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Development and examination of self-assembled nanoformulation of biotinylated paclitaxel derivative

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1. General information

1.1. Materials

Biotinylation of paclitaxel and formation of nanosystems was carried out with the following compounds and reagents: paclitaxel (**PTX**) (Taihua Natural Plant Pharmaceutical Co., Ltd, China), biotin (vitamin B_7) (Baoji Guokang Bio-Technology Co., Ltd., China), N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCl) (Sisco Research Laboratories Pvt. Ltd, India), 4-dimethylaminopyridine (DMAP) (Chimmed, Russia), polyvinyl alcohol (PVA 9-10 kDa, 80% hydrolized) (Sigma-Aldrich, Japan). Ethanol (95% v/v) was used as received. N,N-Dimethylformamide (DMF) was purified by vacuum distillation over P_4O_{10} before use. Phosphate buffered saline (PBS) was prepared by dissolving tablets (MP Biomedicals, LLC, France) in distilled water. Preparative normal-phase column chromatography was performed on silica gel 60 (40-63 μm) (Chimmed, Russia). Analytical TLC was performed on Sorbfil normal phase plates (TLC silica gel 5-17 μm F254) (IMID Co., Ltd., Russia). Spots on TLC plates were visualized using a UV lamp. Biotin quantitation was carried out using 4'-hydroxyazobenzene-2-carboxylic acid (HABA) (Thermo Fisher Scientific, USA) and avidin (Thermo Fisher Scientific, USA). For PVA quantitation, boric acid (Chimmed, Russia), iodine (Chimmed, Russia) and potassium iodide (Chimmed, Russia) were used.

1.2. Instruments

^{1}H and ^{13}C NMR spectra were recorded at room temperature on Bruker AVANCE 500 spectrometer with the residual solvent peak as an internal standard, namely $CHCl_3$ in $CDCl_3$ (7.26 ppm for ^{1}H NMR and 77.16 for ^{13}C NMR). Analytical HPLC (drug and impurity contents measurements) was performed on a Milichrom-A02 chromatographic system (EkoNova, Russia). The HPLC measurement conditions: column – 120 × 5 mm ProntoSIL 120-5-C18, 5 μm; detection – UV detector, 230 nm; temperature – 35 °C; injection volume – 2 μl; mobile phases – A: H_2O, B: MeCN; flow rate – 100 μl/min; mobile phase B 60% isocratic mode for 10 min, gradient – 60% → 80% in 1 min, then gradient – 80% → 60% in 1 min. Colorimetric assay was carried out on UV-1900i spectrophotometer (Shimadzu, Japan).

The pH value was measured using an ST2100-E pH meter with an ST210 electrode (Ohaus, USA). Centrifugation was performed using a Tagler SM-12 centrifuge with a RU-06 rotor (TAGLER, Russia). Lyophilization was performed using a LAB-1F-50 lyophilizer (LabFreez, China).

2. Synthesis of PTX-B7 conjugate

2.1. Synthetic procedure

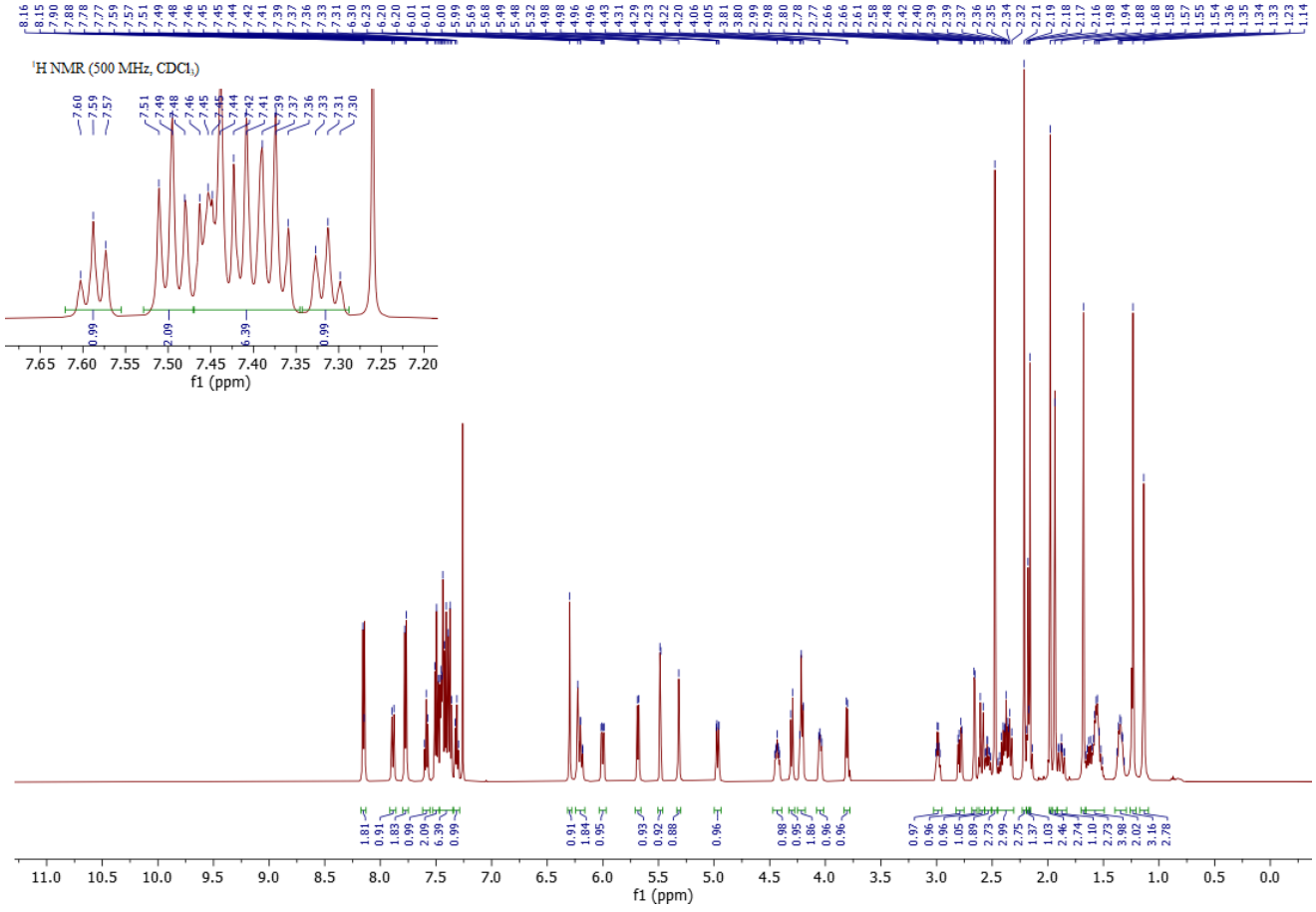
In a 10-ml round-bottom flask were placed 256 mg (0.3 mmol) of **PTX** and 37 mg (0.3 mmol) of DMAP. The compounds were dissolved in 2 ml of DMF, then 81 mg (0.33 mmol) of biotin and 69 mg (0.36 mmol) of EDCl were added successively with vigorous stirring. The reaction mixture was stirred at ambient temperature overnight, then diluted with 50 ml of EtOAc and washed successively with 50 ml of 1 M HCl, 3 × 50 ml of H_2O and 50 ml of saturated NaCl solution. The organic layer was dried over anhydrous sodium sulfate and evaporated. The residue was purified by column chromatography (10 g of SiO_2, 3 × 3 cm, Me_2CO/HexH (1:1 v/v) → Me_2CO). The collected fractions containing the desired product were evaporated in vacuo, which afforded **PTX-B$_7$** conjugate as a white powder (269 mg, 83%). R_f 0.31 (Me_2CO).

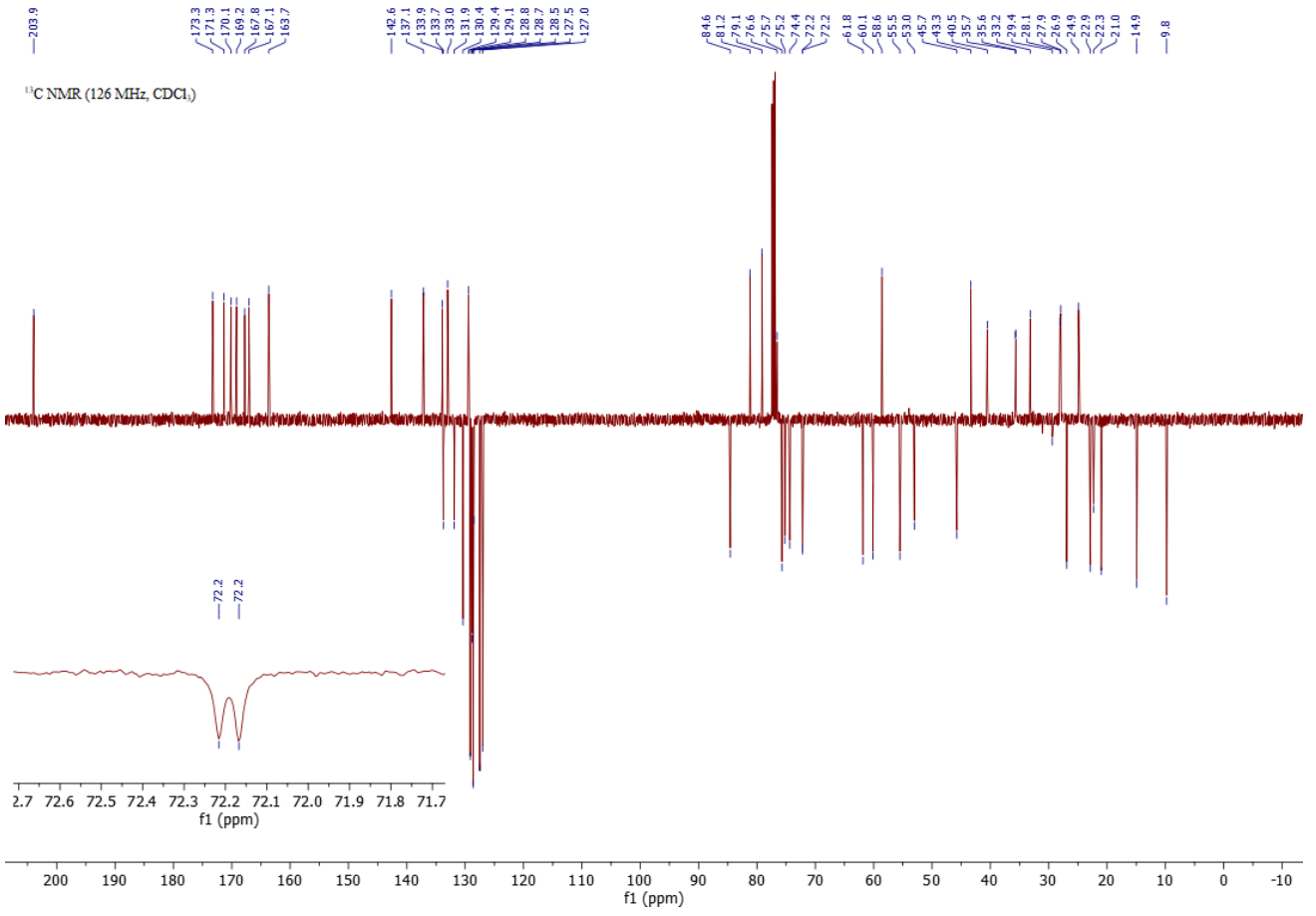
^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.15 (d, J = 5.0 Hz, 2H), 7.89 (d, J = 9.3 Hz, 1H), 7.77 (d, J = 7.0 Hz, 2H), 7.59 (t, J = 7.4 Hz, 1H), 7.50 (t, J = 7.6 Hz, 2H), 7.48 – 7.34 (m, 6H), 7.31 (t, J = 7.2 Hz, 1H), 6.30 (s, 1H), 6.24 – 6.16 (m, 2H), 6.00 (dd, J = 9.3, 3.8 Hz, 1H), 5.68 (d, J = 7.2 Hz, 1H), 5.48 (d, J = 3.8 Hz, 1H), 5.32 (s, 1H), 4.97 (dd, J = 9.6, 2.6 Hz, 1H), 4.43 (ddd, J = 11.0, 6.7, 4.3 Hz, 1H), 4.30 (d, J = 8.2 Hz, 1H), 4.25 – 4.18 (m, 2H), 4.08 – 4.01 (m, 1H), 3.81 (d, J = 7.2 Hz, 1H), 2.99 (td, J = 7.3, 4.6 Hz, 1H), 2.79 (dd, J = 13.0, 4.9 Hz, 1H), 2.66 (d, J = 4.3 Hz, 1H), 2.59 (d, J = 12.8 Hz, 1H), 2.54 (td, 1H), 2.48 (s, 3H), 2.46 – 2.31 (m, 3H), 2.21 (s, 3H), 2.17 (t, J = 3.4 Hz, 1H), 2.16 (s, 1H), 1.98 (s, 2H), 1.94 (s, 3H), 1.88 (ddd, J = 13.6, 11.0, 2.6 Hz, 1H), 1.68 (s, 3H), 1.68 – 1.49 (m, 4H), 1.40 – 1.30 (m, 2H), 1.23 (s, 3H), 1.14 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ: 203.9, 173.3, 171.3, 170.1, 169.2, 167.8, 167.1, 163.7, 142.6, 137.1, 133.9, 133.8, 133.0, 131.9, 130.4, 129.4, 129.1, 128.8, 128.7, 128.5, 127.5, 127.0, 84.6, 81.2, 79.1, 76.6, 75.7, 75.2, 74.4, 72.2, 72.2, 61.8, 60.1, 58.6, 55.5, 53.0, 45.7, 43.4, 40.5, 35.7, 35.6, 33.2, 28.1, 27.9, 26.9, 24.9, 22.9, 22.3, 20.9, 14.9, 9.8.

The spectral data are in agreement with the previous report.[S1]

2.2. NMR spectra





3. Preparation and characterization of self-assembled nanoparticles

3.1. Preparation of nanoparticles

Nanoparticles (NPs) were prepared by nanoprecipitation technique as follows. The example demonstrates the optimal conditions after optimization. Briefly, 1 ml of a 20 mg/ml **PTX-B$_7$** conjugate solution in ethanol was added dropwise over 2 min at room temperature and vigorous stirring (600 rpm) to 10 ml of a 0.8% (w/v) PVA solution. The NPs suspension was stirred for 5 min, after which it was evaporated from most of the organic solvent on a rotary evaporator at 40 °C (150 rpm). The suspension was then filtered through a syringe filter (FILTSTAR Syringe Filter, glass fiber 25 mm, 1 µm, Hawach Scientific Co., Ltd., China) and poured in 0.5 ml aliquots into glass vials. The samples were frozen at –40 °C and lyophilized.

3.2. Transmission electron microscopy

The morphology of nanostructures was analyzed with JEOLJEM-1400 transmission electron microscope (TEM) (JEOL Ltd., Japan) as described previously [S1]. Briefly, a sample of the **PTX-B$_7$** conjugate NPs in distilled water was diluted to a concentration of approximately 0.1 mg/ml and applied to a copper grid for electron microscopy, precoated with formvar (0.5% (w/v) solution in chloroform). The samples were completely dried in order to remove the solvent. The micrographs were collected on TEM at an accelerating voltage of 120 kV. Contrasting was performed using uranyl acetate.

3.3. Size, polydispersity index and zeta-potential measurements

Particle volume average size (d_v), polydispersity index (PDI) and zeta-potential (ZP) were measured using a Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed under fixed temperature (25 °C) and automatic attenuation level, the equilibrium time was 30 s. To determine the optimal concentration for the measurements of the above parameters, a series of **PTX-B$_7$** NPs suspensions were prepared. For all suspensions d_v, PDI and ZP were measured, all data are presented below (Table S1). The concentration suitable for the measurements was set at 20 µg/ml, since approximately constant parameters were observed near this value (Table S1, marked in grey).

Table S1 Selection of optimal conditions for measuring the volume average particle size, polydispersity index and zeta potential, mean value ± SD (n = 3).

PTX-B$_7$ concentration/µg ml^{-1}	**d_v/nm**	**PDI**	**ZP/mV**
1	285 ± 55	0.546 ± 0.048	–22.5 ± 2.9
5	229 ± 10	0.110 ± 0.016	–20.2 ± 1.2
20	213 ± 1	0.159 ± 0.012	–22.8 ± 0.4
50	246 ± 17	0.134 ± 0.012	–22.5 ± 1.0
100	215 ± 3	0.114 ± 0.011	–12.2 ± 1.0
200	209 ± 7	0.068 ± 0.021	–9.3 ± 0.2
500	226 ± 3	0.086 ± 0.007	–7.8 ± 0.3
1000	235 ± 1	0.084 ± 0.009	–3.2 ± 0.1

3.4. Evaluation of the drug content

To determine the content of the **PTX-B$_7$** conjugate in NPs samples, the lyophilisate was resuspended in 1 ml of water upon sonication. Then, 0.5 ml aliquot was transferred to 5 ml volumetric flask and diluted with 1:1 (v/v) MeCN/H_2O mixture. The conjugate concentration in the test solution was calculated using calibration plot (Figure S1).

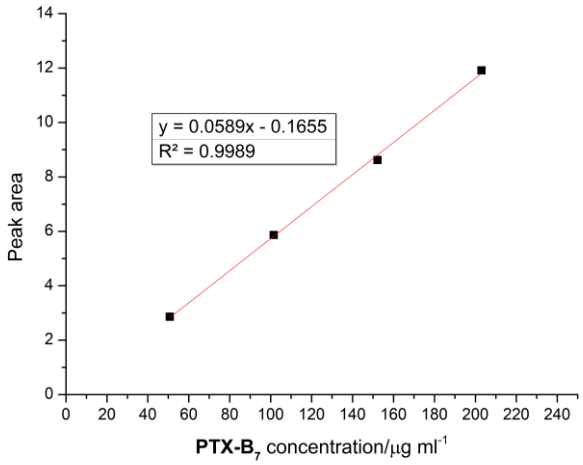


Figure S1 HPLC calibrating plot for **PTX-B$_7$** conjugate.

The conjugate content in the vial was quantified by the following equation (eq. 1):

$$\textbf{PTXB}_\textbf{7}\text{ content (mg)} = \frac{c(\textbf{PTXB}_\textbf{7})V_{VF}V_{sample}}{1000V_{aliquot}} \quad (\text{eq. } 1)$$

Where c(**PTXB$_7$**) – conjugate concentration, found in the final solution (μg/ml); V_{VF} – volume of the volumetric flask (5 ml); V_{sample} – volume of the test sample (1 ml); $V_{aliquot}$ – the aliquot volume (0.5 ml).

3.5. Biotin availability assay

In order to assess the availability of biotin in the conjugate NPs for binding to receptors on the surface of tumor cells, the HABA-avidin assay was used.[S2–S4] To perform the analysis, two reagent solutions were prepared in advance. Solution **(A)** was prepared by dissolving 12.1 mg of HABA in 200 μl of 1 M aqueous NaOH solution, which was then diluted with water in a 5 ml volumetric flask. To prepare solution **(B)**, 5 mg of avidin were dissolved in a 10 ml volumetric flask in 5 ml of PBS (pH 7.1), after which 300 μl of HABA solution **(A)** were added and the volume was brought to the mark with the buffer. The reference solution was prepared by diluting 300 μl of HABA solution with buffer to 10 ml. A 0.1 mM aqueous solution of biotin was also prepared. To prepare the test solution, the contents of the vial with the lyophilisate were quantitatively transferred into a 10 ml volumetric flask and brought up to the mark with water.

Photometric detection was carried out at the fixed wavelength 503 nm. The baseline was established using a solution prepared by mixing 900 μl of the reference solution and 100 μl of water (Figure S2, solution 1). Next, 900 μl of the HABA-avidin complex solution **(B)** was diluted with 100 μl of water (Figure S2, solution 2) and the absorbance was measured relative to the baseline. Next, 100 μl of 0.1 mM biotin solution was diluted with 900 μl of solution **(B)** and allowed to stand for 30 min (Figure S2, solution 3), after which the absorbance was measured. Afterwards, 100 μl of the test solution were diluted with 900 μl of solution **(B)** and allowed to stand for 30 min (Figure S2, solution 4), after which the absorbance was also measured.

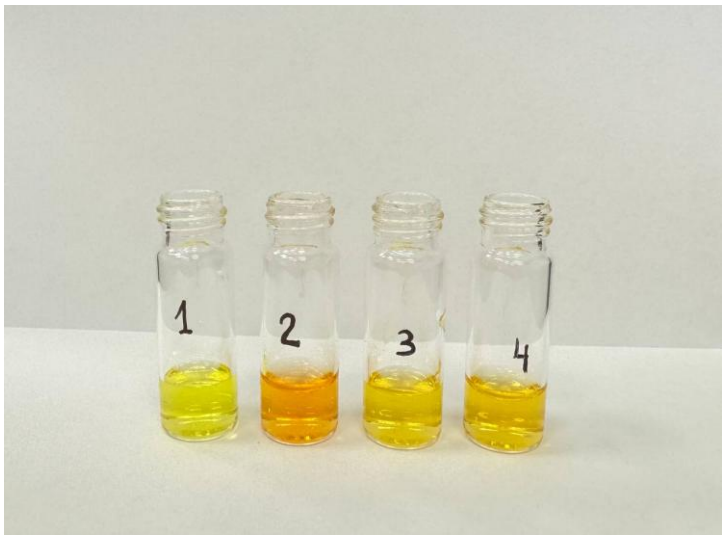


Figure S2 Appearance of the measured solutions (explanations are provided in the text above).

The fraction of biotin potentially available for interaction with receptors in the sample of the conjugate NPs was calculated using the formula (eq. 2):

$$\text{Available biotin fraction (\%)} = \frac{(A_0 - A_{NPs})c_B V_{VF} M}{1000(A_0 - A_B)m_{NPs}} \cdot 100\ \% \quad (\text{eq. } 2)$$

Where A_0 – absorbance of the solution 2; A_B – absorbance of the solution 3; A_{NPs} – absorbance of the solution 4; c_B – biotin concentration (0.1 mM); V_{VF} – capacity of the volumetric flask with the suspension of NPs (10 ml); M – molar weight of the conjugate (1080.21 g/mol); m_{NPs} – average conjugate content in a vial (mg).

3.6. Polyvinyl alcohol quantification

Quantitative assay of PVA in the lyophilisate samples was carried out by spectrophotometric method according to the literature data.[S5,S6] To do this, a 125 mg/l stock solution of PVA (9-10 kDa) in water was prepared. Aliquots of the solution were transferred to 25 ml volumetric flasks to prepare a series of PVA calibration solutions in the concentration range of 10–50 mg/l. Then, 5 ml of 95% ethanol, 7.5 ml of an aqueous solution of boric acid (40 mg/ml), and 1.5 ml of an aqueous solution containing iodine (12.7 mg/ml) and potassium iodide (25 mg/ml) were successively added to each flask. The contents of the flasks were carefully mixed and kept in the dark place at room temperature for 30 minutes, after which they were brought to the mark and mixed. To select the optimal wavelength, the absorption spectrum (Figure S3) of a 50 mg/l PVA solution was measured relative to the reference solution containing all reagents except the analyte.

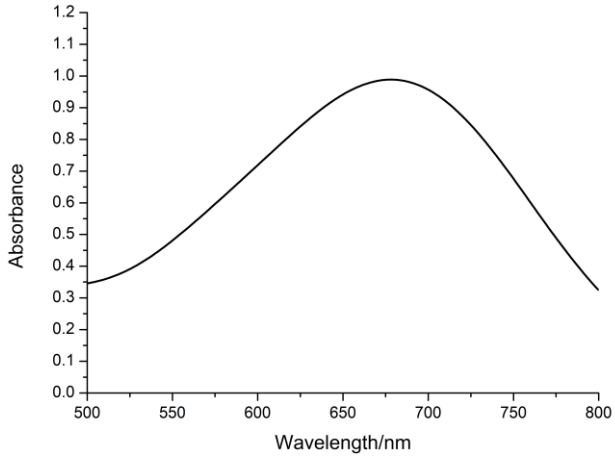


Figure S3 Absorption spectrum of PVA-borate-iodine complex.

For standard PVA solutions, the absorption was measured at 680 nm, which corresponded to the extinction maximum in the visible region. The appropriate calibration plot (Figure S4) is presented below.

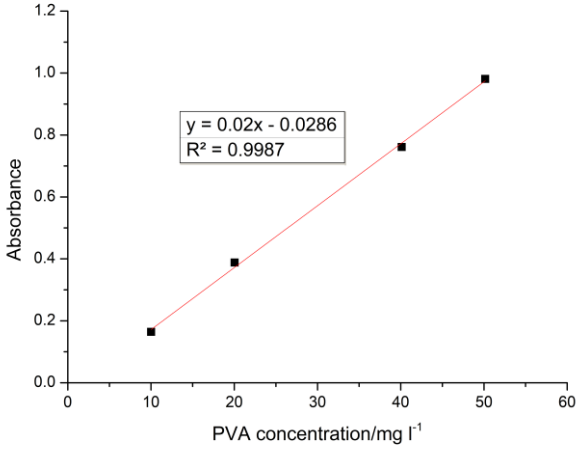


Figure S4 Spectrophotometric calibrating plot for PVA.

To determine the PVA content, the lyophilisate sample was dissolved in 1 ml of 95% ethanol and quantitatively transferred to a 10 ml volumetric flask, brought up to the mark with alcohol and mixed. A 1 ml aliquot was transferred to a 25 ml volumetric flask and diluted with 4 ml of 95% ethanol, 7.5 ml of an aqueous solution of boric acid (40 mg/ml) and 1.5 ml of an aqueous solution of iodine (12.7 mg/ml) and potassium iodide (25 mg/ml), brought up to the mark with water and mixed. After 30 min, the absorption of the solution at 680 nm was measured and the PVA content in the flask was calculated using the following equation (eq. 3):

$$\text{PVA content (mg)} = \frac{c(\text{PVA})V_{VF1}V_{VF2}}{1000V_{aliquot}} \quad (\text{eq. } 3)$$

Where c(PVA) – PVA concentration, found in the final solution (mg/l); V_{VF1} and V_{VF2} – volumes of the volumetric flasks (10 and 25 ml respectively); $V_{aliquot}$ – the aliquot volume (1 ml).

4. Stability tests

4.1. Colloidal stability of nanoparticles in aqueous media

To assess colloidal stability, lyophilisate samples were resuspended in 0.5 ml of water, after which one part of the resulting suspensions was left in a refrigerator at 4 °C, and the other part was placed in a dark place at room temperature (about 22 °C). At certain time intervals, aliquots of the test suspensions were diluted with water 100 times, after which the volume average size and PDI of the NPs were measured (Table S2).

Table S2 Colloidal stability of **PTX-B7** NPs in different dispersion media, mean value ± SD (n = 3).

Dispersion medium	Time, days	Storage temperature			
		22 °C		4 °C	
		d_v/nm	PDI	d_v/nm	PDI
Water	0	202 ± 5	0.112 ± 0.012	187 ± 3	0.088 ± 0.007
	1	191 ± 3	0.099 ± 0.019	198 ± 4	0.109 ± 0.028
	2	190 ± 2	0.174 ± 0.012	190 ± 3	0.086 ± 0.029
	4	191 ± 6	0.126 ± 0.027	196 ± 9	0.125 ± 0.034
	8	213 ± 4	0.149 ± 0.018	191 ± 4	0.067 ± 0.011
0.9% NaCl	0	209 ± 7	0.098 ± 0.067	203 ± 4	0.135 ± 0.017
	1	183 ± 1	0.076 ± 0.031	193 ± 3	0.078 ± 0.029
	2	192 ± 10	0.129 ± 0.014	190 ± 1	0.060 ± 0.034

	4	218 ± 10	0.215 ± 0.012	222 ± 9	0.210 ± 0.015
	8	188 ± 4	0.071 ± 0.024	192 ± 1	0.065 ± 0.029
PBS (pH 7.4)	0	193 ± 2	0.144 ± 0.0122	202 ± 11	0.133 ± 0.015
	1	191 ± 5	0.162 ± 0.011	200 ± 3	0.091 ± 0.026
	2	202 ± 3	0.169 ± 0.006	206 ± 9	0.161 ± 0.017
	4	185 ± 3	0.072 ± 0.029	202 ± 4	0.119 ± 0.019
	8	184 ± 4	0.024 ± 0.011	190 ± 2	0.037 ± 0.011
Phosphate buffer (pH 7.4)	0	185 ± 1	0.079 ± 0.017	192 ± 4	0.085 ± 0.007
	1	189 ± 4	0.115 ± 0.023	193 ± 1	0.101 ± 0.007
	2	199 ± 6	0.144 ± 0.035	190 ± 3	0.098 ± 0.026
	4	197 ± 7	0.084 ± 0.031	213 ± 15	0.145 ± 0.053
	8	191 ± 5	0.053 ± 0.009	190 ± 3	0.075 ± 0.027
5% glucose	0	191 ± 2	0.070 ± 0.029	188 ± 1	0.069 ± 0.027
	1	193 ± 7	0.079 ± 0.052	190 ± 4	0.096 ± 0.020
	2	199 ± 4	0.099 ± 0.032	194 ± 2	0.050 ± 0.030
	4	200 ± 4	0.127 ± 0.018	190 ± 1	0.048 ± 0.040
	8	190 ± 3	0.101 ± 0.013	191 ± 2	0.035 ± 0.012

4.2. Colloidal stability of nanoparticles in FBS solutions

The study of the change in the sizes of nanoparticles under conditions simulating a physiological environment was carried out in PBS (pH 7.4) with differing content of fetal bovine serum (FBS). For this, the lyophilisate in a vial was resuspended in 1 ml of the desired medium and incubated at 37 ℃ in ThermoMixer C (Eppendorf, Germany). The volume average size and PDI were measured by diluting the aliquots of the test samples 50 times with water (Table S3).

Table S3 Colloidal stability of **PTX-B$_7$** NPs in PBS/FBS mixtures, mean value ± SD (n = 3).

Time, h	% FBS	d_v/nm	PDI
0	**0**	203 ± 4	0.092 ± 0.027
	10	233 ± 22	0.106 ± 0.026
	50	233 ± 15	0.219 ± 0.028
	100	213 ± 2	0.127 ± 0.011
1	**0**	203 ± 6	0.093 ± 0.032
	10	245 ± 24	0.131 ± 0.076
	50	203 ± 7	0.215 ± 0.009
	100	227 ± 6	0.326 ± 0.014
2	**0**	202 ± 5	0.075 ± 0.024
	10	208 ± 3	0.123 ± 0.070
	50	198 ± 15	0.233 ± 0.020
	100	282 ± 56 (54%) 65 ± 32 (43%)	0.358 ± 0.032
4	**0**	198 ± 8	0.143 ± 0.018
	10	242 ± 19	0.182 ± 0.005
	50	244 ± 12.4	0.223 ± 0.031
	100	283 ± 83 (54%) 66 ± 41 (39%)	0.379 ± 0.040
6	**0**	203 ± 1	0.095 ± 0.020
	10	244 ± 15	0.335 ± 0.064
	50	257 ± 8	0.208 ± 0.061
	100	245 ± 41 (54%) 50 ± 3 (46%)	0.328 ± 0.028

4.3. Stability of nanoparticles upon storage

Long-term storage stability tests of nanoparticles under accelerated aging conditions were conducted in a test chamber Nuve TK 120 (NÜVE, Turkey). The nanoparticle lyophilisate samples were kept under constant conditions, namely 30 ℃ and 65% relative humidity for 74 days, which is equivalent to storing the NPs at 4 ℃ for 2 years. The volume average size, PDI, drug and impurity contents were measured at certain time intervals below (Figure S5).

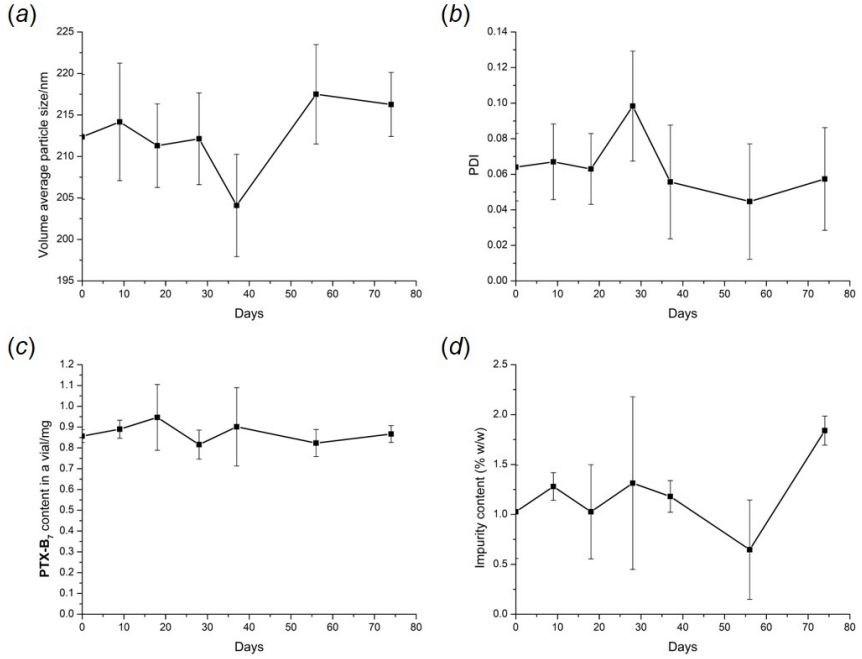


Figure S5 (*a*) Size, (*b*) PDI, (*c*) drug and (*d*) impurity contents plot of **PTX-B$_7$** NPs lyophilisate upon storage under accelerated aging conditions, mean value ± SD (n = 3).

5. Hemolytic activity

The hemolytic activity of **PTX-B$_7$** NPs in comparison with the Paclitaxel-Ebewe® form (6 mg/ml) was assessed spectrophotometrically by the concentration of released hemoglobin as described in the previous report with modifications.[S7] The experiment was approved by the ethics committee of V. P. Serbsky Federal Medical Research Centre of Psychiatry and Narcology of the Ministry of Health of the Russian Federation (no. 5 from 03.04.2024).

The blood samples were obtained from at least 3 donors who had not taken any medications that could affect the experimental results for 2 weeks. Whole human blood was collected in 9 ml vacutainers (K_3-EDTA). Blood samples were centrifuged for 10 min at 18 °C with an acceleration of 900g (Hettich® Universal 320R centrifuge with a 1628 swinging-bucket rotor, Andreas Hettich GmbH & Co. KG, Germany). Erythrocytes were washed twice with PBS and then resuspended in PBS to an erythrocyte content of 4×10^9 cells per ml, which corresponds to the erythrocyte concentration in the blood of a healthy person. **PTX-B$_7$** NPs samples were resuspended in PBS (0.15 M, pH 7.4) to a drug content of 5 mg/ml. Next, a series of 10-fold dilutions of the suspensions in PBS were prepared (Table S4). 100 µl of the corresponding suspension was added to 900 µl of the erythrocyte suspension (4×10^9 cells/ml) to a final drug concentration in the range from 62.5 to 500 µg/ml and incubated in a shaker-incubator at a temperature of +37 °C for 1.5 hours. Dilutions of the comparison drug (Paclitaxel-Ebewe®) were prepared in similar concentrations in PBS (Table S5). After centrifugation of the samples (5 min, 18 °C, 900×g), sodium dodecyl sulfate (0.06% w/w) was added to the supernatant to induce hemichrome formation. Hemoglobin concentration was quantified by measuring the optical density at 540 nm using an EnSpire plate reader (PerkinElmer, USA). The erythrocyte suspension mixed with PBS was used as a negative control (C–), and 1% (w/v) Triton X-100, causing 100% hemolysis, was used as a positive control (C+). The degree of hemolysis was calculated as the ratio of the optical density of the sample to that of the positive control. All experiments were performed in triplicate.

Table S4 Scheme for the preparation of 10-fold suspensions of **PTX-B$_7$** NPs

PTX-B$_7$ concentration/ µg ml^{-1}	**Concentration before dilution/µg ml^{-1}**	**Volume of the initial suspension/µl**	**PBS volume/µl**	**Total volume of the initial suspension/µl**
500	5000	250	0	468.7 **3 vials**
250	2500	125	125	
125	1250	62.5	187.5	
62.5	625	31.2	218.8	

Table S5 Scheme for the preparation of 10-fold solutions of the drug Paclitaxel-Ebewe®

PTX concentration/ µg ml^{-1}	**Concentration before dilution/µg ml^{-1}**	**Volume of the initial suspension/µl**	**PBS volume/µl**	**Total volume of the initial suspension/µl**
500	5000	208	42	390
250	2500	104	146	
125	1250	52	198	
62.5	625	26	224	

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