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Hydrogel containing mPEG-PLGA nanoparticles for the vaginal delivery of saquinavir mesylate against HIV infection

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ABSTRACT

Saquinavir mesylate (SQV) is a protease inhibitor commonly employed for the treatment of human immunodeficiency virus-1 infection. It is generally administered orally as tablets in combination with other antiviral drugs. Another promising route of administration can be represented by the vaginal one through topically applied formulations. This delivery can reduce the first-pass effect in the case of systemic drug adsorption or prevent HIV infection. We propose the formulation of a Carbopol® 974 (C974) hydrogel containing biodegradable mPEG-PL(L)GA nanoparticles (NPs) for the vaginal delivery of SQV, intended both as a prevention and a therapeutic strategy. mPEG-PL(L)GA NPs were incorporated into the C974 polymeric matrix, leading to a reduction of the hydrogel consistency dependent on NPs and C974 concentrations. Despite the moderate drug loading into NPs, the presence of the NPs had an impact on the *in vitro* release of the drug from the hydrogel at pH 5.5 using immersion cells. A higher amount of the drug was released, probably due to the effect of NPs in promoting the incorporation of the drug into the hydrogel at a high SQV dose. These findings can be useful for the development of topically applied hydrogels for SQV delivery, possibly having improved *in vivo* therapeutic outcomes.

1. Introduction

Acquired immunodeficiency syndrome (AIDS) is an infectious disease spread globally among men and women. It is due to the infection of the human immunodeficiency virus-1 (HIV-1) that acts by deteriorating the host immune system. During the last decades, it became an increasing global health, social, and economic problem with more than 38 million patients worldwide (das Neves et al., 2010; UNAIDS, 2023). The incidence of the disease results higher (~54 %) in the female population and in Sub-Saharan African countries. Thanks to the development of antiretroviral therapy, nowadays it is possible to control the disease even if not all the patients have still access to these drugs (Thienemann et al., 2013). Among the active molecules used for the treatment of HIV infection, saquinavir (SQV) is a protease inhibitor that acts by blocking the cleavage of the gag-pol protein substrate in both the virus type HIV-1 and HIV-2 (Vella and Floridia, 1998). SQV is a selective, peptidomimetic HIV protease inhibitor of the hydroxyethylamine class. It has generally been administered orally despite having a limited and variable systemic availability (0.7–4.0 %, depending on the dosage form) through this route, due to its poor aqueous solubility, extensive first-pass metabolism, and even low gastrointestinal tract permeability and absorption. Due to these drawbacks, SQV is therapeutically co-administered with ritonavir as tablet for optimal effect. The presence of adverse effects (nausea, vomiting, diarrhea, abdominal discomfort, prolonged QT and PR electrocardiographic intervals, and serious arrhythmias) has suggested the need for alternative dosage forms and routes of administration to increase the potential of the drug. One approach to avoid the first-pass effect is the formulation of locally-applied drug delivery systems. For instance, a self-nanoemulsifying drug delivery system (SNEDDS)-SQV loaded into polymeric transdermal films was developed and investigated in terms of *ex vivo* permeation and *in vivo* drug bioavailability enhancement (Hosny, 2019).

Different studies have been also reported in the literature regarding the possibility of using the vaginal administration route as an alternative and very promising way for the administration of different types of

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drugs, including antiretroviral ones. It is well known that small molecular antiretroviral drugs are taken up into the tissue where HIV transmission takes place. A gel containing tenofovir (1 %) has been already tested in African women, demonstrating that vaginally applied anti-HIV microbicides can be effective in reducing the transmission of the virus (Karim et al., 2010). Therefore, these drugs have been proposed as microbicides in topical dosage forms (e.g., gels, films, and rings) to prevent HIV infection during sexual intercourse. In the wake of these scientific evidences, Grammen et al. studied an *in vitro* strategy to test the tissue permeation profiles of four microbicide molecules for vaginal administration (tenofovir, darunavir, SQV mesylate, and dapivirine), highlighting the relevance of drug solubility and permeability in microbicide efficacy by testing the influence of formulation excipients on the permeation potential (Grammen et al., 2012). Since these molecules are poorly soluble in an aqueous environment, the addition of solubilizing excipients (e.g., cyclodextrins) in the hydrogel formulation has been employed to increase drug vaginal concentrations, enabling a fast tissue permeation of microbicides. To this extent, polymeric nanoparticles (NPs) were developed for the delivery of microbicide agents for vaginal application (Cutler and Justman, 2008; Das Neves et al., 2013; Ferguson and Rohan, 2011; Mallipeddi and Rohan, 2010). Polymeric NPs opened new opportunities and therapeutic approaches in the field of formulations for topical application. Among the advantages of these formulations compared to conventional ones there are i) protection of labile active molecules, ii) the possibility to control the release, iii) modulation of mucoadhesion, iv) increase of bioavailability, v) active targeting on specific cell subpopulations (Das Neves et al., 2013). Thus, the efficacy of drugs with a narrow therapeutic window or low bioavailability can be significantly improved after nanoencapsulation (Shishir et al., 2018). Polymeric NPs can be formulated using natural or synthetic polymers with a high level of biocompatibility as poly-D, L-lactic acid (PLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PLGA), poly-e-caprolactone (PCL), and poly(methylmethacrylate) (PMM) (Lembo and Cavalli, 2010). The topical microbicide agent PSC-RANTES has been successfully encapsulated in PLGA NPs to facilitate the therapeutic distribution by enhancing mucosal tissue taking-up and providing sustained controlled drug release for the prevention of HIV-1 infection (Ham et al., 2009). Chaowanachan et al. demonstrated the potential of NPs loaded by the combination of tenofovir with either efavirenz or SQV, achieving a synergized drug effect (Chaowanachan et al., 2013). NPs can be easily applied locally thanks to their incorporation into a hydrogel matrix, possibly obtaining a sustained release formulation. Moreover, the presence of NPs allows the incorporation into the hydrophilic gel matrix of poorly soluble drugs. This combined formulation can be particularly effective for vaginal drug administration, promoting contact and permanence with the vaginal tissue, increasing drug residence time, and consequently giving a high concentration of active molecules at the site of action, maximizing their pharmacological activity (dos Santos et al., 2020).

In this work, we employed a biodegradable mPEG-PL(L)GA copolymer for the formulation of NPs intended for the vaginal delivery of SQV mesylate. To prolong the residence time in the vaginal cavity, the NPs were incorporated into Carbopol® 974 hydrogels, a very well-known mucoadhesive polymer (De Souza Ferreira et al., 2017). This formulation can be intended as a prevention strategy, to be applied before sexual intercourse, and/or as a therapeutic treatment to promote drug absorption.

2. Materials and methods

2.1. Materials

Methoxy poly(ethylene glycol) (mPEG; 5 kDa) was purchased from polysciences Europe GmbH (Hirschberg an der bergstrasse, Germany). L-lactide, and glycolide were kindly donated by PURAC Biochem (Gorinchem, The Netherlands). Stannous 2-ethylhexanoate and SQV mesylate (≥98 %, HPLC) were purchased from Sigma-Aldrich (Milan, Italy). Carbopol® 974P NF (C974) was provided by Ashland (Wilmington, DE, USA).

2.2. Methods

2.2.1. mPEG5 kDa-P(L)LGA copolymer synthesis and characterization

mPEG5 kDa-P(L)LGA copolymer was synthesized by ring-opening polymerization (ROP) approach according to the procedure reported in a previous study (Man et al., 2015). Briefly, mPEG 5 kDa was melted and stirred into a Schlenk tube at 80 °C under nitrogen and then (L)-lactide and glycolide were added to the melted polymer by increasing the temperature to 150 °C. Then, stannous 2-ethylhexanoate was added to the mixture and the reaction was run for 4 h. The copolymer was precipitated by adding dichloromethane at room temperature to obtain a solution that was poured into cold diethyl ether. The synthesized copolymer was characterized by proton nuclear magnetic resonance (^{1}H NMR, Bruker Avance 200 MHz) and gel permeation chromatography (GPC, Agilent 1100 series) as reported (Man et al., 2015).

2.2.2. mPEG5 kDa-P(L)LGA NPs preparation

NPs were prepared according to the nanoprecipitation method using acetone as solvent for the polymer and water as anti-solvent (Man et al., 2015). Specifically, the required amount of the copolymer was weighed out and dissolved in 1 mL of acetone at different concentrations (20, 40, and 80 mg/mL copolymer in acetone). These solutions were added at a flow rate of 0.05 mL/min (Aladdin syringe pump, WPI Europe, Germany) to 4 mL of double distilled water at room temperature under magnetic stirring (800 rpm). Then, acetone was removed by evaporation under nitrogen flow to obtain the final NPs concentration of 5 (NPs 5), 10 (NPs 10), and 20 (NPs 20) mg/mL in water. The obtained NPs suspension was extruded ten times through a polycarbonate membrane (Nucleopore membranes, Whatman, NJ, USA) with a pore size of 0.4 μm (Avanti mini Extruder, Avanti polar lipid, AL, USA). To prepare loaded nanoparticles (SQV_NPs), 8 mg of SQV mesylate was dissolved into 0.5 mL of methanol and added to acetone copolymer solution, and then, processed as blank nanoparticles.

2.2.3. NPs characterization (particle size and drug encapsulation)

mPEG5 kDa-P(L)LGA NPs were characterized by dynamic light scattering (DLS). The hydrodynamic diameters (nm) and the polydispersity indexes (PDI) were measured by DLS using a Zetasizer Nano S instrument (Malvern instruments, UK). Briefly, 1 mL of the sample was placed in a disposable cuvette and analysed at 25 °C, after 180 s of equilibration time.

The amounts of SQV mesylate loaded into the NPs were evaluated with an indirect method by placing 500 μL of NPs suspensions in centrifugal filters (modified polyethersulfone membrane with a cut-off 10 kDa, VWR, USA) at 3000 rpm (850 *g*-force) for 20 min. Then, 20 μL of filtered solution were diluted ten times with methanol and analysed by HPLC.

For HPLC analysis (HPLC Agilent 1260 infinity II, Agilent USA), a mixture of methanol and 0.1 % v/v of formic acid in water (mixture ratio 90:10) was used as mobile phase with a flow rate of 1 mL/min in a C18-reverse phase chromatographic column (Agilent ZORBAX eclipse XDB-C18, 250 × 4.6 mm, 5 μm, Agilent, USA). The injection volume was 20 μL and the detection signal was recorded at 238 nm, keeping the analysis system at room temperature.

The encapsulated efficiency (EE%) was calculated by the following equation:

$$EE\ (\%) = \frac{total\ drug - unentrapped\ drug}{total\ drug} * 100$$

Meanwhile, the drug loading (DL%) was calculated by the following equation:

$$DL\ (\%) = \frac{encapsulated\ drug}{weight\ of\ the\ copolymer} * 100$$

2.2.4. Hydrogels preparation

The hydrogels at the concentrations of 0.5 %, 1 %, or 1.5 % w/w of Carbopol® 974 were prepared by dispersing the polymer in water or in the NPs dispersions (5 mg/mL, 10 mg/mL, or 20 mg/mL). Then, the pH of the formulation was adjusted at pH 5 by adding the appropriate amount of triethanolamine to reach the proper reticulation of the Carbopol® 974 and, thus the adequate viscosity. To prepare the hydrogel loaded with SQV mesylate without nanoparticles, 8 mg of the drug was dissolved into 0.5 mL of methanol and then added dropwise into 4 mL of distilled water to obtain the same drug concentration as in the NPs dispersion. The organic solvent was then removed under nitrogen flow. The required amount of Carbopol® 974 to achieve a final concentration of 1 %, or 1.5 % w/w was added to the aqueous dispersions of the drug and, finally, the pH was adjusted to 5 as above.

2.2.5. Rheological analysis

Rheological analyses were performed using a stress-controlled rotational rheometer (Kinexus lab+, Malvern, UK) using a C40/4 cone-plate geometry. Hydrogels were analysed by stress sweep and frequency sweep tests at 37 °C. Stress sweep analysis was performed in the range of 0.5–10 Pa and at a frequency of 1 Hz. For the frequency sweep tests, an increasing frequency in the range of 0.01–10 Hz was applied to the samples at a constant stress (5 Pa). The measured rheological parameters were the elastic modulus (G'), the viscous modulus (G"), and the complex modulus (G*). Analyses were performed in triplicate.

2.2.6. Clarity test

The transparency of the hydrogels containing nanoparticles was evaluated by transmittance analysis (UV-1800 spectrophotometer, Shimadzu, JP). The transmittance was measured at 660 nm and reported as a percentage ($T_{\%}$) with respect to that of the hydrogel without nanoparticles (100 $T_{\%}$).

2.2.7. Release studies

Drug release studies from the hydrogels were carried out according to the immersion cell method (Bisharat et al., 2017). A USP dissolution apparatus 2 (AT7smart, Sotax, CH) and an immersion cell Model A (Enhancer cell, Agilent, USA) were employed. 700 mL of 50 mM acetate buffer pH 4.5 was placed in each of the three vessels, and the medium was equilibrated to 37 ± 0.5 °C to mimic the physiological conditions of the vagina (Lin et al., 2021) and to maintain sink conditions. 0.8 gs of the prepared hydrogels were loaded in the reservoir of the immersion cell and a cellulose membrane of grade 1 (mean pore size 11 μm, Whatman™, UK) or a dialysis membrane with a cut-off 6–8 kDa (Spectra/Por® 1, Spectrum Laboratories, Inc., CA, USA) was placed on the top. The immersion cells made of Teflon have a surface area of 2 cm^2, which represents the hydrogel surface exposed to the release medium (United States Pharmacopeia, 2023). The paddle rotation speed was set at 50 rpm and the distance between the paddle and the immersion cell was adjusted to 4 cm. An aliquot of 1 mL of dissolution media was withdrawn at the following time points: 0, 5, 15, 30, 45, 60, 90, 120, 180, and 240 min. Drug release was monitored by HPLC as reported above. All the experiments were carried out in triplicate.

Release profiles were compared by calculating the model-independent parameters as dissolution efficiency (DE,%) and mean dissolution time (MDT, min) (Costa and Sousa Lobo, 2001). DE represents the area under the release profile up to a certain time expressed as percentage of the rectangle area, describing the 100 % of the release at the same time point. DE over 240 min was calculated using the following equation:

$$DE = \frac{\int_0^t y \cdot dt}{y_{100} \cdot t} \cdot 100$$

Where y is the drug released at the time point t and y_{100} is the maximum amount of the drug released.

MDT represents the mean value of the distribution time relative to the release and it can be calculated from the following equation:

$$MDT = \frac{ABC}{W_\infty}$$

where W_∞ is the asymptote relative to the dissolved drug and ABC is the area between the dissolution curve and W_∞.

Statistical comparison of DE and MDT values was performed through a one-way ANOVA test followed by a Tukey's multiple comparisons test (Prism version 5.0, GraphPad Inc., USA). P values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Synthesis of the mPEG-PLGA block copolymer

The molecular weight of the synthesized mPEG-PLGA block copolymer was determined by ^{1}H NMR and GPC. The number-average molecular weight (M_n) of the synthesized copolymers from ^{1}H NMR was calculated from the ratio of the integration of the signal relative to the methyl group of PLGA and that of the methine group of PEG block and resulted to be 6550 Da. The weight-average molecular weight (M_w) was determined by GPC from the peak area, resulting to be 9165 Da with a PDI of 1.36 (Man et al., 2015).

3.2. mPEG-PLGA NPs characterization

Biodegradable polymeric NPs were prepared using the synthesized mPEG-PL(L)GA according to the nanoprecipitation approach followed by extrusion. The extrusion process was utilized to decrease the particle size and the polydispersity. To optimize the formulation, three different polymeric NPs concentrations were used, namely 5, 10, and 20 mg/mL in water. After the manufacturing process, the NPs dispersions were characterized in terms of particle size distribution by DLS. The measured hydrodynamic diameters, expressed as Z-average (nm) and polydispersity index (PDI) are reported in Table 1. The measured Z-average values ranged from 108 to 160 nm for the unloaded NPs as a function of the copolymer concentration. The NPs size increased after drug encapsulation ranging from 124 to 214 nm and maintaining a polydispersity below 0.25 (Table 1). For loaded NPs, an encapsulation efficiency of 33 %, 41.9 %, and 42.3 % was obtained at the copolymer concentration of 5, 10, and 20 mg/mL. At the same time, the calculated drug loading

Table 1
Z-average (nm), polydispersity index (PDI) from DLS measurements and calculated encapsulation efficiency (%) and drug loading (%) for the prepared NPs (NPs 5, NPs 10, NPs 20) and SQV mesylate NPs (SQV_NPs) at the three copolymer concentrations of 5, 10 and 20 mg/mL. Data are presented as the mean ± standard deviation ($n = 3$).

NPs formulations	Z-average (nm)	PDI	Encapsulation efficiency (%)	Drug loading (%)
NPs 5	108.8 ± 0.8	0.108 ±0.001	–	–
NPs 10	129.2 ± 1.1	0.104 ±0.006	–	–
NPs 20	159.7 ± 1.9	0.110 ±0.018	–	–
SQV_NPs 5	124.2 ± 1.1	0.215 ±0.011	33.0 ± 2.9[a]	13.3 ± 1.2[a]
SQV_NPs 10	153.9 ± 1.2	0.123 ±0.009	41.9 ± 1.1[b]	8.4 ± 0.2[b]
SQV_NPs 20	214.0 ± 2.4	0.229 ±0.021	42.3 ± 0.6[b]	4.2 ± 0.1[c]

Different letters indicate statistically significant differences (ANOVA followed by a Tukey's multiple comparisons test, $P<0.05$).

referred to the weight of NPs, resulted to be 13.3 %, 8.4 %, and 4.2 %, respectively. Therefore, the encapsulation of SQV mesylate in NPs increased over copolymer concentration, despite the encapsulation efficiency of the drug into NPs 20 was only slightly higher than that obtained for NPs 10 and not statistically different.

3.3. Hydrogel rheological characterization

All the prepared C974 hydrogels were characterized from a rheological point of view to understand the possible effect on the gelation properties and consistency exerted by the incorporation of mPEG-PL(L)GA NPs at different concentrations inside the polymeric network. The incorporation of mPEG-PL(L)GA NPs at the analysed concentrations (5, 10, and 20 mg/mL) caused a decrease in the consistency of C974 hydrogels (0.5 %, 1 %, and 1.5 % w/w) as resulted from stress sweep and frequency sweep tests (Fig. 1). The degree of the observed consistency reduction was proportional to NPs concentrations and dependent on C974 concentration. Indeed, this effect was much more pronounced for the 0.5 % w/w C974 hydrogel than for the others prepared at a higher polymer concentration (1 % and 1.5 % w/w). The calculated complex modulus (G*) of 0.5 % C974 hydrogel at a stress of 1 Hz was ~ 500 Pa and it lowered to ~125 Pa in the presence of 20 mg/mL mPEG-PL(L)GA NPs (~75 % decrease). On the other side, the calculated complex modulus (G*) of 1.5 % w/w C974 hydrogel at a frequency of 1 Hz was ~ 810 Pa and it was reduced down to ~530 Pa in the presence of 20 mg/mL mPEG-PL(L)GA NPs (~35 % decrease). The incorporation of NPs also affected the linear viscoelastic region (LVR), which represents the range of applied stresses where the rheological moduli are constant, and therefore, the rheological properties are not dependent on stress. The end of this region is represented by the LVR yield stress value, above which rheological moduli become dependent on the applied stress, and generally decrease due to the internal structure breakdown of the hydrogel as a consequence of the application of larger deformations. The calculated LVR values decrease from ~30 Pa for 0.5 % C974 hydrogels to ~7 Pa for the 0.5 % C974 hydrogels containing NPs (20 mg/mL), while these values remain in the range of 25–30 Pa for all the hydrogel containing NPs at the C974 concentration of 1 % and 1.5 % w/w, confirming the less susceptibility of 1 % and 1.5 % w/w C974 rheological properties in relation to the incorporation of NPs. Differently to other Carbopol-based hydrogels incorporating NPs of a different nature, for which the consistency of the systems was not affected or slightly increased in the presence of nanosystems (Baek and Kim, 2011; Saez et al., 2019), it seems that the incorporation of mPEG-PLGA NPs had a destabilizing effect on the internal structure of the hydrogels as suggested by the lower G* values and shorter LVR. This effect exerted by NPs on the C974 hydrogel could be ascribed to the amphiphilic nature of mPEG-PLGA copolymer. Indeed, mPEG portion of the copolymer is expected to be exposed to water even inside the polymeric network, by altering the hydration state of the C974 polymer itself by competing for binding water molecules. Therefore, the incorporation of amphiphilic NPs affects the rheology of C974 hydrogels similar to that of anti-solvents such as alcohols (Berardi et al., 2022).

Frequency sweep tests (Fig. 2) have evidenced that all C974 hydrogels incorporating nanoparticles showed the typical behavior of cross-linked viscoelastic systems, in which the G' values are much larger than G'' values and rheological moduli (G' and G'') are only slightly dependent on the applied frequency. Despite the values of the G' and G'' moduli decreased as a function of NPs concentration, G' values remained much higher than G'' values in all samples, suggesting that the presence of nanoparticles does not break the internal tri-dimensional organization of the polymeric network. Overall, the effect of the incorporation of NPs up to a concentration of 20 mg/mL, had a marked effect on the rheological properties of 0.5 w/w C974 hydrogels, while a slight effect was only observed for 1 % and 1.5 % w/w C974 hydrogels. Therefore, 1 % and 1.5 % w/w C974 hydrogels were selected for further investigations.

3.4. Hydrogel clarity

The clarity of the prepared hydrogel in the presence of NPs was evaluated by measuring transmittance (Table 2). The Clarity (%) of the hydrogels decreased as a function of the amount of the incorporated NPs. Specifically, at NPs concentrations of 10 mg/mL and 20 mg/mL, 1 % C974 hydrogels were more transparent than 1.5 % C974 hydrogels. This result suggests that free water that is present in the hydrogels can be partially bound on the surface of mPEG-PLGA NPs. Indeed, water is more bounded to C974 polymer chains at the concentration of 1.5 % w/w with respect to the concentration of 1 % w/w, being less available to hydrate the NPs shell.

3.5. Optical microscope on hydrogels

C974 hydrogels (1 % and 1.5% w/w) without NPs appeared as transparent samples through optical microscope, indicating that the polymer is well solvated (Fig. 3A). The observation of the same hydrogels prepared in the presence of NPs (NPs 5, NPs 10 and NPs 20) demonstrated the absence of larger particles or particle aggregates in the micrometric size range, thereby confirming the incorporation of mPEG-PLGA as a nanoparticulate system inside the C974 hydrogels (Fig. 3B). Optical microscopy was also useful to understand the optical appearance of the hydrogels loaded with SQV mesylate (Fig. 3C and 3D). The hydrogel prepared in the presence of 20 mg/mL NPs loaded with SQV (Fig. 3C) did not show any precipitate formation or particle aggregation in the micrometric size (similar to the unloaded hydrogel prepared in the presence of NPs), suggesting that the drug was partially encapsulated inside NPs and partially finely disperse (nanoaggregated) into the polymeric matrix. On the contrary, as shown in Fig. 3D, the hydrogel loaded with SQV mesylate, at the same concentration but without NPs, contained insoluble drug particles, indicating that the presence of mPEG-PLGA NPs had a relevant effect in facilitating the incorporation of

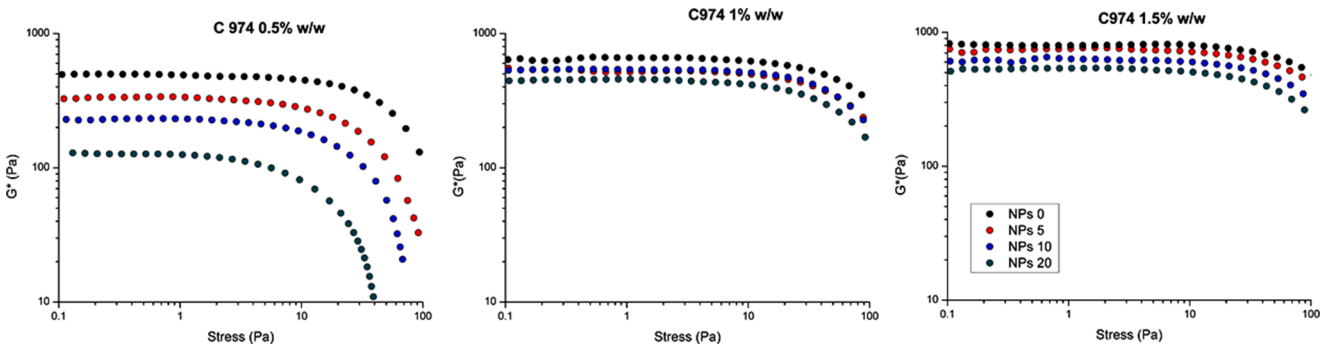

Fig. 1. Stress sweep test ($n = 3$) of the C974 hydrogels (0.5 %, 1 % and 1.5 % w/w) prepared in water (NPs 0) or in the mPEG-PLGA NPs dispersion at different concentrations (5, 10, and 20 mg/mL, namely NPs 5, NPs 10, and NPs 20).

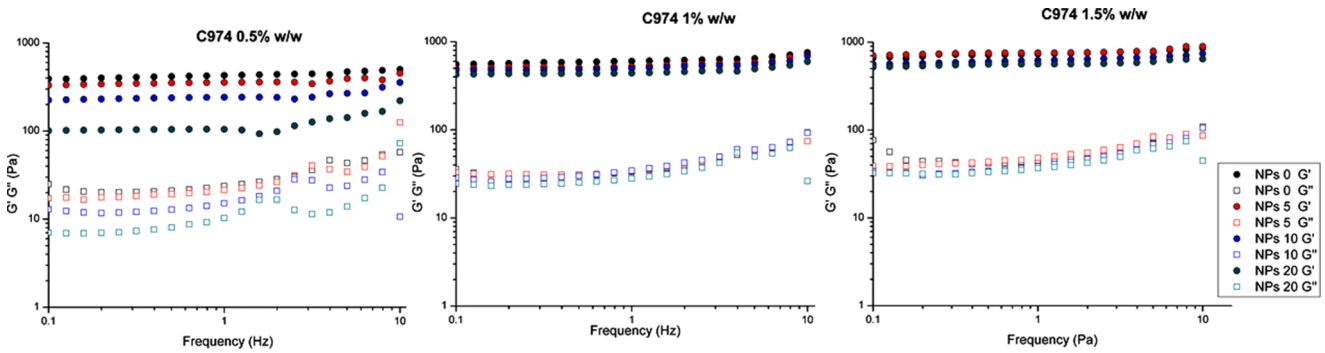

Fig. 2. Frequency sweep test ($n = 3$) of the C974 hydrogels (0.5 %, 1 % and 1.5 % w/w) prepared in water (NPs 0) or in the mPEG-PLGA NPs dispersions at different concentrations (5, 10, and 20 mg/mL, namely NPs 5, NPs 10, and NPs 20).

Table 2
Clarity of the C974 hydrogels (1 % and 1.5 % w/w) prepared in mPEG-PLGA NPs dispersions (5 mg/mL, 10 mg/mL, and 20 mg/mL) with respect to the hydrogel prepared in water as determined by transmittance measurements. Data are presented as the mean ± standard deviation ($n = 3$).

Hydrogel formulations	Clarity (%)
C974 1 %	100
C974 1 % + NPs 5	89.35±1.21
C974 1 % + NPs 10	77.11±2.37
C974 1 % + NPs 20	57.30±2.27
C974 1.5 %	100
C974 1.5 % + NPs 5	90.98±0.70
C974 1.5 % + NPs 10	59.97±0.59
C974 1.5 % + NPs 20	42.84±0.35

the drug inside the C974 hydrophilic matrix.

3.6. SQV release study from hydrogels incorporating mPEG-PLGA NPs

SQV release from mPEG-PLGA NPs incorporating into C974 hydrogel, in comparison to the corresponding hydrogels prepared without NPs, was investigated using the common method for semisolid systems named "immersion cell" (Jug et al., 2018). In most cases, different types of membranes are placed on the top of the cell to avoid coming out the gel from the immersion cell due to paddle rotation. The presence and the porosity of the membrane, placed on the top of the cell and in contact with the hydrogel surface exposed to the receptor medium, have been reported to affect drug release (Bisharat et al., 2017; Lucero et al., 2013). In this study, two membranes (cellulose and dialysis membranes) have been selected on the basis of the porosity to carry out the release experiments. Indeed, the selected cellulose membrane has a mean pore size (from the manufacturer) of ~11 μm, which is a size ~1000 times larger than the Z-average of the prepared NPs. Therefore, using this release apparatus (immersion cell covered by cellulose membrane), the NPs

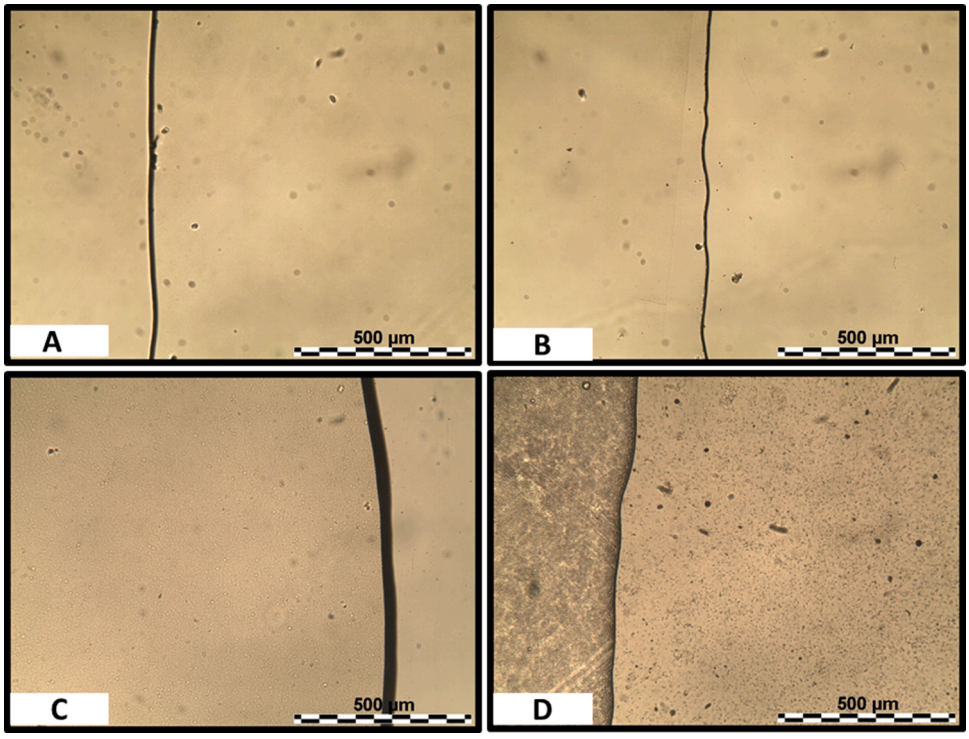

Fig. 3. Optical microscope images (10x of magnification) for the 1 % w/w C974 hydrogel without NPs (A), 1 % w/w C974 hydrogel in the presence of 20 mg/mL NPs (B), 1 % w/w C974 hydrogel in the presence of 20 mg/mL NPs loaded with SQV (C) and 1 % w/w C974 hydrogel in the presence of SQV without NPs (D).

incorporated into the hydrogel are easily released, together with the non-encapsulated drug and, consequently the release should be mainly dependent on the consistency of the hydrogel. On the other side, using a dialysis membrane, NPs should be retained inside the immersion cell due to the pore size (approximately 5–10 nm) and only the free drug should permeate to the release medium. Fig. 4 shows the release profiles obtained for the hydrogels loaded inside an immersion diffusion cell covered with a cellulose membrane. Release profiles from the hydrogel incorporating NPs were similar and SQV mesylate release was mainly dependent on the concentration of C974 in the hydrogels (1% w/w or 1.5% w/w). Indeed, a plateau region in the release for all profiles was reached at around 60 min for 1 % C974 hydrogels (Fig. 4A) and around 100 min for 1.5 % C974 hydrogels (Fig. 4B).

SQV release from 1.5% w/w C974 hydrogels was also investigated using immersion cells covered with a dialysis membrane (Fig. 5A) to focus more on the effect of NPs on SQV mesylate release at the highest tested C974 concentration. A much slower SQV mesylate release was observed for all hydrogels. All profiles showed a linear dependency of SQV mesylate release over time, without reaching any plateau. Specifically, the total amount of the drug released was below 25 % after 240 min. The lower amount of the SQV mesylate release can be attributed to the effect of the membrane that allows the permeation of only the free molecular drug. As for the release conducted using cellulose membrane, no marked differences in terms of SQV mesylate release were observed among NPs-loaded hydrogels, but surprisingly the release from the hydrogel without NPs showed a SQV mesylate release even slower than those from NPs-loaded hydrogels. This result could be explained by considering, as shown in Fig. 3, that in the absence of NPs the drug forms inside gels nanoaggregates, which due to their estimated size larger than the dialysis membrane pore, can retain the drug inside the membrane and reduce the dissolution rate and the release into the medium. Therefore, despite NPs concentration could not have a crucial role in controlling SQV mesylate release, the presence of the NPs can exert a certain effect in promoting the dispersibility of the drug inside the gel and its release in the media. Indeed, after 240 min, SQV mesylate release from the hydrogel without NPs was below 10 %, while in the presence of NPs, the release reached 16–25 %. Some papers report release studies of SQV mesylate from topical formulations as buccal films (He et al., 2021, 2020), a gellified microemulsion (Hosny and Hassan, 2014), and a cubosomal thermogelling dispersion (Hosny, 2020), but only one study was focused on the release of SQV mesylate from a hydrogel incorporating polymeric NPs. In this study, PLGA NPs (size ~190 nm and SQV mesylate encapsulation efficiency ~48 %) were incorporated into 1% w/w hydroxyethyl hydrogel and the release study was conducted at pH 4.6 in microcentrifuge tubes, leading after 4 h to a ~3 % of cumulative release (Yang et al., 2013).

To further assess the effect exerted by NPs, the release study was also performed using NPs dispersion (not incorporated in the hydrogel) placed in a dialysis membrane bag and immersed in the same medium as for the hydrogel (Fig. 5B). As expected, SQV mesylate release from NPs was faster than that from the hydrogel containing NPs, being in the range of 35–47 % after 240 min, thereby underlining the effect of the presence of the C974 polymeric matrix, even in the case of the release conducted using the dialysis membrane. Moreover, as for the release from the hydrogels, this study has confirmed the limited effect exerted by the NPs on the release, being the release profiles from the three tested NPs concentrations comparable up to 60 min and showing a not marked decrease in the drug release only for the SQV_NPs 20 at longer times. The obtained SQV mesylate release from mPEG-PLGA NPs was similar to that reported for other polymeric NPs (Chaowanachan et al., 2013; Yang et al., 2013). Specifically, a cumulative drug release of around 35–40 % was achieved after 4 h in vaginal fluid simulant, using a dialysis membrane, for SQV mesylate loaded PLGA based-NPs prepared by nanoprecipitation and having a comparable size (~190 nm) and encapsulation efficiency (~48 %) (Chaowanachan et al., 2013). Similarly, a ~35 % release of SQV mesylate was observed within 3 h at pH 4.5 from PLGA NPs having a size of ~246 nm and encapsulation efficiency of ~74 % (Yang et al., 2013).

A thorough comparison of the release profiles can be performed by calculating DE and MDT values (Fig. 6). As regards the release studies performed using a cellulose membrane on the top of the immersion cell, the calculated DE for the profiles related to the SQV_NPs formulations incorporated in the hydrogel were lower than that for the NPs free gel, indicating that the presence of NPs can slightly control drug release when a cellulose membrane was employed. However, a statistically significant difference in DE values between NPs free hydrogel and the hydrogel in the presence of NPs was observed only for the hydrogel prepared at NPs 20 mg/mL at both tested C974 concentrations (1% w/w and 1.5% w/w). This suggests that a certain effect on SQV mesylate release at these experimental conditions was exerted only at NPs concentration of 20 mg/mL, which is the NPs formulation having the calculated highest encapsulation efficiency and the lowest drug content for SQV mesylate. On the other side, for the release conducted using the dialysis membrane, the DE values obtained from the release in the presence of NPs were higher than that from the release in the absence of NPs, indicating that the presence of NPs accelerates the release. A statistically significant difference was only observed between the release of SQV mesylate from the hydrogels without NPs and the hydrogels

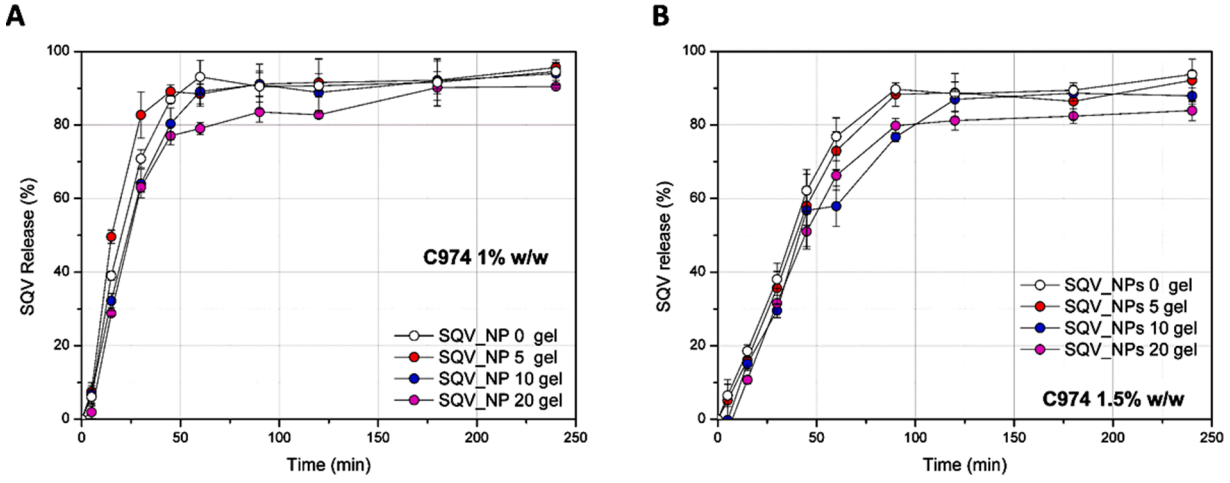

Fig. 4. Release study performed in 50 mM acetate buffer pH 4.5 on C974 hydrogels prepared at the concentration of 1 % w/w (A) and 1.5 % w/w (B) without NPs (SQV_NP 0 gel) and containing SQV-loaded mPEG-PLGA NPs (5, 10, and 20 mg/mL, namely SQV_NPs 5 gel, SQV_NPs 10 gel, and SQV_NPs 20 gel). The release was performed using immersion cells covered with cellulose membrane (mean pore size 11 μm). Data are presented as the mean ± standard deviation ($n = 3$).

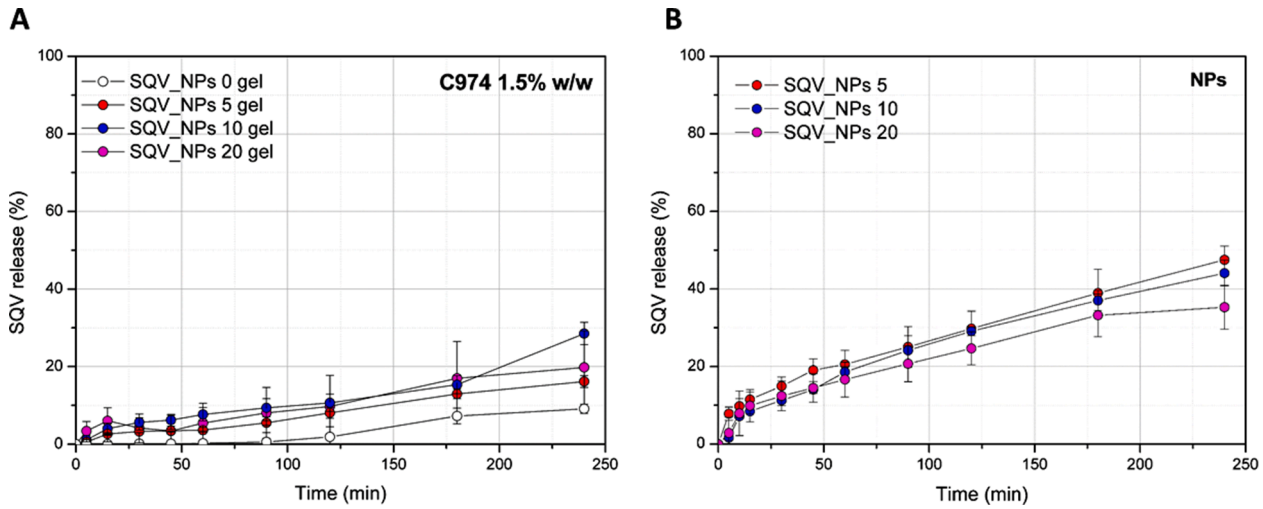

Fig. 5. Release study performed in 50 mM acetate buffer pH 4.5 on C974 hydrogels prepared at the concentration of 1.5 % w/w without NPs (SQV_NP 0 gel) or containing SQV-loaded mPEG-PLGA NPs (5, 10, and 20 mg/mL, namely SQV_NPs 5 gel, SQV_NPs 10 gel, and SQV_NPs 20 gel). The release was performed using immersion cells covered with dialysis membrane (cut-off 6–8 kDa) (A). Release study performed in 50 mM acetate buffer pH 4.5 on SQV-loaded mPEG-PLGA NPs (5, 10, and 20 mg/mL namely SQV_NPs 5, SQV_NPs 10, and SQV_NPs 20) in a dialysis bag (cut-off 6–8 kDa) (B). Data are presented as the mean ± standard deviation (n = 3).

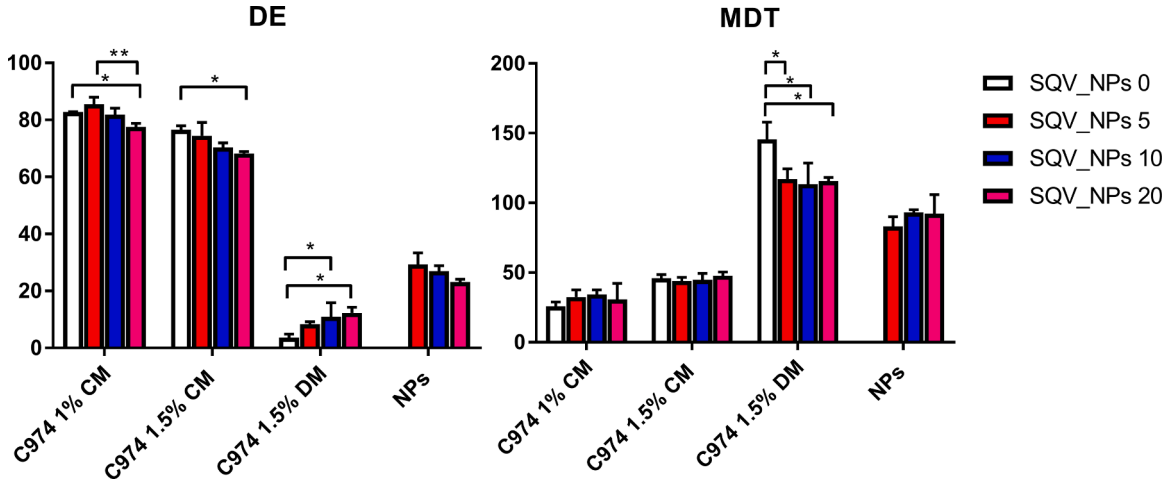

Fig. 6. Dissolution efficiency (DE, %) and mean dissolution time (MDT, min) calculated for the release studies performed at different conditions: 1 % w/w and 1.5 % w/w C974 containing SQV-loaded mPEG-PLGA NPs at different polymer concentrations (5, 10, and 20 mg/mL namely SQV_NPs 5, SQV_NPs 10, and SQV_NPs 20) using immersion cell covered with cellulose membrane (CM) or dialysis membrane (DM); SQV-loaded mPEG-PLGA NPs without C974 were also evaluated in a dialysis bag (NPs). Data are presented as the mean ± standard deviation (n = 3). **0.005 < P < 0.01; *0.01 < P < 0.05.

prepared at NPs concentrations of 10 mg/mL and 20 mg/mL, highlighting the effect exerted by NPs on promoting the dispersion of the drug.

Statistically significant differences in MDT values were observed only for the SQV release conducted using the dialysis membrane. In this case, the MDT value from the NPs-free hydrogel was statistically different from those calculated in the presence of NPs.

For any release study, including that conducted on SQV-NPs not incorporated in the hydrogels, no statistically relevant differences in the calculated DE and MDT values as a function of the mPEG-PLGA copolymer concentration were recorded, probably related to not marked differences in SQV mesylate encapsulation efficiency measured for NPs prepared at different copolymer concentrations.

4. Conclusions

The present work proposes the formulation of topical C974 hydrogels for the delivery of SQV mesylate, as a prevention or a therapeutic strategy for HIV infection, containing mPEG-PLGA NPs. Despite the moderate loading of the drug inside NPs, the presence of mPEG-PLGA NPs had a relevant effect in promoting the incorporation of the drug in the hydrophilic environment of the hydrogel, probably increasing its apparent solubility. As a consequence, the use of NPs in the formulation did not reduce markedly the release rate of the drug but led to a higher amount of the drug released from the hydrogel, when a dialysis membrane was placed on the top of the immersion cell used as release apparatus. From the dialysis membrane, the free drug at the molecular state can only diffuse, differently from the other tested cellulose membrane that allowed, in addition to the free drug, also the diffusion from the hydrogel of the drug-loaded NPs. Indeed, using the same immersion cells covered on the top with the cellulose membrane, the drug release from the hydrogel resulted to be not dependent on the presence of the NPs, but mainly dependent on the consistency of the polymeric matrix itself. The *in vitro* findings of this study can be helpful for the development of topical SQV based-hydrogels, having also an impact on the *in vivo* bioavailability of the drug. However, further studies are necessary

to assess the administrability and efficacy of the proposed formulation such as drug permeation assays, biocompatibility studies and antiviral activity tests.

CRediT authorship contribution statement

Mattia Tiboni: Writing – original draft, Methodology, Investigation. **Marco Cespi:** Data curation, Methodology, Formal analysis. **Luca Casettari:** Supervision, Conceptualization. **Giovanni Filippo Palmieri:** Supervision. **Diego Romano Perinelli:** Writing – original draft, Methodology, Investigation, Conceptualization, Data curation. **Giulia Bonacucina:** Supervision, Writing – review & editing, Resources.

Data availability

Data will be made available on request.

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