

Tenofovir alafenamide fumarate loaded long-acting microsphere for HIV pre-exposure prophylaxis

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ABSTRACT

The Human Immunodeficiency Virus (HIV) continues to be a major global health issue, with 38.4 million people infected at the end of 2021. The daily dosage regimen of Anti-retroviral (ARV) treatment leads to poor treatment adherence and patients' non-compliance. Tenofovir and its prodrugs, such as Tenofovir alafenamide (TAF) are generally used for the Pre-Exposure Prophylaxis (PrEP) of HIV. The current work focusses on the development of long-acting TAF loaded Poly-Lactic-co-Glycolic Acid (PLGA) Microspheres (MS) to improve the treatment adherence and patient compliance. MS were formulated using the industrially scalable in-line homogenizer approach using the solid-in-oil-in-water (S/O/W) emulsion solvent evaporation method. The surface morphology study carried out by SEM indicated the formation of dense, non-porous and spherical MS. The Differential Scanning Calorimetry (DSC) and X-ray Diffractometry (XRD) study revealed the formation of an amorphous form of TAF in the MS. The *in vitro* release study indicated the lower initial burst release of TAF and sustained release of TAF up to 15 days from the MS. The cytotoxicity and HIV inhibitory studies in the reporter TZM-bl cell line with different HIV-1 strains indicated the safety and effectiveness of TAF loaded PLGA MS. Thus, the long-acting MS would be a better alternative as a PrEP measure for HIV infection.

1. Introduction

Globally, HIV has claimed 40.1 million (33.6–48.6 million) lives so far, and every year an estimated 1.5 million new cases are diagnosed, indicating HIV to be a serious life threat for the human race [1]. The rate of infection among the males and the females remains tantamount, although the consequences of women getting infected appear to be more serious, due to the possibility of the infection being transmitted from mother to child. The anti-retroviral (ARV) treatment in such patients, therefore, remains a cornerstone approach to improve the life expectancy [2,3]. Recently many ARV drugs have been used in the PrEP of HIV by oral, transdermal, and vaginal routes, tenofovir (TFV) being one of those important drug candidates and is currently administered as an oral solid dosage form. Since the daily oral administration of TFV containing ARV formulation is known to show high efficacy but poor adherence, sustained-release long-acting dosage forms for pre-exposure

prophylaxis (LA-PrEP) offer an alternative solution [4,5]. Such long-acting formulations reduce the dosing frequency, maintain a therapeutic concentration for a prolonged period of time and improve adherence to the treatment regimen [4]. Currently, one product is in the market, and another is in clinical trials for LA-PrEP treatment in humans, one of them being cabotegravir, an integrase strand transfer inhibitor, and another being rilpivirine, a non-nucleoside reverse transcriptase inhibitor (NNRTI). At the end of 2021, FDA approved the first long-acting dosage form of cabotegravir for PrEP [6]. These formulations are formulated as a nanosuspension of cabotegravir and a suspension of rilpivirine nanocrystals, respectively, for parenteral administration [4,5].

Tenofovir Alafenamide (TAF) is a prodrug of TFV, either used alone or in combination with other ARV drugs for the treatment of HIV, Hepatitis-B Virus (HBV) and HIV PrEP. TAF is more potent and safer than its previously developed prodrug, Tenofovir Disoproxil Fumarate

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(TDF) with respect to bone and renal toxicity [2,7,8]. TAF holds an upper hand over TDF since TAF converts to TFV intracellularly while remaining stable in plasma, in contrast to TDF, which is less stable and gets immediately converted to TFV upon systemic exposure. In addition to this, TAF is proven to exhibit better antiviral activity and lesser side effects of bone toxicity and bone demineralization [5,8]. For *in vivo* antiviral activity, TFV gets converted into the TFV-diphosphate (TFV-DP) in the cells and therefore, TAF prodrug is known to be 1000 times more potent than the TFV and 10 times more potent than TDF. Along with more potency of TAF, the intracellular half-life of TFV-DP is 48 h in Peripheral Blood Mononuclear Cells (PBMCs) of healthy individuals, making TAF a logical choice for long-acting formulations [4].

The importance and the potential benefits of TAF for HIV PrEP are highlighted in a few recently published articles in the literature [2,4,5]. The available literature highlights the efforts being taken by researchers to deliver TAF by subcutaneous route [2]. A study by Gunawardana et al. in beagle dogs suggested that a dose of 0.14 mg/day would be sufficient to maintain the effective antiviral concentration in human PBMCs [4]. Another recently published study by Schlesinger et al. reports the formulation of an implant for a weekly or monthly delivery [5]. Although such a system serves the purpose of delivering the active for a long period of time, the implant poses some disadvantages like being invasive, danger of device failure, and possibility of adverse reactions [2,9].

Biodegradable polymer based long-acting Drug Delivery Systems (DDSs) are currently in focus for the encapsulation of small molecules, proteins, and peptides [10–12]. These injectable polymeric long-acting delivery systems improve patient compliance by reducing the dosing frequency and maintaining the effective concentration of drugs for a longer period of time (weeks or months) after a single injection [13–15]. The different grades of poly (lactic-co-glycolic acid) polymers (PLGA) are used in the marketed long-acting depot formulations since the polymer is biodegradable and biocompatible in nature and approved by the United State Food and Drug Administration (USFDA) [16–18]. The biodegradable and biocompatible nature of PLGA polymer is due to the hydrolysis products, such as lactic acid and glycolic acid, which are safe, and non-toxic to the body [15,17,18]. The desired release rate and duration of the drug delivery system can be modulated by changing the molecular weight or monomer composition (ratio of lactic acid and glycolic acid) of the polymer, which proves PLGA to be a very effective polymer for such applications [19,20]. Marketed PLGA based depot formulations include *in-situ* gel-forming implants, implants, and microparticles [18]. Among these formulations, microparticles are the most convenient formulation with respect to ease of administration and non-invasive method of administration [18,21,22]. Currently, researchers are working on the development of a continuous process of scale-up for the production of PLGA MS by in-line homogenizer approach, which is a technique used for the improvement of the batch-to-batch variability observed during the preparation of PLGA MS using conventional methods [23,24]. Sharifi et al., demonstrated the use of an in-line homogenizer for the fabrication of naltrexone loaded PLGA microspheres [23]. We previously have shown encapsulation of a glycoprotein, the human chorionic gonadotropin (hCG) hormone, in PLGA polymer to form the hCG loaded PLGA MS, by a modified double emulsion solvent evaporation method using in-line homogenizer [24].

To overcome the problems associated with TAF, for instance, poor treatment adherence and patient non-compliance due to daily dosing, the long-acting PLGA MS by subcutaneous (SC) or intramuscular (IM) route is a promising approach. The current work focused on the development of TAF loaded PLGA microspheres by a modified solid-in-oil-in-water (S/O/W) emulsion solvent evaporation technique using an industrially scalable in-line homogenizer. The effect of high-speed homogenizer on the particle size of TAF and the effect of release medium temperature on TAF release were also investigated. The developed TAF loaded PLGA microspheres were evaluated for its particle size, encapsulation efficiency, surface morphology, *in vitro* release, and residual

surfactant content. The cytotoxicity profile of the developed TAF loaded PLGA microspheres was assessed in the reporter TZM-bl cell line, and the anti-HIV activity was determined against different HIV-1 strains.

2. Materials and methods

2.1. Materials

TAF was obtained from Mylan Pharmaceuticals Pvt. Ltd. (India). The PLGA, Resomer® RG 503 H 50:50 (PLGA 50:50) with Mw 24000-32000 D and Resomer® RG 752 H 75:25 (PLGA 75:25) with Mw 4000-15000 D were purchased from Evonik India Pvt. Ltd. (Mumbai, India) and Sigma Aldrich (Germany) respectively. Pearlitol PF- Mannitol was purchased from Signet Excipients Pvt. Ltd. (India). Sodium azide reagent Plus® and Polyvinyl alcohol (Mowiol® 4-88, Mw ~ 31000) were purchased from Sigma Aldrich (Germany). Acetonitrile (HPLC grade) and Dichloromethane (DCM) were purchased from Merck (India). All the solvents and reagents used were of analytical grade.

2.2. TAF loaded PLGA microspheres preparation

TAF loaded PLGA microspheres were formulated by a S/O/W emulsion solvent evaporation technique. An accurately weighed amount of TAF (9 mg–30 mg) was added in 1.5 mL of polymeric solution containing 300 mg of PLGA in dichloromethane (DCM) and dispersed using a high-speed homogenizer (ULTRA TURRAX®, IKA® T 25 digital, Germany) to form a solid-in-oil (S/O) dispersion. The homogenizer was operated at a speed of 18000 rpm for 1 min. The S/O dispersion was further emulsified with a polyvinyl alcohol aqueous solution (1% w/v) containing mannitol (0–5% w/v) (W) (150 mL) by using an in-line homogenizer at 3000 rpm (KINEMATICA AG, MEGATRON® MT 3100 S, Switzerland) to form S/O/W emulsion. The evaporation of the organic solvent, collection of solidified microspheres and lyophilization of microspheres was carried out according to the established protocol [24]. The flow diagram for the preparation of TAF loaded PLGA microspheres is shown in Fig. 1. Various batches were designed to understand the effect of formulation parameters like the effect of osmogen addition in the external stabilizer solution (B-1 and B-2), polymer grade (B-2 and B-3), drug loading (B-2, B-4 and B-5) and batch size (B-6). For B-6 batch, the TAF, PLGA polymer, DCM, and stabilizer solution quantities were taken as 25 mg, 500 mg, 2.5 mL and 250 mL, respectively. The composition of different batches is given in Table 1.

2.3. Microscopy study

To study the effect of high-speed homogenizer on the particle size of TAF, an optical microscopy study was performed. The plain TAF, TAF in dichloromethane homogenized at 18000 rpm for 1 min by high-speed homogenizer and S/O dispersion prepared by operating high-speed homogenizer at 18000 rpm for 1 min were observed under the optical microscope (Mono mini, micron OPTIK, Canada) at 10X resolution. Also, the secondary emulsion was observed under the optical microscope at 100X resolution. The eye-piece and stage micrometer were calibrated for the determination of particle size.

2.4. Encapsulation efficiency determination

An accurately weighed amount of 5 mg of freeze-dried TAF loaded PLGA microspheres were added to an eppendorf tube, and MS were dissolved in 0.4 mL of acetonitrile by sonicating it for 1 min. After the dissolution of microspheres completely, 1.6 mL of mobile phase mixture in an equal ratio (1:1) was added for precipitation of PLGA polymer while TAF remained in the aqueous phase. For the separation of TAF from this dispersion, centrifugation (Remi Laboratory Instruments, India) was performed at 12,000 rpm for 5 min, and the supernatant containing TAF was analyzed by the HPLC method.

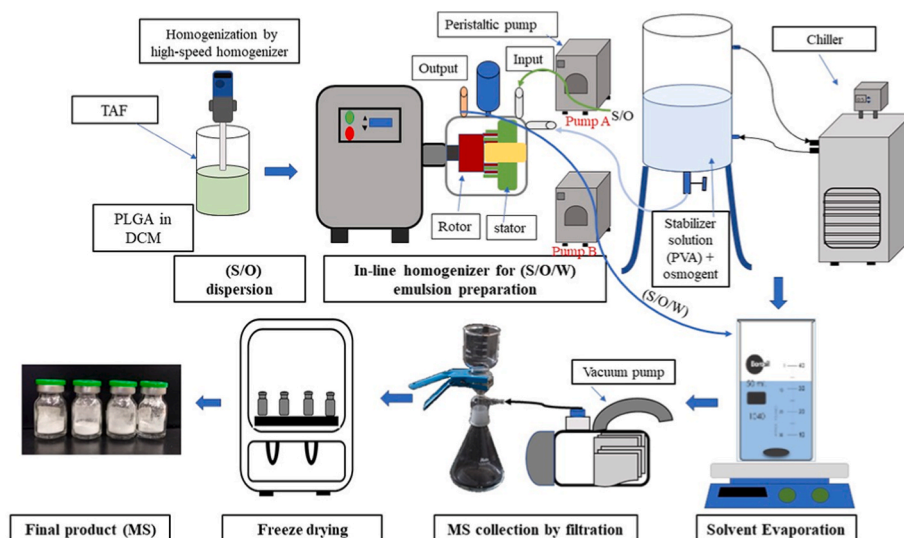


Fig. 1. Flow diagram of TAF loaded PLGA microspheres preparation.

Table 1

Composition of TAF loaded PLGA microspheres batches.

Batches	Polymer	TAF loading theoretical (% w/w)	TAF loading actual (% w/w $\pm$ SD)	Mannitol in external phase (% w/v)	Particle size Volume weighted mean, D _[4,3] (μ m)	Encapsulation Efficiency (%)
B-1	PLGA 75:25	4.76	2.30 $\pm$ 0.006	-	24.4 $\pm$ 0.42	48.36 $\pm$ 0.13
B-2	PLGA 75:25	4.76	2.58 $\pm$ 0.039	5	23.5 $\pm$ 0.37	54.29 $\pm$ 0.82
B-3	PLGA 50:50	4.76	0.397 $\pm$ 0.017	5	29.21 $\pm$ 0.85	8.35 $\pm$ 0.37
B-4	PLGA 75:25	2.91	1.32 $\pm$ 0.01	5	23.4 $\pm$ 0.18	45.32 $\pm$ 0.62
B-5	PLGA 75:25	9.09	3.67 $\pm$ 0.094	5	26.5 $\pm$ 0.14	40.44 $\pm$ 1.03
B-6	PLGA 75:25	4.76	1.86 $\pm$ 0.061	5	27.67 $\pm$ 0.26	39.06 $\pm$ 0.69

The Waters alliance HPLC system (Waters, USA) equipped with a UV-Visible detector, and Empower Pro software was employed for the analysis, and C18 column (Inertsil ODS-3, 150 mm $\times$ 4.6 mm, 5 μ m) was used. Mobile phase A comprised of ammonium acetate buffer having pH 6 (990 mL): acetonitrile (10 mL) and mobile phase B contained ammonium acetate buffer, pH 6 (500 mL): acetonitrile (500 mL) with gradient flow of 20% mobile phase A and 80% mobile phase B for 10 min. The HPLC parameters, such as flow rate of 1 mL/min, injection volume of 10 μ L and detection wavelength of 260 nm were set for analysis. To find out the unknown concentration of TAF, the standard calibration curve equation obtained using the concentration range of 1–60 μ g/mL with R^2 value of 0.999 was used. The measurement of encapsulation efficiency of microspheres batches was performed in triplicate.

The following equations (1)–(3) were used for the calculation of TAF loading and encapsulation efficiency of microspheres batches,

$$\text{Theoretical drug loading} = \frac{\text{drug (total)}}{\text{drug (total) + polymer}} \times 100 \quad \text{Equation (1)}$$

$$\text{Actual drug loading} = \frac{\text{drug (encapsulated)}}{\text{weight of microspheres}} \times 100 \quad \text{Equation (2)}$$

$$\text{Encapsulation efficiency} = \frac{\text{actual drug loading}}{\text{theoretical drug loading}} \times 100 \quad \text{Equation (3)}$$

2.5. Particle size determination

Particle size distribution of microspheres was determined by a Malvern Mastersizer instrument (Mastersizer equipped with Hydro 2000MU (A), Malvern Instruments, UK). The microspheres were dispersed in distilled water (refractive index 1.33) as a measuring medium. The particle size distribution was presented as d (0.1), d (0.5), and d (0.9), where d represents the volume size diameter, indicating that 10%, 50%, and 90% (volume distribution) of the microparticles were below the given sizes respectively [25]. Also, the span value of the microspheres particle size distribution was calculated for the determination of the distribution pattern. The obtained particle size of the microsphere batches was represented as the average volume-weighted mean D [3,4]. The particle size measurement was carried out in triplicate.

2.6. Surface morphology

Surface morphology of the developed microspheres was performed by Scanning Electron Microscopy (SEM) (Zeiss MERLIN Field Emission SEM). The MS sample was coated with a sputter-coating with gold by cathode spraying (POLARON, UK) and visualized at an accelerating voltage of 17 kV under the SEM.

2.7. In vitro release study

Sample and separate method was used to perform the release study of TAF loaded PLGA microspheres [26]. The formulation containing TAF

loaded PLGA microspheres was weighed accurately (7–10 mg) and was further incubated in a 2 mL microcentrifuge tube containing 1 mL of phosphate buffer saline (PBS) solution, pH 7.4. Sodium azide (0.02% w/v), which acts as an antimicrobial agent was added to PBS, to prevent any microbial growth during the release study. The microcentrifuge tubes were then placed in an orbital shaker (IKA KS 3000i control, Germany) with continuous shaking (100 rpm) at 37 °C. Additionally, to check the effect of temperature on the release of TAF from the microspheres (B-6 batch), *in vitro* release study was also performed at 25 °C [4]. The drug released at various time points was determined by collecting the aliquots from the microcentrifuge tubes after the centrifugation (Remi PR-24 Research compufuge, India) performed at 2000 rpm for 3 min, and the supernatant was collected and analyzed by HPLC method. Immediately an equal quantity of fresh PBS, pH 7.4 (1 mL) was added to the microcentrifuge tubes to replace the withdrawn supernatant. The experiment was performed in triplicate.

2.8. Thermal analysis

Differential Scanning Calorimetry (DSC) was performed by using a DSC analyzer (STA7300, Thermal Analysis System, HITACHI, Japan). An accurately weighed quantity of the plain drug, Physical Mixture (PM) and TAF loaded PLGA microspheres (B-2) were placed and sealed in ceramic pans. Samples were heated at a constant rate of 10 °C/min in the temperature range of 30–300 °C. Flushing of inert nitrogen gas was maintained at a rate of 10 mL/min for all the runs.

2.9. X-ray diffractometry

X-ray Diffractometry (XRD) study was performed by using a D2 PHASER X-ray diffraction system (Bruker AXS, Bruker, Germany) installed with a radiation source (Cu K α). The analysis was carried out in the range of 2 θ values between 5° and 60° with a step increment of 0.03° and a scanning speed of 0.02 s. The operating voltage and current were set as 30 KV and 10 mA, respectively. XRD study was performed for plain drug, physical mixture, and TAF loaded PLGA microspheres (B-2).

2.10. Residual surfactant analysis

For the determination of residual surfactant in the MS, a previously reported spectrophotometric method was used [27]. A 100 mg of TAF loaded PLGA microspheres (B-2) were weighed into a 1.5 mL microcentrifuge tube and 1 mL of distilled water was added to it. The formed dispersion of microspheres was sonicated for a period of 15 min. The dispersion was centrifuged for 5 min at 12000 rpm, and 50 μ L of the supernatant was added into the well of a 96-well microtiter plate. Subsequently, 75 μ L of boric acid solution and 15 μ L of iodine solution were added to the well, and the mixture was made up to a final volume of 250 μ L with distilled water. After the incubation of the microtiter plate at room temperature, the absorbance of the samples was quantified by a microtiter plate reader (Biotek Synergy™ H1, United States) at 690 nm. The PVA concentration was calculated by a standard linearity equation.

2.11. Cell line studies

2.11.1. Cell lines

The AIDS Research and Reference Reagent Program (ARRRP), a division of the National Institutes of Health (NIH), USA, provided the TZM-bl and HEK 293T cell lines. The HeLa-derived TZM-bl cell line consistently expresses the CCR5, CD4, and CXCR4 receptors. By activating luciferase or β -galactosidase reporters in response to HIV expression, the cells make simple and quantitative HIV screening possible. This cell line was utilized to test the formulation's anti-HIV effectiveness *in vitro*. The pseudotyped virus was propagated in this investigation using HEK 293T cells. The culture conditions for both cell lines were the same. Their propagation was carried out in Dulbecco's

Modified Eagle Medium (DMEM), supplemented with FBS (10% v/v), Penicillin (100 U/mL), L-glutamine (2 mM), Streptomycin (100 g/mL), and HEPES buffer (25 mM) solution. The cell lines were preserved at 37 °C with 5% v/v CO₂.

2.11.2. Virus stocks propagation

- HIV-1 Primary isolates

Both HIV strains employed in the study, HIV1_{VB28} (CCR5 tropic, Subtype C, ICMR-NARI, India) and HIV1_{UG070} (CXCR4 tropic, Subtype D, NIH ARRRP, USA), were propagated by earlier descriptions in the literature [28,29]. The virus stocks were cultured in pHa-human peripheral blood mononuclear cells (PBMCs), and the HIV-1 p24 antigen detection kit, Advanced Bioscience Laboratories (ABL), Inc., USA, was used to measure viral growth.

- The pseudotyped virus

The full-length HIV-1 NL4-3 molecular clone (pNL4-3) was obtained from the NIH ARRRP, USA. The plasmid was amplified by employing TOP10 competent cells (Invitrogen, USA; *E. coli* DH5 α). In a nutshell, TOP10 competent cells were incubated overnight at 37 °C on an incubator shaker in Luria Bertani broth that had been boosted with ampicillin (100 g/mL). The plasmid was isolated using a Qiaquick™ spin miniprep kit (Qiagen, USA), and quantified using a Nanodrop UV spectrophotometer. A fresh medium was added to the medium prior to transfection. Subsequently, 1.5 g of plasmid DNA was transfected into HEK 293T cells (4×10^5 /well) in a 6 well plate (Techno Plastic Products, Switzerland), using X-tremeGene HP transfection reagent following the manufacturer's instructions (Roche, Switzerland). The cell-free supernatant from each virus stock was collected, centrifuged, filtered, and stored at –80 °C. After titrating each virus stock, the TCID₅₀ (50% Tissue-Culture Infective Dose) was calculated.

2.11.3. In vitro cell viability assay

TZM-bl cells (10^4 cells/well) were plated in 96-well microplates and incubated at 37 °C in a 5% v/v CO₂ atmosphere overnight. The following day, the medium was changed to a serum-free media containing each compound (TAF, MS, and/or TAF-released PLGA MS (g/mL)), and the plates were incubated for 48 h. Each well received 20 μ L of MTT (5 mg/mL), after which the plates were incubated for 4 h. The formazan crystals were then dissolved with the addition of 200 μ L DMSO. The ability of cells to reduce the indicator dye was determined using a plate reader set to 560/590 nm (excitation/emission). A non-linear dose-response regression analysis was used to get the CC₅₀ (concentration demonstrating 50% cytotoxicity).

2.11.4. Assessment of HIV-1 inhibition

The inhibitory activity of different concentrations (μ g/mL) of TAF-released PLGA MS against HIV-1 strains in TZM-bl cells was determined using luciferase quantification of cell lysates at different time points (0, 1/2, 1, 2, 4, 6, 12, 24, 30, 36, 48 h, and 3 days).

Before infection, the 96-well microplates were seeded with 10^4 cells per well of the D10 medium (DMEM containing 10% heat-inactivated FBS), and the plates were kept in a 5% v/v CO₂ chamber overnight at 37 °C. The following day, cells were treated to different concentrations (μ g/mL) of TAF-released PLGA MS (different time points) for 1 h in order to facilitate the internalization of the infectious agent. The pre-titrated virus stock (pNL4.3, HIV1_{VB28}, or HIV1_{UG070}) was introduced to each well after the treatment at a concentration of 400 TCID₅₀/mL. The positive control was TZM-bl cells infected with the virus in the absence of the drug, whereas the negative control was TZM-bl cells cultured in the absence of the virus and drug (100% inhibition). A humidified 5% v/v CO₂ chamber was used to incubate the plates for 48 h at 37 °C [30].

After incubation, cells were lysed with Britelite plus reagent

(PerkinElmer, USA), and luciferase expression was measured in Relative Luminescence Units (RLUs) using a Luminometer (Victor 3, PerkinElmer, USA). The HIV-1 inhibition dose-response curves for TAF-released MS with different HIV-1 strains were plotted.

2.12. Stability study

The stability study of TAF loaded PLGA microspheres (B-6) was carried out for three months as per the ICH guidelines Q1A (R2) [31]. TAF loaded PLGA microspheres were packed in 5 ml transparent glass (clear USP type-I glass) vials closed with a rubber closure and crimped with an aluminium seal. The microspheres samples were stored at $5 \pm 3^\circ\text{C}$ for long-term stability and $25 \pm 2^\circ\text{C}/60\% \text{ RH} \pm 5\% \text{ RH}$ for accelerated conditions. Different parameters such as appearance, encapsulation efficiency, particle size, and *in vitro* release were observed at initial (0), one- and three-month intervals.

2.13. Statistical analysis

Statistical analysis was carried out for all the experiments. The experimental results data were expressed as mean $\pm$ standard deviation (SD) ($n = 3$ or specifically indicated). Statistical analysis was carried out using Mann-Whitney *U* Test and one-way ANOVA (GraphPad Prism software 9.2.0) and significance was expressed in terms of *p*-value < 0.05 . Similarity factor (f_2 value) was used for dissolution profile comparison, and a value of $f_2 \geq 50$ indicates the similarity between two dissolution profiles.

3. Results and discussion

3.1. TAF loaded PLGA microspheres preparation

The solubility of TAF in the aqueous phase and organic phase is an important parameter for the selection of the microspheres preparation method. The single emulsion (O/W) solvent evaporation method is generally used for hydrophobic drugs which are in a solution state in the organic phase. Whereas the double emulsion ($W_1/O/W_2$) solvent evaporation method is a widely reported technique for the fabrication of microspheres of water soluble drugs, proteins, and peptides [32–34]. Although TAF is soluble in a few organic solvents, it is insoluble in dichloromethane, which is a preferable solvent for microspheres preparation. Also, the solubility of TAF in the water is not sufficient to be used in the double emulsion solvent evaporation method. Therefore, in the current research work, the S/O/W emulsion solvent evaporation method was explored. Considering the scale-up of microspheres drug delivery systems, an in-line homogenizer approach was used for the preparation of TAF loaded PLGA microspheres with respect to secondary emulsion preparation [23]. The optimization of process parameters with respect to desired emulsification was carried out by the addition of primary dispersion (S/O) to the external aqueous phase (W) in the in-line homogenizer. The two peristaltic pumps were used for the addition of these phases to avoid manual errors. The desired release profile was achieved by selecting synthetic biodegradable PLGA polymers with different monomer compositions of lactic acid and glycolic acid (50:50 and 75:25) (PLGA 50:50 and PLGA 75:25) [16]. The most widely used solvent for solubilization of PLGA polymer in microspheres preparation is dichloromethane. It is a volatile class II solvent, and forms the solid microspheres during solvent evaporation process. Owing to its favorable properties, dichloromethane was chosen for the preparation of TAF loaded PLGA microspheres. A polymeric surfactant, polyvinyl alcohol was selected as a stabilizer and it is easily removed from the microparticles surface after washing with water [16,35,36]. A very commonly observed challenge in the formulation of depot microspheres is the presence of adsorbed drug on the outer surface of microspheres, which results in burst release. Therefore, mannitol (osmogen) was added to the stabilizer solution to form dense, smooth surface

non-porous microspheres, which reduced high burst release [37,38].

3.2. Microscopy study

To check the effect of high-speed homogenizer on the particle size of TAF in the polymeric solution, a microscopic evaluation was performed. The microscopic image of the plain drug, TAF in dichloromethane after high-speed homogenization, TAF in polymeric solution after high-speed homogenization and secondary emulsion are shown in Fig. 2, A-D respectively. The plain drug average particle size was found to be $64.40 \pm 0.39 \mu\text{m}$ (Fig. 2A). After the high-speed homogenization of plain drug in dichloromethane, a reduction in particle size ($24.59 \pm 1.27 \mu\text{m}$) was observed (Fig. 2B). This phenomenon is obvious as the high speed of homogenization generates higher energy for the reduction of particle size of larger particles into smaller particles. A visual inspection of the polymeric solution reflected the clustering of the TAF particles (Fig. 2E), which highlighted that the solution was not homogenous and therefore required a means to homogenize it using energy. A uniform homogenous emulsion was obtained (Fig. 2F), and a further reduction in particle size ($7.59 \pm 0.38 \mu\text{m}$) was observed after subjecting the polymeric solution containing TAF and PLGA in DCM to high-speed homogenization using a high-speed homogenizer (s/o dispersion) (Fig. 2C). This might be attributed to the additional solubilizing property of PLGA, in addition to its role as a matrix forming polymer [39]. Further, it was found that emulsifying the s/o dispersion using in-line homogenizer resulted in the formulation of an emulsion having particle size $33.74 \pm 1.52 \mu\text{m}$ (Fig. 2D), which correlates with the particle size obtained with Mastersizer instrument.

3.3. Determination of encapsulation efficiency

The drug loading and encapsulation efficiency of TAF loaded PLGA microspheres is shown in Table 1. With an increase in the percentage of mannitol in the external aqueous phase, higher encapsulation of TAF was observed in the PLGA microspheres. The B-1 batch without mannitol in the external aqueous phase had an encapsulation efficiency of $48.36 \pm 0.13\%$, whereas upon increasing the mannitol concentration to 5%, enhancement in the encapsulation efficiency to $54.29 \pm 0.829\%$ (B-2) was observed. The reason behind the increased encapsulation efficiency after mannitol addition in the external aqueous phase is the osmotic effect of mannitol. The addition of osmogen in the stabilizer solution increases the osmotic pressure, prevents the diffusion of TAF from the organic phase to the external aqueous phase, and forms dense, non-porous microspheres [24,36,37]. The change in PLGA grades from 75:25 to 50:50 (B-3) also decreased the encapsulation of TAF in the microspheres. This might have been observed due to the lesser hydrophobic nature of PLGA 50:50 than PLGA 75:25, which might have resulted in a lesser interaction between the drug and the polymer. A 75% lactide of PLGA 75:25 results in better non-polar intermolecular interactions with hydrophobic moieties (aromatic and ester groups) of the drug, resulting in higher encapsulation efficiency. Therefore, PLGA 75:25 was further selected for the optimization of the release profile. An increase in the batch size has got an effect on the formulation properties, the microspheres batch (B-6), showed a decrease in the encapsulation efficiency compared to B-2.

3.4. Particle size determination

The particle size of TAF loaded PLGA MS of all the batches was found within the range of $20\text{--}30 \mu\text{m}$ (volume weighted mean), and the *d* (90) of all batches was below $100 \mu\text{m}$, as shown in Table 1. For easy administration of microspheres by the subcutaneous or intramuscular route, the particle size should be below $100 \mu\text{m}$ [18]. The particle size distribution graph of TAF loaded PLGA MS batch is shown in Fig. 9B. The span value was found to be below 1.8, which represented the narrow particle size distribution of all the microspheres batches.

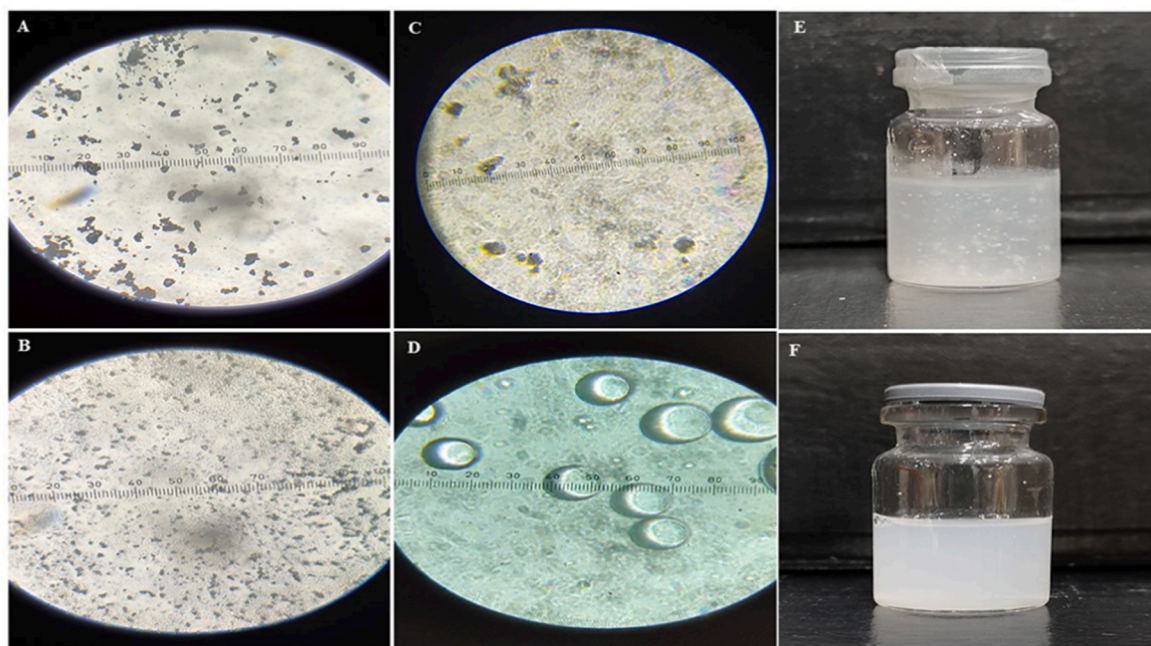


Fig. 2. (A) microscopic image of plain drug TAF at 10X resolution, (B) microscopic image of plain drug TAF in dichloromethane after high-speed homogenization at 18000 rpm for 1-min (10X resolution), (C) microscopic image of s/o dispersion at 100X resolution, (D) microscopic image of secondary emulsion at 100X resolution, (E) TAF in polymeric solution before high-speed homogenizer, (F) TAF in polymeric solution after high-speed homogenizer.

3.5. Surface morphology

The scanning electron micrographs of plain drug and TAF loaded microspheres are shown in Fig. 3. The pure drug showed irregularly shaped crystals, whereas TAF loaded PLGA microspheres showed spherical shaped microspheres. TAF loaded PLGA microspheres (B-2) prepared with the addition of osmogen in the stabilizer solution formed dense microspheres with a smooth outer surface, compared to the B-1 batch prepared without the addition of mannitol. This was observed due

to the osmogen effect of mannitol which formed the dense and smooth surface microspheres [24,38].

3.6. In vitro release study

The graphs of *in vitro* release profiles of TAF loaded PLGA microspheres are given in Fig. 4A. The plain TAF in phosphate buffer saline, pH 7.4, was completely dissolved within a day. The TAF loaded PLGA MS batches were found to release TAF till 15 days which indicated the

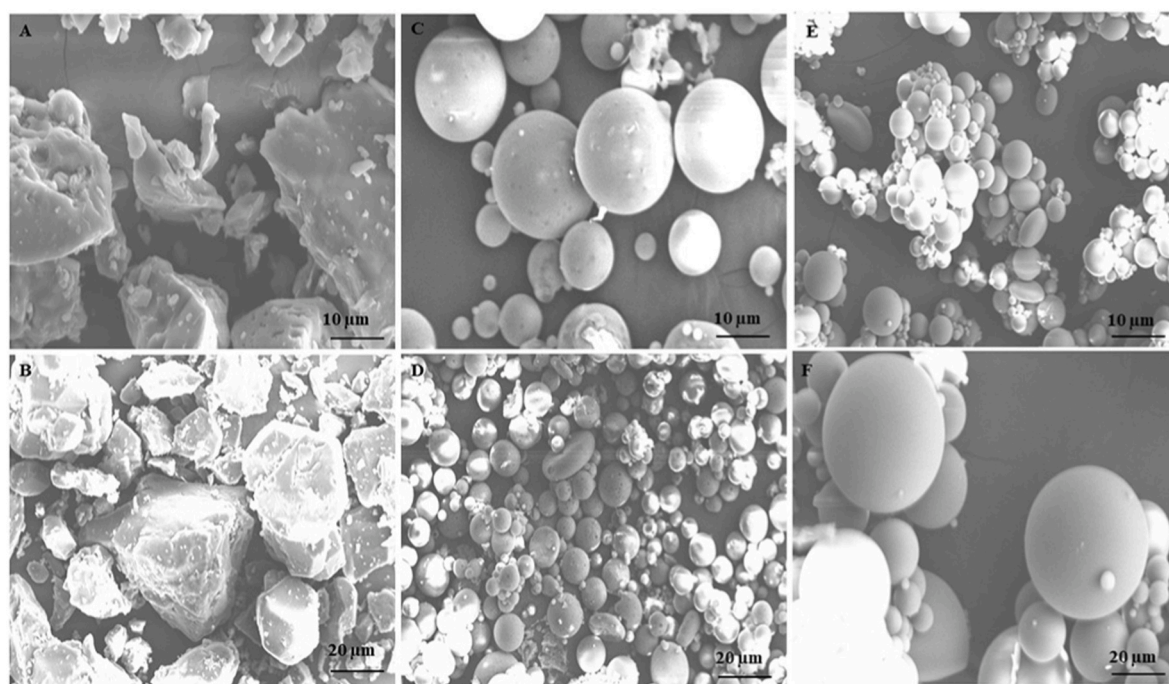


Fig. 3. Scanning Electron Micrographs of; (A-B) plain drug TAF, (C-D) TAF loaded PLGA microspheres of B-1 batch without osmogen, (E-F) TAF loaded PLGA microspheres of B-2 batch with osmogen.

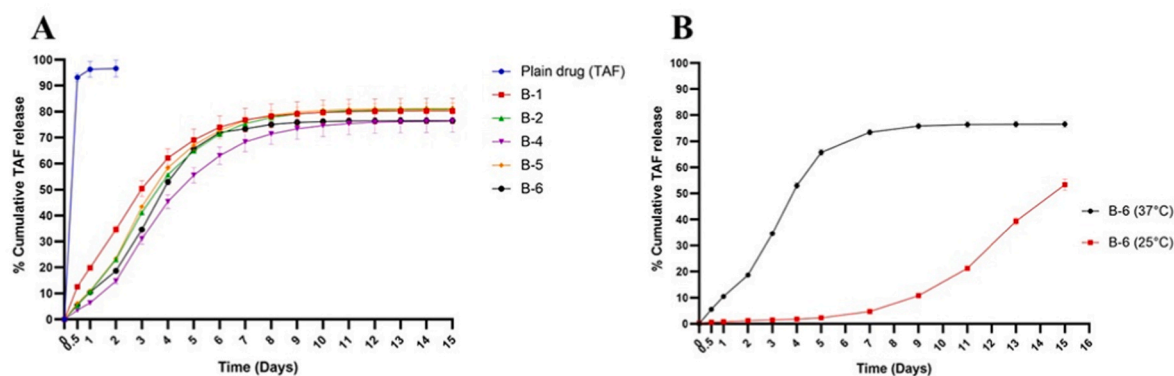


Fig. 4. *In vitro* release of TAF loaded microspheres batches, (A) *In vitro* release at 37 °C, (B) *in vitro* release of microspheres B-6 batch at 25 °C and 37 °C.

sustained release potential of the microspheres. Microsphere batch (B-1) without osmogen in the stabilizer solution showed a higher initial burst release ($19.82 \pm 0.88\%$), whereas the addition of osmogen in the stabilizer solution in the B-2 batch showed reduced initial burst release ($10.60 \pm 0.34\%$). The reduction in initial burst release was observed due to the formation of dense, non-porous microspheres, as reported before [24,37,38]. The decrease in TAF loading from 4.76% to 2.91% in the B-4 batch showed a reduction in initial burst release ($6.31 \pm 0.53\%$). These results were observed since a higher concentration of polymer was available for retardation of initial burst release in B-4 as compared to higher TAF loading in B-2 batch. A further change in TAF loading from 4.76% to 9.09% in the B-5 batch did not show a significant effect in the release profile. However, the encapsulation efficiency of the B-5 batch was found to be significantly lower as compared to the B-2 batch owing to a lesser amount of the matrix forming polymer. In addition to the dissolution studies carried out at the physiological temperature (37 °C), the study was also performed at room temperature (25 °C) to understand the effect of temperature on the release profile, as mentioned by Gunawardana et al. in their study on subdermal implant of TAF [4]. The change in temperature of the dissolution medium from 37 °C to 25 °C in B-6 batch showed a significant decrease in drug release from the microspheres (Fig. 4B). An increase in the temperature of the dissolution medium accelerated the degradation of the PLGA polymer as reported previously [40].

3.7. Thermal analysis

The DSC graph of TAF, PLGA polymer, physical mixture and TAF

loaded PLGA microspheres is shown in Fig. 5. The sharp endothermic peak around 132 °C indicated the melting point of TAF which is due to its crystalline nature. The PM showed a slightly weak but sharp endothermic TAF peak due to the dilution effect of PLGA. The representative endothermic peak of TAF was absent in the TAF loaded PLGA microspheres, which confirmed the encapsulation of TAF in its amorphous form. The conversion of TAF from crystalline to amorphous form might have resulted due to the solubilization ability of PLGA polymer [39]. This conversion is predicted to have taken place during the secondary emulsification and solidification of microspheres.

3.8. X-ray diffractometry

The XRD graph of TAF, physical mixture and TAF loaded PLGA microspheres is presented in Fig. 6. The characteristic peaks of TAF indicated the crystalline nature of the drug. The physical mixture showed a significant decrease in the peak intensities, although a few sharp peaks were observed. On the other hand, the characteristic peaks of TAF were absent in TAF loaded PLGA microspheres, confirming the encapsulation of TAF in the amorphous form.

3.9. Residual surfactant analysis

Surfactants are essential for the stabilization of emulsion during the preparation of nano/microparticles. Polymeric water-soluble polymer, PVA is a popular stabilizer used for the preparation of micro/nano-particulate delivery systems. As per the literature, the PVA solution (5% w/v) given by the SC route causes anaemia in the rats [13,41].

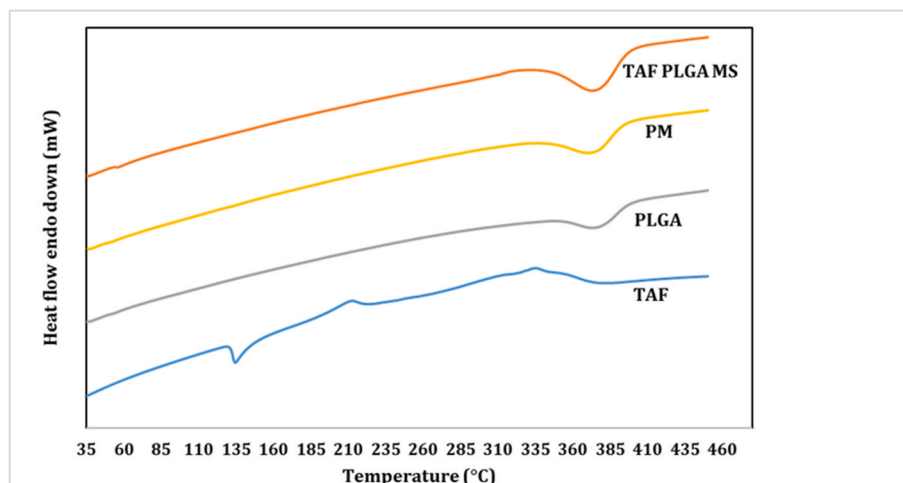


Fig. 5. DSC graph of TAF, PLGA polymer, physical mixture and TAF loaded PLGA microspheres.

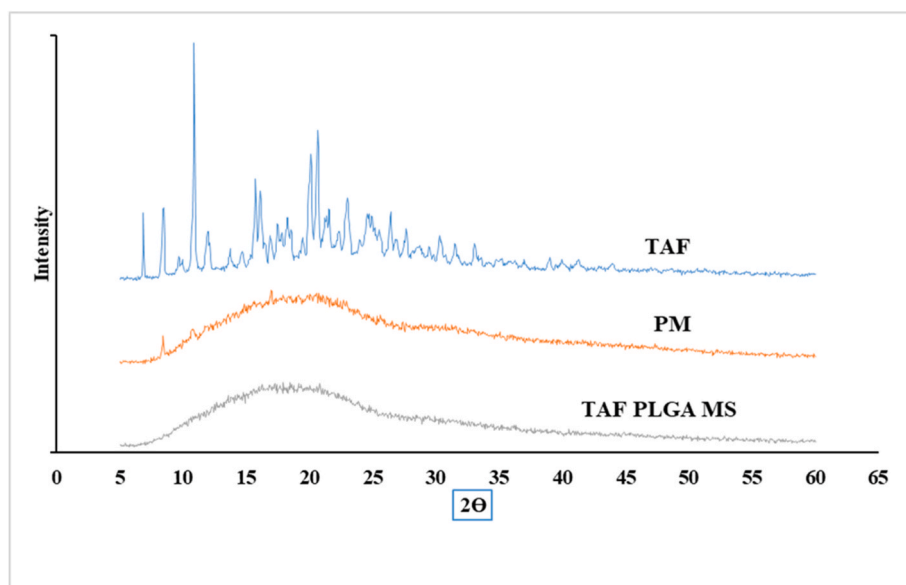


Fig. 6. XRD graph of TAF, physical mixture and TAF loaded PLGA microspheres.

Therefore, avoidance of any residual surfactant in the optimized formulation is a necessity. The microspheres dispersion was filtered for separation of microspheres, and the residual surface-attached PVA was removed by repeated washing with distilled water. The residual PVA was not detected in the freeze dried TAF loaded PLGA MS.

3.10. Cytotoxicity study using MTT assay

The cytotoxic effect of free TAF (based on the maximum released amount within 48 h), MS, and equivalent TAF loaded within PLGA MS on TZM-bl cells was evaluated using the MTT assay after 48 h of exposure. Fig. 7 demonstrates the CC_{50} ($\mu\text{g/mL}$) values of TAF, MS, and TAF-loaded PLGA MS, which were found to be $228.57 \pm 10.43 \mu\text{g/mL}$, $>10000 \mu\text{g/mL}$, and $1439.02 \pm 273.21 \mu\text{g/mL}$, respectively. The drug vehicle or placebo MS control showed 50% cytotoxicity over 10 mg/mL, suggesting that MS alone is not cytotoxic. Additionally, the developed formulation (TAF-loaded PLGA MS) demonstrated less cytotoxicity ($p \leq 0.001$) compared to TAF at an equivalent minimum released concentration within 48 h. This could be attributed to the slow exposure of the cells to the released TAF from PLGA MS during the experimental period. Since anti-HIV-1 inhibition was measured at well lower doses that were cytotoxic, hence it could not expect toxicity to confound the outcome of the antiviral activity assays.

3.11. Effectiveness of drug released by PLGA microspheres on HIV-1 inhibition

To ensure TAF-loaded PLGA MS retained reproducible bioactivity against HIV-1, three different HIV-1 strains were employed. A consistent HIV-1 suppression up to 4 h was observed when treated with the TAF-released PLGA MS (Fig. 8). This indicated the initiation of inhibition of HIV infectivity followed by a progressive decline in the inhibition, until the release of TAF PLGA MS up to 3 days. Unusually/Surprisingly, there was no contrasting difference between the negative control (no drug/no virus) compared to the TAF-released MS at various time points (Mann-Whitney U Test, $p \geq 0.05$). It is worth mentioning that the formulation was capable of suppressing the infectivity of all HIV-1 strains significantly compared with virus control (Mann-Whitney U Test, $p \leq 0.0001$ for all HIV-1 strains). Based on this, it can be hypothesized that the enhanced potency of TAF formulated with MS is due to the sustained release of TAF at the site of the action. Though, the amount of released TAF is higher than the half maximal inhibitory concentration (IC_{50}) value of the TAF, which was found to be $\sim <0.04 \mu\text{g/mL}$ against all HIV-1 strains (data not shown). Therefore, it can be envisaged that the TAF formulated with MS could be a good option for providing a prolonged therapeutic effect and higher intracellular drug concentration for inhibiting the reverse transcriptase action and halting HIV lifecycle

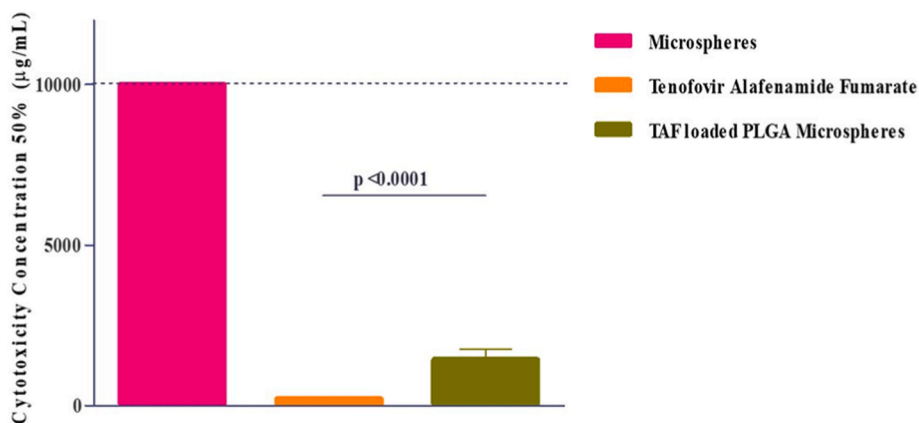


Fig. 7. *In vitro* cell viability assessment, the graph presents the fifty percent cytotoxicity concentration (CC_{50} , $\mu\text{g/mL}$) of free MS, free TAF, and TAF loaded PLGA MS in TZM-bl cells. ($p \leq 0.001$, the significant difference is in comparison with the drug control, TAF).

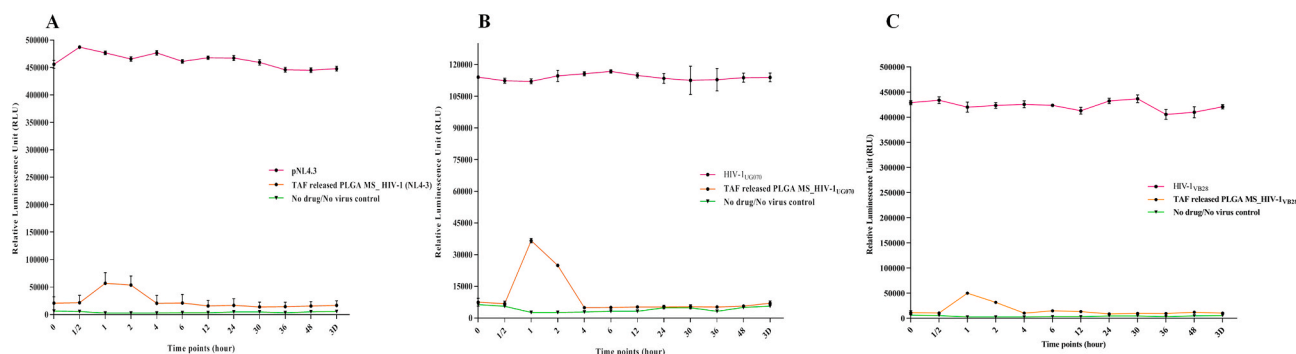


Fig. 8. The HIV-1 inhibition curves for; (A) HIV(NL4.3), (B) HIV-1_{UG070}, and (C) HIV-1_{VB28}, and in presence or absence of the TAF released from PLGA MS in compared to the negative control (no drug/no virus). The results reveal significant HIV-1 infection suppression after 48 h of treatment.

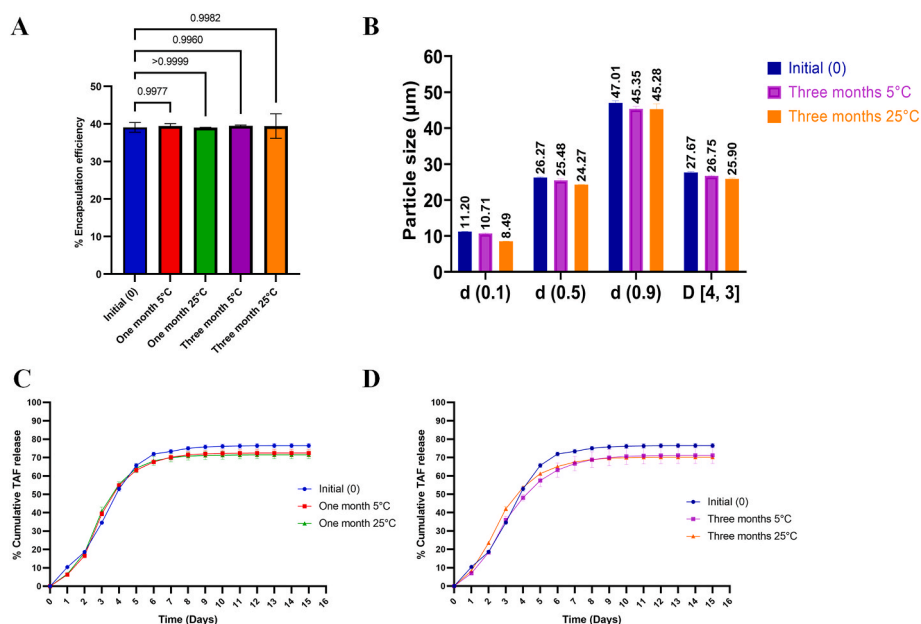


Fig. 9. Stability studies results of TAF loaded PLGA microspheres batches, (A) Encapsulation efficiency, (B) particle size distribution, (C) *in vitro* release after one month, (D) *in vitro* release after three months.

at the transcription stage.

3.12. Stability study

The TAF loaded PLGA microspheres (B-6) batch was also assessed for the encapsulation efficiency, particle size, and *in vitro* release after one and three months of storage at $5 \pm 3^\circ\text{C}$ and $25 \pm 2^\circ\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ as per the ICH guidelines [31]. No significant change was observed in appearance, particle size, encapsulation efficiency (ANOVA, $p \geq 0.05$) and *in vitro* release (ANOVA, $p > 0.05$; $p = 0.81$, Initial vs. 5°C three month; $p = 0.89$, Initial vs. 25°C three month) of microspheres when compared with the initial measurements of the freshly prepared MS. The similarity factor values (f_2 value) for *in vitro* release after one month of storage at $5 \pm 3^\circ\text{C}$ and $25 \pm 2^\circ\text{C}/60\% \text{RH} \pm 5\% \text{RH}$ were 78.14 and 74.35, and three months were 62.92 and 62.33 respectively, considering initial *in vitro* release as a reference. The results of the stability assessment of microspheres evaluation are shown in Fig. 9.

4. Conclusion

It can be concluded from this study that the TAF loaded PLGA MS demonstrated excellent/exceptional activity as compared to the free drug. In this study, our results highlighted new opportunities to design

an efficient cargo for delivering ARV. The developed TAF loaded PLGA MS enhanced the therapeutic concentration of the TAF at the site of the action. *In vitro* cytotoxicity and anti-HIV studies revealed that the formulation had lower cytotoxicity while also being significantly efficacious in inhibiting HIV-1 infection. Process and formulation parameters were optimized with respect to desired encapsulation efficiency, particle size, and *in vitro* release. All these efforts were made to enhance the potential of the TAF-loaded PLGA MS to improve patient compliance by lowering dosing frequency. Our study thus offers TAF-loaded PLGA MS as a promising therapeutic approach for controlling HIV infection.

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Credit author statement

Manoj A. Pawar: Conceptualization, Methodology, Investigation, Data curation, C-writing- Original draft preparation, Visualization, Investigation, Software. Leila Fotooh Abadi: Conceptualization, Methodology, Data curation, C-writing- Original draft preparation,

Visualization, Investigation, Software. Satish V. Rojekar: Conceptualization, Investigation, Formal analysis, Writing – review & editing. Ankita N. Yawalkar: Investigation, Formal analysis, Writing – review & editing. Smita S. Kulkarni: Supervision, project administration, validation, guidance, fund acquisition. Pradeep R. Vavia: Supervision, project administration, validation, guidance, fund acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

- Visualization, Investigation, Software, Satish V. Rojekar: Conceptualization, Investigation, Formal analysis, Writing – review & editing. Ankita N. Yawalkar: Investigation, Formal analysis, Writing – review & editing. Smrita S. Kulkarni: Supervision, project administration, validation, guidance, fund acquisition. Pradeep R. Vavia: Supervision, project administration, validation, guidance, fund acquisition.
- ### Declaration of competing interest
- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- ### Data availability
- Data will be made available on request.
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