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# Nanoformulation of antiretroviral drugs enhances their penetration across the blood brain barrier in mice☆

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**Abstract**

Eradication of virus by sanctuary sites is a main goal in HIV management. The central nervous system (CNS) is a classic model of sanctuary where viral replication occurs despite a complete viral suppression in peripheral blood. In recent years, nanotechnologies have provided a great promise in the eradication of HIV from the CNS. We hereby demonstrate for the first time that the structurally complex antiretroviral drug enfuvirtide (Enf), which normally is unable to penetrate the cerebrospinal fluid, is allowed to cross the blood brain barrier (BBB) in mice by conjugation with a nanoconstruct. Iron oxide nanoparticles coated with an amphiphilic polymer increase Enf translocation across the BBB in both *in vitro* and *in vivo* models. The mechanism involves the uptake of nanoconjugated-Enf in the endothelial cells, the nanocomplex dissociation and the release of the peptide, which is eventually excreted by the cells in the brain parenchyma.

*Key words:* HIV sanctuaries; Enfuvirtide; Blood brain barrier; PMA-coated nanoparticles

## Background

Current antiretroviral treatment regimens suppress plasma HIV-1 RNA and DNA below detectable levels in a consistent proportion of subjects.[1] However, functional cure and eradication are still beyond our possibilities. One obstacle to such goals is represented by the difficulty to achieve therapeutic antiretroviral concentrations within sanctuary sites where HIV-1 has been shown to compartmentalize. Such sites include the genital tract, the gut-associated lymphoid tissue, the lymph nodes, tissue macrophages and the central nervous system (CNS).[2-4] In particular, the CNS is considered one of the most important viral reservoirs. This is mainly due to the presence of macrophages that promote the inflammatory escalation with subsequent astrogliosis and neurodegeneration, thus establishing the so-called NeuroAIDS,[5] responsible for neurocognitive disorders

*Abbreviations:* AF660, Alexa Fluor 660; BBB, blood brain barrier; CNS, central nervous system; DLS, dynamic light scattering; ECM, endothelial cell medium; Enf, enfuvirtide; Epf, epifluorescence; FI, fluorescence intensity; FD40, FITC-Dextran 40; FITC, fluoresceinamine; HAART, highly active anti-retroviral therapy; ICP-OES, inductively coupled plasma optical emission spectrometry; MRP, multidrug resistance-associated protein; $P_{app}$, apparent permeability coefficient; PBS, phosphate buffer saline; PFA, paraformaldehyde; PMA, poly(isobutylene-alt-1-tetradecene-maleic anhydride); RBMVECs, rat brain microvascular endothelial cells; RT, room temperature; SE, standard errors; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TEER, transendothelial electrical resistance.

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with different grades of severity (AIDS dementia complex). From a clinical point of view, NeuroAIDS is a real challenge since the blood brain barrier is poorly crossable by most antiretroviral drugs.[2]

In the effort toward viral eradication, one of the most promising strategies is to treat this latent-T cell reservoir, so that resting cells may be induced to release virions and reactivate,[6-9] while preventing HIV-1 entry in uninfected CD4+ T cells. With this aim it would be important to design new therapeutic strategies to direct antiretroviral drugs in these HIV sanctuaries, both to reduce T-cell mediated delivery of the virus into the sanctuaries and to directly act on HIV-sensitive CD4+ cells inside these sites (*i.e.* microglial cells of brain)[4].

Nanotechnology is an emerging multidisciplinary field that has the potential to offer a radical change in the treatment and monitoring of HIV/AIDS.[10-13] The potential advantages in using nanoparticles for HIV infection treatment include the capacity to incorporate, encapsulate, or conjugate a variety of drugs in order to target specific cell populations, grant long-term drug release, and penetrate into sanctuary sites. With regard to the CNS, the employment of nanotechnology could allow antiretroviral drugs to effectively reach this reservoir,[14] thus preventing the replication of the virus and reducing the damages induced by the infection.

In current clinical practice, the first-line antiretroviral therapy is generally constituted by a combination of two nucleoside reverse transcriptase inhibitors (NRTI) with a non-nucleoside reverse transcriptase inhibitor (NNRTI), such as a protease inhibitor or an integrase inhibitor. Conversely, fusion inhibitors are much less used because of some well-known limitations such as production time and costs, difficult administration (subcutaneous injection twice daily) and adverse effect profile.[15] Therefore, fusion inhibitors are only used in case of resistance or failure of the HAART. Enfuvirtide (Fuzeon™ from Roche Laboratories Inc. and Trimeris Inc.) is a 36-amino acid peptide that targets multiple sites in gp41, a HIV glycoprotein responsible for the fusion with CD4+ cells.[16-18] Enfuvirtide (Enf) inhibits HIV-1-mediated cell-cell fusion and transmission of cell-free virus while it does not have substantial activity against HIV-2.[19-22] Because of its unfavorable pharmacological profile, with a half life of approximately 2 h and a high molecular weight (4.5 kDa), Enf is particularly indicated to provide a proof of concept of the improved access of antiretroviral drug to HIV sanctuaries by nanoformulation. Indeed, Enf does not penetrate the BBB because of its complex structure, and is therefore not detectable in cerebrospinal fluid (CSF).[23]

Aim of our study is to demonstrate the ability of iron oxide nanoparticles coated with PMA amphiphilic polymer (MYTS) to enhance the permeation of a high-weighted molecule, such as Enf, across the BBB both in *in vitro* and *in vivo* models, and propose a mechanism for drug delivery across the endothelium.

## Methods

### *Nanoparticle design*

Magnetic nanoparticles (MNP) were synthesized by solvothermal decomposition in organic solvent from organometallic precursors according to Park et al. protocol.[24] MNP were transferred to water phase using a fluorescent labeled amphiphilic polymer (PMA).[25] Fluorescent-PMA was obtained with fluoresceinamine 1.0 M (0.5 mL in DMSO) was added to a 0.5 M PMA in $CHCl_3$ (5 mL) and the mixture was left overnight at RT. Part of this solution (20 μL) was added to MNP (1.5 mg in $CHCl_3$). The organic solvent was evaporated and sodium borate buffer (SBB, pH 12, 20 mL) was added obtaining a stable nanoparticle dispersion which was concentrated in Amicon tubes (100 kDa filter cutoff) by centrifuging at 3500 rpm for 20 min. The nanoparticles were washed twice with water resulting in green labeled PMA-coated nanoparticles highly soluble in aqueous media (MYTS). MYTS were reacted with an amino-linker useful for Enf immobilization on the nanoparticles. Enf was previously labeled with AF660 dye (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The final double labeled Enf-MYTS are schematically represented in Figure 1, *A*.

### *Characterization of the BBB in vitro model*

The setting of the BBB *in vitro* model, based on a co-colture of RBMVECs and astrocytes, is described in Supplementary materials. Before each experiment, we checked the trans-BBB electrical resistance by an EVOM2 Epithelial tissue Volt/Ohmmeter connected to an Endohm-24SNAP cup (WPI, Germany), obtaining a suitable value on 90% of the inserts. Moreover, the trans-BBB apparent permeability coefficient of FITC-Dextran 40 (FD40) was determined by measuring the flux of the molecule from the upper to the lower chamber of three BBB systems at 1 h, 2 h and 3 h from the addition of 1 mg $mL^{-1}$ FD40 in the upper compartment. The flux through the RBMVECs single layer or through the empty insert was used as control. The amount of FD40 recovered in the lower compartment was determined spectrofluorimetrically and the $P_{app}$ was calculated from the mean flux (see Supplementary materials).

### *In vitro trans-BBB permeation*

The permeation of (AF660)Enf, (FITC)MYTS or (AF660)Enf-MYTS(FITC) across the BBB was assessed on the *in vitro* model described above, using four inserts for each experimental condition. The two formulations were added to the upper chamber and, after 4 or 7 h of incubation, a defined volume of ECM was collected by both the upper and the lower chambers. The fluorescence intensity of the samples was measured spectrofluorimetrically. For an exact comparison between the trans-BBB permeation of free and MYTS-conjugated Enf (5 μg $mL^{-1}$), the FI of the two formulations was used for normalization, and the final amount of Enf-MYTS in the upper chamber was 0.1 mg $mL^{-1}$.

ICP-OES was also used to quantify the amount of MYTS iron in the collected samples.

Reported results are representative of one of three independent experiments.

### *Plasma concentration measurements in mice*

Plasma concentration of free or conjugated Enf was determined upon intravenous injection of AF660-labeled Enf (0.2 μg $g^{-1}$ body weight) or Enf-MYTS (12.5 μg $g^{-1}$ body weight) in Balb/c mice. We treated four mice per experimental condition and repeated the experiment twice (for a total of eight