Supporting Information

Assessing the *in Vivo* Targeting Efficiency of Multifunctional Nanoconstructs Bearing Antibody-Derived Ligands

Luisa Fiandra,[b] Serena Mazzucchelli,[b] Clara De Palma,[b] Miriam Colombo,[a,c] Raffaele Allevi,[a] Silvia Sommaruga,[b] Emilio Clementi,[a] Michela Bellini,[c] Davide Prosperi,[c,d,] Fabio Corsi[a,b,]**

[a]Dipartimento di Scienze Biomediche e Cliniche "Luigi Sacco", Università di Milano, via G.B. Grassi 74, 20157 Milano, Italy; [b]Ospedale L. Sacco, via G. B. Grassi 74, 20157 Milano, Italy; [c]NanoBioLab, Dipartimento di Biotecnologie e Bioscienze, Università di Milano-Bicocca, piazza della Scienza 2, 20126 Milano, Italy; [d]Laboratorio di Biofisica e Nanomedicina, Polo Tecnologico, Fondazione Don Gnocchi IRCCS-ONLUS, 20148 Milano, Italy.

Chemicals and instrumentation for MNP sysnthesis

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Water was deionized and ultrafiltered by a MilliQ apparatus from Millipore Corporation (Billerica, MA) before use. Dynamic light scattering (DLS) measurements were performed at 90° with a 90Plus Particle Size Analyzer from Brookhaven Instruments Corporation (Holtsville, NY), working at 15 mW of a solid state laser (λ = 661 nm). Viscosity and refractive index of pure water were used to characterize the solvent. Nanoparticles were dispersed in the solvent and sonicated in a S15H Elmasonic apparatus (Elma, Singen, Germany) before analysis. Final sample concentration used for measurements was typically of 5 mg mL^{-1}.

Synthesis of surfactant-coated Fe_3O_4 nanoparticles (MNC)

For the synthesis of MNC, the iron-oleate complex was first prepared by reacting metal chlorides and sodium oleate in a mixture solvent composed of ethanol, distilled water and hexane. The resulting solution was heated to 70 °C and kept at that temperature for 4 h. When the reaction was complete, the upper organic layer containing the iron–oleate complex was washed three times with distilled water in a separatory funnel. After washing, hexane was evaporated off, resulting in iron–oleate complex in a waxy solid form. For the nanocrystals synthesis, 40 mmol of the iron-oleate and 5.7 g of oleic acid (20 mmol) were dissolved in 200 g of 1-octadecene at room temperature. The reaction mixture was heated to 320 °C and kept at that temperature for 30 min. The resulting solution containing the nanocrystals was then cooled to room temperature, ethanol (100 mL) was added and the black precipitate was centrifuged. The supernatant was discarded and the product washed several times with ethanol to remove the unbound surfactant. The resulting oleylamine-coated magnetite nanocrystals were dispersed in chloroform (MNC).

Synthesis of PMA-coated Fe_3O_4 nanoparticles functionalized with SPDP (MNP-PDP)

An aliquot of 0.5 M PMA [24] (63 μL) was added to MNC (4.6 mg in $CHCl_3$), the mixture was homogenized and the solvent was then evaporated at reduced pressure. Sodium borate buffer (SBB, pH 12, 10 mL) was added obtaining a clean nanoparticle dispersion, which was concentrated in Amicon tubes (100 kDa filter cutoff) by centrifuging at 3500 rpm for 1 h. Finally, the nanoparticles were washed twice diluting with SBB in the same way and concentrated (each centrifuge cycle was 20 min at 3500 rpm) to a final volume of 200 μL. The nanoparticle solution, was reacted with 0.1 M EDC (18 μL) for 2 min, then 0.05 M 2,2-(ethylenedioxy)bis(ethylamine) (EDBE, 9 μL in deionized water) was added and stirred for 2 h. Next, the nanoparticle dispersion was concentrated and washed two additional times with water as described above. Nanoparticles were shaken for 4 h in the presence of *N*-succinimidyl-3-[2-pyridyldithio]-propionate (SPDP, 690 μL, 10 mg mL^{-1} in DMSO), concentrated and washed twice with water. In this way, we determined that about 70% of available amino groups were converted into thiol-reactive groups. For the *in vitro* studies with the cells, we used fluorescein-labeled nanoparticles obtained using a 0.5. M FITC-labeled PMA. To PMA in $CHCl_3$ (5 mL) was added 1.0 M fluoresceinamine (0.5 mL in DMSO) and the mixture was left overnight at room temperature. For the control set of *in vivo* experiments, we used AF660-labeled MNP as described in the below section.

Synthesis of fluorescent pegylated MNP for *in vivo* experiments (MNPP)

AF660-labeled MNP-PDP (AF660-MNP-PDP) were obtained by shacking MNP-PDP (10 mg) in deionized water (1 mL) with Alexafluor-660 succinimidyl ester (1 mg in 500 μL water) in the dark. AF660-MNP-PDP solution (1 mg), was reacted with excess PEG500-SH for 2 h. The excess of PEG was removed by concentration at 3500 rpm through 100 kDa Amicon filters. The concentrated solution was washed twice and the nanoparticles were then recovered as a stock solution in phosphate buffer saline (PBS).

Cell cultures

MCF7 and MDA-MD-468 cells were cultured in 50% Dulbecco's Modified Eagle's Medium (DMEM) and 50% F12, supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (50 UI mL^{-1}) and streptomycin (50 mg mL^{-1}) at 37 °C and 5% CO_2 in a humidified atmosphere and subcultured prior to confluence using trypsin/EDTA. Cells culture medium and chemicals were purchased from EuroClone.

Production of xenograft tumor models

MCF7 cells, grown as described above, were injected in eleven-week old female Balb/c nude mice after insertion on the neck of estrogen pellets by using a trocar. All tumor injections were done 2 days after pellet placing. MCF7 (10^7 cell for each animals) were suspended in growth medium and mixed with Matrigel high factor (BD, Biosciences) in 3:1 ratio and injected into mammary fat pad of mice. Animals were observed and tumor formation was recorded at least thrice per week; tumors were allowed to grow up to 8 mm in diameter before nanocomplex injection.

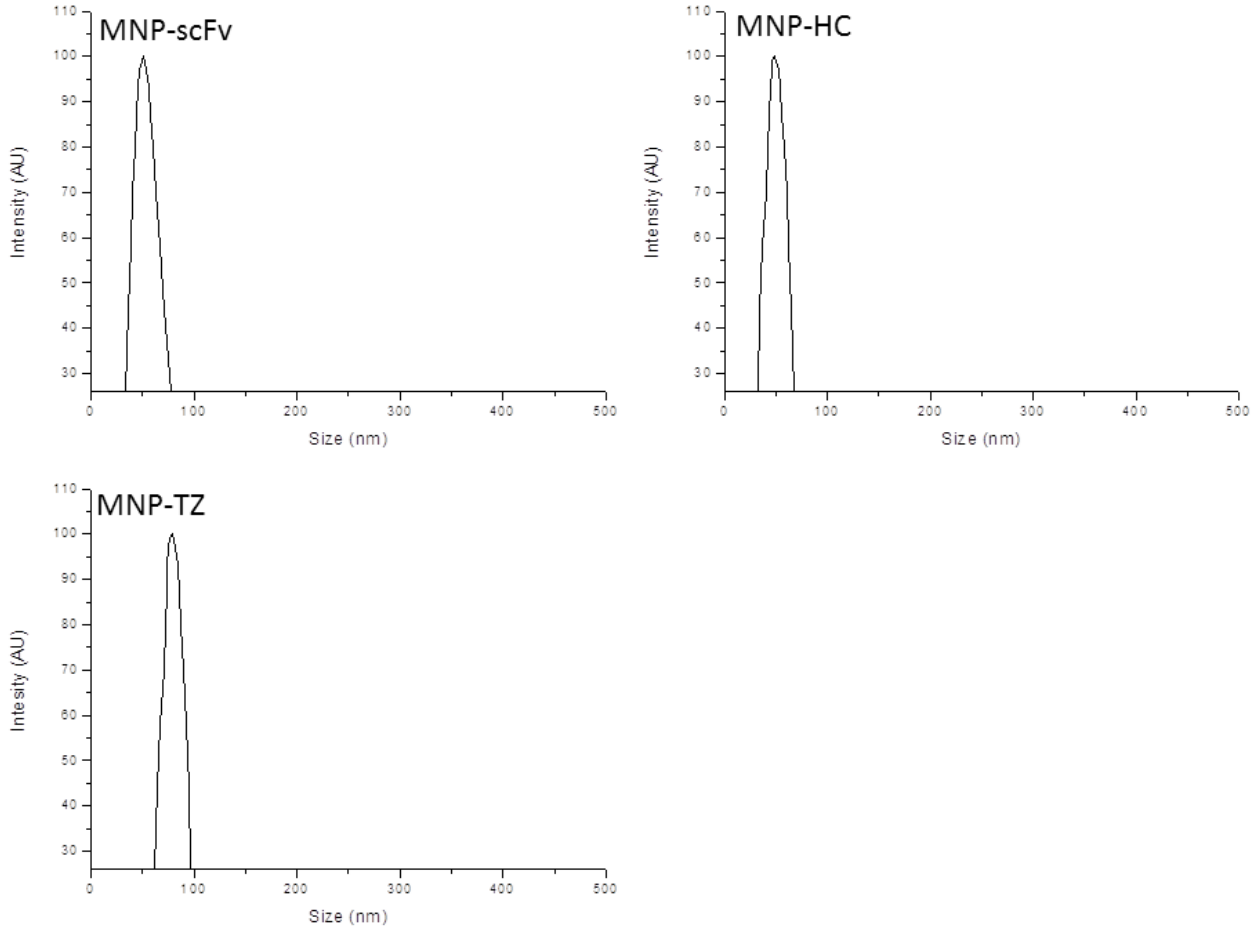

Figure S1. Size distribution of MNP-scFV, MNP-HC and MNP-TZ determined by dynamic light scattering in PBS, pH 7.4. 10 μg mL^{-1}.

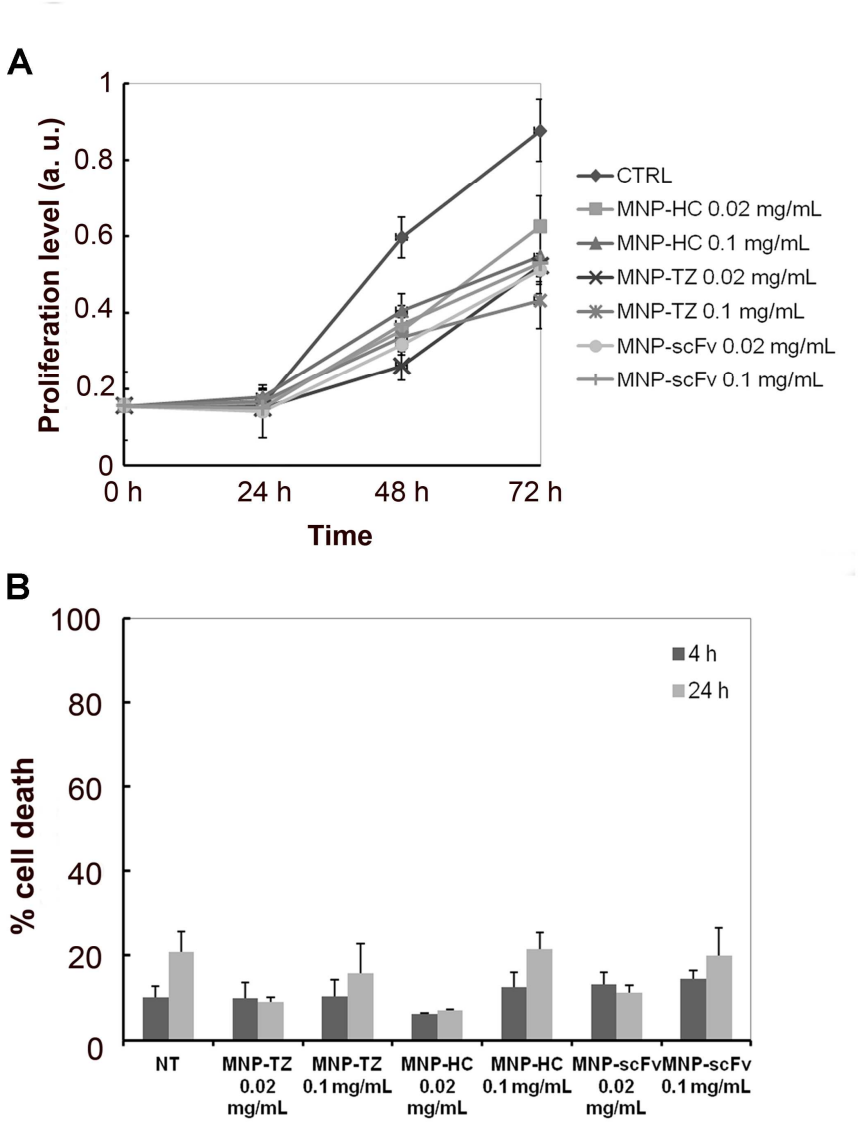

Figure S2. Toxicity *in vitro* assay. MCF-7 cells were exposed to MNP-HC, MNP-TZ and MNP-scFv (0.02 mg mL^{-1} and 0.1 mg mL^{-1}) up to 72 h. Cell proliferation was tested by measuring the conversion of MTT into formazan. CTRL represents untreated control. B) Cell death assay. MCF-7 cells were treated with MNP-HC, MNP-TZ and MNP-scFv (0.02 mg mL^{-1} and 0.1 mg mL^{-1}) for 4 h and 24 h (dark and light grey, respectively). Cell death was assessed by measuring the exposure of Annexin V and the incorporation of 7-aminoactinomycin D and evaluated by flow cytometry. An untreated sample has been reported as negative control. All results are expressed as Means ± standard error (S.E.) of 5 individual experiments.

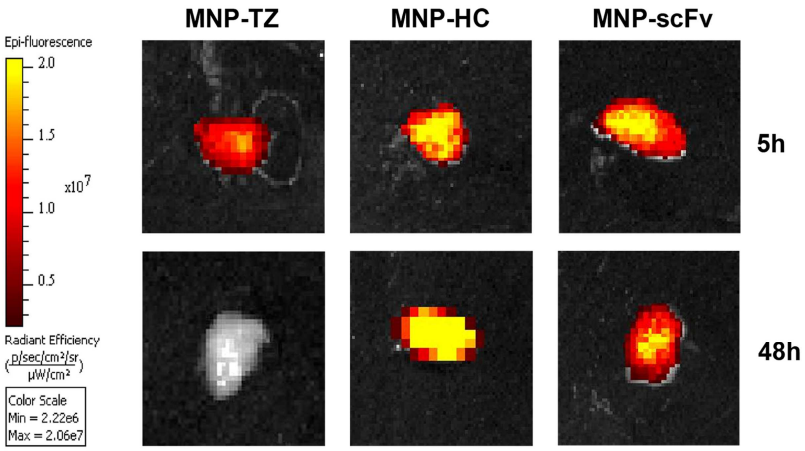

Figure S3. Epifluorescence images of MCF7 tumors isolated by mice exposed for 5 and 48 h to MNP-TZ, MNP-HC or MNP-scFv labeled with AF660.

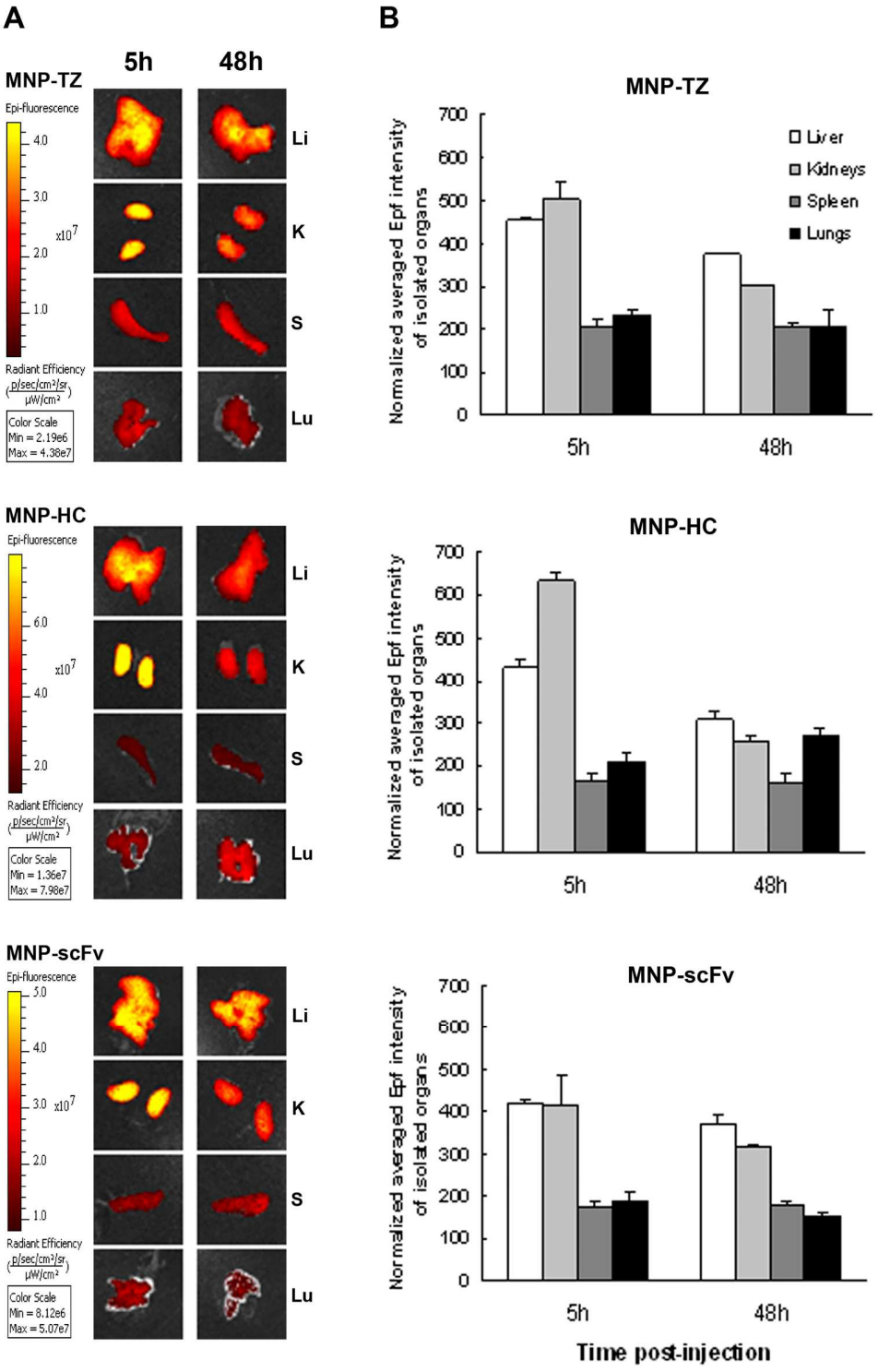

Figure S4. (A) Epifluorescence images of isolated liver (Li), kidneys (K), spleen (S) and lungs (Lu) at 5 and 48 h postinjection of MNP-TZ, MNP-HC or MNP-scFv labeled with AF660. (B) Averaged epifluorescence intensity of the isolated organs. Mean ± S.E. of 3 different samples for each experimental condition.

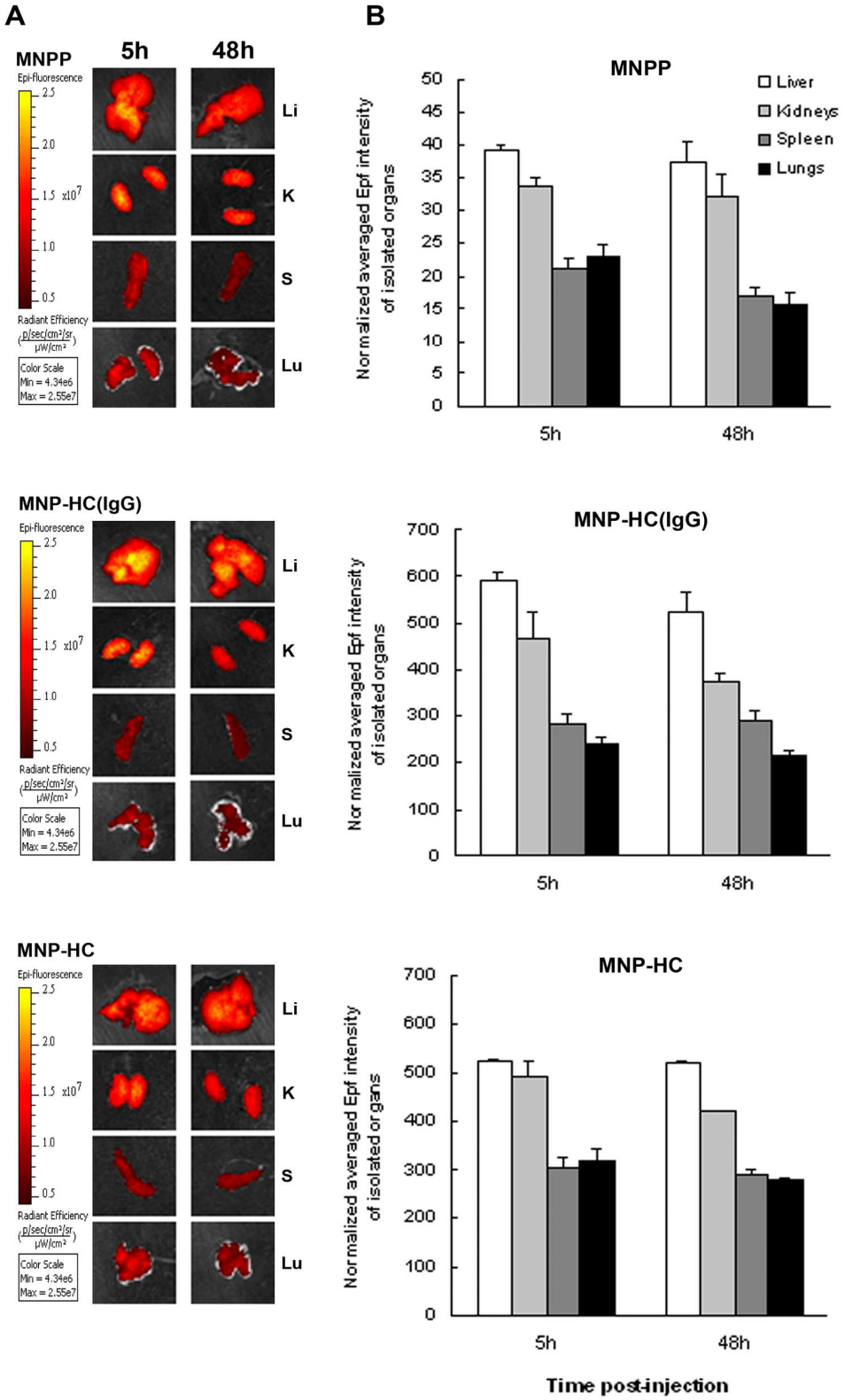

Figure S5. (A) Epifluorescence images of isolated liver (Li), kidneys (K), spleen (S) and lungs (Lu) at 5 and 48 h postinjection of MNPP, MNP-HC or MNP-HC(IgG) directly labeled on PMA shell. (B) Averaged epifluorescence intensity of the isolated organs. Mean ± S.E. of 3 different samples for each experimental condition.