Triple-Modal Imaging of Magnetically-Targeted Nanocapsules in Solid Tumours *In Vivo*

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Supplementary methods

Synthesis of $PLGA_{18KDa}$-$PEG_{3.5KDa}$-NH_2 (conjugate 3)

$PLGA_{18KDa}$-$PEG_{3.5KDa}$-NH_2 conjugate was prepared as we described previously (Supplementary Scheme S1a) [1]. Briefly, to activate the carboxylic group of $PLGA_{18KDa}$-COOH **(1)**, $PLGA_{18KDa}$-COOH (700mg, 0.0389 mmol) with NHS (17.9 mg, 0.1556 mmol) and DCC (32.0 mg, 0.1556 mmol) were dissolved in anhydrous dichloromethane ($PLGA_{18KDa}$-COOH: NHS: DCC stoichiometric molar ratio 1: 4: 4). The reaction mixture was stirred under nitrogen for 24 h at room temperature. The resultant solution was filtered and added drop-wise to a mixture of NH_2-$PEG_{3.5KDa}$-NH_2 **(2)** (680.6 mg, 0.194 mmol) and triethylamine (53.2 μL, 0.3889 mmol) in anhydrous dichloromethane ($PLGA_{18KDa}$-COOH: NH_2-$PEG_{3.5KDa}$-NH_2: triethylamine stoichiometric molar ratio 1:5:10). The reaction mixture was stirred under nitrogen for 24 h at room temperature. The resultant solution was then added drop-wise to the diethyl ether in an ice bath to precipitate the polymer products and remove dicyclohexylurea (DCU) by-product, excess NHS and DCC. The precipitated polymer, $PLGA_{18KDa}$-$PEG_{3.5KDa}$-NH_2 **(3)**, and excess NH_2-$PEG_{3.5KDa}$-NH_2 was dried under vacuum, i.e. dissolved in DMSO and dialysed (MWCO 10 KDa) against distilled water for 4 days to remove excess NH_2-$PEG_{3.5KDa}$-NH_2. Water was changed every 10 min in the first hour and then every 6 h. The resultant solution inside the dialysis membrane was lyophilised and stored at -20 °C. Polymer structure and $PEG_{3.5KDa}$ content were assessed by ^{1}H-NMR and FT-IR.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.05-5.2 (*m*, CH_2 PLGA), 4.75-4.85 (*m*, CH PLGA), 3.5-3.6 (*m*, CH_2 PEG), 1.42-1.6 (*m*, CH_3 PLGA).

Synthesis of NH_2-$PEG_{3.5KDa}$-DTPA (conjugate 4)

NH_2-$PEG_{3.5KDa}$-DTPA conjugate was prepared according to the method reported previously with some modifications (Supplementary Scheme S1b)[2]. NH_2-$PEG_{3.5KDa}$-NH_2 **(2)** (350.0 mg, 0.1 mmol) and triethylamine (27.4 μL, 0.2 mmol) were dissolved in anhydrous dichloromethane. A solution of DTPA dianhydride (13.1 mg, 0.033 mmol) in anhydrous dichloromethane (NH_2-$PEG_{3.5KDa}$-NH_2: triethylamine: DTPA stoichiometric molar ratio 3:1:6) was added drop-wise to the reaction mixture at 0 °C. After addition was complete, the solution was stirred under nitrogen for 2 h. The solvent was then removed under reduced pressure and the solid obtained was dissolved in chloroform and precipitated in diethyl ether to remove un-reacted DTPA. The solid obtained was subsequently dissolved in sodium acetate buffer (0.1 M, pH 4.6) and loaded onto a SP-Sephadex C25 cation-exchanger column and eluted with sodium acetate buffer affording NH_2-$PEG_{3.5KDa}$-DTPA **(4)** product. The elution of various products was monitored using TLC stained by Ninhydrin reagent and potassium permanganate reagent. NH_2-$PEG_{3.5KDa}$-NH_2 and the mono-substituted NH_2-$PEG_{3.5KDa}$-DTPA were Ninhydrin positive. The solution of conjugate **4** was dialysed (MWCO 2 KDa) against distilled water and lyophilised. The obtained product was then dissolved in ammonium acetate buffer (pH 5.5) and then loaded onto a reverse phase C18 Sep-Pak® Vac column using a gradient elution to further remove un-reacted DTPA (if precipitation step did not remove excess DTPA). Mixtures of water/acetone at different ratios, i.e. 100/0, 80/20, 60/40, 20/20, 0/100 were used as the eluent. The product was monitored using TLC stained by Ninhydrin reagent. The obtained NH_2-$PEG_{3.5KDa}$-DTPA was stored at -20°C and characterised by ^{1}H-NMR and FT-IR before further use.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 3.5-3.6 (*m*, CH_2 PEG).

Synthesis of $PLGA_{18KDa}$-$PEG_{3.5KDa}$-DTPA (conjugate 5)

$PLGA_{18KDa}$-COOH **(1)** (350 mg, 0.02 mmol) was activated using NHS (9.2 mg, 0.08 mmol) and DCC (14.5 mg, 0.08 mmol) in anhydrous dichloromethane ($PLGA_{18KDa}$-COOH: NHS: DCC stoichiometric molar ratio 1: 4: 4). The reaction mixture was stirred under nitrogen atmosphere for 24 hours at room temperature. The product was then precipitated by addition of diethyl ether to remove DCU, un-reacted DCC and NHS. The obtained product (360 mg, 0.02 mmol) was dissolved in anhydrous dichloromethane. A mixture of excess of NH_2-$PEG_{3.5KDa}$-DTPA **(4)** (350 mg, 0.1 mmol) and triethylamine (27.4 µL, 0.2 mmol) was added to the activated polymer at $PLGA_{18KDa}$-COOH: NH_2-$PEG_{3.5KDa}$-DTPA stoichiometric molar ratio 1:5. The reaction mixture was stirred under nitrogen atmosphere for 24 h at room temperature. The solution was dialysed (MWCO 10 KDa) against distilled water and lyophilised. The obtained $PLGA_{18KDa}$-$PEG_{3.5KDa}$-DTPA **(5)** was stored at -20°C and characterised by ^{1}H-NMR and FT-IR.

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.05-5.2 (*m*, CH_2 PLGA), 4.75-4.85 (*m*, CH PLGA), 3.5-3.6 (*m*, CH_2 PEG), 1.42-1.6 (*m*, CH_3 PLGA).

Magnetic properties of SPION and *m*-NC

The magnetic properties of the SPION and *m*-NC were assessed and compared using superconducting quantum interference device (SQUID) magnetometry. SQUID samples were mounted using soft gelatine capsules (size 4) and plastic straws, and measurements were made at room temperature (300K). For the liquid sample (*m*-NC), a small piece of cotton wool was placed at the bottom of the capsule, onto which 5µL of sample was dispensed and allowed to dry (ca. one hour) using a super-absorbant-polyer (SAP). For the dry sample (SPION), approximately 10 mg of material was placed at the bottom of the capsule. In both cases the remaining space in the capsule was filled with cotton wool. Magnetisation curves

were recorded using a Quantum Design MPMS-VSM Magnetometer (San Diego, USA) between ± 7 Tesla.

Shelf-life stability of *m*-NC

The NC and *m*-NC suspensions were stored at 4 °C at their original concentration at time of formulation. Stability of both types of NC was assessed for three months by the measurement of the size, Zeta potential, colour and phase separation.

Encapsulation efficiency of ICG in *m*-NC

The encapsulation efficiency of ICG in *m*-NC was assessed using an UV/fluorescence spectrometer (Varian, Cary Eclipse, Mulgrave, Vic., Australia). Prior to quantification, *m*-NC-ICG suspensions were purified by a PD-10 desalting column (size exclusion chromatography) and eluted in PBS buffer to remove any free ICG. NC suspensions before and after purification were diluted in DMSO (1/19, v/v) to rupture the NC structure. The excitation/emission wavelengths used for ICG were 790/820 nm. The actual loading was expressed as the amount of encapsulated dye per gram of NC. The encapsulation efficiency was expressed as the percentage of the encapsulated dye to the total amount of dye added to the formulation. All measurements were performed in triplicate and expressed as mean ± SD (n=3).

Interaction of free ICG and *m*-NC-ICG with serum proteins

The interaction of ICG after encapsulation into the NC with serum was determined in a 96-well-plate study using an IVIS® Lumina III *in vivo* imaging device (Caliper Life Sciences, Perkin Elmer, USA). Briefly, free ICG, ICG spiked NC, NC-ICG, *m*-NC-ICG with SPION loading of 1.85 and 7.02% w/w (SPION/NC) were transferred to a black 96-well plate (200

μL) at ICG concentration ranging between 0 and 30 μg/ml. The plates were then placed into the dark chamber of the imaging device. Photographic and fluorescence images were captured using a sequential acquisition mode with ICG filter (680, 700, 720, 740, 760, 780/845 nm for excitation/emission wavelength). The collected fluorescence emission signals were stored in efficiency units. The obtained images were analysed using Living Image® 4.3.1 Service Pack 2 Software and the linear ranges of free ICG, ICG-spiked NC and encapsulated ICG were determined.

In vitro cytotoxicity studies (MTT assay)

The CT26 murine colon carcinoma (CT26, ATCC®, CRL-2638TM) were cultured in Advanced RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 1% L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) with 5% CO2 and 95% air at 37 °C. The CT26 cells were seeded in 96-well plates and incubated with non-PEGylated and PEGylated NC/ m-NC in complete medium for 24, 48 and 72 h. Cell viability was examined by MTT assay. Briefly, the supernatant of each well was removed at the above time points and replaced with 120 μL of MTT solution (at a concentration of 5 mg/ml in PBS with a further 1: 6 dilution in media prior to use). Cells were incubated for 3 h at 5% CO2 and 95% air at 37 °C. Formazan was then solubilised in 200 μL of DMSO and was incubated for 5 min at 37°C. The absorbance was read at 570 nm using a FLUO star OPTIMA plate reader (BMG Labtech). Results were expressed as the percentage cell survival (mean ± SD) and calculated using the Equation (1):

% Cell viability = (A570 nm of treated cells/ A570 nm of native cells) x 100%. (1)

References

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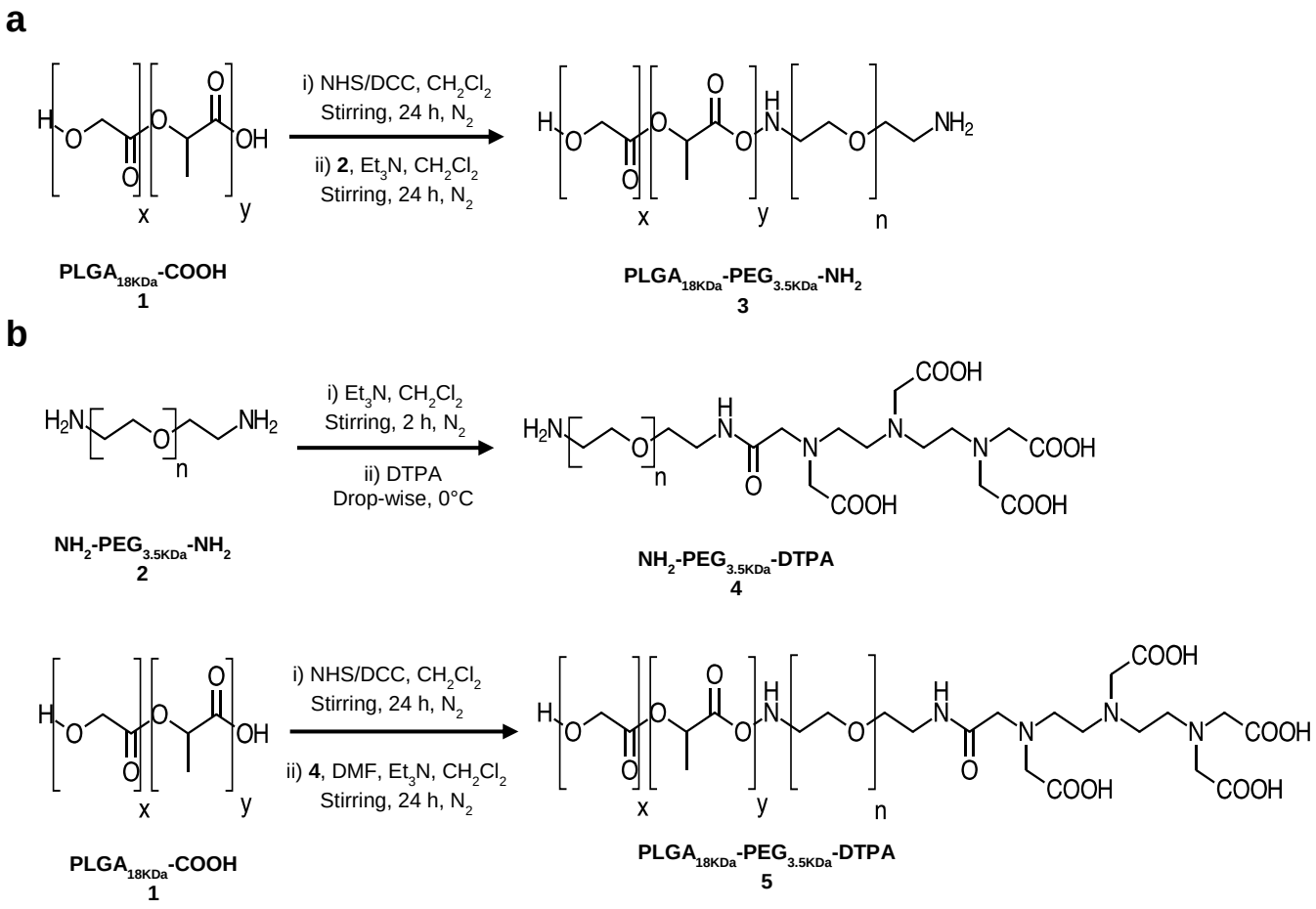
Supplementary scheme



Scheme S1. Synthetic routes of polymeric conjugates in nanocapsule preparation. a, $PLGA_{18KDa}$- $PEG_{3.5KDa}$-NH_2 was synthesised and used to formulate the PEGylated NCs. **b**, $PLGA_{18KDa}$-$PEG_{3.5KDa}$-DTPA was synthesised and used to facilitate radio-labelling of the PEGylated NCs through chelation of indium-111 with DTPA.

Supplementary figures

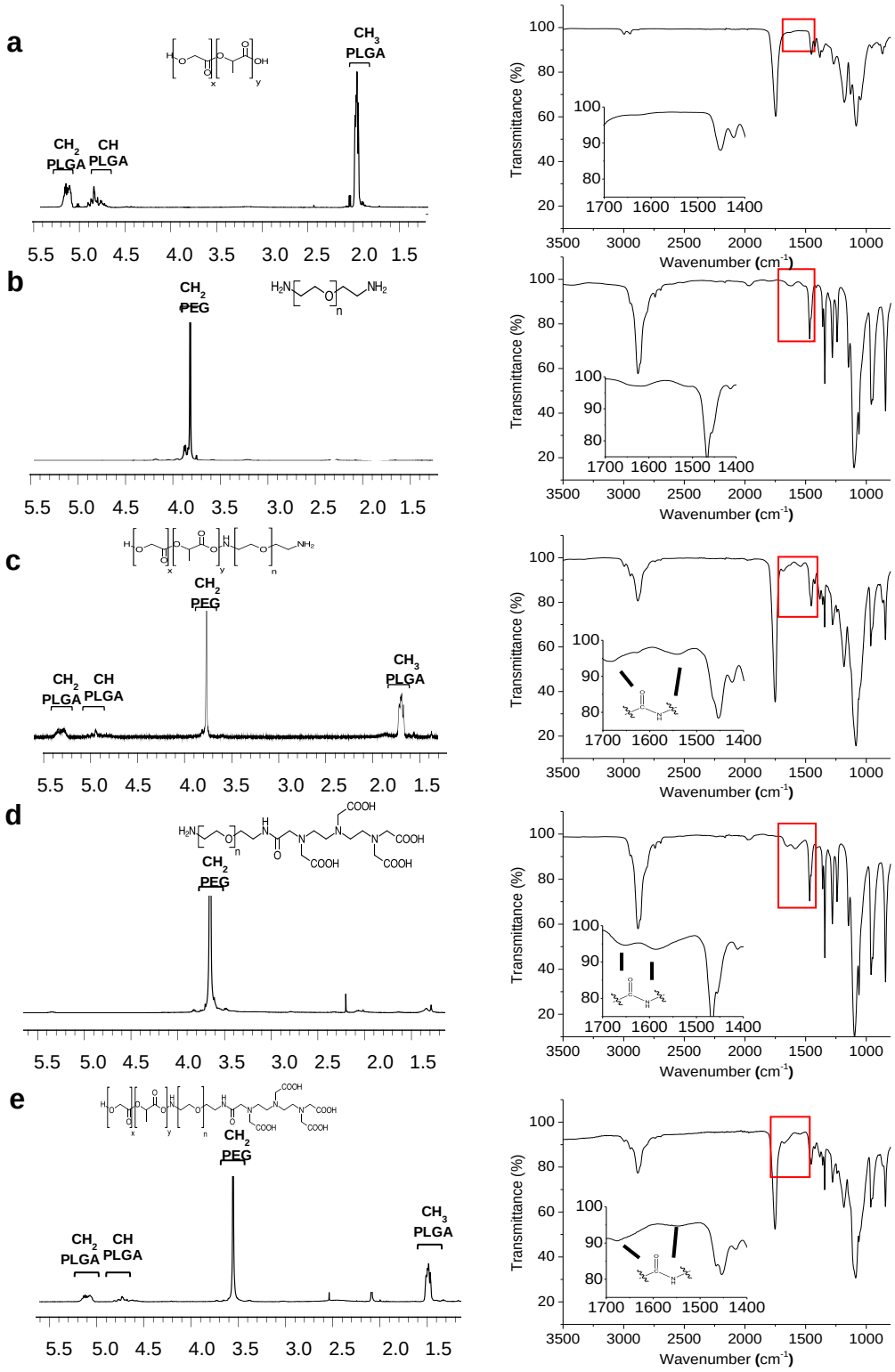


Figure S1. Characterisation of the synthesised conjugates by ^{1}H-NMR and FT-IR. ^{1}H-NMR spectra ($CDCl_3$) and FT-IR spectra (ATR mode) of **a** compound **1** ($PLGA_{18KDa}$-COOH), **b** compound **2** (NH_2-$PEG_{3.5KDa}$-NH_2), **c** conjugates **3** ($PLGA_{18KDa}$-$PEG_{3.5KDa}$-NH_2), **d** conjugates **4** (NH_2-$PEG_{3.5KDa}$-DTPA), **e** conjugates **5** ($PLGA_{18KDa}$-$PEG_{3.5KDa}$-DTPA), respectively. Insets show enlargement of amide region (1700-1400 cm-1), which was absent in **a** and **b**.

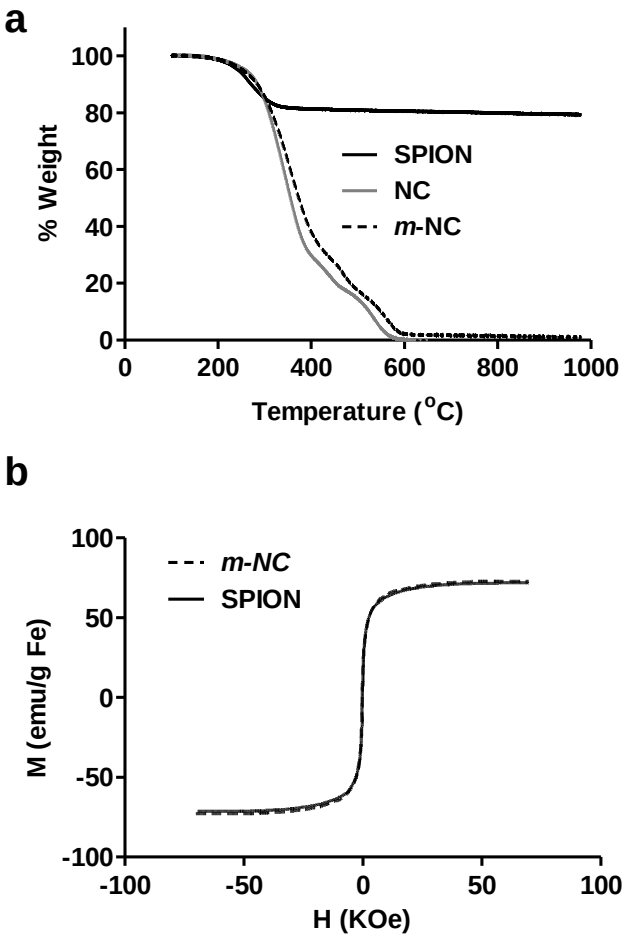


Figure S2. Magnetic properties and thermogravimetric analysis (TGA) of SPION and *m*-NC. a, Thermogravimetric analysis of oleic acid coated SPION, NC and *m*-NC. *m*-NCs were prepared using 1.85% w/w SPION/*m*-NC. Thermogravimetric analysis (TGA) was performed from 100 °C to 1000 °C, with a temperature ramp of 10 °C/min under compressed air condition. A 19.5% weight loss (at 600 °C) was observed for SPION corresponding to the oleic acid coating content. Weight (%) remained for NC, *m*-NC was ~0.01% and 1.84% at 600 °C, respectively. **b,** Magnetisation curve of SPION and *m*- NC as a function of field measured at 300 K. The magnetic properties of the SPION and *m*-NC were assessed and compared using superconducting quantum interference device (SQUID). The magnetisation curve was recorded using a Quantum Design (San Diego, USA) MPMS-VSM between ± 7 Tesla. The obtained values of saturation magnetisation of SPION and *m*-NC were both around 72 emu/g Fe.

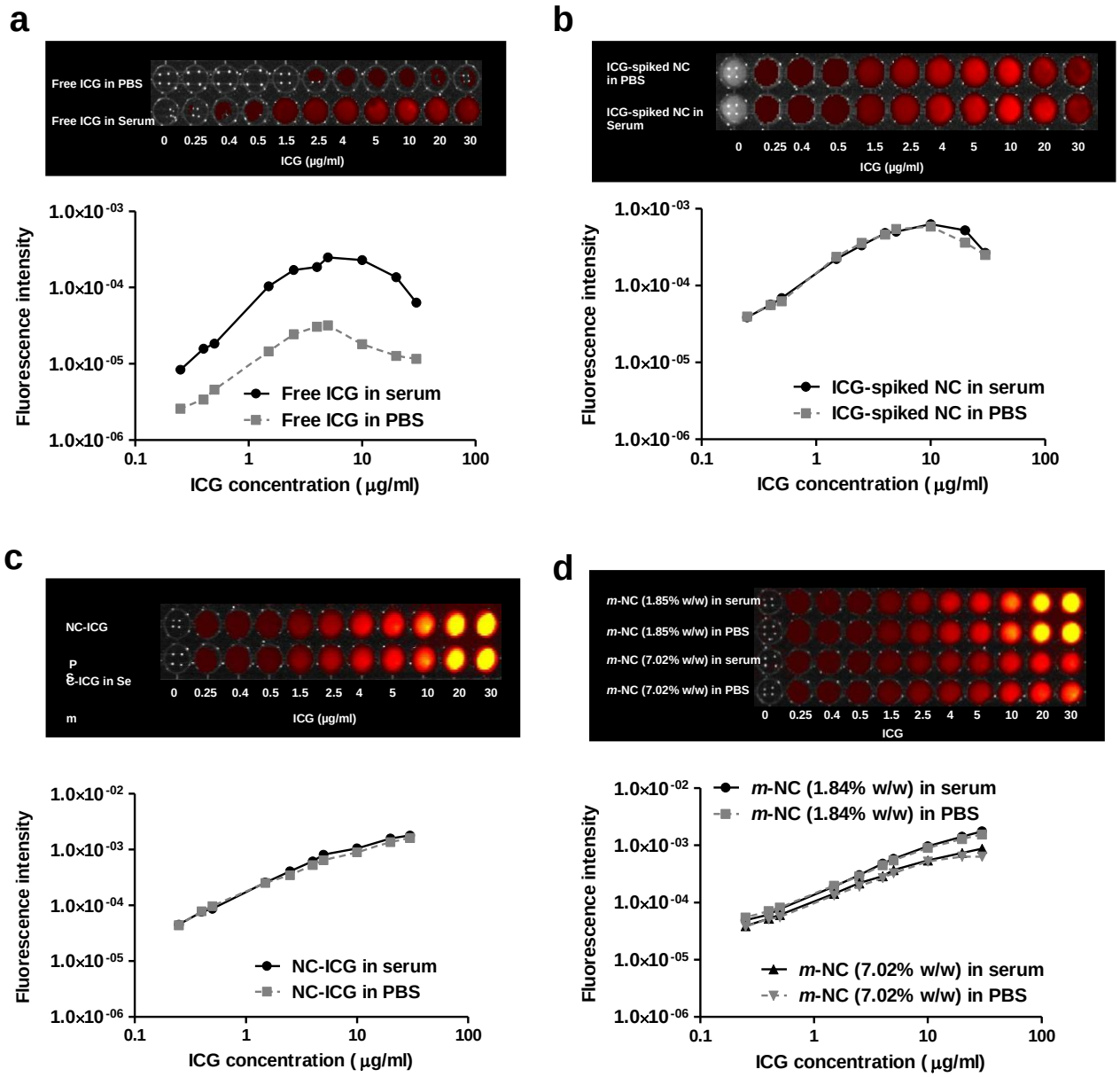


Figure S3. Well-plate study investigating the effect of serum, encapsulation in NC and the presence of SPION on ICG fluorescence. Wells were sampled with ICG, ICG-spiked NC, NC-ICG and *m*-NC-ICG at different concentrations increasing from 0 to 30 µg/ml of ICG in PBS or 50% (v/v) serum. Photographic field and fluorescence images were overlaid. Fluorescence intensity was plotted as a function of ICG concentration in a logarithmic scale and is shown for **a** ICG, **b** ICG-spiked NC, **c** NC-ICG and **d** *m*-NC-ICG with SPION loading of 1.85% and 7.02% (w/w, SPION/NC) (n=3, error bars are too small to be seen). Self-quenching of ICG alone and ICG-spiked NC was observed at concentrations over 2.5 µg/ ml while no quenching was seen when ICG was encapsulated into NC at the concentrations studied. Encapsulation of ICG also increased the linear fluorescence range up to 5 µg/ ml. A reduction in fluorescence intensity was observed with SPION loading of 7.02% compared to 0% and 1.85%. All images were obtained by IVIS Lumina® III at exposure time: 8 s; binning factor: 4; f number 2, field of view: E-25 cm; λex: 680, 700, 720, 740, 760 and 780 nm; λem: 845 nm. Data were analysed by Living Image® 4.3.1 Service Pack 2 software.

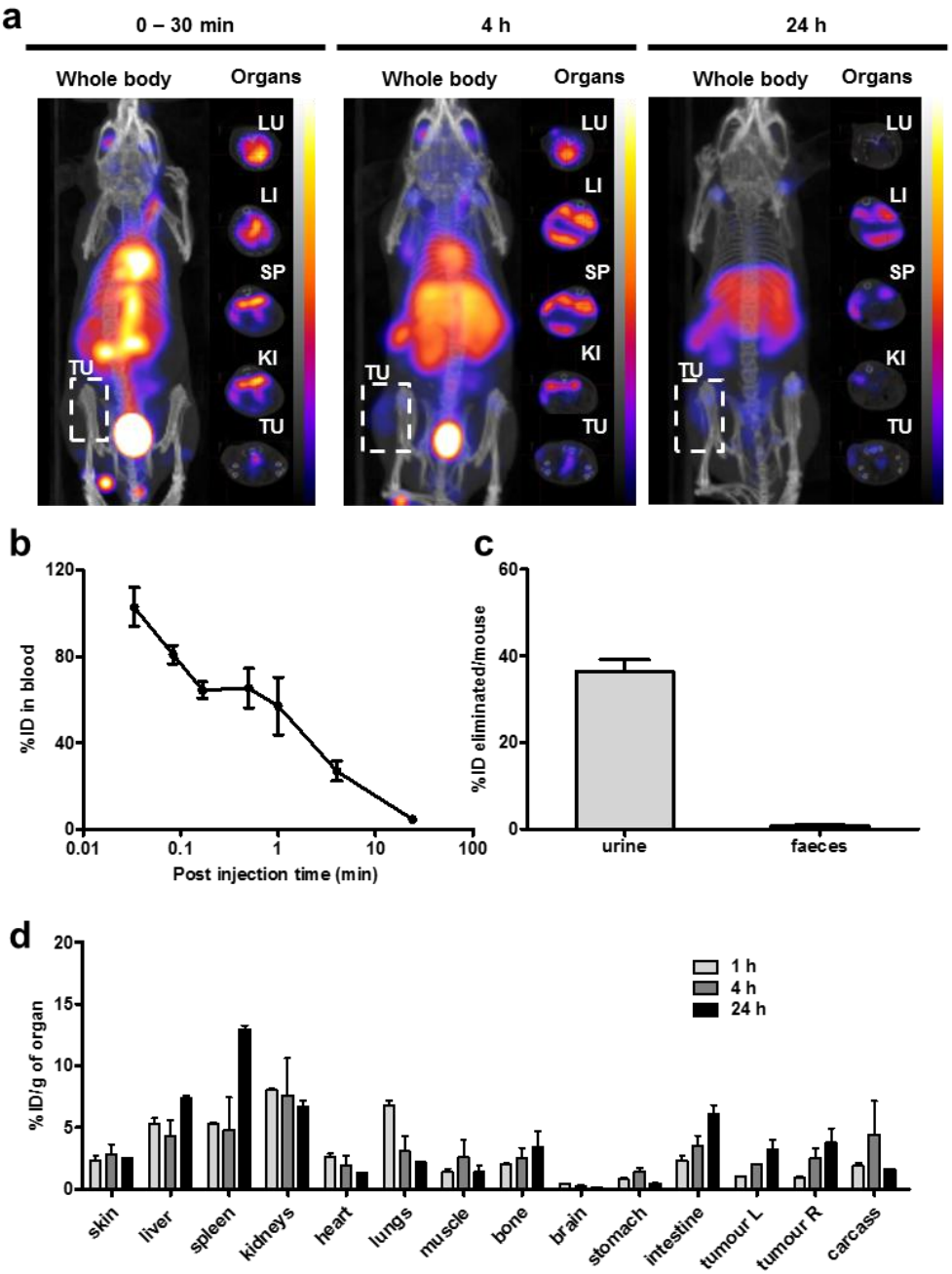


Figure S4. ***In vivo*** **SPECT/CT imaging and organ biodistribution studies of** ***m*****-NC-^{111}In in CT26 tumour-bearing balb/c mice (no magnet exposure)**. Mice were i.v injected with *m*-NC-^{111}In at a dose of 312.5 mg polymer/Kg (containing 31.25 mg of $PLGA_{18KDa}$-$PEG_{3.5KDa}$-DTPA/Kg) and 125 mg SPION/Kg (8-10 MBq per mouse). Mice for SPECT/CT imaging with tumour inoculated at one side only while tumour inoculated bifocally for gamma scintigraphy studies. Organs were excised at 1, 4 and 24 h post-injection. **a,** Whole body 3D SPECT/CT imaging at 0-30min, 4 and 24 h post-injection with scanning time of 30 min each. Cross-sections were from lung (LU), liver (LI), spleen (SP), kidney (KI) and tumour (TU) at equivalent time points. Tumours are marked in dashed square. **b,** Blood clearance profile. **c**, Excretion profile at 24 h. **d,** Organ biodistribution profile values expressed as % ID/g of tissue. Tumour accumulation was observed at 4 h post injection and was enhanced over time for *m*-NC-^{111}In with 3.62 ± 1.42% ID/g of organ at 24 h. Results are expressed as mean ± S.D. (n=3).

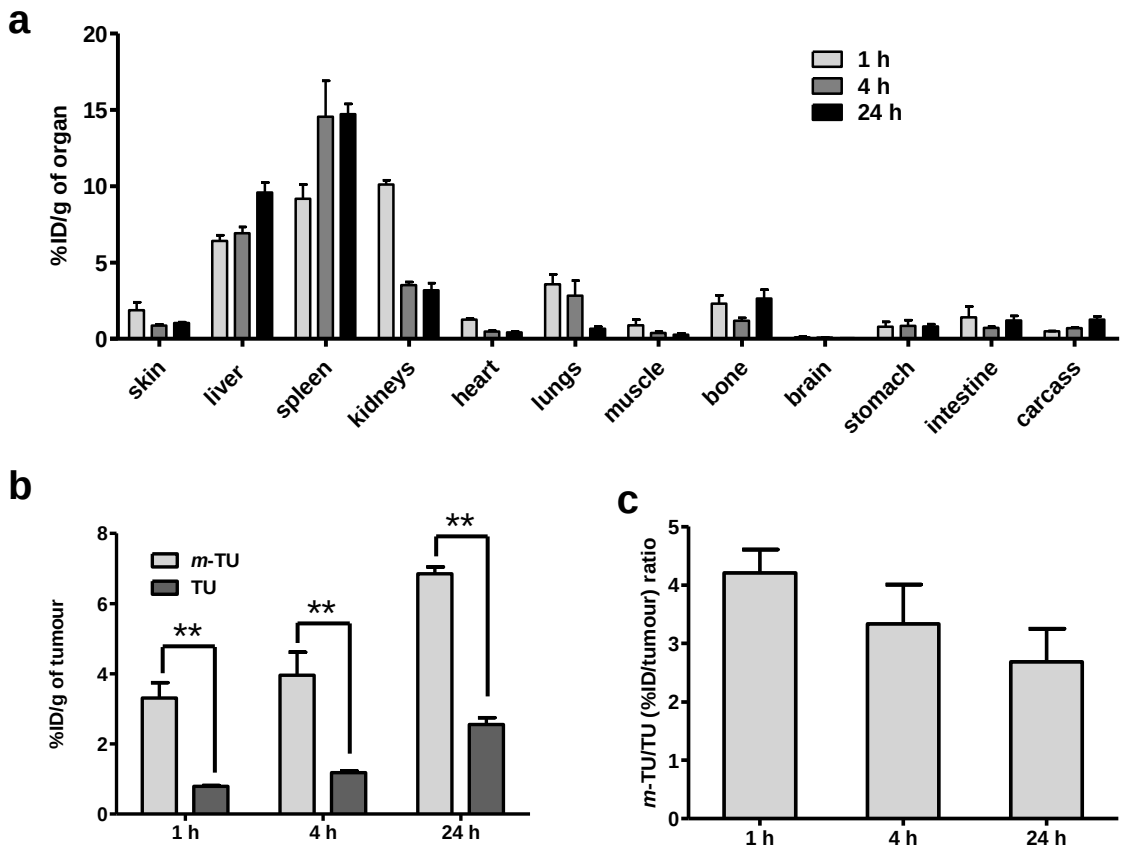


Figure S5. Organ biodistribution profiles of *m*-NC-^{111}In in CT26 tumour-bearing balb/c mice under the influence of magnetic field after whole body perfusion and gamma counting. Mice were i.v injected with *m*-NC-^{111}In at a dose of 312.5 mg polymer/Kg (containing 31.25 mg of $PLGA_{18KDa}$-$PEG_{3.5KDa}$-DTPA/Kg) and 125 mg SPION/Kg. A permanent magnet (0.515 T) was applied at one tumour site (*m*-tumour) for 1 h. mice were subjected to whole body saline perfusion before sacrifice. Organs were excised at 1, 4 and 24 h post-injection. **a,** Organ biodistribution profile with values expressed as % ID/g of organ. **b,** Quantification of uptake at the magnetically targeted site (*m*-TU) and non-magnetically targeted site (TU) at different time points. **c,** Fold increase in % ID/g of tumour upon application of magnetic field. Significant increase in tumour uptake was shown upon application of a magnet at all time (**P < 0.01). Results are expressed as mean ± S.D. (n=4).

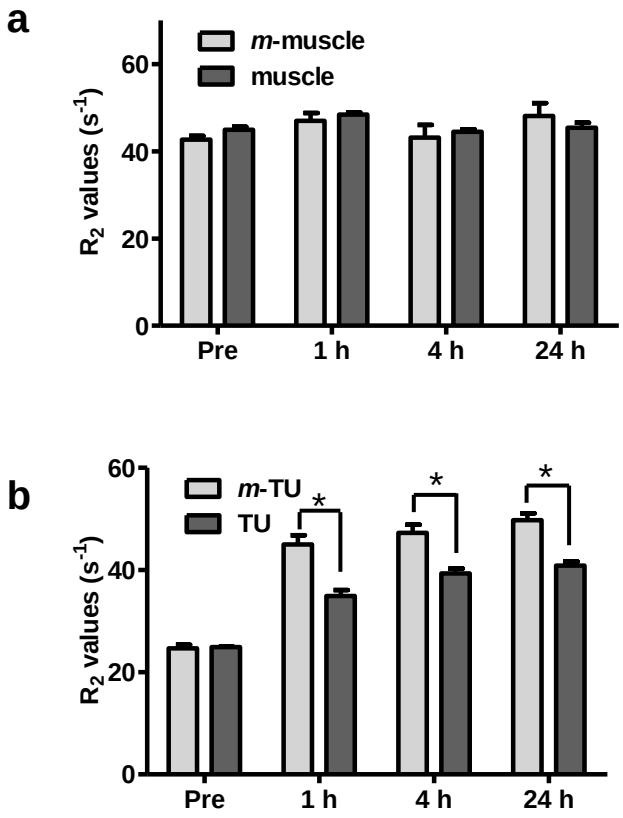


Figure S6. R_2 relaxation rate measurements of *m*-NC in CT26 tumour-bearing balb/c mice under the influence of magnetic field. Mice were i.v injected with *m*-NC at a dose of 312.5 mg polymer/Kg and 125 mg SPION/Kg. A permanent magnet (0.515 T) was applied at one tumour site (*m*-TU) for 1 h. **a,** R_2 relaxation rate at muscles adjacent to the magnetically targeted tumour (*m*-muscle) or the non-magnetically targeted tumour (muscle) over time. **b,** R_2 relaxation rate at magnetically targeted tumour (*m*-TU) and non-magnetically targeted tumour (TU) over time. Significant enhancement in R_2 relaxation rate was seen in *m*-TU compared to TU (* $p<0.05$, ** $p<0.01$). No significant changes in R_2 relaxation rate were observed in both muscle sides. Results are expressed as mean ± S.D. (n=3).

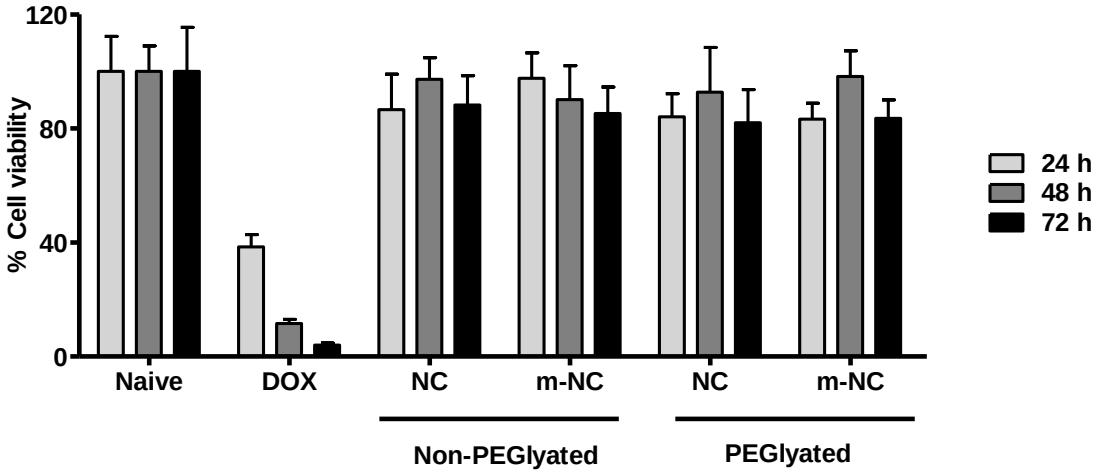


Figure S7. Cytotoxicity of NC and *m*-NC in CT26 murine colon carcinoma cells *in vitro*. CT26 cells were incubated with four types of NCs for 24, 48 and 72 h, at a final polymer concentration of 110 nM. Cell viability was determined by MTT assay and no significant reduction to naive cells was seen in all types of NCs up to 72 h. Results were expressed as mean ± SD (n=5).

Supplementary movie

Movie S1. Cryo electron tomography of the *m*-NCs. A volume rendering 3D visualization of the *m*-NC as well as an xy orthoslice through the 3D reconstruction are presented.