Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.


**Supporting information for**

# Development of cationic peptide-based hydrogels loaded with iopamidol for CEST-MRI detection

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# Supplementary Experimental data

## Chemicals

Protected Nα-Fmoc-amino acid derivatives, coupling reagents and Rink amide MBHA (4-methylbenzhydrylamine) resin was purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). All other chemicals were purchased from Merck (Milan, Italy), Fluka (Bucks, Switzerland), or LabScan (Stillorgan, Dublin, Ireland) and, unless stated otherwise, they were used as delivered. Peptides solutions were prepared by weight and dissolving the peptides in double distilled water. Sodium chloride, potassium chloride, sodium phosphate dibasic, potassium phosphate monobasic and all other chemicals were purchased from Sigma Aldrich (LLC -Merck KGaA, Darmstadt, Germany) and used without further purification. PBS (Phosphate Buffered Saline) was prepared containing 0.137 M sodium chloride and 100 mmol $L^{-1}$ or 10 mmol $L^{-1}$ phosphate (pH 7.3 ± 0.1, osmolarity 280 ± 10 mosm/L).

## Peptide Solid Phase Synthesis and characterization

Ac-K1 and Ac-K2 peptides were synthesized according to standard SPPS (solid-phase peptide synthesis) procedures using the Fmoc/tBu strategy.[1] The Rink amide MBHA resin (substitution 0.73 mmol/g) was selected as the solid-phase support to provide amidate peptides at the C-terminus. Both peptides were synthetized using a scale of 0.20 mmol in DMF.

The resin was allowed to swell for 30 min in the reactor, then the Fmoc group was deprotected by treating the resin with 30% (*v/v*) piperidine in DMF (two cycles of 10 minutes). The coupling of each amino acid was performed by adding a 2-fold molar excess of the protected Fmoc-amino acid, with equimolar amounts of 1-hydroxybenzotriazole (HOBt), benzotriazol-1-yl-oxytris-pyrrolidino-phosphonium (PyBOP), and a 4-fold molar excess of diisopropylethylamine (DIPEA) in DMF/NMP. All couplings were performed twice for 40 min. After the coupling of the last amino acid, the Fmoc group was removed, and the N-terminus acetylated with a solution of pyridine/acetic anhydride (4/4.7 *v/v*) in DMF (two treatments for 10 minutes). Crude peptides were fully cleaved by treating the resin for 3 h at room temperature with a TFA (trifluoroacetic acid) /TIS (triisopropylsilane)/H2O (92.5/5/2.5 *v/v/v*) mixture. All the peptides were then precipitated with cold ether and freeze-dried for three times.

Purification was achieved using preparative RP-HPLC with a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481detector, using a Phenomenex (Torrance, CA, USA) C18 column. $H_2O$/0.1% TFA (A) and $CH_3CN$/0.1% TFA (B) were used as elution solvents with the concentration of (B) increasing from 30 to 80% over 30 min at a flow rate of 20 mL/min. The purity of Ac-K1 and Ac-K2 products was confirmed by analytical RP-HPLC analysis performed by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA, USA), with a C18-Phenomenex column eluting with (A) and (B) at a flow rate of 1 mL / min. The chosen analytic method provided an increase in (B) percentage from 5 to 70 % occurring in 10 minutes. Identity of peptides was assessed by MS spectrometry conducted using a LTQ XL Linear Ion Trap Mass Spectrometer, ESI source.

Ac-K1: tR = 11.62 min, MS (ESI+): m/z 640.82 calcd. for C30H56N8O7: [M+H+] = 641.4 u.m.a.

Ac-K2: tR = 11.51 min, MS (ESI+): m/z 640.82 calcd. for C30H56N8O7: [M+H+] = 641.5 u.m.a.

### CELLS CULTURE

Cell culture media and supplements (RPMI, DMEM, FBS, Glutamine, pen/strep, MycoAlert™ Mycoplasma Detection Kit) were purchased from Lonza Sales AG-EuroClone SpA (Milano, Italy). Experiments were carried

out using three different cell lines: i) GL261 (murine glioma), ii) TS/A (murine breast carcinoma), and iii) 3T3-NIH (murine fibroblasts). GL261 and 3T3-NIH cell lines were purchased from ATCC (American Type Culture Collection). They were grown in DMEM medium (Dulbecco'modified Eagle medium) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol $L^{-1}$ glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were seeded in 75-$cm^2$ flasks at density of ca. $2\times10^4$ cells/$cm^2$ in a humidified 5% $CO_2$ incubator at 37 °C. At confluence, they were detached by adding 1 mL of Trypsin-EDTA solution (0.25 % (*w/v*) Trypsin- 0.53 mmol $L^{-1}$ EDTA). TS/A murine breast cancer cells were derived from a spontaneous mammary adenocarcinoma which arose in a retired breeder BALB/c female, as previously reported.[2] They were grown in RPMI (Roswell Park Memorial Institute) 1064 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol $L^{-1}$ glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were seeded in 75-$cm^2$ flasks at density of ca. 2×104 cells/$cm^2$ in a humidified 5% $CO_2$ incubator at 37 °C. At confluence, they were detached by adding 1 mL of Trypsin-EDTA solution (0.25 % (*w/v*) Trypsin- 0.53 mmol L-1 EDTA). All used cells were negative for mycoplasma as tested by using MycoAlert™ Mycoplasma Detection Kit. All cell media and supplements were purchased from Lonza Sales AG-EuroClone SpA, Milano (IT).

### Cells viability: MTT assay

To study the biocompatibility of empty hydrogels on GL261, TS/A, and 3T3-NIH cells, an elution assay (according to ISO 10993-5 guidelines) was performed with cells cultured in the proper medium previously conditioned by hydrogels. The elution test was performed by preparing 200 µL of each hydrogel under sterile conditions; 2 washes of 2 hours each were performed twice by adding 1 mL of complete medium (ratio gel/extracting media 1/5 *v/v*) and the gels were then incubated overnight with 1 mL of fresh complete medium. The medium was harvested the next day for the elution test. No color change was detected after incubation of the medium, and pink coloration of the gel after the extraction of the medium indicates permeability. To assess the toxicity of the system, MTT assay was used. For this purpose, cells were seeded into 96-well tissue culture plates ($10^4$ cells for plate) 24 h before the experiment. Then, they were incubated with complete medium that was previously conditioned by the presence of the hydrogel to be tested. After the incubation time (*overnight*), the medium was removed, cells washed and re-incubated in the presence of fresh medium supplemented with 0.5 mg/mL MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma Aldrich) for 4 h in a humidified 5% $CO_2$ incubator at 37 °C. Then, the MTT containing solution was removed and the plates were filled with DMSO (0.1 mL for plate) for ½ h at room temperature, under gentle agitation, for allowing solubilization of formazan crystals. The absorbance of the resulting-colored solutions was quantified using a 96-multiwell iMark Bio-Rad microplate Reader (λ = 570 nm). The percentage of viable cells was calculated based on control blank cells by using the following formula:

$$\text{Viable cells \%} = (\text{AbsT}/\text{Abscnt}) \times 100$$

Where AbsT is the mean absorbance of Treated cells and Abscnt is the mean absorbance of control untreated cells (after subtraction of absorption of empty plates as background). Cells experiments were repeated four times and data reported as mean value ± standard deviation.

## Supplementary references


1 P. R. Hansen and A. Oddo, *Methods Mol Biol.* 2015, **1348**, 33-50.

2 P. Nanni, C. De Giovanni, P. L. Lollini, G. Nicoletti and G. Prodi, *Clin. Expl. Metastasis*, 1983, **1**(4), 373-380.

## Supplementary Figures

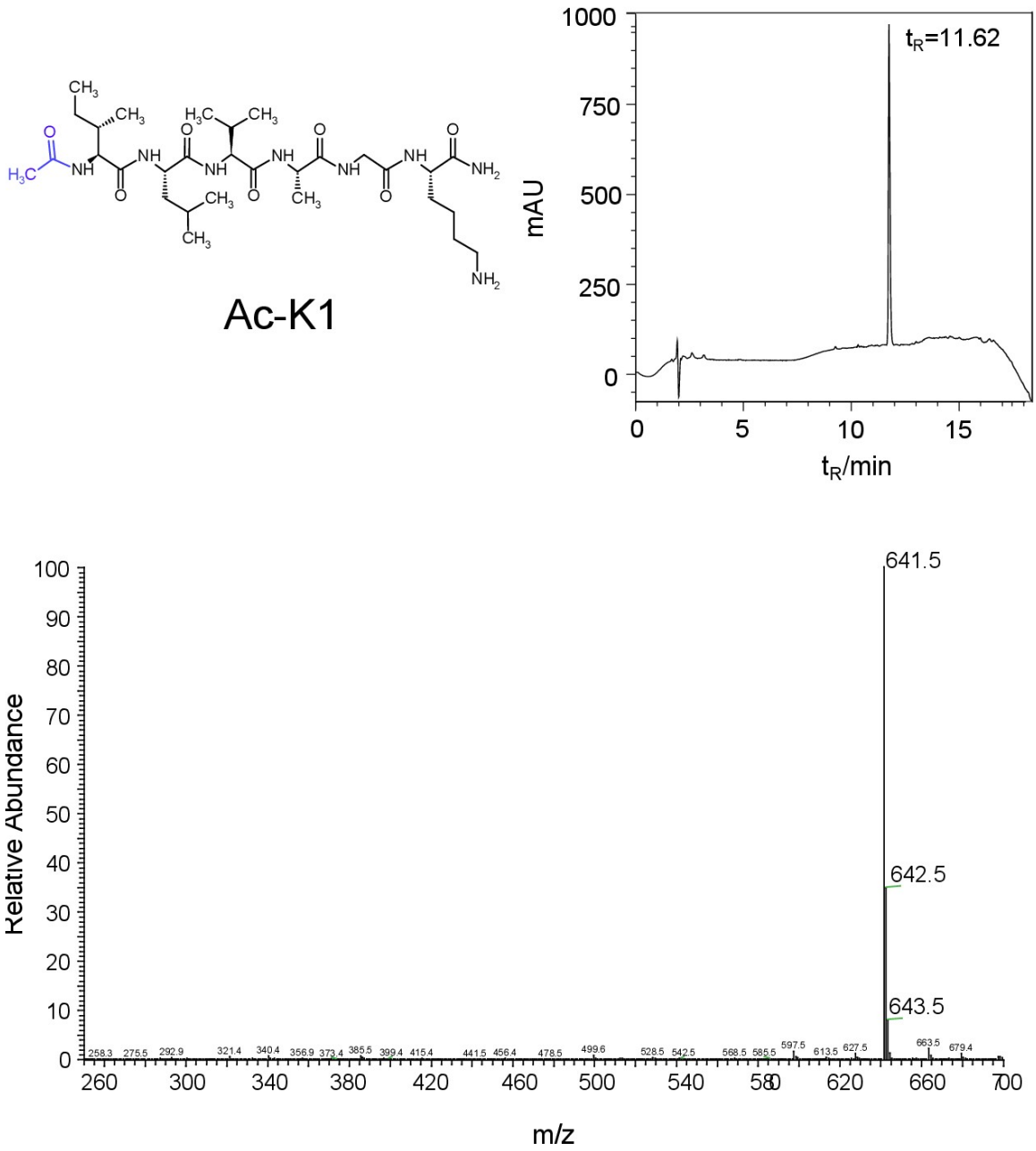
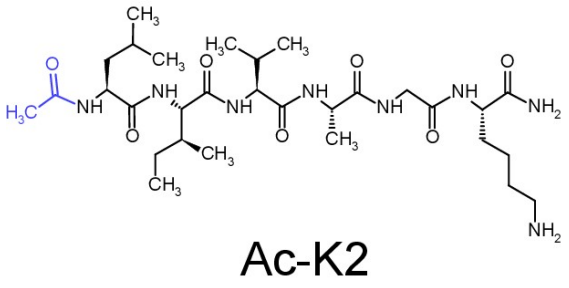
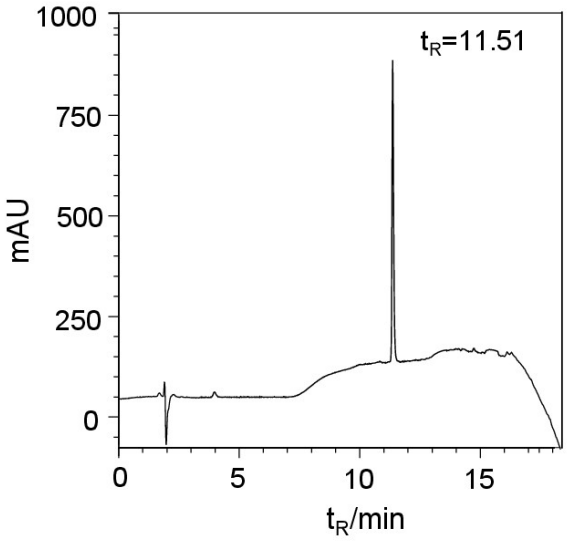



**Figure S1:** Chemical structure of Ac-K1 peptide with the corresponding RP-HPLC chromatogram and ESI mass spectrum



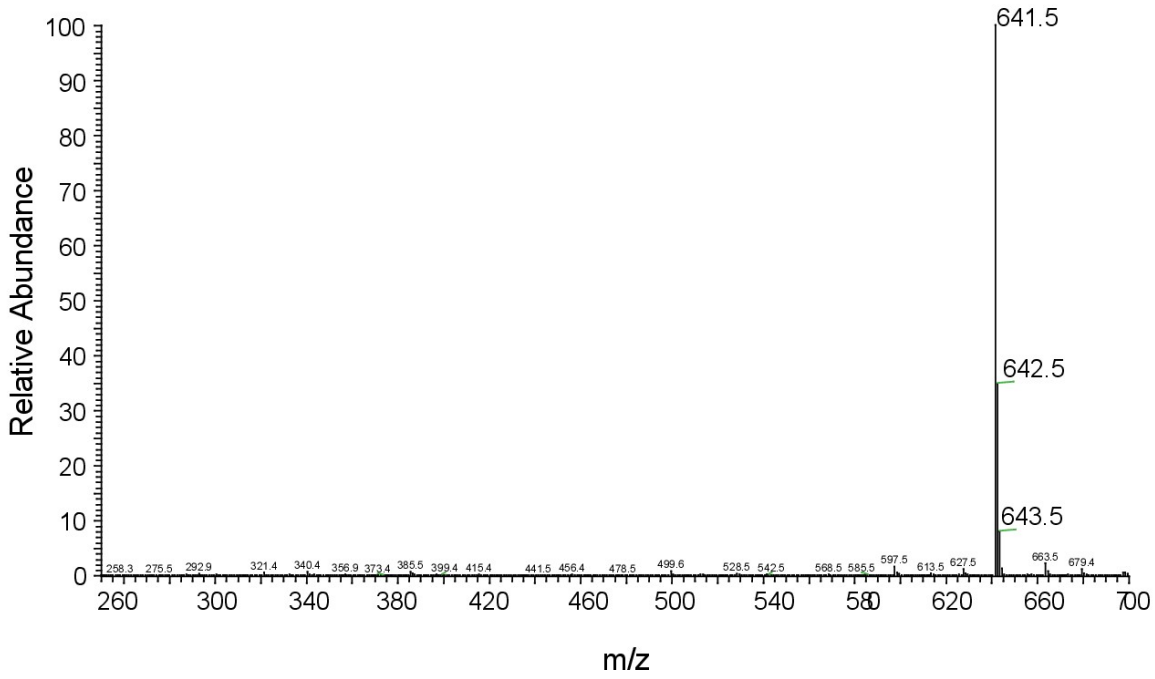




**Figure S2:** Chemical structure of Ac-K2 peptide with the corresponding RP-HPLC chromatogram and ESI mass spectrum

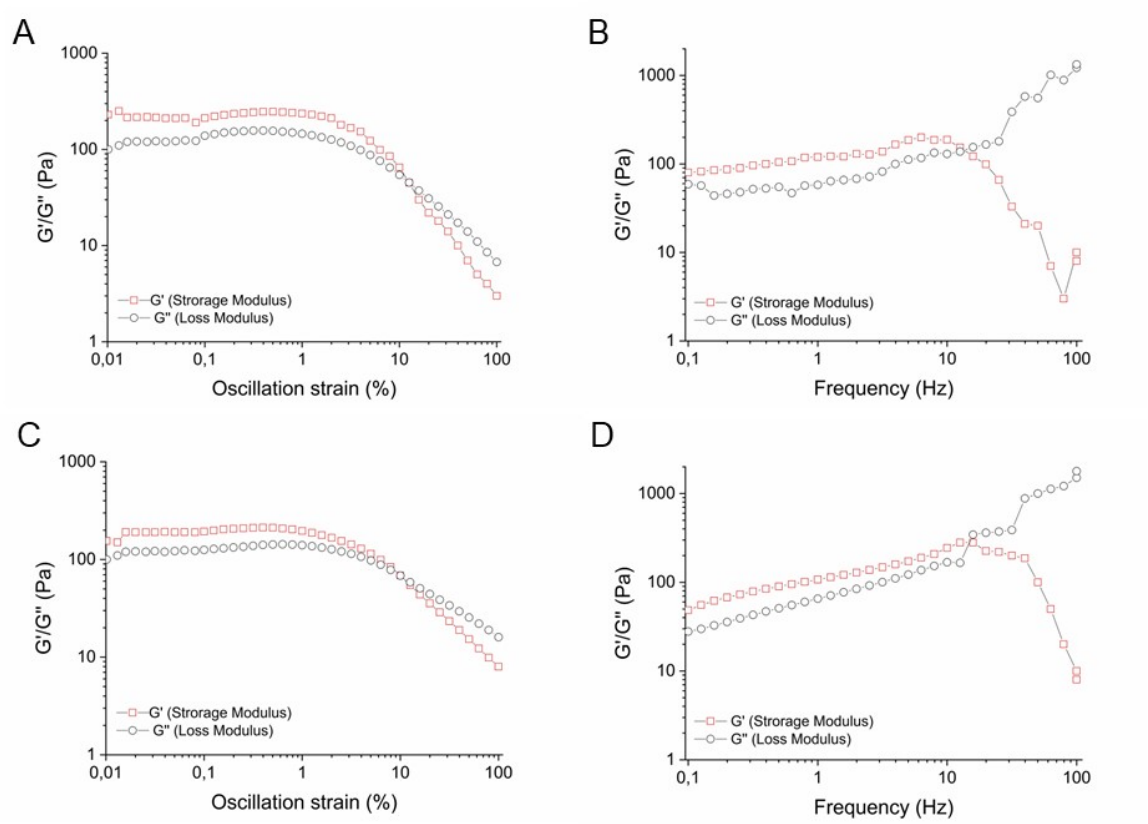



**Figure S3:** Rheological characterization of Ac-K1 (A, B) and Ac-K2 hydrogels (C, D). Dynamic strain sweep oscillatory test performed at 1 Hz frequency (A,C) and dynamic frequency sweep oscillatory test at 0.1 % strain (B,D).

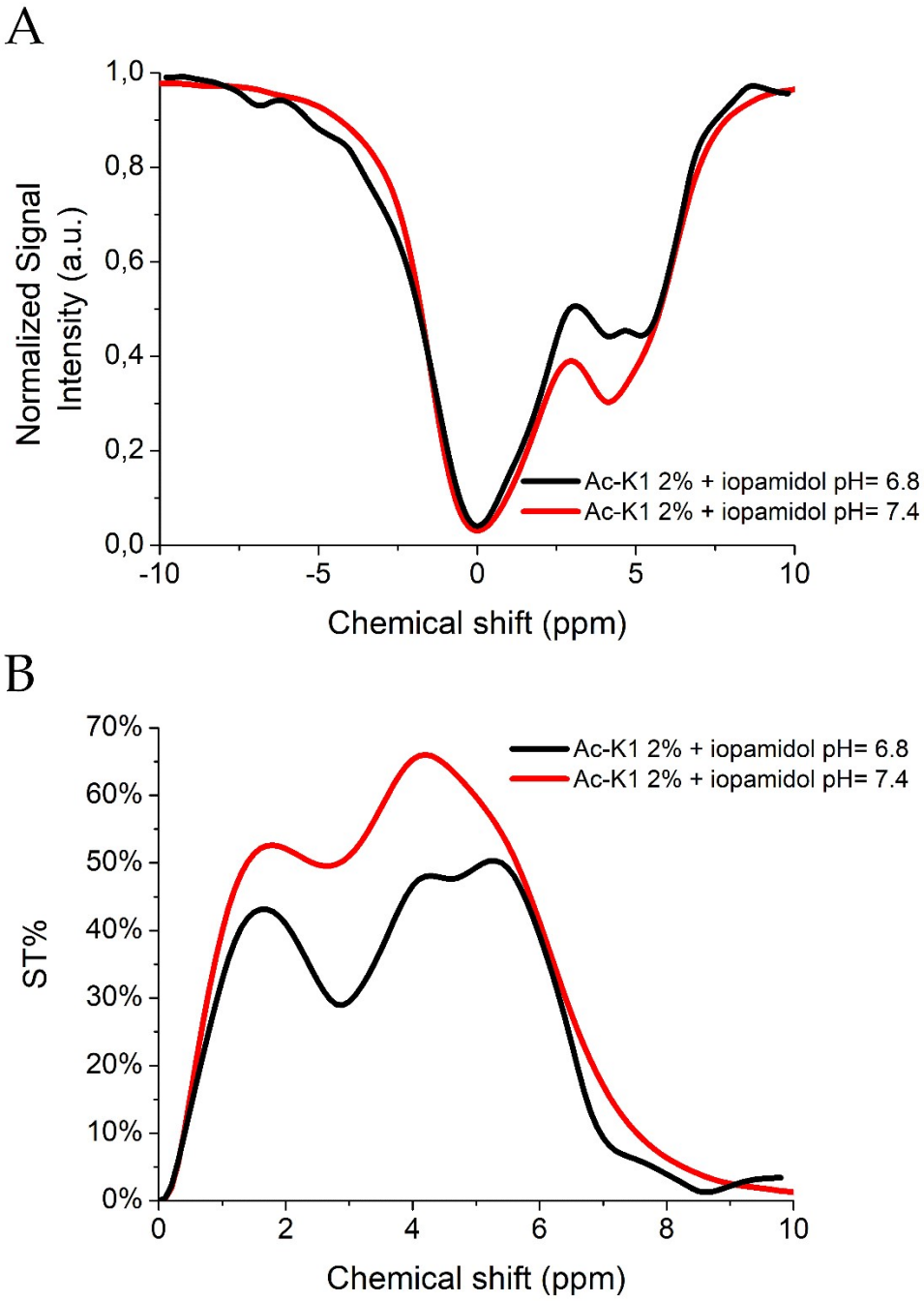



**Figure S4:** (A) Z- and (B) ST%- spectra of Ac-K1 2% w/v loaded with iopamidol at pH 6.8 and pH 7.4 ([iopamidol] = 223 mM)

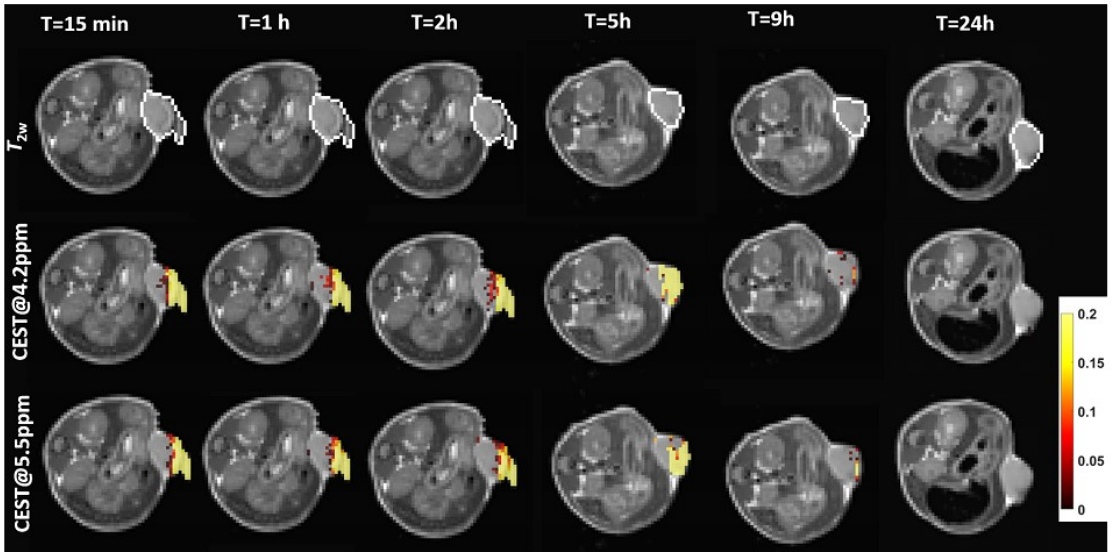



**Figure S5:** $T_{2w}$ and $CEST_{@4.2ppm}$ and $CEST_{@5.5\ ppm}$ MR images of the second mouse upon intratumor administration of Ac-K1 hydrogel at variable time (from t=15 min to t=24h).

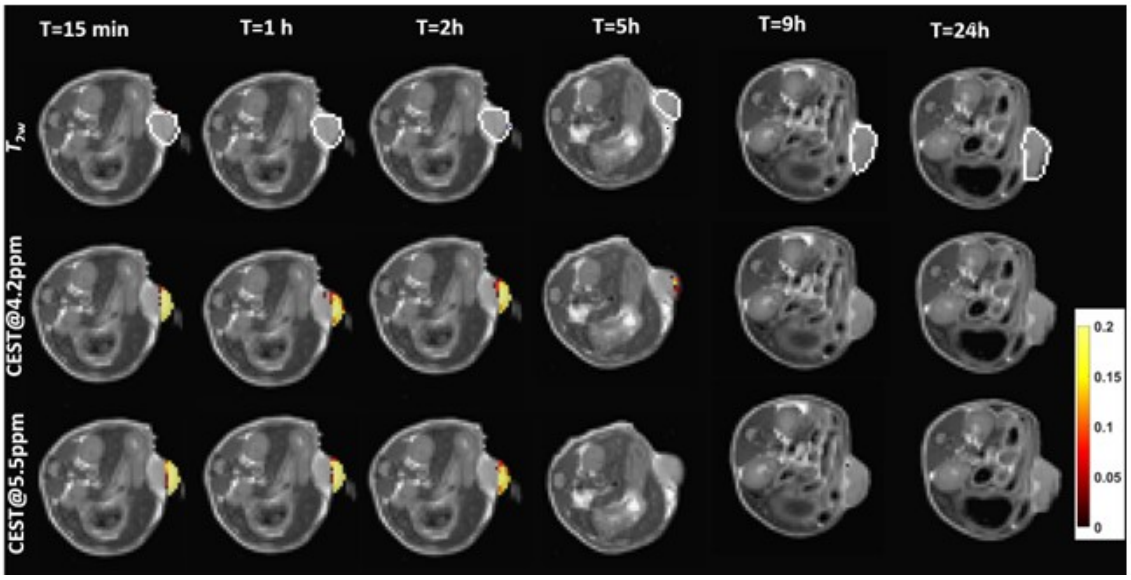



**Figure S6:** $T_{2w}$ and $CEST_{@4.2ppm}$ and $CEST_{@5.5\ ppm}$ MR images of the third mouse upon intratumor administration of Ac-K1 hydrogel at variable time (from t=15 min to t=24h).