

Supporting information

Unveiling the power of liquid chromatography in examining a library of degradable poly(2-oxazoline)s in nanomedicine applications

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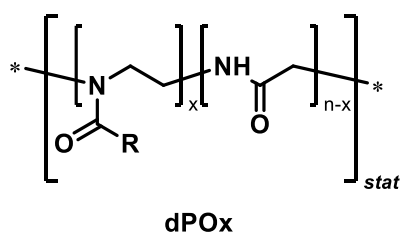
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1. Materials

Table S1. Characterization data of the polymers applied in this study. Molar masses (M_n) and dispersity values \bar{D} were obtained by SEC in two solvents: Polar dimethylacetamide (DMAc) with 0.21 wt.% LiCl and hydrophobic chloroform (CHCl_3) with 2 vol.% iso-propanol and 4 vol.% triethylamine, using a calibration with polystyrene standards and RI-detection.¹

Polymer	$M_{n,\text{theor.}}$ [g mol ⁻¹]	SEC, DMAc		SEC, CHCl ₃	
		$M_{n,\text{SEC}}$ [g mol ⁻¹]	\bar{D}_{SEC}	$M_{n,\text{SEC}}$ [g mol ⁻¹]	\bar{D}_{SEC}
dPMeOx	4200	5420	3.35	-	-
dPEtOx	4600	2400	1.57	540	1.52
dPPropOx	5000	2880	1.77	650	1.95
dPButOx	5400	2560	1.54	680	1.93
dPPentOx	5800	1800	1.97	830	1.79
dPHexOx	6100	1850	2.05	750	1.13
dPHeptOx	6500	1920	1.85	760	2.18
dPOctOx	6900	2020	1.93	1060	1.93
dPNonOx	7300	2060	1.78	890	2.20



R = Me, Et, Prop, But, Pent, Hex, Hept, Oct, Non

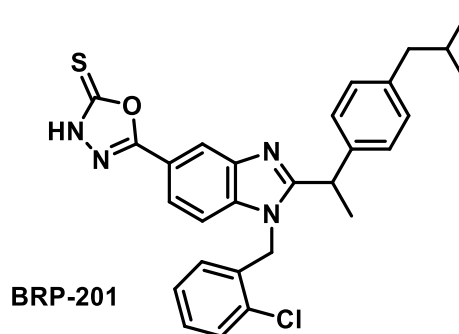


Fig. S1. Schematic representation of the structure of the poly(2-*n*-alkyl-2-oxazoline-*stat*-glycine) referred to as dPOx in the current study and the drug BRP-201.

2. Methods

2.1. Nanoparticle (NP) formulation

2.1.1. Formulation screening

Nanoprecipitation of all the dPOx polymers (see **Table S2**) dissolved in acetone was performed using the following polymer concentrations: 1, 2.5, 5, 10, and 20 mg mL⁻¹. A total volume of 0.5 mL of the respective polymer solution was injected with a 1 mL syringe into a vial that contained 3 mL of purified water (no surfactant) under continuous stirring at 800 rpm. After the suspensions were left for solvent evaporation for 8 hours the vials were closed and stored at 4 °C until further usage.

2.1.2. dPNonOx NPs in larger batches for degradation studies

To study the influence of surfactant on particle stability, dPNonOx NPs were formulated by nanoprecipitation with and without PVA. For that, dPNonOx was dissolved in 1 mL of acetone at a concentration of 20 mg mL⁻¹ and injected to either 15 mL of pure water or 15 mL of water containing 0.05% (m/v) PVA using a syringe pump (Aladdin AL1000-220, World Precision Instruments, Friedberg, Germany) at a flow rate of 2 mL min⁻¹. The suspensions were left under constant stirring at 800 rpm for 30 min at room temperature. Subsequently, the NPs were purified by ultrafiltration using a Vivaspin® 20 tube (Sartorius, Göttingen, Germany) at 5,000 rpm tempered at 18 °C for 60 min (5804 R Centrifuge with rotor A-4-44, Eppendorf, Hamburg, Germany). The Vivaspin® 20 tube was primed with 5 mL water before use. After centrifugation, the samples were rinsed with 10 mL water and centrifuged for a further 45 min. In the end, the samples were removed and filled up to 2 mL with purified water. To study enzymatic degradation of dPNonOx NPs over a longer period of time (68 days), dPNonOx NPs were formulated utilizing an initial polymer concentration of 10 mg mL⁻¹ in acetone and no additional surfactant PVA in the aqueous phase.

2.1.3. Drug-loaded (BRP-201) dPNonOx NPs

Drug-loaded dPNonOx NPs were formulated by nanoprecipitation utilizing PVA as surfactant. For that, dPNonOx was dissolved at a concentration of 5 mg mL⁻¹ or 20 mg mL⁻¹ in acetone. To obtain drug-containing NPs, a 10 mg mL⁻¹ BRP-201 drug stock solution in DMSO was added to the polymer solution to reach a final drug/polymer ratio of 3.0% (w/w). The organic phase (V = 2 mL for the 5 mg mL⁻¹ dPNonOx stock solution or V = 1 mL for the 20 mg mL⁻¹ dPNonOx stock solution) was injected to an aqueous phase (V = 15 mL of purified water), containing 0.05% (w/v) PVA, using a syringe pump (Aladdin AL1000-220, World Precision Instruments, Friedberg, Germany) at a flow rate of 2 mL min⁻¹. The formulations were repeated three times (n = 1, 2, 3). The NP suspensions were left under constant stirring at 800 rpm for 30 min to allow the evaporation of acetone.

The NPs were purified by ultrafiltration using a Vivaspin® 20 tube (Sartorius, Göttingen, Germany) at 5,000 rpm tempered at 18 °C for 60 min (5804 R Centrifuge with rotor A-4-44, Eppendorf, Hamburg, Germany). The Vivaspin® 20 tube was primed with 5 mL water before use. After centrifugation, the samples were rinsed with 10 mL water and centrifuged for a further 45 min. In the end, the samples were removed and filled up to 2 mL (n = 1) or 4 mL (n = 2, 3) with purified water.

The mass of the NPs was determined using a MYA 11.4Y microbalance (Radwag Waagen, Hilden, Germany) and the final particle concentration was calculated by dividing the mass of the lyophilizate by the volume of the dispersion used for lyophilization. The final dPNonOx NP concentrations were in the range between 1.8 and 10.4 mg mL⁻¹ depending on the resuspension volume utilized. The final NP dispersions were stored at 4 °C until further usage.

3. Results and discussion

3.1. Liquid chromatography of dPOx library

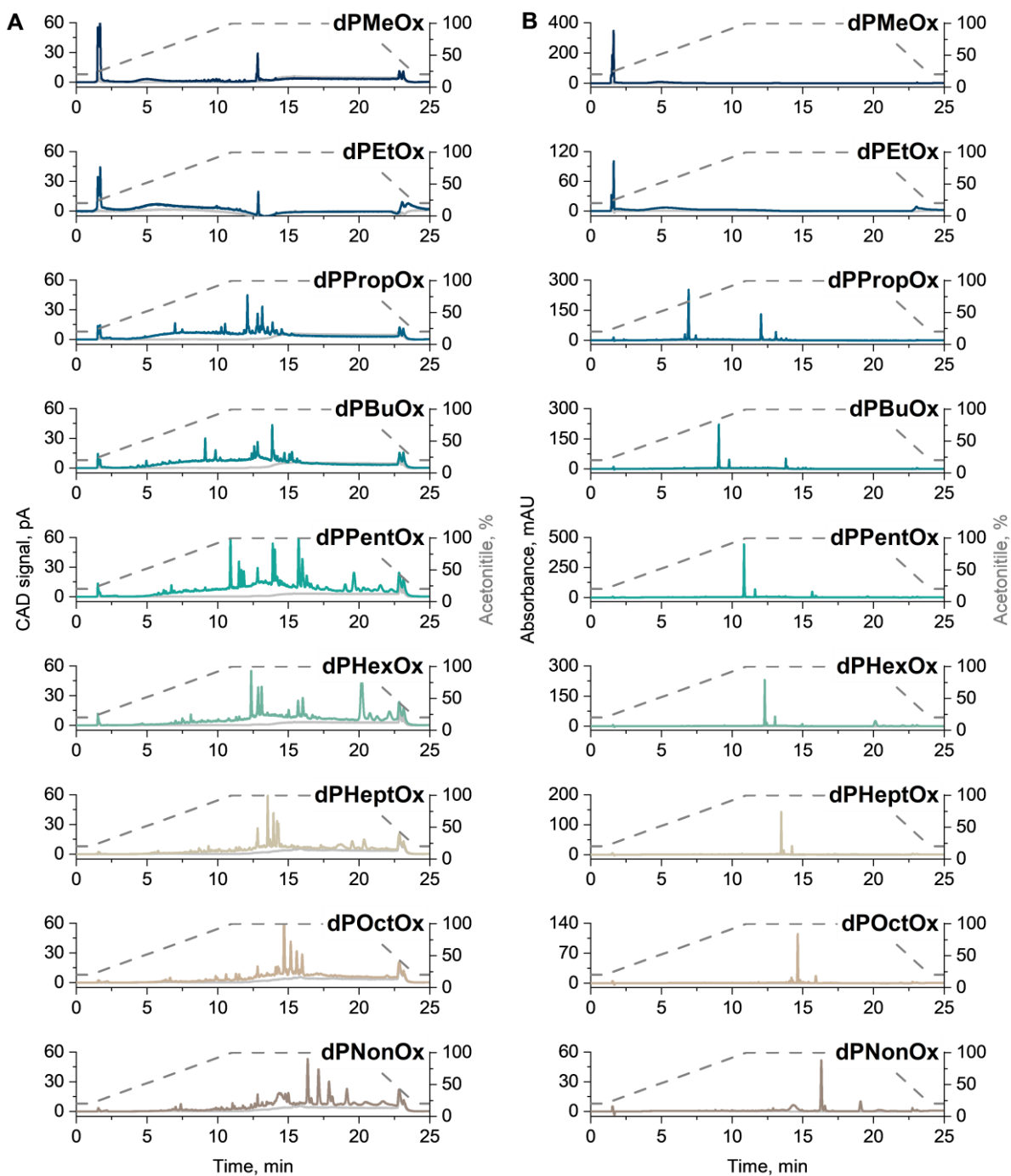


Fig. S2. Overlaid liquid chromatography elugrams of dPOx and the solvent blank (light gray line) recorded by (A) CAD and (B) DAD at 290 nm. The dashed gray lines indicate the gradient programming.

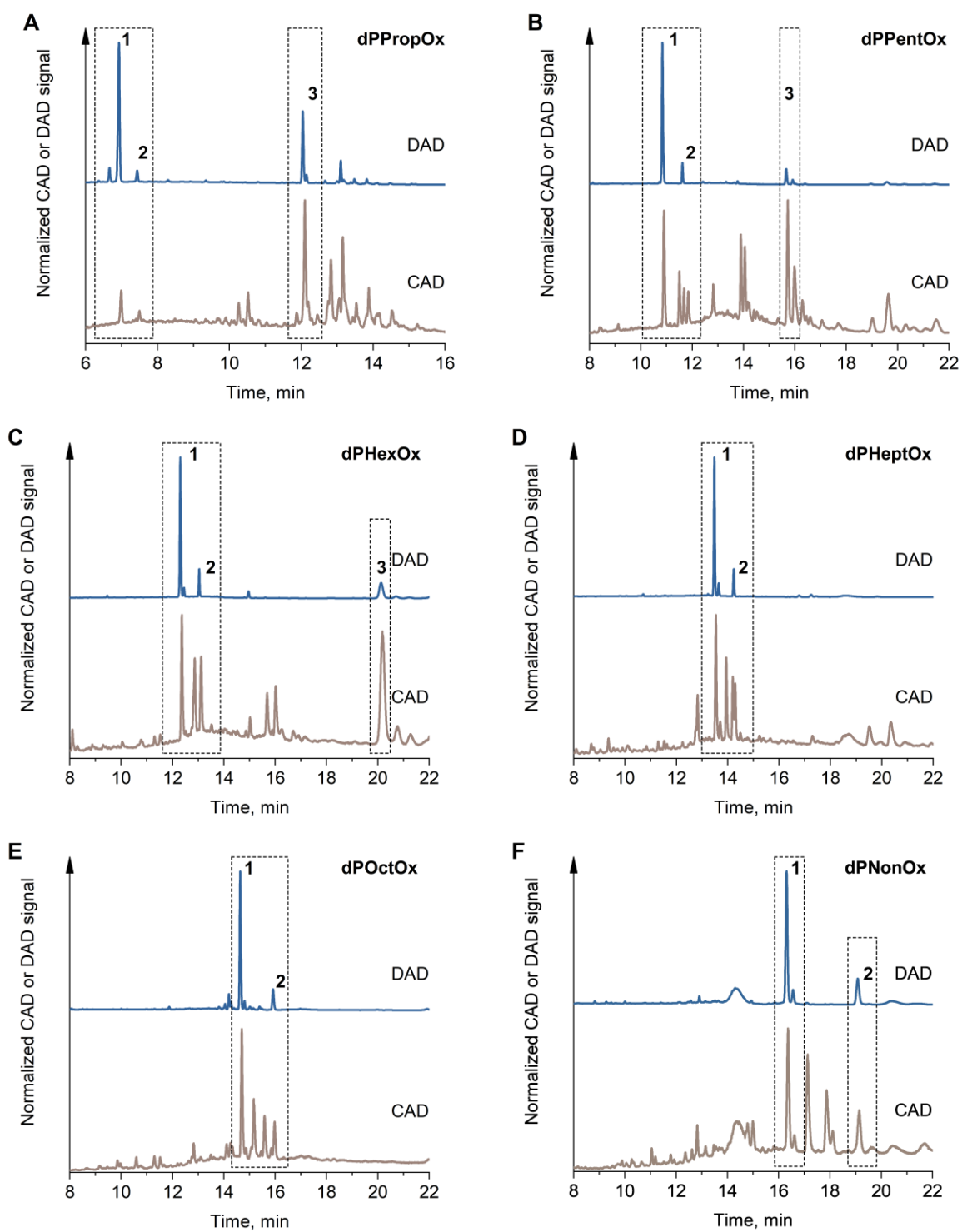


Fig. S3. Overlaid elugrams of dPOx recorded via DAD and CAD. Retention times of peaks indicated with 1, 2, 3 were used for establishing the hydrophobicity row (**Fig. 2D** in the main body text).

3.2. *In vitro* cytotoxicity of dPOx

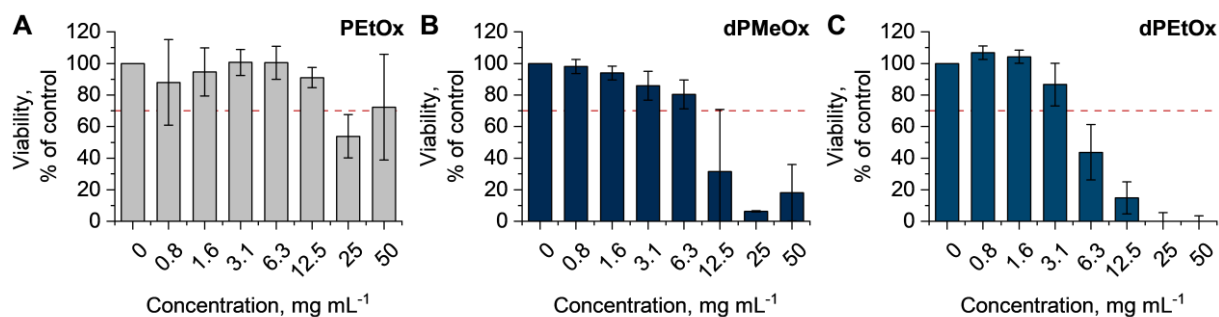


Fig. S4. Relative metabolic activity of cells treated with (A) PETox and hydrophilic dPOx, (B) dPMeOx, and (C) dPEtOx. Data are presented as cell viability values obtained with the L929 cell line via a PrestoBlue™ assay. Potential cytotoxicity of the polymers was tested up to 50 mg mL⁻¹ of polymer dissolved in water. The values are given as percentage of cell viability normalized to the untreated cell control set as 100%. Data are expressed as mean ± standard deviation (SD) of the measurements performed in six technical replicates with at least three independent determinations. The red dashed line indicates the 70% viability value which displays the common threshold marker for estimating a sample as cytotoxic.

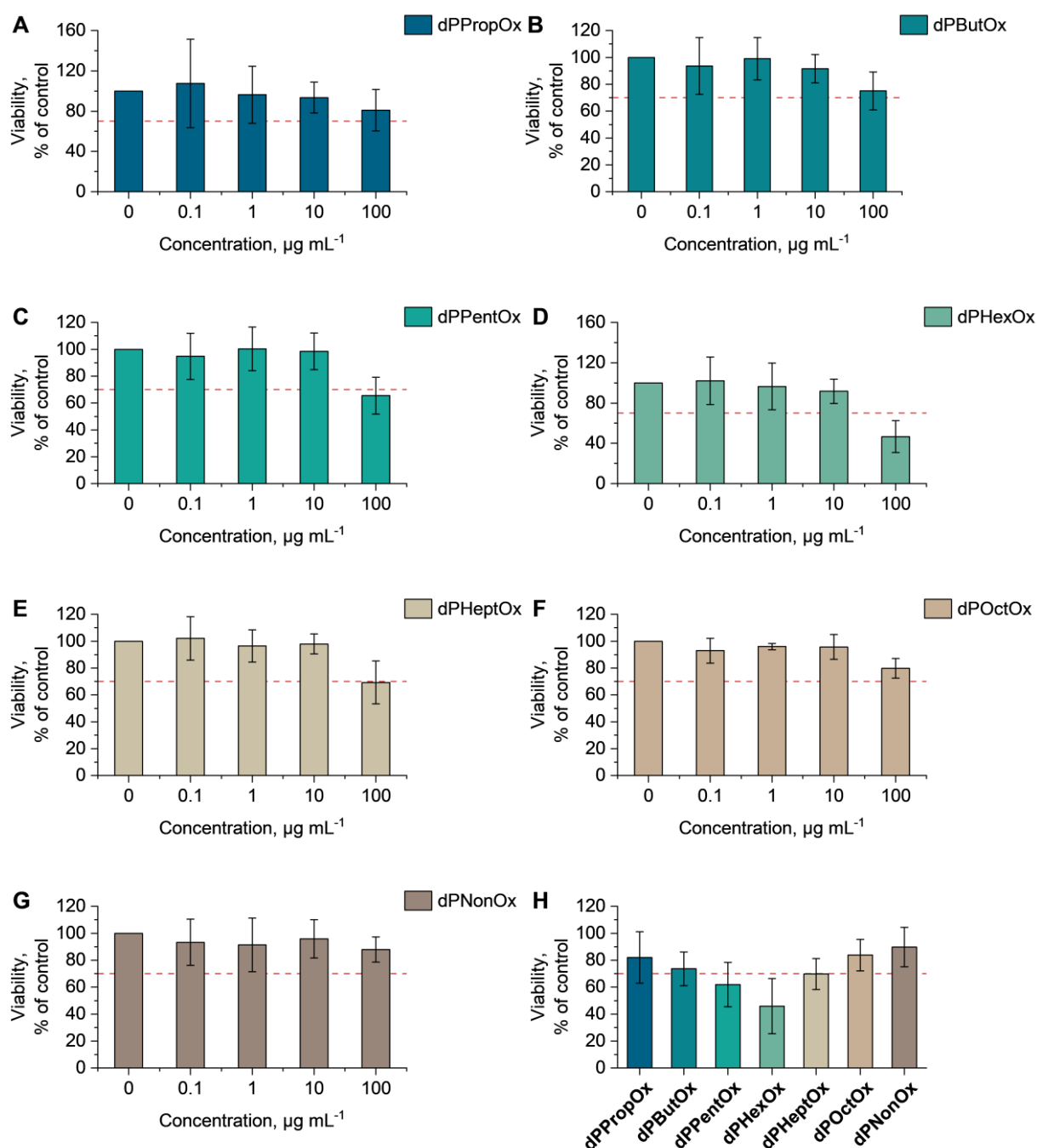


Fig. S5. Relative metabolic activity of cells treated with dPOx presented as cell viability values obtained in L929 cell line via PrestoBlue™ assay. **(A-G)** Cytotoxicity of dPOx for different polymer concentrations (0, 0.1, 1, 10, 100 $\mu\text{g mL}^{-1}$) in DMSO presented in % of untreated control (DMSO). **(H)** Potential toxicity capacity of hydrophobic dPOx estimated at a concentration of 100 $\mu\text{g mL}^{-1}$ given as percentage of cell viability normalized cells treated with the DMSO control set as 100%. Red dashed lines indicate the 70% viability value which displays the common threshold marker for estimating a sample as cytotoxic. Data are expressed as mean \pm SD of measurements performed in six technical replicates with at least three independent determinations.

3.3. Formulation ability of dPOx

Table S2. Nanoparticle properties (size, polydispersity index (PDI), and zeta-potential) obtained by DLS and ELS for hydrophobic dPOx formulated by nanoprecipitation with different initial polymer concentrations in acetone.

c_p [mg mL ⁻¹]		dPPropOx	dPButOx	dPPentOx	dPHexOx	dPHeptOx	dPOctOx	dPNonOx
20	d_h [nm]	159	169	175	168	190	191	183
	PDI	0.10	0.08	0.07	0.08	0.08	0.11	0.12
	ζ [mV]	36	36	35	46	42	42	38
10	d_h [nm]	129	141	149	152	159	170	173
	PDI	0.11	0.07	0.08	0.11	0.08	0.08	0.10
	ζ [mV]	31	32	30	36	40	41	39
5	d_h [nm]	117	119	119	129	137	151	158
	PDI	0.10	0.11	0.11	0.08	0.08	0.09	0.10
	ζ [mV]	31	36	29	36	39	37	36
2.5	d_h [nm]	99	101	109	108	110	104	117
	PDI	0.11	0.13	0.10	0.10	0.11	0.12	0.13
	ζ [mV]	29	30	25	33	33	34	28
1	d_h [nm]	124	83	100	90	87	85	84
	PDI	0.19	0.17	0.08	0.11	0.11	0.11	0.16
	ζ [mV]	16	25	27	27	24	24	28

c_p – initial polymer concentration in acetone used for nanoprecipitation;

d_h [nm] – particle size expressed as hydrodynamic diameter = Z-average value as well as polydispersity index (PDI) determined by DLS (1:10 dilution with purified water);

ζ [mV] – zeta-potential obtained by ELS measurements (1:10 dilution with purified water).

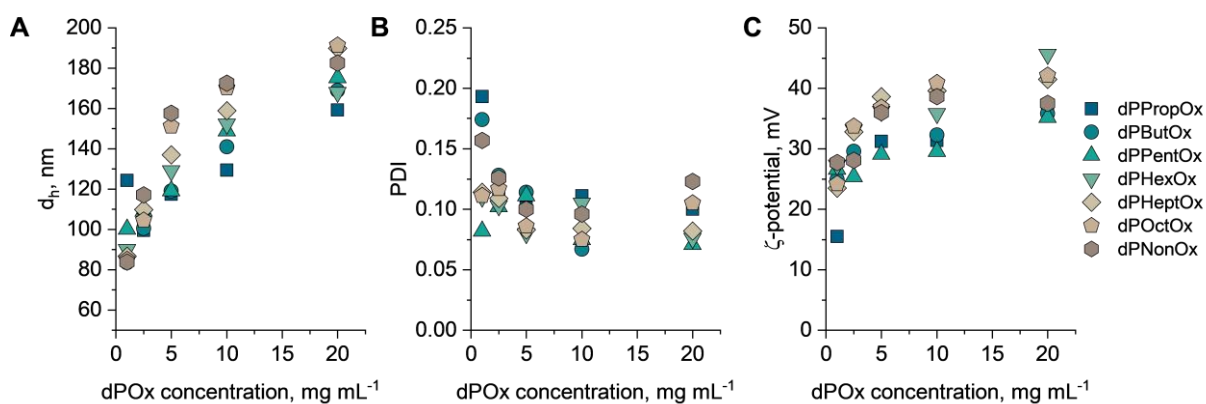


Fig. S6. Properties of the NPs obtained by the nanoprecipitation screening of all dPOx polymers formulated with an initial polymer concentration between 1 and 20 mg mL⁻¹ in acetone ($n = 1$, one formulation): (A) NP sizes (d_h , Z-average) and (B) polydispersity index (PDI) values were obtained by DLS. The intensity-based size distributions are depicted in Fig. S7. (C) Zeta-potential values were obtained by ELS measurements.

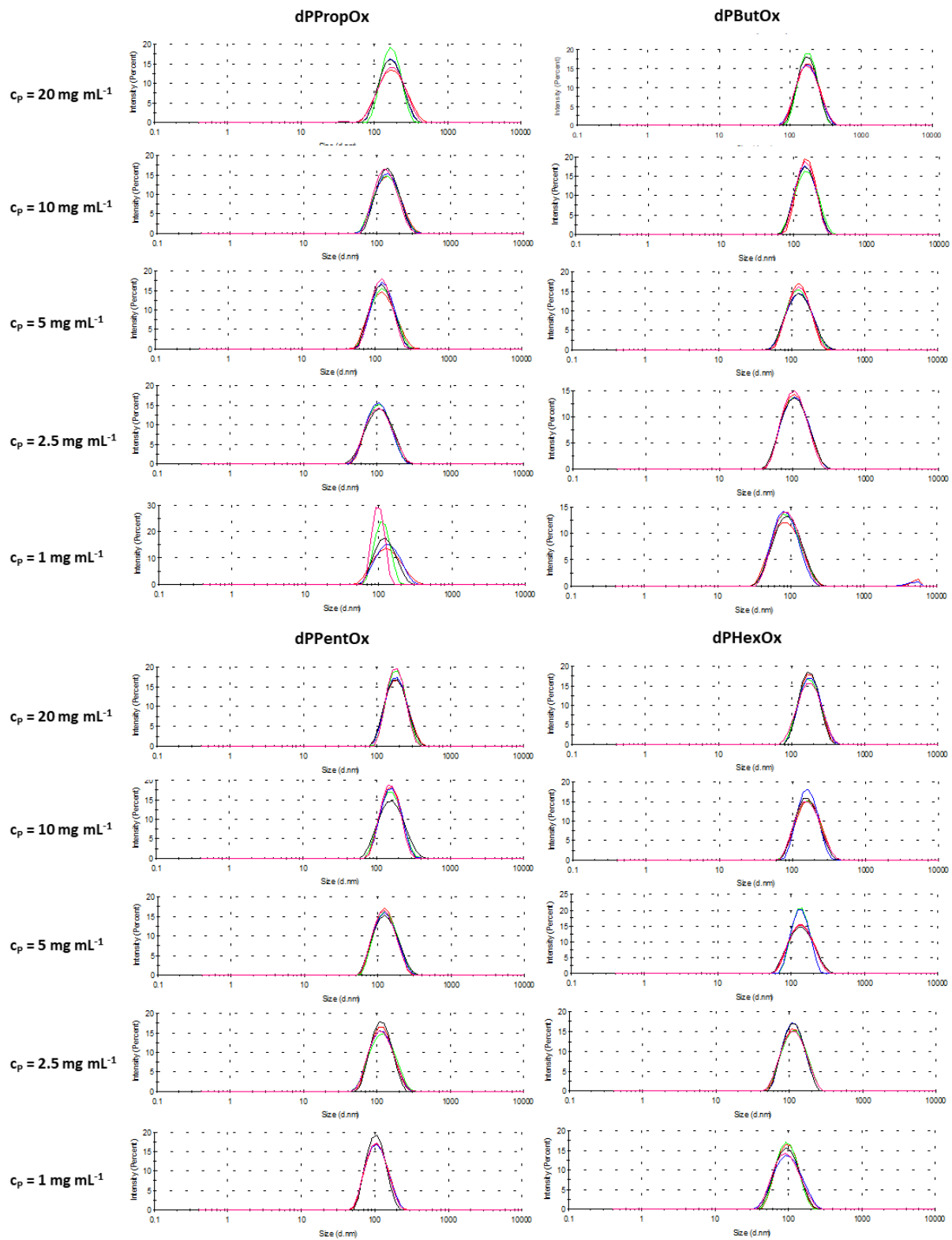


Fig. S7. Intensity-based size distributions obtained by DLS for the NPs formulated with different initial concentration of dPOx (c_p): $n = 1$ (one formulation), overlay of five repetitive measurements.

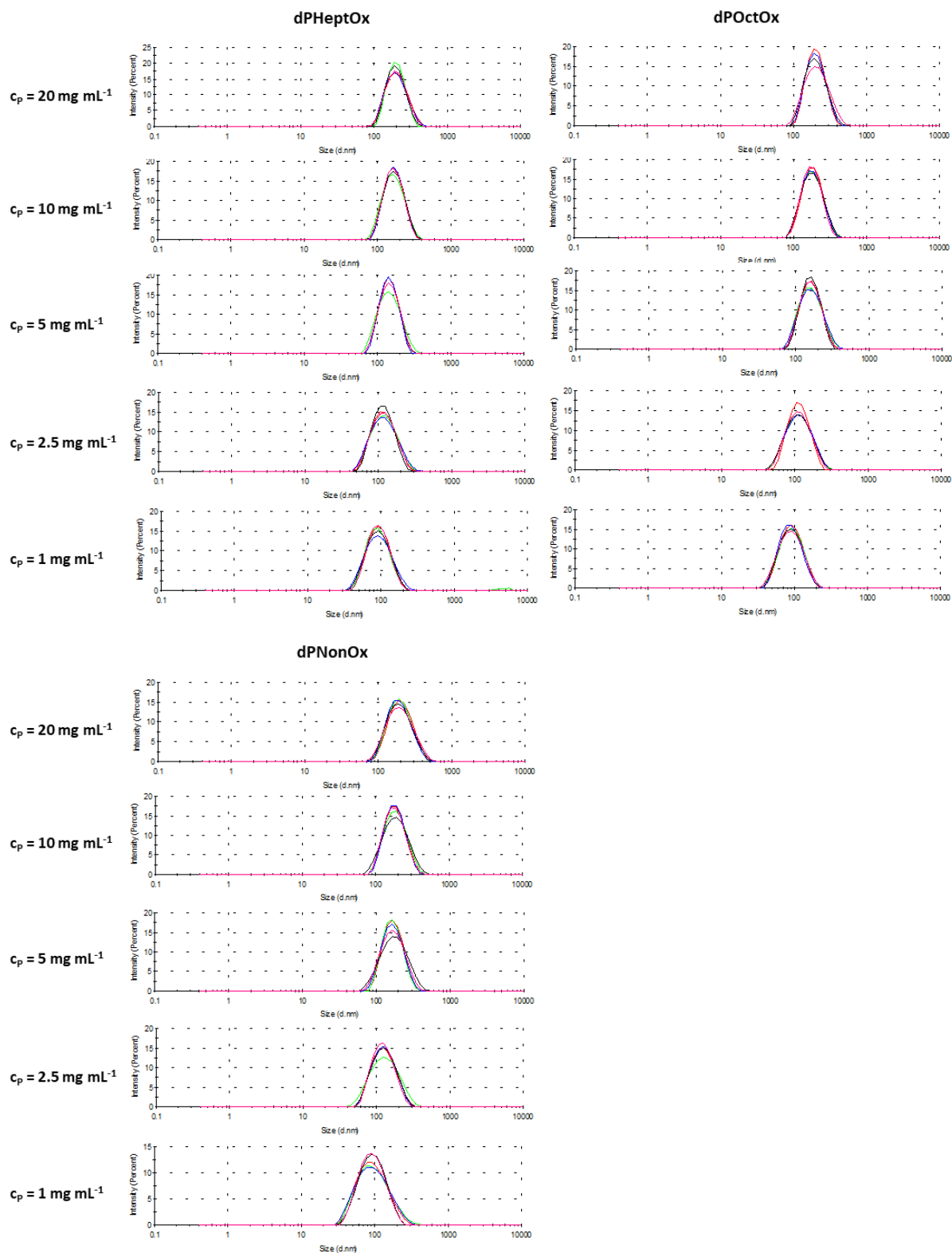


Fig. S7 (continued). Intensity-based size distributions obtained by DLS for the NPs formulated with different initial concentration of dPOx (c_p): $n = 1$ (one formulation), overlay of five repetitive measurements.

3.4. Formulation of dPNonOx NPs

Table S3. Main particle characteristics of dPNonOx NPs formulated by nanoprecipitation with and without PVA. The values were obtained by DLS and ELS measurements.

Sample	c_p^a [mg mL ⁻¹]	PVA, wt. %	d_h^b [nm]	PDI ^b	ζ^c [mV]
dPNonOx NPs with PVA	20	0.05	174	0.050	40.3
dPNonOx NPs without PVA	20	-	175	0.025	37.9

^a c_p – initial polymer concentration in acetone during nanoprecipitation.

^b d_h [nm] – hydrodynamic diameter of particles = Z-average value as well as polydispersity index (PDI) determined by DLS (1:100 dilution with purified water).

^c ζ [mV] – zeta-potential obtained by ELS (1:100 dilution with purified water).

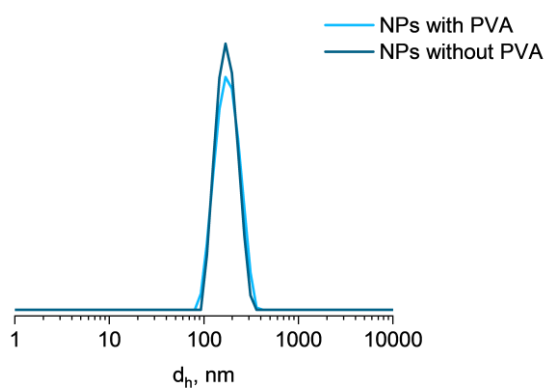


Fig. S8. Overlay of intensity-based size distributions obtained by DLS for dPNonOx NPs formulated with and without PVA ($n = 1$, one formulation).

3.5. *In vitro* degradation of dPNonOx NPs monitored by liquid chromatography

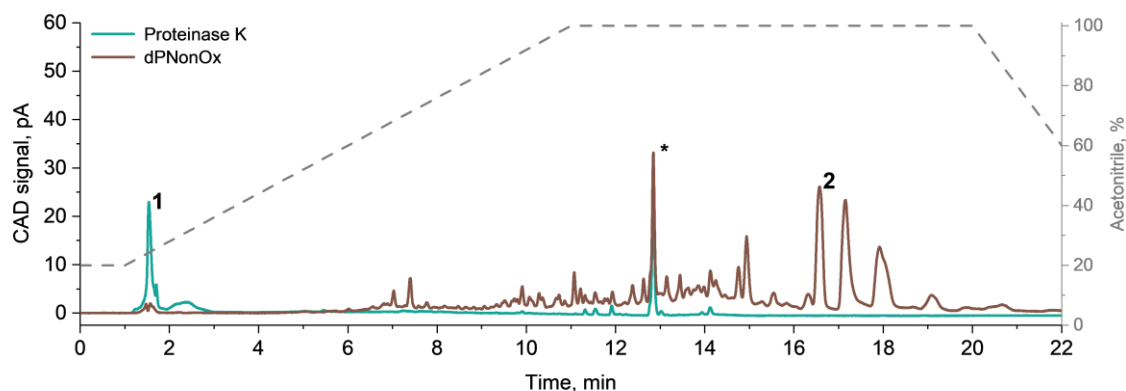


Fig. S9. Overlaid elugrams of Proteinase K (0.5 mg mL^{-1} in 50/50 (v/v) $\text{CH}_3\text{CN}/\text{H}_2\text{O}$) and dPNonOx (1.0 mg mL^{-1} in CH_3CN) recorded by CAD. The dashed gray line indicates the CH_3CN gradient elution program. Specific peaks are indicated as follows: **1** – Proteinase K, **2** – dPNonOx, * – the non-characteristic peak observed in all runs irrespective of which sample was analyzed and was not used for further characterization and data interpretation.

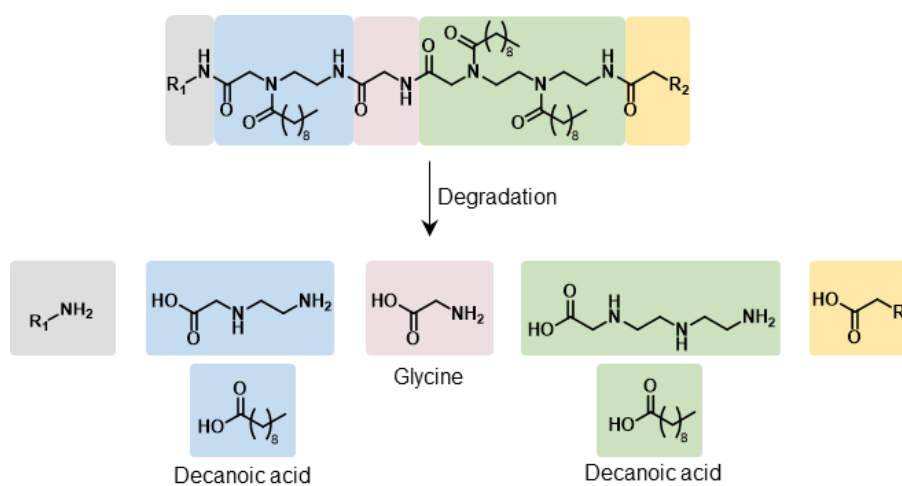


Fig. S10. Possible degradation products of an exemplified structure of dPNonOx under proteinase K action.²

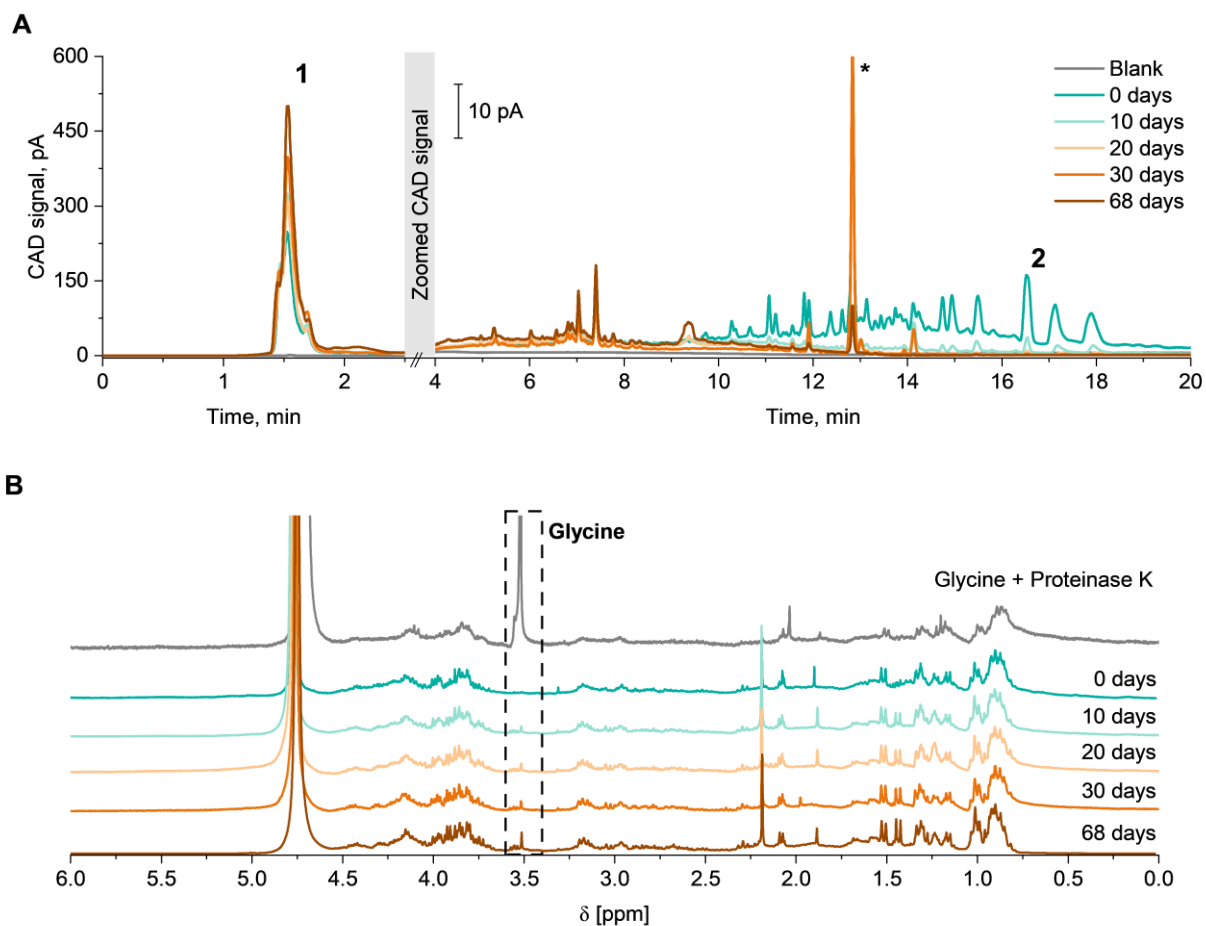


Fig. S11. Enzymatic degradation of dPNonOx NPs under proteinase K action in a mass ratio 1:4 (particle:proteinase K). **(A)** Elugrams of dPNonOx NP suspension stored with proteinase K over 68 days and recorded by CAD. The non-characteristic peak indicated with a * was observed in all runs irrespective of which sample was analyzed and was not used for further characterization and data interpretation. **(B)** Overlay of ^1H NMR spectra (300 MHz, D_2O) of the lyophilized dPNonOx NP suspension stored with proteinase K in a mass ratio 1:4 (particle:proteinase K). The individual normalized spectra are stacked for clarity. The dashed black box indicates the glycine singlet at 3.7 ppm.

3.6. BRP-201-loaded dPNonOx NPs

Table S4. Chromatographic characteristics for Peak I and Peak II (indicated in Fig. S8) of BRP-201 containing and empty dPNonOx NPs as well as dPNonOx standards. The data from three lyophilized NP samples (n=1, 2, 3) of one formulation were compared.

Sample	Peak I ^a height, mAU	Peak II ^b height, mAU	Ratio ^c	Mean ratio \pm SD ^d
dPNonOx NPs + BRP-201				
n=1	50.55	9.11	5.55	5.7 \pm 0.3
n=2	53.10	8.80	6.03	
n=3	50.25	9.22	5.45	
dPNonOx NPs				
n=1	45.98	9.77	4.71	5.2 \pm 0.4
n=2	48.49	9.32	5.20	
n=3	49.94	8.81	5.67	
dPNonOx standards				
0.2 mg mL ⁻¹	0.56	4.28	0.13	0.16 \pm 0.01
0.5 mg mL ⁻¹	1.59	9.8	0.16	
1.0 mg mL ⁻¹	3.06	18.81	0.16	
1.2 mg mL ⁻¹	3.73	22.78	0.16	
1.5 mg mL ⁻¹	4.51	27.26	0.17	
2.0 mg mL ⁻¹	5.99	35.99	0.17	
2.5 mg mL ⁻¹	7.41	43.93	0.17	
3.0 mg mL ⁻¹	8.90	52.39	0.17	

^aPeak I elutes at $t_R = 9.9$ min.

^bPeak II elutes at $t_R = 10.2$ min.

^cRatio was calculated as Peak I height divided by Peak II height.

^dMean ratio values are presented with standard deviation (SD) values.

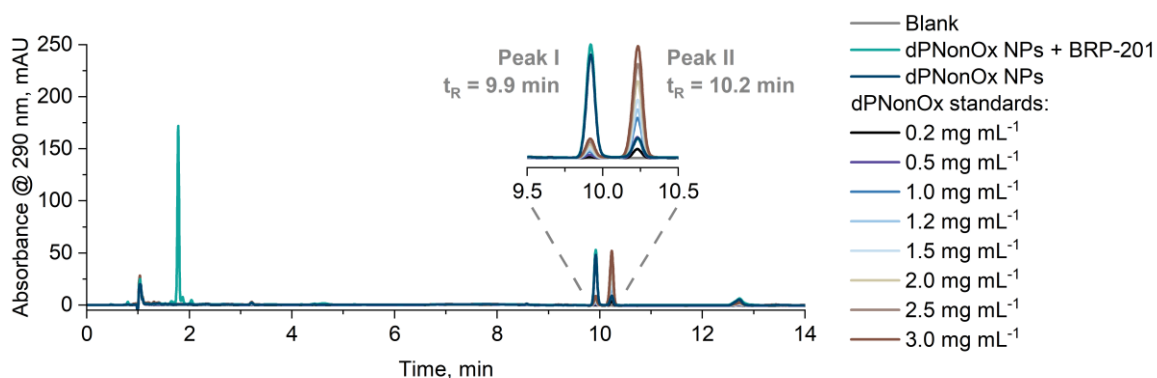


Fig. S12. Overlaid elugrams of BRP-201 containing dPNonOx NPs as well as dPNonOx standard at different concentrations recorded by DAD at 290 nm (polymer absorbance maximum). Peak at 1.8 min refers to BRP-201. Solvent composition in all the samples: 200 μ L DMSO and 800 μ L 85/15 (v/v) CH₃CN/H₂O. Measurement conditions: flow rate 1.5 mL min⁻¹, isocratic hold for 5 min at 85% (v/v) of CH₃CN in the mobile phase followed by a linear gradient of CH₃CN (from 85% (v/v) to 100%) in 2 min.

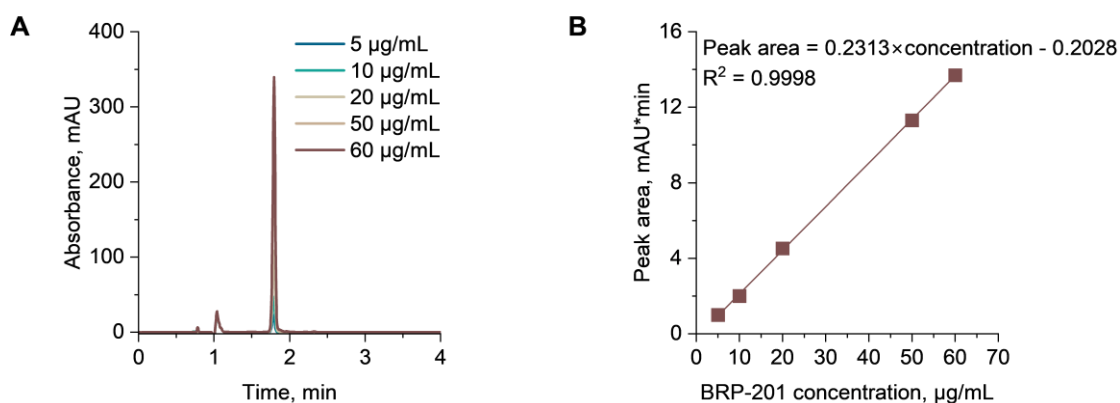


Fig. S13. (A) Overlaid elugrams of BRP-201 (5 to 60 $\mu\text{g mL}^{-1}$) recorded by DAD at 312 nm. (B) Calibration curve for BRP-201 presented by plotting peak areas as a function of analyte concentrations. Data were fitted linearly.

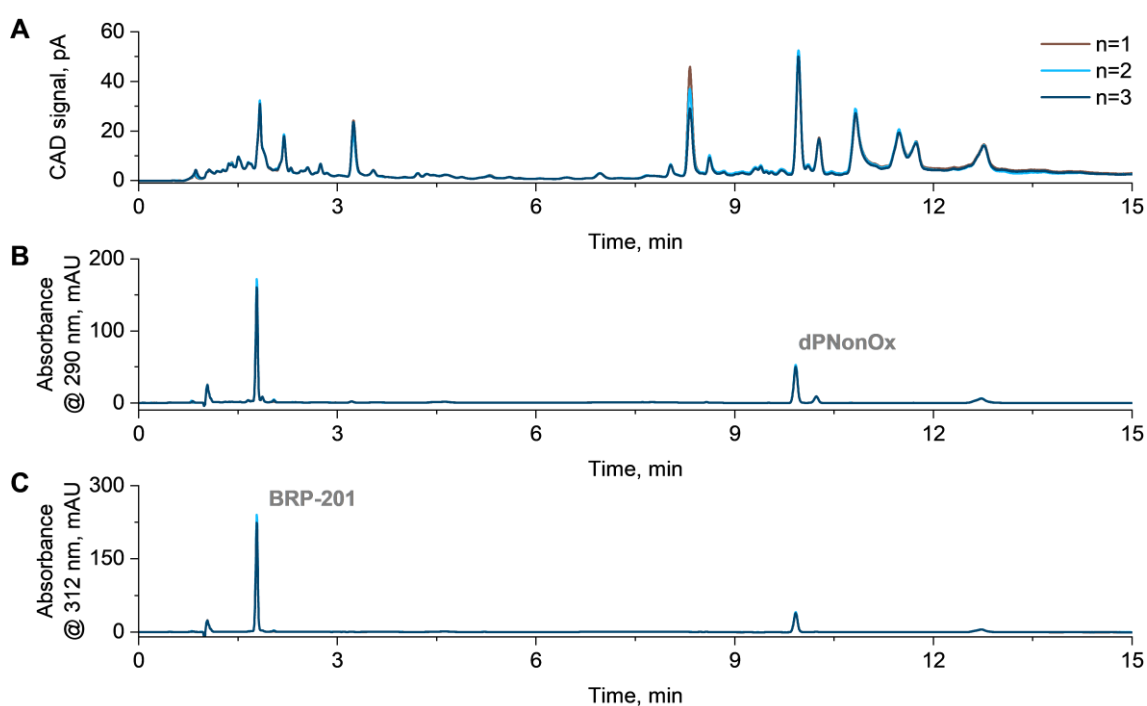


Fig. S14. Overlaid elugrams of three samples of lyophilized dPNonOx NPs loaded with BRP-201 ($n=1, 2, 3$) of one formulation batch recorded by (A) CAD and DAD at (B) 290 nm (polymer absorbance maximum) and (C) 312 nm (drug absorbance maximum). Peak at 1.8 min refers to BRP-201. Solvent composition in all the samples: 200 μL DMSO and 800 μL 85/15 (v/v) $\text{CH}_3\text{CN}/\text{H}_2\text{O}$. Measurement conditions: flow rate 1.5 mL min^{-1} , isocratic hold for 5 min at 85% (v/v) of CH_3CN in the mobile phase followed by a linear gradient of CH_3CN (from 85% (v/v) to 100%) in 2 min.

Table S5.1 Repeatability of chromatographic parameters including standard deviation (SD) and coefficient of variation (CV^a) for BRP-201 and dPNonOx retention time and peak area values calculated from individual injections of three samples of lyophilized dPNonOx NPs loaded with BRP-201 (n=1, 2, 3) of one formulation batch.

dPNonOx NPs	BRP-201 (DAD @ 312 nm)		dPNonOx (DAD @ 290 nm)		Loading capacity, %
	Retention time, min	Peak area, pA*min	Retention time, min	Peak area, pA*min	
n = 1	1.783	9.021	9.923	3.545	1.61
n = 2	1.783	9.588	9.920	3.708	1.68
n = 3	1.783	8.950	9.920	3.504	1.57
SD	0.0	0.285	0.001	0.088	0.046
CV^a	0.0	0.031	0.0	0.025	0.029

^aCV for a parameter (x – LC, retention time or peak area) is calculated according to the equation:

$$CV = SD/x_{average}$$

Table S6. Main particle characteristics of BRP-201 loaded dPNonOx NPs after purification.

Code	c_p^a [mg mL ⁻¹]	Drug	n	d_h^b [nm]	PDI ^b	ζ^c [mV]	LC ^d [%]
dPNonOx NPs + BRP-201	5	BRP-201	1	173	0.02	4.4	-
			2	166	0.03	28	1.6
			3	166	0.04	26	1.3
dPNonOx NPs	5	-	1	163	0.07	42	-
			2	176	0.04	41	-
			3	182	0.03	36	-
dPNonOx NPs + BRP-201	20	BRP-201	1	206	0.07	20	-
			2	204	0.08	32	1.8
			3	220	0.04	30	1.9
dPNonOx NPs	20	-	1	240	0.09	29	-
			2	211	0.05	42	-
			3	237	0.09	40	-

^a c_p – initial polymer concentration in acetone during nanoprecipitation. The initial drug load was set to 3 % (w/w) referred to polymer.

^b d_h [nm] – hydrodynamic diameter of particles = Z-average value as well as polydispersity index (PDI) determined by DLS (1:100 dilution with purified water).

^c ζ [mV] – zeta-potential obtained by ELS (1:100 dilution with purified water).

^dLC [%] – loading capacity values determined by liquid chromatography.

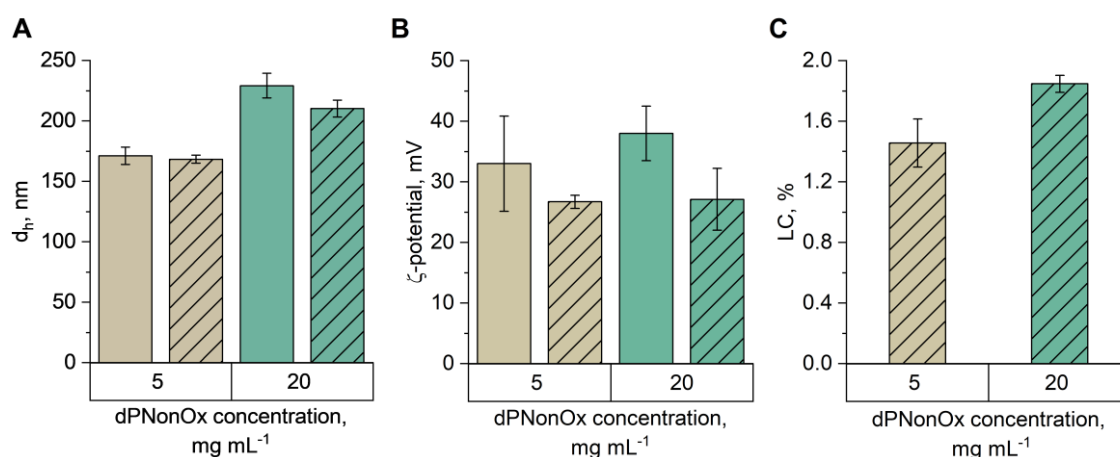


Fig. S15. Properties of the empty (solid bars) as well as BRP-201-loaded (dashed bars) dPNonOx NPs obtained by the nanoprecipitation of dPNonOx with initial polymer concentration (c_p) of 5 and 20 mg mL⁻¹ in acetone ($n = 3$, three formulations). **(A)** Mean average particle sizes ($n = 3$) plotted with a standard deviation (error bars). The intensity-based size distributions are present in **Fig. S16**. **(B)** Zeta-potential values obtained by ELS measurements from three repetitive formulations. **(C)** Drug loading values for BRP-201 obtained by liquid chromatography measurements of BRP-201 containing dPNonOx NPs presented as loading capacity LC, %.

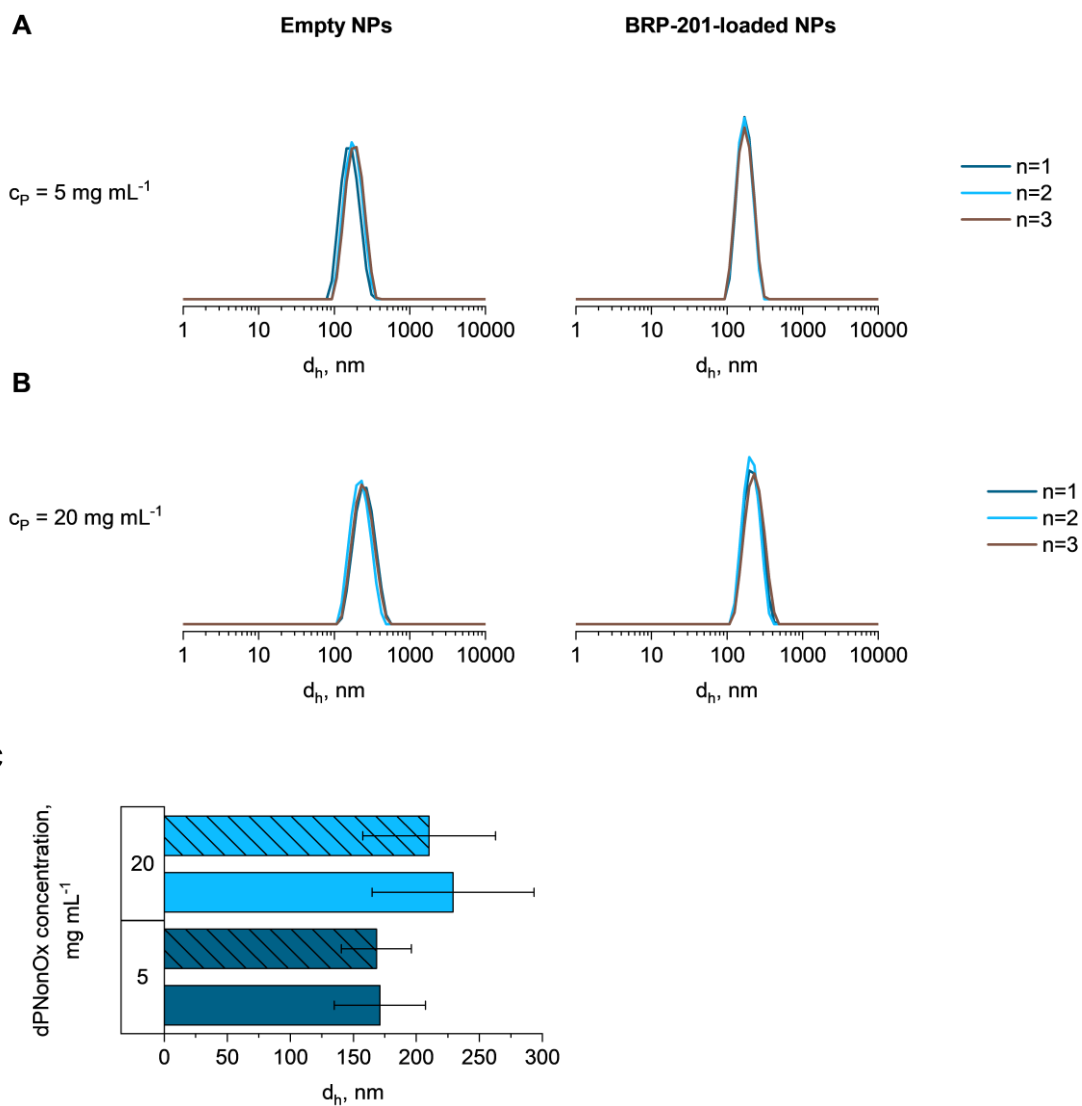


Fig. S16. Overlay of intensity-based size distributions of the empty and drug-loaded (BRP-201) dPNonOx NPs formulated with different initial polymer concentration (c_p) of **(A)** 5 and **(B)** 20 mg mL^{-1} in acetone ($n = 3$, three formulations). **(C)** Mean average NP sizes the empty (solid bars) as well as BRP-201-loaded (dashed bars) dPNonOx NPs ($n = 3$) plotted with a standard deviation of particle sizes σ (indicated error bars) calculated from the PDI by $\sigma = \sqrt{PDI} \times d_h$.

3.7. 5-LOX activity and membrane integrity assay in neutrophils

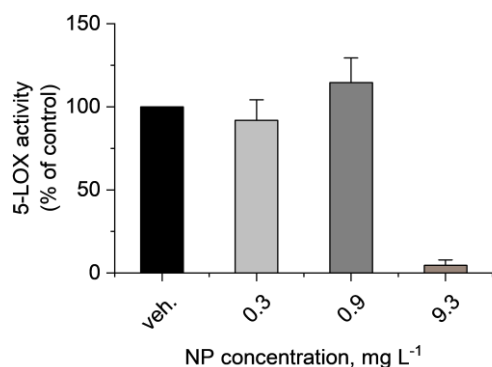


Fig. S17. Inhibition of 5-LOX product formation by blank (unloaded) dPNonOx NPs. Neutrophils were preincubated for 15 min at 37 °C with PBS (vehicle, veh.) and blank dPNonOx NPs at concentrations, which correspond to the concentrations of drug-loaded dPNonOx NPs containing 0.01, 0.03 or 0.3 μ M of BRP-201 in Fig. 6. Afterward, the neutrophils were stimulated with 2.5 μ M A23187. After 10 min, the reaction was stopped, and 5-LOX products were extracted via solid phase extraction (SPE) and analyzed by reversed-phase liquid chromatography. Values are given as 5-LOX products (LTB₄, trans-LTB₄, epi-trans-LTB₄, and 5-HETE) in percentage of control. For statistical analysis matched one-way ANOVA with Tukey's multiple comparisons test was used; * $p < 0.05$.

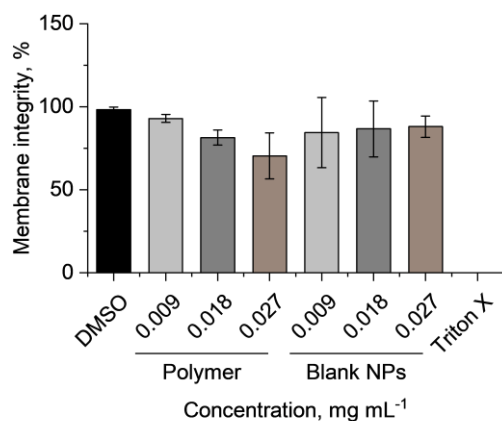


Fig. S18. Membrane integrity determined by LDH release assay. For determination of cytotoxicity of blank (unloaded) dPNonOx NPs as well as untreated dPNonOx, LDH release was monitored after 3 h of incubation time. Free BRP-201 was tested at 0.3 μ M. The results are shown as mean \pm standard error of mean in bar charts (three repetitive measurements are indicated with empty spheres on the graph, $n = 3$).

4. References

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