SUPPLEMENTARY MATERIALS

Imaging Mass Spectrometry Differentiates Effects of Doxorubicin Formulations on Non-targeted Tissues

Authors

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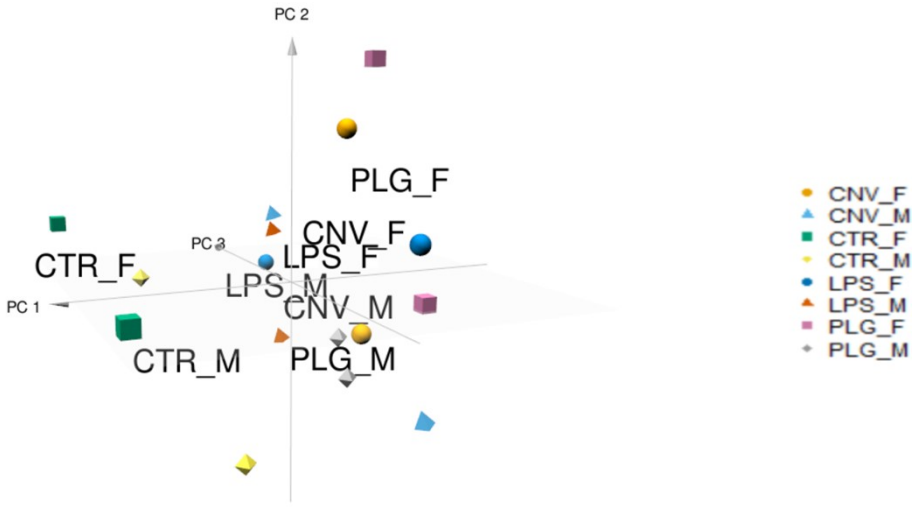
If not stated otherwise abbreviations used here correspond to the ones given in the accompanying article.

S1. Detailed PCA



Each polygon represents a mass spectrum coming from the individual animal. 80.9% of data variance has been explained using 3 principle components. Class labels are placed at centroids corresponding to each group. Mass spectra were recorded by application of IMS on kidney cortex samples of 4 groups of rats: animals treated with CNV, LPS and PLG doxorubicin dosage forms and a CTR group. m/z values averaged over all recorded pixels were used. 2 M and 2 F animals per group were enrolled in the study.

PCA of mass spectra demonstrates existance of chemical differences between controls and treatments (separated along the PC1 axis). Gender differences are also visible (separated along the PC2 axis). It also demonstrates similar response to all treatments (dispersed along PC2 and PC3 axis). It is worth of noticing that gender differences make more impact on the mass spectra than the selection of dosage form does.

S2. Complete List of Putative Annotations of Fingerprint m/z Values

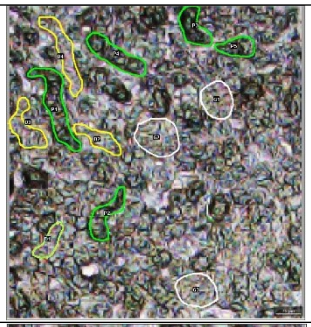
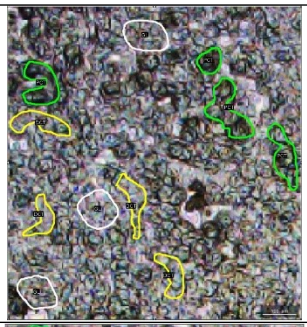
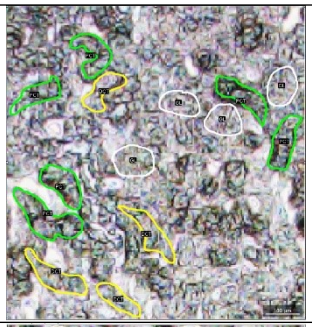
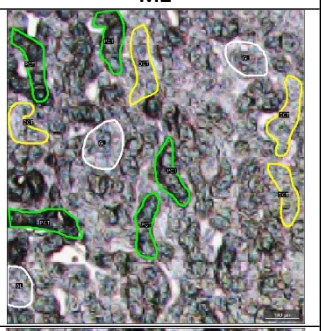
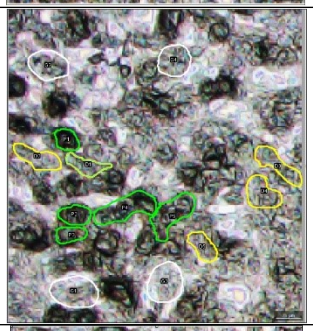
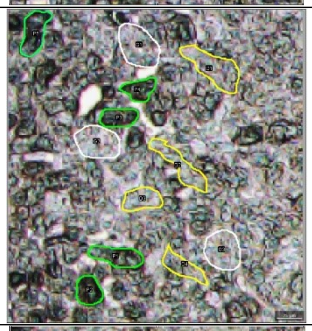
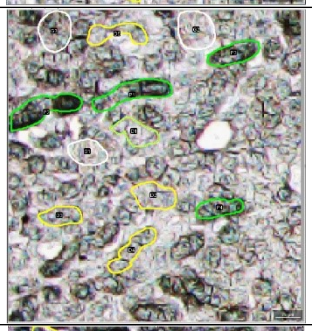
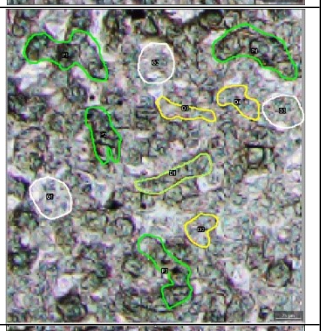
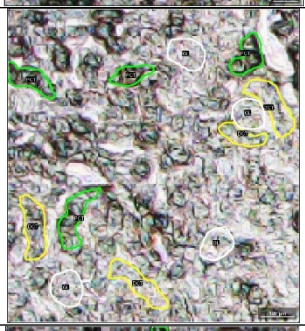
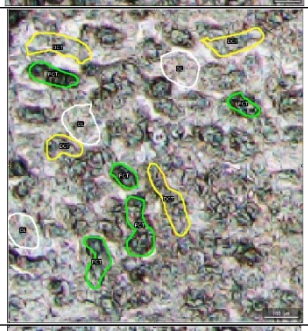
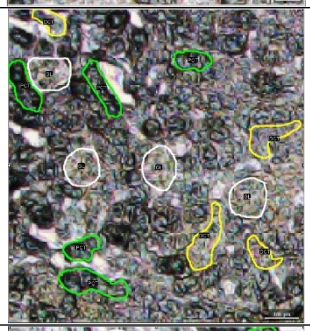
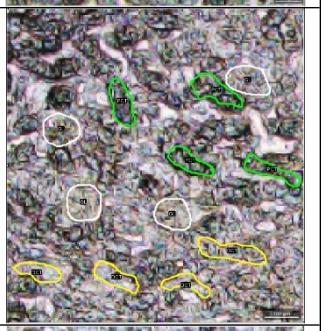
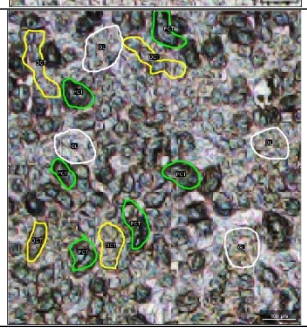
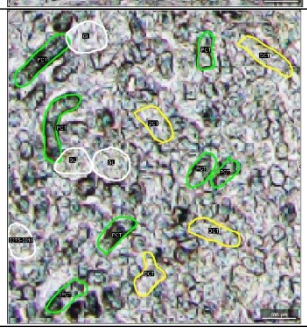
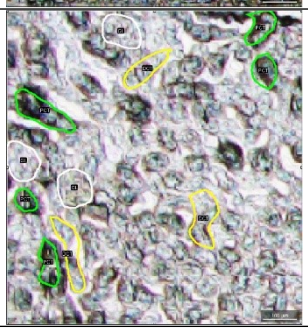
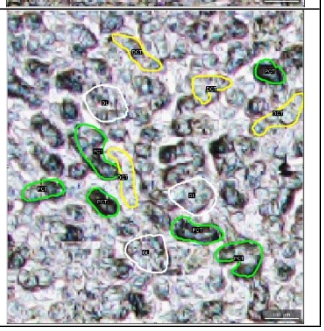
Formulation	m/z	HMDB search hits (± 50 ppm)/ Putative metabolites
CNV	283.072	Multiple endogenous compounds
	291.109	Multiple endogenous compounds
	304.112	Multiple endogenous compounds
	319.097	Multiple endogenous compounds
	334.151	Multiple endogenous compounds
	340.117	Multiple endogenous compounds
	368.112	Phosphatidylserine
	374.161	Tryptophyl-Phenylalanine
	375.135	cyclic N-Acetylserotonin glucuronide
	448.306	Glycocholic acid/3a,7b,12a-Trihydroxyoxocholanyl-Glycine
	455.081	17-Beta-Estradiol-3,17-beta-sulfate
	458.095	No endogenous compounds
	468.143	Tetrahydrofolic acid
	744.454	Multiple endogenous compounds
	753.592	Multiple endogenous compounds
	758.127	No hits
	797.473	Multiple endogenous compounds
	815.619	Multiple endogenous compounds
	841.550	Multiple endogenous compounds
	845.514	Multiple endogenous compounds
	850.603	Multiple endogenous compounds
	856.149	No endogenous compounds
LPS & PLG	204.122	L-Acetylcarnitine*
	322.110	Multiple endogenous compounds
	365.288	Multiple endogenous compounds
	377.128	Multiple endogenous compounds
	377.166	LysoPA(i-12:0/0:0)/1-dodecanoyl-glycero-3-phosphate
	382.063	No endogenous compounds
	383.172	Multiple endogenous compounds
	383.211	Multiple endogenous compounds
	400.928	No hits
	426.017	Multiple endogenous compounds
	432.023	No endogenous compounds
	448.082	No endogenous compounds
	449.024	No endogenous compounds
	478.365	Multiple endogenous compounds
	504.350	LysoPC(18:1(9Z)/0:0) isomers
	505.360	LysoPA(i-24:0/0:0)
	512.177	Multiple endogenous compounds
	585.030	No endogenous compounds
	623.009	No endogenous compounds
	623.071	No endogenous compounds
	666.845	No hits
	679.907	No hits
	683.862	No hits
	701.874	No hits
	709.921	No hits
	721.876	Guanosine 3'-diphosphate 5'-triphosphate
	723.538	Multiple endogenous compounds
	735.575	Multiple endogenous compounds
	743.041	No hits
	744.901	No hits
	756.537	Multiple endogenous compounds
	761.013	No hits
	765.899	No hits
	769.661	Multiple endogenous compounds
	771.510	Multiple endogenous compounds
	775.998	No hits
	777.007	No hits
	788.907	No hits
	789.854	No endogenous compounds
	791.911	No endogenous compounds
	800.029	No hits
	804.602	Multiple endogenous compounds
	804.843	No hits
	806.052	Coenzyme A
	829.519	Multiple endogenous compounds
	833.677	Multiple endogenous compounds
	878.612	Multiple endogenous compounds
	895.196	No endogenous compounds
	896.629	Multiple endogenous compounds
	897.616	Multiple endogenous compounds
	898.604	Multiple endogenous compounds
	910.999	No hits
	912.001	No hits
	913.005	No hits
	913.096	No hits
	914.010	No hits
	914.101	Multiple endogenous compounds
	929.030	No hits
	992.014	No hits
Adducts: +H+, -H2O+H+, +Na+, +K+		

* Difference between L-Acetylcarnitine in CNV and LPS&PLG cases is negligible i.e. numerical error

S3. Light Microscopy Images with Depicted Glomerules (G), DCT and PCT

Color coding: G – White; DCT – Yellow; PCT – Green.

Images used for IMS in the 200-650 Da range

	F1	F2	M1	M2
CNV				
LPS				
PLG				
CTR				

Images used for IMS in the 650-1000 Da range

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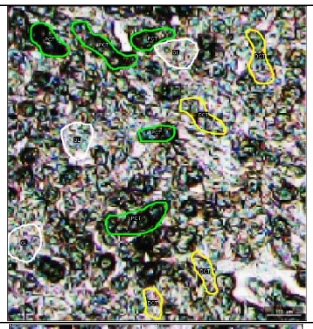
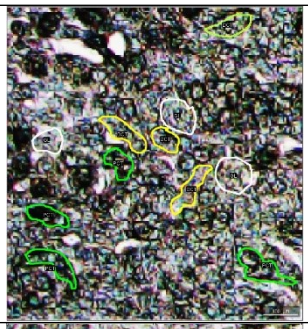
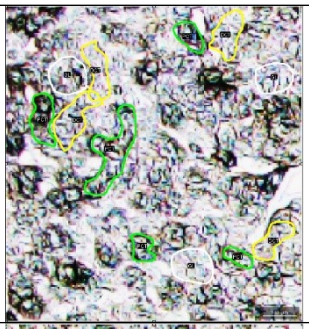
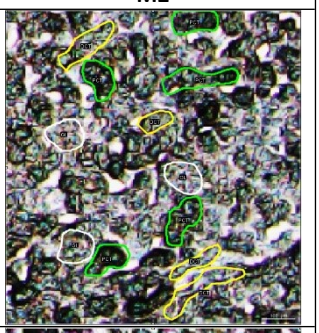
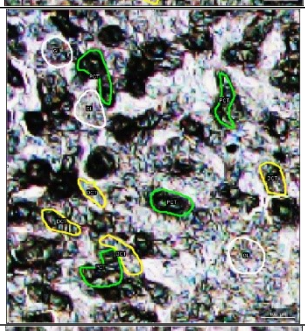
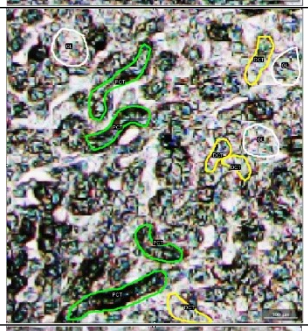
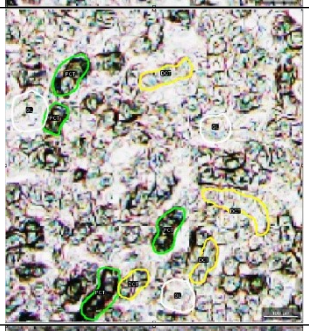
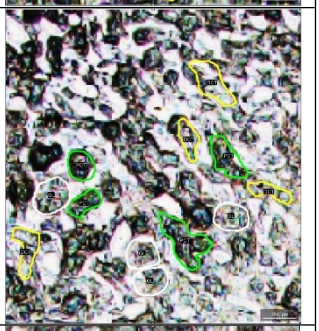
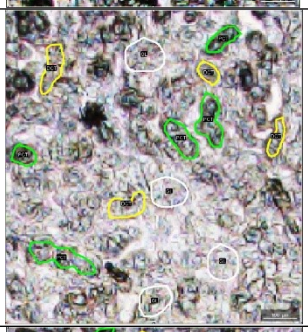
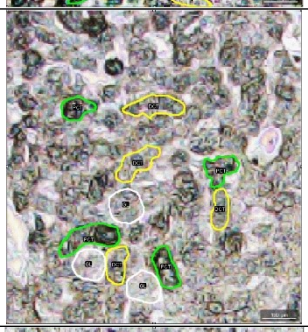
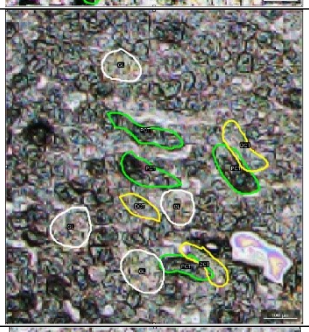
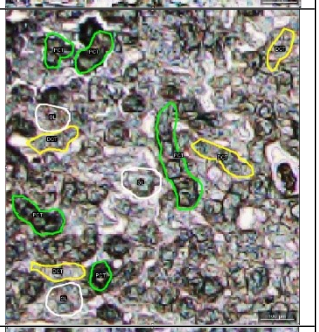
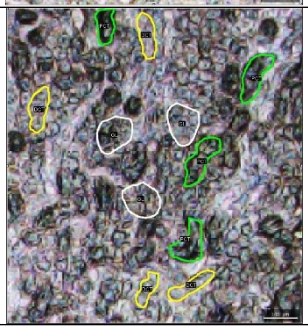
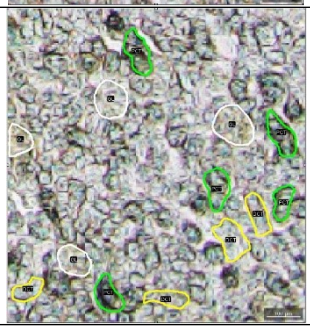
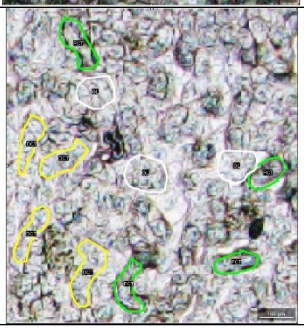
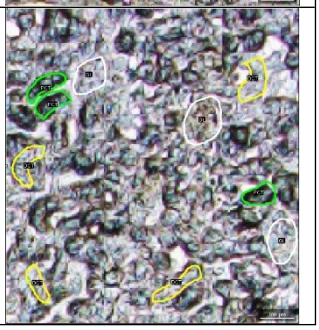
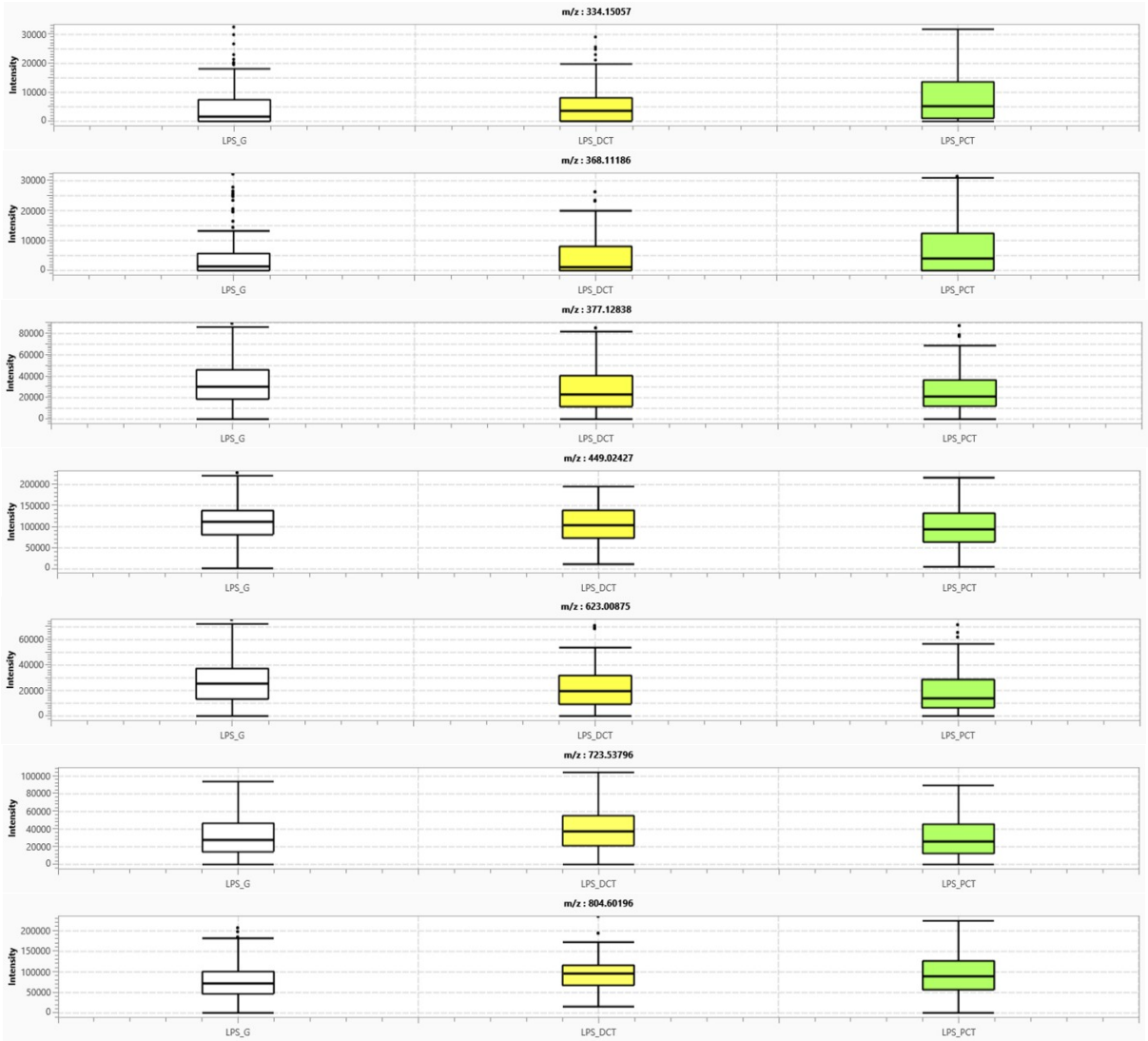
CNV				
LPS				
PLG				
CTR				

S4. List of Tissue Specific m/z Values Associated with Administration of LPS

G stands for “glomeruli”.



S5. Physicochemical Characterization of Nanoformulations

Both, LPS and PLG, nanoformulations of DOX were characterized regarding their shape, size and size distribution and surface charge after dilution with saline. The particles were visualized by transmission electron microscopy (TEM) using the microscope TEM 902A (Carl Zeiss Meditec, Jena, Germany) operating in bright-field mode with an acceleration voltage of 80 kV. The images were captured using the attached Canon PowerShot S50 camera. The samples were prepared by droplet deposition on a Formvar®-coated copper grid. Hydrodynamic diameter (d_H) and diameter distribution were determined by dynamic light scattering (DLS). The measurements were performed using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a green laser (532 nm), at a detection angle of 173°. d_H was obtained from the size by intensity distribution function as an average of 10 measurements. Zeta (ζ) potential values were measured by electrophoretic light scattering (ELS) using the same instrument. ζ potential was determined from the measured electrophoretic mobility using the Henry equation with Smoluchowski approximation, and the average of 3 measurements is reported. Both DLS and ELS measurements were conducted at 25 °C after 50-fold dilution of the samples in saline. All data was processed using the Zetasizer software (6.32; Malvern Instruments, Malvern, UK).

Results

DOX nanoformulations were characterized using the standard techniques for evaluating size, size distribution and ζ potential. TEM micrographs (Fig. S5) show LPS DOX as uniform dispersed liposomes of spheroid shape and around 100 nm in size, while PLG DOX are hard spheres roughly twice the size. According to DLS measurements, both nanoparticle types were uniform in terms of d_H with a monomodal distribution. LPS DOX were smaller in size with an average d_H of 126.2 ± 1.4 nm, while PLG DOX were larger (252.4 ± 12.7 nm (Table S5). The ζ potential was negative in both cases, with -48.3 ± 0.8 mV in LPS DOX case and -12.9 ± 0.4 mV in PLG DOX case.

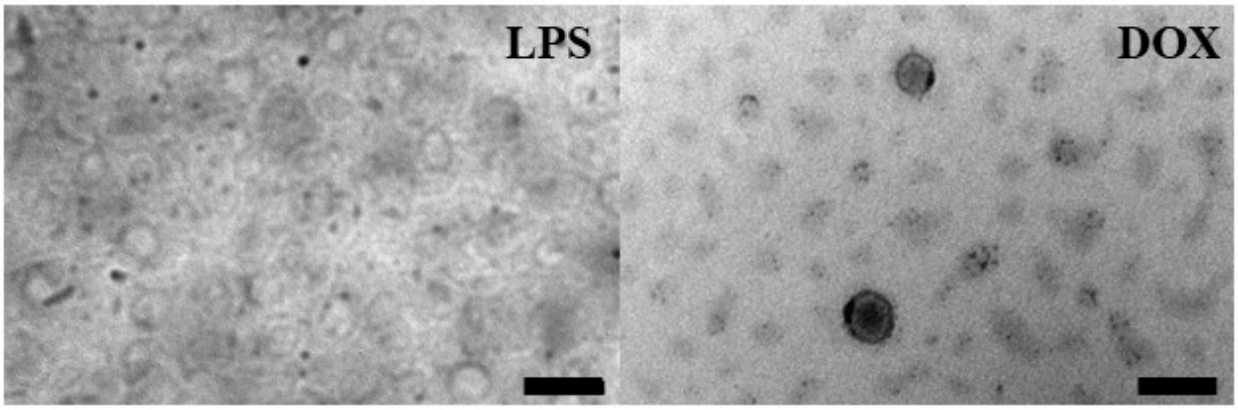


Figure S5. Transmission electron micrographs of LPS (left) and PLG (right). Scale bars are 200 nm.

Table S5. Physico-chemical characteristics of LPS and PLG formulations. hydrodynamic diameters (d_H, nm) were obtained by DLS, and z potential (mV) was measured using the ELS method. All measurements were done at 25 °C.

Formulation	d_H /nm (% Intensity)	ζ potential / mV
LPS	126.2 ± 1.4 (100%)	-48.3 ± 0.8
PLG	252.4 ± 12.7 (100%)	-12.9 ± 0.4

S6. Animal Experiments

Healthy Wistar rats of both sexes, aged 12 weeks and weighing 320-350 g body weight (b.w.) in case of males (M) and 190–220 g b.w. in case of females (F) were used in the study. Animals were bred under specific pathogen free (SPF) conditions at the Animal Breeding Unit, Institute for Medical Research and Occupational Health (IMROH), Zagreb, Croatia. They were acclimated in the controlled environment (temperature: 23 ± 2 °C; humidity: 55 ± 7% and light: 12 h light/dark cycle) and fed with standard GLP certified food (Mucedola, 4RF21, Italy) and water *ad libitum*. Each of the 3 experimental groups and a control group consisted of 2 M and 2 F animals (N=4x4). The dose of 3 mg DOX per kg of b.w., either in CNV, LPS or PLG dosage form, was administered intraperitoneally. Experimental groups received 4 doses of DOX, in 5-day intervals. The control group (CTR) received one dose of saline solution in volume equivalent to those given to experimental groups. 120 h after the last administration of DOX animals were sacrificed by exsanguination under general anesthesia using a cocktail of anesthetics (Narketan (Vetoquinol UK Ltd.) at 80 mg/kg b.w. and Xylapan (Vetoquinol UK Ltd.) at 12 mg/kg b.w.). Kidneys were immediately collected, transferred to the experimental tubes and frozen in liquid nitrogen. The samples were then stored at -70°C until further analysis.

S7. Average Mass Spectra

Mass spectra of the kidney cortex averaged over each group of animals are given below.