Luminescent PLGA Nanoparticles for Delivery of Darunavir to the Brain and Inhibition of Matrix Metalloproteinase-9, a Relevant Therapeutic Target of HIV-Associated Neurological Disorders

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ABSTRACT: Human immunodeficiency virus (HIV) can independently replicate in the central nervous system (CNS) causing neurocognitive impairment even in subjects with suppressed plasma viral load. The antiretroviral drug darunavir (DRV) has been approved for therapy of HIV-infected patients, but its efficacy in the treatment of HIV-associated neurological disorders (HAND) is limited due to the low penetration through the blood–brain barrier (BBB). Therefore, innovations in DRV formulations, based on its encapsulation in optically traceable nanoparticles (NPs), may improve its transport through the BBB, providing, at the same time, optical monitoring of drug delivery within the CNS. The aim of this study was to synthesize biodegradable polymeric NPs loaded with DRV and luminescent, nontoxic carbon dots (C-Dots) and investigate their ability to permeate through an artificial BBB and to inhibit in vitro matrix metalloproteinase-9 (MMP-9) that represents a factor responsible for the development of HIV-related neurological disorders. Biodegradable poly(lactic-co-glycolic) acid (PLGA)-based nanoformulations resulted characterized by an average hydrodynamic size less than 150 nm, relevant colloidal stability in aqueous medium, satisfactory drug encapsulation efficiency, and retained emitting optical properties in the visible region of the electromagnetic spectrum. The assay on the BBB artificial model showed that a larger amount of DRV was able to cross BBB when incorporated in the PLGA NPs and to exert an enhanced inhibition of matrix metalloproteinase-9 (MMP-9) expression levels with respect to free DRV. The overall results reveal the great potential of this class of nanovectors of DRV for an efficacious treatment of HANDs.

KEYWORDS: darunavir, PLGA nanoparticles, carbon dots, blood–brain barrier, MMP-9, HANDs

1. INTRODUCTION

The human immunodeficiency virus type one (HIV-1) invades early the central nervous system (CNS) causing a spectrum of neurological symptoms known as HIV-associated neurocognitive disorders (HANDs). In general, HANDs include disorders of various degrees, the most severe form of which is represented by HIV-associated dementia (HAD), and they are a consequence not only of the direct effect of virus on host cells but also of a cascade of processes that result in chronic inflammation. Within the CNS, HIV infects astrocytes and microglia inducing cell activation and release of inflammatory and neurotoxic molecules such as cytokines, chemokines, and matrix metalloproteinases (MMPs), which exacerbate the inflammatory state contributing to neuronal damage.

In the last decades, the use of combination antiretroviral therapy (cART), based on the administration of two or three combined antiretroviral drugs (ARVs), has brought a break-through in the management of HIV-1 infection, although HANDs still represent a challenge in the clinical and pharmacological field. The persistence of HANDs may depend on several factors including the chronic state of inflammation and immune activation due to the reduced efficacy of therapy in CNS reservoirs.

The CNS is a hard anatomical sanctuary for the treatment of HANDs given the presence of the blood–brain barrier (BBB), which prevents the ARVs from reaching their targets with efficacious therapeutic concentrations. In addition, the...
amount of antiretrovirals that reaches the CNS is also influenced by plasma protein binding, molecular size, lipophilicity, and ionization.

Therefore, currently, the challenge in the treatment of HANDs is to achieve adequate drug levels in the CNS without causing drug-related neurotoxic effects. To overcome the issues related to the presence of BBB, several conventional and alternative therapeutic strategies have been exploited, but research efforts are still needed for the development of drug delivery systems that result noninvasive and safe for human health. In this context, nanotechnology enables the fabrication of different, properly designed nanostructured delivery systems suited for their use as noninvasive tools able to effectively improve the bioavailability of ARVs toward the CNS viral reservoirs, escaping the physiological mechanisms of the BBB and, therefore, ensuring efficacious therapeutic drug concentrations. Interestingly, polymeric nanoparticles, characterized by biodegradability and high degree of biocompatibility, represent attractive candidates for the delivery of specific therapeutic compounds to CNS.

Here, nanof ormulations composed of poly(lactic-co-glycolic) acid (PLGA) nanoparticles (NPs), co-encapsulating luminescent carbon dots (C-Dots) and the antiretroviral drug darunavir (DRV) were designed and proposed for the brain delivery of ARVs. The presence of luminescent C-Dots in the nanof ormulations was expected to provide optically traceable nanovehicles for the optical monitoring of the drug delivery. Indeed, C-Dots represent safe and efficient optical probes for biolabeling and bioimaging applications, thanks to their very low toxicity and high chemical stability.

DRV belongs to the class of HIV protease inhibitors (PI) that have marked a significant turning point in the management of HIV infection. Among second-generation PI, DRV has been proved to be characterized by a high efficacy, and in 2013, it was authorized by the Food and Drug Administration (FDA) for pediatric patients older than 6 years. Unfortunately, DRV has an intermediate CNS penetration effectiveness score (3), low bioavailability if administered by oral route, and limited solubility in aqueous and lipid media. Consequently, several nanocarriers, such as solid lipid nanoparticles (SLN) or solid self-microemulsifying drug delivery systems, have been explored to increase the bioavailability of DRV and its accumulation in the brain.

Based on these premises, this work aims to investigate the ability of the luminescent DRV-loaded PLGA NPs to cross an in vitro model of BBB and exert their therapeutic efficacy on MMPs that have been identified as key mediators in various HIV-infection-associated diseases, including neurological injury. Matrix metalloproteinases are neutral Zn^{2+}-dependent endopeptidases belonging to the metzincin superfamily that have as major targets the components of the extracellular matrix (ECM) and represent important factors involved in physiological and pathological processes.

Among MMPs, MMP-9 plays a crucial role in viral dissemination, sanctuary consolidation, tissue damage, and the development and progression of neuroAIDS. Elevated MMP-9 levels have been detected in serum and cerebrospinal fluid from patients with HIV-related neurological diseases and HIV-positive patients. Several ex vivo and in vitro studies have demonstrated that MMPs can represent an important therapeutic target in course of HIV infection. Inhibition of MMP-9 expression has been proved in blood mononuclear cells from HIV-infected subjects under antiretroviral therapy, suggesting that the beneficial effects of cART may be in part due to its ability to inhibit MMPs. Different studies have reported that several ARVs, in particular HIV protease inhibitors, possess extravirological properties, that are independent of their ability to block HIV replication.

Among these effects, recently, Latronico et al. have demonstrated that DRV is able to inhibit in vitro MMP-9 levels and expression in astrocytes through the inhibition of signaling transduction pathways involved in the regulation of the MMP-9 gene. Consequently, strategies capable of increasing the delivery of ARVs to the CNS could provide therapeutic benefits to patients affected by HANDs also through the inhibition of MMP-9.

The results of this study highlight that the PLGA NPs are able to deliver DRV through the BBB with an efficiency higher than that found for the free drug, preserving its inhibitory activity on MMP-9, suggesting their potential use for the treatment of HANDs. The use of the designed and obtained drug delivery nanocarriers is expected to improve pharmaco-
kinetics and protect easily degradable ARVs, that often have short in vivo half-lives, thus reducing drug administration doses and, consequently, toxicity.

2. RESULTS AND DISCUSSION

The BBB is a critical checkpoint between systemic circulation and brain parenchyma acting as a physical and metabolic barrier and represents the main obstacle in the treatment and diagnosis of neurological diseases. To overcome the issues deriving from the presence of the BBB, several therapeutic strategies have been exploited; however, many of them have proved unsuccessful. The main hurdle concerns the research for controllable drug delivery that is noninvasive and safe for human health. In this scenario, nanotechnology may have a relevant clinical impact in neuroscience, offering alternative noninvasive tools for the delivery of drugs and other therapeutic agents to specific targets into the brain parenchyma.36 In this study, optically traceable PLGA NPs containing luminescent C-Dots were designed, prepared, and characterized by evaluating their size, shape, colloidal stability, and DRV encapsulation efficiency and, finally, assessing their suitability for exploitation as DRV delivery nanovectors to the brain (Figure 1). In particular, their safety and ability to pass through the BBB, as well as their effectiveness to inhibit MMP-9, which represents a pathogenic key factor in course of neuroAIDS, were investigated by a systematic in vitro study.

2.1. PLGA-Based Nanoformulations: Preparation and Characterization. Co-encapsulation of C-Dots and DRV in the polymeric matrix of PLGA NPs was achieved by exploiting the oil-in-water (O/W) emulsification–solvent evaporation approach. As a first step, the synthesis of oil-dispersible luminescent C-Dots was performed by means of a one-step procedure: citric acid was employed as a carbon precursor, while ODE and HDA were employed as a high-boiling solvent and a N-containing surfactant, respectively.37 The transmission electron microscopy (TEM) investigation proved the formation of spheral-shaped C-Dots characterized by an average size of about 3.5 nm (σ = 23%), as clearly displayed in Figure 2B. UV−vis absorption and photoluminescence (PL) spectroscopy were used to perform their optical characterization (Figure 2A). In Figure 2A (inset), the absorption spectrum of C-Dots exhibits a strong signal in the UV region, ascribable to the π−π* transitions of the sp² domain of the carbogenic core, a well-defined peak centered at 350 nm and an absorption tail extending from 400 nm up to the visible portion of the spectrum, related both to the edge and surface chemical groups and to the molecular fluorophores formed during the carbonization process.37 PL spectroscopic measurements that were performed on the “as-synthesized” C-Dots by varying the excitation range between 320 and 500 nm confirmed their established excitation-wavelength-dependent fluorescence57 and revealed the presence of a wide asymmetric emission band originating by the contribution of different energy states that contribute to the overall C-Dots fluorescence.57,58 The maximum fluorescence peak position red-shifts, and the related PL intensity changes with the increase of the excitation wavelength. A higher PL QY was achieved by exciting the C-Dot dispersion in chloroform at 410 nm, reaching a value of 36% (Table 1, Supporting Information).

For the preparation of luminescent PLGA NPs loaded with DRV, the hydrophobic drug and C-Dots were mixed in chloroform, and subsequently, the resulting organic phase was added to an aqueous solution containing Pluronic F-68. Nanosized droplets were formed by homogenization; after completely evaporating the organic solvent, the NPs were extensively purified by ultracentrifugation. Pluronic F-68, an amphiphilic and nonionic co-polymer, having a central polyoxypropylene block, bound on both sides to hydrophilic chains of polyoxyethylene, was selected as a biocompatible surfactant to coat the NP surface. The use of surfactants, such as Pluronic F-68 or Tween 80, as surface coating of polymeric NPs represents a successful approach for the exploitation of polymer-based NPs that mimic the low-density lipoproteins (LDL) into the brain, thus potentially representing effective drug delivery vehicles to CNS.39−43 Interestingly, different in vivo studies, reported in the literature, have indicated that Pluronic F-68 ensured a brain distribution and therapeutic efficacy of surfactant-coated PLGA NPs higher than that found using Tween 80, when the NPs were administrated via intravenous injection.44 Nanoformulations at different drug loads were obtained by varying the initial drug feed (0.5, 1, 5, and 10 mg) at a fixed initial C-Dot concentration (2.5 mg/mL). The actual amount of drug embedded in the PLGA NPs
was quantified by UV–vis absorption spectroscopy analysis. In the preparation of polymeric NPs, an increase in the starting amount of DRV induced a decrease of drug encapsulation efficiency and an increase of the actual drug load, as quantified by EE% and DL% values (Figure 3A). The obtained results suggested that the maximum value of drug loading was reached when the starting drug feed was 10 mg, with an actual DRV concentration of 100 ± 2 μg/mL in the resulting nanoformulation. Thus, this last formulation was selected, among all of those prepared starting from different initial drug feed, for further investigation, as it ensured a higher DL% when DRV was incorporated in the polymeric matrix of PLGA NPs. Moreover, the actual amount of C-Dots incorporated in the nanoformulation, obtained starting from a DRV feed of 10 mg and an initial C-Dots concentration of 2.5 mg/mL, was evaluated by a procedure based on PL measurements, described in detail in Section 4, and estimated to be 0.28 ± 0.03 mg/mL.

Figure 3. (A) Characterization of DRV-loaded luminescent PLGA NPs: DRV encapsulation efficiency (EE%), DRV loading (DL%), size, and morphology. EE% and DL% of four different DRV/C-Dot/PLGA NP samples, obtained starting from different initial amounts of DRV. (B) Representative size distribution by intensity and TEM micrographs, obtained (C) without and (C1, D) with staining for two increasing staining times, namely, (D) 30 and (C1) 60 s of the DRV/C-Dot/PLGA NPs sample prepared starting from 10 mg of DRV (B, C) and at a fixed C-Dots concentration of 2.5 mg/mL, and (E) DRV/C-Dot/PLGA NPs schematic sketch.
Investigation on specific physical–chemical characteristics, such as morphology, size, and surface charge, that strongly influence NP ability to cross the BBB, was performed on the selected nanoformulation by TEM, dynamic light scattering (DLS), and ζ-potential measurements. DLS analysis revealed that DRV/C-Dot/PLGA NPs were characterized by an average hydrodynamic size (expressed as diameter) of about 120 nm (PDI 0.171 ± 0.053) and a homogeneous and monomodal size distribution (Figure 3 B). TEM investigation proved the formation of spherical NPs having size ranging from 40 to 170 nm, thus resulting well correlated with the data obtained from DLS measurements (Figure 3C,C1(inset),D).

In vivo study reported by Kulkarni et al. demonstrated that Pluronic F68-coated PLGA NPs, with an average size of 252 nm, were able to reach the brain with a brain distribution percentage of 6.2%, via intravenous injection.44,45 Therefore, the prepared PLGA nanoformulations, coated with Pluronic F68 and characterized by an average hydrodynamic diameter of 120 nm, can represent promising nanocarriers for the in vivo delivery of DRV to the CNS, as NPs larger than 150 nm resulted to have more limitations in crossing the BBB and reaching a relevant brain distribution.12

A representative close-up of the TEM micrograph of a single DRV/C-Dot/PLGA NPs, obtained with a staining time of 30 s, reported in Figure 3D, evidences the presence of C-Dots, appearing as small black spots localized within the dark gray polymeric matrix, thus highlighting their successful incorporation in the PLGA NPs (Figure 3D,E). The ζ-potential measurements on the nanoformulations provided a value of −47.9 ± 1.3 mV that indicated their relevant colloidal stability in aqueous media and the presence of a negative charge on the NP surface, which should better preserve the BBB integrity and enhance brain uptake with respect to cationic NPs.46

The PL emission spectra of the C-Dots encapsulated in the PLGA-based nanoformulation redispersed in organic solvent (chloroform) as a function of the excitation wavelength are reported in Figure 4A (see Section 4). The spectroscopic measurements finally proved that the peculiar PL emitting

Figure 5. Effect of empty PLGA NPs, C-Dot-only-containing NPs, and C-Dot- and DRV-containing NPs on astrocyte cell viability. In the top panel, representative images show the morphology of astrocytes observed by phase contrast microscopy (50× magnification) after 24 h treatment with empty PLGA NPs or NPs containing C-Dots only (C-Dot/PLGA NPs) or C-Dots and DRV (DRV/C-Dot/PLGA NPs) at the indicated concentrations. In the bottom panel, the graph reports the cell viability, assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, expressed as a percentage of surviving cells to untreated astrocytes in serum-free Dulbecco’s modified Eagle’s medium (DMEM) as control (CTRL, 100%). The doses of the preparations of NPs resulting in a cell survival below 60% were considered toxic. Data represent mean ± SD of n = 3 experiments on different cell populations.
properties of the C-Dots are retained after their incorporation in the complex polymeric matrix and in the presence of the drug. However, the manipulation of the C-Dots for their incorporation in the PLGA NPs resulted in a slight decrease of their PL quantum yield (QY), whose maximum value results in 27% when a 410 nm excitation was used. Such a variation in the PL QY is typically observed in the case of luminescent NPs, such as colloidal inorganic QDs and C-Dots, when postsynthesis processing procedures are required to make the "as-synthesized" nanostructures, originally dispersed in organic solvent, dispersible in aqueous medium. Indeed, changes in surface chemistry and/or dispersing medium and, in general, in the NP surrounding environment can induce even a drastic modification of the optical properties of the fluorescent probe, ultimately leading to the detrimental deterioration of their emission features. The slight reduction in QY observed in the case of DRV/C-Dot/PLGA nanosystems can be here ascribed mainly to the processing procedure and to changes in the medium composition due to the presence of PLGA and DRV residuals. The radiative decay dynamics of C-Dots, before and after their co-encapsulation with DRV in PLGA NPs, was measured with time-resolved (TR) PL spectroscopy. According to the literature, both the TR-PL decays of C-Dots can be best fitted by a three-exponential decay, providing average lifetimes of 12.2 ± 0.08 and 13.2 ± 0.06 ns for the C-Dots dispersed in the organic solvent and in the aqueous medium after their encapsulation in the PLGA nanoformulation, respectively. The observed variation in the decay rate and the faint modification of the corresponding lifetime, especially of the longer component, can be ascribed to the different environmental surrounding experienced by the surface states predominating the overall fluorescence of the C-Dots and to major changes in the dielectric constant of the dispersing medium that finally affects the energy distribution of the electronic excited states involved in the emission process.

Figure 6. Effect of empty PLGA NPs, C-Dot-containing NPs, and C-Dot- and DRV-containing NPs. In the top panel, the representative images show the morphology of the bEnd3 observed by phase contrast microscopy (50× magnification) after 24 h treatment with empty PLGA NPs, for C-Dot-only-containing NPs (C-Dot/PLGA NPs), and C-Dot- and DRV-containing NPs (DRV/C-Dot/PLGA NPs) at the indicated concentrations. In the bottom panel, the graph reports cell viability, assessed by the MTT test, expressed as a percentage of surviving cells compared to untreated astrocytes in serum-free DMEM as control (CTRL, 100%). The doses of the preparations of NPs resulting in a cell survival below 60% were considered toxic. Data represent mean ± SD of n = 3 experiments on different cell populations.
The in vitro release of DRV from the optically traceable PLGA NPs was monitored by UV−vis spectroscopy, and a sustained release of DRV up to 43 ± 6% was recorded over 24 h (Figure 4C).

2.2. Evaluation of the Effect of PLGA-Based Nanoformulations on the Cell Viability of Astrocytes and Endothelial Cells. A preliminary investigation was carried out on astrocytes and bEnd 3, the cell types that were used to set up the artificial model of BBB, to evaluate the effect of the PLGA-based nanoformulations on cell viability and, consequently, identify the nontoxic concentrations for further in vitro experiments.

As shown in Figure 5, no significant differences in cell survival can be observed between astrocytes treated for 24 h with empty PLGA NPs and C-Dots/PLGA NPs, thus suggesting that both NP preparations resulted nontoxic to astrocytes at all tested concentrations. Conversely, the incorporation of DRV in the luminescent polymeric nanovectors induced a reduction in cell viability of astrocytes treated with DRV/C-Dot /PLGA NPs at concentrations higher than 10 μg/mL DRV, although the microscopic observation did not evidence signs of cellular suffering. Anyhow, the cell viability of astrocytes was found to be always higher than 60%, when treated with DRV/C-Dot /PLGA NPs for 24 h, in the entire tested NP concentration range.

In the case of bEnd3 cells, MTT assay evidenced that all of the tested different PLGA-based nanoformulations did not affect cell survival in the whole investigated NP concentration range (5−400 μg/mL) (Figure 6B), as also confirmed by the microscopic observation that did not show any appreciable...
morphological differences in the bEnd3 cells after their 24 h incubation with empty PLGA NPs, C-Dots/PLGA NPs, or DRV/C-Dot/PLGA NPs (Figure 6A). Our findings were found to be in accordance with results, already reported in the literature, concerning the cytotoxicity of different PLGA-based formulations in the concentration range of 0.075–8000 μg/mL.44

2.3. Validation of the BBB Artificial Model by Evaluating Transendothelial Electrical Resistance (TEER) and Permeability. To assess the ability of NPs to convey the DRV through the BBB, an artificial blood–brain barrier (BBB) model was set up. Although the in vitro models of BBB are known not to possess all of the characteristics of the in vivo BBB, they offer interesting opportunities to study the uptake and drug delivery in a less expensive way than with in vivo experiments and reducing animal testing. The BBB model was selected among the reported artificial BBB models51 so as to most satisfactorily reproduce the structural and physical characteristics of in vivo BBB. This model was set up by co-culturing mouse bEnd3 cells and primary astrocytes on luminal and abluminal sides, respectively, of poly(ethylene terephthalate) (PET) membrane insert with a pore size of 0.4 μm (Figure 7A). Such a pore size is assumed appropriate for allowing a direct contact between endfeet of astrocytes and endothelial cells (ECs) that is a prerequisite for the development of tight intercellular junctions (TJ).52,53 Starting from day 4 of cocultures, the formation of TJ was monitored by measuring the transendothelial electrical resistance (TEER) daily. Figure 7B shows that TEER values increased until reaching a peak of 55 ± 5 Ω·cm² on day 5 of culture, when both the astrocyte and bEnd3 monocytes and the co-cultures reached the confluence. The resistance recorded in the co-cultures maintained elevated values in the range of 35–55 Ω·cm² until day 7, in accordance with the TEER values reported in the literature for the same in vitro BBB model.54,55 Conversely, TEER values of astrocyte and bEnd3 monocytes showed a significant decrease starting from day 5.

These results highlighted that, with respect to BBB models consisting of endothelial cell monolayers, the presence of astrocytes allowed the BBB to maintain its structural characteristics for a longer time. Astrocytes, indeed, contribute to the induction and maintenance of BBB phenotype through the secretion of factors that influence the features of the brain endothelial cells (ECs) promoting expression and assembly of intermolecular junctions and localization of brain EC transporters.24,56,57

In addition, the evaluation of BBB paracellular permeability, detected at day 5 of culture, using fluorescein isothiocyanate–dextran (FITC–D) and calculated as apparent permeability coefficient (P_a), evidenced that P_a values of the co-cultures (3.50 ± 0.02 × 10^{-6} cm/s) were significantly lower (p < 0.001) than that of the CTRL (8.30 ± 0.01 × 10^{-6} cm/s), represented by inserts coated with PLL and collagen Type I without cells (Figure 7C), suggesting that the ECs possessed junctional complexes. The semithin and ultrathin sections of insert membranes containing the co-cultures confirmed the presence of a uniform layer of bEnd3 on the luminal side, as well as of astrocytes on the abluminal side (Figure 7D,E).

2.4. Ability of DRV, Free and Encapsulated in PLGA Nanoparticles, to Cross the Artificial BBB. The ability of PLGA NPs to convey the antiretroviral drug DRV through the BBB was evaluated. To this end, on day 5 after the preparation of the artificial BBB, when it still presented the physical and chemical characteristics typical of an intact BBB, the inserts were treated with DRV/C-Dot/PLGA NPs at the nontoxic concentration of 150 μg/mL, 15 μM, and 25 μg/mL for NPs, DRV, and C-Dots, respectively. For comparison, in the same set of experiments, the inserts were treated with 15 μM free DRV. After 3 h incubation, the supernatants were collected from the lower and upper chambers, respectively, to quantify the concentrations of NPs and DRV. No significant changes were recorded for the TEER values, before and after PLGA NP crossing the BBB model, thus indicating that the physiological integrity of its tight junctions was preserved (data not shown).

The C-Dots PL measured in the basolateral chamber was used for the determination of the endothelial permeability (P_e) of DRV/C-Dots/PLGA NPs. The analysis indicated that P_e was equal to (9.9 ± 0.5) × 10^{-5} cm/s. The DRV percentage, free and incorporated in the luminescent PLGA NPs, that permeated through the BBB was evaluated by UV–vis absorption spectroscopy, with respect to its initial amount in the upper chamber. As shown in Figure 8, the DRV percentage that crossed the BBB was found to be significantly (p < 0.001) higher when the drug was incorporated in PLGA NPs (38.0 ± 2.1%, corresponding to 5.7 ± 0.31 μM) than for free DRV (9 ± 0.1%, corresponding
to 1.32 ± 0.01 μM). Literature data indicate that the concentration of DRV in the cerebrospinal fluid (CSF) of HIV-positive subjects was 100-fold lower than that detected in plasma. One of the possible factors that affect the passage of ARVs across the BBB may be the molecular pumps present on the endothelial cells. In particular, the expression of active efflux transporters, such as the P-glycoprotein, can partially contribute to determine suboptimal concentrations of DRV in the brain. Indeed, large lipophilic drugs such as protease inhibitors may be prevented from entering the brain having strong binding affinities to drug efflux transporters expressed at the BBB. The current strategies clinically explored to ensure a boosted therapeutic efficacy of DRV relies on its combination with drugs that, having a higher binding affinity (i.e., ritonavir) to P-glycoprotein, occupy a wide proportion of the efflux transporter binding sites slowing down the efflux rate of the co-administered PI, thus promoting its brain entry. Remarkably, several studies indicated that the possible mechanism underlying the transendothelial transport of surfactant-coated NPs into the brain involves the selective adsorption of the apolipoproteins E and B from the blood, after NP intravenous administration. Since apolipoprotein E plays an active role in the delivery of LDL into the brain, surfactant-coated NPs could mimic the LDL and undergo transcytosis mediated by LDL receptor, located on the surface of the endothelium that forms the BBB.

2.5. Effect of DRV, Free or Encapsulated in PLGA Nanoparticles, on MMP-9 Release from Astrocytes. Astrocytes, the most copious type of glial cells in the CNS, aside from contributing to the development and functioning of the BBB, play a crucial role for the maintenance of brain homeostasis and are hallmark of different neurological diseases such as HANDs. Harmful insults, such as HIV infection, may activate astrocytes promoting tissue repair or exacerbating inflammatory reactions and tissue damage. Activated astrocytes are well known to contribute to the pathogenesis of HIV-1-associated neurocognitive impairment through the production of neurotoxic factors such as MMP-9, resulting in the perpetuation of an inflammatory response. Therefore, MMP-9 may be considered a therapeutic target in HANDs. In a previous study, we have proved that several ARVs, including DRV, are able to inhibit MMP-9 levels in LPS-activated astrocytes according to mechanisms that are independent of their antiretroviral activity. The reason we used astrocytes stimulated with LPS rather than those stimulated with HIV or with HIV fragments derived from the observation that this model well describes the indirect activation of astrocytes related to the residual immune activation present in patients with suppressed viremia following ARV treatment. Furthermore, this model best reproduces the systemic immune activation associated with HIV, linked to the increase in

Figure 9. Effect of DRV, free or encapsulated in PLGA NPs, on MMP-9 release from LPS-activated astrocytes. (A) Representative zymographic gel of the analysis of cell culture supernatants from astrocytes activated with LPS (10 μg/mL) and simultaneously treated for 24 h with DRV/C-Dot/PLGA NPs or free DRV (DRV) at the indicated concentrations. Positive and negative controls were represented from LPS-stimulated astrocytes and unstimulated and untreated astrocytes in serum-free DMEM (CTRL), respectively. (B) Histogram representing MMP-9 levels expressed as % in comparison with LPS, calculated after scanning densitometry and computerized analysis of gels. The values represent mean ± SD of n = 3 experiments performed on different cell populations; * indicates values statistically significant different in comparison with LPS (one-way ANOVA followed by Dunnet’s post hoc test; *p < 0.05).
circulating levels of LPS, as a consequence of microbial translocation from the damaged gastrointestinal tract, which can be responsible for the upregulation of MMP-9 by various cell types including glial cells. Therefore, here, such a suitable experimental model was used to evaluate the ability of DRV to maintain its inhibitory effect against MMP-9 after its encapsulation in the luminescent PLGA NPs. To do this, astrocytes, seeded in 96-well plates, were activated with LPS, simultaneously treated with DRV/C-Dot/PLGA NPs or with free DRV at concentrations comparable to those detected in plasma and CSF of HIV-positive subjects, as reported in Latronico et al.\textsuperscript{35}

As shown in the representative zymogram in Figure 9A, MMP-9 was absent in the negative control (CTRL), and it was induced by treatment with LPS (LPS). Conversely, astrocyte treatment with DRV/C-Dot/PLGA NPs determined a significant reduction of MMP-9 levels at the concentration of 150 μg/mL (corresponding to 15 μM DRV), which was comparable to that observed in cells treated with free DRV (Figure 9B). Finally, the inhibitory effect of DRV, delivered by PLGA NPs, on the MMP-9 expression was evaluated on astrocytes after BBB crossing. For these experiments, a DRV concentration within the range of doses detected in the plasma of HIV patients (3–20 μM) was chosen. The quantitative zymographic analysis of supernatants collected from the lower chamber after 24 h of incubation at 37 °C, 5% CO\textsubscript{2} from astrocytes. (C) Histograms showing MMP-9 levels expressed as % in comparison with LPS: scanning densitometry and computerized analysis of gels were carried out for their calculation. The values of MMP-9 levels are reported as mean ± SD (n = 3 replicates of different experiments). Values characterized by statistically significant difference in comparison with LPS (one-way ANOVA followed by Dunnet’s post hoc test) were indicated by the symbols * (p < 0.05) and ** (p < 0.001).

3. CONCLUSIONS

The ensemble of the results achieved in this work has proven that luminescent PLGA NPs, characterized by a high degree of biocompatibility, are able not only to deliver DRV through the BBB with an efficiency higher than that found for the free drug but also to retain the inhibitory activity of DRV toward MMP-9, which represents an important therapeutic target in the course of HIV infection, after passing through the BBB.

The retained inhibitory activity of DRV incorporated in PLGA-based NPs toward MMP-9 highlights the great potential of these nanosystems for the treatment of not only HANDs but also other neurological disorders that could clinically benefit the inhibition of MMPs, with improved therapeutic efficacy and reduced toxicity.

Although these results have been achieved using an in vitro model, they represent a necessary prerequisite before their validation in an in vivo model.

4. EXPERIMENTAL SECTION

4.1. Materials. Citric acid (anhydrous), 1-octadecene (ODE, technical grade 90%), 1-hexadecylamine (HDA, 98%), Pluronic F-68 (synonyms: Lutrol F-68, Poloxamer 188) solution, poly(lactic-co-glycolic) acid (PLGA), Resomer (RG 502 H, lactic/glycolic acid molar ratio of 50/50, M\textsubscript{w} 7000–17 000), gelatin, DNase 1, poly-l-lysine, trypsin, lipopolysaccharide (LPS), Trypan Blue, 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT), and fluorescein isothiocyanate-dextran (FITC–D, average molecular weight 3000–5000) were purchased from Sigma (St. Louis, MO). 20,70-Dichlorofluorescein diacetate (DCFH-DA) was from Calbiochem, San Diego, CA. Standard proteins and R-250 Coomassie Brilliant Blue were purchased from Bio-Rad (Hercules, CA). Uranyl acetate dihydrate, paraformaldehyde (reagent grade, crystalline), glutaraldehyde solution (50 wt % in H\textsubscript{2}O), osmium tetroxide solution, LR white acrylic resins, and toluidine blue were purchased.
from Sigma-Aldrich. Collagen I high concentration, RAT TAIL, and Transwell cell culture inserts were from Corning (New York). DRV was provided by Silag GA (Schaffhausen, Switzerland). Anti-glia fibrillary acidic protein (GFAP) antibodies (RRID: AB_2294571) were purchased from Serotec (Oxford, U.K.). Brain-immortalized endothelial cell line (bEnd3) were from American Type Culture Collection (Manassas, Va.). All solvents used were purchased from Sigma-Aldrich, and they were of analytical grade. Milli-Q gradient A-10 system (Millipore, 18.2 MΩ-cm, organic carbon content <2 μg/L) was used for the preparation of all aqueous solutions. Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and penicillin and streptomycin were provided by Thermo Fisher Scientific (Waltham, MA).

4.2. Synthesis of Luminescent C-Dots. Thermal carbonization of citric acid was performed in the presence of ODE as a high-boiling solvent and HDA as a surface ligand and nitrogen source to synthesize colloidal C-Dots. Reactants were previously dried and degassed. The synthesis was carried out in an inert atmosphere of nitrogen using a Schlenk line. HDA (1.5 g) was solubilized in 26 mL of ODE under vacuum at 110 °C for 30 min. Subsequently, the mixture was heated above the citric acid decomposition temperature (153 °C). Then, citric acid (1 g) was quickly added to the reaction mixture, at a temperature of 200 °C. After 3 h, the reaction was stopped by lowering the temperature to 25 °C. Several washing cycles with acetone were performed to purify the resulting C-Dots, which were finally dispersed in organic solvent (chloroform).37

4.3. Preparation of Poly(lactic-co-glycolic) Acid Nanoparticles Co-encapsulating C-Dots and Darunavir. For the preparation of the NPs loaded with C-Dots and DRV (DRV/C-Dot/PLGA NPs), 3 mL of organic solution containing PLGA (20 mg/mL), C-Dots (25 mg/mL), and deionized water (1 mL) were added dropwise to 27 mL of a 4% (w/v) Pluronic F-68 aqueous solution. The two phases were homogenized to form an emulsion by performing a low-speed magnetic stirring. Cellulose acetate membrane with an average pore size of 0.2 μm was used to separate the emulsion and the receptor chambers. PBS dispersion (500 μL) containing DRV/C-Dot/PLGA NPs (1 mg/mL in terms of DRV concentration) was added as the donor solution. PBS solution (9 mL) contained the receptor chamber. At scheduled times, aliquots of the receptor solution were analyzed by UV−vis absorbance spectroscopy to evaluate the DRV content and using a calibration curve. For this purpose, standard aqueous solutions containing DRV in the concentration range of 5−25 μg/mL were prepared. The in vitro release experiments were performed in triplicate.

4.4. Determination of Drug Loading and Encapsulation Efficiency. The encapsulation efficiency (EE%) and loading values of each in vitro release study of DRV from PLGA-based nanoformulations in PBS (50 mM, pH = 7.4) at 37 °C under magnetic stirring. Cellulose acetate membrane with an average pore size of 0.2 μm (Fisher Scientific Milano) was placed between the donor and receptor chambers. PBS dispersion (500 μL) containing DRV/C-Dots/PLGA NPs (1 mg/mL in terms of DRV concentration) was introduced in the donor chamber. PBS solution (9 mL) was introduced in the receptor chamber. At scheduled times, 400 μL of receptor solution was taken within 24 h and an equal volume of PBS was added in the receptor chamber. Each collected solution fraction was analyzed by UV−vis absorbance spectroscopy to evaluate the DRV content and using a calibration curve. For this purpose, standard aqueous solutions containing DRV in the concentration range of 5−25 μg/mL were prepared. The in vitro release experiments were performed in triplicate.

4.8. Setting of bEnd3 and Astrocyte Cultures. Brain-immortalized mouse endothelial cell line (bEnd3) was maintained at 37 °C, 5% CO2 in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Astrocytes were obtained from neocortical tissues of Wistar rat pups at postnatal days 0−2 (Harlan Laboratories srl, Udine, Italy). Procedures involving animals were carried out in compliance with the directives of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of University of Bari, Italy (Permit Number: 23-98-A). All experiments were designed to minimize the number of animals used and their suffering. For the experiments, 8 litters of 12 pups were used. The pups were obtained from five females (RRID: RGD_737960), which were treated with chloroform (1 mL) to promote the rupture of PLGA NPs, and subsequently, the organic solvent was completely removed by evaporation under nitrogen flux. Finally, the dried samples were solubilized in methanol (0.5 mL) and UV−vis absorbance measurements (PerkinElmer Lambda 20 UV VIS Spectrophotometer) were performed on the resulting solutions. A calibration curve was plotted by preparing standard methanol solutions containing DRV in the concentration range of 5−25 μg/mL and C-Dots at the same concentration of the PLGA NPs samples. The calibration curve was obtained by reporting in the graph the UV−vis absorbance values at 266 nm versus DRV concentrations. The encapsulation efficacy (EE%) values of drug loaded in PLGA-based nanoformulations were obtained as follows

\[
EE\% = \left( \frac{W_r}{W_i} \right) \times 100
\]

where \(W_r\) is the total amount of DRV in the PLGA NPs and \(W_i\) is the total amount of DRV introduced in the organic phase during preparation.

The drug loading (DL%) is the effective amount of drug (in weight) incorporated in the particles system, and it is calculated as

\[
DL\% = \left( \frac{W_r}{W_{PN}} \right) \times 100
\]

where \(W_{PN}\) represents the weight of PLGA NPs.

4.5. DLS Investigation and ζ-Potential Measurements. Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, U.K., was employed to evaluate the mean hydrodynamic size (reported as intensity mean diameter), polydispersity index (PDI), and ζ-potential values of the NPs.68 Data are referred to as mean ± standard deviation (n = 3 replicates).

4.6. TEM Investigation. Transmission electron microscopy (TEM) analysis was performed using a Jeol JEM-1011 microscope, provided with an Olympus QuemaCamera (11 Mpx). The samples were prepared by depositing on a carbon-coated Cu grid (400 mesh) a drop (5 μL) of C-Dots or PLGA NPs dispersion, in chloroform or in aqueous solution, respectively. After solvent evaporation, the grid was placed on the top of a drop of an aqueous phosphotungstic acid solution 2% (w/v) for 30 or 60 s. As a final step, the grid was washed with ultrapure water and stored in a vacuum chamber until TEM measurements. Size statistical analysis, expressed as C-Dots average size and relative standard deviation (r%), was performed using the ImageJ analysis program.

4.7. In Vitro Release Study. A Franz diffusion cell was used to perform the in vitro release study of DRV from PLGA-based nanoformulations in PBS (50 mM, pH = 7.4) at 37 °C under magnetic stirring. Cellulose acetate membrane with an average pore size of 0.2 μm (Fisher Scientific Milano) was placed between the donor and receptor chambers. Aqueous PBS dispersion (500 μL) containing DRV/C-Dots/PLGA NPs (1 mg/mL, 0.1 mg/mL in terms of DRV concentration) was introduced in the donor chamber. PBS solution (9 mL) was introduced in the receptor chamber. At scheduled times, 400 μL of receptor solution was taken within 24 h and an equal volume of PBS was added in the receptor chamber. Each collected solution fraction was analyzed by UV−vis absorbance spectroscopy to evaluate the DRV content and using a calibration curve. For this purpose, standard aqueous solutions containing DRV in the concentration range of 5−25 μg/mL were prepared. The in vitro release experiments were performed in triplicate.
were breeding and mated in the animal facility of the Department of Biosciences, Biotechnologies and Biopharmaceutics of University of Bari (Italy). All animals were maintained at room temperature (22°C) and humidity of 40–50% on a 12:12 h light/dark cycle. One day after birth, the pups were euthanized by exposure to carbon dioxide (CO2) and sacrificed by rapid decapitation. Primary glial cell cultures were prepared from newborn brains as described by Di Bi et al.9 Briefly, neocortical tissues were cleaned of meninges and blood vessels, then minced, and incubated for 10 min at 37°C with 0.25% trypsin and 0.01% DNase in DMEM. After centrifugation, the cells were plated in PLL-coated flasks (75 cm²) at a density of 1.5 x 10⁵ viable cells/flask in DMEM, 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and maintained at 37°C in a 5% CO2. After 10 days of culture, astrocytes were separated from microglia and oligodendrocytes by mechanical dislodging.

Immunostaining for GFAP was used to assess the purity of astrocyte cell culture, thus revealing that more than 98% of the cells were GFAP-positive in all of the preparations.

4.9. Evaluation of Cell Viability. Confluent astrocytes and bEnd3 were seeded in 96-well plates and treated with empty PLGA NPs, C-Dots/PLGA NPs, or DRV/C-Dot /PLGA NPs (NPs concentration ranging from 5 to 400 μg/mL). The cells were incubated for 24 h with different formulations and then rinsed with PBS. The cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.70 PBS. The cell viability was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.70

4.10. Setting Up the In Vitro Blood–Brain Barrier Model. The in vitro model of BBB was set by co-culturing mouse bEnd3 cells and primary astrocytes on inserts with a diameter of 10.5 mm containing a track-etched poly(ethylene terephthalate) (PET) membrane (0.4 µm pores). Before cell seeding, the abluminal side and luminal side of the insert membrane were coated, respectively, with poly-L-lysine (PLL) and collagen type I rat tail (500 μg/mL).

Astrocytes were plated at a density of 3.5 x 10⁵ cells/cm² on the abluminal side using the insert flipped back. To allow adherence, after 2 h of incubation, the inserts were placed in a 12-well plate in DMEM, 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin, and incubated at 37°C, 5% CO2. After 3 days, when astrocytes had reached almost 80% confluence, bEnd3 were plated on the luminal side of the inserts at a density of 2.3 x 10⁵ cells/cm². The controls (CTRL) were represented by PLL- and collagen-coated inserts and inserts containing monoculture of astrocytes or bEnd3. Measurements of transepithelial electrical resistance (TEER) were carried out using an epithelial voltmeter, daily, starting from day 4 of co-culture to determine the formation of a functional and intact BBB.

To have an effective estimation of the resistance, the average TEER value of the CTRL inserts was subtracted from the resistance of inserts containing the co-cultures or the monocultures of astrocytes or bEnd3 cells. The obtained values of TEER were expressed as Ω·cm².

The paracellular permeability of BBB was detected at day 5 of culture, when the mean TEER values recorded in the co-culture BBB reached a value of the CTRL inserts was subtracted from the resistance of inserts coated with PLL and collagen Type I without cells. The control (CTRL) inserts coated with PLL and collagen Type I without cells. The controls (CTRL) represented by inserts without cells treated under the same conditions. After 3 h of incubation at 37°C, 5% CO2, supernatants were collected and analyzed by ELISA. The TEER was checked to evaluate the integrity of BBB.

The endothelial permeability (Pf) of DRV/C-Dot /PLGA NPs was calculated by recording the C-Dots PL spectra.80 The samples recovered from the upper and lower chambers of the transwell were lyophilized, and then treated with 1 mL of chloroform. The inorganic salts derived from the culture medium were removed by centrifugation (1500g for 15 min). Subsequently, the supernatants were collected and analyzed by ELISA. The calibration curve, previously described in Section 4.3, was used to estimate the C-Dots concentration in the samples. The Pf value of DRV/C-Dot /PLGA NPs across the in vitro BBB model was calculated by evaluating the permeability through blank insert without cells and the permeability across the insert containing cells, as reported in the literature.46,71,72 The supernatants were dried under N2 flux, treated with 0.5 mL of methanol, and finally spectroscopically analyzed by UV–vis absorption spectrophotometry at a wavelength of 266 nm, to detect DRV. A calibration curve obtained by preparing DRV solutions in the concentration range of 5–25 μM was used to determine the amount of DRV, both in the free form and incorporated in C-Dot /PLGA NPs. The amount of DRV, recovered in the lower chamber of the transwell, after transmigration through the artificial BBB, was calculated as a percentage with respect to its initial amount detected in the upper chamber of transwell.

4.13. Treatment of LPS-Activated Astrocytes with Free DRV or DRV/C-Dot /PLGA NPs. Confluent astrocytes, seeded in 96-well plates, were washed twice with serum-free DMEM, activated with LPS (10 μg/mL), and simultaneously treated with DRV/C-Dot /PLGA NPs at the concentrations of 5 μg/mL and 150 μg/mL, corresponding to 0.4 and 15 μM of DRV, or with free DRV at the concentrations, corresponding to those reached in DRV/C-Dot /PLGA NPs. Negative and positive controls were represented, respectively, from nonactivated and untreated astrocytes in serum-free DMEM (CTRL). LPS-activated astrocytes were collected and stored at −20°C until used for zymographic analysis. Cell viability was assessed evaluated by MTT.

4.14. Evaluation of the Effect of DRV, Free or Encapsulated into PLGA Nanoparticles, on MMP-9 Released by Astrocytes after BBB Crossing. Simultaneously with the setup of BBB, 1.7 x 10⁵ astrocytes were plated at the bottom of the 12-well plate containing the transwell inserts. After BBB formation, confluent astrocytes, at the bottom of the wells, were washed with serum-free DMEM and activated with 10 μg/mL of LPS. Simultaneously, in the upper chamber of inserts, containing the artificial BBB, 150 μg/mL DRV/C-Dot /PLGA NPs (containing 15 μM DRV and 25 μg/mL C-Dots) were added. Free DRV (15 μM) was added in other wells. Cells activated with LPS represented the positive control (LPS), while...
nonactivated and untreated cells represented the negative control (CTRL).

After 24 h incubation at 37 °C and 5% CO₂, astrocyte supernatants were recovered from the lower chamber and a zymographic analysis was performed.

4.15. Detection of MMP-9 by Zymographic Analysis. Matrix metalloproteinase-9 in cell culture supernatants was detected by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis zymography as reported in Latronico et al. Briefly, 50 µL of culture supernatant was analyzed in 7.5% polyacrylamide slab gels copolymerized with 0.1% (w/v) gelatin. After the electrophoretic run, the gels were incubated in washing buffer (2.5% (w/v) Triton X-100/10 mM CaCl₂ in 50 mM Tris pH 7.4) for 20 min and then incubated at 37 °C in developing buffer (1% (w/v) Triton X-100/50 mM Tris–HCl/10 mM CaCl₂, pH 7.4) for 24 h. MMP-9 activity was detected as a band of digestion on a blue background on the gels and was quantified, after scanning densitometry, by computerized image analysis using the Image Master 1D program (Pharmacia Biotech, Uppsala, Sweden). Levels of MMP-9 were expressed as optical density (OD) (OD/OD) × 100%

% levels = [(OD_sample/OD_positive control)×100]

4.16. Photophysical Investigation. A Cary 5000 (Varian) UV/vis/NIR spectrophotometer and a Fluorolog 3 spectrofluorimeter (HORIBA Jobin-Yvon) were used to record the UV–vis absorption or PL emission spectra, respectively. PL emission absolute QY of C-Dots and of the luminescent nanoformulations dispersed in solution was evaluated using a “Quantaphot” (HORIBA Jobin-Yvon) integrating sphere coated by Spectralons. Time-correlated single photon counting (TCSPC) measurements were performed with a FluoroHub (HORIBA Jobin-Yvon) to investigate fluorescence lifetime of C-Dots, bare or PLGA nanoformulations. A picosecond laser diode (NanoLED 375L) emitting τ ≈ 80 ps pulses at a 1 MHz repetition rate was used as the excitation source. The PL signals were detected by a picosecond photon counter (TBX ps Photon Detection Module, HORIBA Jobin-Yvon) with a temporal resolution of ~200 ps.

4.17. Statistical Analysis. Parametric one-way analysis of variance (ANOVA) followed by the Dunnett’s multiple comparison post hoc test was used to compare transendothelial electrical resistance (TEER) and MMP-9 levels under different setting conditions. Student’s t-test was used to compare the apparent permeability coefficient (Pₐ) of fluorescein isothiocyanate–dextran (FITC–D) and the endothelial permeability (Pₑ) of DRV/C-Dots/PLGA NPs and free DRV.

“n” represents the number of independent experiments performed with different cell cultures. Data from at least three different experiments, with every data point in an individual experiment representing triplicate measurements, were used for statistical analysis. Data were analyzed by GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.1c00436.

Absolute QYs (%) of C-Dots in an organic solvent before and after their encapsulation in PLGA NPs (PDF)

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T.L. and F.R. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors acknowledge the Bilateral project CNR-RFBR (Russia) “Mesoporous silica nanocarriers incorporating plasmonic Cu₂₋₃S nanocrystals, fluorophores, and 5-fluouracil and functionalized with FZD10 antibody for the targeted photo-induced therapy of colorectal cancer” joint research projects in the triennium 2021–2023 and the Italian National project PON TITAN “Nanotechnology for cancer immunotherapy”, 2021–2023 AR501_00906. The authors also acknowledge Dr. Anna Fasano for TEM characterization of the in vitro BBB model.
**ABBREVIATIONS USED**

HIV, human immunodeficiency virus; CNS, central nervous system; HAND, HIV-associated neurological disorders; HAD, HIV-associated dementia; BBB, blood–brain barrier; MMP, matrix metalloproteinase; MMP-9, metalloproteinase-9; HIV-1, human immunodeficiency virus; DRV, Darunavir; cART, combination antiretroviral therapy; ARV, antiretroviral drug; PI, protease inhibitor; FDA, Food and Drug Administration; C-Dots, carbon dots; NP, nanoparticle; PLGA, poly(lactide-glycolic)acid; SLN, solid lipid nanoparticles; LDL, low-density lipoproteins; QY, quantum yield; TR, time-resolved; TEER, transendothelial electrical resistance; Pe, apparent permeability; Pe, endothelial permeability; CSF, cerebrospinal fluid; EC, endothelial cell; FITC–D, fluorescein isothiocyanate–dextran; PLL, poly-L-lysine; PL, photoluminescence; EE, encapsulation efficacy; DL, drug loading; PDI, polydispersity index; PET, poly(ethylene terephthalate); ODE, 1-octadecene; HAD, 1-hexadecylamine; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCDFH-DA, 20,70-dichlorofluorescein diacetate; GFAP, anti-glia fibrillary acidic protein; PBS, phosphate buffer solution; TEM, transmission electron microscopy; Dynamic light scattering; RT, room temperature; DDS, solid dodecyl sulfate; OD, optical density; TCSPC, time-correlated single photon counting.

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