Supporting Information

Polymer nanoparticle engineering for podocyte repair. From in vitro models to new nanotherapeutics in kidney diseases.

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Materials

ε-caprolactone (CL, 99%), 2-hydroxyethyl methacrylate (HEMA, ≥ 97%), 2-ethylhexanoic acid tin(II) salt ($Sn(Oct)_2$, ~ 95%), potassium persulfate (KPS, ≥ 99%), poly(ethylene glycol) methyl ether methacrylate (PEGMA, molecular weight 2080 Da), Rhodamine B (RhB, >95%), N,N'-dicyclohexyl carbodiimide (DCC, 99%), acetonitrile (anhydrous, > 99.8%) and 4-(dimethylamino) pyridine (DMAP, > 99%), 3-sulfopropyl methacrylate potassium salt (SPMAK, 98%) and [2-(methacryloxy)ethyl]trimethyl ammonium chloride (methacrylate choline chloride (MACC), 80% w/w in water), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH, 97%), sodium dodecyl sulfate (SDS>99%) were purchased from Sigma Aldrich and used without further treatment. Dexamethasone (DEX,>98%) was also supplied by Sigma Aldrich.

Synthesis of HEMA-CL_3 macromonomer

All NPs used in this work were obtained from HEMA-CL_3 macromonomer (HEMA grafted to 3 repeating units of CL), which was synthesized according to literature[1]. Briefly, 30 mg of $Sn(Oct)_2$ were mixed with 3.8 g of HEMA in a 10 mL vial under magnetic stirring at room temperature until full dissolution of $Sn(Oct)_2$ was obtained. Meanwhile, 10g of CL were heated up to 130°C in a stirred flask and the temperature was controlled by an external oil bath. The HEMA/$Sn(Oct)_2$ solution was then added to the flask, and the reaction was carried out for 2 hours.

Characterization of HEMA-CL_3 macromonomer

HEMA-CL_3 macromonomer characterization was carried out by ^{1}H-NMR spectroscopy. The sample was dissolved in $CDCl_3$ and the spectra were recorded on a 400 MHz apparatus (Brucker, Germany).

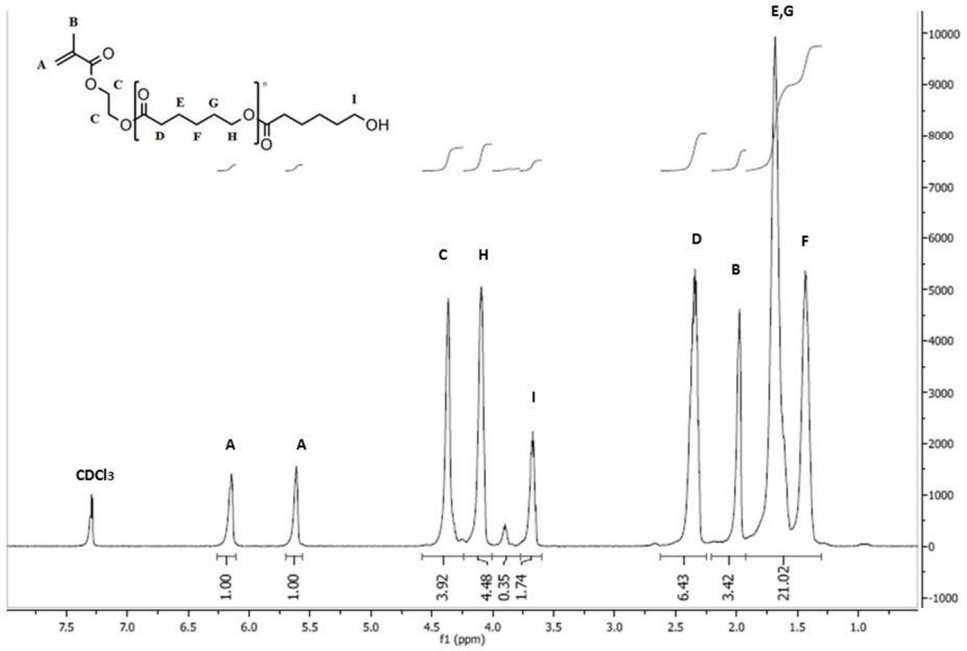

Figure S1. 1H-NMR spectrum of the HEMA-CL_3 macromonomer

According to the spectrum in Figure S1 the number of repeating unit can be evaluated as follows:

$$Mw = MwHEMA + MwCL\left[\frac{H(methylene\ proton\ signal)}{I(terminal\ methylene\ proton\ signal)} + 1\right]$$

where MwHEMA are the molecular weight of HEMA and ε-caprolactone, respectively. The term in brackets represents the average number of CL repeating units of the oligomer. According to Figure S1, H is representative of the total number of repeating CL units, while I is the total number of terminal groups.

Synthesis of fluorescent HEMA-RhB monomer

HEMA-RhB was prepared through a DCC coupling as reported in the literature[2, 3]. 1g of RhB together with 0.325 g of HEMA were dissolved in 30 mL acetonitrile under magnetic stirring at room temperature. 0.43 g of DCC and 13 mg of DMAP were dissolved in 20 mL of acetonitrile; this solution was added dropwise to the reacting mixture in 20 minutes. The reaction was carried out in the dark at 40°C under magnetic stirring for 24h and then quenched in an ice bath for 10 minutes. Dicyclohexylurea, the side product of the coupling reaction, was eliminated from the solution through filtration and finally acetonitrile was removed using rotatory vacuum.

Characterization of HEMA-RhB monomer

In order to confirm the structure and the molecular weight of the obtained purified HEMA-RhB monomer, MALDI ESI analyses were carried out with a maXis ESIQ-TOF (Bruker, Switzerland) mass spectrometer equipped with an automatic syringe pump for sample injection (KD Scientic, US). The ESI-Q-TOF mass spectrometer was running at 4500 V, with desolvation temperature of 200 C. The mass spectrometer was operated in the positive ion mode; nitrogen was used as nebulizer and drying gas. The standard electrospray ion (ESI) source was used to generate ions and chloroform (CH_3Cl) was used as a solvent. Sixty shots from each spot were averaged to obtain one mass spectrum. The ESI-Q-TOF MS instrument was calibrated in the m/z range 50–1300 with the use of an external calibration standard supplied by Agilent. The results are reported in Figure S2[1].

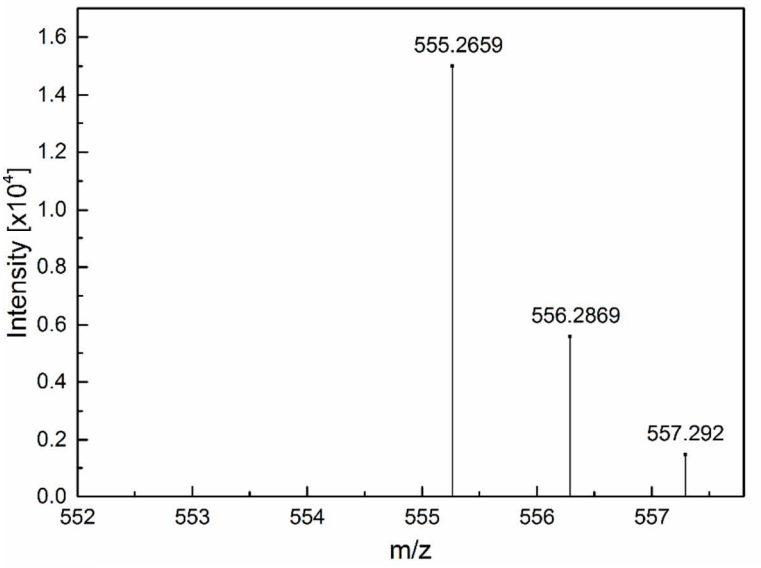

Figure S2: ESI spectrum of the HEMA-RhB macromonomer.

The data confirm the good purity of the produced material, with the main peak at 555.2 Da which corresponds to the macromonomer with the addition of a sodium atom (+ 23 Da), and the chlorine atom coming from the RhB chloride is not present (-35 Da)[2].

Polymer Characterisation

NP2 nanoparticle suspension was dried under air stream and the resulting dried polymer was dissolved and analyzed via GPC and NMR without further purifications. Results are reported in Figure S3.

From 1H-NMR it is possible to appreciate the presence of both PEG and CL3 repeating units, with the protons of the methyl group of the polymeric backbone (1.2 – 0.80 ppm). Quantitative monomer conversion was confirmed by the disappearance of the vinyl protons (6.2-5.6 ppm).

GPC analysis reported a monomodal molecular weight distribution. A number average molecular weight (M_n) of 18.5 kDa and a dispersity (M_w/M_n) of 2.1 was determined.

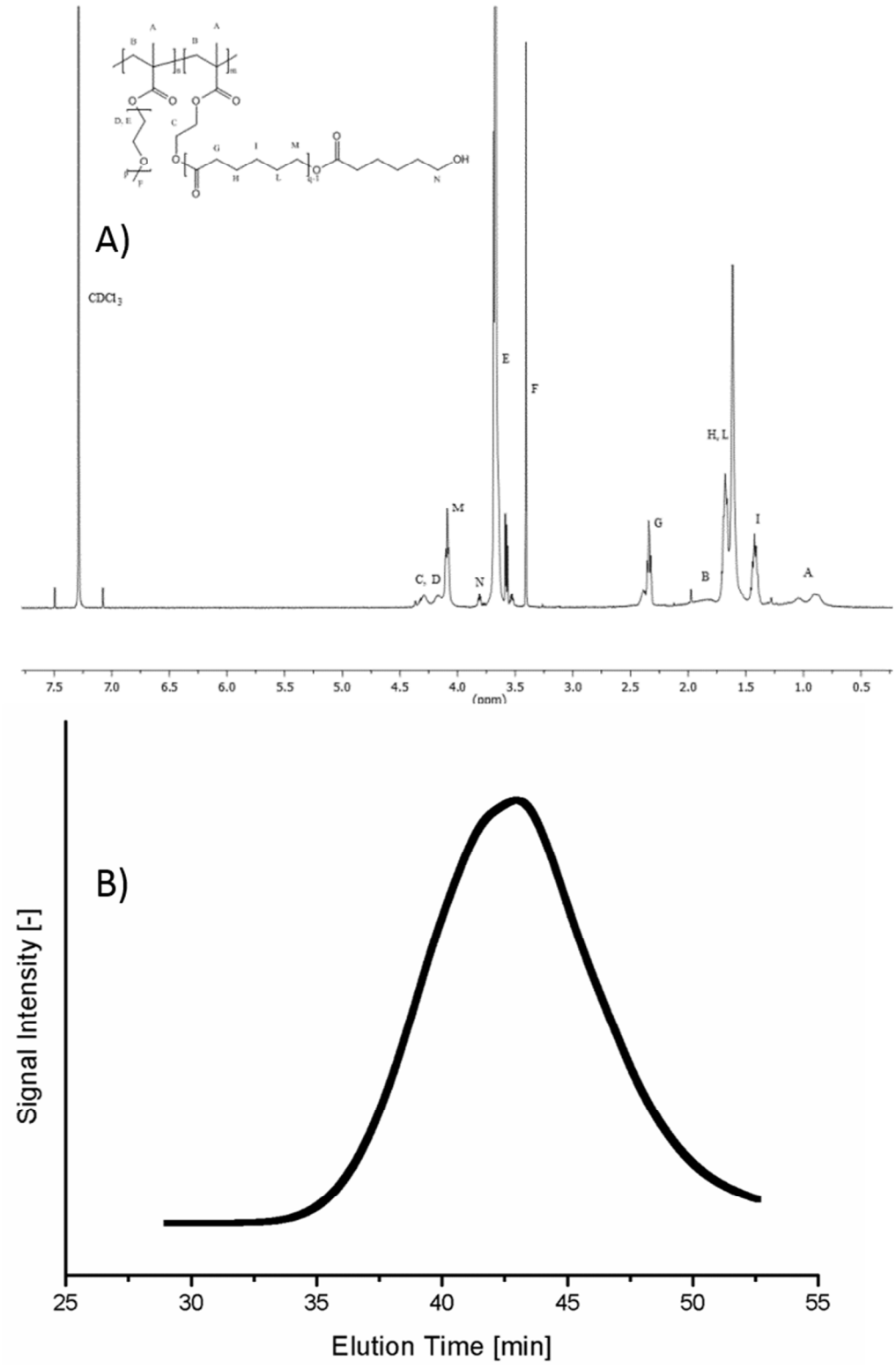

Figure S3: ^{1}H-NMR spectrum of dried polymer from NP2 nanoparticles in $CDCl_3$ (A) and GPC chromatogram (in THF) of the same material (B). A number average molecular weight (M_n) of 18.5 kDa and a dispersity (M_w/M_n) of 2.1 was determined.

Transmission Electron Microscopy (TEM) Characterisation

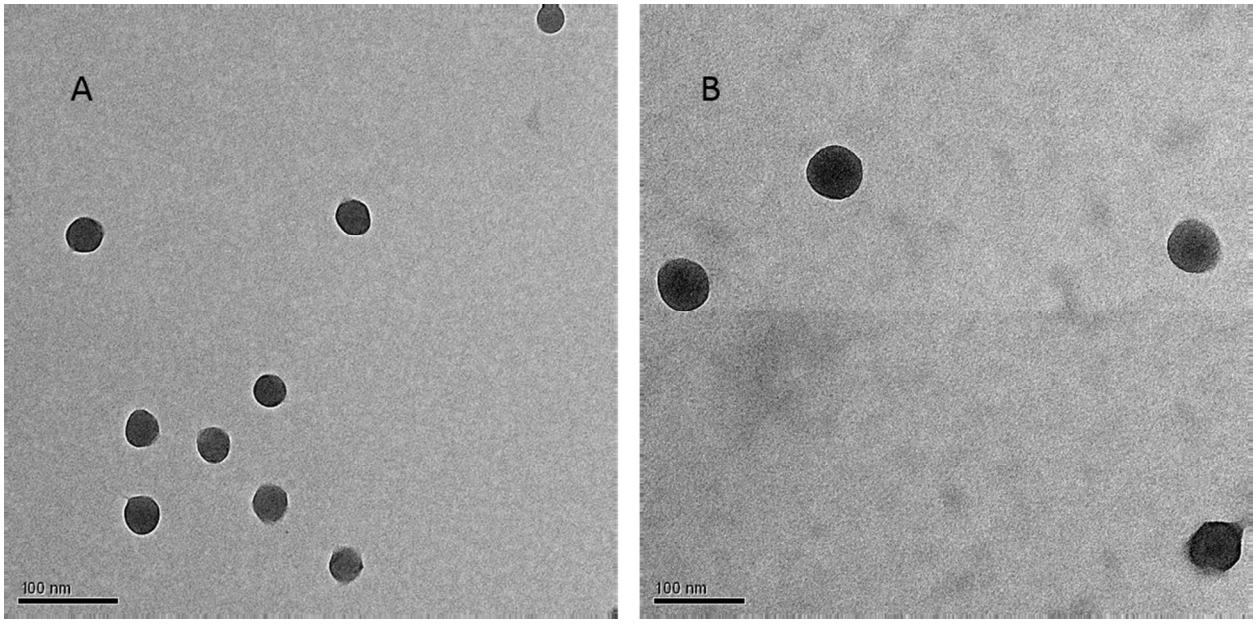

Figure S4: TEM pictures of sample NP5 and NP6.

LDH Cytotoxicity

NPs cytotoxicity was measured using LDH-Cytotoxicity Colorimetric Assay Kit (BioVision Incorporated). Briefly 6000-8000 per well of conditionally immortalized murine kidney podocytes SV1 (CLS Cell Line Service Ltd, Eppelheim, Germany) were plated on a 96-well plate and cultured at 37°C in DMEM/F-12 medium supplemented with 10% FCS, 5 µg/mL transferrin, 10-7M hydrocortisone, 5 ng/mL sodium selenite, 0.12 U/mL insulin, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mM L-glutamine, and without γ-interferon for 3-4 days. Then, the culture medium was replaced by medium containing different concentration of NPs (0.01-2mg/mL) which was incubated with cells for 24 hours. For positive control (high control), 10 µL of cell Lysis solution was added and incubated for 24 hours, while the low control was referred to cells incubated only with standard medium. At the end of incubation, the plate was

gently shacked for some minutes and centrifuged at 600 x g for 10 min. 10 µL of culture medium from each well was transferred into a new optically clear 96-well plate, and 100 ul of LDH Reaction Mix was added to each well and incubated at room temperature for 1 to 2 hours (depending on the colour development). The absorbance of all controls and samples was measured with 450 nm filter using SAFAS Spectrophotometry (Monaco). The cytotoxicity was calculated using the equation: Normalised Cytotoxicity = (Test sample-Low control)/(High control-Low control) ; Low control : normal cells; High control : cells treated with lysis buffer.

Nanoparticle uptake.

Methods

6000-8000 per well of SV1 cells were plated on a 96-well plate and cultured at 37°C without γ-interferon for 3-4 days. The culture medium was replaced by medium containing different concentration of NPs labelled with rhodamine (0.01 – 2 mg/mL) which was incubated with cells for 24 hours. At end of incubation, the supernatants were removed and the cells were thoroughly washed three times with PBS. The intracellular NPs as well as the NPs in supernatant was measured using SAFAS Spectrophotometry at 540 nm of Excitation and 584 of Emission wavelength. To adjust the cell plating difference among the wells, Janus Green cell normalization stain was performed. Briefly, after Spectrophotometry reading, cells were fixed with 4% of Paraformaldehyde, stained with 1X Janus Green Solution (Abcam) for 5 minutes at room temperature. After thoroughly wash, 0.1 mL of 0.5 M HCl per well was added and incubated with cells for 10 minutes. The optical density at 595 nm was measured using SAFAS Spectrophotometry.

Results

The effect of nanoparticle concentration on the internalisation process was assessed by measuring the percent uptake by fluorescence analysis. PEGylated nanoparticles (NP1-NP4) showed limited uptake at any size and concentration (% uptake <10% at 0.2-2mg/mL, Figure S5,A). On the other hand, negatively charged nanoparticles (NP8-NP10) showed a marked percent uptake at low concentration (NP10 reached a 60% uptake at concentration < 0.1mg/mL, Figure S5,B). The concentration dependence of the percent uptake (which is high at 0.1mg/mL and low at 1mg/mL) may indicate an energy-dependent endocytic mechanism, since podocytes are effective in internalising a large fraction of nanoparticles at low concentration but tend to saturate at higher particle concentrations. Small particles (NP8, 33nm) showed lower percent uptake when compared to particles bigger than 60nm (NP9, NP10), whereas no relevant difference were appreciated between nanoparticles at a size range of 60-120nm (figure S5,B). When the particle size was fixed (NP1, NP8, NP11, 40nm range) the uptake was dependent on particle surface (figure S5,C); at particle concentration of 0.1mg/mL the percent uptake varied from ~10% for PEGylated nanoparticles (NP1) to ~40% for negatively charged NPs (NP8), and increased up to ~60% for positively charged NPs (NP11). Moreover, positively charged NP11 also showed much higher percent uptake compared to NP1 and NP8 even at a concentration of 2mg/mL, and this offset may be due to a concurrent passive mechanism which involve electrostatic interactions and particle adsorption to cell membrane[4].

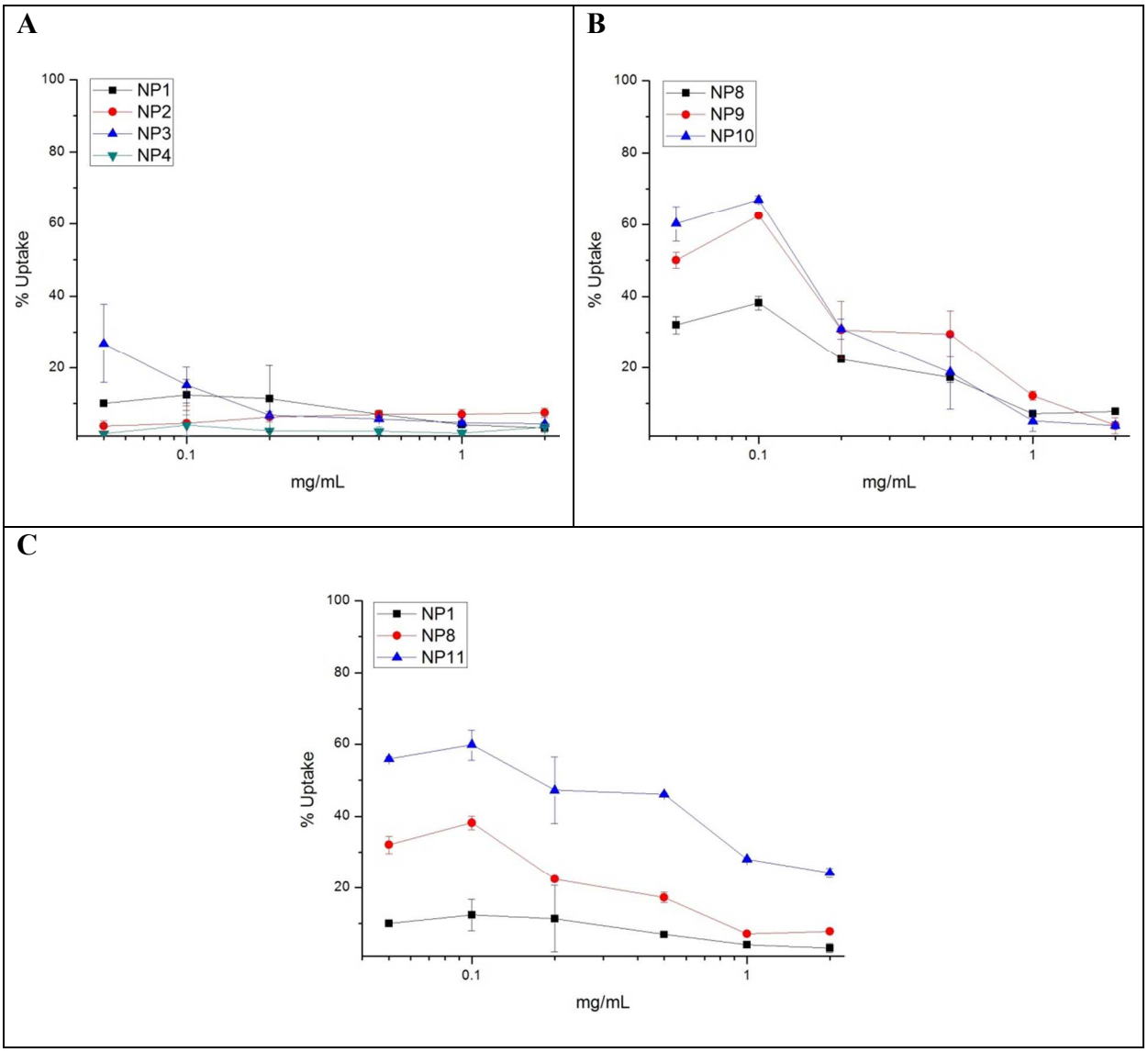

Figure S5. Percent uptake by podocytes of nanoparticles NP1-NP11 incubated for 24h at different concentrations (0.05-2mg/mL). A) Comparison between PEGylated NPs at different size (NP1 37nm, NP2 40nm, NP3 70nm, NP4 118nm). B) Comparison between negatively charged NPs (NP8 33nm, NP9 64nm, NP10 117nm). C) Comparison between small PEGylated nanoparticles (NP1), small negatively charged NPs (NP8), and small positively charged NPs (NP11).

Co-culture system and assessment of permeability

1μm porous Biopore membranes (Millicell hanging cell culture inserts, made of Polyethylene Terephthalate (PET), Millipore, Milan, Italy) were coated on both sides with collagen type IV, as previously reported[5]. 1.5 x 10^5 of endothelial cells (EOMA, CRL-2586, LGC Standards S.r.l., Italy) were seeded on the lower side of the membrane and exposed to VEGF 5 ng/mL for one week before 65000 SV1 cells were seeded on the upper side of the membrane and incubated in their own medium.

In order to assess albumin permeability, cells were carefully washed on both sides with PBS. Podocyte damage was induced by adding to the podocyte medium Adriamycin (doxorubicin hydrochloride, 0.8 μM) and incubating for 24 hours. Then, the upper (podocyte) compartment was filled with either with standard medium (as control) or with DMEM/F-12 containing DEX-loaded NP8 nanoparticles (DEX 5% w/w, total nanoparticle concentration range 2÷100 μM) and the system was incubated for 24÷48hr. Afterwards, the lower (endothelial) compartment was filled with DMEM/F12 supplemented with 40mg/mL of Bovine serum albumin (BSA, Sigma-Aldrich). Medium was taken from the upper compartment after 2 hours incubation and albumin content was measured by spectrometry using the DC protein assay kit (Bio-Rad, Milano, Italy). Results were expressed as percentage of BSA permeability versus control conditions. Coomassie-stained gels were used to confirm that BSA was the only protein present in the medium, excluding the contribution from cellular proteins.

Stability of fluorescent nanoparticles with podocytes at different incubation times

The chemical stability of our fluorescent nanoparticles was confirmed by monitoring the localisation and shading of red fluorescence at different incubation times (Figure S6). After 96h incubation, red staining was still evident and compartmentalised in the cell body, and the reduced amount of red spots per cell should be mostly ascribed to cell division and proliferation during the time of incubation.

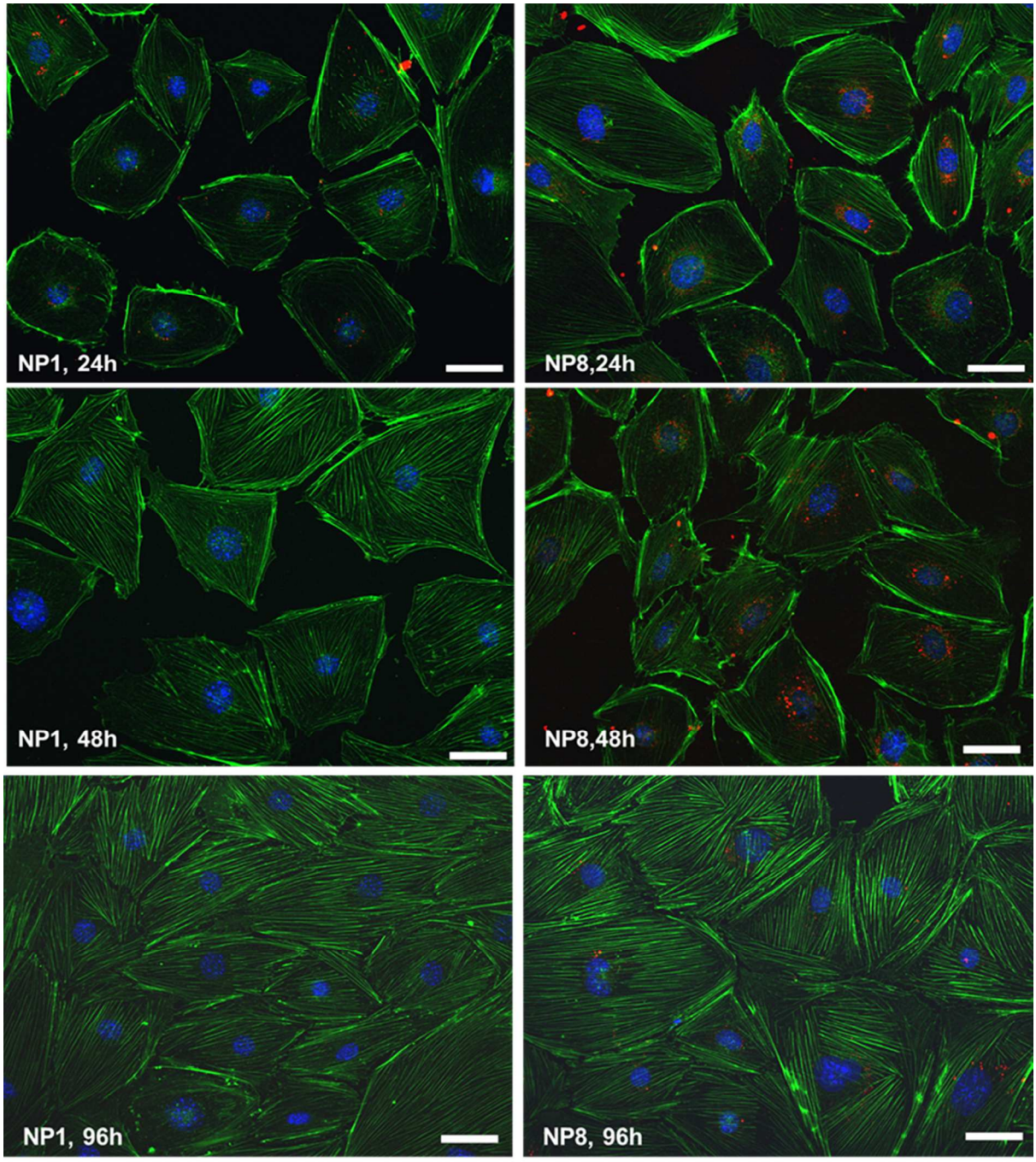

Figure S6. Fluorescence of uptaken nanoparticles (NP1, NP8) by podocytes at different incubation time (24-96)h. Scale bar 50 µm.

References

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