Supplementary Material

Near infrared dye-labeled polymeric micro- and nanomaterials: *in vivo* imaging and evaluation of their local persistence

Gracia Mendoza[a,b†*], *Isabel Ortiz de Solorzano*[a,b,c†], *Inmaculada Pintre*[a], *Sara Garcia-Salinas*[a,b,c], *Victor Sebastian*[a,b,c], *Vanesa Andreu*[a,b], *Marina Gimeno*[d], *Manuel Arruebo*[a,b,c]

[a]Department of Chemical Engineering. Aragon Institute of Nanoscience (INA), University of Zaragoza, Campus Río Ebro-Edificio I+D, C/ Poeta Mariano Esquillor S/N, 50018-Zaragoza, Spain

[b]Aragon Health Research Institute (IIS Aragón), 50009 Zaragoza, Spain

[c]Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, 28029-Madrid, Spain

[d]Department of Animal Pathology, University of Zaragoza, C/ Miguel Servet 177, 50013-Zaragoza, Spain

[†]Both authors contributed equally to this work

[*]Address correspondence to gmmenc@unizar.es

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Materials

Poly(lactic-co-glycolic) acid (PLGA) 50:50 (MW 24-38 kDa), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl) 99%, N-hydroxysuccinimide (NHS) 98%, triethylamine 98%, 6-aminocaproic acid (titration powder), 1,6-hexanediamine 98%, N-isopropylacrylamide ≥99%, N-isopropylmethacrylamide 97%, acrylamide >99%, N,N'-methylenebis(acrylamide) 99%, ammonium persulfate ≥98%, Span® 80, polyN-isopropylacrylamide (PNIPAM) amine terminated (PNIPAM-NH_2) average Mn 5000 and IR-820 80% dye were purchased from Sigma-Aldrich (Germany). The solvents Hexadecane, ReagentPlus®, 99%, dimethylformamide anhydrous (DMF) 99.8%, ethyl acetate (AcEt), dichloromethane (DCM) and acetone HPLC were also acquired from Sigma-Aldrich (Germany) while methanol (HPLC quality) was obtained from Acros Organics (Belgium). Free endotoxin water was purchased from Alfa-Aesar (UK) while paraformaldehyde 4% in PBS was acquired from Affymetrix (UK) and absolute ethanol from Merck (Germany).

Dialysis membranes benzoylated with an average flat width of 9 mm and molecular weight cut off of 2 kDa were purchased from Sigma-Aldrich (Germany). Silica gel with particle size 63-100 μm (150-230 mesh ASTM) was purchased from Riedel-de Haën (Germany).

Synthesis of PLGA and PNIPAM tagged materials

Synthesis of IR820-COOH

A round bottom flask containing 150 mg of IR-820 (80% dye, 0.141 mmol, 1 eq.) and 63 mg of 6-aminocaproic acid (0.480 mmol, 3.4 eq.) was purged with argon prior to the addition of 5 mL of dry DMF and 0.1 mL of triethylamine. The green solution was

stirred and heated in a pre-warmed oil bath at 85 °C for 3 h under argon atmosphere and protected from the light. The mixture upon reaction turned into a deep blue colour and after completing the reaction time the stirring was stopped, the reaction was cooled down and the solvent evaporated under vacuum to dryness. The crude was purified by column chromatography using a polarity gradient (ethyl acetate-methanol 7:3 to 3:2). The efficiency of the reaction was 78.7% obtaining 105 mg of IR820-COOH.

Synthesis of IR820-NH_2

A round bottom flask containing 150 mg of IR-820 (80% dye, 0.142 mmol, 1 eq.) and 20.52 mg of 1,6-hexanediamine (0.142 mmol, 1 eq.) was purged with argon. 25 mL of dry DMF and 0.1 mL of triethylamine were added. The green solution, under argon atmosphere, was protected from the light, stirred and heated in a pre-warmed oil bath at 85 °C for 3 h. After completing the reaction time the stirring was stopped, the blue mixture was cooled down and the solvent evaporated under vacuum to dryness. The crude was purified by column chromatography using a polarity gradient (ethyl acetate-methanol 7:3 to pure methanol) obtaining an efficiency of 47.7% (63 mg of IR820-NH_2).

Synthesis of PLGA-IR820

The carboxylic group of the polymer was first activated through reaction with EDC and NHS followed by the coupling reaction with IR820-NH_2. Then, 100 mg of PLGA (3.2 μmol, 1 eq) were stirred with 6.1 mg of EDC (32 μmol, 10 eq.) and 3.7 mg of NHS (32 μmol, 10 eq) in 1 mL of dry DMF under argon atmosphere at room temperature. After 2 h of reaction, a solution of IR820-NH_2 was prepared under argon atmosphere in a round bottom flask, protected from the light with 3.6 mg of IR820-NH_2 (3.8 μmol, 1.2 eq.) dissolved in 134 μL of triethylamine-dry DMF (20 μL in 2 mL of DMF, 15.2 μmol, 4 eq.) and 100 μL of dry DMF. The content of the vial was then added to the dye mixture

and later washed with a total amount of 600 µL of dry DMF. The mixture was stirred at room temperature overnight protected from the light. After, the solvent was evaporated to dryness under vacuum and the solid obtained was purified dissolving it in acetone (3 mL) and precipitated with methanol (30 mL) for 3 times. 93 mg of polymer were obtained (90% efficiency).

Synthesis of PNIPAM-IR820

In this procedure, the carboxylic group of IR820-COOH was first activated through reaction with EDC and NHS followed by the coupling reaction with PNIPAM. In a round bottom flask protected from the light, 20.6 mg of IR820-COOH (21.8 µmol, 1.2 eq.) were stirred with 16.8 mg of EDC (87.6 µmol, 4.8 eq.) and 10 mg of NHS (86.9 µmol, 4.8 eq.) in 1 mL of dry DMF under argon atmosphere at room temperature. After 2 h of reaction, a solution of PNIPAM was added under argon atmosphere over the activated IR820-COOH. PNIPAM (100 mg, 18.2 µmol, 1 eq.) was dissolved in 800 µL of dry DMF and 6.8 µL of triethylamine (92.5 µmol, 5 eq.) added as a solution in DMF (40 µL TEA in 1 mL of DMF, 170 µL of the solution). The vial was washed with 500 µL of dry DMF and the solution added to the reaction mixture. The mixture was stirred at room temperature overnight and protected from the light. After, the solvent was evaporated to dryness under vacuum, methanol was added to remove the remaining DMF in the crude reaction and the solid obtained was purified by dialysis against water for 3 days. After lyophilisation, 86 mg of PNIPAM-IR820 were obtained (78% efficiency).

Preparation of fluorescent labeled polymeric nanoparticles

Labeled PLGA by the amino-modified IR820 (PLGA-IR820-NH2), PLGA encapsulating the NIR dye (PLGA-IR820 encapsulated), tagged PNIPAM by the acidic-modified IR820 added during the polymer synthesis (PNIPAM-IR820-COOH) or after

the synthesis (PNIPAM-IR820-COOH post-synthesis), labeled PNIPAM microgels by the fluorophore (PNIPAM-IR820 microgels) and tagged PNIPAM microparticles (MPs) by IR820 (PNIPAM-IR820 microparticles) were prepared as follows. The tagged nanoparticles (NPs) were synthesized with the pre-labeled materials (PLGA IR820-NH_2 NPs and PNIPAM-IR820-COOH NPs) or were first synthesized and then labeled with the modified dye (PNIPAM-IR820-COOH post-synthesis NPs) or with the commercial IR-820 (PLGA-IR820 encapsulated NPs, PNIPAM-IR820 microgels and PNIPAM-IR820 microparticles).

Preparation of PLGA nanoparticles

50 mg of the pre-synthetized PLGA-IR820-NH_2 polymer were dissolved with 150 mg of Pluronic-F68 in 5 mL of ethyl acetate. 10 mL of water were then added to the solution and the mixture was sonicated 25 sec in an ice bath with a sonicator probe at 40% of amplitude. The cloudy suspension was stirred for several hours until the complete evaporation of the organic solvent. The remaining aqueous suspension was centrifuged (10 min, 4500 rpm) to concentrate NPs which were finally resuspended in water to a final volume of 10 mL.

The encapsulation of IR-820 in PLGA (PLGA-IR820 encapsulated NPs) was performed following also the procedure explained above but with slight modifications. Commercial PLGA-COOH was used and 625 µg of IR-820 were added to the initial ethyl acetate solution. The cleaning centrifugation step was performed twice in order to remove the not encapsulated dye.

Preparation of PNIPAM nanoparticles

25 mg of the corresponding PNIPAM polymer and 125 mg of Pluronic-F68 were dissolved in 5 mL of chloroform and subsequently 10 mL of water were added. The mixture was sonicated for 25 sec with a sonicator probe at 40% of amplitude in an ice

bath. The cloudy suspension was stirred at 700 rpm for several hours to evaporate chloroform. The remaining aqueous phase was centrifuged for 10 min at 4500 rpm to remove the supernatant containing the polymeric material not incorporated into the NPs. NPs were then resuspended in water to a final volume of 10 mL.

Procedure to functionalize pre-synthesized PNIPAM nanoparticles

The carboxylic acid group of IR820-COOH was activated prior to the reaction with PNIPAM. 4.5 mg of the dye (4.54 μmol, 1 eq.) were stirred for 1 h at room temperature with 8.7 mg of EDC (45.4 μmol, 10 eq.) and 5.23 mg of NHS (45.4 μmol, 10 eq.) in 1 mL of water. The activated dye was then added to 10 mL of the PNIPAM nanoparticle suspension (25 mg of PNIPAM, 4.54 μmol, 1 eq.) and the mixture was stirred at room temperature overnight. NPs were finally centrifuged for 10 min at 4500 rpm, the supernatant was removed and the pellet containing the NPs was resuspended in 10 mL of water and centrifuged again to discard the excess of dye and EDC/NHS.

Preparation of PNIPAM microgels

PNIPAm microgels were synthetized following the protocol of Timko et al [1]. Briefly, N-isopropylacrylamide (0.6 g), N-isopropylacrylmethylamine (0.8 g), acrylamide (50 mg) and N,N'-methylenebisacrylamide (80 mg) were dissolved in 150 mL distilled deionized water (DDI) previously degassed with argon at least for 15 min. The solution was again degassed through vacuum-argon cycles and heated to 70 ºC. Ammonium persulfate initiator (5 mL of 20 mg/mL solution in DDI) was then added to the solution with high stirring. After 6 h under argon atmosphere, the reaction was stopped and the particles were dialyzed against DDI water to remove unreacted monomer following lyophilisation.

Preparation of PNIPAM microparticles

PNIPAM microparticles (MPs) were prepared following Choi et al protocol [2] with some modifications. To synthetize PNIPAm MPs of an average diameter of around 400 μm, a coaxial microreactor was used. The disperse aqueous phase flow with the monomer (NIPAM 20% w/w) and crosslinker (BIS 5% w/w) was 10 μL/min while the hexadecane continuous phase flow with Span 80 (5% w/w) as surfactant and DEAP as photoinitiator (5% v/v) was 100 μL/min. Photopolymerization was carried out with a 365 nm LED (UV light, 4.6W) to accelerate the polymerization step.

Loading of IR-820 commercial dye in PNIPAM microgels and microparticles

Microgels or MPs were dispersed in water with a final concentration of 5 mg/mL and a polymer/dye weight ratio of 1:10 and kept in contact with the commercial IR-820 dye for 3 days. Then, they were washed twice by centrifugation to discard the non-encapsulated dye and resuspended in water in the case of microgels or in methylcellulose in the case of MPs to obtain homogenous dispersions with a final concentration of 5 mg/mL.

Characterization of tagged polymeric micro- and nanomaterials

The formation of micro- and NPs as well as the modified IR-820 were monitored by UV-vis spectroscopy through a double-beam UV-vis spectrophotometer (PerkinElmer Lambda 35). PLGA and PNIPAM tagged micro- and nanomaterials were characterized regarding shape and size by using a scanning electron microscope (SEM) Inspect F50 (FEI Co., LMA-INA, Spain) operating at an accelerating voltage of 10-15 keV. The lower critical solution temperature (LCST) of the thermoresponsive polymer PNIPAM, un-modified and modified with the dye, was analysed by turbidimetry. In addition, Fourier transform infrared spectroscopy (FTIR) was performed on a Bruker Vertex 70 equipped with a DTGS detector and ATR Golden Gate Diamond. The scans were

developed in the range of 4000-400 cm^{-1}. Mass spectrometry of the dyes was carried out in a Microtof-Q equipment whereas NMR spectroscopy was developed in a Bruker ARX-300 equipment to completely characterize the products obtained.

Supplementary references

[1] B.P. Timko, M. Arruebo, S.A. Shankarappa, J.B. McAlvin, O.S. Okonkwo, B. Mizrahi, et al., Near-infrared-actuated devices for remotely controlled drug delivery, Proc. Natl. Acad. Sci. 111 (2014) 1349–1354. doi:10.1073/pnas.1322651111.

[2] C.-H. Choi, J.-H. Jung, D.-W. Kim, Y.-M. Chung, C.-S. Lee, Novel one-pot route to monodisperse thermosensitive hollow microcapsules in a microfluidic system., Lab Chip. 8 (2008) 1544–51. doi:10.1039/b804839h.

SUPPLEMENTARY TABLES

Table S1: IR820 dye concentration in the final materials (Mean ± SD; n = 4)

	PLGA-IR820 encapsulated	PLGA-IR820-NH_2	PNIPAM-IR820-COOH	PNIPAM-IR820-COOH post-synthesis	PNIPAM-IR820 microgels	PNIPAM-IR820 microparticles
Dye concentration (µg/mL)	32.2 ± 3.7	21.0 ± 0.5	3.9 ± 0.3	12.0 ± 3.5	28.5 ± 6.4	48.6 ± 4.5

Table S2. Distribution of cell cycle phases for the different cell lines studied when treated with the dye-labelled materials synthesized (1 mg/mL)

	Cell cycle phases	Control	PLGA-IR820 encapsulated	PLGA-IR820-NH_2	PNIPAM-IR820-COOH	PNIPAM-IR820-COOH post-synthesis	PNIPAM-IR820 microgels	PNIPAM-IR820 microparticles
mMSCs	**G1**	42.04	60.45	31.94	36.41	36.13	26.71	30.43
	S	30.53	21.11	40.35	35.94	44.53	30.80	37.80
	G2	27.43	18.44	27.71	27.65	19.34	42.49	31.77
Macrophages	**G1**	25.37	50.28	36.35	36.69	41.41	56.36	28.04
	S	46.53	29.88	31.61	33.69	21.62	22.33	48.30
	G2	28.10	19.85	32.04	29.62	36.98	21.31	23.66
Monocytes	**G1**	50.82	45.00	40.78	41.23	47.02	45.35	45.64
	S	30.32	35.65	39.65	43.45	23.49	34.18	32.22
	G2	18.85	19.35	19.57	15.32	29.49	20.48	22.04
U251MG	**G1**	50.24	63.86	62.88	61.94	71.05	42.64	44.40
	S	34.21	29.82	29.65	30.43	13.23	39.14	36.78
	G2	15.56	6.32	7.47	7.63	15.72	18.22	18.82
Fibroblasts	**G1**	65.42	74.85	62.91	54.34	34.15	64.95	59.49
	S	4.92	14.46	9.21	6.36	37.51	19.54	21.03
	G2	29.66	10.70	27.88	39.29	28.34	15.50	19.48

SUPPLEMENTARY FIGURES

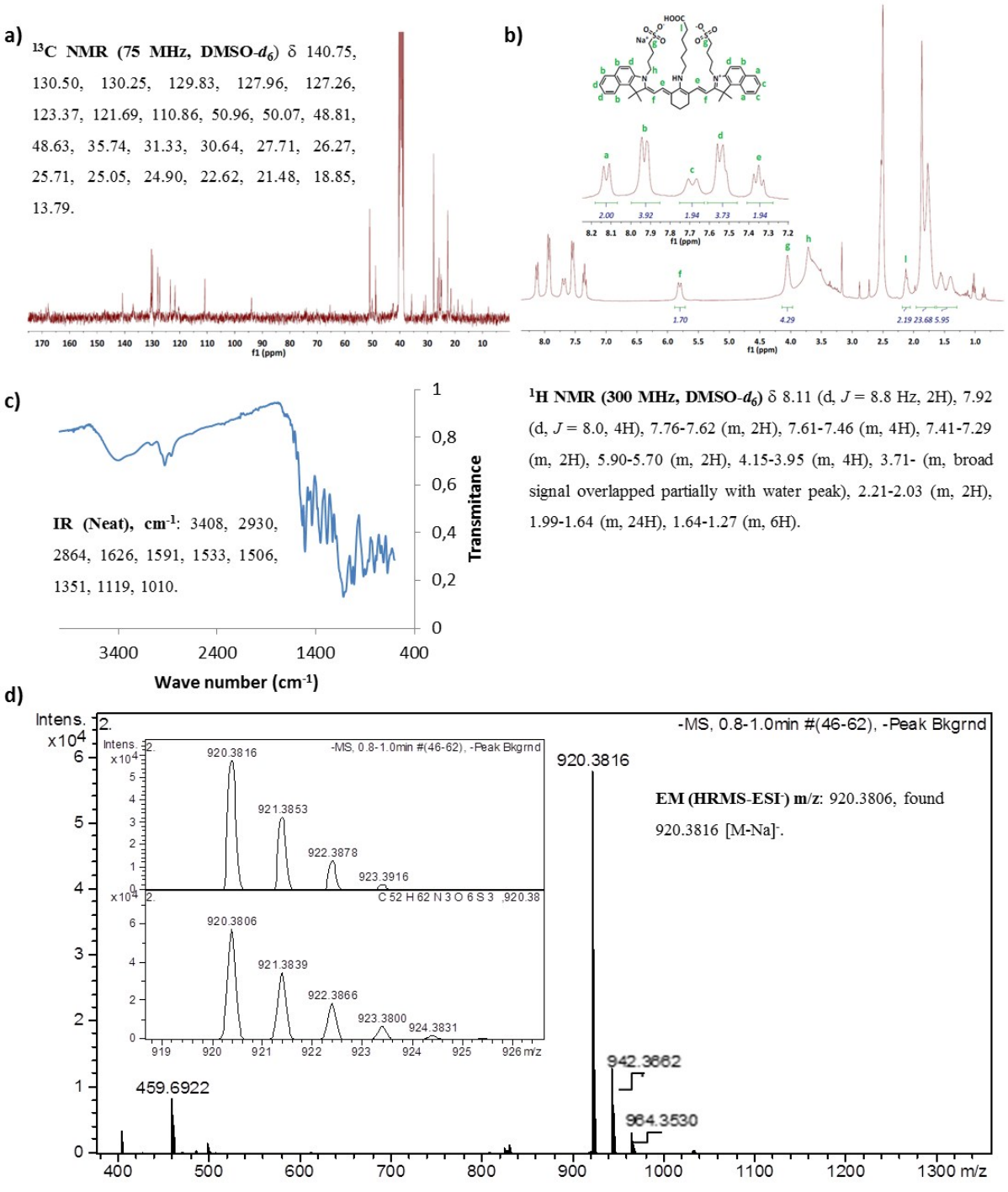


Figure S1. Characterization of modified IR820-COOH: a) NMR spectroscopy of ^{13}C; b) NMR spectroscopy of ^{1}H; c) FTIR; d) Mass spectrometry

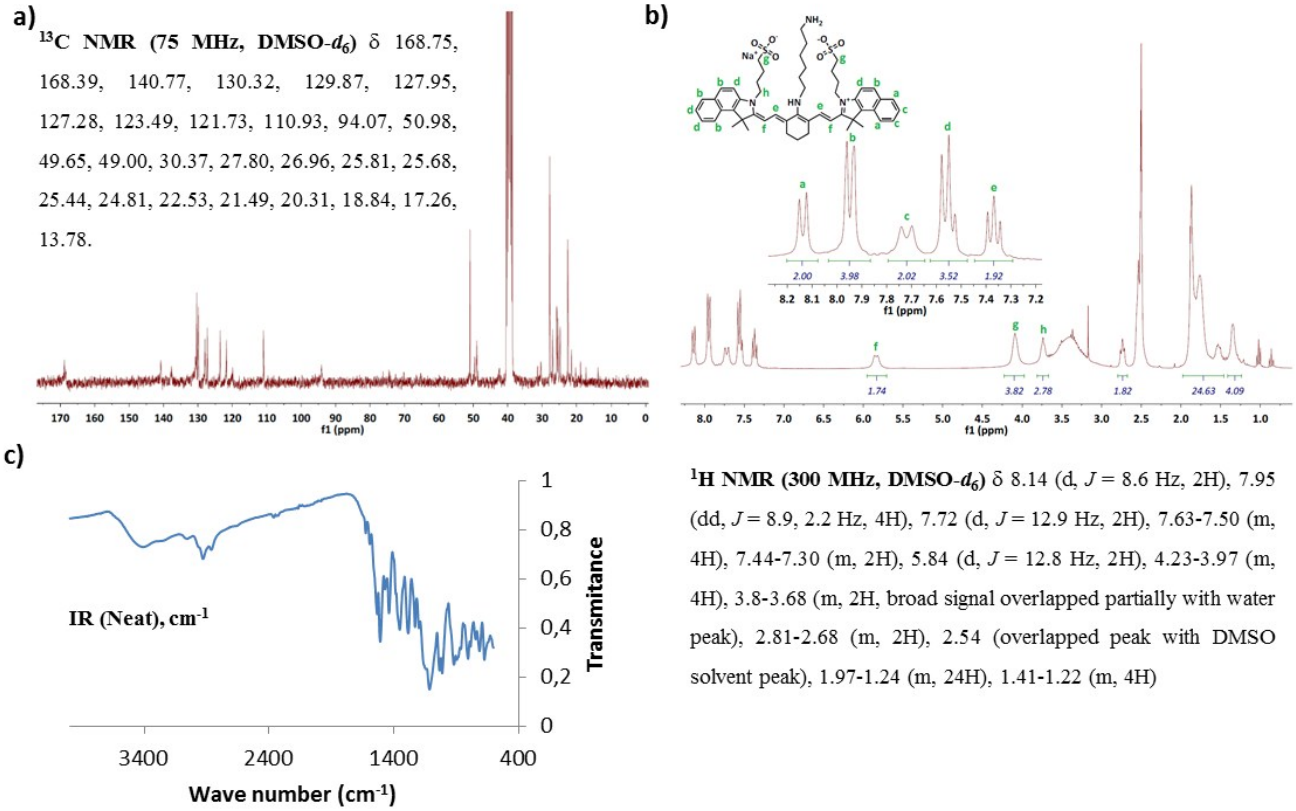


Figure S2. Characterization of modified IR820-NH_2: a) NMR spectroscopy of ^{13}C; b) NMR spectroscopy of ^{1}H; c) FTIR; d) Mass spectrometry

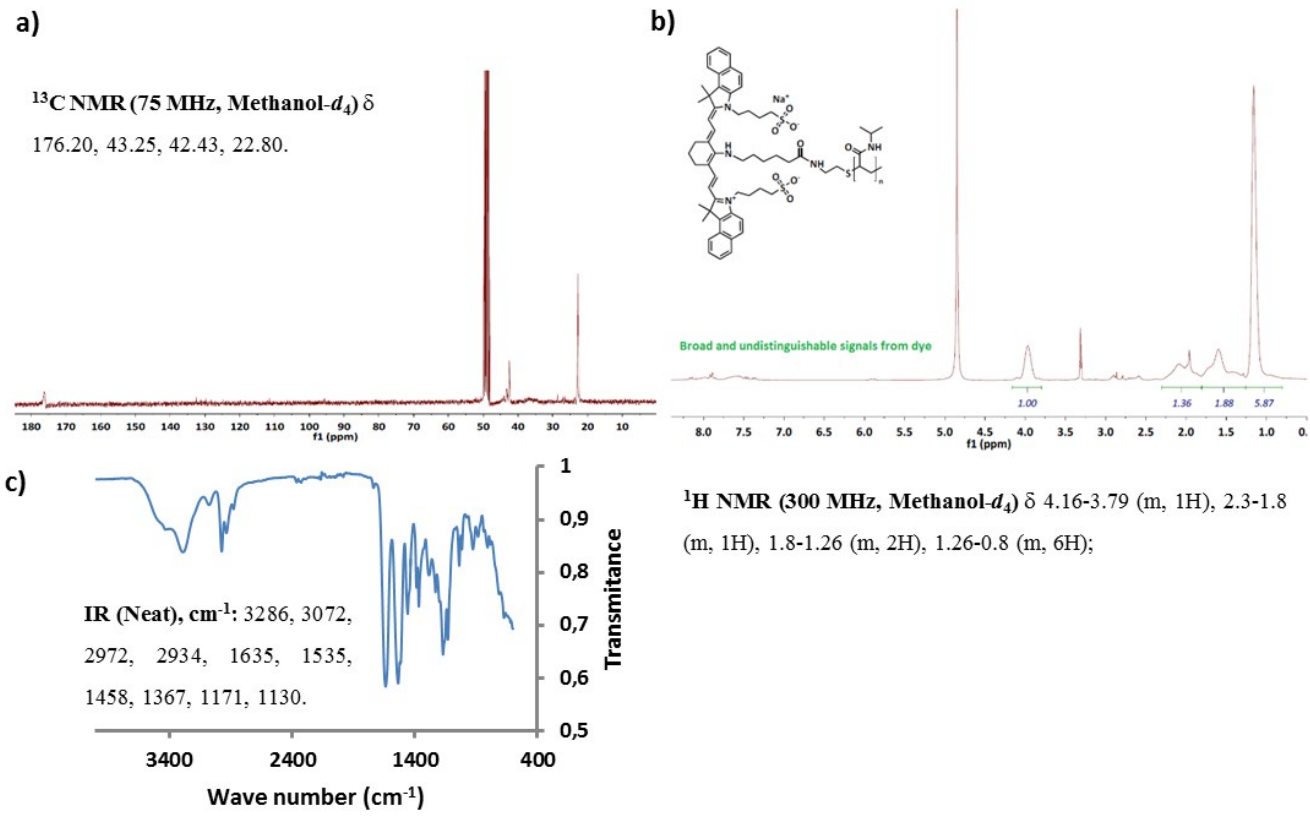
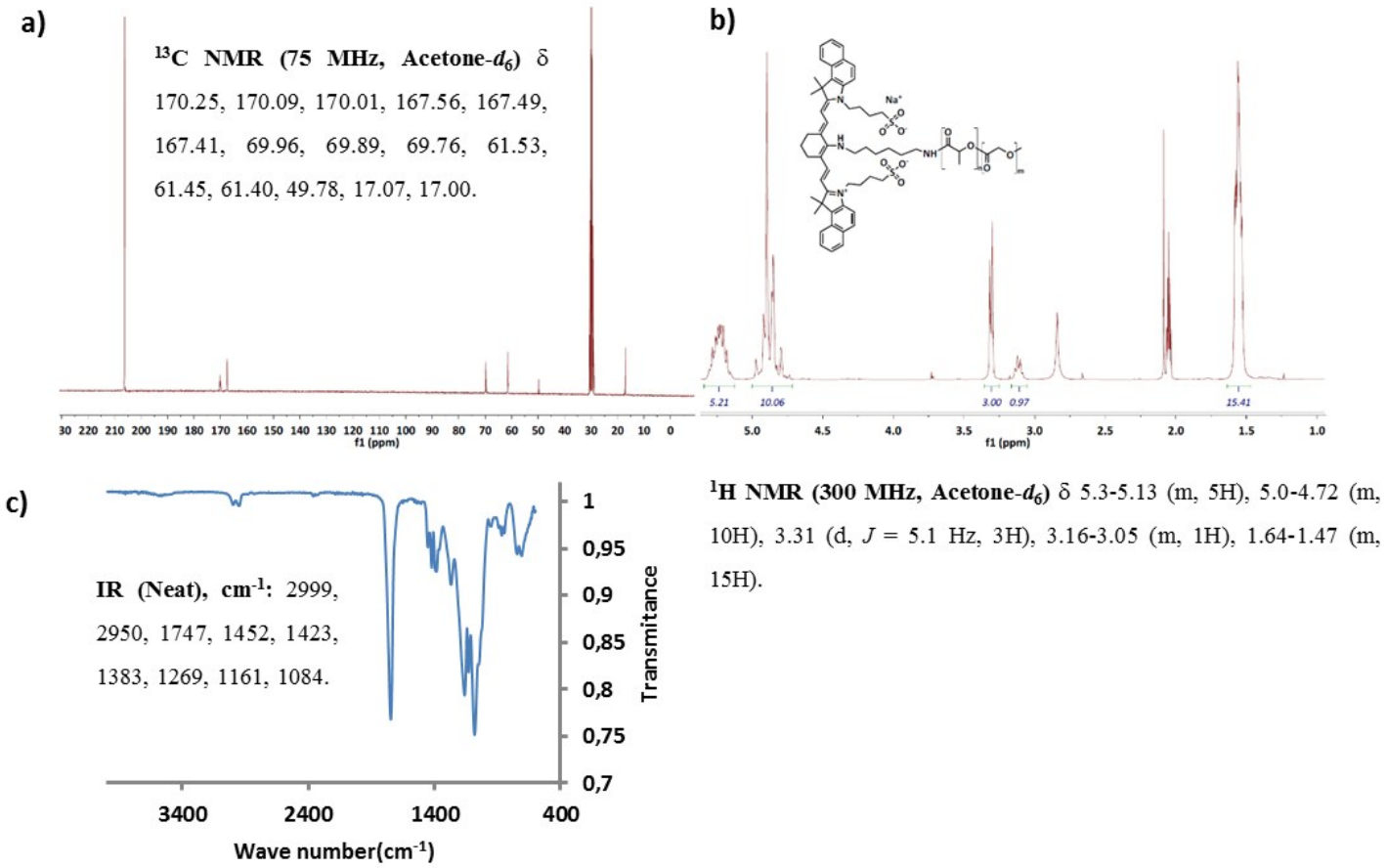


Figure S3. Characterization of PNIPAM-IR820-COOH: a) NMR spectroscopy of ^{13}C; b) NMR spectroscopy of ^{1}H; c) FTIR



^{1}H NMR (300 MHz, Acetone-d_6) δ 5.3-5.13 (m, 5H), 5.0-4.72 (m, 10H), 3.31 (d, J = 5.1 Hz, 3H), 3.16-3.05 (m, 1H), 1.64-1.47 (m, 15H).

Figure S4. Characterization of PLGA-IR820-NH$_2$: a) NMR spectroscopy of ^{13}C; b) NMR spectroscopy of ^{1}H; c) FTIR

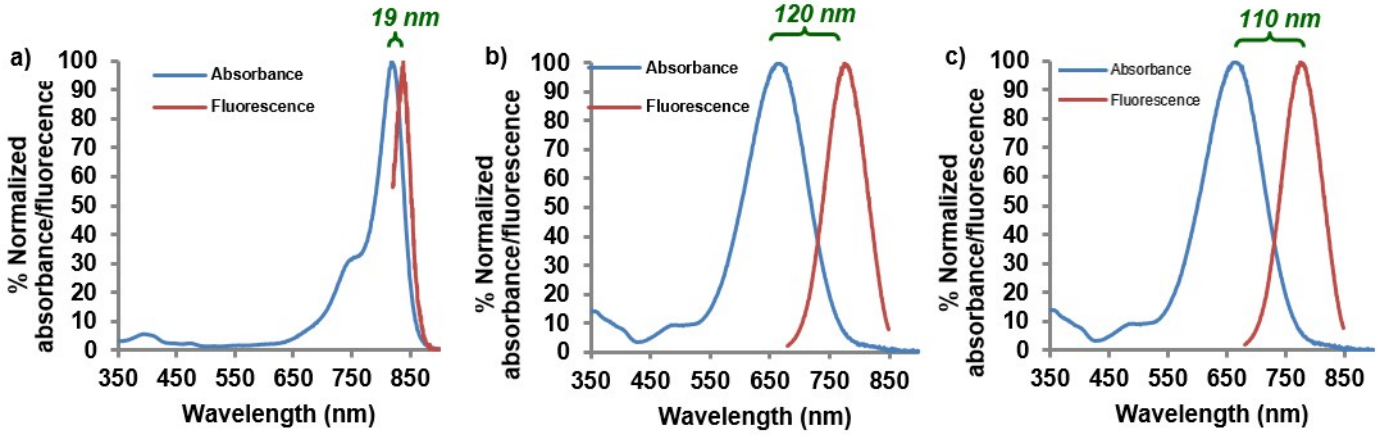


Figure S5. Normalized absorbance and fluorescence spectra in methanol: a) IR-820; b) IR820-COOH; c) IR820-NH_2.