanoparticles (SLNs) are made of lipids that are solid at body temperature, such as fatty acids and triglycerides [17]. The method of preparation usually involves heating to liquefy the lipids. Upon cooling, SLNs separate and can be readily dried. They are stabilized with emulsifiers and co-emulsifiers, such as polysorbates, poloxamers, fatty acid co-esters, lecithin and bile salts. The amount of stabilizer used must be enough to prevent aggregation without reducing drug uptake. The proper selection of lipids and surfactants can affect the particle size, long-term stability during storage, drug loading and behaviors of release. SLNs are less toxic and easier to scale up than synthetic polymer NPs. Furthermore, the state of the lipids depends upon the characteristics of the medium (e.g., temperature, pH), which allows for better controlled release, safety and efficacy. Common disadvantages of SLNs are unpredictable gelation tendency and inherent low incorporation rates resulting from the crystalline structure of the solid lipid [22].

2.6 Dendrimers

Dendrimers are nanosized, radially symmetric molecules with homogenous, well-defined and monodisperse structures, which typically have a symmetric core, an inner shell and an outer shell. These polymers are shaped like the head of a tree and have two unique characteristics, namely, a globular structure and polyvalency, which is found in many naturally occurring systems. Tomalia *et al*. reported the synthesis of the first family of dendrimers, known as poly(amido amine) (PAMAM), which is one of the most successfully used dendrimers [23]. One of the dendrimer-based formulations was designed specifically with HIV and HSV antiviral activity. Vivagel®, a carbomer gel containing a dendrimer (SPL7013), has demonstrated efficacy against human immunodeficiency virus and herpes simplex virus in *in vitro* and animal models. Phase I clinical trials have been conducted, and the results concerning its safety were generally favorable [24-25].

2.7 Cyclodextrin derivatives

Cyclodextrins are nanometric biomaterials consisting of a-1,4-linked cyclic glucopyranose oligomers, and they are synthesized by enzymatic action on hydrolyzed starch. The most common (so-called "native") forms of cyclodextrins are α–, β–, and γ-cyclodextrin, consisting of six, seven or eight glucopyranose units, respectively. They have a characteristic toroidal shape formed by linkages of multiple glucose units, thereby creating a hydrophilic exterior and a hydrophobic cavity. This distinctive structure can form inclusion complexes with compounds whose geometry and polarity are compatible with that of their cavity. The production of drug/cyclodextrin complexes has become one of the most extensively investigated approaches to improve the stability, solubility, dissolution rate and bioavailability of drugs. Indeed, cyclodextrins have been employed to overcome the main biopharmaceutical drawbacks of antiviral agents [26]. Cyclodextrin-based nanosponges are hyper-

cross-linked polymers that have cyclodextrin units as their building blocks, and they are characterized by their marked capacity to encapsulate a great variety of substances [27].

2.8 Nanoemulsions

Nanoemulsions are biphasic systems consisting of oil nanodroplets in the order of 100 nm. A typical nanoemulsion contains oil, water and an emulsifier. The addition of an emulsifier is critical for the creation of small droplets as it decreases the interfacial tension (i.e., the surface energy per unit area) between the oil and water phases of the emulsion [28]. Nanoemulsions are potential tools for improving the oral bioavailability of poorly aqueous soluble drugs. Self-nanoemulsifying drug delivery systems (SNEDDS) are anhydrous homogenous liquid mixtures consisting of oil, surfactant, drug and co-emulsifier or solubilizer, which spontaneously form oil-in-water nanoemulsions of approximately 200 nm or less upon dilution with water under gentle stirring [29]. These systems offer a number of advantages including decreased dose and dosing frequency (due to improved bioavailability), high drug-loading efficiency and high stability. However, questions regarding the physical and chemical stability of drugs solubilized in lipid and excipients mixture have not yet been adequately addressed [30]. Successful large-scale production of the SNEDDS exemplified for two HIV protease inhibitors ritonavir (Norvir®, Abbott Laboratories,Abbott Park, IL, USA) and saquinavir (Fortovase®, Roche Pharmaceuticals, Nutley, NJ, USA), has generated considerable interest.

2.9 Nanosuspensions

Nanosuspensions or nanocrystals are nanoformulations comprising 100% pure drug nanoparticles with sizes in the nanoscale range, generally stabilized by surfactants or polymers. These drug nanodelivery systems are derived from traditional pharmaceutical technology. Nanosuspensions are usually obtained in liquid media by bottom-up or top-down methods or a combination of both techniques. They have

been designed to enhance the solubility, dissolution rate and bioavailability of drugs via various administration routes. Due to their small sizes, nanosuspensions can also be considered nanotechnology-based drug delivery systems for nanomedicine products. Many nanocrystal formulations have been developed to increase the bioavailability of drugs and to decrease adverse side effects [31]. Currently, 9 nanocrystal-based drug products are on the market.

2.10 Hydrogel based nanocarriers

Hydrogels are swellable polymer networks, cross linked together either by physical or chemical cross-linking, with high fluid absorption capacity. The use of these versatile, highly biocompatible structures for drug delivery has been extensively studied. Some hydrogels exhibit a high drug loading efficiency and display prolonged drug release. Moreover, drug release from hydrogel nanoparticles can be controlled physically or chemically. Hydrogels at the nanoscale have a large surface area for multivalent conjugations and subsequent surface modifications. The targeting capacity can be enhanced by coupling targeting ligands to the nanoparticle surface [32]. Efforts are being made to functionalize hydrogel-based nanoformulations for brain delivery to eradicate neuro-AIDS. Owing to their biodegradability and biocompatibility, bio-polymeric nanogels have shown excellent potential as targeted drug carriers for the central nervous system. Nanogels might be modified using hydrophobic moieties or many such compounds with the ability to enhance the permeability of BBB by crossing the tight junctions between endothelial cells. Moreover, nanogel surface charge and hydrophobicity have a great influence on plasma protein adsorption, affecting the uptake via transcytosis [33].

Hydrogels have been also explored for intravaginal drug delivery to prevent sexual transmission of HIV and other vaginal infections, providing excellent convenience for frequent usage and prolonged drug release. They could be topically applied over the vulvovaginal area to achieve a high bioavailability at the targeted tissues and potentially reduce the side effects associated with oral

formulations [34]. Thermosensitive hydrogels provide a method for convenient administration, showing the ability to spread along the vaginal canal after injection and coat the vaginal epithelium by hydrogel formation. Furthermore, the formed hydrogel acts as a drug reservoir releasing the drug that would be absorbed by epithelia or directly penetrated into virus to prohibit virus propagation [35].

Nanolipogels are an emerging platform being investigated as an alternative to the more widely used liposomes or polymer nanoparticles. These dual structure nanoparticles have a distinct lipid bilayer encompassing a polymer core, and they have been synthesized by UV-induced gelation of a hydrogel network within liposomes using various processes. Nanolipogels have been used to incorporate physico-chemically diverse agents within the lipid bilayer and in the hydrogel core, showing high encapsulation efficiency and controlled release kinetics. Studies have demonstrated the biological utility of nanolipogels for use in HIV infection chemoprophylaxis as topical microbicides [36].

2.11 Stimuli responsive drug delivery systems

On-demand drug delivery is becoming feasible through the design of stimuli-responsive systems that recognize specific triggers and react in a dynamic way. Nanoscale stimuli-responsive systems are able to control drug release in response to specific stimuli, either exogenous (variations in temperature, magnetic field, ultrasound intensity, light or electric pulses) or endogenous (changes in pH, enzyme concentration or redox gradients). They can take advantage of specific microenvironmental changes associated with pathological situations such as neoplastic diseases, ischemia, inflammatory diseases or infections [37].

Smart nanoparticles with stimuli responsive release are proposed for delivery of anti-retroviral agents, exploiting as triggers semen, enzymes, endosomal escape, temperature and magnetic field [38]. Novel stimuli-responsive strategies are provided especially in the development of vaginal nanocarriers for the prevention of sexually transmitted infections. Among them are Eudragit® S100-based vaginal delivery

systems for Tenofovir. The presence of Eudragit®, a pH-sensitive methacrylic acid/methyl methacrylate copolymer (soluble at pH 7.6 but insoluble at pH 4.5), enabled the release of drug depending on environment pH changes as occurring upon intravaginal ejaculation and, therefore, increased the release of Tenofovir from nanoparticles in simulated seminal fluid [39]. Furthermore, Huang et al. developed pH-sensitive cellulose acetate phthalate (CAP) nanofibers to deliver tenofovir disoproxil fumarate- or etravirine, and fibers dissolved quickly in the presence of human semen releasing the drug payload [40]. Moreover, many enzymes in the semen, like hyaluronidase and prostate specific antigen, can act as triggers for the delivery of drugs on contact with semen.

2.12 Toxicology aspects

To date, the toxicity of nanomaterials delivering antiviral drugs has been studied predominantly *in vitro* or to assess the component biocompatibility [41]. Generally, *in vivo* biodistribution studies of injected nanoformulations have revealed high accumulation of drugs in various organs without investigating the toxicity profile after prolonged administration. Furthermore, the effects of oral nanoformulations on gut microbiota is also an important aspect to take into account when analyzing possible modulation on the metabolic activity of enteric bacteria after treatment. Studies of immunogenicity, hypersensitivity reactions or other adverse immune effects of orally or intravenously administered drug delivery systems used to cure infections are poor and require more attention [42]. Increasing evidence suggests a potent immune modulatory role of nanoformulations, especially lipid nanoparticles and engineered liposomes used as vaccine adjuvants, in order to boost antigen-specific immune responses [43-45]. In the past decade, nanoformulated vaccine candidates have been developed and tested *in vivo* against viral infections, such as respiratory syncytial virus and HIV. Interestingly, nanoparticles efficiently enter the lymphatic system, and through classic receptor-mediated endocytosis particles smaller than 150 nm are endocytosed by macrophages and dendritic cells and antigens are presented for following

activation of T cells. A different effect on the immune response has been observed according to the nanoparticle size: a stronger CD4 or CD8 T cell response has been promoted by a particle size major or minor than 50nm, respectively [46].

Cellular uptake and interaction of nanocarrier are affected by their composition, size, shape, and surface properties (area, porosity, charge, surface modifications, coating) [47,48]. The biological activity and therapeutic efficacy achieved by drug loaded in nanosystems depend on these different physico-chemical parameters. They are likely to modify biological responses, such as translocation across epithelia to other organs, induction of oxidative stress, binding to proteins and receptors, and localization in cellular organelles as mitochondria.

In regard to the safety of human exposure to nanomaterials, genotoxicity studies, including the Ames test (the Salmonella typhimurium reverse mutation assay), mouse lymphoma assay (*in vitro* mammalian assay) and chromosome aberration assay (in vivo rodent assay), are crucial. These studies have often revealed a high genotoxicity especially by inorganic nanoparticles [49].

Furthermore, nanomaterials should be tested on primary cells and cell lines to test their genotoxic potential to induce strand breaks and oxidized DNA lesions and to affect the cell cycle progression

3. Nanoformulations for approved drugs against specific human viral infections

3.1 HIV infection

The human immunodeficiency virus (HIV) is the cause of acquired immunodeficiency syndrome (AIDS) and represents a major public health issue and cause of mortality globally [50]. At present, there is no effective vaccine for HIV or a way to eradicate the established infection due to its peculiar pathogenesis: 1) HIV has a high mutation rate that promotes immune escape and antiviral resistance; 2) the HIV replication cycle requires integration of the viral genome (provirus) in cellular DNA; and 3) cellular HIV reservoirs (i.e., latently infected CD4+ memory T cells, macrophages and hematopoietic

cells) and anatomical HIV reservoirs (i.e., mucosa, brain, lymph nodes) are created where low levels of viral replication are maintained even in the presence of treatment [51].

As shown in Table 1, there are currently 29 approved antiretroviral drugs to treat the infection [52], belonging to five different classes based on the phase of replication affected: 1) nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) [53]; 2) non-nucleoside reverse transcriptase inhibitors (NNRTIs) [54]; 3) integrase inhibitors [55]; 4) protease inhibitors (PIs) [56]; 5) fusion/entry inhibitors [57]. To reduce the incidence of viral resistance and increase drug efficacy, the gold standard of care for treatment of HIV infection involves the use of a combination of at least three antiretroviral therapy (ART) drugs belonging to different classes, which are administered orally at least once a day. Only one anti-HIV drug, the oligopeptide Enfuvirtide, requires subcutaneous administration [58]. Despite the potent activity of antiretroviral products, many challenges remain in eradicating HIV from infected cells due to the toxicity and poor bioavailability of approved anti-HIV drugs, drug-drug interactions, achieving efficacious drug concentrations in viral reservoirs, poor patient compliance, development of drug resistance and systemic side effects. To overcome these problems, different ART nanodelivery approaches have been developed in the last several decades, and they have been described in recent reviews [59-62].

Here, we highlight the most promising nanotechnology strategies for treatment of HIV infection, taking into account the key limitations of the drugs (see Table 2). It is worth noting that the majority of the marketed antiretroviral drugs are administered orally. Consequently, the new oral nanodelivery systems should be able to overcome the various challenges raised either by the physico-chemical properties of the drug or by the conditions of the gastrointestinal tract [63]. To address poor water solubility problems, nanocrystal formulations could be used [31]. The formulation rationale is that nanoparticles produce a huge increase of the surface area, resulting in a significant increase of the drug dissolution rate, consequently improving the oral bioavailability. Moreover, nanocrystals have mucoadhesion

properties, thus prolonging the retention time in the intestinal mucosa. Drug nanosuspensions, consisting of 100 % pure drug nanocrystals, can be easily translated into clinical studies because they can be obtained with low amounts of excipients and exploiting current pharmaceutical manufacturing processes. This type of nanoformulation can be considered a tool useful for traditional and innovative product production. Liquid nanocrystal nanoformulation can permit the administration of antiretrovirals to pediatric patients and to patients with swallowing problems. Nanocrystals are also able to mask the drug's unpleasant taste, favoring the patient's compliance considering that HIV medicinal products are for chronic use.

Nanocrystals as innovative nanodelivery systems can be used to obtain sustained release [31]. For example, paliperidone nanocrystals is a long acting injectable formulation on the market. Recently, two antiretroviral drug nanocrystals have been considered for clinical phases, one developed by Janssen (rilpivirine LA) [64], and the other prepared by ViiV/Healthcare (cabotegravir LA) [65. Both are long-acting formulations produced by the nanotechnology top-down process of wet bead milling. The versatility of nanocrystals can permit the delivery of different therapeutics for a synergistic therapy. Intriguingly, an efficient targeted combination treatment might be obtained by the co-delivery of different drug nanocrystals.

An interesting nanoART formulation consisting of a nanocrystal mixture of atazanavir, efavirenz, and ritonavir was prepared by high-pressure homogenization, followed by *in vitro* characterization. The results demonstrated that physical characteristics such as particle size, surfactant coating, surface charge and shape affected cell uptake and antiretroviral efficacy [66]. In addition nanocrystals can be used as such or surface-modified. Nevirapine nanocrystals were developed and their surfaces were modified with albumin, polysaccharides or polyethylene glycol to enhance drug targeting potential after intravenous administration to rats [67].

Polymers, either natural or synthetic, are key components of nanodelivery systems. They can be used as solid matrices, self-assembled aggregates, or coating materials (as reported for nanocrystals), according to their chemical structure and preparation process of nanomedicines. The interest of these materials is mainly related to their ability to protect the encapsulated drugs and to control the release kinetics.

Polymer-based nanosystems, such as nanoparticles and micelles, represent a versatile platform for improving HIV infection therapy.

The use of surfactant micelles is a technological strategy commonly used to increase the drug solubility. Interestingly, polymer micelles have a high solubilization capability and an increased stability with respect to surfactant micelles, as recently demonstrated with anticancer drugs.

The encapsulation in polymer micelles can govern the drug release kinetics and protect the dug from the external environment. The application of polymer micelles for the delivery of anti-HIV drugs was widely investigated.

Chiappetta *et al*. [68] studied polymeric micelles of different poly(ethylene oxide)-poly(propylene oxide) (PEO-PPOs) block copolymers for the treatment of pediatric patients which encapsulated efavirenz with increased aqueous solubility up to 34 mg/mL (drug solubility = 4µg/mL). Moreover, good oral bioavailability was obtained after the administration of the nanoformulation to rats. The highest bioavailability was obtained with smaller micellar size [68]. Subsequently, the oral pharmacokinetics of efavirenz-loaded micelles was evaluated in adult healthy volunteers in comparison to suspension and oil solution of the drugs. The micellar system showed a 3-fold increase in the oral bioavailability [69].

To expand the application of polymeric micelles to mucosal administration routes, Sosnik *et al.* [70] generated mucoadhesive thermo-responsive chitosan-g-poly(N-isopropylacrylamide) polymeric micelle-encapsulated indinavir, and a significant 24-fold increase in aqueous solubility was obtained

449 [70]. Other architectures and compositions are currently under investigation to improve the residence
450 time of drug-loaded micelles.
451 Various types of nanoparticles encapsulating antivirals, have been studied using polymers approved by
452 the regulation agencies, such as PLGA and PCL. Polymer nanoparticles can improve the performance
453 of antiviral drugs. In particular, they can provide the sustained release of the encapsulated drug, thus
454 decreasing the administration frequency – which is a feature important for chronic treatments.
455 Interestingly, efavirenz-loaded poly(epsilon-caprolactone) (PCL) nanoparticles have been obtained by a
456 spray-drying method and compared with efavirenz-loaded micelles and pure drug nanocrystals. The
457 encapsulation within polymer nanoparticles significantly increased the maximum concentration in
458 plasma and the oral bioavailability [71]. PLGA-based NPs containing didanosine have been developed
459 to study their uptake by macrophages *in vitro*, and sustained drug release was demonstrated for up to 60
460 days [72].
461 The co-delivery of two or more drugs in the same polymer nanocarrier is an attractive strategy for
462 obtaining a combination therapy. Combinations of ART drugs from different classes have been proved
463 to offer sustained efficacy, durability and long-term safety. Destache *et al.* [73] developed PLGA NPs
464 to simultaneously encapsulate ritonavir, lopinavir and efavirenz, and evaluated their phagocytosis into
465 monocyte-derived macrophages. The sustained release of drugs from the PLGA nanocarrier showed
466 high levels of antiretrovirals in cells until day 28 without cytotoxicity. Furthermore, PLGA NPs were
467 tested for efficacy *in vivo* after intraperitoneal injection in mice. The sustained release of the drugs was
468 confirmed, as the nanoformulated antiretrovirals were detected in blood and organs up to 35 days after
469 administration [73].
470 Another interesting approach is the development of lipid nanoformulations such as SNEDDS for
471 improving oral drug delivery. This type of formulation was designed to overcome the stability problems
472 of emulsions. They can be reconstituted in water just before the administration or administered filled

in capsules, being an anhydrous system able to form o/w emulsions in the gastrointestinal environment [30]. In this regard, Patel *et al*. developed SNEDDS consisting of Maisine 35-1 as oil for improvement of oral bioavailability of nelfinavir mesylate [74]. The *ex vivo* drug release through rat intestinal membrane was much faster from both liquid (reconstituted) and solid SNEDDS nanoformulations in comparison with the administration pure drug suspension, suggesting the enhancement in oral permeability. Some products based on this nanotechnology are on the market [63].

Moreover, other kinds of lipid-based drug carriers have been proposed to enhance the oral bioavailability of antiretrovirals. Tenofovir loaded-cationic phosphatidylcholine proliposomes have been formulated with different stearylamine levels to improve the drug permeability for oral delivery. Proliposomes with 5% and 15% stearylamine presented enhancements of permeability by 16.5-fold and 5.2-fold, respectively [75].

Liposomes, due to their fluid lipid bilayer structure, have been successful in improving oral bioavailability of drugs. They can better adhere to biomembranes, form mixed-micelle structures with bile salts in the gut to increase the solubility of poorly-soluble drugs and are suitable candidates for lymphatic uptake [76]. To increase low bioavailability of saquinavir and increase delivery to mammalian cells, a novel liposome formulation was prepared and compared to PEGylated liposomes. PEGylated liposomes showed more sustained drug release, which persisted for 50 hours, and they were less cytotoxic than non-PEGylated liposomes or free drug [77].

Various lipid based formulations have been studied to enhance the intestinal lymphatic uptake after oral administration as an alternative pathway to reach blood circulation. The lymphatic absorption via Peyer's patches can protect the drug from the epatic first-pass metabolism [78]. Previously, Cavalli et al. [79,80] demonstrated that SLNs administered intraduodenally to rats were mainly targeted to the lymph. This strategy was exploted also with antivirals. SLNs loaded with lopinavir, which has very poor oral bioavailability, were formulated to target intestinal lymphatic vessels. The lipid formulation

increased the cumulative percentage dose into the lymph of rats, as well as the drug's bioavailability, most likely by crossing the gut via endothelial intercellular gaps [81,82]. Gaur *et al.* [83] developed efavirenz-loaded SLNs to enhance the oral bioavailability of this poorly water-soluble drug. The nanoformulation exhibited a 5.32-fold increase in peak plasma concentration and a 10.98-fold increase in the area under the curve compared with the drug aqueous suspension after oral administration [83].

Notably, one of the significant challenges in treating HIV infection is the abolishment of cellular and anatomical reservoirs of HIV, where viruses can persist and drug concentrations are suboptimal [60,84]. Different delivery strategies have been explored to improve targeting on the critical sites of HIV infection such as macrophages, lymph nodes and the brain [60,85-87]. Macrophage uptake of drugs depends on the different physical characteristics of nanocarriers such as particle size, surfactant coating, surface charge and shape. Polymeric NPs have been the principal nanoformulation investigated for drug uptake in target cells of HIV-1 infection.

To optimize macrophage targeting specificity, suitable ligands can be conjugated to nanocarrier surfaces, such as small molecules that are able to bind to target cell receptors [62,88]. The mannose receptor of macrophages may also serve as drug delivery cellular portals for nanocarriers. Thus, mannosylated poly(ethylene glycol)-conjugate nanocarriers have recently been explored in cellular uptake studies [89].

Atazanavir-encapsulated galactosylated liposomes were prepared to target the lectin receptors present on macrophages. The nanoformulations resulted in increased uptake by alveolar macrophages and better drug distribution in the lymph nodes, liver, spleen and lungs compared with free atazanavir (AZT). In addition, no toxicity was observed after intravenous injection in rats, confirming that the galactosylated liposomes might be a potential system for targeted drug delivery [90].

One of the main limitations in using AZT lies in their poor intracellular activation by cellular kinases into their active triphosphorylated forms. The administration of tri-phosphorylated molecule (AZT-TP)

may bypass the metabolic triphosphorylation by cellular kinases in cell cytoplasm, but these derivatives do not diffuse intracellularly because of their too hydrophilic feature. Several nanoformulations directly encapsulating the tri-phosphorylated molecule have been studied. Hillaireau *et al.* [91,92] prepared aqueous-cored poly(isobutylcyanoacrylate) nanocapsules as carriers to overcome the cellular delivery of AZT-TP. To prevent leakage of the drug through the nanocapsule membrane, due to its relatively low molecular weight, AZT-TP was complexed to cationic polymers, such as chitosan or polyethylenimine. Nanocapsules efficiently delivered AZT-TP *in vitro* to macrophages, reaching relevant cellular concentrations for therapeutic purposes [91,92].

Furthermore, chitosan nanoparticles based on ionic interactions between chitosan and AZT-TP were developed by Giacalone *et al*. for macrophage targeting [93]. They showed very high drug loading (up to 44% w/w) and increased *in vitro* delivery in murine macrophages compared with the free molecule [93].

It is known that, even at effective plasma drug concentrations, insufficient drug exposure to lymphoid tissue may be one of the key factors in the inability to completely eliminate residual virus.

A number of nanoformulations to enhance drug accumulation in the lymph nodes are currently being evaluated [94]. Among them, an attractive approach for the sustained and targeted delivery of anti-HIV drugs is the use of surface-modified liposomes.

Zidovudine-loaded liposomes have been engineered by incorporating charges (positive or negative using stearylamine and dicetyl phosphate, respectively) or the site-specific ligand mannose to enhance uptake and localization of the liposomes in the lymph nodes and spleen, and increasing quantities of the drug were detected in these organs [95].

A lipid-drug nanoparticle containing lopinavir, ritonavir, and tenofovir was developed and administered as a single subcutaneous dose in primates, resulting in a 50-fold higher intracellular drug concentration in the lymph nodes compared with free drug. After 7 days, persistent levels of the drugs were detected

in the blood, and therefore an improvement on what is achieved using current oral therapies [96]. Endsley *et al.* [97] designed novel peptide-coated lipid NPs for targeting IDV on CD4-expressing cells. Four candidate peptides showed marked binding specificity to CD4 and were used to anchorate on lipid NPs to be targeted in the lymph nodes [97]. Terminal cysteine containing candidate peptides were conjugated to lipid nanoparticles through maleimide-linked phopholipids for targeting to CD4 cells. Interestingly, two peptides bounded on lipid nanoparticles showed a CD4 selectivity in a peptide dose dependent manner. The results proved the key role played by pepetide-tagged nanoparticles for targeting delivery.

Roy *et a*l. [98] developed an efavirenz-loaded polymer-based Pluronic nanocarrier, which was bioconjugated with anti-M-cell-specific antibodies to target the microfold cells in the gut-associated lymphoid tissue. A significantly improved sustained release and increased anti-HIV activity of the targeted loaded drug were observed compared with the free drug [98].

However, the treatment of virus reservoirs in the central nervous system (CNS) remains a challenge.

The brain is one of the main HIV reservoirs due to the inability of antiretroviral drugs to reach effective concentrations in the CNS.

Delivering therapeutics across blood-brain barrier (BBB) have been deeply investigated. Nanoparticle chemical modification or physical triggers can be used to enhance drug penetration to CNS [99]. Nanoparticle features can be tuned to favour the BBB passage, particularly exploiting their surface functionalization. The binding of specific ligands such as peptides, glutathione, transferrin and transferrin antibody, lectins and lactic acid, to the nanoparticle surface have been extensively explored to achieve drug delivery to the brain [100]. Interestingly, to increase the brain targeting efficacy nanoparticles can be functionalized with apolipoprotein E modified peptide responsible for low-density lipoprotein receptor (LDL) binding, highly expressed in the brain [101].

Different strategies have been explored to increase the penetration of ART across the BBB, as summarized by Nair *et al.* [102]. To overcome the limitations of CNS delivery, a valid approach is to eradicate the treatment of neuroAIDS, as reported in a number of recent review articles [102-106]. The design of specific sized and structured nanocargo able to cross the BBB and reach the HIV brain reservoir is one of the goals of nanomedicine. For these purposes nanodelivery systems triggered by external physical stimuli might have great potential for future treatments, i.e. magnetic fields or ultrasound [107].

Polymeric NPs, properly modified to obtain an effective passive or active permeability across BBB, have been designed for drug delivery to the CNS.

The delivery of stavudine, zidovudine, and lamivudine across an *in vitro* BBB model has been explored using both methylmethacrylate-sulfopropylmethacrylate (MMA-SPM) and poly(butyl cyanoacryalate) (PBCA)-based NPs [108]. More recently, Kuo *et a*l. [109] have investigated the ability of MMA-SPM nanoparticles with grafted RMP-7, a synthetic linear pseudopeptide agonist of bradykinin type II (B2) receptor, to deliver stavudine, delavirdine, and saquinavir across the BBB in a co-culture model containing human brain-microvascular endothelial cells and human astrocytes [109]. Tat-peptide-conjugated ritonavir-loaded PLGA/PLA based NPs, formulated using an emulsion–solvent evaporation technique, delivered drug to neural cells, both with and without Tat-peptide conjugation [110].

The transferrin receptor is highly expressed in the brain; therefore transferrin-conjugated nanocarriers are internalized by endocytosis. Transferrin-grafted poly(lactide-co-glycolide) nanoparticles have been developed to deliver the nevirapine across the BBB into human brain microvascular endothelial cells [107]. Lamivudine loaded on PAMAM dendrimer, and mannose-capped poly(propyleneimine)-PPI dendrimers, exerted stronger anti-HIV activity than free drug solution, with a 21-fold increase in cellular uptake [112].

Liposomes have been extensively investigated for delivery of anti-HIV drugs to the brain [113]. The physico-chemical features of lipid systems allow them to cross the BBB easily, and their ability to reach the CNS can be further increased by surface engineering with BBB targeting moieties or cell-penetrating peptides (CPPs) [114].

Interestingly, a saquinavir-loaded flaxseed oil-based nanoemulsion was developed for brain delivery. When orally administered to mice, it had the ability to cross the BBB, probably due to the small size of the lipid-based nanocarrier [115].

Nasal administration as a means to deliver therapeutic agents preferentially to the brain has gained significant recent interest. Intranasal delivery is a non-invasive approach that allows direct access of the therapeutic substances to the brain, bypassing the BBB. Animal and human studies have shown that drugs can be transported directly from the nasal cavity to the CNS via the olfactory epithelium and/or the trigeminal nerve system [116]. A nanoemulsion was prepared for the intranasal saquinavir mesylate delivery to the brain. A higher *in vivo* concentration of saquinavir in the brain was detected after intranasal administration of the nanoemulsion compared with intravenous administration [117].

More recently, Chiappetta *et al.* developed PEO–PPO polymeric micelles loaded with efavirenz for targeting on the CNS. After intranasal administration in rats, an increased accumulation of micelles in the brain was detected, and a 5-fold relative exposure index (the ratio between the area under the curve in the CNS and the area under the curve in the plasma) was calculated compared to intravenous administration [118].

Finally, the use of magnetic nanoformulations allows targeting on specific anatomic sites by applying an external magnetic field and determination of site specific dosing through magnetic resonance imaging [119]. Magnetoliposomes loaded with 3′-azido-3′-deoxythymidine-5′-triphosphate (AZTTP) were developed for targeted delivery across the BBB and consisted of magnetic nanoformulations encapsulated into liposomes, to escape the reticuloendothelial systems. Magnetoliposomes, packed in

monocytes/macrophage, increased the *in vitro* BBB transmigration of the drug and did not affect the BBB integrity. Furthermore, they maintained antiviral activity and resulted in a sustained AZTTP release for two weeks [120]. Jayant *et al*. [121] engineered magnetic nanoparticles for sustained release of Tenofovir by a layer-by-layer assembly of dextran sulfate and drug, exploiting the interaction between the negative sulphate group of dextran and positive charged drug. These magnetic nanoformulations showed a stronger antiviral activity in infected astrocytes and greater ability to cross the BBB compared with the free drug [121]. HIV latency-breaking agents such as vorinostat can be packaged into ultrasmall magnetic nanoparticles in conjunction with tenofovir in order to simultaneously reactivate and kill HIV in a sustained manner for 5-7 days across the BBB. Nanoformulation showed a good BBB transmigration ability with marked *in vitro* antiviral efficacy in primary human astrocytes, with good cell viability after HIV infection [122].

There is a critical requirement to select an efficient and biocompatible nanocarrier, which can be externally guided for on-demand delivery of cargo across the BBB. Magneto-electro carriers (MENCs) of BaTiO3@CoF2O4 (BTO@CFO) were explored for on-demand controlled release of anti-HIV drugs as potential therapy against NeuroAIDS. After administration MENCs were uniformly distributed inside the brain, and were non-toxic to the brain and other major organs and did not affect hepatic, kidney and neurobehavioral functioning [123].

633 **3.2 Herpes virus infections**

634 Herpes viridae is a family comprising several human pathogens divided into three different subfamilies
635 (α, β and γ): herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) and Varicella Zoster virus
636 (VZV) belong to the α-herpes virus subfamily; human cytomegalovirus (HCMV), human herpes virus
637 6 (HHV-6) and human herpes virus 7 (HHV-7) belong to the β-herpes virus subfamily; while Epstein-
638 Barr virus (EBV) and Kaposi's sarcoma-associated herpes virus (KSHV) belong to the γ-herpes virus
639 subfamily. Following primary infection all these viruses establish life-long latency in different cell
640 types and tissues and they are all able to reactivate at a later point in time, leading to asymptomatic or
641 symptomatic infections. The approved drugs shown in Table 3 treat the acute symptomatic infections
642 but they are unable to eradicate the latent infections. Various nanomedicines are currently under
643 investigation for the treatment of herpetic infections (see Table 4).

644 Acyclovir (ACV) is the drug of choice for treating HSV infections. However, due to its short half-life
645 and incomplete absorption it must be taken in its oral dosage form five times daily (up to 1200
646 mg/day), and the dosage interval for intravenous formulations is 8 h.

647 Infections caused by HSV are incurable, so that the main goal of antiviral therapy with ACV is to
648 inhibit the replication of the virus and thereby prevent associated epithelial damage [124]. Currently,
649 the treatments available for herpes simplex are conventional tablets and topical gel for application on
650 outbreaks. The drugs that are commonly used for herpes simplex are acyclovir, valaclovir and
651 famciclovir.

652 ACV was the first antiviral to be licensed for the treatment of HSV infections, and it is the drug of
653 choice for the treatment of epidermal, ocular or systemic herpetic infections, despite the fact that most
654 of its currently available dosage forms, that is, tablets, suspension, cream, fail to achieve suitable levels
655 at target sites following oral, local, or parenteral administration [125]. The main reason is that
656 Acyclovir is a class III drug according to the Biopharmaceutics Classification System [126]: it is

657 slightly soluble in water, has a short plasma half-life, its absorption from gastrointestinal tract is slow
658 and incomplete, and oral bioavailability ranges from only 10% to 30%. As a consequence, higher doses
659 are prescribed, resulting in systemic toxicity and adverse reactions [126]. Also in local therapy, ACV
660 bioavailability is low and highly variable, associated with low retention at the vaginal mucosa and poor
661 patient compliance, and requires frequent administrations [127-129].
662 For these reasons, the development of efficient nanoformulations of ACV is of increasing importance.
663 Moreover, the increased incidence of sexually transmitted diseases (STDs) raises concern about the
664 limitations of currently available therapies. Among various STDs, the infections with human *Herpes*
665 *simplex* virus type-1 and type-2 remain among the most common [130].
666 Over the course of the past decade, different types of nanoparticles based on cyclodextrin have been
667 developed to improve the physico-chemical features of ACV [131-133]. In general, cyclodextrin-based
668 formulations allow high loading and the sustained release of ACV; in some cases, they also improve its
669 *in vitro* antiviral efficacy.
670 Recently, Eudragit RLPO®, a copolymer of ethyl acrylate, methyl methacrylate and a low content of
671 methacrylic acid ester with quaternary ammonium groups, has been selected for preparation of ACV
672 loaded nanoparticles [134]. *In vitro* studies show that this formulation provides sustained drug release
673 over a period of 24 h.
674 Amany and colleagues [135] prepared stealth PLGA nanoparticles loaded with ACV with the aim of
675 extending the drug plasma half-life after intravenous administration [135]. In another study, PLGA
676 surface tailored nanoparticles were prepared for targeted hepatic delivery of ACV with the purpose of
677 minimizing side effects [136]. Galactose, actively taken up by asialoglycoprotein receptors (ASGP-R),
678 was conjugated to PLGA nanoparticles, to exploit the exclusive presence of ASGP-R on hepatocytes
679 for liver targeting.

In vitro, the galactosylated nanoparticles resulted in less haemolysis and successfully exhibited a sustained release pattern. *In vivo* studies showed an improved bioavailability, increased residence time, and enhanced delivery of ACV to the liver upon galactosylation [136].

Nano-niosomes have also been used to encapsulate ACV and were found to increase ACV effectiveness and decrease its side effects [137].

In regard to ACV vaginal therapy of HSV-2 sexually transmitted infections, high drug concentrations in genital tissues are desirable. Unfortunately, the incomplete coverage and short duration of action limit the effectiveness of vaginally administered drugs, including ACV. The use of mucoadhesive polymers such as poly(acrylic acid) derivatives, in the preparation of delivery systems, may overcome these limitations. For instance, mucoadhesive ACV-containing liposomes coated with mucoadhesive polymers (Carbopol® or chitosan) have been developed [138]. In recent years, efforts have been made to develop new types of mucoadhesive polymers that form a covalent bonding with the mucous membrane, producing a stronger bio-adhesion compared to conventional polymeric mucoadhesive materials. In this regard, Yandrapu *et al.* [139] developed new thiolated dendrimers for mucoadhesive drug delivery. The thiolated dendrimers were synthesized by conjugating PAMAM dendrimer with cysteamine and further encapsulated with ACV [139]. The thiolated dendrimers showed sustained release of ACV in addition to a high mucoadhesion capability.

The dendrimer SPL7013 has been formulated in a mucoadhesive carbopol gel (VivaGel®) for use as a topical microbicide in the prevention of HIV and HSV infections. Potent antiviral activity against HIV-1 and HSV-2 was observed following vaginal administration of VivaGel® in human [25].

An interesting strategy to enhance the accumulation of nanoparticles is the design of mucus penetrating systems. Ensign prepared mucus-penetrating particles (MPPs) for mucosal drug delivery in order to enhance the distribution and increase the retention in the vagina *in vivo* [140]. MPPs provided uniform

703 distribution over the vaginal epithelium in mice, whereas conventional nanoparticles were aggregated
704 by the animals' vaginal mucus, resulting in poor distribution.
705 Solid lipid nanoparticles can play an important role as ocular delivery system [141]. In 2013, Seyfoddin
706 and colleagues [142] developed two lipid formulations (namely solid lipid nanoparticles and
707 nanostructured lipid carriers) to improve the ocular bioavailability of ACV [142]. The high
708 encapsulation efficiency, superior physical properties and good release profile obtained from
709 nanostructured lipid carriers indicated that this formulation could be used as a potential ocular drug
710 delivery system for ACV.
711 Interestingly, solid lipid nanoparticles and nanoemulsions were studied for enhancing dermal delivery
712 of ACV by Jain [143]. Mechanism of topical permeation and dermal distribution studies suggested that
713 pilosebaceous route was followed by solid lipid nanoparticles for skin penetration, without major
714 morphological changes on rat skin surface.
715 Another class of nanocarriers which has been investigated for the topical dermal drug delivery of ACV
716 are nanoemulsions. Schwarz and colleagues [144] optimized several water/oil/water nanoemulsions for
717 the dermal application of ACV by using the phase inversion temperature method [144].
718
719 Nanoformulations have been also proposed for the two derivatives of Acyclovir, i.e. penciclovir and
720 valaciclovir. For example, SLNs have been prepared and evaluated as nanocarriers for the topical
721 delivery of penciclovir to prolong the drug release [145].
722 Ganciclovir (GCV) is the the first choice antiviral treatment for human cytomegalovirus infections.
723 However, the low bioavailability and short half-life of GCV necessitate the development of a carrier. In
724 a recent study, guanosine-based GCV was used to synthetize hydrophobic GCV-poly(caprolactone)
725 (GCV-PCL), which was then grafted with hydrophilic chitosan to form amphiphilic copolymers for the
726 preparation of stable micelles [146]. Solid lipid nanoparticles were also investigated as drug delivery

systems for GCV. Intriguingly, borneol can be used as penetration enhancer. Recent studies demonstrated that borneol increased the permeation of drugs across BBB and enhanced their distribution in the brain tissue [147]. Ren prepared borneol-modified and non-borneol ganciclovir-loaded SLNs and showed that borneol could enhance the transport of ganciclovir (GCV) incorporated in solid lipid nanoparticles to the brain in mice after their intravenous administration [148].

GCV plays an important role also in the treatment of ocular HCMV infections. Akhter [149] used the reverse-phase evaporation technique to prepare GCV mucoadhesive nanoemulsions, chitosan nanoparticles, and mucoadhesive niosomal dispersions [149]. All three formulations were successfully shown to be non-irritant and non-toxic. These results may therefore be useful for the development of GCV formulations for the treatment of ocular infections.

Then, Akhter and colleagues [150] prepared nano-niosomes in order to improve the oral bioavailability of GCV. *In vitro* release studies confirmed a sustained release profile of the niosomal dispersions; whereas *in vivo* studies in rats revealed a five-fold increment in GCV bioavailability after the oral niosome administration compared with the standard tablet formulation [150].

Foscarnet is a pyrophosphate analog mainly used for the treatment of GCV-resistant HCMV infections in patients with AIDS or in transplant recipients. A novel nanoparticulate system for foscarnet delivery was developed by Russo *et al.* [151]. Nanoparticles were obtained by ionotropic gelation of chitosan induced by foscarnet itself, acting as an ionotropic agent. Crosslinked nanoparticles showed a controlled drug release, and foscarnet released from nanoparticles maintained the antiviral activity of the free drug when tested *in vitro* against HCMV.

3.3 Influenza virus infection

Seasonal orthomyxoviruses cause influenza in humans every year, affecting approximately 5% to 10% of the global adult population annually and associated with 250,000 to 500,000 deaths every year. To date, three classes of anti-influenza drugs have been approved: inhibitors of the ion channel matrix 2 (M2) protein, known as adamantanes, inhibitors of neuraminidase, that are analogues of sialic acid, blocking the release of progeny viruses from infected cells and, finally, a recent inhibitor of the viral RNA polymerase (Table 5). Neuraminidase inhibitors, like oral Oseltamivir or inhaled Zanamivir, are the main drugs currently recommended for antiviral treatment. Zanamivir presents poor oral bioavailability (approximately 2%), and it is therefore administered by inhalation. However, the inhalation route is not recommended in patients with chronic respiratory conditions. Different approaches to increasing the oral bioavailability of Zanamivir have been investigated.

Cao *et al*. prepared Zanamivir-loaded SLNs, which achieve relatively high entrapment efficiency, using a double emulsion solvent evaporation method [152]. Wang *et al*. developed dendrimer-cyclodextrin conjugates encapsulated with different guest molecules, including amantadine hydrochloride. The combination of PAMAM dendrimer and CDs can improve the drug loading ability of dendrimer and make amantadine hydrochloride suitable for dendrimer-based drug delivery systems. Indeed, they demonstrated that the drug is encapsulated within β-cyclodextrin cavity in the dendrimer conjugate [153].

3.4 HBV infection

Despite the availability of a prophylactic vaccine, Hepatitis B virus (HBV) causes 780,000 deaths every year and 240 million people were infected in 2014. The current approved therapy for chronic infection is based on immunomodulating peptides, such as interferon-α, and nucleos(t)ide analogues targeting the HBV polymerase.

Orally administered polymerase inhibitors have certain drawbacks, including the poor oral bioavailability, as reported for Adefovir dipivoxil, and long-term treatment-induced viral resistance. Several drug delivery systems have been developed to improve the activity of the existing anti-HBV drugs (Table 5). While nanocarriers for interferon-α are reported in the paragraph relative to Hepatitis C virus, nanoformulations for the delivery of nucleos(t)ide analogues are described below.

To improve the bioavailability of adefovir dipivoxil, Dodiya and colleagues prepared and studied SLNs and nanosuspensions *in vivo* [154]. Both nanoformulations exhibited good bioavailability of 52% and 78%, for the nanosuspension and SLNs, respectively. Furthermore, higher accumulation of adefovir dipivoxil was detected in the liver and other organs after 24 hours after administration.

Du and colleagues [155] prepared nanoassemblies of an adefovir lipid derivative with cytochrome P450-triggered drug release with the purpose of improving hepatocyte targeting [155]. Cytochrome P450-induced cleavage thus provides a way to trigger the release of the active drug within hepatocytes. To ensure hepatocyte targeting, a galactose–ended polyoxyethylene fatty ether was added to assemblies. After its intravenous administration into infected mice, the resulting nanoassemblies showed long circulation times, liver targeting, and very high anti-HBV activity.

Stearic acid-g-chitosan oligosaccharide micelles, able to self-aggregate in aqueous medium, were prepared and loaded with Lamivudine stearate, the prodrug of Lamivudine. The resulting micelles were characterized by high entrapment efficiency, drug loading, and pH-sensitive drug release. Low cytotoxicity and high levels of antigen expression inhibition, and DNA replication inhibition were found compared with the free drug [156].

3.5 HCV infection

The approved treatment for chronic hepatitis C virus (HCV) infection is the combination of PEGylated interferon-α (IFN), ribavirin and protease inhibitors, such as boceprevir, telaprevir or sofosbuvir (Table 5). A great deal of research has focused on the development of a carrier for ribavirin to improve its therapeutic effect and safety [157]. Clinical use of ribavirin is often accompanied by severe side effects, like hemolytic anemia, which occurs in approximately 50% of treated patients. Regarding liver targeting of hepatitis treatments, nanotechnologies may provide important tools with which to promote the selective accumulation of the drug in the target cells; in turn, decreasing their toxic effects in other tissues, improving drug efficacy and reducing administration frequency, with consequent increases in patient compliance.

Craparo *et al*. developed polymer-based targeting micelles bearing galactose moieties on the surface for the liver-targeted delivery of ribavirin [158-159]. Polymeric micelles were prepared starting with a galactosylated polylactide-polyaminoacid conjugate (PHEA-EDA-PLA-GAL copolymer) able to self-assemble into micelles. In order to increase the entrapment within the system, ribavirin tripalmitate was synthesized as a hydrophobic prodrug. No toxic effects on cell viability and good hemocompatibility were observed. The specificity of ribavirin tripalmitate-loaded PHEA-EDA-PLA-GAL micelles against HepG2 was demonstrated *in vitro* by their ability to be internalized in HepG2 cells and the enhanced accumulation of prodrug compared with non-galactosylated systems.

Biodegradable nanoparticles encapsulating ribavirin have also been developed with the aim of modulating the pharmacokinetics of the drug [160]. Ribavirin monophosphate-loaded nanoparticles were prepared from a mixture of poly(D,L-lactic acid) homopolymer and arabinogalactan (AG)–poly(l-lysine) conjugate. The stable retention of ribavirin by the nanoparticles within the systemic circulation can prevent accumulation of ribavirin in erythrocytes, and in turn reduce haemolytic anaemia.

822 Ribavirin was shown to accumulate in the liver of mice following the intravenous administration of the
823 drug-loaded nanoparticles.
824 Hashim and colleagues [161] prepared ribavirin niosomes with the aim of investigating the influence of
825 niosomal encapsulation upon the capacity to target the drug on the liver in rats. Niosomal formulations
826 significantly increased the ribavirin liver concentration (6-fold) in comparison with ribavirin-free
827 solution following intraperitoneal administration of a single dose of 30mg/kg.
828 Interferon-α (IFNα) has enormous potential as an anti-viral treatment for Hepatitis C, but its clinical
829 success is hampered by its limited bioavailability, relatively narrow therapeutic index, and short half-
830 life following parenteral administration. To achieve better tolerability, a PEGylated-IFNα formulation
831 has been developed with better pharmacokinetics.
832 Liu and colleagues [162] investigated a method to obtain IFNα-loaded nanoparticles favourable for the
833 preservation of IFNα-2b integrity and biological functionality [162]. The antiviral activity of the
834 polysaccharide nanoparticles was shown to be highly preserved (above 97%) both *in vitro* and *in vivo*.
835 Li and co-workers encapsulated IFN-α into SLNs using the double emulsion solvent evaporation
836 method. Antiviral assays demonstrated that SLNPs preserved the bioactivity of IFN-α after
837 encapsulation; i.e. it maintained its antiviral activity [163].
838
839 **4. Future challenges**
840 Many strategies using nanomedicines have been proposed in order to improve the current treatment of
841 viral infections. Although numerous efforts have been made in the field of nanotechnology-based drug
842 delivery, and although pre-clinical and clinical results are encouraging, a number of problems remain
843 that need to be addressed in the future (Figure 4). First, a crucial challenge for the clinical application
844 of nanomedicines concerns their safety. Suitable long-term toxicity studies in animal models are
845 necessary, especially in relation to their use in the treatment of chronic infections. Particular attention

should be paid to immune system compatibility, complement activation, and coagulation effect. The design of novel biocompatible and biodegradable nanomaterials might overcome some toxicity drawbacks. However, some of the advantages associated with nanocarriers, such as the reduced drug doses and the possible oral administration, may themselves limit the undesirable side effects. In addition, nanomedicine limitations can be related to poor drug loading capability and nanotoxicology issues. In this regard, more research is needed to provide the robust preclinical data necessary to justify clinical trials of biocompatible nanoformulations with good biodistribution profiles, sustained release dosages, and no significant side effects. Another challenge to address in the future is the design of nanocarriers with high drug payload. High drug amounts within a nanosystem involve the use of less amounts of excipients that may have safety problems. In addition, nanomedicine may be able to exploit innovative administration routes, such as pulmonary and nasal, to overcome administration limitations.

An important property of a nanocarrier should be its persistent stability for a sufficient length of time, both *in vitro* and *in vivo*, to assure safety and its localization to the sites of interest and to minimize toxicity of the liver and kidneys. In this regard, it is crucial to integrate nanotechnology with physiological and pharmacological knowledge, and with an understanding of drug distribution and metabolism in specific tissues. Pharmacokinetics studies play a key role. Stability should be also assured during storage and administration.

Nanocarriers with modified drug release kinetics, such as sustained release systems, will be able to decrease the number of administrations required and doses. Currently, long-acting injectable delivery systems represent an important solution for problems caused by patient incompliance with infrequent dosing, thereby guaranteeing better long-term adherence especially in chronic diseases [164]. Another important challenge is related to the manufacturing of nanomedicines. Scaling-up their preparation method might not be easy and may prove to be expensive. Therefore, investments will need to be made in industrial processes in order to provide large batches of nanoproducts and render them cost-effective.

However, the potential for nanocarriers to reduce the required doses might compensate for the cost increase required for technological upgrades.

In addition, the regulatory aspects of nanomedicines should be taken into account to translate in industrial products, suitable to clinical application.

Treatment of viral infections could be substantially improved in the future by the development of "smart" delivery systems, such as stimuli sensitive nanocarriers. The design of triggered release nanocarriers allows on-demand purchase of the drug. For this purpose, responsive polymers might be used as a key component, either in self-assembling copolymer aggregates or as a coating on the surface of nanoparticles and nanocrystals, as reported previously. Their ability to undergo rapid structural changes in their network in a triggered manner and to exhibit the desired drug release has been exploited to design smart nanosystems. The literature offers some initial evidence supporting the proof of concept of such "smart" delivery systems. For example, Duan et al. [165] proposed pH-responsive atazanavir or darunavir-loaded lipid nanoparticles as a long-acting targeted antiretroviral therapy [165]. Additionally, purposely surface-modified nanocarriers might allow antiviral accumulation in infected sites. Targeted delivery is a strategy to be exploited for addressing specific tissues, such as lymph nodes.

Moreover, the future design of nanocarriers targeted on specific viral nanostructures is an important milestone, and it will be achieved by studying the mechanisms of interaction between viruses and nanoparticles. Deeper knowledge about the selective interactions can be exploited in strategies aimed at weakening the viral activity and in the therapy of infections. A possible challenge to targeting the delivery of drugs by nanomedicine is the possible induction of drug resistant mutations in not targeted anatomical sites where drugs reach suboptimal doses.

892 Nanodelivery of combinations of several drugs might be preferable, especially against viruses
893 characterized by a high degree of genetic variability, like HIV-1. Therefore, “combo” delivery systems
894 will be one of the future goals.
895 In the future, new antiviral drug candidates continue to be developed and subjected to in-depth
896 investigations. These candidates may be proteins, siRNA or antisense oligonucleotides; and purposely
897 tuned nanomedicines will be required for their protection and delivery. Nanoformulations are needed
898 for their administration. Although these studies are generally still in their early stages, encouraging
899 results have been obtained. The development of microbicides against viral sexually transmitted
900 diseases has attracted much research [166]. In this context, RNA interference (RNAi) provides a highly
901 valid tool for downregulating the expression of viral and host mRNA targets required for viral infection
902 and/or replication. The latest advances made in RNAi technology towards the treatment of HIV-1
903 infection have been recently summarized [167]. Currently, two RNAi products for HBV treatment from
904 Arbutus Biopharma are undergoing clinical trials: ARB-1467 (Phase II) and ARB-1598 (Phase I) [168].
905 Finally, advanced nanomedicine will be considered. A novel approach now emerging is the cell-based
906 drug delivery system**,** which entails the use of cells as carriers of drug-encapsulated nanoformulations.
907 In particular, macrophages represent a proof of concept of this mechanism. They have been used as
908 cellular vehicles because of their ability to migrate to lymph nodes or travel across the BBB, ensuring
909 the sustained release of drugs over an extended period of time.
910
911 **5. Conclusion**
912 In this review the most common nanomedicine strategies for the delivery of approved antivirals have
913 been described. Future nanomedicine design should address many challenges, such as high payload,
914 target delivery, and toxicity issues, to overcome the limitations of current conventional formulations. A
915 further understanding of the nanoparticle behavior either *in vitro* or *in vivo* might accelerate their

pharmaceutical development. A multidisciplinary approach of research with collaborative industrial partners may permit the faster translation of nanoformulations to clinics and on the market for patients.

6. Expert Opinion

Considering the continual occurrence of viral infections**,** new drug delivery strategies are required to treat and eradicate viral diseases. Nanomedicine formulations can play multiple roles in achieving this goal by improving the biopharmaceutical properties of approved antiviral drugs. Moreover, nanocarriers can enable the delivery of drugs to specific infection sites.

Moreover, it is the specific physico-chemical properties of nanocarriers that underlie their ability to overcome biological barriers or interact with virus nanostructures being in the same size range. In addition, nanoparticles with engineered surfaces can act as multifunctional systems able to interact with selective receptors able to inhibit viral infections.

Interestingly, the peculiar properties of nanomedicines, such as nanoscale size and surface charge, can be exploited to improve the mucosal permeability of encapsulated drugs. Recently, numerous efforts have been made in using nanotechnology to efficiently deliver and cross the mucosal barrier, mainly thanks to the development of mucoadhesive and mucopenetrating nanoparticle systems [59,140,169]. Mucoadhesive particles may protect encapsulated drugs from the low gastric pH and proteolytic enzymes, improve the residence time in the gastrointestinal tract and, as a consequence, the bioavailability of orally administered drugs. In particular, polymer nanoparticles have been studied for mucoadhesion, cell targeting and uptake, exploiting hydrophobic interactions, van der Waals interactions and polymer chain interpenetration with mucin. For these reasons, mucopenetrating

940 nanoparticles able to penetrate the adherent mucus layer and thus be retained longer seem more suitable
941 for oral and vaginal delivery than mucoadhesive nanosystems. However, it must be taken into account
942 that, during infection, the properties of mucus may be altered and nanoformulations could be
943 transported differently.
944 To overcome some limitations of antiviral drugs, the co-delivery of multimodal therapeutics for
945 "combo" therapy, previously investigated for cancer therapy, can be also proposed with nanomedicine.
946 Much research attention has been directed over the last few years to developing antiviral drug delivery
947 systems for combinations of drugs or a minimum of at least two drugs. This issue is most relevant to
948 infections that require the simultaneous administration of multiple drugs to prevent the generation of
949 resistance and viral rebound. Instead of the co-encapsulation of several antiviral drugs in the same
950 nanoparticle, an alternative approach could consist of the administration of multiple single drug-loaded
951 nanocarriers. The advantages of this approach are: better encapsulation efficiencies and drug loading
952 associated with the single drugs; greater ease in obtaining the correct ratio of drugs; and better patient
953 compliance.
954 Numerous efforts have also been made to optimize nanoformulations in order to improve their ability
955 to cross the BBB by reducing particle size, by means of surface engineering and BBB transmigration
956 studies. Nanomedicines can also provide the delivery proteins and biotech products, such as siRNA, by
957 various administration routes, avoiding aggregation and chemical degradation, as well as stability
958 during storage and administration [168].
959 Children constitute the most challenging population for infection treatment, such as HVI or RSV.
960 Interestingly, nanomedicine could be an useful platform for the development of effective therapeutic
961 systems and appropriate dose adjustment for children and special patients with swallowing problems.
962 The advantages offered by long-acting injectables over oral formulations include eliminating the need
963 for daily oral tablet administration and therefore first–pass metabolism, lower antiviral doses with

consequently reduced hepatic and renal toxicities, and prevention of resistance. Long-acting injectable products have been successfully applied in the treatment of infectious diseases, most notably chronic HIV infection, promoting compliance and patient adherence and reducing the burden of regimens requiring daily treatment. Some long-acting injectable nanosuspensions of nonnucleoside reverse transcriptase inhibitors, and integrase inhibitors are currently in clinical study and are highly anticipated medications for the prevention of HIV infection [170]. Pharmacokinetic studies in humans have demonstrated sustained drug concentrations after intramuscular administration at 4 weekly and 8 weekly intervals of rilpivirine long-acting injectable nanosuspension [171]. Preliminary *in vivo* studies have shown that intramuscular cabotegravir can prevent simian/HIV acquisition from rectal, vaginal, and intravenous challenge. Currently, there are two ongoing Phase II studies assessing cabotegravir as a preexposure prophylaxis agent in humans [172]. Crystalline nanoparticle formulations for the co-administration of rilpivirine and cabotegravir have progressed into phase II clinical trials as long-acting intramuscular maintenance therapy [173]. Nanomedicine production is generally more complex than traditional small molecule based drugs. It is worth noting that complex formulations present unique chemistry, manufacturing and control (CMC) properties, in particular when changes are needed for manufacturing scale-up. The development of nanotherapeutic products should be optimized with the Quality by Design (QbD) approach to identify critical process parameters and material attributes. For several nanotechnologies, the scaling up of manufacturing production processes using current pharmaceutical technologies can be used.

Finally, the possibility of reducing the required number of antiviral doses might compensate for the increased costs associated with technological upgrades.

Based on these premises, nanomedicines have the potential to become a revolutionary solution for the treatment of viral diseases, although some issues still need to be addressed and solved for the successful translation of nanotech products onto the market.

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Figure legend

Figure 1. Antiviral targeting strategies of drugs at different steps of the viral life cycle.

Figure 2. Overview of main drawbacks of antivirals and nanotechnological strategies to overcome limits of antiviral therapy

Figure 3. Administration routes of nanosystems studied in *in vivo* experiments and animal models used according to viral infections

Figure 4. Future challenges of nanoformulations for antiviral drugs