Original Article

Development of Docetaxel-loaded Folate-modified Poly(lactic-co-glycolic acid) Particles

Yuri I. Poltavets, Vasilisa V. Zavarzina, Sergey L. Kuznetsov, Anna A. Krasheninnikova, Danil O. Dronov, Nadezhda V. Gukasova, Valentina G. Shuvatova, Vadim Yu. Balabanyan[1]

Laboratory of Nanocapsules and Targeted Delivery of Drugs, National Research Centre "Kurchatov Institute", Moscow, Russia, [1]Department of Pharmaceutical technology, Faculty of Fundamental Medicine, Lomonosov Moscow State University, Moscow, Russia

Abstract

Background: Poly(lactic-co-glycolic acid) (PLGA) particles with small vector molecules are used for targeted delivery of anticancer agents. To be effective, they must be small, noncytotoxic, sterile, and stable. **Aim:** The aim of this study was to prepare docetaxel-loaded folate-modified PLGA-based nanoparticles (FD-Dtx-NPs) and to assess their as parenteral folate-receptor targeted delivery systems during γ-sterilization and long-term storage. **Materials and Methods:** NPs were prepared by oil/water single emulsion-solvent evaporation method and simultaneous loading of polymer particles with docetaxel and folic acid derivative. NPs' physicochemical characteristics and antitumor activity were assessed. **Findings:** FD-Dtx-NPs presented uniform characteristics over repeated measurements: ~250 nm size, <0.100 polydispersity index, and >2.5% docetaxel content in the finished lyophilizate. The observed slow docetaxel release from FD-Dtx-NPs was acceptable for proposed usage. γ-irradiated NPs were sterile under all tested protocols and maintained their physicochemical properties at a 10-kGy cumulative dose, 0.500 Gy/s dose rate, and 5.57-h exposure. No significant differences were observed in physicochemical characteristics of FD-Dtx-NPs over 12 months. Finally, FD-Dtx-NPs showed a high anticancer activity *in vitro*. **Conclusion:** The proposed method generates FD-Dtx-NPs with reproducible characteristics, high activity, and elevated stability during the long-term storage. Results of γ-sterilization and stability studies may be valuable for the development of polymer-based drugs.

Keywords: *Docetaxel, folic acid, γ-irradiation, long-term storage, nanoparticles, poly(lactic-co-glycolic acid), stability*

Introduction

Synthetic polymers, such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA), and their copolymers, poly(lactic-co-glycolic acid) (PLGA), have been approved by the Food and Drug Administration and are widely used as carriers in drug delivery.[1,2] PLGA particles provide sustained drug delivery, biocompatibility, biodegradability, and no cytotoxicity,[2-5] which is key for parenteral administration.

Most polymer-based particles for antitumor drug delivery have optimal sizes of up to 300 nm.[6] The polydispersity index (PdI) serves as an indicator of sample quality.[7] Another important criterion is particle size distribution, as differences in particle size reflect the Active Pharmaceutical Ingredient (API) content and, consequently, pharmacological activity.

Targeted delivery systems in the form of PLGA particles with anticancer agents and small vector molecules are being actively developed.[8,9] Promising candidates used as vectors in such systems are low-molecular-weight (MW) biomolecules, which is also called "small molecules," including vitamins, hormones, and peptides. It is a well-known fact that tumor cells have an increased need for small molecules to satisfy their metabolic requirements, therefore strongly expressing the corresponding receptors.[10,11] In this case, the presence of a vector fragment provides more selective transport of the particle because of receptor-mediated interaction and increases the effect of nonselective transport of particles to the tumor area achieved via enhanced permeability and retention..[12] Covalent binding of vector molecules is used extensively to decorate targeted delivery systems, but it is hampered by low yield and difficult purification from side products.[13,14] A rational way to obtain vectorized particles through simultaneous loading of polymer particles with docetaxel and folic acid derivative was proposed in our recent work.[15]

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Address for correspondence:
Mr. Yuri Poltavets, Laboratory of Nanocapsules and Targeted Delivery of Drugs, National Research Centre, "Kurchatov Institute", Moscow, Russia.
E-mail: y.p.rovizor@gmail.com

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One of the technical difficulties for the industrial use of polymer nanoparticles (NPs) is to achieve the sterility of the finished product. In general, biopharmaceuticals and polymer-based NPs are produced under aseptic conditions but are not subjected to terminal sterilization. As these NPs are heat-sensitive, they are normally sterilized by filtration; however, 250-nm particles cannot be filtered through a 0.22-μm filter, but only through a 0.45-μm one. In this case, only γ-sterilization is acceptable for vectorized NPs.[16]

Long-term stability of NPs is very important while developing potential drugs because shelf-life depends on the stability of the finished product.[16] For polymer-based NPs, their stability depends on the production method and on the presence or absence of sterilization stage.

Thus, development of parenterally administered vectorized NPs must ensure suitable formulation, sterility, apyrogenicity, and long-term stability. Additionally, particles intended for intravenous administration preferably must be <300 nm in size and have a PdI of <0.15.

The main objective of this work was to prepare docetaxel-loaded folate-modified PLGA-based NPs (FD-Dtx-NPs) and to study their anticancer activity and characteristics, particularly γ-sterilization and long-term stability.

Materials and Methods

The materials used in this study were as follows: 50/50 poly(D,L-lactic-co-glycolic acid), ester terminated (PLGA 50:50), and PURAC® PDLG 5004 (inherent viscosity 0.41 dL/g) from Purac Biomaterials (Amsterdam, Netherlands); docetaxel trihydrate (Dtx) (MW = 44 kDa), pharm. EP from Qilu Pharmaceutical (Jinan, China); polyvinyl alcohol (PVA) (87%–90% hydrolyzed; average MW = 30,000−70,000 Da) and trifluoroacetic acid (TFA) from Sigma-Aldrich, St. Louis, MO); methylene chloride ($MeCl_2$), dimethyl sulfoxide (DMSO), and dimethyl formamide (DMF), purriss. from Chimmed (Moscow, Russia); folic-acid dodecylamide (FD) by the IREA Institute (Moscow, Russia); and acetonitrile (MeCN) from Scharlab (Barcelona, Spain). The solutions were made up with distilled and deionized water throughout the all experiments. All other chemicals were of analytical grade reagents and used as received.

FD-Dtx-NPs were prepared by single emulsion method. All phases were previously filtered using 0.45-μm nylon membrane filters. Briefly, 10-mg Dtx and 200-mg PLGA were dissolved in 5-mL $MeCl_2$. A 1-mg/mL FD solution in DMSO, DMF, or similar polar aprotic solvent was obtained separately, and 200 μL of it was added to the Dtx-PLGA solution (organic phase). Then, 5-mL organic phase was added to 35-mL 0.25% PVA aqueous solution and emulsified by repeated sonication using a Vibra-Cell VCX 750 sonicator, equipped with #630-0220 probe and #630-0420 6-mm diameter microtip (Sonics & Materials, Newtown, CT). Sonication was performed in a 100-mL glass beaker on ice at 45 J, 45% (108 μm) probe amplitude, 2-s/2-s pulsation (pulse/clear), and 1-min sonication time (2-min overall pulse/clear with 1-min rest). Solvent was eliminated under constant stirring for 16 h at room temperature and laminar airflow. Finally, 78-mg NaCl in 5-mL water was added to the formed suspension. Before lyophilization, the suspension was filtered through a single-layer filtrating tissue filter (nylon or similar) and frozen in liquid nitrogen. Dry particles were collected after freeze-drying and stored in presterilized tightly closed plastic containers at 4°C.

FD-free nanoparticles (Dtx-NPs) were obtained as described above, except for using 200-μL aprotic solvent instead of FD solution.

Each lyophilizate weighted ~360 mg, or ~96% of the initial amount of dry substance. Yield can be improved by scaling up the process.

Dtx and FD contents in NPs were measured by using high-performance liquid chromatography (HPLC) (LC 1200 Series; Agilent, Santa Clara, CA), with a reversed-phase ReproSil-Pur® C18-Basic Column (250 mm × 4.6 mm, 5 μm). Briefly, 10-mg freeze-dried NPs were dissolved in 1-mL MeCN/DMSO (50:50 v/v) in a sonic bath for 5 min, centrifuged at 16,000 rpm for 5 min, and analyzed by HPLC with 0.01% TFA in water (phase A) and 0.01% TFA in MeCN (phase B). A linear gradient consisting of 45% phase B at 0 min and 100% phase B at 20 min was established at a flow rate of 1.0 mL/min, injection volume of 2 μL, and 45°C. The effluent was detected at 220 nm with a UV/Vis detector. Approximate retention time for Dtx and FD was 10 min and 9 min, respectively [Figure 1]. The Dtx content was determined quantitatively by comparison to a standard curve, whereas the FD content was determined only qualitatively because it showed no linearity for the determined quantities. The chromatography system was considered suitable if there were ≤1.5% relative standard deviation of Dtx peaks; ≥2 resolution between FD and Dtx peaks; ≥40,000 column efficiency for Dtx and ≥15,000 theoretical plates for FD; and ≤1.1 tailing factor for Dtx and ≤2.0 for FD.[17]

To determine the total amount of Dtx in the lyophylizate, 50 mg of the latter was dissolved in 1-mL DMSO, diluted in 10-mL MeCN, and filtered through a 0.45-μm polyvinylidene fluoride syringe filter. A 20-μL sample was evaluated in triplicate by HPLC for each variation.

To measure Dtx content in NPs, 50-mg lyophilizate was mixed in 5-mL water using a vortex, the suspension was centrifuged at 25,000× *g* for 30 min at 4°C, and 4.75 mL of supernatant was carefully collected. Another 4.75 mL of water was added to the residue, resuspended again, and centrifuged. This process was repeated twice. Finally, the residue (washed NPs) was lyophilized and Dtx content was determined in triplicate. The residual aqueous phase did not significantly interfere with the results, because the calculated dilution factor was 0.0625%, whereas the standard error for Dtx determination was 3%.

Entrapping efficacy (EE) was calculated as follows:

$$EE = \frac{Dtx_{\text{encapsulated(twice washed NPs)}}}{Dtx_{\text{actual amount in the sample}}} \times 100\%.$$

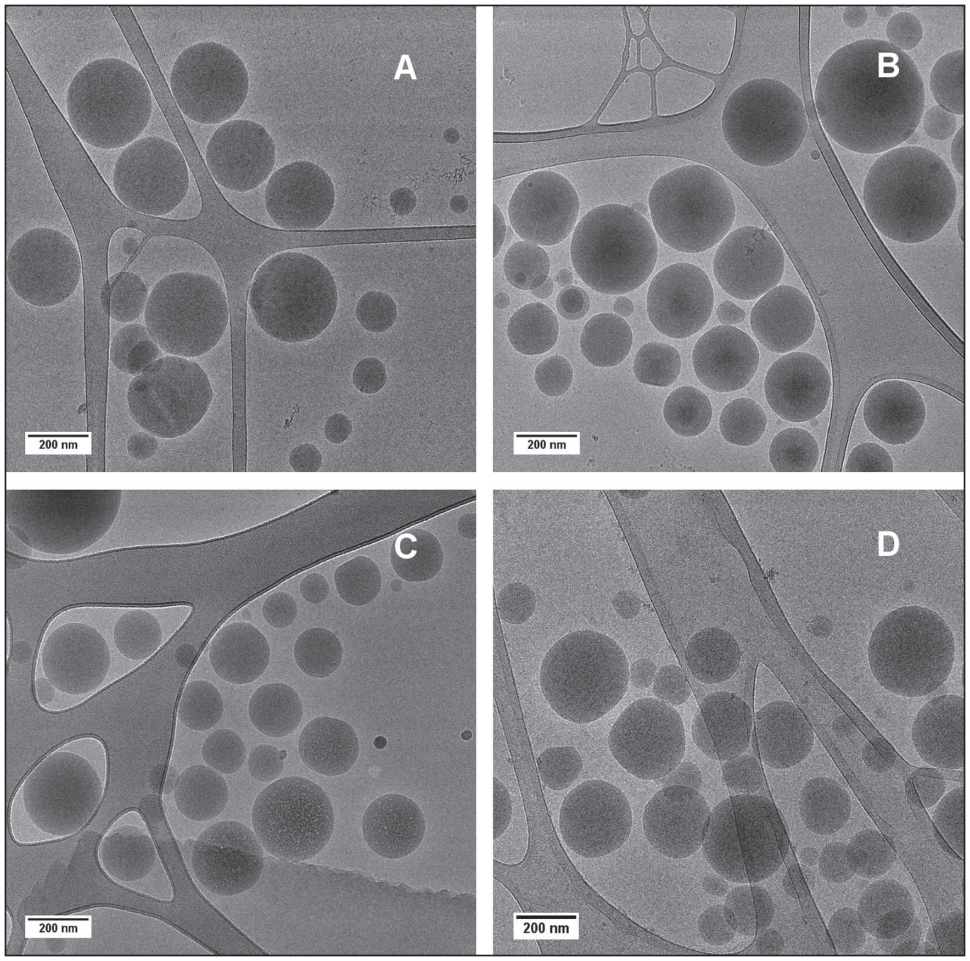

Figure 1: TEM images of NPs: (A) intact NPs, (B) NPs after 10-kGy γ-sterilization, (C) NPs after six-month storage, and (D) NPs after 12-month storage

Particle size, PdI, and zeta potential of NPs (0.2 mg/mL in deionized water) were measured by laser light scattering (Zetasizer ZS 3600; Malvern Instruments Ltd., Malvern, UK) at 25°C and were calculated from the average of three measurements.

The morphological examination of NPs was performed by transmission electron cryomicroscopy (TEM). Samples were diluted in 1-mL purified water, vortexed for 1 min, and placed on a previously hydrophilized supporting grid. After vitrification in liquid ethane, samples were transferred in liquid nitrogen to the pressing station and placed in a cassette holder under cryogenic conditions. Imaging was performed using a Titan Krios TEM FEI (Thermo Fisher Scientific, Waltham, MA) at ×5,000−18,000 magnification in a low-dose mode using a Falcon II electron detector.

Different batches of NPs were weighted (50 mg) and transferred to 5-mL glass aluminum-sealed cap vials (La-Pha-Pack GmbH, Langerwehe, Germany). Samples were γ-irradiated at ~22°C using a ^{60}Co source (GUT-200M; National Research Centre "Kurchatov Institute", Moscow, Russia) to 10, 15, and 20 kGy. Each cumulative dose was calculated based on varied dose rate (Gy/s) and exposure time (h).

Powder X-ray diffraction patterns were obtained using a SmartLab X-ray diffractometer (Rigaku Corporation, Tokyo, Japan), with Cu−Kα radiation (1.54 Å) within the 2θ range of 5−60° at 45 kV and 200 mA. The beam was formed by a parabolic mirror and a double monochromator Ge (220).

Sterility was tested by the direct inoculation method.[18] γ-irradiated NP samples (800 mg) were suspended in 20-mL sterile distilled water and added to fluid thioglycollate or soybean-casein digest medium at a ratio of 1:10 in triplicates. The inoculated media were incubated for at least 14 days at 32.5 ± 2.5°C and 22.5 ± 2.5°C, respectively, and observed periodically. The sterility of the samples was confirmed by the absence of microbial growth occurred after 14 days.

Endotoxins were determined by the gel clot Limulus Amebocyte Lysate (LAL) test using Endosafe LAL-reagent (Charles River Endosafe, Wilmington, MA) according to the manufacturer's instructions. Equal parts (100 μL) of Endosafe LAL-reagent (LAL sensitivity of 0.03 EU/mL standardized by control standard endotoxin) were mixed with different dilutions of lyophilizate samples in LAL reagent water (Pyrotest, Russia) and incubated for 60 ± 2 min at 37 ± 1°C. The reaction was positive if the formed gel clot remained stable when the tube

was inverted by 180°. Endotoxin content was normalized per mg lyophilizate sample.

In-vitro Dtx release studies were performed by the dialysis bag diffusion technique. Briefly, 1-mL NP suspension containing 78-mg NPs was placed in a dialysis membrane bag of 12,000−14,000 Da molecular weight cutoff (OrDial D14; Orange Scientific, Braine l'Alleud, Belgium) and dialyzed against 2000 mL of phosphate-buffered saline containing 0.5% Tween 80 at 37°C, pH 5.5 or pH 7.4, on an orbital shaker at 50 rpm. The NP mass-to-buffer volume was calculated so not to exceed the solubility limit of Dtx (8.7 μM).[19] The samples were then quantitatively transferred from dialysis bags to glass vials, frozen, and lyophilized. The Dtx content in the lyophilized samples was determined by HPLC and a time-dependent curve of Dtx release from NPs was calculated.

A 47-day study was performed to evaluate the stability of NPs stored light protected at 37 ± 0.1°C under static conditions. Immediately after 6, 12, 18, 24, 36, and 47 days of storage, samples were analyzed for particle size, PdI, zeta potential, concentration that inhibited cell survival by 50% (IC_{50}), and Dtx content. A long-term study over 12 months evaluated the stability of NPs stored light protected at 5 ± 3°C under static conditions. Samples taken after 3, 6, 9, and 12 months of storage were analyzed the same way as for the 47 day the stability study.

HeLa human cervical carcinoma cells were obtained from the Russian collection of cell cultures in St. Petersburg. Cells were cultured in plastic flasks (Corning, Corning, NY) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 50-μg/mL gentamicin in a CO_2 incubator at 37°C in a humidified atmosphere containing 5% CO_2. Cells were passaged twice a week using trypsin−ethylenediaminetetraacetic acid. Cells were seeded in folic-acid-free DMEM supplemented with 10% FBS and 50-μg/mL gentamicin in 96-well plates one before the experiment for attachment and adaptation. To test the anticancer activity of NPs *in vitro*, Dtx and NPs were added in a wide range of concentrations and incubated for 24−72 h under standard conditions. Cell survival was measured by the MTT assay.[20] Two hours before the end of incubation, 50-μL MTT at 1 mg/mL in culture medium was added to each well. After color development, the medium was removed, the precipitated formazan crystals were dissolved in 150-μL DMSO, and color intensity was measured by absorbance at 570 nm using an iMark™ microplate reader (Bio-Rad, Hercules, CA). Cell survival was assessed as a percentage of the untreated control and IC_{50} was determined. The test was repeated three times.

Results and Discussion

This study describes a rational method for incorporating FD as a vector molecule into PLGA particles. NPs were prepared by oil/water single emulsion-solvent evaporation method. Polymer particles were loaded simultaneously with Dtx and FD. Vectorization was simplified by adding a solution of previously obtained FD to the nonpolar phase of the emulsion, without additional washing of NPs. The proposed simplified method of vectorized NP production repeatedly yielded particles with uniform characteristics [Table 1].

The Dtx EE value (90%) was consistent with the data described in other literatures. FD-Dtx-NPs and Dtx-NPs had very similar size, PdI, zeta potential, and EE [Table 1], which represent an advantage of the proposed method. Particles had an average size of <250 nm and low polydispersity, which is optimal for parenteral drug administration.[6] Zeta potential of all NPs was relatively small, but it was sufficient for detection in the presence of a strong electrolyte such as sodium chloride. A zeta potential close to neutral is desirable for targeted drug delivery systems,

Table 1: Basic characteristics of NPs

Name	Dtx content (%)	Average diameter (nm)	PdI	Zeta potential[a] (mV)
Dtx-NPs	2.69 ± 0.08	237.4 ± 2.45	0.071 ± 0.025	−2.35 ± 0.62
FD-NPs	–	235.3 ± 6.14	0.111 ± 0.031	−1.66 ± 0.63
FD-Dtx-NPs				
01	2.61 ± 0.09	253.2 ± 3.58	0.088 ± 0.041	−2.23 ± 0.120
02	2.59 ± 0.08	251.2 ± 1.44	0.095 ± 0.013	−2.38 ± 0.174
03	2.57 ± 0.07	253.7 ± 2.72	0.077 ± 0.023	−1.44 ± 0.214
04	2.66 ± 0.08	250.0 ± 2.46	0.069 ± 0.014	−1.98 ± 0.128
05	2.85 ± 0.09	254.0 ± 1.68	0.044 ± 0.022	−2.71 ± 0.257
06	2.68 ± 0.07	251.3 ± 1.95	0.065 ± 0.023	−2.93 ± 0.246
07	2.55 ± 0.08	249.2 ± 1.00	0.073 ± 0.013	−2.06 ± 0.231
08	2.63 ± 0.08	247.5 ± 2.83	0.088 ± 0.012	−2.46 ± 0.105
09	2.68 ± 0.07	248.9 ± 1.41	0.054 ± 0.040	−3.57 ± 0.506
10	2.85 ± 0.09	251.8 ± 3.30	0.080 ± 0.027	−2.23 ± 0.269
Mean (1–10)	2.67	251.1	0.073	−2.40
SD (1–10)	0.10	2.87	0.026	0.597
RSD % (1–10)	3.75	1.14	35.6	24.9

SD = standard deviation, NPs = nanoparticles, FD = folic-acid dodecylamide, Dtx = docetaxel, PdI = polydispersity index
All values are expressed as mean ± SD of three independent experiments
[a]Zeta potential deviation in each single determination is ±4.2 mV

as it reduces the risk of toxic effects on healthy cells.[21] For larger polymer-based particles, NP stability in water derives mostly from PVA (surfactant) content, which, in the case of blood, is serum albumins. Electrostatic interactions play only a minor role in this case.

All examined NPs had a regular spherical shape. The average diameter of NPs measured by dynamic light scattering correlated with TEM results in all cases (γ-treatment, storage) [Figure 1]. X-ray diffraction revealed no reliable distinctions between FD-Dtx-NPs and Dtx-NPs, with particle structure being "predominantly amorphous." All samples were characterized by a series of diffraction peaks with identical 2θ values and similar intensity. A comparison between typical NP diffraction patterns with those of individual components [Figure 2] indicated that the former differed drastically from that was expected by simply adding up the latter. Specifically, Dtx peaks disappeared from FD-Dtx-NP spectra, suggesting that Dtx was present in its amorphous rather than crystalline state, as described for other NP formulations.[22,23]

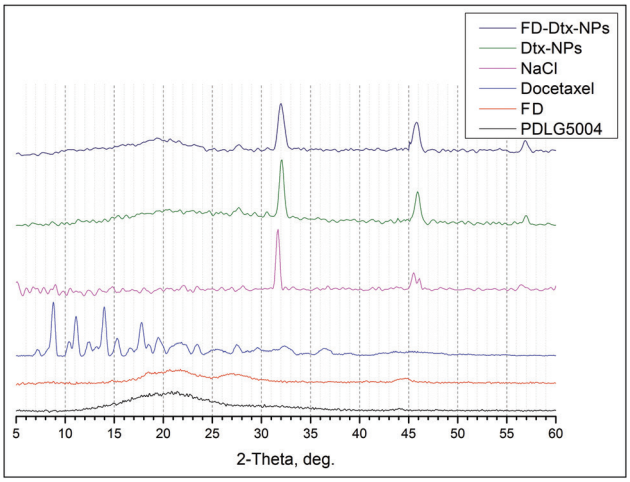

Figure 2: X-ray diffractograms for NPs and individual components

According to ISO 11137-2:2013[24] standards, sterility is achieved with a uniform dose of 25 kGy, but for plastic devices, with lower γ-tolerance, the recommended dose can be 15 kGy. Here, we used doses of 10, 15, and 20 kGy, all of which excluded any secondary radioactivity in treated devices. As shown in Figure 3 and Table 2, NPs were distributed in groups with similar cumulative dose and exposure time. Generally, Dtx content decreased with longer or more severe γ-irradiation, whereas particle size and spherical shape remained mostly unchanged, as confirmed by TEM [Figure 1B]. Accordingly, Dtx content was the most sensitive indicator, which can be explained by both the direct destructive effect of γ-irradiation and induced hydrolysis of Dtx by residual water.

All γ-treatment protocols resulted in sterile NPs and fell within the desired parameters of sterilization: cumulative dose ≤10 kGy, dose rate ~0.500 Gy/s, and exposure time ~5.57 h. Further

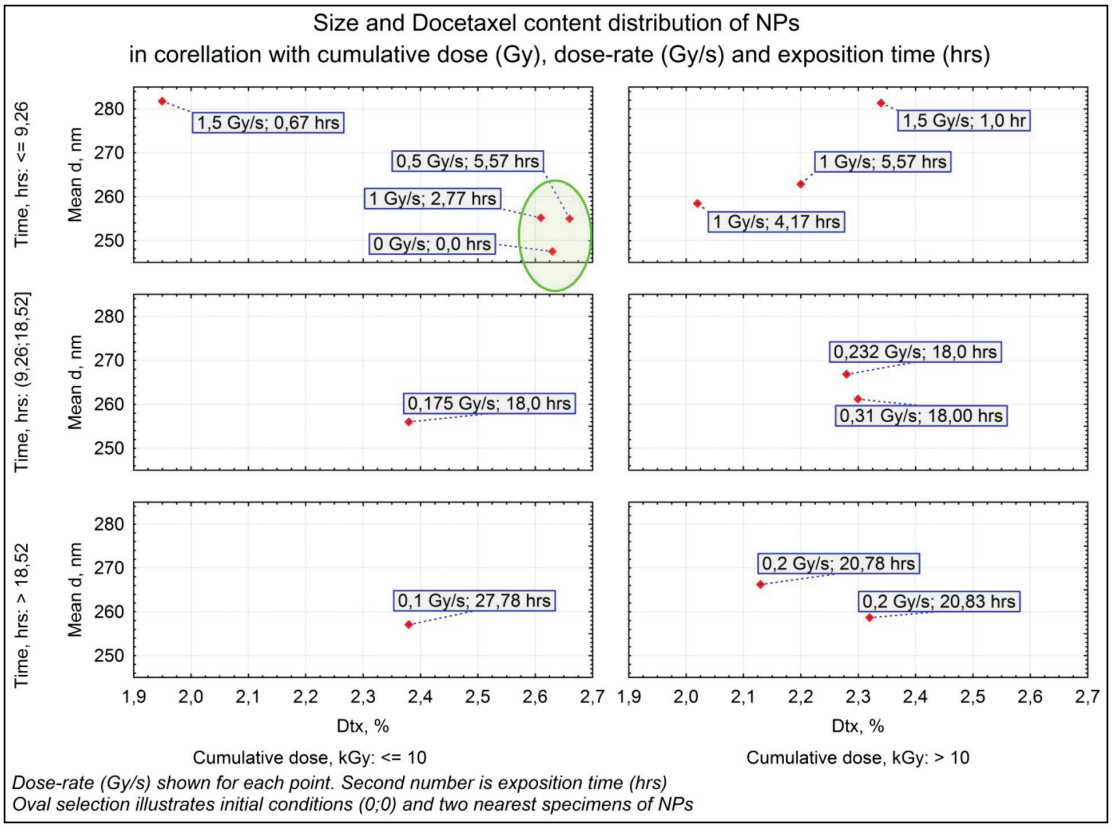

Figure 3: Distribution characteristics of γ-irradiated NPs relative to cumulative dose and exposure time. Dose rate (Gy/s) and exposure time (h) are shown for each point. The oval selection shows initial conditions (0.0 h) and two nearest specimens of NPs

Table 2: Characteristics of γ-treated NPs

Batch#	Cumulative dose (kGy)	Exposure time (h)	Dose rate (Gy/s)	Average diameter (nm)	PdI	Dtx content (%)
1	10	0.67	1.500	281.7	0.176	1.95
2	10	2.77	1.000	255.1	0.088	2.61
3	10	5.57	0.500	254.9	0.087	2.66
4	10	18.00	0.175	256.0	0.080	2.38
5	10	27.78	0.100	257.1	0.078	2.38
6	15	1.00	1.500	281.3	0.191	2.34
7	15	4.17	1.000	258.4	0.097	2.02
8	15	18.00	0.232	266.8	0.120	2.28
9	15	20.83	0.200	258.6	0.112	2.32
10	20	5.57	1.000	262.8	0.110	2.20
11	20	18.00	0.310	261.2	0.115	2.30
12	20	27.78	0.200	266.2	0.135	2.13
Reference[a]	**0**	**28.00**	**0.000**	**247.5**	**0.088**	**2.63**

NPs = nanoparticles, Dtx = docetaxel, PdI = polydispersity index
All values are expressed as the median of two independent experiments
[a]Data for unprocessed FD-Dtx-NPs stored at the same conditions for 28 h

studies will establish whether a dose of <10 kGy is suitable for sterilization in this case.[25]

Figure 4 shows *in-vitro* Dtx release profiles of FD-Dtx-NPs at pH 7.4 and pH 5.5. The pH value in tumors can vary within this range.[26] Both profiles are characterized by an initial rapid release period followed by a continuous and slower release that is typical of PLGA-based NPs.[27-29] The sustained Dtx release up to 48 hrs was obtained for FD-Dtx-NPs and did not reveal any significant difference at pH 7.4 and pH 5.5. In comparison to pH value, more important factors affecting the drug release from NPs are MW of polymers, poly-D-lactide:polyglycolide blocks ratio, particle size, encapsulated drug physico-chemical properties.[30] Only 11.3% of Dtx, presumably localized on the surface of NPs, was released in the first hour; 28.61% ± 2.77% (pH 7.4) and 25.57% ± 2.51% (pH 5.5) of Dtx were released over the following 48 h. Such a release profile is desirable for a drug delivery system targeting cancer because it minimizes post-administration acute general toxicity, which occurs with clinically applied Dtx (e.g., Taxotere™, Laval (Quebec), Canada),[31] and highlights one of the main benefits of polymer-based formulations.[32,33]

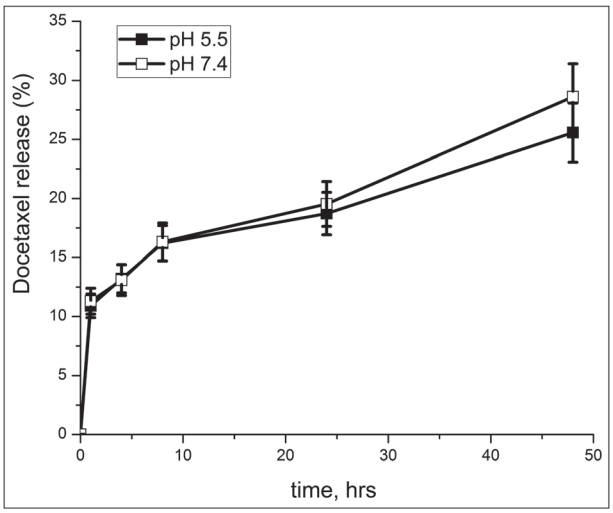

Figure 4: Dtx release profile for FD-Dtx-NPs at 37°C (the initial amount of Dtx in the sample was taken as 100%) at pH 7.4 and 5.5

In-vitro anticancer activity of FD-Dtx-NPs was investigated in HeLa cells characterized by a high level of folate receptor-α expression.[34] Survival of HeLa cells after a 72-h exposure was measured by the MTT assay [Figure 5]. FD-Dtx-NPs showed higher anticancer activity as compared to free Dtx, with IC_{50} of 2.54 ± 0.20 nM and 4.25 ± 0.33 nM, respectively. Higher cytotoxic effects of Dtx-loaded folate-modified PLGA NPs on folate receptor expressing HeLa cells are in agreement with other reports with folate delivery systems for paclitaxel and carboplatin on this cell line.[35,36] The expected mechanism of action of NPs is associated with receptor-mediated endocytosis by tumor cells,[14] release of Dtx from NPs, and Dtx toxicity to the cellular microtubule network.[37] FD was successfully used as a vector molecule to obtain target drug delivery systems based on liposomes and micelles.[11,38] As shown in Figure 5, the release of docetaxel can last even after the particles have entered tumor cells. Hence, we assume that NPs remain pharmacologically active for some time even after the cells' death. Particles containing excess FD will have a targeted effect and can potentially affect further tumor cells.

Long-term and accelerated studies of stability were performed over 12 months at 5 ± 3°C and 65% ± 5% relative humidity, and 24 months with 1.5 months of real storage, respectively. Accelerated storage can estimate possible changes in NPs that develop slowly over time. Storage time calculations were based on Van't Hoff rule: the rate of chemical reactions increases by twofold or more for each rise of 10°C in temperature. Accordingly, shelf-life (C) at storage temperature (t_{st}) is related to experimental shelf-life (C_E) at an elevated storage temperature (t_e) by the following relationship:

$$C = K \times C_E,$$

where the correspondence coefficient $K = A^{\frac{t_{st}-t_e}{10}}$. The temperature coefficient of the chemical reaction rate (A) is 2.5 and K is 18.77 for accelerated storage at 37°C.

In-vitro anticancer activity of FD-Dtx-NPs was calculated as the ratio of IC_{50} values of FD-Dtx-NPs and free Dtx: $IC_{50}^{t_i} / IC_{50}^{Dtx}$, where $IC_{50}^{t_i}$ is IC_{50} of FD-Dtx-NPs for the designated storage time (t_i) and IC_{50}^{Dtx} is the IC_{50} value of free Dtx. The relative error of IC_{50} determination was ~10%.

All results from storage measurements are summarized in Table 3. Accelerated storage revealed a significant drop in anticancer activity (IC50) and Dtx content, but particle size and polydispersity, at elevated temperature still similar compared to normal conditions (2–8°C). As PLGAs are amorphous substances with strict temperature-dependent properties, the high temperature could have affected polymer-based NPs.[39] In contrast, changes in Dtx content and lower activity were common to other storage conditions.

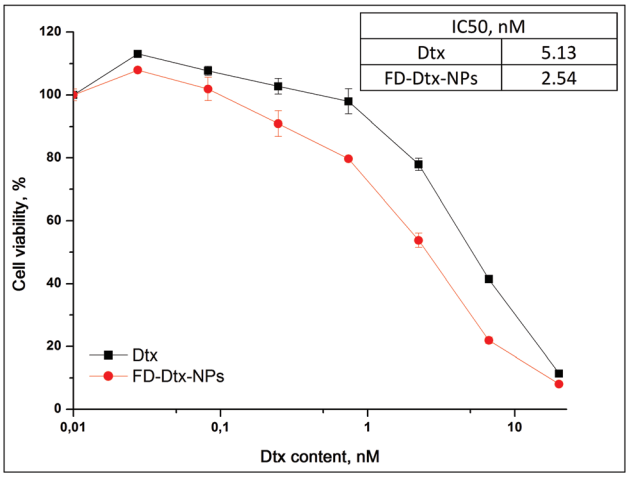

Figure 5: Survival of cancer cells following a 72-h exposure to Dtx and FD-Dtx-NPs

The long-term stability study revealed no significant changes in FD-Dtx-NP characteristics. After 12 months, average size, PdI, and Dtx content were 251.0 ± 2.208 nm, 0.065 ± 0.026, and 2.54± 0.070%, respectively, as confirmed by TEM [Figure 1]. PdI was <0.100, indicating a strictly monomodal size distribution. The zeta potential was close to neutral and went from −1.98 mV to −7.17 mV in 12 months. However, it was considered unchanged because zeta deviation was ±4.2 mV for each determination. NP activity was stable after nearly six months and substantially exceeded that of free Dtx; however, by 9−12 months, it became close to that of Dtx [Table 3]. Consequently, six months represent the guaranteed shelf-life of these NPs.

Endotoxin content in all PLGA-based NP samples before and during storage was <0.192 EU/mg, which meets the requirements of most Pharmacopoeias (EP, USP, and State Pharmacopoeia of the Russian Federation) for safe intravenous administration (5 EU/kg/h).[40] Low endotoxin levels represent the cumulative result of using injection-grade materials, rational technology, and the absence of pyrogen-like degradation products during the long-term storage.

The stability results show that the ingredients in the particles are stable and do not react with each other or with the environment under selected storage conditions. Thus, the proposed targeted polymer particles are superior to liposomal and micellar

Table 3: Comparative storage data

Storage time (days)[a]	Dtx content (%)	Average diameter (nm)	PdI	Zeta potential[b] (mV)	$IC_{50}^{t_i} / IC_{50}^{Dtx}$
Long-term storage, 5 ± 3°C					
0[b]	2.66 ± 0.078	250.0 ± 2.458	0.069 ± 0.014	−1.98 ± 0.128	0.598
90	2.57 ± 0.071	253.1 ± 1.457	0.081 ± 0.014	−1.50 ± 0,093	0.650
180	2.68 ± 0.055	253.7 ± 2.221	0.084 ± 0.006	−1.80 ± 0.138	0.674
270	2.63 ± 0.051	256.1 ± 2.415	0.054 ± 0.019	−0.731 ± 0.082	0.903
360	2.54 ± 0.070	251.0 ± 2.208	0.065 ± 0.026	−7.14 ± 0.675	0.972
Short-term storage, 37 ± 0.1°C					
0[b]	2.59 ± 0.067	251.2 ± 1.442	0.095 ± 0.013	−2.38 ± 0.174	0.688
6 (3 months)	2.61 ± 0.067	879.8 ± 343.4	0.842 ± 0.173	−1.12 ± 0.164	0.630
12 (6 months)	2.61 ± 0.073	595.1 ± 175.1	0.684 ± 0.127	−1.01 ± 0.310	0.747
18 (9 months)	2.66 ± 0.076	570.5 ± 79.0	0.700 ± 0.063	−1.21 ± 0.162	0.890
24 (12 months)	2.58 ± 0.047	617.0 ± 70.86	0.726 ± 0.104	−1.31 ± 0.068	1.117
36 (18 months)	2.48 ± 0.069	450.4 ± 67.97	0.558 ± 0.103	−3.52 ± 0.191	1.290
47 (24 months)	1.81 ± 0.053	490.8 ± 38.12	0.645 ± 0.108	−2.08 ± 0.594	1.457

Dtx = docetaxel, PdI = polydispersity index, IC_{50} = concentration that inhibits cell survival by 50%
All values are expressed as mean ± standard deviation of three independent experiments
[a]Accelerated storage, the months of storage under normal conditions
[b]Initial conditions
[c]Zeta deviation in each single determination is ±4.2 mV

delivery systems, as these NPs can keep their morphology, avoid aggregation, and maintain API content for much longer. Indeed, the proposed shelf-life of FD-Dtx-NPs was only limited by the biological activity of Dtx.[27,41] In our work we performed longer storage, reaching one year. Such long-term storage is of interest for drug development based on PLGA NPs.

Conclusion

A rational design for incorporating FD as a vector molecule into PLGA particles containing Dtx has been proposed. All FD-Dtx-NPs presented uniform size, polydispersity, surface charge, and Dtx content. The incorporation of FD boosted the NPs' anticancer activity *in vitro* against tumor cells overexpressing the folate receptor. Within one year of storage, physicochemical parameters of the NPs remained unchanged and their anticancer activity was equal to that of free Dtx. Moreover, the desired parameters of sterilization provided adequate sterility while minimally affecting NP characteristics. Thus, the described method of FD-Dtx-NP preparation allows the production of NPs with elevated anticancer activity, simple sterilization, long-term storage stability, and optimal characteristics for parenteral dosage.

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Conflict of interest

There are no conflicts of interest.

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