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Doxorubicin delivery systems based on polypeptide nanoparticles for subcutaneous administration in cancer therapy

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S1. Synthesis of copolymers

The previously developed protocols for the synthesis of random and block-polypeptides were applied.[S1,S2] Briefly, synthesis of polymers was carried out using 4 wt% solution of monomer(s) in dioxan for 96 h at 22 °C. The polymers were precipitated into diethyl ether for three times and air dried. The benzyl protective groups of Ser and Glu were deprotected with the use of 0.1 M solution of trifluoromethanesulfonic acid in trifluoracetic acid for 3 h at room temperature. Deblocked polymers were diluted with DMF and purified by dialysis against water using dialysis bags with molecular weight cut off equal to 1000 (Orange Scientific, Belgium). All reagents applied for polypeptide synthesis were purchased from Sigma-Aldrich (Germany) and used as received. Organic solvents were purchased from Vecton (Russia) and distilled before use according to standard procedures.

S2. Characterization of copolymers

^{1}H MNR analysis of protected copolymers was carried in DMSO-d_6 using a Bruker Avance-400 Spectrometer (Bruker, Germany). The spectrum of P(Glu-*co*-Phe) can be found in ESI to our previous paper.[S2] The spectrum of PSer-*b*-PGlu is shown in **Figure S1**.

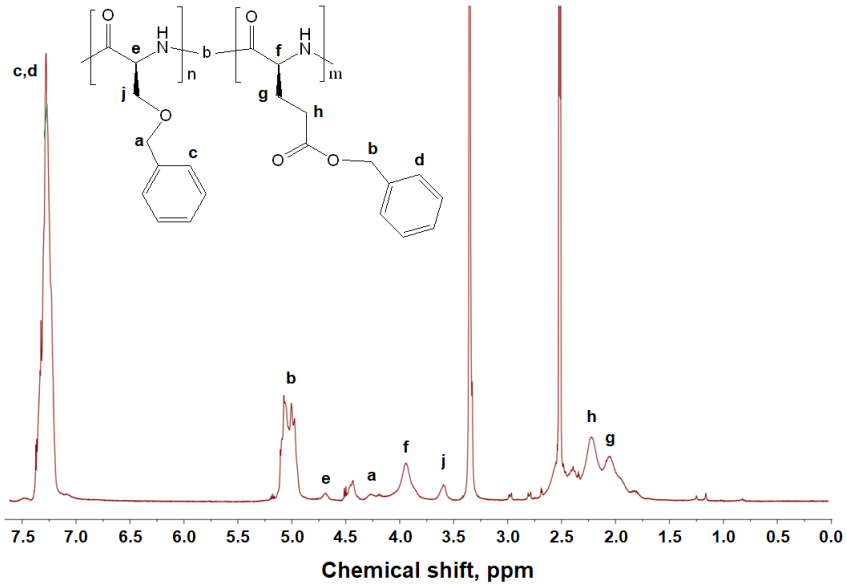


Figure S1 ^{1}H NMR spectrum of PSer-*b*-PGlu (DMSO-d_6, 25 °C).

SEC was performed in DMF containing 0.1 M LiBr at 40 °C using Shimadzu LC-10 HPLC system (Shimadzu, Japan) with refractometric detection. Number average and weight average molecular weights were calculated regarding the calibration plot built for poly(methyl methacrylate) standards (Sigma-Aldrich, Germany). An example of SEC trace of PSer-*b*-PGlu is shown in **Figure S2**.

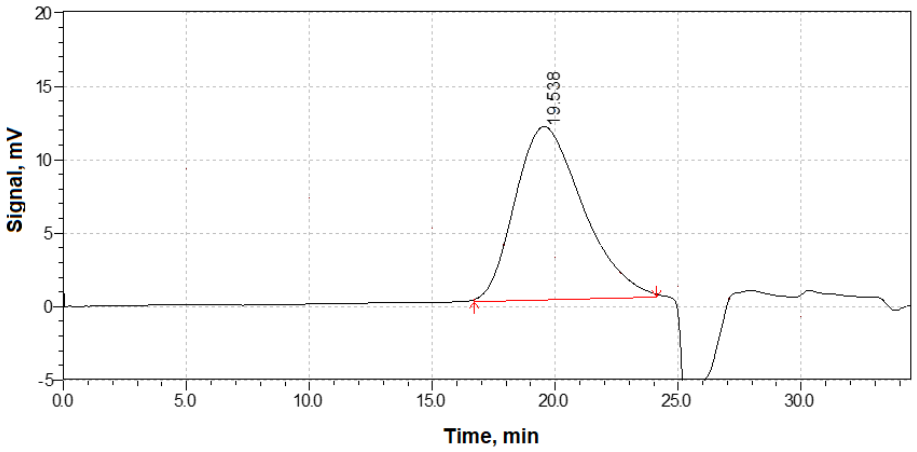


Figure S2 SEC trace of the protected PSer-*b*-PGlu copolymer (DMF + 0.1 M LiCl, 40 °C). The chromatogram reflects the unimodal distribution of the polymer sample. The reverse signal is a system peak.

The copolymer composition was determined by reversed-phase HPLC analysis of free amino acids obtained after total acidic hydrolysis of polypeptides. The hydrolysis was carried out in 6 M HCl with 0.0001% phenol in vacuum-sealed ampoule for four days.[S1] The hydrolysates were analyzed using LCMS-8030 Shimadzu system with triple quadruple mass-spectrometry detection (LC-MS) (Shimadzu, Japan) equipped with 2 × 150 mm Luna C18 column packed with 5 μm particles. The isocratic elution mode was applied and 0.1% acetonitrile/HCOOH in a ratio

5/95 wt% was used as eluent. The mobile phase flow rate was equal to 0.3 ml min^{-1}. The calibration plots obtained for standard solutions of amino acids (Glu and Ser) as well as results of HPLC-MS analysis are presented in **Figure S3** and **Table S1**.

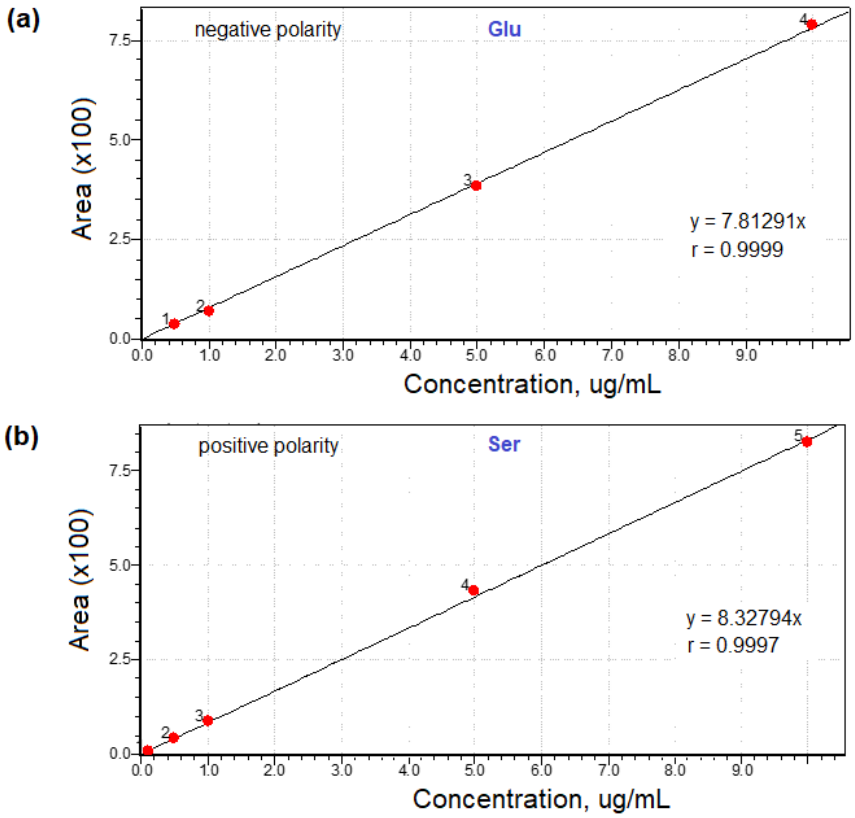


Figure S3 Calibration plots built for standard solutions of amino acids: (*a*) Glu and (*b*) Ser.

Table S1 Results of HPLC analysis of amino acids in hydrolysates of PSer-*b*-PGlu (n = 3). Results are presented as mean.

Amino acid	*m/z*	Concentration/μg ml^{-1}	Sample mass/mg	Amount/μmol
Glu	146.1	1.366	1.366	9.2
Ser	106.1	0.607	0.607	5.8

S3. Preparation and characterization of nanoparticles

Nanoparticle dispersions were prepared by redispersion of the lyophilized copolymers in aqueous media (deionized water or 0.1 M PBS) at concentrations of 1–5 mg ml^{-1} under short-time ultrasonication (30 s) performed by an ultrasound probe Sonopuls HD2070 (Bandelin, Germany) at a 20% power.

TEM analysis was carried out with the use of a Jeol JEM-2100 (Tokyo, Japan) microscope operated at an acceleration voltage of 160 kV and Cu grids (300-mesh) covered with carbon and formvar and staining with 2% uranyl acetate solution (*w*/*v*). Sample concentration was 0.5 mg ml^{-1}. After sample application, the dried grid was stained for 30–60 s and used for measurements

after 18 h. ImageJ software (USA) was used to calculate the average particle diameter from 3 to 5 TEM images for each sample (10–20 nanoparticles).

DLS and ELS measurements were performed using Zetasizer Nano-ZS (Malvern Instrument Ltd., UK) equipped with a He–Ne laser beam at 633 nm and a detection angle of 173°. Sample concentration was 0.5 mg ml^{-1}. Zeta potential was measured in 0.1 M PBS (pH 7.4).

S4. Preparation of DOX-loaded nanoparticles

Doxorubicin hydrochloride (DOX, ≥99%) used in the work was purchased from Bld Pharmatech (China). The formulations were prepared by adding an aliquot of DOX to a dispersion of nanoparticles in 0.1 M PBS (pH 7.4), followed by vigorous stirring for 30 min in a thermostatic shaker at 25 °C and further incubation at 4 °C overnight. Supernatant with free DOX was removed by centrifugation at 15 000 rpm for 10–15 min. Nanoparticles were washed with water for 4 times. All supernatant fractions were combined together, freeze-dried and dissolved in a 1.5 ml of deionized water. The content of DOX in a sample was determined spectrophotometrically at a wavelength 480 nm. Drug load was determined as the difference between the initial and unencapsulated amounts of DOX.

Table S2 DOX load and encapsulation efficacy depending on initial DOX amounts.

DOX amount/ μg	Drug load/ μg mg^{-1} of nanoparticles	Encapsulation efficacy (%)
P(Glu-*co*-Phe)		
200	170 ± 9	85
300	274 ± 19	91
400	363 ± 21	91
500	459 ± 15	92
1000	972 ± 28	97
PSer-*b*-PGlu		
200	188 ± 6	94
300	283 ± 9	94
400	379 ± 11	95
500	490 ± 10	98
1000	991 ± 9	99

S5. Release study

Freshly prepared DOX formulations were dispersed in the appropriate medium (1 mg ml^{-1}) and incubated in a thermostated shaker at 37 °C. At a specified time, the supernatant containing the released DOX was separated by centrifugation and analyzed as described in footnote 7. The withdrawn portion of the supernatant was replaced with an aliquot of fresh buffer/blood plasma and the procedure was repeated for further time points. The loss of initial DOX was included in the calculation and the cumulative percentage of DOX release was determined.

S6. Cell viability

Viability of MCF-7 cells was determined using 2×10^4 cells placed into biosensing substrate of the RTCA iCELLIgence System (ACEA Biosciences, USA). The electrical impedance was measured as a function of cell state in dynamics.

Viability of A549 cells was determined using 2×10^4 cells seeded into 96-well plate via standard MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

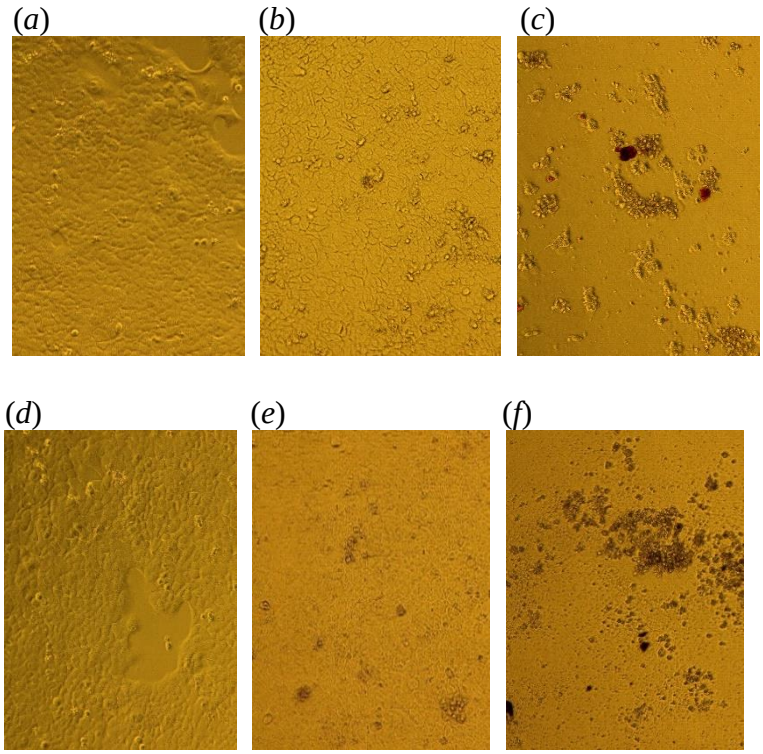


Figure S4 A431 cell images obtained by Primo Vert (Carl Zeiss, Germany) optical microscope (×100) after 3 days of incubation with (*a*)–(*c*) PSer-*b*-PGlu and (*d*)–(*f*) P(Glu-*co*-Phe), containing various DOX concenterations: (*a*),(*d*) 0, (*b*),(*e*) 0.05 and (*c*),(*f*) 2 μg ml^{-1}.

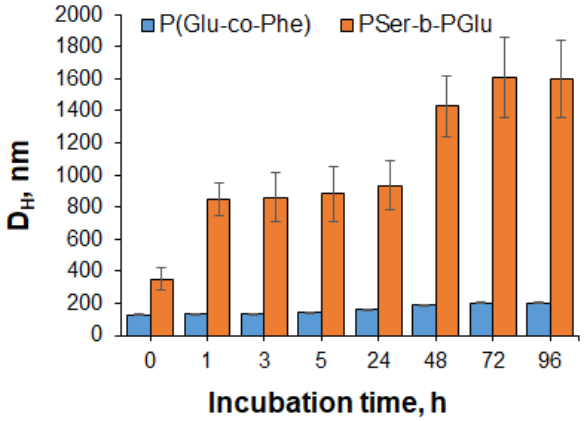


Figure S5 Stability of polypeptide nanoparticles over time in cell culture medium (DMEM) at 37 °C.

S7. Experiments *in vivo*

Animals were kept in a conventional vivarium in polypropylene cages of 5 heads each, at an air temperature of 20–22 °C, relative humidity of 50–60%, with a 12-hour light/dark cycle. Mice received complete briquette feed (4RF18 prolonged keeping formula for rodents, Mucedonia, Italy) and drinking water without restriction. The experimental and control groups of mice were examined regularly. The activity of animals was evaluated daily, they were weighed, their temperature was measured, the condition of hair coat, feed and water intake were also monitored.

The DOX formulation was injected in 1.0 ml of 5% glucose solution with the use of 21-gauge needles under general anesthesia. For this, Zoletil (0.05 ml per 0.1 kg of body mass) and Rometarum 20 mg ml^{-1} (0.0125 ml per 0.1 kg of body mass) were applied intramuscularly. Animals that died in the experiment were autopsied with liver and other altered organs and tissues taken. The tumor volume was measured for all animals in experiment. The standard hematoxylin and eosin histological study was performed after mice death. Microscopic analysis was performed using a Nikon Eclipse E200 light microscope (Nikon, Japan) equipped with a Nikon DS-Fi3 digital camera.

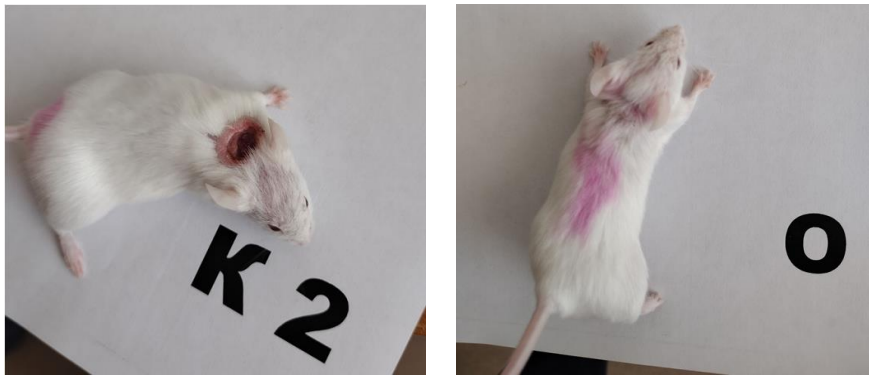
Tumor growth inhibition (GI, %) was calculated as[S3]

$$GI = \frac{Vc - Ve}{Vc} \times 100, \qquad (S1)$$

where V_c is the average volume of a tumor in the control group, and V_e is the average volume of a tumor in the experimental group.

Efficacy index (EI) was calculated as

$$EI = \frac{Ve}{Vc} \qquad (S2)$$



(*a*) (*b*)

Figure S6 Images of mice after subcutaneous DOX administration as (*a*) free drug and (*b*) DOX nanoformulation.

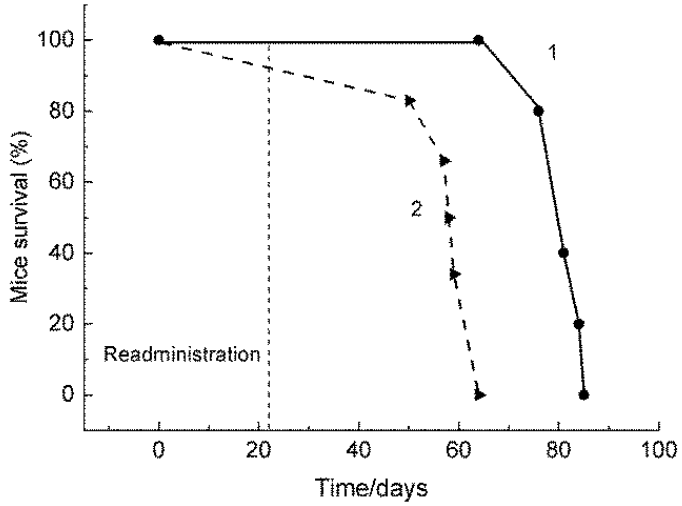


Figure S7 FVB/N mice survival after two subcutaneous administrations of 5 mg DOX per mouse in (*1*) experimental and (*2*) control groups.

References

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