Effect of kidney disease and vascular calcification on the circulation and distribution of tetracycline-functionalized polymer nanoparticles

Vanessa Dos Passos Maio[1], Roth-Visal Ung[2], Karine Greffard[1], Nicolas Gaudreault[1], Sylvain Picard[2], Fabrice Mac-Way[2] and Nicolas Bertrand[1]*

[1]CHU de Québec Research Center-Université Laval, Centre mère-enfant Soleil Hospital, Endocrinology and Nephrology Unit, Faculty of Pharmacy, 2705 Laurier Blvd, Quebec City, G1V 4G2, Canada.

[2]CHU de Québec Research Center-Université Laval, Hôpital de l'Enfant-Jésus, Endocrinology and Nephrology Unit, Faculty and Department of Medicine, 2260 boul. Henri-Bourassa, Quebec City, Quebec, G1J 5B3, Canada.

*Correspondence to:

Nicolas Bertrand

CHU de Québec Research Center-Université Laval

2705 Laurier Blvd, T4-13

Quebec City, QC, G1V 4G2, Canada

Tel +1 (418) 525-4444 ext 46302

nicolas.bertrand@pha.ulaval.ca

Supplementary Information

1. Synthesis of Cy5.5 or Cy5 terminated PLA

An excess of amine-terminated PLA_{20k}, synthesized by a ring-opening polymerization method, as described previously [1], was conjugated with N-hydroxysuccinimidyl ester of cyanine 5.5 (Cy5.5-NHS: λ_{ex} 684 nm , λ_{em} 710 nm; Lumiprobe, Hunt Valley, USA). Briefly, 300 mg of PLA_{20k} (0.03 mmol, 1 eq) were dissolved in 1.2 mL of anhydrous dichloromethane. Anhydrous DIPEA (27 µL, 0.16 mmol, 5.3 eq) was added and mixed. In a black tube, 6 mg of Cy5.5-NHS (0.008 mmol; 0.3 eq) was dissolved in 600 µL of anhydrous dichloromethane. This solution was added to the vial containing the PLA and DIPEA. The reaction was conducted at room temperature, protected from light, for 18 hours. The fluorescent polymer was recovered by four precipitations in cold ether and dried overnight under vacuum. The same method was employed to obtain Cy5-PLA_{20k} using Cy5-NHS (Cy5-NHS: λ_{ex} 646 nm , λ_{em} 662 nm; Lumiprobe, Hunt Valley, USA).

2. Synthesis of [^{14}C]-mPEG$_{10k}$ (used in Figure 3)

Radiolabeled PEG was synthesized via acetylation of PEG_{10k}-diamine (Laysan Bio, Arab, Alabama) (Laysan Bio, Arab, Alabama) using [^{14}C]-acetyl chloride (#ARC-0180, 54 µCi/µmol, American Radiolabeled Chemicals, St-Louis, Missouri), as previously described [2]. Briefly, PEG_{10k}-

diamine (12 mg, 1.2 μmol) was dissolved in 200 μL of dichloromethane in a glass vial, followed by the addition of N,N-diisopropylethylamine (1.5 mg, 10 μmol) and [^{14}C]-acetyl chloride (50 μL of an 18.5 μmol/mL solution, 0.9 μmol, 50 μCi). The reaction mixture was stirred at room temperature for 4 h. The polymer was then concentrated by evaporating dichloromethane and precipitated twice in cold diethyl ether. After drying, the reaction yielded a white solid (9.5 mg, 78%), with a radiochemical recovery of 84% (42 μCi). The labeled polymer was diluted with non-radioactive polymer synthesized using the same procedure and analyzed by gel permeation chromatography (GPC) using an Agilent 1260 Infinity 2 system equipped with a diode array detector (G7115A), a refractive index detector (G7162A), and a fraction collector (G1364C). Fractions corresponding to each peak were collected and analyzed by scintillation counting (Wallac 1411 Liquid Scintillation Counter), confirming that >90% of the radioactivity was associated with the polymer [2].

3. ^{1}H-NMR and GPC characterization of polymers

Polymers were dissolved in deuterated chloroform ($CDCl_3$). Spectra were acquired at 25°C and chemical shifts are expressed in ppm relative to $CDCl_3$ (7.26 ppm). The products obtained in steps 1, 2 and 3 of the polymer synthesis were analyzed at 400 MHz in a Bruker Advance 400 (Billerica, MA, USA) (Supplementary Figures S1, S2 and S3). (Billerica, MA, USA). The the product from step 4 was acquired on a more sensitive Bruker Advance NEO 300 model. (Supplementary Figure S4). The number average molecular weight (Mn) of PLA (Supplementary Table 1) was estimated by comparing the methylene and methine protons of PLA to the methylene protons of the PEG macro-initiator. The integration of PEG's methylene protons was referenced using the molecular weight reported by the supplier.

The product from each synthesis step, dissolved in THF, was analyzed by gel permeation chromatography (GPC) using an Agilent 1260 Infinity II system. Separation was performed on an Agilent ResiPore PL1113-6300 column (300 × 7.5 mm), with THF as the mobile phase at a flow rate of 1 mL/min over 30 minutes. The column temperature was maintained at 30 °C. A sample volume of 100 μL was injected, and detection was carried out using an Agilent 1260 G7162A refractive index detector. A calibration curve ranging from 370 to 364,000 g/mol (R^2 = 0.988071), generated using a polystyrene standard (Agilent EasiVial Polystyrene PS-M Standard reconstituted in THF), was used to determine the number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity index (Mw/Mn) for each analyzed product (Supplementary Table 1).

4. Nanoparticle uptake analysis in normal and calcified cells using flow cytometry

Primary human aortic smooth muscle cells (HASMC) PCS-100-012, were grown in Vascular Cell Basal Medium PCS-100-030 supplemented with 5% of fetal bovine serum (Vascular Smooth Muscle Cell Growth kit PCS-100-042), and antimicrobial/antimycotic solution containing phenol red. All these materials were purchased from ATCC (Manassas, USA). Cells were kept in a humidified incubator at 37 °C and 5% CO_2 until 70–80% confluence. Targeted nanoparticles (Tet-NP) and non-target nanoparticles (NP) were prepared with Cy5-PLA, as described above and diluted in the culture media right before the treatment. Twenty-four hours before treatment, the cells were seeded in 12-well plates (140,000 cells/well). Each well was added with 1 mL media and incubated for 24 h to allow cell attachment. After the incubation, the media in each well was replaced and 10 μL of nanoparticle solution was added and mixed with a pipette. The cells were incubated with six different concentrations (0.1 ug – 10 ug/well) of each kind of nanoparticle for 24 h before cell uptake analysis by flow cytometry. The cell viability was assessed with a viability marker (FVS 510, BD Biosciences, San Jose, USA). Unstained and single-stained (Cy5 or FSV 510) sample controls were also analyzed for compensation calculations. All experiments were conducted on a BD FACSCanto II (BD Biosciences, Mississauga, ON, Canada) using a 633 nm laser (660/20 filter) and a 405 nm laser (filter 525/50). The data was analyzed using FlowJo V10 software (Tree Star Inc., BD, Ashland, OR, USA).

To determine the nanoparticle uptake by calcified cells, the calcification of HASMC was induced. The same medium cited above but supplemented with calcium (2.7 mM) and phosphate (2.5 mM) (CaP media), as described previously [3], was used. Briefly, the cells were seeded in 12-well plates (20,000 cells/well) with 1 mL of normal media and incubated. Forty-eight hours later, the normal media was replaced by the CaP media at a volume of 1 mL/well. The treatment media was replaced on alternate days. On the sixth day, the nanoparticles solutions (Tet-NP and NP) were added, and the cells were incubated for 24 h. To identify the presence of cell calcifications, a von Kossa staining was conducted with 5% silver nitrate and UV light exposure, followed by 5% sodium thiosulfate, and counterstained with nuclear fast red solution (N3020, MilliporeSigma, Oakville, Canada). The presence of calcium deposition was confirmed when black spots were observed on cell monolayers using an EVOS AMG microscope (Life Technologies, Carlsbad, CA, USA) at a magnification of 20×. The nanoparticles uptake by the calcified cells was assessed by flow cytometry following the same procedure described for the non-calcified cells.

6. Vitamin K quantification by HPLC for nanoparticle characterization.

Vitamin K was quantified in the nanoparticle suspensions by an HPLC method adapted from Hacene, Loiseau et al. [1]. To analyze nanoparticle formulations, 20 μL of the suspension were added in a black tube, and 490 μL of acetonitrile was added. The tube was vortexed for 10 seconds. Next, 490 μL of ethanol was added, and the tube was vortexed for 10 seconds again. The solution was further diluted in a 1:1 volume per volume mixture of acetonitrile and ethanol to obtain around 5 μg/mL vitamin K solution and then analyzed by HPLC as follows. The system used was an Agilent liquid chromatography system (California, USA) equipped with OpenLAB CDS software (Version 2.6), a 1260 DAD WR diode array detector, a 1260 FLD Spectra fluorescence detector, a 1260 Infinity II degasser, a 1260 Infinity II quaternary pump, a 1260 Infinity II vial sampler, a 1260 Infinity II multicolumn thermostat, and a C18 column (4.6 mm × 100 mm, 3.5 μm particle size, ZORBAX Eclipse Plus®, Agilent). The mobile phase at an isocratic flow rate (1 mL/min) consisted of a 53:42:5 volume per volume mixture of acetonitrile, tetrahydrofuran, and water over

6.0 minutes of run. The temperature of the column was set at 25 °C, 80 µL of samples were injected and vitamin K was detected at 245 nm. Linearity of a 6-point calibration curve was achieved for concentrations ranging from 0.3 to 10 µg mL^{-1} with a quantification limit above 0.3 µg mL^{-1} for vitamin K. For mean calculations, values under half of the quantification limit were set at 0 µg mL^{-1}.

7. Vitamin K quantification by HPLC for pharmacokinetic experiment

For the quantification of vitamin K in the rat plasma, a more sensitive method adapted from Marinova et al. [4], with fluorometric detection, was used. In this method, the quinone form of vitamin K1 is converted into its fluorescent hydroquinone form by post-column zinc reduction. The same Agilent system was used with a C18 column (4.6 mm×150 mm, 3 µm particle size, SiliaChrom® Plus, Silicycle, Canada). A post-column was dry-packed with zinc powder (<10 µm, Sigma-Aldrich, St. Louis, USA) and connected between the analytical column and the fluorescence detector. Fluorescence detection was carried out at an excitation wavelength of 244 nm and an emission wavelength of 430 nm. During analysis, the column temperature was maintained at 22°C and the autosampler temperature was kept at 10°C. The mobile phase consisted of water 5%, acetonitrile 10%, tetrahydrofuran 42% and zinc acetate methanolic solution 43% (1.1 g zinc acetate, 10 mL acid acetic and 10 mL water par litre of methanol). The flow rate was 0.8 mL/min over 8 min. Linearity of a 12-point calibration curve was achieved for concentrations ranging from 5 to 2000 ng mL^{-1} with a quantification limit above 5 ng mL^{-1} for vitamin K.

8. Vitamin K quantification by HPLC in the aorta

For vitamin K quantification in the aorta, we used the Vitamin K1 HPLC kit from Immundiagnostik AG (Bensheim, Germany), following the manufacturer's procedure for sample preparation and analysis. All procedures were conducted to minimize exposure to light. Briefly, approximately 70 mg of aortic tissue was placed in a 1.5 mL microtube along with 10 µL of the internal standard. The tissue was homogenized using a disposable plastic pestle, which was then rinsed twice with 100 µL of acetonitrile. Subsequently, 900 µL of PREC was added. The tube was placed in an ultrasonic bath for 10 minutes. The contents of the tube were then transferred to a 15 mL tube, followed by the addition of 900 µL of PREC, and vortexed for 30 seconds. Extraction was performed using a vortex mixer by adding 1.6 mL of water and 4 mL of ELUSOL for 1 min. After centrifugation (300 g; 5 min), 3.2 mL of the organic layer was evaporated under a stream of nitrogen at 45°C. The lipid extract was dissolved in the mobile phase provided in the kit, and the solution was injected into the same chromatographic system mentioned above, equipped with a post-column zinc reactor. The chromatographic column used (C18 Superspher RP 18, 4 µm, 125 x 4.6 mm) and the zinc powder were also provided in the kit. The detection wavelengths were 248 nm excitation and 418 nm emission.

Product	PLA Mn via CH (g/mol)	PLA Mn via CH_3 (g/mol)	Polymer Mw/Mn	Polymer Mw (g/mol)	Polymer Mn (g/mol)
Step 1	9000	8900	2.2	-	-
Step 2	10300	10200	1.3	16900	12800
Step 3	9500	9500	1.3	23000	18400
Step 4	9200	9100	1.3	18400	14400

Supplementary Table 1. Number-average molecular weight (Mn) of PLA calculated from NMR spectra, and weight-average molecular weight (Mw), Mn, and polydispersity index (Mw/Mn) obtained by GPC at each synthesis step.

t	Rat 2	Rat 6	Rat 9	Rat 10	Rat 22	Rat 33
h	%ID/g	%ID/g	%ID/g	%ID/g	%ID/g	%ID/g
0.25	3.668	2.232	3.351	3.138	3.445	3.353
0.5	3.158	2.050	3.984	3.020	3.334	3.281
1	3.319	2.227	3.728	3.023	3.750	3.087
2	3.439	1.964	2.966	3.219	3.484	2.672
4	2.932	1.707	2.537	2.637	3.105	2.467
6	1.974	1.694	2.317	2.668	2.572	2.231
12	1.896	1.260	1.698	1.711	2.678	1.774
24	1.395	1.021	1.204	1.114	1.251	1.275

Supplementary Table 2. Plasma targeted tetracycline nanoparticles concentration (% of injected dose/gram of blood) in the CKD+VC group (n = 6).

t	Rat 14	Rat 17	Rat 18	Rat 19	Rat 20
h	%ID/g	%ID/g	%ID/g	%ID/g	%ID/g
0.25	3.065	3.388	3.496	2.460	2.296
0.5	3.086	2.897	3.559	2.745	2.200
1	2.939	2.496	2.978	3.598	2.723
2	2.579	2.833	3.058	2.258	2.182
4	2.411	2.671	2.765	2.175	1.875
6	3.005	2.203	2.060	2.117	1.626
12	1.760	1.405	1.462	1.431	0.897
24	1,364	1,219	1,174	1,064	0,842

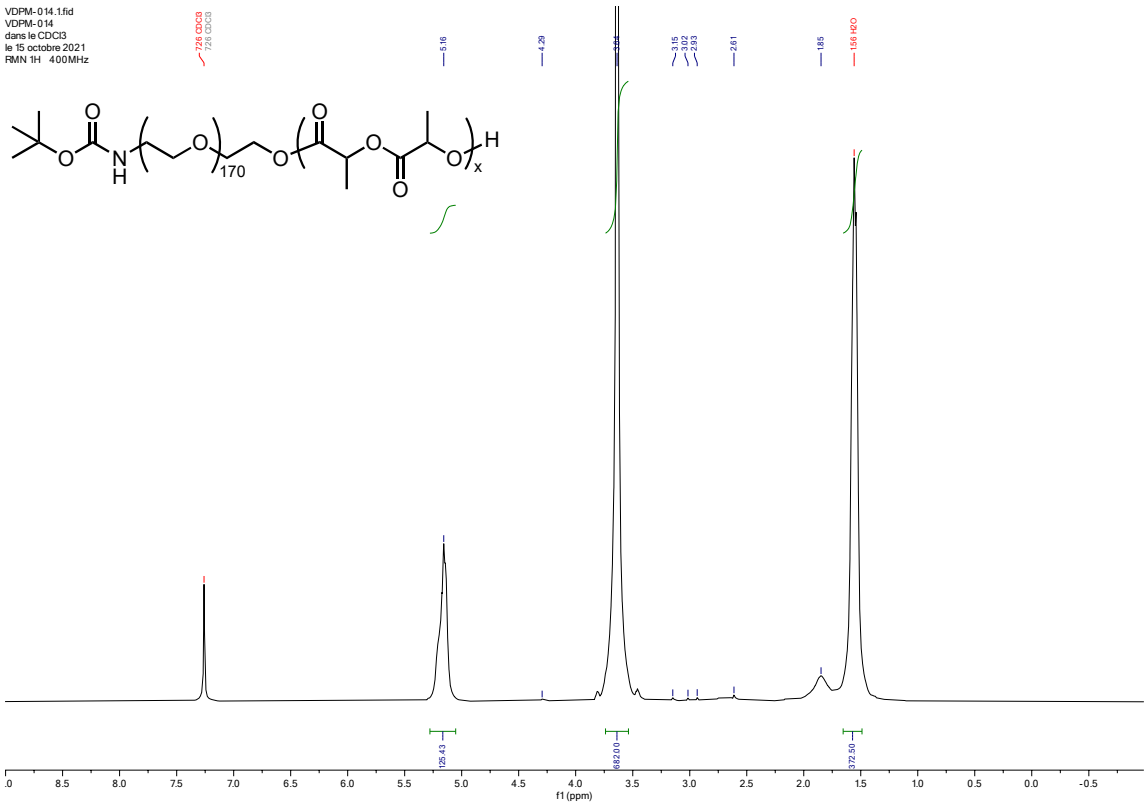
Supplementary Table 3. Plasma targeted tetracycline nanoparticles concentration (% of injected dose/gram of blood) in the CKD group (n = 5).

t	Rat 31	Rat 23	Rat 34	Rat 35	Rat 36
h	%ID/g	%ID/g	%ID/g	%ID/g	%ID/g
0.25	3.028	3.520	2.550	3.165	3.767
0.5	3.187	3.574	2.649	3.147	3.639
1	2.615	3.443	2.620	3.186	3.168
2	2.700	2.935	2.274	2.822	2.907
4	2.329	2.530	1.935	2.433	2.948
6	2.028	2.926	1.704	2.167	2.348
12	1.409	1.859	1.015	1.583	1.625
24	1.239	1.428	0.878	1.147	1.112

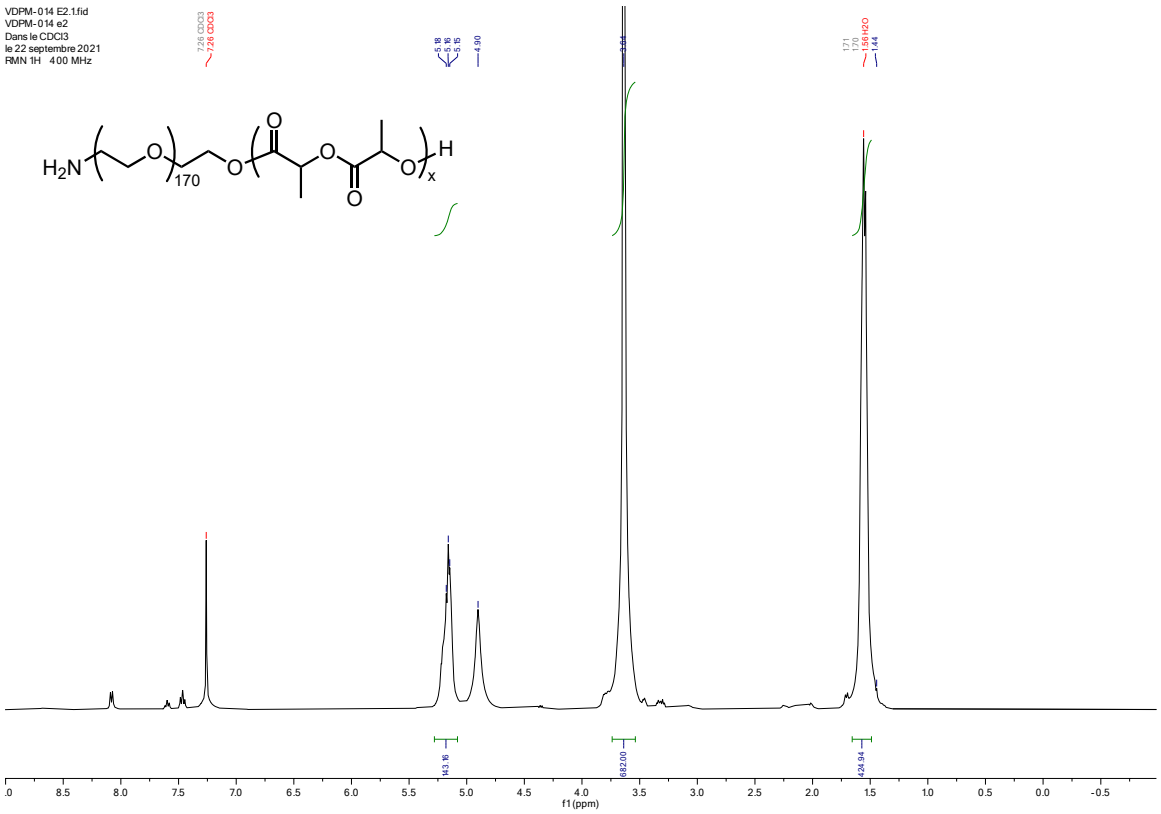
Supplementary Table 4. Plasma targeted tetracycline nanoparticles concentration (% of injected dose/gram of blood) in the Healthy group (n = 5).

		Organic Phase								
Figure	**Type of NPs**	**Tet_1-$PEG_{7.5k}$-PLA_{9k}**	**PEG_{5k}-PLA30k**	**$PLGA_{95k}$**	**PLA_{20k}-Cy5.5**	**PLA_{20k}-Cy5**	**^{14}C-PLGA**	**Average size (nm)**	**PDI**	**Vitamin K in NPs (wt%)**
1C and 2A	Targeted (Tet-NP)	80%	-	-	-	-	<0.2% 0.2 µCi/mg of NPs	114 ± 3.4	0.105 ± 0.011	-
	Targeted (Tet-NP)	75%	-	-	-	-	<0.2% 0.2 µCi/mg of NPs	101 ± 1.5	0.094 ± 0.013	-
	Targeted (Tet-NP)	50%	-	-	-	-	<0.2% 0.2 µCi/mg of NPs	95 ± 1.8	0.095 ± 0.018	-
	Targeted (Tet-NP)	20%	-	-	-	-	<0.2% 0.2 µCi/mg of NPs	98 ± 0.5	0.105 ± 0.011	-
	Targeted (Tet-NP)	10%	-	-	-	-	<0.2% 0.2 µCi/mg of NPs	95 ± 1.2	0.081 ± 0.013	-
	Non-targeted (NP)	-	80%	20%	-	-	<0.2% 0.2 µCi/mg of NPs	86 ± 0.3	0.089 ± 0.017	-
2A and 2C	Targeted (Tet-NP)	80%	-	-	20%	-	-	128 ± 2.5	0.219 ± 0.011	-
	Non-targeted (NP)	-	80%	-	20%	-	-	131 ± 0.53	0.137 ± 0.004	-
2B	Targeted (Tet-NP)	80%	-	20%	-	-	<0.2% 0.2 µCi/mg of NPs	112 ± 1.4	0.112 ± 0.011	-
2D	Targeted (Tet-NP)	80%	-	-	-	20%	-	129 ± 6.1	0.164 ± 0.007	-
	Non-targeted (NP)	-	80%	-	-	20%	-	135 ± 0.44	0.244 ± 0.006	-
5	Targeted (Tet-NP)	50%	30%	20%	-	-	<0.3% 0.3 µCi/mg of NPs	94 ± 0.76	0.095 ± 0.014	-
6	Targeted (Tet-NP) Day 1	80%	-	-	20%	-	-	131 ± 2.6	0.168 ± 0.008	9,9%
	Targeted (Tet-NP) Day 2	80%	-	-	20%	-	-	124 ± 2.8	0.173 ± 0.011	7.8%
	Targeted (Tet-NP) Day 2	80%	-	-	20%	-	-	148 ± 1.1	0.160 ± 0.017	10.2%
	Non-targeted (NP) Day 1	-	80%	-	20%	-	-	129 ± 1.1	0.177 ± 0.006	11.0%
	Non-targeted (NP) Day 2	-	80%	-	20%	-	-	125 ± 0.4	0.186 ± 0.023	8.6%
	Non-targeted (NP) Day 3	-	80%	-	20%	-	-	126 ± 1.4	0.184± 0.06	10.8%

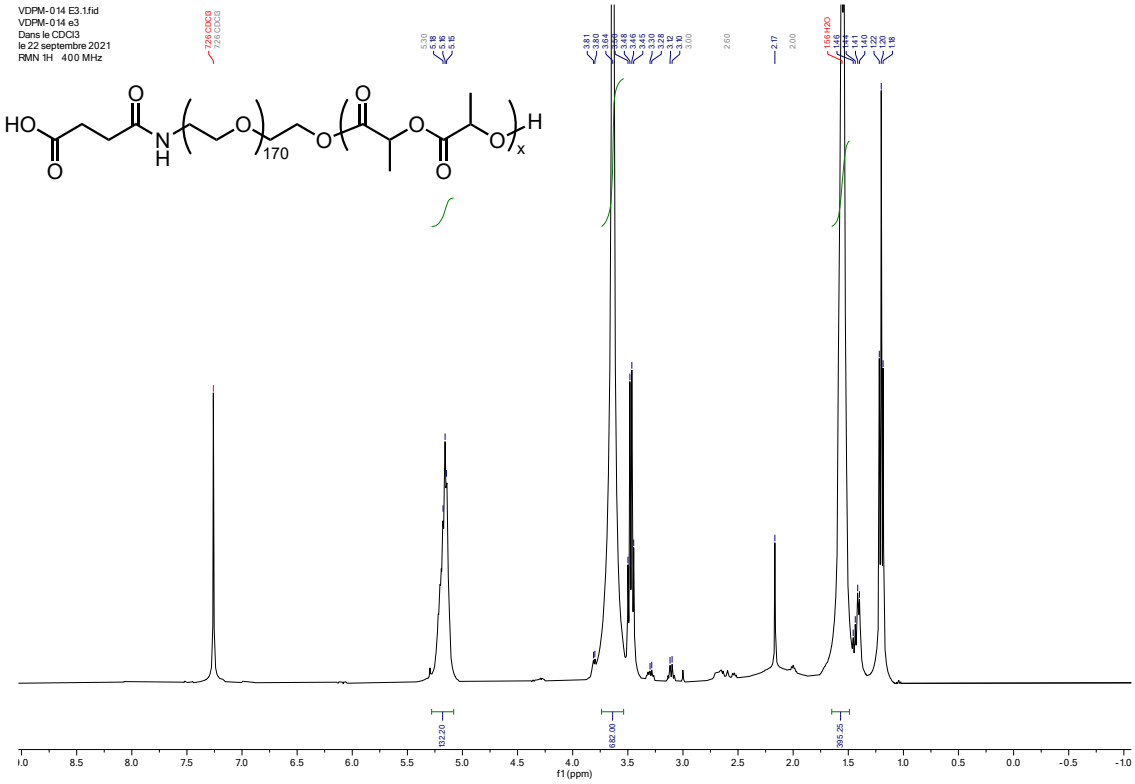
Supplementary Table 5. Composition and characteristics of nanoparticles (NPs) corresponding to each figure. All nanoparticles (NPs) were prepared by nanoprecipitation: 1 mL of organic phase added dropwise to 9 mL of water under stirring at 1600 rpm at room temperature (See 2.2 Preparation of nanoparticles). Values represent mean ± SD (n=3).



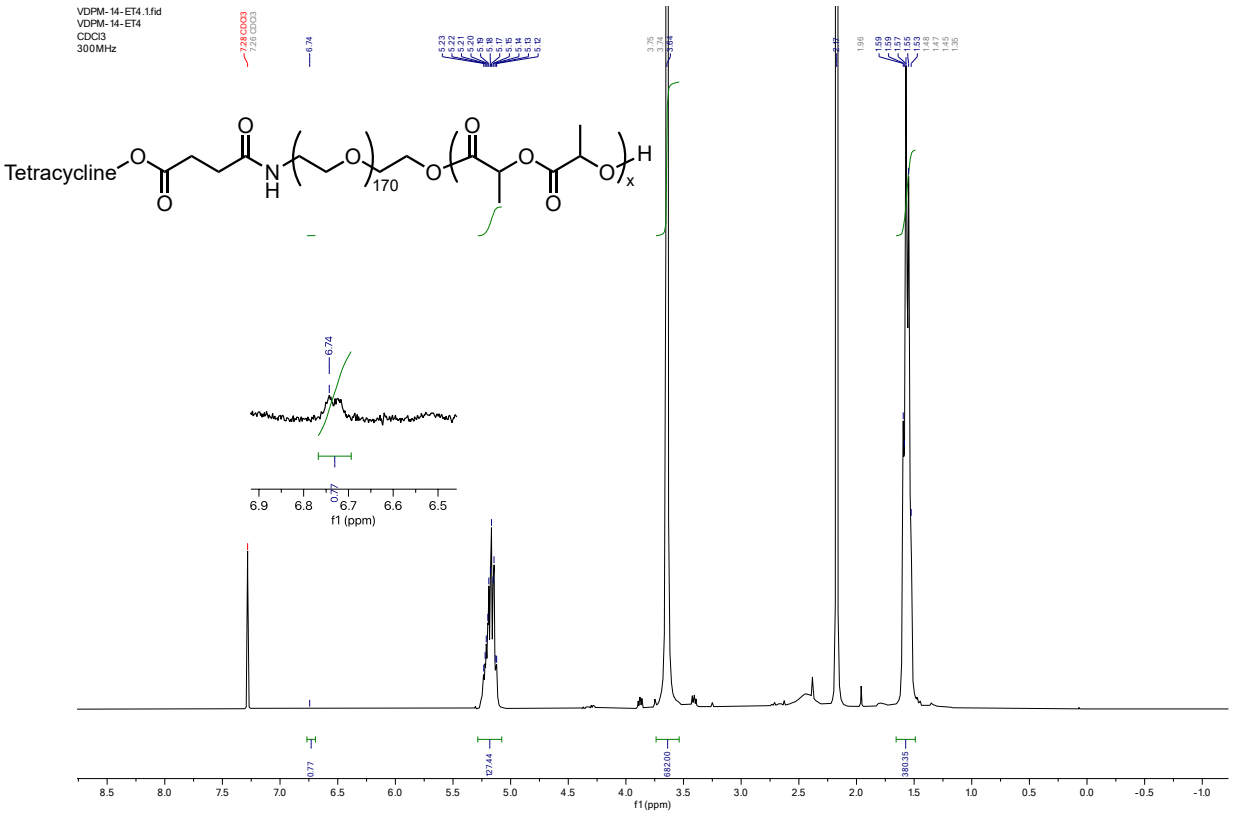
Supplementary Figure 1. Step 1: t-Boc-N-PEG-PLA ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C) δ= 5.28-5.08 (m, 125H; OC<u>H</u>(CH_3)CO), 3.64 (s, 682H; OC<u>H_2</u>C<u>H_2</u>), 1.65-1.49 (m, 372H; C(C<u>H_3</u>)H)



Supplementary Figure 2. Step 2: H_2N-PEG-PLA ^{1}H-NMR (400 MHz, $CDCl_3$, 25 °C) δ= 5.28-5.08 (m, 143 H; OC<u>H</u>(CH_3)CO), 3.64 (s, 682H; OC<u>H_2</u>C<u>H_2</u>), 1.65-1.49 (m, 425H; C(C<u>H_3</u>)H).

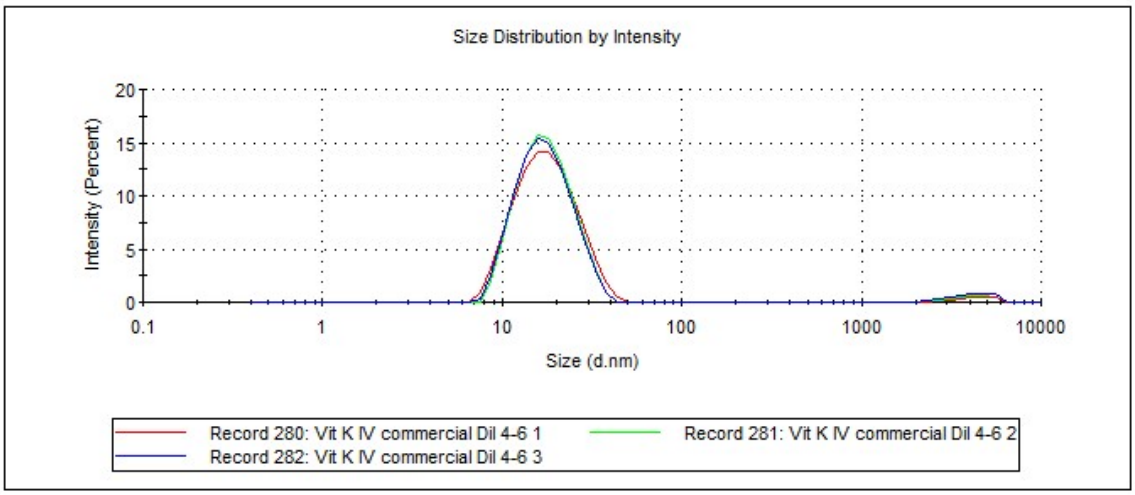


Supplementary Figure 3. Step 3: COOH-PEG-PLA ^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C) δ= 5.28-5.08 (m, 132 H; OC<u>H</u>(CH_3)CO), 3.64 (s, 682H; OC<u>H_2</u>C<u>H_2</u>), 1.65-1.49 (m, 395H; C(C<u>H_3</u>)H).

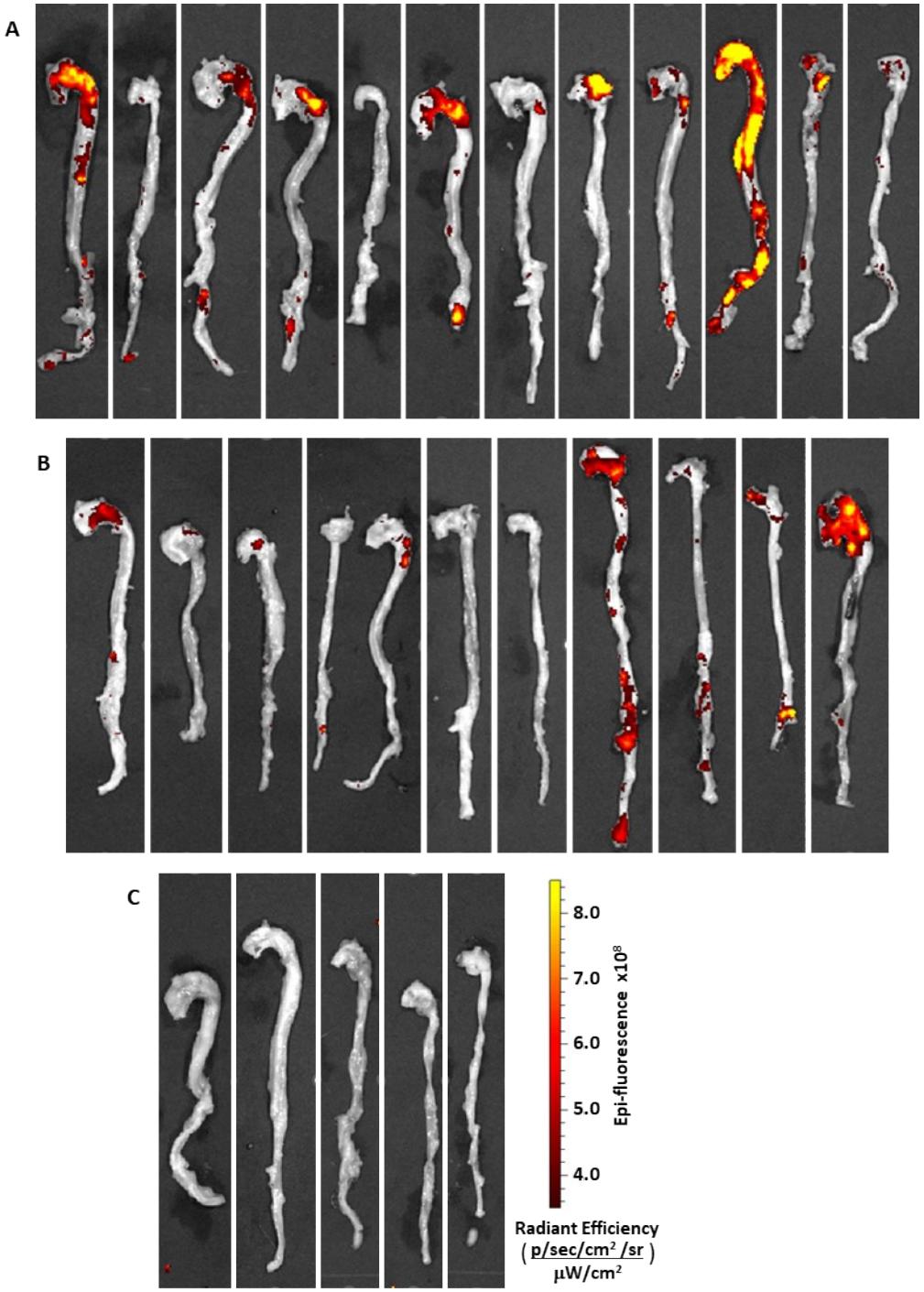


Supplementary Figure 4. Step 4: Tet-PEG-PLA ^{1}H-NMR (300 MHz, $CDCl_3$, 25 °C) δ=6.69-6.67(br, 0.7H; Aryl C<u>H</u>); 5.28-5.08 (m, 128H; OC<u>H</u>(CH_3)CO), 3.64 (s, 682H; OC<u>H_2</u>C<u>H_2</u>), 1.65-1.49(m,380H ; C(C<u>H_3</u>)H)

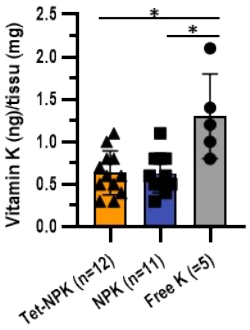
			Size (d.nm):	% Intensity:	St Dev (d.nm):
Z-Average (d.nm):	16.64	Peak 1:	18.49	97.8	6.933
PdI:	0.177	Peak 2:	4347	2.2	943.3
Intercept:	0.949	Peak 3:	0.000	0.0	0.000
Result quality :	Good				



Supplementary Figure 5. Vitamin K1 emulsion size (Z-average) and size distribution (polydispersity index - PDI) measured by dynamic light scattering (DLS) at 22 °C with a 173 backscatter angle, using a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA).



Supplementary Figure 6. IVIS aortas' 48 h after formulation injection. **A.** Targeted tetracycline nanoparticles with vitamin K (Tet-NPK). **B.** Non-targeted nanoparticles with vitamin K (NPK). **C.** Vitamin K emulsion (Free K).



Supplementary Figure 7. Vitamin K content in the aortas 48 h after formulation injection, determined by HPLC. Targeted tetracycline nanoparticles with vitamin K (Tet-NPK), non-targeted nanoparticles with vitamin K (NPK), and vitamin K emulsion (Free K). Values represent the mean ± SD from n animals. ANOVA with Tukey's post hoc test: *$p < 0.05$.

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