# Endosome-escapable magnetic poly(amino acid) nanoparticles for cancer diagnosis and therapy

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## Materials

Iron (III) acetylacetonate, benzyl ether, oleic acid (90%), oleylamine (> 70%), 1,2-hexadecanediol (90%), L-aspartic acid, mesitylene, sulfolane, aminoethanol, octadecylamine, 1,3-Dicyclohexylcarbodiimide, 4-dimethylaminopyridine, trifluoroacetic acid, triisopropylsilane and chloroform were purchased from Sigma-Aldrich and were used as received. Phosphoric acid, dimethyl sulfoxide, and N,N-dimethylformamide were purchased from Junsei. Dimethyl sulfoxide-$d_6$ (DMSO-$d_6$) used in NMR experiments were Sigma-Aldrich products.

## Synthesis of the histidine and octadecyl grafted poly(amino acid)s

The precursor polymer, poly-(succinimide) (PSI), was synthesized via acid-catalyzed polycondensation of L-aspartic acid using phosphoric acid as the catalyst. Purified PSI (0.97 g, 10 mmol succinimide unit) was dissolved in water-free DMF (7 ml), followed by aminolysis with 10 mole % of octadecylamine at 70$^{o}$C for 25h and aminolysis with of 120 mole % of 2-amino-ethanol at room temperature 6 h. (PHEA-g-$C_{18}$). The reaction mixture was precipitated in cold ether and dried in vacuo at 50$^{o}$C. The product was dialyzed against DDI water, and freeze-dried. For fluorescence study, FITC modified with hexamethylenediamine was added to the reaction mixture. The grafting mole % of FITC was less than 2.

Histidine moieties were grafted on PHEA-g-$C_{18}$ by a DCC-mediated reaction. One equivalent of Nα-Boc-L-histidine and three equivalents of DCC and DMAP were added to DMF solution of PHEA-g-$C_{18}$. The reaction mixture was stirred at room temperature for 2 days. The insoluble products were filtered out, and the clear reaction mixture was precipitated into cold ether. The product was reacted with TFA/TIS/water (95/2.5/2.5vol.%) solution for the deprotection of BOC groups of conjugated Nα-Boc-

L-histidine. The reaction mixture was precipitated into cold ether. The product was dissolved in dimethyl sulfoxide, extensively dialyzed against DDI water for 3days, and then freeze-dried

**Synthesis of as-synthesized iron oxide nanocrystals**

6 nm sized iron oxide nanocrystals were synthesized using a thermal decomposition method. Briefly, iron (III) acetylacetonate (2 mmol), 1,2-hexadecanediol (10 mmol), oleic acid (6 mmol), oleyl-amine (6 mmol), and benzyl ether (20 mL) were mixed in a three-neck round flask under an $N_2$ atmosphere. Next, the mixture was heated to 200 $^oC$ for 2 h and further heated to 300 $^oC$ for 1 h under reflux. After the mixture was cooled to room temperature, excess ethanol was used to wash the reactant. Nanocrystals were collected by centrifugation.

**Preparation of histidine-conjugated magnetic poly(amino acid) nanoparticles (H-MPNs)**

Mixture of PHEA-g-$C_{18}$-His solution in water (50 mg in 5 mL) and iron oxide nanocrystals solution in chloroform (10 mg in 0.2 mL) was vortexed at 3000 rpm for 30 minutes and followed by sonication for 5 minutes to generate an emulsion. Chloroform was evaporated using a rotary evaporator and unloaded iron oxide nanocrystals were removed by centrifugation at 7000 rpm for 10 minutes. H-MPNs were selectively collected by centrifugation at 15000 rpm for 1 hour.

**Preparation of doxorubicin-loaded H-MPNs**

Doxorubicin·hydrochloride (DOX·HCl) (10 mg) was stirred with 2 equivalents of triethylamine in DMSO (1ml) for 12h. The 0.15 mL of DOX solution was added drop-wise into 3 mL of H-MPNs (15 mg) solution at room temperature and shook for 30 min. The mixture was extensively dialyzed against PBS buffer (pH7.4) and then freeze-dried.

The amount of entrapped DOX in aggregates was determined by spectrophotometric measurement at 481 nm after dissolving aggregates in DMSO.

**Characterization histidine-conjugated magnetic poly(amino acid) nanoparticles (H-MPNs)**

Transmission electron microscopy (TEM) was obtained using a Philips CM-200 instrument operating at 200 kV. A solution of H-MPNs containing 0.1% (w/v) phosphotungstic acid (PTA; a negative stain) was placed on a copper grid covered with a formvar carbon membrane. Hydrodynamic sizes of nanoparticles were measured by dynamic light scattering employing the particle size option in the Malvern instrument (Zetasizer nano zs). Fourier transform infrared (FTIR) spectra were recorded using a Spectrum GX & AutoImage instrument (Perkin-Elmer) at room temperature. The saturation of magnetization was evaluated using a vibrating-sample magnetometer (Lakeshore, model 955287(A)). The atomic weight percentage of Fe in each PAION was measured by inductively-coupled plasma atomic emission spectrometer (ICP-AES, model : Jarrell Ash IRIS-AP, Thermo) for further in vitro study.

**Drug release experiment**

5 mg of DOX-loaded H-MPNs and MPNs in a buffer (1 ml) solution was sealed in a dialysis membrane (MWCO 12,000) and incubated in a PBS buffer (pH 7.4, 20 ml) and an acetate buffer (pH 5.2, 20 ml) at 37°C. The whole medium was taken and replaced with a fresh buffer at every time interval. The concentration of released DOX was quantified by an UV spectrophotometer (Jasco) at 480 nm. The release experiment was performed in triplicate, and the obtained values were used to calculate a mean value and standard deviation

**MRI Phantom study**

A total of $1 \times 10^6$ cells were seeded on each well of a six-well plate and grown for 24 h. The cells were then incubated with a medium containing H-MPNs. After 24 h, the medium was removed and then the cells were washed with a PBS solution. The labeled H-MPNs and control cells were, respectively, detached from the well using a trypsin/EDTA solution and harvested by centrifugation at 9000 rpm for 3 min. The cells were resuspended in a PBS solution containing 1% paraformaldehyde, followed by incubation at 4 °C for 2 h. The cells were subsequently washed with PBS solution and again harvested at 9000 rpm for 3 min. Cell pellets were finally suspended with 2% solution of low melting agarose, solidified at room temperature, and kept at 4 °C. All MR imaging experiments were performed using a 4.7 T clinical MRI instrument (Bruker BioSpec 47/40). The parameters were as follows: TE = 7.4 ms, TR = 8000 ms, FOV = 5.5 × 5.5 cm, matrix = 128 × 128, slice thickness = 2 mm.

**Prussian blue staining**

To demonstrate ferric iron on HeLa cells, $2.5x10^4$ cells were seeded on each well of 8-chamber slide (Nunc, Roskilde, Danmark) and grown for 24 h. Then the cells were incubated with H-MPNs containing medium. After 6 h, the medium was removed then cells were washed with PBS solution. The cells were generally fixed with 4% paraformaldehyde (Sigma-Aldrich, MO,USA) for 20 min at room temperature. After removing the fixing agent, the cells were washed and stained according to manufacturer's protocol (Prussian Blue Iron Stain Kit, Polyscience, PA, USA). Briefly, the H-MPNs-labelled and control cells were incubated with 1:1 mixture of 4% potassium ferrocyanide and 4% hydrochloric acid for 20 min, and washed with distilled water several times. To stain nuclei of the cells, Nuclear Fast Red solution (Polyscience, PA, USA) was treated to the cells for 5 min and then rinsed in running tap water for 1

min. After drying cells, cover slip was mounted by using mounting medium (DAKO, CA, USA) and then the cells were observed using light microscopy.

**Cell viability: MTT assay**

$5x10^3$ HeLa cells were seeded on each well of a 96-well plated and cultured for 12 h at $37^oC$. Various concentrations of H-MPNs or MPNs in a serum-free medium were then treated to the cells for 24 h. After incubation, the medium containing H-MPNs was exchanged with a fresh medium, and 10 μL of MTT regent (5 mg/mL of stock solution) were added to each well. After culturing for 2 h, The 100 μL of DMSO were added to each well at room temperature. The absorbance of sample was measured by using a microplate reader (Bio-rad model 680) at 570 nm. The viability of cells was determined as the percentage of viable cells of the untreated control and analyzed in triplicate.

**Confocal microscopy**

$2 \times 10^3$ cells were seeded on each well of a u-slide 8-well microscopy chamber slides (ibidi Integrated Biodiagnostics, Munich, Germany) and incubated at 37 $^oC$ for 12 hr. The cell medium was replaced with 0.125 mg/mL of H-MPNs or MPNs solution and discarded after an appropriate incubation at 37 $^oC$. The cells were washed twice with PBS and submerged in a fixation and permeabilization solution (BD biosciences). After storage for 20 min, the cells were washed twice with PBS and treated with Hoechst 33342 to stain the nuclei of the cells for 20 min. The cells were washed twice with PBS and visualized by a Deltavision RT (Applied Precision Technologies, Issaquah, WA).

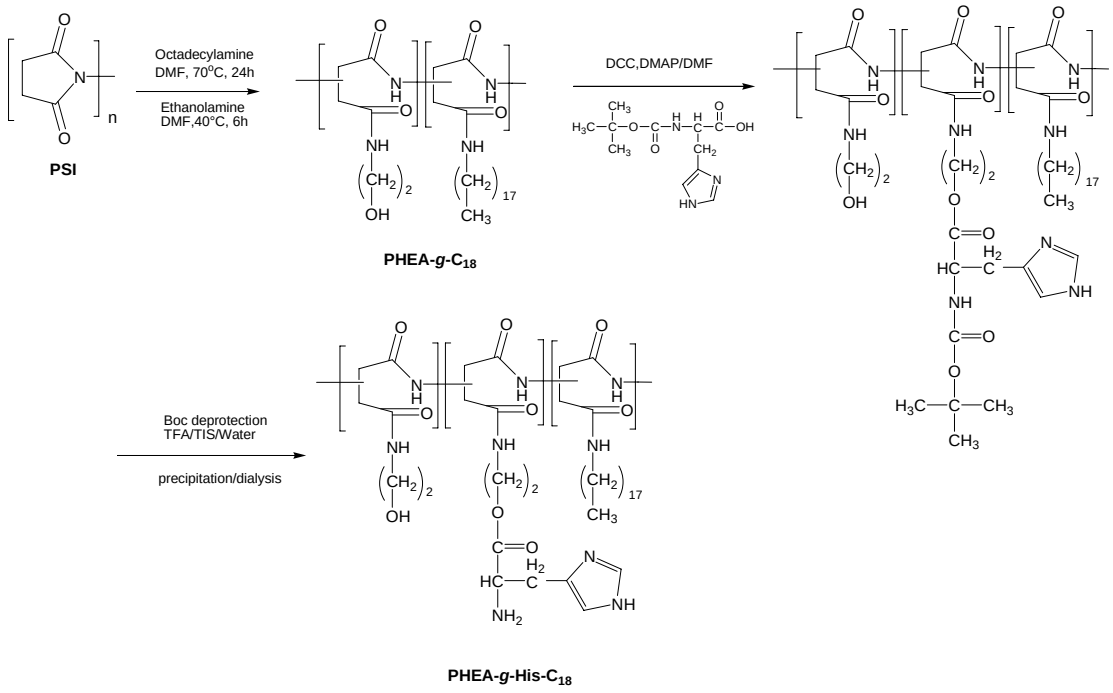


**Fig. S1**. Synthetic route and molecular structure of PHEA-*g*-$C_{18}$-His.

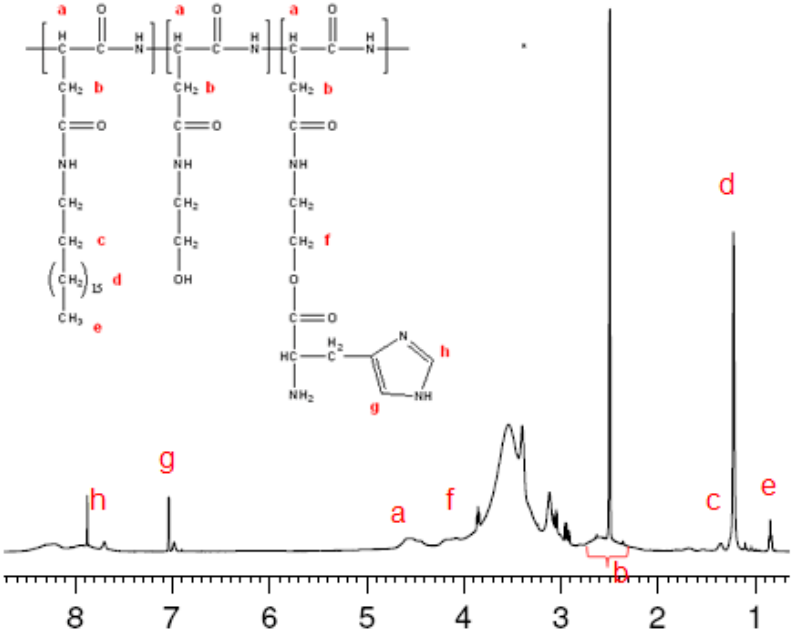


**Fig. S2.** $^{1}$H NMR spectra of PHEA-*g*-$C_{18}$-His in DMSO-$d_6$

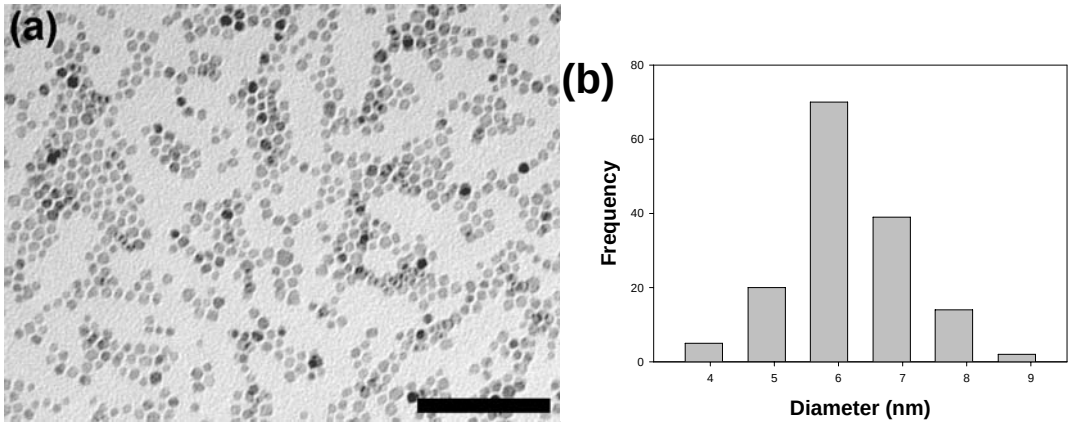


**Fig. S3.** (a) TEM image and (b) size histogram of as-synthesized iron oxide nanocrystals. Scale bar represent 200 nm.

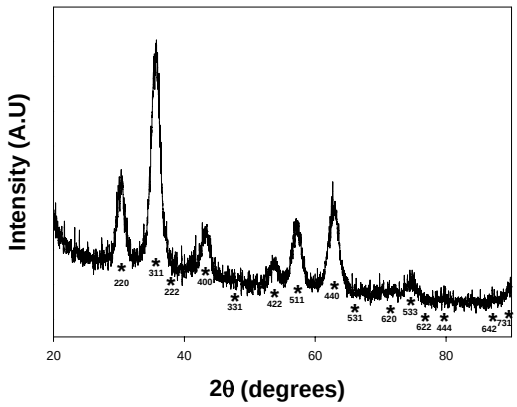


**Fig. S4.** X-ray diffraction patterns of 6 nm sized iron oxide nanocrystals.

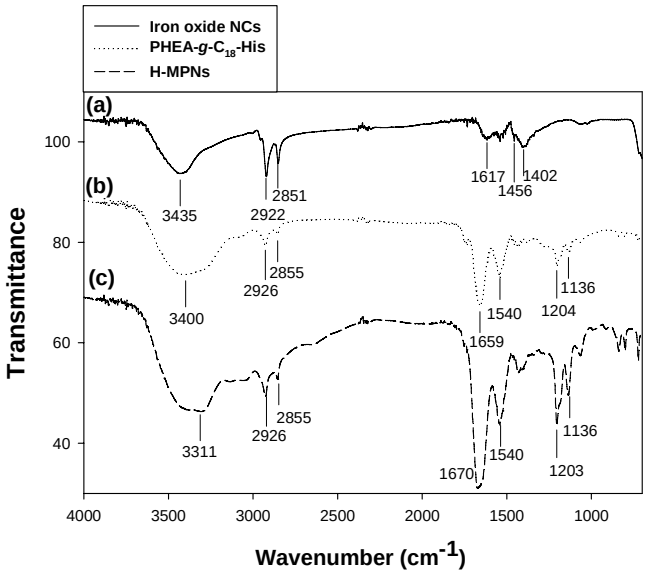


**Fig. S5.** FT-IR spectra of (a) 6 nm sized iron oxide nanocrystals, (b) PHEA-*g*-$C_{18}$-His and (c) H-MPNs.

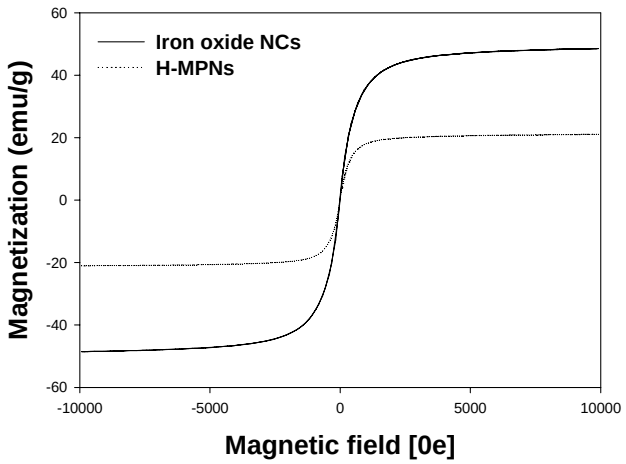


**Fig. S6.** Magnetization curves of iron oxide nanocrystals and H-MPNs at room temperature.

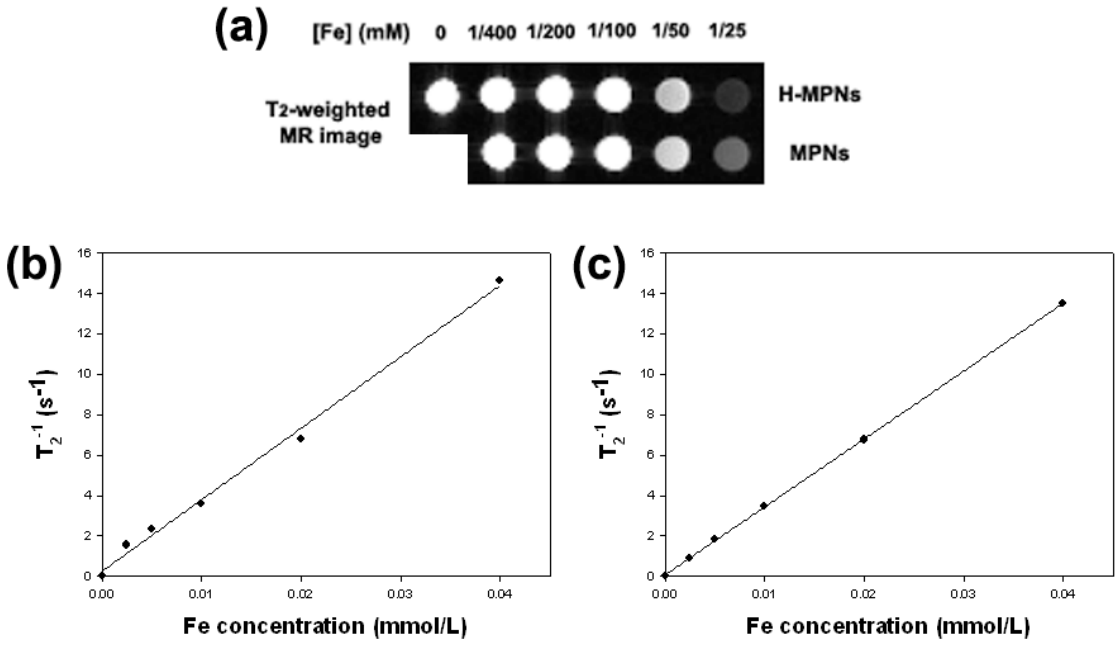


**Fig. S7** (a) $T_2$-weighted MR images of H-MPNs and MPNs in aqueous solution with various concentrations at 4.7 T. (b) graph of $R_2$ against the iron concentration in H-MPNs. (c) graph of $R_2$ against the iron concentration in MPNs.

**Table S1.** $T_2$ relaxation time of HeLa cells without or with H-MPNs and MPNs.

| | Control | 10 μg/mL | 20 μg/mL |
|---|---|---|---|
| H-MPNs | 147.6 ms | 59.6 ms | 50.5 ms |
| MPNs | | 70.7 ms | 55.3 ms |

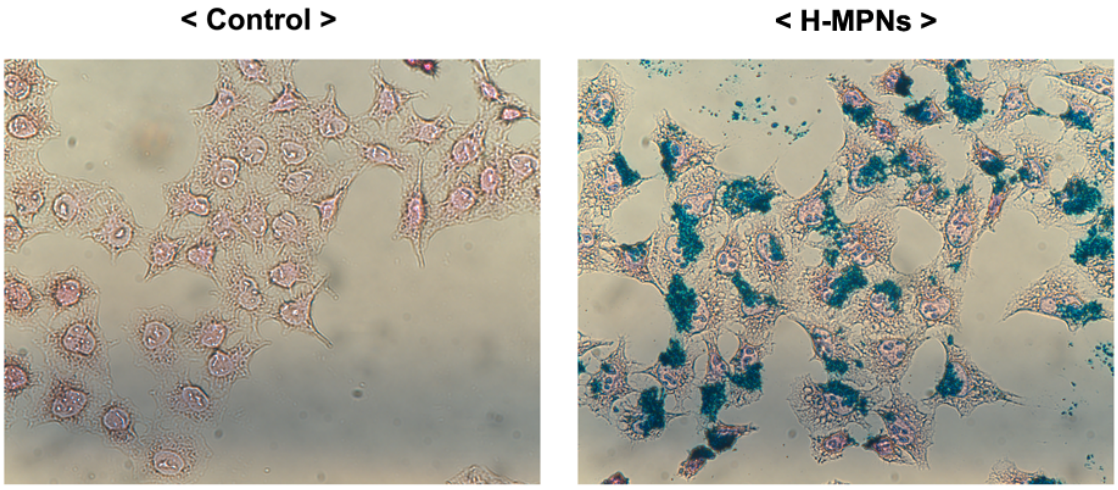


**Fig. S8**. Prussian blue staining images of cancer cells treated with DOX-loaded H-MPMs.

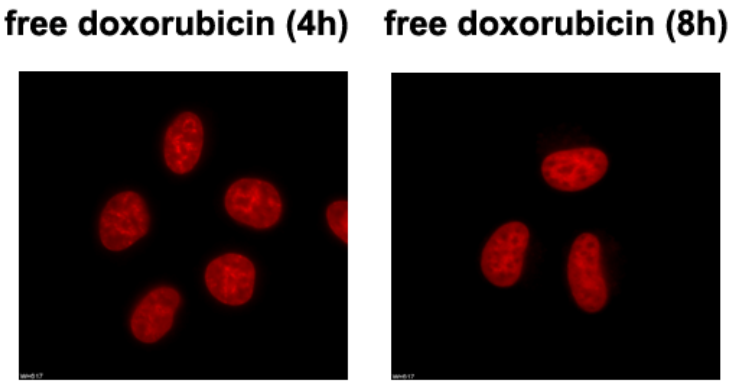


**Fig. S9**. Confocal laser scanning microscopy images of HeLa cells incubated with free doxorubicin.

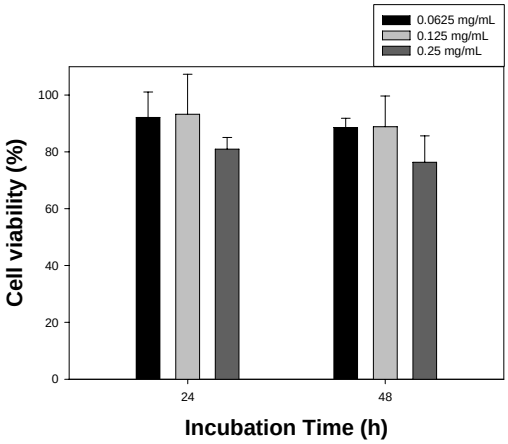


**Fig. S10.** Cell viability of HeLa cells treated with various concentrations of H-MPNs by a MTT assay for 24 h and 48 h.

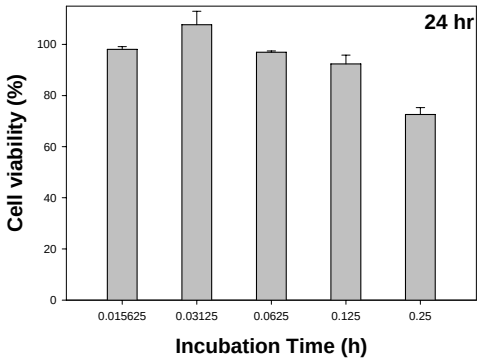


**Fig. S11.** Cell viability of HeLa cells treated with various concentrations of MPMs, ranging from 0.015625 mg/mL to 0.25 mg/mL, by a MTT assay for 24 h.