Thermosensitive and mucoadhesive pluronic®/hydroxypropylmethylcellulose hydrogel containing the mini CD4 M48U1 is a promising efficient barrier against HIV diffusion through macaque cervico-vaginal mucus

Running title: The double barrier against HIV diffusion

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To be efficient, vaginal microbicide hydrogels should form a barrier against viral infections and prevent virus spreading through mucus. Multiple particle tracking was used to quantify the mobility of 170 nm fluorescent-labeled COOH-modified polystyrene particles (COOH-PS) into thermosensitive hydrogels composed of amphiphilic triblock copolymers with block compositions EO$_n$-PO$_m$-EO$_n$ (where EO refers to ethylene oxide and PO to propylene oxide) containing mucoadhesive hydroxypropylmethylcellulose (HPMC). COOH-PS were used to mimic the size and the surface charge of HIV-1. Analysis of COOH-PS trajectories showed that particle mobility was slowed-down by pluronic® hydrogels in comparison with Cynomolgus macaque cervico-vaginal mucus and hydroxyethylcellulose hydrogel (HEC 1.5 wt%) used as negative controls. Formulation of the peptide mini CD4 M48U1 used as anti-HIV-1 molecule into a mixture of pluronic® F127 (20 wt%) and HPMC (1 wt%) did not affect its anti-HIV-1 activity in comparison with HEC hydrogel. The IC50 was 0.53 µg/mL (0.17 µM) for M48U1/HEC and 0.58 µg/mL (0.19 µM) for M48U1/F127/HPMC. The present work suggests that hydrogels composed of F127/HPMC (20/1 wt%) can be used to create efficient barrier against particle diffusion in comparison to conventional HEC hydrogels.

**Keywords:** pluronics, hydrogel, thermally responsive material.
Introduction

According to the latest (2008) WHO and UNAIDS global estimates, among the 35 million people worldwide living with the human immunodeficiency virus (HIV), 50% are women. The first step in HIV infection by the vaginal route involves virus diffusion through vaginal mucus followed by the interaction of viral envelope proteins with their receptors on the surface of the vaginal host cells. Mucus is an entangled viscoelastic gel that coats the surfaces of the vaginal tract. It provides the outermost barrier against viral infections. The idea exposed in this work is to form a physical barrier composed of thermosensitive and mucoadhesive hydrogel against HIV diffusion limiting thus virus attachment to mucosal surfaces of the vagina. The thermosensitive property of \( (\text{ethylene oxide})_{98}(\text{propylene oxide})_{67}(\text{ethylene oxide})_{98} \) (EO\(_{98}\)PO\(_{67}\)EO\(_{98}\)) block copolymer denoted pluronic\textsuperscript{®} F127 or poloxamer P407 is particularly interesting for the design of vaginal microbicides acting as a physical barrier. Under certain concentration, this system is fluid at room temperature and in the form of gel at body temperature (37°C) (1). Contrarily to semi-solid hydrogels, the administration of the formulation in the form of a solution (for instance, via syringes or adequate device) facilitates its spreading on the mucosa, while hydrogel layer on the mucosal surface could allow the formation of a physical barrier against virus diffusion. Mucoadhesion of pluronic-hydrogels was further improved by adding hydroxypropylmethylcellulose (HPMC) as mucoadhesive polymer (1).

Previous works reported different techniques for the investigation of this barrier effect by using viruses or virus-like particles. Diffusion chambers and Transwell-Snapwell chambers were used for hydrogel samples. The principle is based on donor-receptor duality. The sample containing tracking substance such as polystyrene particles is placed in a donor chamber and...
their passage in a receiver chamber filled with an appropriate liquid media is observed (2, 3). However, this technique did not allow controlling parameters such as thickness and uniformity of the sample layer, influence of preparation and handling on the structure of the sample and optimal diameter of the pore to prevent blockage with hydrogel sample (4). Fluorescent-labeled probe observations using fluorescence recovery after photobleaching (FRAP), Single or Multiple Particle Tracking (SPT/MPT) and Fluorescence correlation spectroscopy (FCS) are other techniques that present the advantage of being “soft techniques” without effect on the structure of the sample (5). These methods demonstrated the utility of using fluorescence-techniques to study the transport and the stability of particles in different media. Particularly, Single or Multiple Particle Tracking can give information at individual particle scale, while fluorescence microscope equipped with laser (FRAP) method gives ensemble-average data. These techniques are based on FRAP or photo diodes (FCS) as light source and acousto-optic filters or modulators. For SPT a fast and sensitive camera is added to the system. This camera permits to analyze dynamic phenomena like particle movements in a medium by showing each particle by a dot. The size of the dot is related to the resolution of the objective. It is possible to calculate particle positions with sub-resolution precision using algorithms that can calculate the centers of the dots (6).

The main objective of this work was to show that measuring the barrier effect of hydrogels is possible with “soft techniques”. We tried to correlate this barrier effect with pluronic® hydrogel intrinsic structure versus negative controls composed of macaque cervico-vaginal mucus (CVM), hydroxyethylcellulose (HEC) hydrogel and water. HEC hydrogel is very commonly used as a carrier of antiviral drugs in clinical studies on vaginal microbicides (7). Drug-free HEC hydrogel failed to prevent HIV transmission using macaque models (8, 9).
In the present work, multiple particle tracking was used to quantify the mobility of 170 nm fluorescent-labeled and negatively-charged COOH-modified polystyrene particles (COOH-PS) into pluronic®-based hydrogels. COOH-PS were used to mimic the size and the surface charge of HIV-1. The size of HIV-1 virions ranges from 120 to 180 nm, while mature HIV-1 size ranges from 140 to 220 nm (10). COOH-PS particles are negatively-charged mimicking thus the anionic HIV-1 virion envelope (4). Furthermore, multiple particle tracking showed that transport rates of COOH-PS particles are comparable to HIV in non-ovulatory mucus (11).

Finally, the anti-HIV-1 activity of the miniCD4 M48U1 formulated into hydrogels was evaluated. This peptide was used as HIV-1 molecule that target initial step of viral attachment to CD4 cell receptor to block the viral entry (12-14).

Materials and methods

Ethical statement

Adult cynomolgus macaques (Macaca fascicularis) were imported from Mauritius and housed in the facilities of the “Commissariat à l’Energie Atomique et aux Energies Alternatives” (CEA, Fontenay-aux-Roses, France). Non-human primates (NHP, which includes M. fascicularis) are used at the CEA in accordance with French national regulation and under national veterinary inspectors (CEA Permit Number A 92-032-02). The CEA is in compliance with Standards for Human Care and Use of Laboratory of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-01. All experimental procedures were also conducted accordingly to European guidelines for animal care (European directive 86/609, “Journal Officiel des Communauté Européennes”, L358, December 18, 1986). The use of NHP at CEA is also in accordance with recommendation
with the newly published European Directive (2010/63, recommendation N°9). No suffering was specifically associated with atraumatic vaginal fluid sampling in macaques. The animals were used under the supervision of the veterinarians in charge of the animal facility.

**M48U1 synthesis**

The M48U1 peptide containing a p-(cyclohexylmethoxy)phenylalanine residue at position 23 was synthesized at Pepscan Presto Inc. (Lelystad, The Netherlands) by solid phase peptide synthesis and purified after refolding by reverse-phase high performance liquid chromatography as described elsewhere (15). M48U1 structure has been previously described (13).

**Preparation of hydrogels**

HEC hydrogel (1.5 wt%) was prepared by adding 1.5 g of gelling polymer HEC (Natrosol 250 M Pharm, Aqualon, USA) to a vial containing citrate buffer (5 mM, pH 4.5). The volume was then completed to 100 g by citrate buffer. The final formulation was mixed with a mechanical stirrer until complete dissolution of HEC.

Pluronic®-based hydrogels were prepared by weight according to the so-called “cold method” (1, 16, 17).

For the preparation of pluronic-based hydrogels (F127 wt% and F127/F68 22.5/2.5 wt%), pluronic® powders (BASF, Ludwigshafen, Germany) were gradually added under magnetic agitation at 4°C to citrate buffer. The different preparations were denominated by two numbers indicating the weight percentage of pluronic® F127 and pluronic® F68, respectively.

For the preparation of formulations composed of F127/HPMC (20/x wt%) and F127/F68/HPMC (22.5/2.5/x wt%), HPMC powder (Methocel® K4M series MM87091702 K,
from Colorcon) was gradually added under magnetic agitation at 4°C to citrate buffer. The proportion of HPMC in the formulation denoted x was varied as: (0, 0.2, 0.5, 0.8 and 1 wt%). After complete dissolution of HPMC, pluronic® powders were gradually added to this phase in the same conditions of temperature and agitation. Preparations composed of F127/F68/HPMC were denominated by three numbers indicating the w/w percentage of F127, F68 and HPMC, respectively.

Noteworthy, F68 was added to modulate gelling temperature \( T_{gel} \), while HPMC was added to improve hydrogel mucoadhesion (1).

M48U1 (3 mg/g) was formulated by adding the peptide to hydrogels. The hydrogels were homogenized by magnetic mixing (18).

To investigate the effect of the pH on \( T_{gel} \), the pH was adjusted to 7 using NaOH solution (Sigma-aldrich, Saint-Quentin Fallavier, France).

In all cases, after complete dissolution of pluronic® powders, each formulation was hermetically sealed and stored during 48 h at 4°C to eliminate foam and air bubbles.

**Rheological characterization of pluronic®-based hydrogels**

All rheological measurements were carried out on a CSL 100 controlled stress rheometer (Carri-Med, Rhéo Champlan, France). The geometry was a stainless steel cone/plate (diameter 40 mm, angle 2° and gap 54 µm), which provided homogeneous shear of the sample. The cone was equipped with a solvent trap to limit evaporation during measurement. Thanks to Pelletier diodes placed in the lower plate, it was possible to perform temperature sweeps from 0 to 70°C with a precision of 0.1°C. Oscillatory (or dynamic) experiments were
carried out. A sinusoidal shear was applied to the sample where the stress $\tau(t)$ and the strain $\gamma(t)$ were defined as follows:

$$\tau(t) = \tau_0 \cos(\omega t) \quad \text{Eq. 1}$$

$$\gamma(t) = \gamma_0 \cos(\omega t - \delta) \quad \text{Eq. 2}$$

$\tau_0$ and $\gamma_0$ are, respectively, the maximal amplitudes of the stress and strain, $\omega = 2\pi N$, with $N$ the frequency, $\omega$ the shear pulsation, and $\delta$ the phase angle stress/strain.

From the phase angle, one could define various dynamic viscoelastic quantities, and especially the elastic (or storage) modulus $G'$ (Eq. 3) and the viscous (or loss) modulus $G''$ (Eq. 4).

$$G' = \frac{\tau_0}{\gamma_0} \cos \delta \quad \text{Eq. 3}$$

$$G'' = \frac{\tau_0}{\gamma_0} \sin \delta \quad \text{Eq. 4}$$

The higher the $G'$ value, the more pronounced the elastic character and conversely, the higher $G''$, the more pronounced the viscous properties.

Hydrogel dynamic viscosities ($\eta$) were calculated from the viscous modulus $G''$ according to equation Eq.5:

$$\eta = \frac{G''}{2\pi} \quad \text{Eq. 5}$$

Viscosities were evaluated at two different temperatures:

- At 17°C: before gelification, all the formulations are in the form of viscous liquid.
At 37°C: after gelification, the formulations are in the form of highly viscous gel.

The effect of HPMC addition on $T_{gel}$ and $\eta$ of pluronic®-based hydrogels was investigated after progressive addition of HPMC (0, 0.2, 0.5, 0.8 and 1 wt%) to hydrogels composed of F127 (20 wt%) and F127/F68 (22.5/2.5 wt%).

All rheological results are the means of $n = 3$ experiments.

**Viscosity measurements of HEC hydrogel**

Dynamic viscosity of HEC hydrogel was determined with a RS 600 controlled stress rheometer (19) (Haake, Rhéo, France) at 25°C and 37°C. The geometry used was the stainless steel cone/plate (35/0.5°, truncation 29 μm) which provided homogenous shear of the samples and high values of the shear rate.

**Evaluation of hydrogel cytotoxicity**

Evaluation of hydrogel cytotoxicity was first conducted on HeLa cells (from UMR-S 756 Inserm and University Paris-Sud, Faculty of Pharmacy, Châtenay-Malabry, France). The cells were grown in Dulbecco's modified Eagle's minimum essential medium (DMEM) with L-glutamine supplemented with 10% heat-inactivated fetal calf serum and 1% Amino-Acid Non-Essential at 37°C in an atmosphere containing 5% CO$_2$. Cell lines were harvested from the flask using trypsin (0.5 mg/mL) with ethylenediamine tetraacetic acid (EDTA) (0.2 mg/mL), washed once with culture medium and seeded into culture plates at the appropriate cell densities (150,000 cell/well) before being incubated for 24 h at 37°C in an atmosphere containing 5% CO$_2$. For maintenance, cells were passaged weekly using 0.02% trypsin in Ca$^{2+}$-Mg$^{2+}$-free phosphate buffer saline (PBS, 0.01 M, pH 7.4 at 25°C) containing 3 mM EDTA. The culture medium was changed every two days. Confluent HeLa cells were
washed twice with PBS, and then put in contact with hydrogels. The plates were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the contact period, cells were washed twice with sterile PBS before analysis. Quantification of viable cells was conducted with Trypan blue numeration in Malassez cell.

Hydrogel cytotoxicity was also conducted using the fully-differentiated enterocyte-like Caco-2/TC7 clone cells (20, 21) and on the mucin-secreting HT-29/MTX cell subpopulation (21, 22).

Caco-2/TC7 cells (from UMR-S 756 Inserm and University Paris-Sud, Faculty of Pharmacy, Châtenay-Malabry, France) were grown in DMEM with 25 mM glucose, supplemented with 15% heat-inactivated fetal calf serum and 1% Amino-Acid Non-Essential. HT-29/MTX cells (from UMR-S 756 Inserm and University Paris-Sud, Faculty of Pharmacy, Châtenay-Malabry, France) were grown in DMEM (25 mM glucose) supplemented with 10% heat-inactivated fetal bovine serum. For maintenance, cells (Caco-2/TC7 or HT-29/MTX) were passaged weekly using 0.02% trypsin in Ca²⁺-Mg²⁺-free PBS containing 3mM EDTA.

Experiments and cell maintenance for Caco-2/TC7 and HT-29/MTX were carried out at 37°C in an atmosphere containing 10% CO₂. The culture medium was changed daily. For Caco-2/TC7 cells, assays were performed with cells at passages between 15 and 32. Fully differentiated Caco-2/TC7 cells were obtained after 15 days in culture while HT-29/MTX cultures were used at late postconfluence after 21 days in culture. Evaluation of hydrogel cytotoxicity was achieved after 24 h of direct contact with the cells. Cells were prepared in 24-well culture plates at 75,000 cell/well for Caco-2/TC7 and at 125,000 cell/well for HT-29/MTX. Before contact with hydrogels, confluent cells were washed twice with PBS. The plates were incubated 24 h at 37°C in a humidified atmosphere containing 10% CO₂. At the end of the contact period, cells were washed two times with sterile PBS before analysis.
Whatever the cell line used, citrate buffer and simulated vaginal fluid (SVF) were used as controls. SVF was prepared by adding under stirring 900 mL of distilled water contained in a beaker, NaCl (3.51 g), KOH (1.4 g), Ca(OH)$_2$ (0.22 g), bovine serum albumin (0.018 g), lactic acid (2.00 g), acetic acid (1.00 g), glycerol (0.16 g), urea (0.40 g), and glucose (5.00 g). The stirring was maintained until complete dissolution. The pH of the mixture was then adjusted to 4.5 using HCl and the final volume was adjusted to 1 L. All reagents for SVF preparation were from Sigma-aldrich (Saint-Quentin Fallavier, France) and were of analytical grade.

**Videomicroscopy and multiple-particle tracking**

Particle transport rates were measured by analyzing the trajectories of fluorescent-labeled carboxylate-modified polystyrene particles (COOH-PS, 170 nm size, PS-Speck™ Microscope Point Source Kit yellow-green fluorescent beads from In vitrogen™, Molecular Probes® Eugene, Oregon, USA) in the hydrogels (F127/HPMC 20/1 wt%, F127/F68/HPMC 22.5/2.5/1 wt%, HEC 1.5 wt%) and in macaque CVM.

Macaque CVM was collected from 24 naïve female *cynomolgus macaques* (*Macaca fascicularis*). The results were compared to the particle trajectories in water. Microscopic observations were made using an inverted AxioObserver Z1-Colibri (Zeiss, Germany) videomicroscope equipped with a CCD HSm camera (9.9 µm pixel size) under optimal incubation conditions provided by a XL incubator. Time lapse images were acquired with a Plan-Apochromat 63X/1.4 NO oil-immersion objective lens, a 470 nm LED for excitation and a BandPass 505-550 nm filter to collect the emission of fluorescence. The data sets were processed using ImarisTrack 7.1.1 (Bitplane AG, Zurich) and excel software. Experiments were carried out in 8-well glass chambers (Labtek, Campbell, CA), where 10 µL of 1/10 diluted particle solutions were added to 250 µL of pluronic® hydrogels, HEC hydrogel,
macaque CVM or water and incubated for 2 h at 37°C before microscopic observation.

Trajectories of n=10 particles were analysed. Movies were captured with Axiovision 4.8 (Zeiss, Germany) software at a temporal resolution of 210 ms for 30 s. The coordinates of particle centroid were transformed into time-averaged MSD (Eq. 6 and Eq. 7) from which distributions of MDSs were calculated.

$$\Delta x_1 (\Delta t) = x_2 - x_1$$  \hspace{1cm} \text{Eq. 6}

where $$x_1 = x (t_1)$$, $$x_2 = x (t_1 + \tau)$$ and $$\tau$$ is a fixed time lag

$$\langle \Delta r_1 (\tau) \rangle^2 = \langle \Delta x_1 (\tau) \rangle^2 + \langle \Delta y_1 (\tau) \rangle^2$$  \hspace{1cm} \text{Eq. 7}

**Evaluation of anti-HIV-1 activity of the formulations**

HIV-1<sub>ada</sub> strain (NIBSC, London, UK) stocks were prepared (23) in the lymphoblastoid T<sub>CD4</sub>+ C8166 cell line (a kind gift from Dr. Stefano Butò, ISS, Rome, Italy). All viral stocks were titrated using an HIV-1 gag p24 antigen ELISA kit (Biomerieux, Marcy L’Etoile, France) at 1000 ng/mL of the HIV-1 gag p24 protein. The peripheral blood mononuclear cells (PBMCs) were separated from healthy donor peripheral blood using a Ficoll gradient (Ficoll-Histopaque, Pharmacia, Uppsala, Sweden) and seeded in RPMI1640 (GIBCO, Gaithersburg, MD, USA) plus 10% FCS (GIBCO) and 2 mM L-glutamine (GIBCO) medium at 5x10<sup>5</sup> cells/mL. PBMCs were activated by PHA (5 µg/mL; Sigma, St Louis, MO, USA) plus IL-2 (10 U/mL; Pierce, Rockford, IL, USA) treatment for 72 h. The medium was replaced every three days with fresh one.

Scalar concentrations of M48U1/HEC, or M48U1/F127/HPMC were pre-incubated with HIV-1<sub>ada</sub> strain (5 ng/mL of HIV-1 gag p24) in RPMI1640 for 1 h at 37°C or 4°C respectively, and then added to PHA+IL2-activated PBMCs that were adjusted to a final
density of $1 \times 10^6$ cells/mL for 2 h at 37°C. Final M48U1 concentrations were 0.1, 1, 3 and 10 µg/mL. After four washes in PBS, cells were seeded at $1 \times 10^6$ PBMCs/mL into fresh medium represented by RPMI1640 plus 10% FCS and 2 mM L-glutamine with scalar dilutions either of M48U1/HEC, or M48U1/F127/HPMC. As control, the same protocol of pre-incubation was applied on untreated cultures or samples treated by relative hydrogel dilutions without M48U1. To analyse HIV replication, HIV-1 gag p24 content was evaluated at day 7 post-infection in culture supernatants using HIV-1 p24 antigen ELISA kit (Biomerieux, Marcy l’Etoile, France). The PBMC viability was evaluated by the Trypan Blue exclusion at day 7 using the same scalar concentrations of M48U1/HEC, or M48U1/F127/HPMC indicated above. Untreated cell cultures were used as control.

Results and discussion

Our strategy for the prevention of vaginal HIV transmission consists on the design of a barrier composed of pluronic® hydrogels able to better immobilize viral particles containing the peptide mini CD4 M48U1. This barrier could hinder HIV diffusion through mucus and its subsequent interaction with epithelial cells. Formulations composed of F127/HPMC (20 wt%) and F127/F68/HPMC (22.5/2.5/1 wt%) exhibited thermosensitive properties, evaluated by measuring the variation of elastic and viscous moduli when the temperature is progressively increased (Fig.1). Both formulations are fluid at room temperature facilitating their application and spreading within the vaginal cavity, while they form a highly viscous hydrogel above sol-gel transition temperature promoting prolonged contact-time with the vaginal mucosa at body temperature. We have previously demonstrated that addition of HPMC improved mucoadhesion of pluronic® hydrogels (1). HPMC addition did not have an impact on $T_{gel}$ variation (Table 1).
The pH of pluronic® hydrogels was 4.5 within the range of the normal, premenopausal vaginal pH. During coitus, vaginal secretions are temporarily neutralized by the alkaline pH of semen. Results on Table 2 showed that T_gel was not significantly modified when the pH increased from 4.5 to 7.

In the present work, the ability of pluronic® hydrogels to slow-down virus mobility was estimated by studying the trajectories of fluorescent-labeled carboxylate-modified polystyrene particles (COOH-PS, 170 nm size) using high-resolution multiple particle tracking in comparison with their movements in HEC hydrogel, CVM and water, used as negative controls.

**Particle mobility in water**

The 2D trajectories of COOH-PS particles in water exhibited a typical profile of the Brownian motion (Fig. 2 panel 1). The transport rates of different particle formulations in water were quantified by their time scale-dependent geometric ensemble-average mean square displacement (MSD) (Fig. 3). The slope $\alpha$ of each MSD curve was determined by fitting MSD to the equation $\text{MSD} = 4 D_0 \tau^\alpha$, where $D_0$ is the time scale-independent diffusion coefficient and $\tau$ is the time scale. $\alpha$ ranges from 0 for completely immobile particles, to 1 for unobstructed Brownian diffusion, such as that of particles in water. Thus, a decrease of $\alpha$ value indicates increasing obstacle to particle movement. The slope of the MSD curves presented in Table 3 showed that $\alpha$ equals 1 as expected for pure unobstructed Brownian diffusion of particles in water.

**Particle mobility in CVM**
The analysis of COOH-PS trajectories in CVM showed that 54% of particles were strongly slowed-down in mucus in comparison with water (Fig. 2, panel 2), while 46% of particles were not immobilized by mucus and exhibited Brownian or near-Brownian trajectories (Fig. 2, panel 3).

Mucus is mainly composed of water (95%) but also contains salts, lipids, enzymes and proteins. The most important constituent of mucus is mucin. Mucin content usually ranges between 2 and 5% by weight. The immobilization of 54% of COOH-PS particles in mucus could be attributed to adhesive and low-affinity bonds of these particles with the hydrophobic domains of mucin.

The mobility of 46% of particles could be due to the heterogeneous structure of mucus and their diffusion through the large pores of mucus. Mucin pore size is heterogeneous varying from 20 to 3000 nm depending on the technique used. It was evaluated at (650 ± 150) nm using freeze-substitution fixation technique and transmission electron microscopy (24). Scanning and transmission electron microscopy after dimethylsulfoxide-mediated glutaraldehyde fixation evaluated the mesh spacing between mucin fibers at 20-200 nm (25, 26). More recently, mucus pore size was estimated to 340 nm by multiple particle tracking (27).

**Particle trajectories in hydrogels**

COOH-PS particle displacement was 16-fold lower in HEC than in water (Fig. 4, panel 2 and Table 3). Dynamic viscosity of HEC 1.5 wt% hydrogel determined at a shear rate of 0.1 s$^{-1}$ was 3200 mPa.s at 25°C and 1900 mPa.s at 37°C, which is much higher than the dynamic viscosity of water (1 mPa.s at 25°C and 0.6947 mPa.s at 37°C) and could partly explain this reduced mobility.
Particles were strongly immobilized in both pluronic® hydrogels; F127/HPMC (20/1 wt%) and F127/F68/HPMC (22.5/2.5/1 wt%). This was evidenced by the highly constrained, non-Brownian time-lapse traces of COOH-PS particles observed in Fig. 4 (panels 3 and 4). The results at a time scale of 1 s, showed that MSD and the average diffusivity of particles were 1320 and 590-fold slower in F127/HPMC and F127/F68/HPMC hydrogels than in HEC (Table 3).

Higher dynamic viscosity of pluronic® hydrogels (Table 1) in comparison with HEC hydrogel partly explains these differences. The two types of hydrogels are also different from a structural point of view. HEC is an ether of cellulose consisting in uncharged linear chains. Concerning pluronic® formulations: at low temperatures, before T_gel, copolymers exist in the form of unimers in low-viscous aqueous solution. Upon heating, the dehydration of F127 unimers takes place and they begin to associate together to form micelles composed of hydrophobic core formed by PPO blocks surrounded by the hydrophilic PEO blocks. When the temperature is increased above T_gel, pluronic® micelles arrange themselves in a well-organized crystalline structure. Small Angle X-Ray diffraction experiments have showed that the diameter of each micelle was about 22 nm, and the distance between F127 20 wt% micelles was about 21 nm at a temperature higher than T_gel (28). This organization results from lower mobility of COOH-PS particles into pluronic®-based hydrogels in comparison with HEC hydrogel.

The two types of pluronic® hydrogels F127/HPMC and F127/F68/HPMC exhibited different behaviors regarding the mobility of COOH-PS particles. This mobility was 2-fold faster in hydrogel composed of F127/F68/HPMC than F127/HPMC. This result was unexpected because hydrogel composed of pluronic® mixture F127/F68/HPMC (22.5/2.5/1 wt%) was more viscous that hydrogel composed of F127/HPMC (20/1 wt%) at
These findings suggest that high viscosity of hydrogels is not the main parameter controlling particle mobility. In a previous work conducted in our research group, the effect of F68 on the molecular organization of F127 micelles has been studied (28). MicroDSC experiments unambiguously demonstrated that the two mixed pluronics F127 and F68 did not form mixed micelles but resulted on a segregation of the two kinds of pluronics (28). F68 is considered to be more hydrophilic than F127, its micellization temperature is higher. At 37°C, no micellization was detected at a concentration of F68 lower than 10 wt%.

F127 formulations are more stable and more homogenous, while the presence of F68 results in a disruption of the crystalline structure of F127 micelles. This disorganization due to F68, resulted in a faster mobility of COOH-PS particles observed in Fig. 3 and Fig. 4.

These results clearly suggest that the relative efficiencies of hydrogels to hinder particle mobility are intimately related to their molecular structure. Hydrogel composed of F127 and HPMC is more suitable to be used as a physical barrier against HIV diffusion than the hydrogel composed of F127/F68 and HPMC.

**Cytotoxicity**

The non-toxicity of the hydrogels was *in vitro* demonstrated using HeLa cells (Fig. 5). HeLa cells are non-oriented cells derived from the human cervical carcinoma. Experiments evaluating hydrogel cytotoxicity were also conducted using the fully-differentiated Caco-2/TC7 clone cells and the mucus-secreting HT-29/MTX cell subpopulation. Although Caco-2/TC7 are not derived from vaginal tissues, they were used here as a model of differentiated cells forming monolayers of polarized cells while HT-29/MTX cells were used as mucus-secreting cell model. HT-29/MTX produce mucus secreted by the specialized goblet cells that cover the apical cell surface (for review see reference 29).
Whatever the cell model used, cell viability was found more than 80% for all samples. These results were in accordance with data from the literature on the toxicity of pluronic® hydrogels. Pluronic® F127 has been approved by the FDA for use as food additives and pharmaceutical ingredients. Moreover, pluronic® F127 hydrogel showed non-cytotoxicity towards pig vaginal mucosa (30) indicating the biocompatibility of the pluronic® F127-based hydrogels reported here.

Anti-HIV-1 activity of hydrogels containing M48U1

The antiviral effect of scalar concentrations of M48U1/F127/HPMC was evaluated in comparison with M48U1/HEC (Table 4). The analysis of HIV-1 gag p24 content at day 7 post-infection in cell supernatants by ELISA technique demonstrated that M48U1 formulated into HEC or F127/HPMC hydrogels (3-10 μg/mL, 1-3.3 μM) effectively inhibits HIV infection (Table 4). Here, the effect of hydrogel formulation of M48U1 antiviral activity is investigated for the first time. The viability of activated PBMCs treated with M48U1/HEC or M48U1/F127/HPMC scalar concentrations with antiretroviral effects was not significantly affected when compared with the untreated cell cultures (Figure 6).

In conclusion, this investigation suggests that F127/HPMC (20/1 wt%) hydrogel could be suitable for its use as topical microbicide. Inclusion of anti-HIV molecule into F127/HPMC (20/1 wt%) hydrogel represents an original strategy combining the effect of two barriers; mechanical and pharmacological against HIV diffusion.

References


**Figure legend**

**FIG. 1.** Typical profile of the variations of the elastic (G’, ○) and viscous (G”, ●) moduli, as a function of temperature. Experiments performed with hydrogels composed of F127/HPMC (20/1 wt%) (A) and F127/F68/HPMC (22.5/2.5/1 wt%) (B).

**FIG. 2.** Panel 1 represents typical trajectories of fluorescent-labelled COOH-modified polystyrene particles in water. Panel (2) represents typical trajectory of fluorescent-labelled COOH-modified polystyrene particles that are stationary in CVM. Panel (3) represents the trajectory of the particles that are mobile in CVM. The trajectories were obtained at 37°C.

**FIG. 3.** Comparison of average mean square displacements (MSD) as a function of time scale of COOH-PS particles in (┃) water, (×) HEC (1.5 wt%) and (■) macaque CVM that are stationary in mucus, (★) F127/HPMC (20/1 wt%) and (♦) F127/F68/HPMC (22.5/2.5/1 wt%). The data were obtained at 37°C.

**FIG. 4.** (A) Examples of trajectories of fluorescent-labelled COOH-modified polystyrene particles in water (Panel 1), HEC (1.5 wt%) (Panel 2), F127/F68/HPMC (22.5/2.5/1 wt%) (Panel 3) and F127/HPMC (20/1 wt%) (Panel 4). A zoom of panels 2, 3 and 4 is presented on (B). The trajectories were obtained at 37°C.

**FIG. 5.** Percentages of cell viability of HeLa (■), Caco2/TC7 (■) and HT29-MTX (■) after 24 h of contact with F127/HPMC (20/1 wt%) and F127/F68/HPMC (22.5/2.5/1 wt%) hydrogels, in comparison with HEC and SVF. Control was composed of citrate buffer 5 mM, pH 4.5. Data are the mean of three determinations ± SD.
FIG. 6. Trypan blue analysis of activated PBMC viability treated by scalar concentrations of M48U1/HEC (■) and M48U1/F127/HPMC (■) at day 7. M48U1/HEC and M48U1/F127/HPMC at M48U1 concentrations with anti-retroviral activity are not toxic for PBMCs. Data were expressed as the means (±SD) of viable cells relative to untreated controls (set to 100%) obtained from three independent experiments in duplicate.
TABLE 1: Effect of HPMC proportion (x wt%) on $T_{gel}$ and $\eta$ of hydrogels composed of F127 (20) wt% or F127/F68 (22.5/2.5) wt%.

<table>
<thead>
<tr>
<th>HPMC x wt%</th>
<th>F127/HPMC (20/x wt%)</th>
<th>F127/F68/HPMC (22.5/2.5/x wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{gel}$ (°C)</td>
<td>$\eta$ (Pa.s) at 17°C</td>
</tr>
<tr>
<td>0</td>
<td>22 ± 1</td>
<td>0.057 ± 0.001</td>
</tr>
<tr>
<td>0.2</td>
<td>21 ± 1</td>
<td>0.078 ± 0.001</td>
</tr>
<tr>
<td>0.5</td>
<td>21 ± 1</td>
<td>0.094 ± 0.002</td>
</tr>
<tr>
<td>0.8</td>
<td>20 ± 1</td>
<td>0.129 ± 0.003</td>
</tr>
<tr>
<td>1</td>
<td>21 ± 1</td>
<td>0.200 ± 0.015</td>
</tr>
</tbody>
</table>

$N = 1$ Hz. $\eta$ was calculated from $G''$ according to the equation: $\eta = \frac{G''}{2\pi}$. Data are the mean of three determinations ± SD.
**TABLE 2:** Effect of pH on $T_{gel}$ of hydrogels composed of F127/HPMC (20/1 wt%) or F127/F68/HPMC (22.5/2.5/1 wt%).

<table>
<thead>
<tr>
<th>pH</th>
<th>F127/HPMC (20/1 wt%)</th>
<th>F127/F68/HPMC (22.5/2.5/1 wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>21 ± 1</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>22 ± 1</td>
<td>21 ± 1</td>
</tr>
</tbody>
</table>

$N = 1$ Hz. Data are the mean of three determinations ± SD.
TABLE 3: Values of MSD, $\alpha$ and $D_{eff}$ of COOH-PS particles in different media. The slope $\alpha$ of each MSD curve was determined by fitting MSD to the equation $MSD = 4D_0\tau^\alpha$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MSD $\mu m^2$ at 1 s</th>
<th>$\alpha$</th>
<th>$D_{eff} (\mu m^3/s)$ at 1 s</th>
<th>$D_{eff}/D_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.09</td>
<td>1</td>
<td>0.26</td>
<td>1</td>
</tr>
<tr>
<td>HEC (1.5 wt%)</td>
<td>0.07</td>
<td>56 $10^{-7}$</td>
<td>0.017</td>
<td>0.065</td>
</tr>
<tr>
<td>macaque CVM$^a$</td>
<td>1.66 $10^{-4}$</td>
<td>0.070 $10^{-3}$</td>
<td>3.95 $10^{-3}$</td>
<td>15.19 $10^{-3}$</td>
</tr>
<tr>
<td>F127/F68/HPMC (22.5/2.5/1 wt%)</td>
<td>1.18 $10^{-8}$</td>
<td>0.020 $10^{-3}$</td>
<td>2.80 $10^{-3}$</td>
<td>10.76 $10^{-3}$</td>
</tr>
<tr>
<td>F127/HPMC (20/1 wt%)</td>
<td>0.53 $10^{-4}$</td>
<td>0.009 $10^{-3}$</td>
<td>1.27 $10^{-3}$</td>
<td>4.88 $10^{-3}$</td>
</tr>
</tbody>
</table>

$^a$- Data reported for particles stationary in mucus.
TABLE 4: Antiviral activity of miniCD4 M48U1 against HIV-1_{ada} infection.

<table>
<thead>
<tr>
<th>M48U1 concentration µg/mL (µM)</th>
<th>M48U1/HEC</th>
<th>M48U1/F127/HPMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 10</td>
<td>100 ± 8</td>
</tr>
<tr>
<td>0.1 (0.033 µM)</td>
<td>85 ± 9</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>1 (0.33 µM)</td>
<td>37 ± 6</td>
<td>42 ± 6</td>
</tr>
<tr>
<td>3 (1 µM)</td>
<td>4 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>10 (3.3 µM)</td>
<td>2 ± 1</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>IC50 µg/mL (µM)</td>
<td>0.53 (0.17 µM)</td>
<td>0.58 (0.19 µM)</td>
</tr>
<tr>
<td>95% CI µg/mL</td>
<td>0.2010-1.441</td>
<td>0.1765-1.923</td>
</tr>
</tbody>
</table>

HIV-1_{ada} were pre-incubated for 1 h at 37°C or 4°C with scalar concentrations of M48U1/HEC or M48U1/F127/HPMC, respectively, in RPMI1640, and challenged with activated PBMCs for 2 h at 37°C. HIV replication was monitored by HIV-1 gag p24 ELISA and values were determined from cell culture supernatants at day 7 post infection. Data were expressed as the means ± standard deviations (± SD) of HIV-1 gag p24 amount to untreated control (set to 100%). Three experiments in duplicate were performed.