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Sodium Acetate Coated Tenofovir-Loaded Chitosan Nanoparticles for Improved Physico-Chemical Properties

Albert N. Ngo,

Laboratory of Future Nanomedicines and Theoretical Chronopharmaceutics, Division of Pharmaceutical Sciences, University of Missouri-Kansas City, New Health Sciences Building, 2464 Charlotte Street, Kansas City, MO 64108, USA

Miezan J.M. Ezoulin,

Laboratory of Future Nanomedicines and Theoretical Chronopharmaceutics, Division of Pharmaceutical Sciences, University of Missouri-Kansas City, New Health Sciences Building, 2464 Charlotte Street, Kansas City, MO 64108, USA

James B. Murowchick, and

Department of Geosciences, University of Missouri-Kansas City, 420 Flarsheim Hall, 5110 Rockhill Rd., Kansas City, MO 64110, USA

Bi-Botti C. Youan[✉]

Laboratory of Future Nanomedicines and Theoretical Chronopharmaceutics, Division of Pharmaceutical Sciences, University of Missouri-Kansas City, New Health Sciences Building, 2464 Charlotte Street, Kansas City, MO 64108, USA

Abstract

Purpose—It is hypothesized that sodium acetate (SA) can be used for *in situ* coating of drug loaded chitosan NPs for improved physico-chemical properties.

Methods—Tenofovir (TFV) is used as a model drug. Uncoated chitosan NPs are prepared by ionic gelation. SA is generated *in situ* from half neutralization of acetic acid with sodium hydroxide, and coats chitosan NPs during freeze-drying. The NPs physico-chemical properties [e.g. particle mean diameters (PMD) and zeta potential (ζ), EE%, drug release profile, morphology] are characterized by dynamic light scattering, spectrophotometry, Korsmeyer-Peppas model, transmission electron microscopy (TEM), respectively. Melting point (MP), non-aqueous titration, Fourier transform infrared (FTIR) analysis, and X-ray powder diffractometry (XRD) pattern evaluated the SA coated chitosan NPs. The NPs cytotoxicity on macrophages Raw 264.7 is assessed by neutral red, resazurin, nitrite oxide (NO) and cytokines assay.

Results—Collectively, FTIR, ζ , XRD, MP, and TEM data confirmed that SA coats chitosan NPs. The PMD range is 136–348 nm (uncoated) and 171–379 nm (coated) NPs. The ζ values range is +24.3–28.5 mV (uncoated) and 0.1–3.1 mV (coated). The EE% ranges from 5.5–11.7 % (uncoated NPs) and increased up to 8–17 fold (86.3–92.7% after coating). The SA also prevents NPs

[✉]Laboratory of Future Nanomedicines and Theoretical Chronopharmaceutics, Division of Pharmaceutical Sciences, University of Missouri-Kansas City, New Health Sciences Building, 2464 Charlotte Street, Kansas City, MO 64108, USA. youanb@umkc.edu, Tel: 816-235-2410, Fax: 816-235-5779.

aggregation's during the freeze-drying. The core-shell NPs exhibited a sustain release of TFV following anomalous transport mechanism ($R^2 \sim 0.99$). Coated NPs are non-cytotoxic (cell viability $\sim 100\%$) and without any proinflammatory response.

Conclusions—These SA coated chitosan NPs may be useful for (i) efficient encapsulation, (ii) masking tastes, (iii) controlling the release and improving solubility of drug.

Keywords

Coated Chitosan Nanoparticles; Efficient encapsulation; Sodium acetate; Tenofovir; Water soluble drugs

INTRODUCTION

Sodium acetate (SA), is approved by European food control authorities, and is an edible salt that is added to food as a seasoning. It has a wide range of applications. Firstly, it is used as antibacterial additive and preservative in food compounds; it is a inhibitor of gram-negative bacteria, and fungi that grow in food (1–3). Secondly, oysters shelf-life can be extend through dipping in SA (4). SA is generally prepared by mixing an aqueous solution of sodium carbonate or hydroxide with an aqueous solution of acetic acid. The recovery of SA, in this aqueous solution, is generally either by crystallization or evaporation using techniques such as spray drying (5). Beside its widespread use in food industry, up-to-day, little is known about the coating property of SA for pharmaceutical nanoformulations.

Chitosan is a polysaccharide obtained from the deacetylation of chitin and have been used as a nanocarrier for novel drug delivery system because of its biodegradable and biocompatible properties(6). Among chitosan based nanocarriers, chitosan crosslinked with polyanion triphosphate (TPP) based nanoparticles (NPs) have been widely used for the nanoencapsulation of HIV/AIDS microbicide such as tenofovir (TFV) (7, 8). The solubility in water, logP and its oral bioavailability of TFV are 13.4 mg/mL, -1.1 , and, 25–39%, respectively. TFV is a BCS class III drug(9). *The phosphonic acid group of TFV has two pKa values of 2 and 6.8, respectively (10). Below pH = 5, the two basic amino groups are protonated and thus positively charged and the phosphonic acid group has a charge of -2 above pH = 7 (11).*

However, the nano-encapsulation process using chitosan-TPP ionic gelation, and a highly water soluble drug such as TFV, has several limitations. *Firstly*, the encapsulation efficiency (EE %) of a highly water soluble drug, such as TFV is typically very low. For instance, Meng and al. encapsulated only 5.83% of TFV in chitosan NPs (7). *Secondly*, chitosan NPs exhibits an initial burst release (12) leading to a failure to sustain release, and to protect drugs. *Thirdly*, the freeze drying process is not effective for chitosan based NPs in absence of cryoprotectant. This leads to the aggregation of NPs (13)

It is hypothesized that *in situ* formation of SA, can be used to uniformly coat chitosan NPs and dramatically increase the physico-chemical properties (e.g. improvement of the freeze drying process, EE %, non-aggregation of NPs without the use of cryoprotectant, physical stability, and sustained drug release profile). This hypothesis is tested with supporting

physico-chemical characterization of the NPs (e.g. particle mean diameters (PMD), EE%, zeta potential (ζ), Fourier transform infrared spectroscopy (FTIR), X-ray powder diffractometry (XRD), and transmission electron microscopy (TEM)), and in vitro cell culture for cytotoxicity assessment (e.g. assessment of cell membrane integrity, mitochondrial activity, assessment of nitric oxide (NO) or cytokines production). In addition, both the non-aqueous titrations (*perchloric acid and lithium methoxide titrations*) and the melting point assessment of the pure salt, SA used as a control, and prepared in this study using the freeze drying technique provide an additional proof to confirm the nature of the salt coated chitosan NPs.

MATERIALS

Chitosan, high molecular weight (source: crab shells, % deacetylation degree > 75%, viscosity = 800 – 2000 cps), sodium diacetate (SD), lipopolysaccharide (LPS), sodium acetate anhydrous (SAA), sodium acetate trihydrate (SAT), lithium methoxide (LM) in methanol (1M), methanol, acetonitrile, acetic anhydride, perchloric acid (PA), crystal violet, calcium chloride dihydrate, Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified eagle medium (DMEM), neutral red (NR), acetic anhydride, and *potassium hydroxide* are purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium triphosphate pentabasic (Na₅TPP), hydrochloric acid, sodium hydroxide, ethanol, and glacial acetic acid reagents are supplied by Fisher Scientific (Pittsburgh, PA, USA). The murine macrophage RAW 264.7 (TIB-71) and fetal bovine serum (FBS) are purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Tenofovir (TFV) is purchased from Pichemicals (Zhang Jiang Hi Tech Park, Shanghai, China). Griess reagents is supplied by Promega (Madison, WI, USA).

All chemicals used in the study are of analytical grade and used as received without further purification.

Sodium acetate (SA), the pure salt used as control is currently prepared *in situ by sublimation*, in this study, using the freeze drying technique after half neutralization of glacial acetic acid with sodium hydroxide.

METHODS

Method of preparation of chitosan-TPP nanoparticles

Chitosan-TPP NPs is prepared according to ionic gelation method (7) with a modification of the process. Firstly, chitosan is dissolved in 2% V/V aqueous solution of glacial acetic acid (pH~2.3) so that the final concentration of chitosan is 2 mg/ml for 24 hours. Secondly, volume (V) ~2.2 ml of sodium hydroxide (2M) is added to 20 ml of the aqueous chitosan solution to raise the pH of the solution from ~2.3–2.94 to 4.76 which is the pKa of acetic acid to produce enough acetate ion while keeping the pH of the solution acidic a crucial condition to keep both, protonating the amino groups of chitosan polymer, and soluble chitosan polymer. Thirdly, 2 mg, 4 mg and 6 mg of TFV powder are added to 3 different beakers containing, TPP aqueous solution (V = 4 ml, 2mg/ml) respectively along with a blank formulation to form the mixture TPP-TFV aqueous solution. The pH is adjusted in the

range (5.60–5.99) with a few drop of hydrochloric acid (2M) to minimize hydroxide ion amount in TPP aqueous solution respectively. Then, the mixtures TPP-TFV aqueous solution (V=4 ml), with a total amount of TFV = 2 mg, 4 mg, and 6 mg are added dropwisely into three chitosan solutions (pH = 4.76, V = 20 ml) and named formulation F1, F2 and F3, respectively, along with a blank formulation as shown in Table I. Thus, chitosan NPs is formed instantaneously by ionic gelation. *At this stage of the process, the uncoated NPs is formed.* Fourthly, after 2–6 hours of continuous stirring, the colloidal solution, is directly frozen at –20°C for 12 hours, *without centrifugation to avoid the loss of TFV in the supernatant and subsequently freeze dried using the freeze dryer Freezone model* (Labconco corporation, Kansas City, Missouri, USA) without any addition of cryoprotectants. *The coated NPs is obtained after the freeze drying process.*

The schematic representation of the process of preparing coated chitosan NPs with SA is shown in Fig. 1.

Particle size and zeta potential analysis

Uncoated chitosan NPs are suspended by sonication (Qsonica LLC, Newtown, CT, USA) *after centrifugation* in deionized water, or SA coated chitosan NPs are straightforwardly dissolved in deionized water. Particle size expressed as particle mean diameters (PMD), and polydispersity index (PDI) are measured through dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments Ltd, Worcestershire, UK) at the temperature of 25°C. Samples with PDI < 0.05, are considered monodispersed according to the National Institute (7).

The zeta potential (ζ) of both, uncoated and coated NPs suspended in deionized water is determined by the zeta potential analysis mode of the instrument. Nanosphere™ size standard (59 ± 2.5 nm) and zeta potential standard (68 ± 6.8 mV) are used to calibrate the instrument before the ζ analysis.

Encapsulation efficiency (EE %) determinations

EE% determination of uncoated chitosan NPs—The final amount of TFV entrapped into the uncoated chitosan NPs after centrifugation is calculated from the difference between the total amount of TFV initially used and the amount of drug found in the supernatant after encapsulation process. The free drug amount in the supernatant is measured using UV spectrophotometer (Spectronic Genesys 10 Bio, Thermo Electron Corporation, WI, USA) at a wavelength of 260 nm. The drug EE% is calculated as follow:

$$EE\% = \frac{\text{Total amount of TFV} - \text{free TFV}}{\text{Total amount of TFV}} * 100 \quad (1)$$

The standard curve of TFV absorbance (Y), used is: $Y = 0.0448X$ ($R^2 = 0.9994$), where X = concentration of TFV (μg/ml).

EE% determination of coated chitosan NPs—The amount of TFV entrapped in the core-shell NPs after freeze drying, is measured at 260 nm using the above UV spectrophotometer. Briefly, 1 mg/ml of different formulations (F1, F2, and F3), along with

the appropriate blank are dissolved in 2 M HCl up to 23 hours at room temperature and followed by 1 hour of sonication *to accelerate the dissolution of chitosan NPs* using the sonicator (Qsonica LLC, Newtown, CT, USA). Then, the dissolved NPs solutions are centrifuged (14, 000 rpm, 10°C) for 20 min using refrigerated microcentrifuge (VWR, Radnor, PA) *to remove any eventual traces of undissolved debris of chitosan NPs*. Finally, the total amount of TFV entrapped, is recorded at 260 nm along with the appropriate blank, used to setup the baseline. The drug EE% is calculated as follow:

$$EE\% = \frac{\text{Total amount of TEV entrapped}}{\text{Total amount of TFV}} * 100 \quad (2)$$

The same standard curve of TFV (equation 1) is used to assess the total amount of drug in the NPs because, the media does not shift the calibration curve based on preliminary screening.

Fourier transform infrared (FTIR) spectroscopy—The FTIR is used to confirm the chemical nature of the salt coating chitosan NPs after freeze drying. Powdered samples are deposited on the crystal for analysis at room temperature.

The spectra are recorded on an Agilent Cary 630 FTIR spectrometer with a software microlab version 4 (AGILENT Technologies, Santa Clara, CA, USA) in a wavenumber range of 500–4000 cm⁻¹, and at a resolution of 4 cm⁻¹.

X-ray powder diffractometry (XRD)—XRD is carried out to pinpoint the crystallinity of the NPs coated with SA. The powder XRD scans are performed using a MiniFlex automated X-ray diffractometer (Rigaku, The Woodlands, TX) at room temperature. Ni-filtered Cu K-alpha radiation is used at 30 kV and 15 mA. The diffraction angle is covered from 2θ = 5° to 2θ = 60° with a step size of 0.05° /step, and a count time of 2.5 s/step (effectively 1.1° /min for approximately 46 minutes/scan). The diffraction patterns are processed using Jade 8+ software (Materials Data, Inc., Livermore, CA). The relatives intensities of the diffracted beams which are directed by the position of atoms can be estimated using the following equations (14):

$$I = |F|^2 p \left(\frac{1 + \cos^2 2\theta}{\sin^2 \theta} \right) \quad (3)$$

where, F is defined as follows,

$$F = \sum_i^N f_n e^{2\pi i(hu_n + kv_n + lw_n)} \quad (4)$$

where, I = relative intensities of the diffracted beams, F = Structure factor for hkl reflection in terms of atom position in 3 dimensions with defined coordinates (u, v, w), θ = Bragg angle, and p = the multiplicity factor.

Non aqueous titration of acetate ion in the salt with perchloric acid (PA)—PA

solution (0.015 M) in glacial acetic acid, used as a titrant, is used to determine the molar mass of the salt coating chitosan NPs. Three different samples, sodium acetate anhydrous (SAA), sodium acetate trihydrate (SAT) and sodium diacetate (SD) with a known molar mass (M) are used as a control to validate the molar mass of SA. Crystal violet (dye) (0.0005 % w/v, 100 µl), dissolved in acetonitrile, is used to determine the equivalence point. Briefly, approximately 10 mg of the appropriate salt is dissolved in 20 ml of acetonitrile, followed by addition of 100 µl of the dye, under continuous stirring. Then the amount of the acetate in salt is titrated with PA until the color of the solution changed from purple to green (15). The molar mass of the salt is calculated using the following equation assuming the purity of the salt is ~100%:

$$M = \frac{m}{C_a V_a} \quad (5)$$

where, M = the molar mass found of the salt, m = mass (g) of the salt C_a = concentration of PA (0,015 M), and V_a = volume of PA added to reach the equivalence point.

The molar mass M is corrected and term molar mass corrected (M_c) to find the true molar mass of the different salt using as a reference the known molar mass of SAA. The following equation allows us to find M_c :

$$M_c = M * \frac{\text{expected molar mass of SAA}}{\text{average molar mass found of SAA}} \quad (6)$$

Non aqueous titration of acetic acid content in the salt with lithium methoxide (LM) in methanol—LM solution (0.015 M) in methanol, used as a titrant, is used to titrate

the acetic acid content if any of the new salt SA dissolved in methanol (16). Glacial acetic acid in methanol (0.006 M, 20 ml), SAA~10 mg, SD~10 mg, dissolved in methanol are used as controls. Briefly, the appropriate salt (~10 mg, 20 ml) *dissolved in methanol* or glacial acetic acid solution (0.006 M, 20ml) diluted in methanol, is titrated with LM using a pH-meter. The titration curve, pH = f (Vb) of the change in pH values, due to the addition of the LM is plotted against the volume Vb; (Vb = volume of LM added). Microsoft Excel 2013 is used to fit the non-aqueous titration curve.

Method of determination of melting point (MP)—The melting point of SA salt is measured to determine its purity. Commercial available SAA, SAT, and SD are used as controls. Briefly the appropriate salt is packed into a Kimble Chase capillary melting point tube made of borosilicate glass, 1.5–1.8 × 90 mm, purchased from (Fisher Scientific, USA). The tube is gradually heated in a MEL-TEMP capillary melting point apparatus (Sigma Aldrich, USA) and the temperature is measured with a Fluke 51 II digital thermometer containing a thermocouple probe (Fluke, USA) as a range from the appearance of the first drop of liquid to a complete melt of the salt.

Morphological analysis—The surface morphology of both uncoated and coated chitosan NPs is visualized with the transmission electronic microscopy (TEM). To get the specimens, the drops of coated or uncoated NPs suspension are placed on a copper grid with a carbon support film and air dried. The NPs are viewed under a scanning transmission electron microscope CM12 (FEI, Hillsboro, OR, USA) at 80 kV accelerating voltage. Digital images are acquired with an ORIUSTM SC 1000 11 Megapixel CCD camera (Gatan, Pleasanton, CA, USA).

In vitro drug release study—*The respective amount of 25.00 mg, 12.50 mg and 8.33 mg of the formulation F1, F2 and F3 suspended in 4 ml of Tris-HCl buffer (9.1 mM, pH = 7.51) or citrate buffer (1M, pH = 4.2). These environments simulate the pH condition of the mixture of human seminal fluid and human vaginal fluid (HVF), and that of HVF alone, respectively (8). These suspensions are placed into a cellulose ester membrane dialysis bag (Spectra/Por® Float-A-LyzerG2, MWCO 3.5–5 KD, Spectrum Laboratories Inc. Rancho Dominguez, CA, USA). The dialysis bag is then dipped into a tube of 50 ml total capacity containing 24 ml of the appropriate buffer. The whole system is incubated in a thermostatically controlled shaking (50 rpm) water bath (BS-06, Lab Companion, Seoul, Korea) at 37 °C. At set time intervals (0, 3, 6, 9, 12, 24... 120 hours), 1ml of the buffer solution outside the dialysis bag is removed and replaced by fresh buffer solution to maintain a sink condition. The concentration of the drug released from the NPs in the outer tube solution is determined by a UV spectrophotometer at 260 nm as indicated in the EE% determination section. Each experiment is run in triplicate. In addition, the release curve is fitted with Korsmeyer-peppas model (17) to understand the release mechanism of TFV from the NPs, using the following equation;*

$$\frac{M_t}{M_\infty} = at^n \quad (7)$$

where, $\frac{M_t}{M_\infty}$ represents the fractional drug release, a is a constant combining structural and geometric features of the drug dosage form, and n typify the release mechanism (e.g. fickian diffusion ($n=0.5$); anomalous diffusion ($0.5 < n < 1.0$); case II transport ($n=1$) and super case II transport ($n > 1$)).

Macrophage RAW 264.7 culture—These cells are grown and maintained in a monolayer culture, in 75 cm² culture flasks (Techno Plastic Product, Switzerland), at 37°C in a humidified atmosphere of 5% carbon dioxide (CO₂) and 95% air.

Exposure protocol—The NPs are freshly suspended in DMEM/FBS 5% at 1000 µg/ml, dispersed by sonication (VWR, model 150 D; VWR International, West Chester, PA, USA) for 10 minutes, sterilized for 30 minutes under UV light (18), and diluted 1:1, 1:10, 1:100 and 1:1000. Macrophages RAW 264.7 (2×10^5 cells/100 µl/well) are seeded in 96-well culture plates (growth surface: 0.34cm²) and incubated for 48 hours. Then, the cells are subjected for 24 hours to the NPs at 1, 10, 100 and 1000 µg/ml corresponding to 0.3, 3, 30 and 300 µg/cm², respectively. Wells containing cells without NPs are used as the negative

controls. As a positive control, the macrophages are treated with lipopolysaccharide (LPS) (10 µg/ml), a well-known activator, to stimulate an inflammation (19).

Assessment of cell membrane integrity—The cell membrane integrity is measured using the specific accumulation of the vital dye neutral red (NR) in lysosomes (20). After exposure to the NPs, the cells are washed twice with Dubelcco's phosphate buffer saline (DPBS), and then 100 µl of fresh medium containing 50 µg/ml NR is added to each well and incubated for 3 hours. Then, the cells are washed twice with DPBS, and the dye is extracted with 1% acetic acid/50% ethanol (v/v). The plate is shaken for 15 minutes in the dark to solubilize all the NR crystals prior to the fluorescence intensity measurement (530–560 nm excitation, 590 nm emission) using a DTX 800 multimode microplate reader (Beckman Coulter, Brea, CA, USA).

Assessment of mitochondrial activity—The mitochondrial activity is examined using a resazurin assay (Sigma-Aldrich, St. Louis, MO, USA) (21). After exposure to the NPs, the cells are washed twice with DPBS, fresh medium is added, and then 10 µl of resazurin (0.1 mg/ml in DPBS) is added to each well. The assay plate is shaken for 30–60 seconds and incubated for 3 hours. Afterwards, the plate is shaken for 30–60 seconds prior to determining the fluorescence intensity (530–560 nm excitation, 590 nm emission) using the above microplate reader.

Assessment of intracellular nitrogen species: nitric oxide (NO)—NO production in culture supernatant, a measure of inducible NO Synthase (iNOS) activity, is examined via nitrite accumulation measurement, the stable end product of the autoxidation of NO in aqueous solution(20). After the indicated exposure period to the NPs, cell supernatants are collected and centrifuged at $1,000 \times g$ for 10 minutes to remove cellular debris and particulate materials. Then, 50 µl of the supernatant is placed into a new plate and mixed with the Griess reagents according to the manufacturer's instructions. Absorbance is measured at 540 nm using the microplate reader and nitrite concentration is calculated using the sodium nitrite standard curve.

Multiplex immunoassay analysis of cytokines secretion—After macrophages RAW 264.7 exposure to the NPs, cell free supernatants are harvested and analyzed for cytokines using a high-sensitivity multiplexed bead-based immunoassay (Milliplex MAP Mouse Cytokine/Chemokine Magnetic Bead Panel, Millipore Corp., Billerica, MA; and Luminex MAGPIX instrument, Luminex Corp., Austin, TX, USA). Supernatants from untreated cells (negative control) and LPS-treated cells (positive control) are also evaluated. Four cytokines, [e.g. interleukins (IL), including IL-1α, IL-1β, IL-6 and IL-7] levels are measured according to the manufacturer's protocol. Briefly, premixed magnetic beads conjugated to antibodies for all 4 analytes are mixed with equal volumes of supernatants (25 µl) in 96-well plates. Plates are protected from light and are incubated on a microplate shaker overnight at 4°C. Then, magnetic beads are washed twice with 200 µl of wash buffer, and detection antibodies are added to each well. The mixtures are incubated at room temperature for 1 hour. Streptavidin-phycoerythrin conjugate compound is added to each well, and the mixtures are incubated for 30 min at room temperature. The magnetic beads

are washed and resuspended in wash buffer for 5 min, and plates are assayed on the Magpix system with xPONENT software. The median fluorescence intensity is analyzed using a 5-parameter logistic method from a standard curve of each respective analyte to determine the concentration of the cytokines in supernatants. These assays are run in duplicate.

Statistical analysis—All values are expressed as mean $\pm$ standard deviations. One-way analysis of variance (ANOVA) in combination with all pairs Tukey's HSD (Honestly Significant Difference) post-test are used to find means of data that are significantly different from each other. All statistical analysis is carried out using JMP software version 10, (SAS Institute, Cary, North Carolina, USA). A P-value below 0.05 is considered statistically significant and allows the rejection of the null hypothesis.

RESULTS

Nanoformulation physico-chemical properties

Particle mean diameters (PMD), zeta potential (ζ) and polydispersity (PDI)

analysis—The PMD for the uncoated or coated blank and the three different formulations before and after freeze drying respectively, (F1, F2 and F3) and the ζ and PDI are shown in Table II. The PDI of the NPs is overall conserved before and after freeze drying (Table II) suggesting that the coating salt prevents aggregation of the NPs. However, the PMD *in general* increases slightly due to the additional thickness of the shell, (SA) on the core chitosan NPs as shown in Table II. Fig. 2 shows the uncoated NPs PMD distribution (Fig 2, A1, B1, C1, and D1), before freeze-drying and coated NPs PMD distribution after freeze-drying (Fig 2, A2, B2, C2 and D2), respectively.

Unlike the zeta potential (ζ) of the uncoated chitosan NPs, which is $\sim$ above + 25 mV (Table II), ζ of coated/freeze-dried NPs ($\sim$ neutral) is consistent with that of the pure salt further confirming coating by SA ($\zeta = -4.87 \pm 0.64$ mV).

Encapsulation efficiency (EE%) determination—The EE% of TFV for different formulations uncoated and coated before and after freeze drying are shown in Table II, respectively. The EE% of uncoated NPs are indeed very low (EE% = 5.5–11.7%) *due mostly to drug loss due to mass transfer of TFV toward the supernatant during the centrifugation process* (7) whereas those coated with SA, after freeze drying using the new process developed in this study are indeed high (EE% $\sim$ 86.3–92.7%) and improved by $\sim$ 8–17 fold.

Morphological analysis—Fig. 3 shows the visualization of the uncoated NPs (**A, B**) and coated NPs (**C, D**) before and after freeze drying respectively. The salt coating chitosan NPs (Fig. 3, D, E, and F) is clearly visible after freeze drying. The coating of SA onto chitosan NPs give a core-shell structure in which the shell is SA and the core is TFV loaded chitosan NPs or chitosan blank NPs as shown in Fig 1 and (Fig. 3 D, E, and F).

Determination of the melting point (MP)—The MP of the different salt of acetic acid are shown in Table III. The melting point of SA (salt prepared using this new method) is identical to the melting point of SAA (supplied by company) suggesting that both SAA and SA are made almost of the same main element which are acetate *anion* and sodium *cation*.

Non-aqueous titration of acetate with perchloric acid (PA)—To further elucidate and confirm the nature of the salt coating the NPs additional analyses are done. SAA, SAT, and SD, are used as controls to find the molar mass (M) of the salt (SA) coated (chitosan NPs using PA as a titrant.

Molar mass corrected (M_C) and M and of the different salt after titration with PA are shown in Table III. M_C (SAA) ~ 82.03 g/mol is identical to M_C (SA) ~ 80.38 g/mol, whereas M_C (SA) is statistically different to both M_C (SAT) and M_C (SD) based on ANOVA test. This result suggests that the composition of SA in term of acetate *anion* and sodium *cation* is similar to that of SAA and not SD (sodium diacetate) although, SA is prepared from half neutralization of acetic acid with sodium hydroxide followed by freeze drying.

Non aqueous titration of acetic acid content in the salt with lithium methoxide (LM)—Fig. 4 shows the titration curve of different salts and acetic acid glacial in methanol with LM used a titrant. The titration curve of both SAA and SA with LM overlaps suggesting that the two salt are the similar in term of acetate ion. There is no inflexion point, whereas the titration curve of both acetic acid in methanol, and SD has an inflexion point. This suggests SD contains indeed acetic acid whereas both SAA and SA don't contain acetic acid.

In vitro drug release profile—Fig. 5 shows the release profile of TFV from different nanoformulations. Table IV gives the value of “n” that characterized the release mechanism of the TFV from the NPs. Sustained release of the drug occurs over a period of 5 days. The drug release mechanism is anomalous transport based on Korsmeyer Peppas model.

FTIR spectral analysis—Fig. 6 shows the FTIR result of the individual component (A = TFV, B = TPP, C = chitosan) used for the preparation of TFV loaded chitosan NPs as well as the three physical mixtures (D = P1, E = P2, F = P3). Indeed, P1, P2, and P3 are the physical mixtures of the three main ingredients, namely TFV, TPP and chitosan, respectively. In Fig. 6, the FTIR spectra of P1, P2, and P3 show the presence of the individual component TFV, TPP and chitosan respectively. The FTIR spectrum of G = SAA is identical of the FTIR spectrum of H = SA. The FTIR spectrums of coated NPs I = “blank”, J = F1, K = F2, and L = F3 are identical to the spectrum of the pure salt SA after lyophilization. Thus, these further confirm the deposition of SA on the surface of chitosan NPs.

The two bands (Fig. 6 G–L) on the FTIR spectrum at 1572.89 cm^{-1} and 1411.55 cm^{-1} derived from the resonance of electron within the carboxylate ion COO^- of SA. This observation is expected because these bonds are transitional between single and double bonds (22). The antisymmetric stretch and symmetric stretch of single bond C-O is found at 1042.48 cm^{-1} and 921.37 cm^{-1} respectively (22). The bands at 3000.04 cm^{-1} and 2940.67 cm^{-1} are also the antisymmetric stretching vibration of CH_3 , and overtone transition from the ground state to the second excited state of the CH_3 symmetric distortion, respectively. These results well characterize SA coating because antisymmetric characteristic IR absorption stretch frequency of both carboxylate (COO^-), and single bond CO present in SA chemical structure are usually higher than to that of symmetric stretch.

X-ray powder diffractometric (XRD)—Fig. 7 shows the XRD pattern of the individual component (**A** = TFV, **B** = TPP, **C** = chitosan) used for the preparation of TFV loaded chitosan NPs as well as the three physical mixtures (**D** = P1, **E** = P2, **F** = P3). P1, P2, and P3 respectively. The physical mixtures P1, P2, and P3 (Fig. 7 D, E, and F) show peaks for the individual components, chitosan, TFV, and TPP, but they don't show peak for SA. This is to be expected for a dry physical mixture of components. The XRD pattern of coated NPs **I** = "blank", **J** = F1, **K** = F2, and **L** = F3 are identical to the pattern of **H** = SA after lyophilization. This confirms further the coating of the salt SA on the surface of chitosan NPs.

Nanoformulation cytotoxicity assessment

Assessment of cell membrane integrity—Fig. 8 A shows the result of the membrane integrity of the cell. The NPs are investigated for their effect on plasma membrane integrity using the NR assay, which enables to distinguish between viable, damaged, or dead cells. The specific accumulation of NR in lysosomes is dependent of an intact plasma membrane and functioning lysosomes. As *shown* in Fig. 8 A, the four nanoformulations (F0 or blank NPs, F1, F2, and F3) do not compromise the cell membrane integrity of the macrophages, as no significant disruption of NR cell uptake is noted, based on both ANOVA test and all pairs Tukey's HSD test. In contrast, upon exposure to the positive control (LPS), a dramatic loss of cell membrane integrity is observed (~ 81%, $p < 0.001$).

Assessment of mitochondrial activity—Fig. 8 B shows the mitochondrial activity of the cell. Resazurin assay is used to evaluate the mitochondrial activity. As illustrated in Fig. 8 B, following 24 hours exposure, the four nanoformulations (F0, F1, F2, and F3) do not cause any impairment of mitochondrial activity in the macrophages based on both ANOVA test and all pairs Tukey's HSD test. In contrast, the positive control (LPS) induces a dramatic decrease of mitochondrial activity by ~98 % ($P < 0.001$).

Assessment of NO production—Fig. 9 shows the nitric oxide released from the cell. The NPs are tested for their potential action in inducing cellular NO production which is considered as a sensitive biomarker for pro-inflammatory response associated to macrophage activation. The secretion of NO by RAW 264.7 cells in the supernatant culture medium is quantified, by measuring nitrites accumulation. As shown in Fig. 9, in the absence of a stimulator the basal levels of nitrites in RAW 264.7 cells are 7.4–8.27 μM . In response to 24 hours stimulation by LPS, inducible NO Synthase (iNOS) is strongly induced in the macrophages as evidenced by the significant accumulation of nitrites in cell culture supernatant 22.54–25.50 μM (Fig. 9) compared to controls (untreated cells) ($P < 0.001$). For the four nanoformulations (F0, F1, F2, and F3), there are no significant effect on nitrite production compared to the basal level (Fig. 9).

Multiplex immunoassay analysis of cytokines secretion—The release of selected cytokines is shown in Fig. 10. The results indicate that in the absence of a stimulator (untreated cells) the basal level of cytokines IL-1 α , IL-1 β , IL-6 and IL-7 is the range of 0.70–5.28 pg/mL (Fig. 10). As expected, LPS-stimulated macrophage highly produces cytokines ($P < 0.001$) compared to untreated cells as follows: IL-1 α (12 fold), IL-1 β (15

fold), IL-6 (8x104 fold), IL-7 (1.5 fold) as shown in (Fig. 10). For the four nanoformulations (F0, F1, F2, and F3), there are no significant production of cytokines compared to the basal level.

DISCUSSION

In this study, sodium acetate (SA) is successfully used to coat chitosan NPs for improved physico-chemical properties. After a thorough, and initial screening, the following unique and novel process is developed to *in situ* coat chitosan NPs with SA to overcome limitations of classical ionic gelation method in four steps. Each step of this preparation process of SA *in situ* coated chitosan NPs is indeed crucial for both the formation of chitosan NPs, and its successful coating by SA.

In the first step, the effective dissolution of chitosan and protonation of its amino groups occurs only in acidic environment (e.g. 2% v/v acetic acid aqueous solution, (pH~2.3–2.94, V = 20 mL, “see method section”) (23). Therefore, SAA salt aqueous solution with a basic pH cannot not be used as an alternative solution to dissolve chitosan polymer.

The second step requires dropwise addition of sodium hydroxide aqueous solution to raise the pH of the chitosan aqueous solution to 4.76 which is the pKa value of acetic acid (24). This enables a selective deprotonation of half glacial acetic acid and produces half acetate ion according to Henderson Hasselbach equation while (25) concomitantly increasing sufficiently the amount of sodium cation in the medium. The pKa value of amino acid groups of chitosan is ~6.3 (26). Based on Henderson Hasselbalch equation, at pH= 4.76 the amino groups of chitosan polymer is still highly protonated.

The third step requires the decrease of the pH of the aqueous solution of TPP in the range of 5.5–5.99. This pH decrease avoids *not only* the competitive binding between hydroxide ion (OH⁻) and TPP, formed instantaneously after the dissolution of Na₅TPP in deionized water (pH > 9) (27) *to the protonated amino groups of chitosan*, and *also it also maintains the full ionization state* of TPP for efficient ionic gelation process. Indeed, triphosphoric acid, the acid form of the polyanion TPP can undergo as many as 5 dissociations, and therefore has 5 pKa values (pKa1 = 1; pKa = 2.2; pKa3 = 2.3; pKa4 = 3.7 and pKa = 8.5) (28). That means, at pH = pKa4 + 2 = 5.7, TPP is almost fully ionized based on Henderson Hasselbalch equation (29) and may crosslink with protonated amino group of chitosan polymer through electrostatic attraction. At this step of the process, the TPP aqueous solution or mixture with the drug (TFV) is also added dropwise into chitosan aqueous solution. The color of the solution changes from colorless to milky (Tyndall effect) indicating the instantaneously formation of the uncoated chitosan NPs through ionic gelation (Fig. S1 B) (30). The EE % of TFV in uncoated chitosan NPs is indeed low before freeze drying (Table II). In fact, the highly water soluble drug escapes the NPs compartment during the centrifugation favoring its concentration in the supernatant phase. This limitation of the drug encapsulation is overcome by avoiding the centrifugation step (7) and by freezing the TFV loaded chitosan NPs suspension at (–20°C), as described in the above method section.

The fourth step is the *in situ* coating on the surface of chitosan NPs by SA during the freeze drying process. Indeed, on the phase temperature-composition diagram, the eutectic point of the binary systems comprised of water and acetic acid, is reached at -26.7°C with 59% w/w acetic acid (31). The triple point of water occurs at the pressure 6.12 mBar and at the temperature of 0.01°C (32) and that of acetic acid occurs at the pressure of 12.7 mbar and at the temperature of 16.68°C , respectively(33). Thus, at the used freeze drying operating condition (pressure ~ 0.06 mBar, and temperature -48°C) with ~ 2.1 % w/w acetic acid, below both the eutectic and triple points, any mixture of ice and acetic acid is solid (31) and are co-sublimated during the lyophilization process. There is a highly uniform, and dense deposition of SA salt on the surface of the NPs (Fig. 3 D, E, and F). The *in situ* formation of SA is due to the electrostatic attraction between the negatively charged acetate *anion* from deprotonated acetic acid and the positively charged of sodium *cation* generated mostly from sodium hydroxide (2M). In fact, this electrostatic attraction between acetate and sodium ions follows Debye-Hückel theory which states that, in a solution(e.g. before freezing the NPs suspension), near a given ion, counter ions are likely to be found and vice versa (34). Thus acetate and sodium ions are found close to each other around chitosan NPs in the nanosuspension. In addition it is well documented that SA has a hydrotropic behavior in aqueous solution with CH_3 , the hydrophobic part, oriented at the solution vapor interface and COO^- , the hydrophilic part, pointed toward the aqueous phase (35). Upon freeze drying, the SA salt is spontaneously formed *in situ* through electrostatic attraction between acetate and sodium on the surface of chitosan NPs. This coating of SA onto chitosan NPs may be attributed to the dual intrinsic surface active and efflorescent behaviours of SA. In fact water and acetic acid are sublimated during the freeze drying and SA migrates onto the porous chitosan NPs to form a coating. The dynamic of adsorption of SA onto chitosan NPs may also be enhanced by both the vacuum (low pressure and temperature) created during the freeze drying. There may be also the “shielding” attraction effect between uncoated chitosan NPs ($\zeta \sim +25$ mv) and SA ($\zeta = -4.87 \pm 0.64$ mV). This new core-shell nanosystem has a core made of TFV loaded chitosan NPs, and the shell made of SA. There is a very visible, distinctive, uniform, and unique coating layer of SA on the surface of chitosan NPs (Fig. 3 D, E, and F).

There are different crystal structure of monobasic acetic acid salt. For instance sodium diacetate (SD) crystalizes in the cubic system (space group = $\text{Ia}\bar{3}$)(36), whereas sodium acetate trihydrate (SAT) crystalizes in the monoclinic system (space group = C2/C) (37). The relative intensities (I) of the two salts (SAA, SA) appear at the same double Bragg's angle (2θ) as shown in (Fig. 7 G and H). The angular positions of both SAA (Fig. 7 G) and SA (Fig. 7 H) of the diffracted beams which defined the shape and size of the unit cell are almost the same as shown in Fig. 7 (38). Thus, both SA and SAA are qualitatively identical as shown in Fig. 7. The XRD results are also consistent with the FTIR and the TEM results strongly confirming the coating of the chitosan NPs with SA. Future crystallographic study will be performed to determine the space group and lattice parameters of SA.

These results are in agreement with the melting point data, used to assess the purity of the new salt. The melting point range of SA is almost identical to the melting point range of SAA (Table III). These results are also consistent with the titration of SA with both PA and LM used as titrants (Table III). Mc (SAA) is identical to Mc (SA). In addition, the titration

curve of both SAA and SA almost overlaps (Fig. 4). This suggests that the salt coating chitosan NPs is indeed SA. The titration curve which does not have an inflexion point (Fig. 4), suggesting that the salt does not contain any acetic acid.

Collectively, the FTIR, XRD, TEM, ζ , MP, and non-aqueous titration data indicate that, the shell coating chitosan NPs is indeed SA salt.

The SA salt coating chitosan NPs exhibits at least four potential advantages.

Firstly, it dramatically increases the EE% of TFV by 8–17 fold (Table II). Indeed there is no drug loss *in the supernatant* because, both the centrifugation for collection and washing of NPs steps are avoided respectively *after the ionic gelation prior freeze drying* (7). But rather, the aqueous solution of acetic acid is leveraged for the efficient encapsulation of TFV. Indeed the *in situ* coating with SA, on the surface of chitosan NPs allows the efficient entrapment of TFV in the core chitosan NPs. *In fact, chitosan NPs suspension is immediately frozen after the step 3* (Fig. 1) *and the removal of both water and acetic acid by sublimation during freeze drying prevents the outward drug diffusion. Concomitantly, during the freeze drying, the intrinsic surface active and efflorescent properties of SA constraint TFV to remain entrapped in chitosan NPs core during the self-assembly of the NPs shell as explained above. This enhanced EE% is not due to the charge of TFV at the final working pH 4.76 condition. Indeed, at this pH, the EE% of uncoated NPs is only 5.5–11.7% because the drug water solubility outweigh its charge-charge interaction with the polymeric matrix and the drug is mainly lost by mass transfer in the supernatant even though the two basic amino groups are positively charged and the phosphonic acid group of TFV has a charge of –1 at pH 4.76* (10, 11)

In (Fig. 6 J, K, and L), the FTIR spectrum for the three formulations shows only the spectrum of SA but not those of TFV. Therefore, TFV is mainly entrapped in the core of NPs. This result is also consistent with the XRD pattern (Fig. 7 J, K, and L) showing only the pattern of SA. These results are consistent with the morphology analysis showing SA coating TFV loaded chitosan NPs (Fig. 3 D, E and F).

Secondly, the coating prevents the aggregation of chitosan NPs [Fig. 3, Fig. S2 B and Table II (PMD, PDI)], whereas there are a formation of a “*cake*” of the “blank” and the three formulations (F1, F2, and F3) using the commonly ionic gelation process after freeze drying (Fig. S2 A) without any use of cryoprotectant in both processes. The non-aggregation of the core-shell NPs may be attribute to both the hydrotropic and efflorescent properties of SA, and the decrease of the surface tension between coated chitosan NPs which could be correlated to the decrease of the ζ by 9–20 fold compared to uncoated chitosan NPs *formed at the step 3 of this specific process* (Table II). The PDI of the NPs is overall conserved after freeze drying as shown in Table II.

Using potassium hydroxide as an alternative base to neutralize acetic acid, there is also no compact aggregation or “caking” of chitosan NPs after freeze drying. Future studies will elucidate if this process applies to other salts of carboxylic acid (5).

Thirdly, the coating NPs exhibits sustained release of TFV as shown in Fig. 5, thus avoiding burst release compared to the uncoated NPs. *It is counter intuitive that, there is a sustained release profile of free TFV solution extends over a period of 20–24 hrs. It is noteworthy that similar release profile was obtained with other drug solution such as diclofenac solution (MW 296 Da) using a cellulose membrane (MWCO 12 kDa). In that study, the release period of solution of diclofenac was also extended over 24 hrs (39). These observations may be attributed to the release rate limiting effect resulting from the interference of the dialysis bag membrane which may slow down the drug diffusion towards the outer phase thus prolonging the time to reach drug concentration equilibrium on either side of the membrane. However, in the same condition and considering the dialysis membrane retardation effect, it clearly appears that the coated NPs exhibited a more sustained release kinetics compared to those of native drug solution and uncoated chitosan NPs for the same amount of drug in the media (~110 µg).*

Based on Korsmeyer Peppas model, (17) the release mechanism is an anomalous transport with $0.61 < n < 0.86$, for the three different formulations (F1, F2 and F3) as shown in Table IV. Therefore, the release of TFV from the chitosan NPS (core) followed a combination of both a fickian-controlled drug release and a swelling-controlled drug release (40) under continuous erosion of SA (shell structure). This erosion is relatively slow and time dependent. SA layer is still visible after 24 hours of incubation in the release media at 37°C (Fig 3 E and F). The thickness of the coating layer can be controlled by acting on the initial concentration of glacial acetic acid aqueous solution and NaOH solutions. Thus, this finding can be potentially used to control and sustain or delay release of bioactive agent in the biological matrix.

Fourthly, it is quite impossible to disperse any portion of the freeze dried un-coated chitosan-TPP “cake” (Fig. S3), in deionized water whereas, the SA coated NPs can easily be dispersed in deionized water with no additional surfactant addition. The possible dispersion of the coated NPs might be explained by the surface active and hydrotropic properties of SA (35) whereas the non-possible dispersion of the “cake” might be explained by the very poor aqueous solubility properties of chitosan at pH 6 or above (41).

Based on International Organization for Standardization (ISO) ISO 10993-5 for cell viability, with 100 % viability assigned to the control, products with cell viability higher than 80% are not cytotoxic. Cytotoxicity level is classified as weak within 80%–60% ; moderate within 60%–40% and strong below 40% respectively (42). Thus, under the current experimental conditions, LPS induces a strong cytotoxicity including, cell membrane damage (viability~19%) (Fig. 8 A), and mitochondrial deficiency (viability~2%) (Fig. 8 B), associated high production of pro-inflammatory mediator NO (Fig. 9) and cytokines IL-1 α , IL-1 β , IL-6 (Fig. 11). In contrast, SA coating chitosan NPs appears to be non-cytotoxic to the macrophages as the core-shell NPs do not impair the cell membrane integrity (viability ~100%) (Fig. 8 A). In addition, the mitochondrial activity is maintained intact (viability ~ 100%) (Fig. 8 B). Moreover, the NPs do not neither induce a proinflammatory response associated to macrophage activation as reflected by a low level of both NO (Fig. 9), and low level of cytokines production (Fig. 10), which are comparable to the basal level.

It is noteworthy that IL-7 has been reported to facilitate HIV transmission through sexual intercourse (43) Thus, this finding suggest that the NPs may not promote HIV infection which could support their potential as a drug delivery template for topical/vaginal microbicide.

CONCLUSION

This study demonstrated for the first time that sodium acetate (SA) can be used to uniformly coat *in situ* chitosan NPs during ionic gelation for improved physico-chemical properties. The coated NPs exhibits higher encapsulation efficiency (86–93%) of water soluble drug such tenofovir. The release of TFV is sustained and the release mechanism is anomalous transport. This unique coating strikingly prevents the chitosan NPs aggregation during the freeze drying process. These NPs are non cytotoxic to the macrophage cell line.

The potential application of these findings are countless; this unique, edible, and highly reproducible coating salt may be potentially useful for, (i) masking the unpleasant taste, (ii) increasing the shelf-life by protecting against moisture, light and microbial growth (preservative), (iii) preventing enzymatic degradation or maintaining activity of proteomics and genomics derived bioactive agents(iv) controlling, sustaining or delaying release of bioactive agent in biological matrix, (v) improving solubility of poorly water soluble drug. Future study will be done to assess those properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

DISCLOSURES

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Abbreviations

ANOVA	analysis of variance
BCS	biopharmaceutics classification system
DPBS	Dulbecco's phosphate buffered saline
DMEM	Dulbecco's modified eagle medium
EE	% encapsulation efficiency percent
F	formulation
FBS	fetal bovine serum

FTIR	Fourier transform infrared
HSD	honestly significant difference
HVF	human vaginal fluid
IL	Interleukin
LM	lithium methoxide
LPS	lipopolysaccharide
M	molar mass
Mc	molar mass corrected
NO	nitric oxide
NPs	nanoparticles
NR	neutral red
PA	perchloric acid
PDI	polydispersity index
PMD	particles mean diameters
SA	sodium acetate
SAA	sodium acetate anhydrous
SAT	sodium acetate trihydrate
SD	sodium diacetate
TEM	transmission electron microscopy
TFV	tenofovir
TPP	polyanion triphosphate
V	Volume
XRD	x-ray powder diffractometry
ζ	zeta potential

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Fig. 1.

Fig. 2. Particle size distributions by intensity of uncoated chitosan NPs (**A1**, **B1**, **C1**, and **D1**) and coated chitosan with SA (**A2**, **B2**, **C2**, and **D2**) respectively for **blank** formulation, **F1**, **F2** and **F3** formulation, respectively.

Fig. 3. Morphological analysis of uncoated chitosan NPs (**A, B**) for the blank formulation *synthesized before freeze drying* and *coated chitosan NPs (C, D) after freeze drying*, respectively. Morphological analysis of coated chitosan NPS (blank) after 24 hours, and incubated at 37°C (Tris HCl buffer pH 7.51 (**E, F**)). Scale bar represents 100 nm for (**B, D, E, F**) and 1000 nm for (**A, C**), respectively.

Fig. 4.
Titration curve of different acetic acid salt with lithium methoxide in methanol.

Fig. 5. In vitro drug release study **A** = formulation F1, **B** = formulation F2 and **C** = formulation F3. Release of coated NPs in tris-HCl buffer pH = 7.51 (solid line blue, dot marker), and in citrate buffer pH = 4.2 (solid line orange, square marker), respectively. Release of tenofovir (TFV) from uncoated NPs (solid line green, fill diamond marker) and release of native TFV solution (solid line purple, fill triangle marker), respectively.

Fig. 6. FTIR spectra of native tenofovir (**A**), pentasodium phosphate (**B**), chitosan (**C**), physical mixture P1 (**D**), physical mixture P2 (**E**), physical mixture P3 (**F**), sodium acetate anhydrous (SAA) (**G**), pure sodium acetate coating chitosan NPs (SA) (**H**), blank formulation (**I**), formulation F1 (**J**), formulation F2 (**K**) and formulation F3 (**L**), respectively.

Fig. 7.

XRD pattern of native tenofovir (**A**), pentasodium phosphate (**B**), chitosan (**C**), physical mixture P1 (**D**), physical mixture P2 (**E**), physical mixture P3 (**F**), sodium acetate anhydrous (SAA) (**G**), pure sodium acetate coating chitosan NPs (SA) (**H**), blank formulation (**I**), formulation F1 (**J**), formulation F2 (**K**) and formulation F3 (**L**), respectively

Fig. 8.

A Percent Raw cell membrane integrity (% control) treated with the different NPs formulations “Blank” (pattern fill, downward diagonal), F1 (pattern fill, dark horizontal), F2 (pattern fill, sphere), and F3 (pattern fill, dark upward diagonal) respectively (n=3). *P<0.05 vs media, **P<0.01 vs media, ***P<0.001 vs media. Fig. 8 **B** Percent Raw cell mitochondrial activity (% control) treated with different NPs formulations “Blank” (pattern fill, downward diagonal), F1 (pattern fill, dark horizontal), F2 (pattern fill, sphere), and F3 (pattern fill, dark upward diagonal), respectively, (n=3). *P<0.05 vs media, **P<0.01 vs media, ***P<0.001 vs media.

Fig. 9. Percent nitrite release from raw when exposed to the different NPs formulation “Blank” (pattern fill, downward diagonal), F1 (pattern fill, dark horizontal), F2 (pattern fill, sphere), and F3 (pattern fill, dark upward diagonal) respectively (n=3). *P<0.05 vs media, **P<0.01 vs media, ***P<0.001 vs media.

Fig. 10. Percent cytokine/interleukin (IL) (A, B, C and D) release respectively for **IL-1 α** , **IL-1 β** , **IL-6** and **IL-7** from macrophage when exposed to the different NPs formulation “Blank” (pattern fill, downward diagonal), F1 (pattern fill, dark horizontal), F2 (pattern fill, sphere), and F3 (pattern fill, dark upward diagonal), respectively (n=2). *P<0.05 vs media, **P<0.01 vs media, ***P<0.001 vs media.

Table I

Physical mixtures and formulation variables

Physical mixture	Blank	P1	P2	P3
Chitosan amount (mg)	40	40	40	40
Sodium pentaphosphate amount (mg)	8	8	8	8
Tenofovir amount (mg)	0	2	4	6
Formulation	Blank	F1	F2	F3
Volume of chitosan aqueous solution in 2% v/v acetic acid (2mg/ml, pH=4.76) ¹ (ml)	20	20	20	20
Volume of aqueous solution of triphosphate (2mg/ml, pH= 5.60–5.99) ² (ml)	4	4	4	4
Amount of tenofovir add into triphosphate aqueous solution (mg)	0	2	4	6

¹ pH raised with (2M, ~2.2 ml) of sodium hydroxide aqueous solution;

² pH decreased with (2M) few drops of hydrochloridric acid aqueous solution

Table II

Particle mean diameters (PMD), zeta potential (ζ), polydispersity index (PDI), and percent encapsulation efficiency (EE %) for different formulations

Formulation	Blank	F1	F2	F3
PMD (uncoated NPs) d. (nm)	348.33±74.64	135.67±1.86	150.67±1.20	155.93±4.34
PMD (coated NPs) d (nm)	165.2±70.01	261.23±118	379.53±130.26	171.53±70.01
EE %	n/a	11.74±0.71	5.52±0.34	6.12±0.10
EE % (	n/a	89.27±0.77	92.74±4.00	86.34±4.53
ζ (mV)	28.47±2.07	25.97±1.07	27.53±2.05	24.3±2.05
ζ (mV)	3.06±1.95	0.08±0.86	2.23±0.68	1.22±0.88
PDI	0.47±0.05	0.30±0.05	0.34±0.07	0.32±0.04
PDI	0.30±0.05	0.24±0.02	0.37±0.14	0.43±0.08

Table III

Melting point and molar mass of different salts of acetic acid

Sample	Sodium acetate anhydrous (SAA)	Sodium Acetate (SA)	Sodium acetate trihydrate (SAT)	Sodium diacetate (SD)
Melting point (°C)	332.5–338	333–338	329–333	325–333
<i>M</i> = Molar mass found (g/mol)	75.57±2.95	74.05±0.39	121.65±1.81	134.81±12.21
<i>Mc</i> = molar mass corrected (g/mol)	82.03±3.2	80.38±0.42	132.05±1.96	146.34±13.26
Expected molar mass (<i>g/mol</i>)	82.03	n/a	136.08	142.09

Table IV

Value of 'n' for the different coated nano-formulations using Korsmeyer-Peppas model

Nanoformulation	Citrate buffer pH 4.2	Tris Hcl buffer pH 7.51
F1	n= 0.86 ($R^2 = 0.970$)	n= 0.78 ($R^2 = 0.991$)
F2	n= 0.61 ($R^2 = 0.974$)	n= 0.79 ($R^2 = 0.998$)
F3	n= 0.71 ($R^2 = 0.999$)	n= 0.63 ($R^2 = 0.994$)

R^2 represents the coefficient of determination after model fitting, and **n** pinpoints the release mechanism of tenofovir.