Electronic Supplementary Material (ESI) for ChemComm.

Supplementary information for

Crosslinkable polymeric contrast agent for high-resolution X-ray imaging of the vascular system

Ngoc An Le[1,+], Willy Kuo[2,3,4,+], Bert Müller[4], Vartan Kurtcuoglu[2,3,*], Bernhard Spingler[1,*]

[1] Department of Chemistry, University of Zurich, 8057 Zurich, Switzerland;

[2] Institute of Physiology, University of Zurich, 8057 Zurich, Switzerland;

[3] National Centre of Competence in Research, Kidney.CH, 8057 Zurich, Switzerland;

[4] Biomaterials Science Center, Department of Biomedical Engineering, University of Basel, 4123 Allschwil, Switzerland

[+] and * These authors contributed equally to this work.

Corresponding author email addresses: vartan.kurtcuoglu@uzh.ch and spingler@chem.uzh.ch

METHODS

General

Unless otherwise stated, all chemicals were of reagent grade and purchased from *Sigma–Aldrich*. All solvents were of analytical grade. The synthesis and characterization of acrylic anhydride were performed in accordance with the literature.[1] Dialysis tubings were purchased from *Sigma–Aldrich* (cellulose, 12 kDa cut-off) and from *Spectrum* (cellulose ester, 100 kDa cut-off). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer. IR spectra were recorded on a Spectrum Two FT-IR Spectrometer (Perkin–Elmer) equipped with a Specac Golden Gate™ ATR accessory. Low resolution mass spectroscopic analysis was performed with a Waters AQUITY-Bruker UPLC-MS system. Elemental microanalyses were performed on a LECO CHNS-932 elemental analyser. Analytical gel permeation chromatography was performed by the analytical service of PSS Polymer (Mainz, Germany) using columns PSS-NovemaMax_F 5µm: Guard + 30Å + 1000Å +1000Å with UV/VIS and differential refractometer RID detectors. An aqueous solution of 0.1 M NaCl and 0.1 vol.-% TFA was used as an eluent. The average molecular weight and the molecular weight distribution of the samples were calculated relative to a standard calibration with pullulan.

Synthesis of the contrast agent

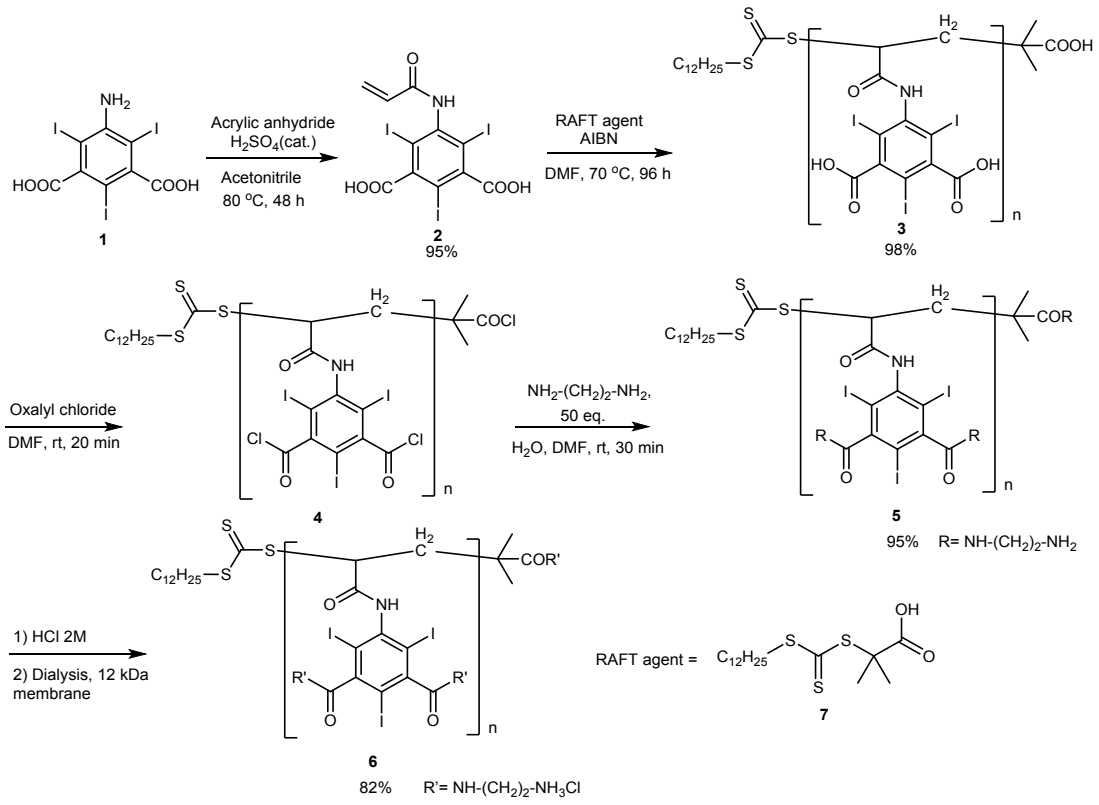

Figure S1. Synthesis pathway for polymeric contrast agent *XlinCA* (**6**) using RAFT polymerization.

2,4,6-triiodo-5-(prop-2-enamido)benzene-1,3-dicarboxylic acid (2)

To a suspension of 5-amino-2,4,6-triiodobenzene-1,3-dicarboxylic acid (**1**) (20 g, 35.8 mmol) and concentrated sulfuric acid (0.04 mL) in acetonitrile (40 mL), acrylic anhydride (12 mL, 107 mmol) was added drop-wise while the reaction mixture was being cooled in an ice bath. The reaction mixture was stirred at 80 °C for 36 h until no 5-amino-2,4,6-triiodobenzene-1,3-dicarboxylic acid (**1**) was detected by UPLC. The mixture was cooled down to room temperature, then filtered under vacuum and washed with acetonitrile (20 mL). The solid was dried in high vacuum for two days to yield 20.8 g (95%) of a white product. Multiple attempts to synthesize compound **2** with the help of the cheaper acryloyl chloride reagent were unsuccessful. UV-Vis spectrum (methanol, λ; nm): 243; IR [KBr, cm^{-1}]: 3247, 3000, 1725, 609, 508; ^{1}H NMR (400 MHz, DMSO-d_6): 5.82 (dd, J^2 =1.8 Hz, J^3cis =10.2 Hz, 1H, H(15)), 6.30 (dd, J^2 =1.7 Hz, J^3trans =17.2 Hz, 1H, H(16)), 6.44 (dd, J^3cis =10.2 Hz, J^3trans =17.1 Hz, 1H, H(17)), 10.22 (s, 1H, H(7)), 14.0 (s, broad, 2H, H(20,23)), proton assignment: see Figure S2. ^{13}C NMR (101 MHz, DMSO-d_6): 169.9 C(18,21), 163.5 C(8), 149.7 C(2,4), 143.8 C(6), 131.5 C(10), 128.1 C(11), 98.6 C(1,5), 87.7 C(3), the carbon assignment was done with the help of ChemDraw Professional 19: see Figure S3. ESI-MS: m/z = 613.74 $[M+H]^+$; Elemental analysis calcd (%) for $C_{11}H_6I_3NO_5$: C 21.56, H 0.99, N 2.29; found: C 21.75, H 0.93, N 2.36.

Poly(2,4,6-triiodo-5-(prop-2-enamido)benzene-1,3-dicarboxylic acid) (3)

2,4,6-triiodo-5-(prop-2-enamido)benzene-1,3-dicarboxylic acid (**2**) (25.12 g, 40 mmol) was dissolved in DMF (40 mL). Then, 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (**7**) (72.8 mg, 0.2 mmol) and AIBN (16.4 mg, 0.1 mmol) were added. The solution was degassed by three freeze-evacuate-thaw cycles and transferred to an oil bath preheated at 70 °C under nitrogen flow. The polymerization was carried out for 96 h under slow stirring and then was quenched by cooling in an ice bath under atmospheric air for 30 minutes (conversion degree 78 %). DMF was removed on a rotovap at 10 mbar, 50 °C and the product was dried under high vacuum for 2 days to give polymer **3** (24.6 g, 95%) as a yellowish solid. The proton nuclear magnetic resonance (NMR) spectrum was measured at the end of the reaction to calculate the conversion degree of the polymerization process, which was determined to be 78 %. It was obtained via the ratio of the integral of the methine proton signal at 2.54 to 2.60 ppm to half of the integral of the carboxylic group proton signal at 13.4 to 14.5 ppm. (*Suppl. Fig. S2*). ^{1}H NMR (400 MHz, DMSO-d_6): 5.82 (dd, J^2 =1.8 Hz, J^3cis =10.2 Hz, 0.22 H), 6.30 (dd, J^2 =1.7 Hz, J^3trans =17.2 Hz, 0.26 H), 6.44 (dd, J^3cis =10.2 Hz, J^3trans =17.1 Hz, 0.22 H), 10.22 (s, 0.26 H), 14.01 (s, broad, 2 H), see Figure S4. Elemental analysis calcd (%) for $C_{15.35}H_{16.15}I_3N_{2.45}O_{6.45}$ (M+1.45 DMF): C, 25.65; H, 2.26; N, 4.77 Found: C, 25.57; H, 2.56; N, 4.41.

XlinCA (**6**)

The polymer **3** was re-dissolved in 40 mL of DMF before the oxalyl chloride-DMF adduct was added in small portions. The oxalyl chloride-DMF adduct was synthesized by adding oxalyl chloride (15 mL) dropwise over 15 min to a solution of 40 mL of DMF in 300 mL of DCM, while the reaction was being cooled in an ice bath. After 15 min, DCM was evaporated giving the mixture of adduct in DMF, then the whole mixture was used to treat the polymer **3**.

The reaction mixture was stirred at room temperature for 30 min. Afterwards, the solution was added quickly to 500 mL of water to precipitate the product. The precipitate was filtered, washed with 100 mL of water, and dried under high vacuum overnight to give the chlorinated polymer **4**.

Chlorinated polymer **4** was dissolved in DMF (100 mL) and the solution was added quickly to the ice-cold mixture of ethylene diamine (100 mL) and water (100 mL) under vigorous stirring. After 30 min, the solvents were evaporated *in vacuo*. Water (50 mL) was added to the residue and the mixture was lyophilized under high vacuum to give **5** (23.4 g, 95 %). Polymer **5** was found not to be soluble in all tested solvents tested solvents (D_2O,

MeOH, DMSO and DMF). Therefore, it was converted to a water-soluble salt form (polymer **6**) without characterisation. **5** was dissolved in HCl solution (100 mL, 2 M). The solution was dialyzed with a 12 kDa membrane against NaCl solution (10 L, 0.2%), changing the solution after 3 h, 8 h and 24 h; then against deionized water (10 L) for 6 h more. Afterwards, pH was readjusted to 7 by adding NaOH 1M solution and the solution lyophilized to give the final product *XlinCA* **6** as a white to yellowish powder (19.2 g, 82 %). IR [KBr, cm^{-1}]: 3420, 3231, 3053, 1647, 1546, 1384, 1351, 1270, 1171, 1030, 618. GPC (M_n= 33700, PDI= 3.16). Unfortunately, no high-resolution ^{1}H-NMR spectra could be obtained for **6**. The ^{1}H-NMR of polymer **6** in D_2O gave broad signals (see Figure S5). Other tested solvents (MeOH, DMSO and DMF) did not dissolve the compound.

GPC measurement of contrast agent *XlinCA* (6)

GPC measurements were done by PSS Polymer Standards Service GmbH, Mainz, Germany.

Sample preparation. About 2 mg of each sample were weighed in on an analytical balance. 2 mL of eluent were added to the sample and left to dissolve at room temperature. After 2 hours, the sample was completely dissolved and could be measured. The sample solution was not filtrated before the measurement and 100 µL were injected by an autosampler (Figure S6).

Calibration and Calculation. Pullulan-standards with different molecular weights were analyzed first in order to get a calibration curve. The calculations of the average molecular weights and the molecular weight distribution of the samples were done by the so-called slice by slice method based on the pullulan-calibration.

Table S1. Calculation of the average molecular weights and the molecular weight distribution. M_n: Number average molecular weight, M_w: Weight average molecular weight, M_z: Size average molecular weight, PDI: Polydispersity index, V_p: Elution volume at peak maximum, M_p: Molecular weight at the peak maximum, Area: Total area under elugram.

	Detector	M_n /Da	M_w /Da	M_z /Da	PDI (=M_w/M_n)	V_p /mL	M_p /Da	Area
_____	UV@230 nm	33700	107000	324000	3,16	22,58	39900	2686,9200
_____	RID	33400	105000	325000	3,16	22,60	39400	16,6182

Mouse husbandry

C57BL/6J mice were purchased from Charles River Laboratories and Janvier Labs and were housed in individually ventilated cages in 12 h light/dark cycles with ad libitum access to water and standard rodent food (Kliba Nafag 3436). All animal experiments were approved by the veterinary office of the canton of Zurich (license number ZH233/15).

Pre-crosslinking of contrast agent

Contrast agent *XlinCA* (5 g) was dissolved in water (20 mL). 150 µL of an aqueous glutaraldehyde solution (25 %) were added and mixed well. The mixture was left to rest at room temperature for 20 min. 30 mL of water were then added, and the solution was dialysed through a 100 kDa dialysis membrane against NaCl solution (5 L, 0.2 %), changing the solution after 3 h, 8 h and 24 h; then against deionized water for 6 h. The solution was centrifuged to remove all insoluble particles and lyophilized to give 4 g of a solid, pre-crosslinked contrast agent.

Transcardial whole body perfusion with polymeric contrast agent

A 9 month old mouse was euthanized with ketamine/ xylazine. The chest cavity was opened, a blunted 21 G butterfly needle inserted into the left ventricle and the right atrium cut as an outlet. Blood was flushed out with approx. 10 mL of phosphate-buffered saline (PBS) and the mouse fixed with 100 mL 4 % formaldehyde and 1 % glutaraldehyde in PBS. The aldehydes were flushed out with 50 mL PBS and quenched with 50 mL 0.5 % glycine in PBS. The mouse was finally flushed with 25 mL PBS and perfused with 14 mL contrast agent solution filtered through a 1.2 µm pore syringe filter (2.7 g of pre-crosslinked above contrast agent in 14 mL H_2O, 100 mg iodine/ml prior to filtering). To close the outlet, 4 % glutaraldehyde in PBS was dripped onto the heart to initiate crosslinking. Gelation of superficial *XlinCA* on the heart and in the thoracic cavity started within 30 s, and did not visibly proceed further after 5 min. The entire mouse was subsequently immersed in 500 mL 4 % glutaraldehyde in PBS.

Transcardial whole body perfusion with vascular casting resin PU4ii

The left ventricle was cannulated as above, and the blood was flushed out with approx. 10 mL of PBS and the mouse fixed with 100 mL 4 % formaldehyde in PBS. Vascular casting was performed using a mixture of 3.7 g 1,3-diiodobenzene (Sigma-Aldrich, USA) with the vascular casting resin PU4ii (vasQtec, Switzerland), which consists of 10 g 2-butanone, 10 g PU4ii resin and 1.6 g PU4ii hardener. The final contrast agent concentration of the PU4ii mixture was 110 mg iodine/ml.

X-ray µCT scans

For the low-resolution comparison scans, the heads of the PU4ii- and *XlinCA*-perfused mice were scanned with 80 µm voxel size using a QuantumFX *in vivo* µCT scanner (PerkinElmer, USA) with an acceleration voltage of 70 kV and a tube current of 200 µA. High-resolution scans were performed on a Nanotom m µCT scanner (General Electric, USA) using an X-ray tube with a water-cooled tungsten target set to an acceleration voltage of 60 kV and a tube current of 310 µA. 1440 projections per height step were acquired with 0.5 s exposure time using a scintillator-coupled flat-panel detector. The mouse was removed from the fixation solution, mounted in a plastic cup using polyurethane foam and scanned with 20 µm voxel size. The skull was scanned in the same configuration with 14.5 µm voxel size. The brain was excised, cut in the middle with a razor blade. The right brain hemisphere and the kidney were embedded in 1% agar in 1.5 mL centrifugation tubes and scanned with 4.4 µm voxel size.

The whole mouse and skull were visualized using Arivis4D 2.12.4 (Arivis, Germany). The brain hemisphere, kidney and adrenal gland were visualized using VGStudio Max 2.1 (Volume Graphics, Germany).

Fractional vessel volume (*FVV*) was estimated by dividing the overall mean intensity values of regions of interests (I_{ROI}) by the intensity values of contrast-agent filled, fully resolved vessels (I_{Ves}), after first subtracting intensity values of non-perfused tissue as background (I_{Bkg}).[2] Regions of interest were measured in the brain, liver and kidneys.

$$FVV = \frac{I_{ROI} - I_{Bkg}}{I_{Ves} - I_{Bkg}}$$

References

1. Z. B. Jian, M. C. Baier and S. Mecking, *J. Am. Chem. Soc.*, 2015, **137**, 2836-2839.

2. A. Garcia-Sanz, A. Rodriguez-Barbero, M. D. Bentley, E. L. Ritman and J. C. Romero, *Hypertension*, 1998, **31**, 440-4.

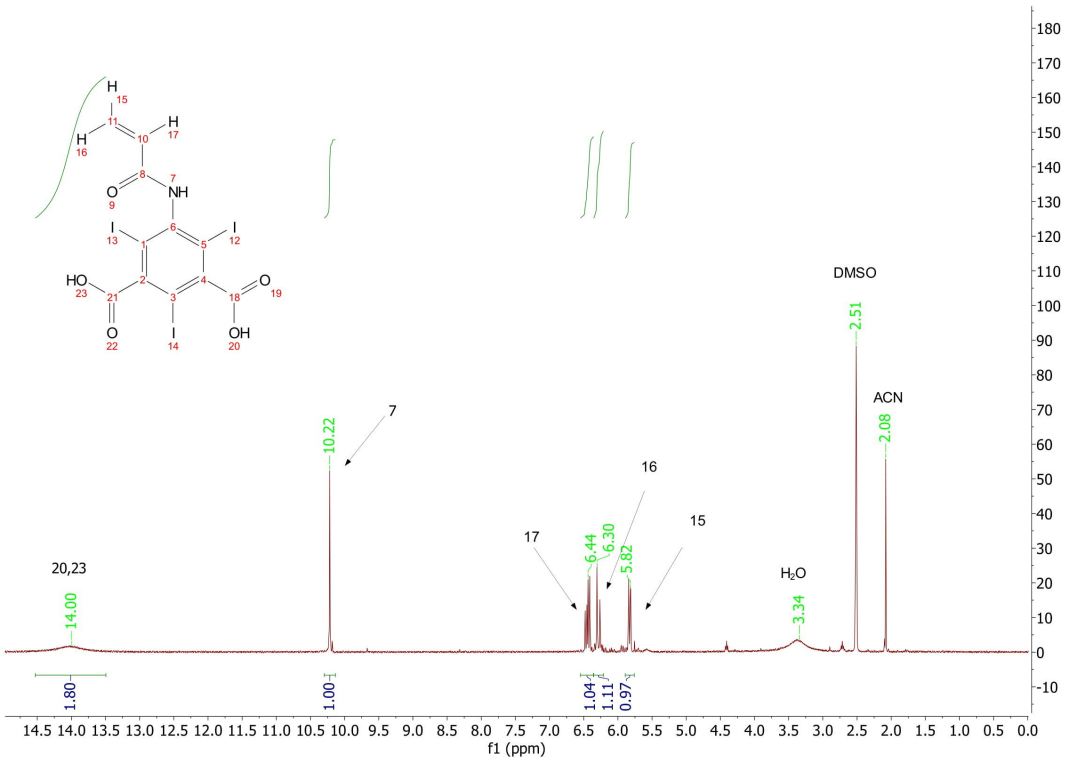

Figure S2. ^{1}H NMR of **2** in DMSO-d_6

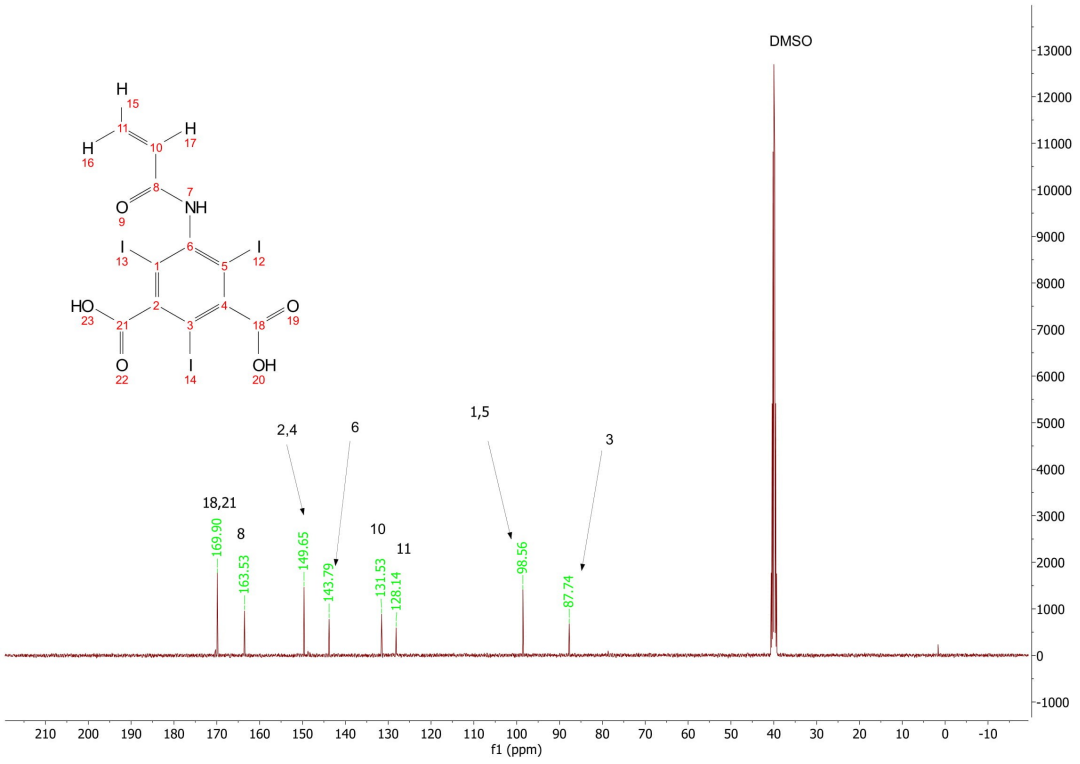

Figure S3. ^{13}C NMR of **2** in DMSO-d_6

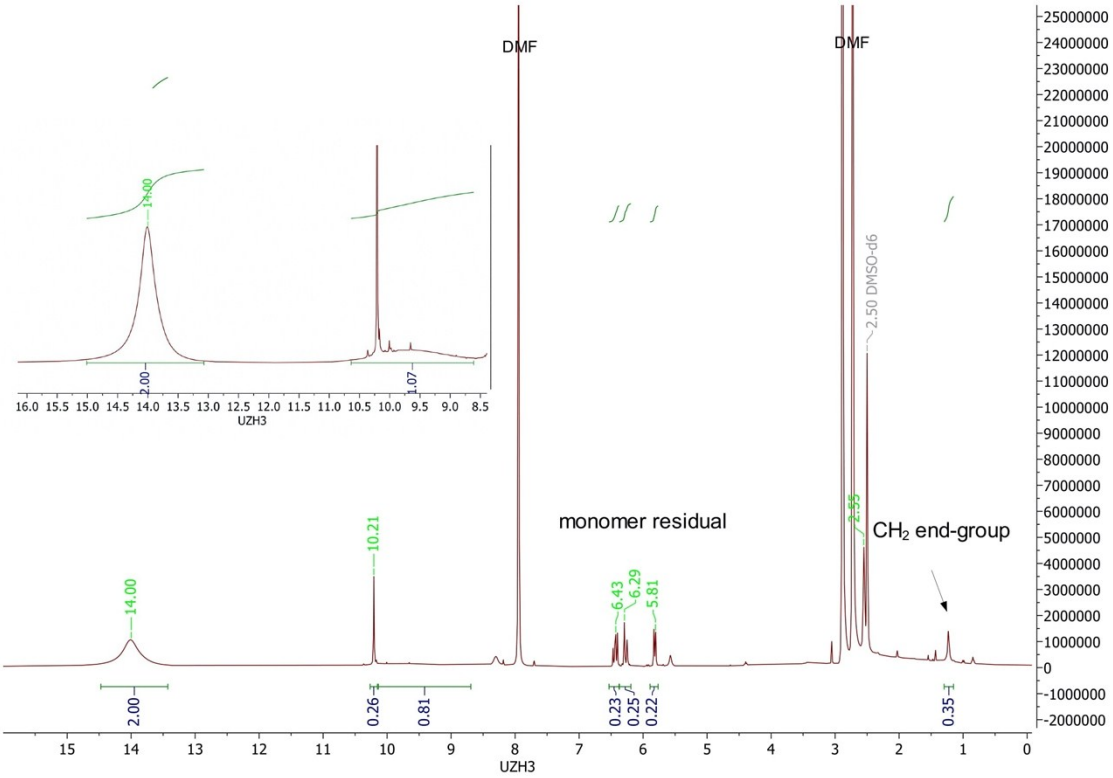

Figure S4. ^{1}H NMR of polymer **3** in DMSO-d_6

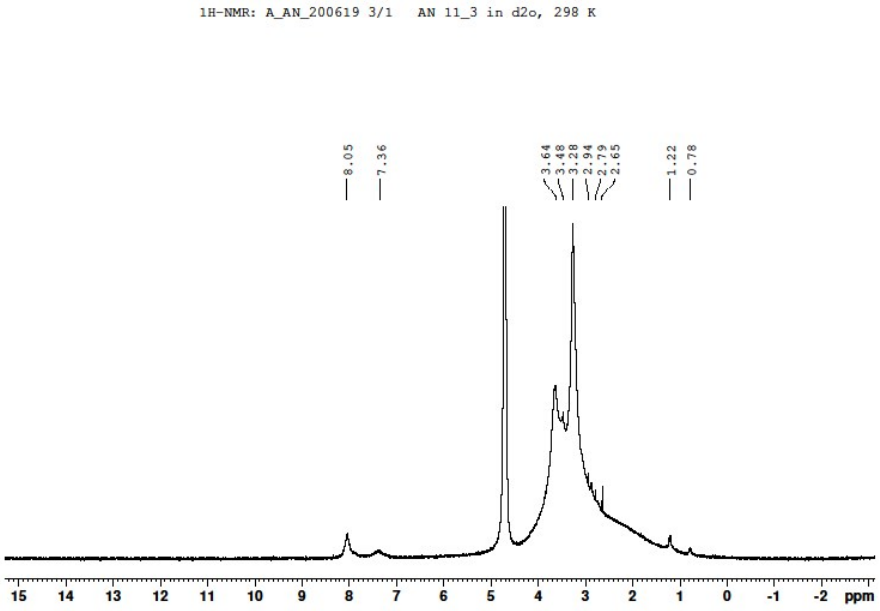

Figure S5. ^{1}H NMR of *XlinCA* (**6**) in D_2O.

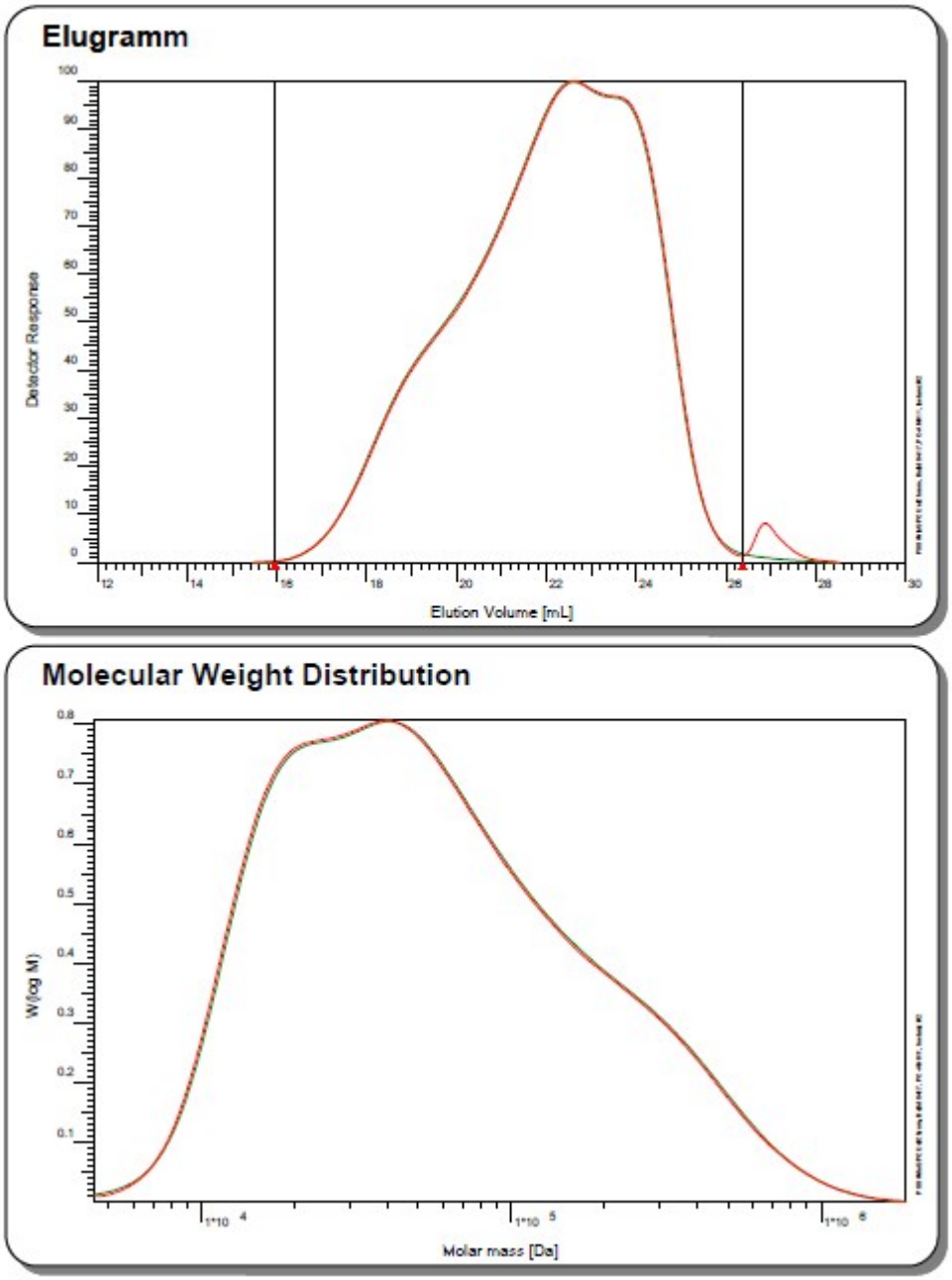

Figure S6. Elugram and molecular weight distribution diagram of *XlinCA* (**6**)

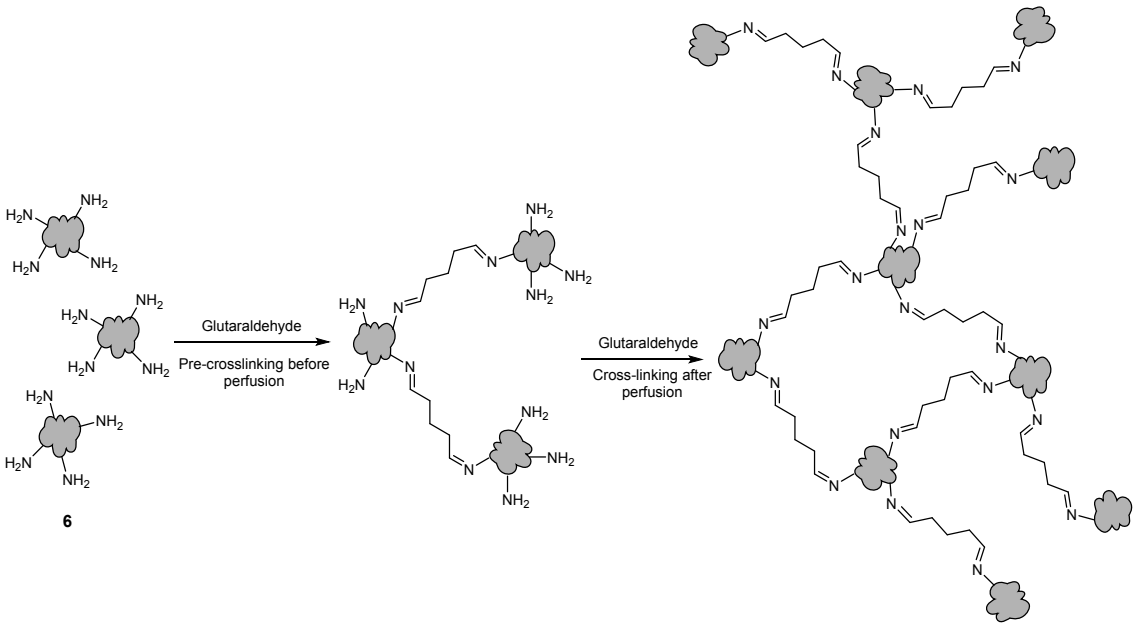

Figure S7. Crosslinking of the contrast agent *XlinCA* (**6**) by imine formation through reaction of amine groups with glutaraldehyde.

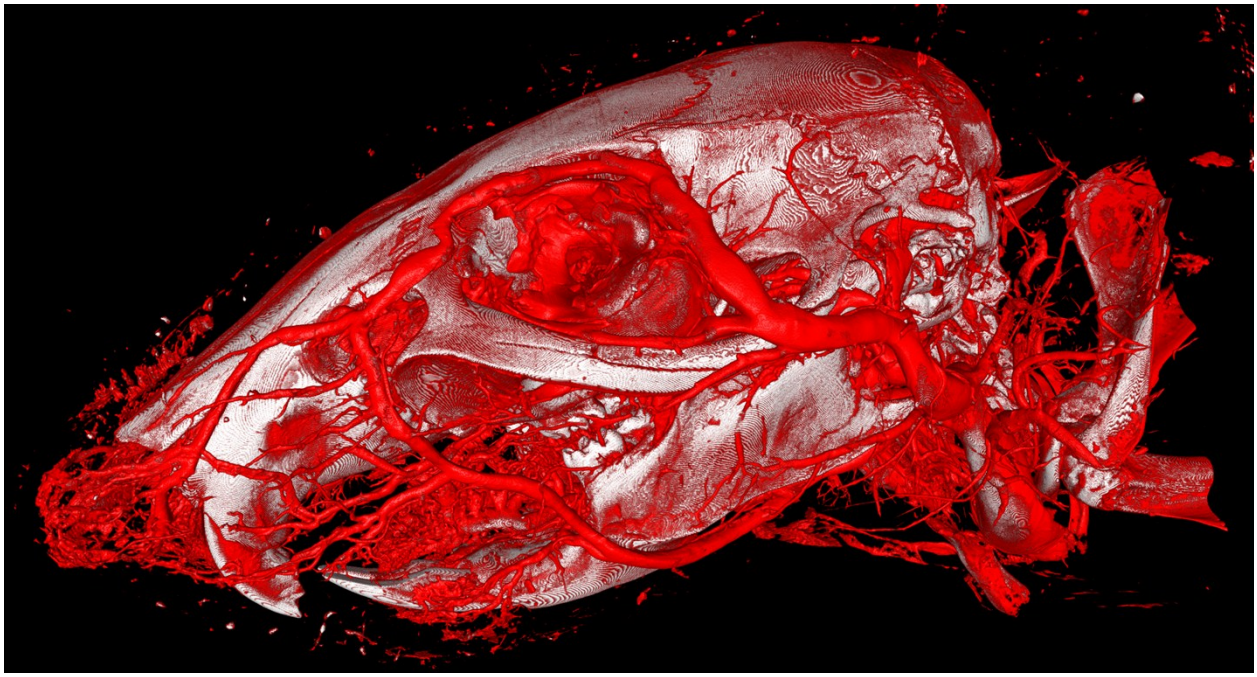

Figure S8. 3D visualization of the skull vasculature perfused with *XlinCA* and imaged with 14.5 µm voxel size using X-ray µCT.

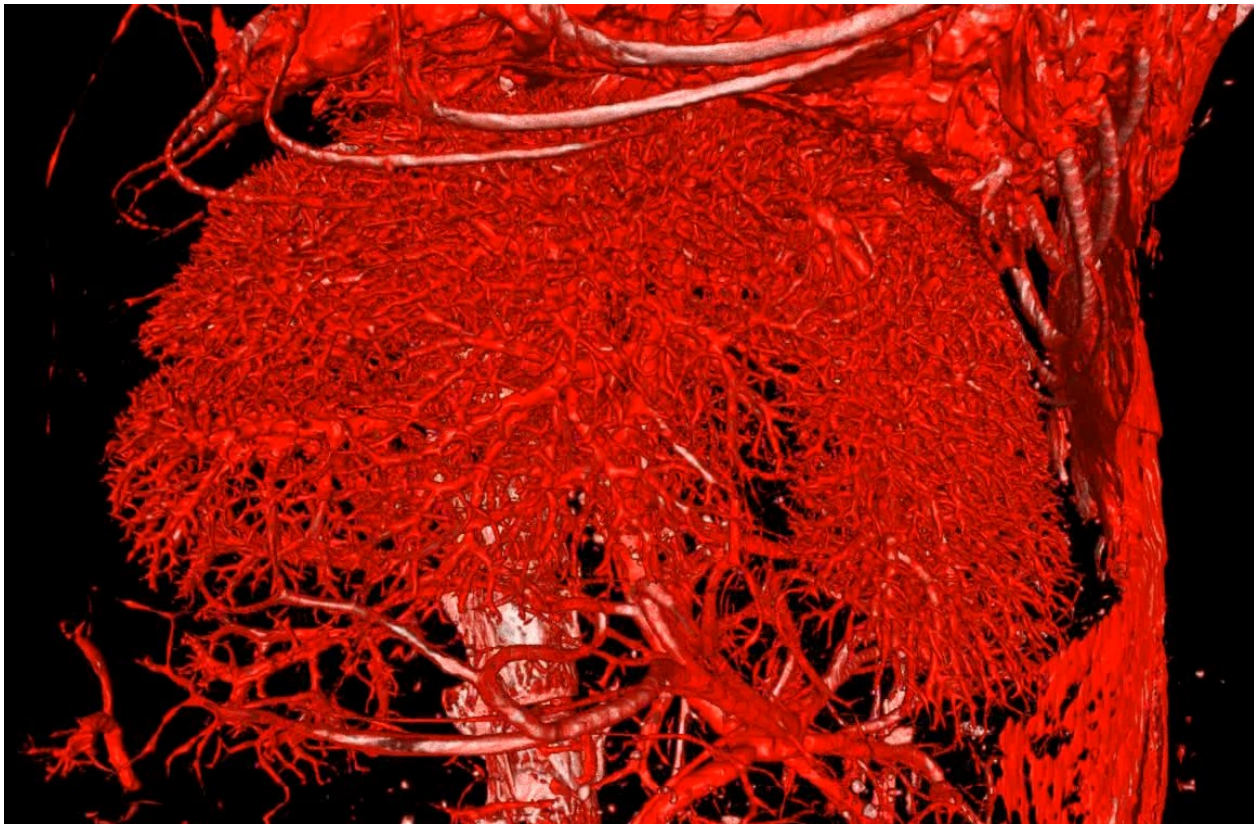

Figure S9. 3D visualization of the liver vasculature perfused with *XlinCA*, derived from the whole mouse μCT dataset acquired with 20 μm voxel size.

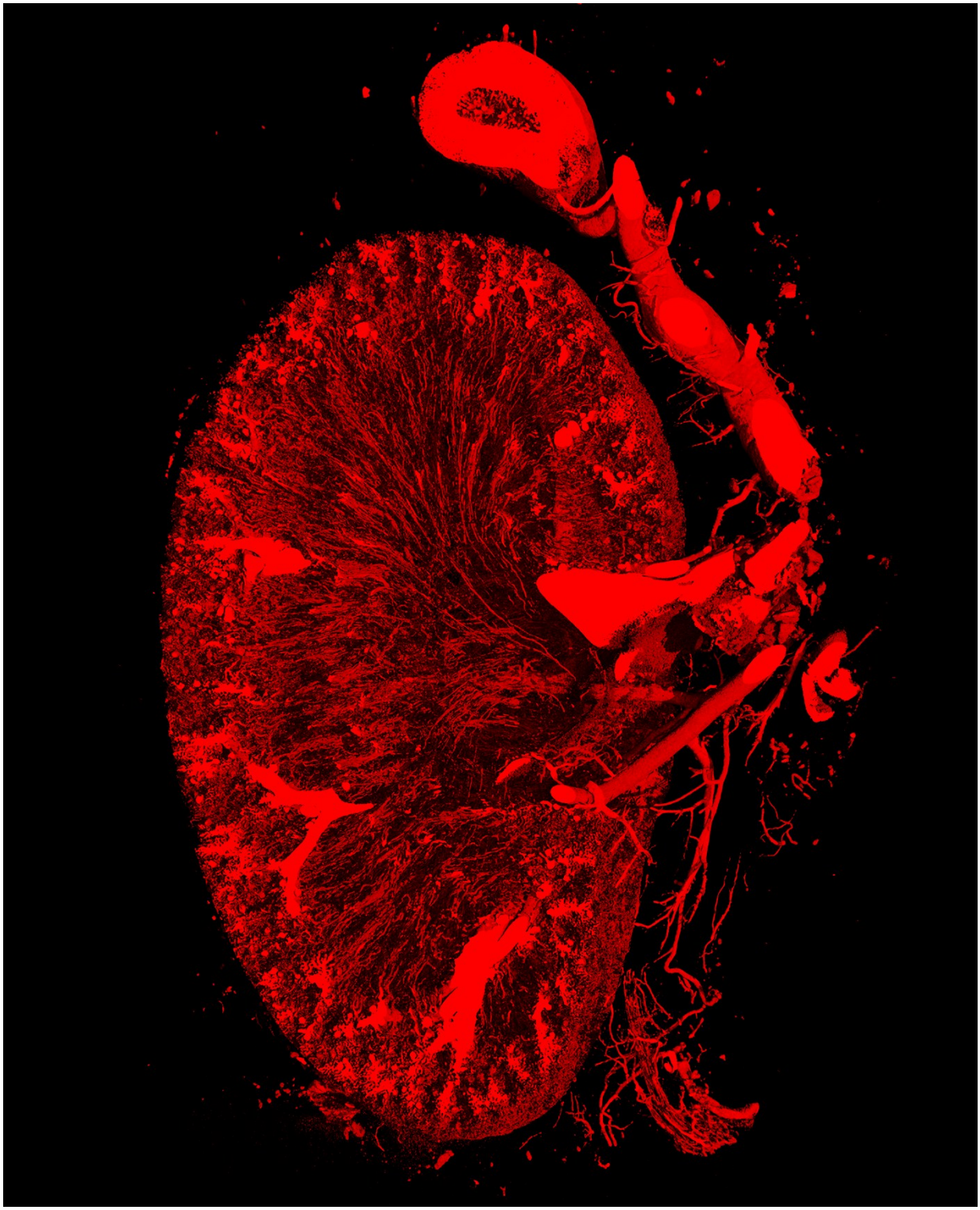

Figure S10. 3D visualization of microvasculature in the kidney and adrenal gland imaged with 4.4 µm voxel size. The organs are well perfused with *XlinCA*, with the exception of parts of the capillaries missing in the renal medulla. This issue is caused by incomplete flushing of blood prior to contrast agent injection, and can be resolved by employing kidney-specific, optimized perfusion protocols.

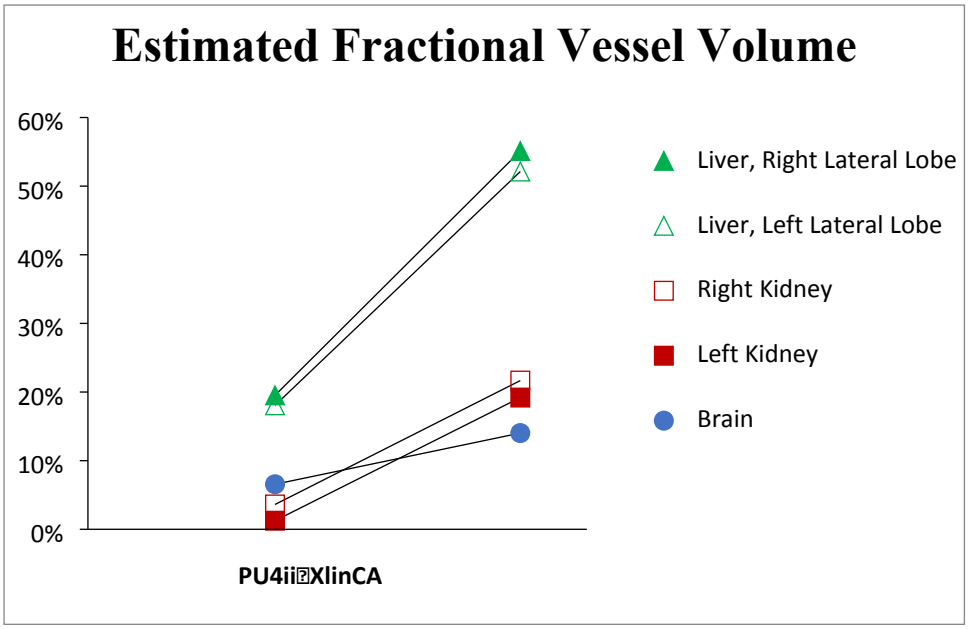

Figure S11. Estimated fractional vessel volume of independently vascularized regions of interest in the brain, liver and kidney. *XlinCA* (**6**) demonstrates higher fractional vessel volume (32.4 % ± 19.6 %, mean ± SD) than PU4ii (9.8 % ± 8.4 %, mean ± SD), suggesting a larger proportion of filled vasculature ($p = 0.01336$, two-tailed paired Student's *t*-test, $n = 5$).

Table S2. Optimization of the synthesis of polymer **3**. The reaction temperature was 70 °C. M: monomer, R: RAFT agent, I: AIBN. MW of the polymers **3** were calculated from the measured GPC data of the corresponding polymers **6**.

conditions	**[M]: [R]: [I] [mmol]**	**Reaction time**	**Precipitation**	**Conversion degree by ^{1}H NMR**	**MW (Da) by GPC**
1	2 : 0.04 : 0.02 (= 100 : 2 : 1)	18 h	no	95%	17500
2	2 : 0.02 : 0.01 (= 200 : 2 : 1)	28 h	no	92%	24600
3	2 : 0.01 : 0.005 (= 400 : 2 : 1)	3 days	no	78%	30400
4	2 : 0.01 : 0.01 (= 400 : 2 : 2)	3 days	no	77%	29200
5	2 : 0.008 : 0.004 (500 : 2 : 1)	4 days	+	no NMR	-
6	2 : 0.0067 : 0.0033 (= 600 : 2 : 1)	5 days	++	no NMR	-
7	2 : 0.0057 : 0.029 (= 700 : 2 : 1)	7 days	+++	no NMR	-
8	2 : 0.005 : 0.0025 (= 800 : 2 : 1)	7 days	+++	no NMR	-