Research Article

Hyaluronidase-Sensitive Nanoparticle Templates for Triggered Release of HIV/AIDS Microbicide *In Vitro*

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Abstract. This study was designed to test the hypothesis that a triggered release of a topical microbicide (tenofovir) from hyaluronic acid nanoparticles (HA-NPs) can be achieved under the influence of hyaluronidase (HAase) enzyme. A fractional factorial experimental design was used to examine the factors [molar concentrations of adipic acid dihydrazide (X_1) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (X_2), volume of acetone (X_3) and reaction time (X_4)] influencing the responses, Y_1 ; particle mean diameter: PMD (nanometers: nm), Y_2 ; polydispersity index: PDI and Y_3 ; zeta (ζ) potential: (millivolts). The amide bond formation between HA and ADH after cross-linking was confirmed by FT-IR and ^{13}C -NMR analyses. These NPs were also characterized for cytotoxicity on a human vaginal epithelial cell line and *L. crispatus*. When formulated with factors X_1 ; 2.49 mM, X_2 ; 9.96 mM, X_3 ; 60 mL, X_4 ; 6 h, HA-NPs exhibited a spherical shape with PMD, PDI, ζ potential, encapsulation efficiency, and drug loading of 70.6 ± 4.1 nm, 0.07 ± 0.02 , -38.2 ± 2.8 mV, $51.8 \pm 2.4\%$ w/w and $26.1 \pm 1.2\%$ w/w, respectively, ($n=3$). Unlike for HA based gel, HAase significantly triggered the drug release and HA degradation from the NPs after 24 h ($\sim 90\%$ w/w and 65% w/w, respectively); whereas, in its absence, these values were $\sim 39\%$ w/w and 26% w/w, respectively. The NPs were non-cytotoxic to human vaginal VK2/E6E7, End1/E6E7 cells and *Lactobacillus crispatus*. These data highlight the potential of HAase-sensitive HA-NPs templates for the controlled and vaginal delivery of anti-HIV/AIDS microbicides.

KEY WORDS: experimental design; hyaluronic acid; hyaluronidase; microbicide; nanoparticles.

INTRODUCTION

Unprotected heterosexual vaginal intercourse has become one of the major routes of human immunodeficiency virus (HIV) infection. In general, women are at greater risk of heterosexual transmission of HIV virus. However, apart from the female condom (expensive and cumbersome to use) (1) at present there are no effective prevention methods for HIV transmission in women. Currently, female-controlled prophylactic methods using microbicides are the major focus among HIV prevention strategies (1–3). These are agents applied within the vagina or rectum to prevent the transmission of sexually transmitted diseases including HIV infections (4). Since human semen is the carrier of HIV virus

transmission during male to female intercourse (5), designing a semen-triggered nanoparticles (NPs) delivery system (due to several advantages offered by nanotechnology in anti-HIV therapy) (6) would have the potential to inactivate or kill the HIV virus prior to exposure and penetration of the vaginal mucosa.

The pH of the cervicovaginal (CV) mucosa with healthy vaginal microflora is acidic (pH 3.5–4.5) due to the presence of lactic acid produced by *Lactobacillus* sp. bacteria (7). Women with bacterial vaginosis (BV) and other genital infections have a higher mean CV pH of 5 to 6 (7,8). The CV pH is also significantly higher (6 to 7) during the menstruation cycle (9). During sexual intercourse, the CV fluid is neutralized (pH 7 to 7.2) by the alkaline pH (7.2 to 8) of human semen within 8 s of arrival of seminal fluid (8,10). A small amount of semen is sufficient to neutralize the acidic pH of CV fluid due to its higher buffer capacity (8). After sexual intercourse, the CV fluid maintains this neutral pH up to 23.5 h (8). However, the time during which the semen traces are detectable in the CV fluid is approximately 48 h (8). The neutralization of CV fluid provides a favorable condition to increase male to female HIV virus transmission. Moreover, the anatomy and physiology of the mucosal barrier changes significantly during the menstrual cycle and in sexually transmitted disease conditions such as BV which could further enhance the virus transmission process (8). These

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observations warrant the need for urgent development of novel methods for the prevention of HIV virus transmission.

In recent years, hyaluronic acid (HA) has been used widely, owing to its biodegradable, non-immunogenic and bio-adhesive properties (11,12). It is a non-sulfated, hydrophilic, naturally occurring anionic muco-polysaccharide made of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, linked through $\beta(1-4)$ and $\beta(1-3)$ glycoside bonds (12,13). HA is hydrolyzable under treatment with the hyaluronidase (HAase) enzyme (14), which is abundant in human seminal fluid (15) as well as other body fluids and tissues (11). The amount of HAase in human semen is entirely dependent on the sperm count. In general, the HAase content of human semen containing 100 million sperms/mL is about 0.36 U/mL with the total amount of 1.08 U considering the average volume of human ejaculate is 3 mL (15). The mechanism of degradation of HA has been reviewed in detail (16). Due to its excellent physicochemical properties, medical and biological functions, HA has also been widely used for NPs designs (12) and other drug delivery applications (17).

Tenofovir (TFV), used as a model microbicide in this study, belongs to the category of antiretroviral drugs under the sub-category of nucleotide reverse transcriptase inhibitors (18). It is a small molecule, hydrophilic drug with a log *P* value of -1.6 (19). The molecular weight of TFV is 287.213 g/mol. The effectiveness and safety of TFV as a vaginal microbicide have been demonstrated (20). TFV formulations such as, vaginal gel (21), vaginal ring (22), solid lipid NPs (23), muco-adhesive chitosan NPs (24), pH responsive NPs (25) and, microspheres (26), intended for the prevention of HIV transmission, have been successfully engineered.

The currently available vaginal dosage forms such as gel and suppository cause a somewhat uncomfortable wetness, lack of vaginal retention, and drug leakage (3). The NPs delivery systems to the vagina may be beneficial by causing much less discomfort and reducing the dosing frequency simultaneously (6). Considering these facts, the present study aimed at developing a NPs delivery system capable of degradation that provides a triggered release of microbicide on exposure to seminal HAase. To achieve this aim, in this study, the nano-formulations containing TFV are synthesized for the first time using HA cross-linked with adipic acid dihydrazide (ADH) and a surfactant-free cross-linking method adapted from the literature (27). The effects of various formulation factors on the physicochemical properties of HA-NPs were analyzed by using fractional factorial experimental design (FFED) (28).

MATERIALS AND METHODS

Chemicals

Tenofovir (99% purity) was purchased from Beijing Zhongshuo Pharmaceutical Technology Development Co. Ltd. (Beijing, China). Hyaluronic acid sodium salt (MW, 11,624 Da) was supplied by Zhenjiang DongYuan Biotech Co., Ltd., (Jiangsu, China). Hyaluronidase (HAase) from bovine testes with a specified activity of 810 U/mg, bovine serum albumin (BSA, Fraction V), N-hydroxysuccinimide (NHS), adipic acid dihydrazide (ADH), sodium D-glucuronate and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide

hydrochloride (EDC) was from Thermo Fisher Scientific Inc. (Rockford, IL, USA).

The human vaginal epithelial cell lines (VK2/E6E7, ATCC:CRL-2616), endocervical epithelial cell lines (End1/E6E7, ATCC:CRL-2615) and *Lactobacillus crispatus* (ATCC:33197) were from the American Type Culture Collection (ATCC, Manassas, VA, USA). The CellTiter 96® Aqueous One Solution Proliferation assay kit with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] reagent and CytoTox- ONE™, lactate dehydrogenase (LDH) cytotoxicity assay kit was from Promega (Madison, WI, USA). Deionized water for all the experiments was obtained from a Millipore Milli-Q water purification system (Millipore Corp., Danvers, MA, USA). All other chemicals were of analytical grades and used as obtained from suppliers.

Experimental Design

Generally, if there are *k* numbers of independent variables (factors), each at two levels (high and low), the full factorial experimental design would require 2^k runs (28). However, if there are four or more factors, it is generally too complicated and also unnecessary to run all the possible combinations of factor levels and experiments. In these conditions, FFED design is useful as it requires only half of the runs (2^{k-1}) instead of the original 2^k runs of a full factorial design (28). The FFED design provides the possibility of obtaining maximum information from the minimal number of experiments. Thus, in this work, the FFED design was employed with *k* value of four as shown in Table I.

Formulation and Drug Loading into HA-NPs and HA-Gel

The HA-NPs were prepared by a surfactant-free cross-linking method adopted from a previous report (27) with some modifications (Supplementary material Reaction

Table I. Independent and Dependent Variables with Their Corresponding Values in the Fractional Factorial Experimental Design (FFED)

Variables	Levels		
	Low	Medium	High
Independent variables (factors)			
Coded values of levels of X_1 , X_2 , X_3 , and X_4	-1	0	+1
Actual values of X_1 , X_2 , X_3 and X_4			
X_1 ; molar concentration of ADH: mM ^a	2.49	6.23	9.96
X_2 ; molar concentration of EDC: mM ^a	2.49	6.23	9.96
X_3 ; volume of acetone: mL ^b	30	45	60
X_4 ; reaction time: hour ^c	2	4	6
Dependent variables (responses)			
Y_1 ; particle mean diameter: PMD, nm			
Y_2 ; polydispersity index: PDI			
Y_3 ; zeta (ζ) potential: mV			

^a Molar concentration with respect to the molar concentration of HA (kept constant at 2.49 mM)

^b Volume of acetone with respect to the volume of aqueous phase (kept constant at 10 mL)

^c Reaction time after secondary acetone addition

Scheme S1). Briefly, 1 mg/mL aqueous solution of HA (10 mL) was prepared by dissolving the HA powder in milli-Q water with continuous stirring for 1 h at room temperature. Acetone was added in a total amount of 40% v/v (primary addition) in the above solution and stirred for 1 h to make sure that all the components are well dispersed. Three different aqueous solutions of EDC, NHS, and ADH (250 μ L each) were separately added to the above solution and stirred for 30 min, respectively, after each addition. The carbodiimide mediated cross-linking was continued by stirring at room temperature for 15 h, leading to the formation of amide bonds between the carboxylic acid groups of glucuronic acid units of HA and the hydrazide groups of ADH. Finally, a secondary addition of acetone occurred to raise the volume three to six times with respect to the initial aqueous phase (X_3). The solution was stirred continuously for various time points. The organic solvent was evaporated by using a rotatory evaporator (BUCHI Labortechnik AG, Flawil, Switzerland).

The colloidal dispersion was ultra-centrifuged, using a Beckman L8-70 M ultra-centrifuge (Beckman Instruments Inc., Palo Alto, CA, USA) at 20,000 rpm and 10°C for 45 min to isolate the NPs. The NPs were purified using dialysis method against 1 L of milli-Q water for 24 h with three water changes. The NPs were then freeze-dried (Labconco Corporation, Kansas City, MO, USA). The process yield of the NPs recovery was determined using mass balance calculation. The soaking method (29,30) was used to encapsulate TFV in the HA-NPs matrix. Typically, 10 mg of purified and freeze-dried NPs were immersed in an aqueous solution of TFV at pH 7 for 3 days at room temperature. The NP/drug ratio in the loading solution was varied from 10:1 to 1:1 (w/w). The percent encapsulation efficiency (%EE) and drug loading (%DL) were determined indirectly from the supernatant after ultra-centrifugation and appropriate dilution using a high-performance liquid chromatography (HPLC) assay (31). The formulation of HA-gel was based on hydration and internal cross-linking reaction of the native HA at the concentration of 2% w/v in water as previously reported (32).

Size Distribution and Zeta (ζ) Potential Analyses

The NPs were analyzed for their particle mean diameter (PMD); nanometers, polydispersity index (PDI), and size distribution by dynamic light scattering (DLS) method. The ζ potential was analyzed by laser doppler velocimetry and phase analysis light scattering methods using Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK). After suspending 100 μ L of NPs dispersion in 1 mL of water, the measurement was undertaken at 25°C. The PMD of the NPs was represented as Z-average diameter following the cumulant model (33). It is noteworthy that estimation of the PMD and PDI was based on the intensity of the light signal processed according to the cumulant method. The cumulants are defined as the coefficients of an expansion of a MacLaurin series (34). The PDI value was given by Eq. (1):

$$PDI = K_2/K_1 \quad (1)$$

The cumulant K_1 is an effective mean diffusion coefficient; whereas, K_2 describes the relative width of the size

distribution if normalized by K_1 (34). The instrument was calibrated by using nanosphere™ of PMD (59.0 $\pm$ 2.5 nm) and ζ potential standards (−68.0 $\pm$ 6.8 mV). According to the National Institute Standard, a sample with a PDI value <0.05 is considered mono-dispersed (35).

Surface Morphology of HA-NPs

The surface morphology of HA-NPs was analyzed by transmission electron microscopy (TEM) using a Philips TEM CM12 instrument (FEI, Hillsboro, OR, USA). The instrument was equipped with the large format (II Megapixel), retractable and fiber-optical coupled SC100 ORIUS® CCD camera (Gatan, Inc., Pleasanton, CA, USA) for digital image acquisition. The samples for the TEM analysis were obtained by placing a drop (10 μ L) of colloid dispersion of the HA-NPs on the carbon-coated copper grid. The excess fluid was removed by a piece of filter paper before samples were completely dried at room temperature and examined for TEM imaging without any further modifications. The TEM observations were performed at an accelerating voltage of 80 kV.

Enzymatic Degradation Analysis of HA-NPs

An uronic acid–carbazole assay (36) was used to analyze the degradation of the HA-NPs under the influence of HAase enzyme. The degradation analysis was performed with the amount of HAase (1.08 U) that is normally present in human ejaculate with an average volume of 3 mL (15). The degradation study was performed at pH 7.1, which was equivalent to the pH condition of vaginal fluid during sexual intercourse (5). The optimum pH for maximum hydrolytic activity of HAase is 4 to 5 (37,38). However, it has been reported that HAase exert its activity at weakly acidic (pH 5 to 6) (38) and alkaline conditions (pH 7 to 8) (37,39). The bovine testicular HAase was used as a model enzyme, since it is commercially available and, like human HAase, it catalyzes the hydrolysis of the β (1–4) glycosidic bonds in HA (40).

Briefly, the HA-NPs (10 mg) were dispersed in 3 mL of the simulant mixture (pH 7.1) of vaginal fluid simulant (VFS, pH 4.2) and seminal fluid simulant (SFS, pH 7.8). These VFS and SFS buffers were prepared according to the previous reports (41,42). The 1:4 ratios of the VFS and SFS, respectively, were used for preparing the simulant mixture, considering that the volumes of normal human vaginal fluid and male ejaculate, respectively (41,42). The pH of simulant buffers was adjusted using 1 M HCl or NaOH solutions, prepared accordingly.

The NPs dispersion was transferred to a dialysis bag (Spectra/Por Float-A-Lyzer G2, MW-CO, 3.5–5 kDa), supplied from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA), and placed inside a dialysis tube containing 20 mL of the simulant mixture. The whole system was then placed in a thermostatic shaking water bath (BS-06, Lab Companion, Seoul, Korea) with 60 rpm at 37°C. Aliquots of 100 μ L solutions were taken from the medium at different time intervals (0, 1, 3, 6, 24, 48, 72, 96, 120 h), boiled for 10 min to inactivate the enzyme and analyzed for the amount of glucuronic acid released. Simultaneously, the fresh simulant mixture was added at the same rate to maintain the sink condition.

The Genesys 10 Bio UV–Vis Spectrophotometer (Thermo Electron Sci. Inst., LLC, Madison, WI, USA) was used for the degradation analysis at 530 nm. The amount of glucuronic acid released at each time point was determined by using the standard curve of sodium D-glucuronate. The curve was found linear in the concentration range of 0–200 µg/mL (square of the correlation coefficient: $r^2=0.99$). The measured amount of D-glucuronic acid was represented as percent cumulative degradation (%w/w) of NPs compared to the degradation of native HA incubated either in the presence or absence of HAase.

In Vitro Drug Release Analysis of HA-NPs

The *in vitro* drug release profile of HA-NPs was compared with that of the HA-gel. The release study was performed with the 10 mg of drug loaded HA-NPs or HA-gel using the dialysis method as explained above for the enzymatic degradation analysis. The blank HA-NPs, HA-gel, and TFV powder were taken as control samples. Aliquots of samples (0.1 mL or 100 microliters) were taken at, 0, 1, 3, 6, 9, 12, 24, 48, and 72 h from the release medium. Simultaneously, an equivalent volume of fresh simulant mixture was added at the same rate to the release medium to maintain the sink condition. The amount of TFV was quantified by using HPLC assay (31).

Cytotoxicity Assays: Effect of the HA-NPs on the Vaginal Epithelial Cells

As the safety of 1% TFV gel has already been determined (20,43), in this study blank HA-NPs (without drug) in the concentration of 1 mg/mL were used to test their effects on the viability (MTS assay) and membrane integrity (LDH assay) of VK2/E6E7 and End1/E6E7 cells using the published protocols (25). Briefly, cells were seed in a 96-well plates in a keratinocyte-serum free medium and were allowed to grow until 80% confluence was reached. The medium was replaced with 100 µL of HA-NPs. The NPs were kept in contact with the cell lines for 48 h. The amount of formazan product formed was determined by adding 20 µL of MTS reagent to culture wells. The wells were incubated for 4 h at 37°C in a humidified, 5% CO₂ atmosphere and the absorbance was measured at 490 nm using a DTX 800 multimode microplate reader (Beckman Coulter, Brea, CA, USA). The medium and 1% Triton X was used as negative and positive controls, respectively. The absorbance was directly proportional to the number of viable cells in the culture. The percent cell viability was determined by Eq. (2):

$$\text{Viability(\%)} = \frac{\text{ABS(test)}}{\text{ABS(control)}} \times 100 \quad (2)$$

Where, ABS (test) and ABS (control) represented the absorbance of the amount of formazan product formed in viable cells after test and control treatments, respectively.

To perform cellular membrane integrity assay (LDH assay), the cells were incubated with 100 µL medium containing the NPs (1 mg/mL) in each well. The plates were incubated at 37°C for 48 h and equilibrated to room temperature for 30 min. One hundred microliters of CytoTox-One™ reagent was added in each well and vortexed for 30 s. The plates were incubated at

25°C for 10 min. Fifty microliters of stop solution was added in each well and the plates were shaken for 10 s. The fluorescence was measured at the excitation and emission wavelengths of 560 and 590 nm, respectively, using microplate reader. The percent cytotoxicity was determined using Eq. (3):

$$\text{Cytotoxicity(\%)} = \frac{\text{Experimental-Background}}{\text{Positive control-Background}} \times 100 \quad (3)$$

Where, experimental, background and positive control represented the fluorescence of the wells with NPs treatment, without NPs treatment and with 1% Triton X treatment, respectively.

L. crispatus Viability Assay

The acidic environment of vagina is maintained by *Lactobacilli*, which lowers the risk of HIV infection by its natural defense mechanism (7). Hence, any microbicide formulation will need to preserve the vagina's natural defense mechanism by not disturbing the *Lactobacilli* viability. This was assessed by using *Lactobacillus* viability assay (25). The *L. crispatus* used as model bacteria was grown in ATCC medium 416 *Lactobacilli* MRS broth (BD, Franklin Lakes, NJ, USA) at 37°C and the viability assay was performed using an established method (44). Briefly, the bacteria density was adjusted to an OD₆₇₀ of 0.06, which corresponded to 0.5 McFarland Standard or 10⁸ CFU/mL (45). *L. crispatus* was seeded in a 96-well plate at a volume of 100 µL and incubated with 100 µL of the NPs suspension for 48 h at 37°C. The bacterial wells treated with 10 µg/mL of commercially available penicillin–streptomycin solution (positive control) from Invitrogen, Carlsbad, CA, USA. After the incubation, 20 µL MTS reagent was added to each well and viability was expressed using Eq. (2) and determined by measuring the absorbance at 490 nm using microplate reader.

Enzymatic Degradation and In Vitro Drug Release Kinetics of HA-NPs

The drug release kinetics of NPs was analyzed by using zero-order, first-order, Higuchi, and Korsmeyer–Peppas models as given by Eqs. (4), (5), (6), and (7), respectively (46).

$$M_t = M_0 + K_0 t \quad (4)$$

$$\log M_t = \log M_0 - K_1 t / 2.303 \quad (5)$$

$$M_t = K_H t^{0.5} \quad (6)$$

$$M_t / M_\infty = k t^n \quad (7)$$

In these equations, M_t is the amount of drug released at time t , M_0 the initial amount of drug in solution, K_0 is zero-order release constant in micrograms per hour K_1 is the first-order release constant in per hour, K_H is Higuchi dissolution constant in micrograms per square root hour, M_t/M_∞ is the

fraction of drug released at time t , M_∞ is the total amount of drug released, k is a kinetic constant and n is the exponent explaining the drug release mechanisms (46). The exponent n value enables to classify the drug release mechanism as Fickian diffusion ($n \leq 0.5$), case-II transport ($n=1$), anomalous transport ($0.5 < n < 1$), and super case-II transport ($n > 1$) (46).

The Eq. (7) was further depicted in a log-transformed Eq. (8):

$$\log M_t / M_\infty = n \log t + \log k \quad (8)$$

The model independent approach was then applied to compare the enzymatic degradation and drug release kinetics of the HA-NPs (test compounds) with HA-gel (reference compound) as per US FDA guidance to industry (46). In the case of HA degradation study, the difference (f_1) and similarity factors (f_2) were calculated, using Eqs. (9) and (10), respectively.

$$f_1 = \left\{ \left[\sum_{t=1}^n |R_t - T_t| \right] / \left[\sum_{t=1}^n R_t \right] \right\} \times 100 \quad (9)$$

$$f_2 = 50 \times \log \left\{ \left[1 + (1/n) \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \times 100 \right\} \quad (10)$$

Where, n is the number of time points, R_t and T_t are the cumulative degradation and drug release (%w/w) at time t for reference (HA-gel) and test compounds (HA-NPs), respectively.

Here, f_1 measures the percent error while f_2 measures the sum-squared error between the test and reference compounds over all time points. The curves with f_1 and f_2 values close to 0 and 100, respectively, are considered similar. Generally, $f_1 < 15$ (0–15) and $f_2 > 50$ (50–100) ensure sameness or equivalence of the two curves (46). For simplicity, the difference and similarity factors are represented as f_1' and f_2' , respectively, for drug release kinetics study.

Data Analyses

JMP™-Release 8.0.2 experimental design software (Cary, NC, USA) was used for the statistical analysis. The values are presented as mean $\pm$ standard deviation (SD) of triplicate determinations ($n=3$), unless otherwise mentioned. Statistical analysis was evaluated using Students t test. The p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Mechanism of Formation of Cross-Linked HA-NPs

The HA-NPs formation was based on cross-linking efficiency of HA, water-HA, water-acetone, and acetone-HA interactions. The rationale for the selection of factors in FFED design (Table I) was based on the fact that the molar concentrations of ADH (X_1) and EDC (X_2) are significant for efficient cross-linking between HA and ADH while the interactions among aqueous phase and organic solvent and HA are critical for NPs formation (27,47). In the aqueous phase, the HA polymer is swollen and takes an expanded coil conformation due to the inter- and intra-molecular hydrogen

bonding among its functional groups and water molecules (48). The repulsive force exerted by the negatively charged carboxylate groups of HA also contributed in its expanded coil confirmation. The acetone was added in two steps (primary and secondary addition), which induced the breakage of the hydrogen bonds of HA. After this step, the carboxylate groups of HA become available to be cross-linked with ADH through amide bond coupling. The adequate ratio (X_3) between the volume of acetone and the aqueous phase initiated the cross-linking reaction and NPs formation. After the secondary addition of acetone, water was diffused into acetone and caused the precipitation of HA with a coil-to-globule transition. Since, HA is cross-linked with ADH, it undergoes molecular transition to form NPs (49). The reaction time (X_4) after the secondary acetone addition, when the color of the solution changed from clear to light blue (Tyndall effect) due to the transformation of HA from extended coil conformation to a compact globular structure shows the NPs formation.

Some methods have been described for the preparation of HA-NPs; such as reversed-phase micro-emulsion (29) and surfactant-free (27) methods. The surfactant-free method has an advantage in terms of avoiding the use of surfactants, which might be deleterious and difficult to remove from the final formulation and could cause significant loss of NPs in the washing steps. However, the modified method described in this work requires less time (< 24 h) compared to surfactant-free method (> 44 h) to produce the NPs. The shorter time was achieved by effective screening of the factors responsible for NPs formation using an experimental design method.

Experimental Data Analysis and Selection of HA-NPs Formulations

The measured values for responses Y_1 (PMD), Y_2 (PDI) and Y_3 (ζ) potential for runs F1 to F12 are summarized in Supplementary material Table S-1. The polynomial Eqs. (11), (12), and (13) for the potential responses Y_1 , Y_2 , and Y_3 , respectively, were developed after the data analysis in order to evaluate the best predictive model for each response in relation to the factors.

$$Y_1 = 122.75 - 1.81X_1 + 0.47X_2 - 4.14X_3 - 12.52X_4 + 9.61X_1X_2 + 21.57X_1X_3 + 17.14X_1X_4 \quad (11)$$

$$Y_2 = 0.179 - 0.007X_1 - 0.067X_2 - 0.016X_3 - 0.013X_4 - 0.0004X_1X_2 + 0.033X_1X_3 - 0.028X_1X_4 \quad (12)$$

$$Y_3 = -38.18 - 0.091X_1 + 0.84X_2 - 0.99X_3 - 1.09X_4 + 0.88X_1X_2 + 1.21X_1X_3 + 0.086X_1X_4 \quad (13)$$

In these equations, the positive coefficients before the terms demonstrated an increasing effect; whereas, the negative coefficients showed a decreasing effect on the selected responses. The interaction terms displayed how the responses changed when the two factors were varied simultaneously.

To confirm the acceptability of the model, analysis of variance (ANOVA) was performed and shown in Supplementary material Table S-2. The acceptability criterion for a good model is a p value in ANOVA analysis <0.01 and RSquare (R^2 , ratio of the difference to the reduced negative log-likelihood values) should be in the range of $0.70 \leq R^2 \leq 0.90$ (23). According to the acceptability criteria, the polynomial equations for Y_1 and Y_2 fit very well with the data modeling. The polynomial Eq. (13) for response Y_3 did not fit well according to the acceptability criteria and was neither appropriate nor considered for subsequent modeling and prediction efforts. This may be due to the fact that NPs were made of HA as a main matrix component, which did not lead to significant changes in the ζ potential (Y_3) values.

To understand the factors effects and significance, Pareto charts were generated as shown in Fig. 1a, b, for the responses Y_1 , and Y_2 , respectively. The values on the x -axis of the Pareto charts represent the standardized effects; the ratio of estimate and the standard error of the factor effect (t ratio values). These t values were obtained on the basis of the estimate of factor effects, which are the coefficients in Eqs. (11), and (12) and the standard error of an effect (50). The obtained t value is compared with a tabulated critical t value ($t_{\text{critical}}=2.78$) as shown in the vertical lines in the Pareto charts. This t_{critical} value is determined at $\alpha=0.05$ for residual degrees of freedom (residual df =number of runs-number of terms-1) (50). In Fig. 1a and b, the absolute t value of the factors whose length of the chart passed the vertical line (t_{critical} at $p<0.05$ and $df=4$) have significant effects on response values.

According to Fig. 1a, Y_1 was significantly affected by interaction of X_1X_2 , X_1X_3 , X_1X_4 (all positively) and X_4 (negatively). The response Y_2 was significantly affected by

X_1X_3 (positively) and X_2 (negatively) as shown in Fig. 1b. By considering only the significant effects, it appeared that X_1 interacted with the factors X_2 , X_3 , and X_4 that increased the PMD and PDI values of the NPs. The physical significance of this is explained later in this manuscript. The relationship among factors and responses was further investigated by constructing a prediction and desirability plot (Fig. 1c).

According to the desirability value of 0.91, one that was very close to the ideal value ($d=1$) (Fig. 1c), the run F13 (coded values: $X_1=-1$, $X_2=+1$, $X_3=+1$, $X_4=+1$; actual values of $X_1=2.49$ mM, $X_2=9.96$ mM, $X_3=60$ mL, and $X_4=6$ h) was selected as the optimized nano formulation which shows Y_1 and Y_2 values of 70.6 ± 4.1 nm and 0.07 ± 0.02 , respectively ($n=3$). Meanwhile, to evaluate the effect of molar concentrations of ADH (X_1) and EDC (X_2) on the enzymatic degradation, drug release, %EE, and %DL of the NPs, the runs F1 (coded values: $X_1=-1$, $X_2=-1$, $X_3=+1$, $X_4=+1$; actual values: $X_1=2.49$ mM, $X_2=2.49$ mM, $X_3=60$ mL, and $X_4=6$ h), and F7 (coded values: $X_1=+1$, $X_2=+1$, $X_3=+1$, $X_4=+1$; actual values: $X_1=9.96$ mM, $X_2=9.96$ mM, $X_3=60$ mL, and $X_4=6$ h) were selected as control formulations. These control formulations F1 and F7 having the lowest and highest molar concentrations of ADH and EDC, respectively. The rationale for this selection was based on the fact that the free carboxylic acid groups on HA are the recognition sites to initiate its degradation by HAase (51). Increasing the molar concentrations of ADH and EDC could affect the cross-linking between HA and ADH. Theoretically, this affects the degradation kinetics of the NPs due to the higher cross-linking density of the carboxylic acid groups that are not favorable for the attack of HAase on its recognition site.

Fig. 1. Pareto charts showing the standardized effects of factors (as defined in the above Table I) and their interactions for responses. **a** Y_1 particle mean diameter (PMD, nanometer). **b** Y_2 polydispersity index (PDI). Bars extending past the vertical lines indicates the values reaching statistical significance ($\alpha=0.05$). Asterisk (*) indicates the significant effect ($p<0.05$) of factors on the responses. **c** Prediction profiler and desirability plot showing the effect of factors, X_1 , X_2 , X_3 , and X_4 , on the responses, Y_1 and Y_2 . The desirability value of 0.91 corresponds to the optimized HA nano formulation (F13) with the coded values of $X_1=-1$, $X_2=+1$, $X_3=+1$, and $X_4=+1$. The coded values listed above correspond with the actual values of $X_1=2.49$ mM, $X_2=9.96$ mM, $X_3=60$ mL, and $X_4=6$ h

Checkpoint Analysis

The resultant model was analyzed for its acceptability and validity with the theoretical optimum run, F13, which shows the maximum desirability value (Fig. 1c), while minimizing both Y_1 and Y_2 values as given in Supplementary material Table S-3. Two randomly selected runs, F14 (coded values: $X_1=-0.5$, $X_2=-0.5$, $X_3=+1$, $X_4=+1$) and F15 (coded values: $X_1=0$, $X_2=0$, $X_3=+1$, $X_4=+1$) were also analyzed to perform checkpoint analysis ($n=3$) to ensure reproducibility of the model. The difference between the predicted and measured values was statistically insignificant ($p>0.05$) as analyzed by the Student's t test.

Spectral Analysis by FT-IR and ^{13}C -CP/MAS NMR Spectroscopy

The cross-linking between HA and ADH was assessed by using Fourier Transform InfraRed (FT-IR) and solid state high resolution ^{13}C cross-polarization magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) analyses (Supplementary material). The results indicated by FT-IR (Fig. S1, Table S-4) and NMR (Fig. S2, Table S-5) analyses confirmed the cross-linking between HA and ADH.

PMD and Size Distribution Analysis of HA-NPs

The PMD and PDI values for the NPs formulations are shown in Table II. The NPs were found stable as the ζ potential value was in the range of -38.2 ± 2.8 mV ($n=3$), which was above (absolute value) the ζ potential limits of (± 30 mV) for a colloidal dispersion to be stable (52).

Enzymatic Degradation Analysis of HA-NPs

The percent cumulative degradation either in the presence or absence of HAase, is shown in Fig. 2a, b, c and d for native HA, F13, F1, and F7 formulations, respectively. It was observed that in the presence of HAase, the degradation of native HA was higher in comparison to formulation F13, F1, and F7. The variability in the degradation kinetics of these samples was due to the different interactions of HAase with its recognition site of HA as explained below.

It is well known that HAase begins its enzymatic action by affecting the free carboxylic acid groups of glucuronic acid units of HA (51). Thus, the molar concentrations of ADH (cross-linking agent) was considered as the major formulation

variable in the experimental design that could have significant effect on the cross-linking density, enzymatic degradation and drug release kinetics of the NPs. It was observed that the enzymatic degradation was highest for the native HA compared to the HA-NPs since, in the native HA most of the carboxylic acid groups were free to interact with HAase. The slower degradation rate for formulation F7 can be explained by the fact that, the molar concentrations of ADH and EDC were highest with the maximum carboxylic acid groups being blocked by forming amide bonds with ADH. However, the degradation of HA and HA-NPs in the absence of HAase might be due to the hydrolytic degradation of HA as previously explained (14,16). Overall, the degradation rate for native HA and HA-NPs was faster in the presence of HAase compared to its absence.

Drug Loading, Encapsulation Efficiency and *In Vitro* Drug Release Analysis of HA-NPs

The %EE, %DL and %yield of HA-NPs calculated after purification and freeze-drying process is given in Table II. It was observed that the %DL of the NPs was increased with increasing drug concentration in the loading solution as also observed by other researchers (53,54). The %DL indeed varied from about 16 to 26% w/w for F13, 13 to 22% for F1 and 5.5 to 11% w/w for F7, when the NPs:drug ratio was varied from 10:1 to 1:1 (w/w) in the loading solution, respectively. The drug loading of HA-NPs was estimated with a constant amount (10 mg) of HA-NPs while the amount of drug was variable. However, it has been reported that the higher the NPs amount or the higher the polymer (HA) concentration in the loading solution, the greater will be the molecular tortuosity and drug diffusion path length within the NPs (a factor which might have contributed to lowering the drug loading) (54,55).

The *in vitro* drug release profile was depicted in Fig. 3a, b, c and d for TFV containing HA-gel and native TFV control, F13, F1, and F7 formulations, respectively. A significantly triggered release of drug ($\sim 90\%$ w/w) was observed from NPs (F13) in the presence of HAase enzyme; whereas, in its absence the value was about 39% w/w after 24 h which was equivalent to 40.3 ± 1.2 and 17.4 ± 0.4 $\mu\text{M}/\text{day}$ ($n=3$) for 1 mg of NPs. It was observed that HA-gel exhibited a burst release and about 78% and 73% w/w of the drug was released in first 6 h in the presence and absence of HAase, respectively. However, about 97% w/w of drug was released from HA-gel either in the presence or absence of HAase after 24 h (Fig. 3a). The results indicated that the drug release rate

Table II. Physicochemical Characterization of HA-NPs

HA-NPs	X_1^a (CV, AV)	X_2^b (CV, AV)	X_3^c (CV, AV)	X_4^d (CV, AV)	Y_1^e , PMD (nm)	Y_2 , PDI	EE ^e (%)	DL ^e (% w/w)	Yield ^e (% w/w)
F13	-1, 2.49	+1, 9.96	+1, 60	+1, 6	70.6 $\pm$ 4.1	0.07 $\pm$ 0.02	51.8 $\pm$ 2.4	26.1 $\pm$ 1.2	93.3 $\pm$ 0.7
F1	-1, 2.49	-1, 2.49	+1, 60	+1, 6	81.5 $\pm$ 2.6	0.21 $\pm$ 0.01	42.0 $\pm$ 1.4	22.0 $\pm$ 0.7	90.9 $\pm$ 0.8
F7	+1, 9.96	+1, 9.96	+1, 60	+1, 6	157.7 $\pm$ 4.0	0.08 $\pm$ 0.02	17.3 $\pm$ 5.3	10.6 $\pm$ 1.8	84.2 $\pm$ 1.1

^a X_1 molar concentration of ADH given in CV coded values and AV actual values in millimolars

^b Molar concentration of EDC, in millimolars

^c Volume of acetone, in milliliters

^d Reaction time, in hours

^e Values are given as mean $\pm$ SD, $n=3$

Fig. 2. Percent cumulative HA degradation (% w/w) either in presence or absence of HAase at pH 7.1. **a** Native HA. **b** F13. **c** F1 and **d** F7 nanoformulations. Results are given as mean $\pm$ SD, ($n = 3$)

was much higher from the HA-gel; whereas, comparatively slow release of TFV was observed in the case of HA-NPs. This might be due to the difference in cross-linking density inside the HA-NPs and HA-gel that significantly affected their enzymatic degradation behavior as explained above. Moreover, other factors such as pH, temperature, the van der Waal and electrostatic interactions between TFV with HA polymeric chains inside the NPs could also affect the drug encapsulation and release profile from the HA-NPs as explained below.

In this study, the drug loading and release analysis was performed at pH 7.1 which is higher than the two pK_a values of TFV at 3.8 and 6.7 (19), and that of HA=2.9 (56). Hence, both TFV and HA had predominantly negative charge at pH 7.1 leading to higher drug release rate observed with HA-gel. However, due to the higher cross-linking density of native HA polymeric chains inside the NPs, there would be increased interactions of TFV with HA through the nonpolar carbon atoms and amine groups of the ADH. This explained a relatively slower drug release rate from the HA-NPs.

Moreover, the drug encapsulation and release profile may also vary with the swelling/deswelling effects depending on the pH of the release medium. At pH 7.1, due to the presence of ionized carboxylate groups belonging to HA, there could be an electrostatic repulsion among the HA

chains (29). This would increase the inter-chain distances and degree of swelling leading also to higher drug release rate and encapsulation (29). This was also observed with HA-gel since there were more free carboxylic acid groups available compared to those of NPs. Accordingly, a decrease in the pH of the drug release medium below the pK_a value of HA would reduce the degree of swelling of HA and thus lowering drug loading and release kinetics from HA-NPs and HA-gel.

Temperature is another factor that could have affected the drug encapsulation and release kinetics from the HA-NPs. In this study, the drug loading was conducted at room temperature considering the fact that HA is stable below 50°C under neutral pH conditions, whereas it undergoes thermal degradation under acidic or alkaline pH in all temperature conditions due to the fragmentation and disintegration of the polymeric chains (57–59). It has been reported that HA undergoes hydrolysis even at neutral pH at 60°C or higher temperature (59). The release assay of the HA-NPs was performed at 37°C to mimic the *in vivo* conditions. However, it has been reported that the temperature has direct effect on the swelling of the NPs and hydrogels due to the increase in kinetic energy of the polymeric chains (60) which can also significantly affect the drug encapsulation and release profile. Therefore, the physicochemical stability of such NPs in both acidic to basic pH in combination with various

Fig. 3. Percent cumulative drug release (% w/w) profile either in presence or absence of HAase at pH 7.1. **a** HA-gel and TFV control, **b** F13, **c** F1, and **d** F7 nanoformulations. Results are given as mean $\pm$ SD, ($n = 3$)

temperature conditions remained to be assessed to better understand the ultimate storage conditions and biological fate.

The effective molar concentration (EC_{50}) of TFV required for *in vitro* anti-HIV activity varies between 5 μ M and 7.4 μ M (61). The HIV virus crosses the CV mucosa barrier in 2–6 h and interacts with the macrophages and dendritic cells (62). Ideally, the amount of TFV required to exhibit its activity should be released from the NPs before the HIV virus crosses the vaginal mucosa between 2 and 6 h. It is noteworthy that although $\sim 30\%$ w/w of the drug is released in 3–6 h, the EC_{50} value of TFV from the optimized formulation (F13) is achieved in that critical timeframe in the presence of HAase; whereas, more than 6 h is required in the absence of HAase. Nevertheless, the potential timely responsiveness and efficacy of these NPs in HIV prevention remain to be confirmed in future *in vitro* and *in vivo* studies.

The higher %EE and DL associated with F13, compared to F1 and F7, can be explained by the effect of the lower cross-linking density of carboxylic acid groups of HA in F13. Thus, there would be higher electrostatic interaction of the positively charged amino group of TFV and the negatively

charged carboxylate group of HA. Also, the introduction of cross-linking into native HA produced porous surfaces, while native HA has a fibrous and irregular structure (63). This might also promote the diffusion of TFV inside the HA matrix and increased the % EE and DL of the NPs. The porosity was further affected by high molar concentration of EDC in the F13 compared to the ADH as previously explained (64).

However, there are certain limitations associated with the current design, for example the HAase triggered drug release from the HA-NPs takes about 24 h to reach 90% w/w instead of desired timeframe of 2–6 h. This might be due to the low concentration of HAase used (only 1.08 U) to mimic the *in vivo* conditions, based on average volume of human ejaculate containing 100 million sperms/mL. The HAase amount is significantly dependent on the sperm density in seminal fluid (15) which varies from 10 to 250 million sperms/mL (65). It is reasonably speculated that the effect of HAase on NPs would be higher with the person having higher sperm count and thus enzyme concentration in clinical practice. Moreover, the HAase activity would further be enhanced

when the normal acidic pH the vagina will be restored after sexual intercourse since HAase has higher activity at acidic pH compared to neutral pH. In addition, incorporating an enzyme-catalyzing agent (e.g., polyamines) as one of the formulation variables (66) in the future nanoformulations could further enhance the HAase activity.

Kinetics of Enzymatic Degradation and *In Vitro* Drug Release Profile from HA-NPs and HA-Gel

The drug release from the NPs was analyzed using various *in vitro* kinetic models as given by Eqs. (4), (5), (6), and (8), respectively. The parameters obtained, based on the release kinetic models, are given in Table III. It was observed that in the absence of HAase the drug release from F13 and F1 followed predominantly the Peppas model, whereas in the presence of HAase, F13 and F1 followed first-order and Peppas models, respectively. The drug release kinetics from HA-gel followed first-order model (r^2 : 0.97), whereas drug release from F7 was predominantly governed by the combination of zero- and first-order kinetic models, either in the presence or absence of HAase.

To determine the drug release mechanism from NPs and gel formulation the Korsmeyer–Peppas model is applied. The decision to use the Korsmeyer–Peppas model is based on its simplicity and efficiency in analyzing the drug release mechanism when more than one form of mechanisms are involved. It is observed that the drug release from F13 and F1 occur by Fickian diffusion ($n < 0.5$), whereas drug release from F7 predominantly followed anomalous transport (diffusion and erosion controlled release) mechanism since $0.5 < n < 1$. The drug release from the HA-gel predominantly followed Super Case-II transport (erosion of polymeric chain and degradation of HA) mechanism since $n > 1$. The f_1 and f_2 values (46) are calculated using Eqs. (9) and (10), respectively, and shown in Table III. The results indicated that there was a significant difference between the HA degradation and drug release kinetics from HA-gel and HA-NPs either in the presence and absence of HAase. Overall, the NPs provide a better control on the drug release compared to the HA-gel. The study suggests that ultimately suspending the TFV-loaded NPs in TFV-loaded HA-gel formulation might be an alternative formulation for effective prevention of the HIV virus transmission. In this case, the gel would provide a bolus dose for immediate drug release before the HIV virus crosses

Fig. 4. **a** Surface morphology: scale bar 500 nm, and size distribution analysis of native HA-NPs (F13) by TEM and DLS analysis, respectively. **b** Surface morphology of formulation F13 in the absence of HAase, after day 1: scale bar, 500 nm, and day 3: scale bar, 200 nm. **c** Surface morphology of formulation F13 in the presence of HAase, after day 1: scale bar, 500 nm and day 3: scale bar 200 nm

the CV mucosa (in about 2–6 h) followed by a semen-triggered drug release from NPs for at least 72 h after sexual intercourse.

Table III. Estimated Parameters: *In Vitro* Drug Release Model and Degradation Kinetics of HA-NPs

HA-NPs	Zero-order release model	First-order release model	Higuchi release model	Korsmeyer–Peppas release model	Model independent analysis	
	K_0^a , (r^2)	K_1^a , (r^2)	K_H^a , (r^2)	k^a , (exponent n , r^2)	Enzymatic degradation f_1 , (f_2)	<i>In vitro</i> drug release f_1' , (f_2')
F13 ^b	1.30 (0.79)	0.08 (0.97)	12.7 (0.93)	0.18, (0.29, 0.93)	32.0, (12.9)	26.4, (13.3)
F13 ^c	0.46 (0.63)	0.01 (0.72)	4.8 (0.85)	0.12, (0.25, 0.98)	35.4, (28.6)	57.3, (–3.1)
F1 ^b	0.89 (0.75)	0.02 (0.88)	8.8 (0.94)	0.18, (0.34, 0.95)	29.7, (14.5)	40.9, (3.8)
F1 ^c	0.49 (0.72)	0.01 (0.80)	4.9 (0.92)	0.17, (0.32, 0.97)	36.8, (28.2)	62.3, (–4.9)
F7 ^b	0.81 (0.99)	0.01 (0.99)	7.1 (0.95)	0.02, (0.78, 0.97)	64.3, (–2.3)	61.4, (–1.5)
F7 ^c	0.47 (0.99)	0.01 (0.99)	4.1 (0.93)	0.02, (0.68, 0.97)	74.5, (12.5)	83.4, (–7.6)

^a Rate constant: K_0 in micrograms per hour; K_1 in per hour, K_H in micrograms per square root hour, k in per hour

^b Presence of HAase enzyme

^c Absence of HAase enzyme

Fig. 5. Cytotoxicity assays: Effect of HA-NPs; F1, F7, and F13 at the concentration of 1 mg/mL for each treatment. **a** Effect on the viability of VK2/E6E7 and End1/E6E7 cell lines (MTS assay). **b** Effect on the lactate dehydrogenase release from VK2/E6E7 and End1/E6E7 cell lines (LDH assay). **c** Effect on the viability of *L. crispatus* (*Lactobacillus* viability assay). Results are given after 48 h of incubation as mean $\pm$ SD, $n=3$. Asterisk (*) indicates the significant difference ($p<0.05$), compared with the positive control (1% Triton X)

Surface Morphology and Size Distribution Analysis of HA-NPs During the Enzymatic Degradation Process

The HA-NPs exhibited spherical shape and small PMD, as analyzed by TEM and DLS analyses, respectively (Fig. 4a). The surface morphology of the NPs during the enzymatic degradation process was analyzed for up to 3 days. As shown in Fig. 4b, the surface morphology in the absence of HAase did not change significantly; whereas, in its presence there was a significant change after 3 days of incubation (Fig. 4c) compared to that of native HA-NPs (Fig. 4a). This was potentially due to the enzymatic degradation of HA at the surface of the NPs, which generated the fragments of HA and caused its transition from globular to coil conformation (48).

Cytotoxicity Analyses and Effects of HA-NPs on Viability of *L. crispatus*

The HA-NPs did not show any statistically significant effect ($p>0.05$) on the viability of VK2/E6E7 and End1/E6E7 cells, compared to the cell culture medium (negative control) as demonstrated by MTS assay (Fig. 5a). Statistically insignificant ($p>0.05$) LDH release and lower percent cytotoxicity were observed from the cells incubated with the NPs for 48 h in comparison to the negative control as well as shown in Fig. 5b. The similar effects for MTS and LDS assays were observed with control formulations F1 and F7. As shown in Fig. 5c, the HA-NPs have no statistically significant effect ($p>0.05$) on the viability of *L. crispatus* bacteria compared to that of cell culture medium. However, there was a statistically significant difference between the treatments and the positive control (1% Triton X) effects. The

data demonstrated that the HA-NPs had negative effect on *L. crispatus* viability.

CONCLUSIONS

The HAase-sensitive HA-NPs loaded with TFV are formulated using FFED design. The NPs were found stable and exhibited a spherical shape as confirmed by TEM analysis. It was observed that HAase enzyme significantly triggered the degradation and drug release of the NPs. The NPs were non-cytotoxic to human vaginal cells and had no effect on *Lactobacillus* viability. Collectively, the results suggested that TFV-loaded HAase-sensitive HA-NPs templates developed in this study have the potential for topical delivery of microbicides for the prevention of sexually transmitted diseases such as HIV/AIDS after successful completion of safety and efficacy studies. In this respect, further *in vivo* studies would be undertaken to characterize the vaginal retention time, safety and immunological/inflammatory responses.

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