

Supplementary Information:

Heparin Modulates the Cellular Uptake of Nanomedicines

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1. Material and Methods:

1.1 Polystyrene nanocarriers' synthesis:

a) PS – CTMA-NH₂

The macro emulsion was composed of an aqueous continuous phase containing 2-aminoethyl methacrylate hydrochloride (3 %wt. to styrene) and cetyl trimethyl ammonium chloride (25 %wt. in water) in sterile Milli-Pore water. The organic dispersed phase contained distilled styrene, BODIPY 523/535 as fluorescent dye, and 2,2'-Azobis(2-methylpropionamidine) dihydrochloride in hexadecane. The continuous phase was added slowly to the dispersed phase under constant stirring. The macro emulsion was stirred for 1 hour at high speed. The emulsion was then passed through a microfluidizer (Microfluidics USA, LM10). The miniemulsion obtained was directly transferred into a flask and let to polymerize under stirring condition for 18 h at 72 °C. The dispersion was purified by filtration followed by successive centrifugations at 13,200 g. The supernatant was removed and the pellet was dispersed in sterile Milli-Pore water. The purification step was repeated twice.

b) PS-Lut-NH₂

The same procedure as PS-CTMA-NH₂ was followed, yet Lutensol AT50 replaced cetyl trimethyl ammonium chloride in the continuous phase.

c) PS-SDS-COOH

For these particles, acrylic acid was used as a co-monomer along with styrene in the disperse phase. In addition, sodium dodecyl sulphate was added to the continuous phase as surfactant. The emulsion and polymerization was carried as described in 1.1a. The purification was done by dialysis for 3 days against Milli-Pore water using 14000 kDa MWCO dialysis bags.

1.2 Primary cells isolation staining

The successful isolation and differentiation was monitored by the expression of monocytes or macrophages markers. Cells seeded in a 6-wells plate were recovered by incubation at 4 °C with 2.0 mM EDTA solution. The pooled cells were then split for each pair of marker tested and incubated with 5.0 µL CD13-PE, CD14-FITC, CD14-APC, CD16-APC, CD44-APC, CD45-APC, and CD68-PE for 30 minutes at 4°C in dark. After incubation, the cells were washed twice. The expression of each marker was assessed by flow cytometry (Partec, ML CyFlow). The quadrants were positioned based on the unlabelled sample to have the bottom right quarter accounting for over 95% of the total population, as shown in Figure S11.

1.3 Cell uptake solution preparation steps

AmBisome® was stored as a powder in a desiccant. On the day before the experiment, 3 mg were dissolved in 3 mL Milli-Pore water to obtain a 3.0 mg/mL solution. F5 liposome was provided by BioNTech as a solution in water at 3.0 mg/mL. In a glass vial, the AmBisome® or the F5 liposome solution were diluted two-fold with Milli-Pore water and DiO-Vybrant or DiD-Vybrant dye was added (1:250). The solution was incubated for 30 minutes under constant agitation (300 rpm) at 30 °C. Then, the solution was transferred to a dialysis tube (Slide-A-Lyzer™ MINI Dialysis Device, 3.5K MWCO) and dialysed overnight in Milli-Pore water. The solution was recovered and transferred to a glass vial and stored at 4 °C until used for cell uptake experiment or for characterization measurement.

Human blood was obtained from healthy donor in accordance to the Declaration of Helsinki from the Department of Transfusion Medicine in Mainz. A serum pool from ten volunteers was pooled, aliquoted and stored at -80 °C until used. The aliquot was thawed on the day of the experiment in a water bath at 37 °C. Ten millilitres human serum were added to 90 mL DMEM media without phenol red, to make a 10% human serum solution. The solution was prepared freshly before each experiment. Unfractionated Na-Heparin (Rotexmedica) for flow cytometry or FITC-heparin (ThermoFisher Scientific) for confocal imaging was added to the 10% human serum solution to a final concentration of 4.0 IU/mL. From this master mix, two-fold dilutions were carried on until a concentration of 0.25 IU/mL was reached (Table S1). The solutions were thoroughly mixed in between dilution steps. This mixture was prepared in LoBind tubes to minimize the adhesion of proteins, lipids and heparin to the tubes walls. The solutions were then stored at 4 °C for maximum 30 minutes before addition to the cells.

Sample	[Heparin] IU/mL	V (Na-hep) μL	V (10% hS) μL	V (FITC-hep) μL	V (10%hS) μL
A	4.0	25 (@ 5000 IU/mL)	31000	25 (@ 170 IU/mL)	975
B	2.0	15000 (from A)	15000	438 (from A)	438
C	1.0	14000 (from B)	14000	375 (from B)	375
D	0.5	12000 (from C)	12000	250 (from C)	250
E	0.25	8000 (from D)	8000	n/a	n/a
F	0	n/a	5x3x1000	n/a	2x200

Table S1. Overview of the dilution volume used for the heparin-serum solution. Na-hep stands for unfractionated heparin, hS stands for human serum, FITC-hep stands for FITC-labelled heparin.

At this point, the attached cells seeded the day prior were washed with PBS. Then, 60 μL of labelled liposome (@ 1.5 μg/μL) or 12 μL of polystyrene nanocarrier solution (@ 10 μg/μL) were added to the respective heparin-serum tubes. One millilitre of the nanocarrier-heparin-serum solutions was added to the cells in triplicate. Thus, each sample was treated with 10 % human serum, various amount of heparin and either 30 μg liposomes or 40 μg polystyrene nanocarrier in triplicate for 3 hours at 37 °C.

1.4 Proteomics analysis of the protein corona around PS nanocarriers

For proteomics analysis, PS NCs were incubated for 1 hour at 37 °C under agitation in a solution containing 10% human serum with/out 1.0 IU/mL heparin. The NCs were washed three times with DPBS. The NC pellet was incubated in Tris-HCl containing 2 % sodium dodecyl sulphate (SDS) for 5 min at 95 °C in order to dissociate the proteins from the NCs. They were spun down; the supernatant was recovered and stored at -20 °C. SDS was removed from the protein sample via Pierce Detergent Removal Spin Columns. Subsequently, proteins were precipitated using the ProteoExtract Protein Precipitation kit according to the manufacturer instruction. Furthermore, protein digestion was carried out as described in details elsewhere.⁷ Finally, the isolated peptide solutions were spiked with 50 fmol/μL Hi3 Ecoli (Waters, Milford, MA) for absolute protein quantification and diluted with 0.1 % formic acid. Measurements were performed on a nanoACQUITY UPLC system coupled to a Synapt G2-Si mass spectrometer and data was analysed with MassLynx 4.1 software. For protein identification, Progenesis QI (2.0) was used and a reviewed human database was downloaded from Uniprot.

1.5 Estimation of the heparin interacting with the liposomes

To estimate the amount of heparin per nanocarrier, we analysed the images obtained by CLSM after incubation of HeLa cells and RAW 264.7 macrophages with heparin, liposomes (F5 and AmBisome®) and human serum.

First, using LAS X software, we manually selected a region containing liposomes, i.e. red clusters as a region of interest. A total of three regions of interest were saved per images and three images were analysed, leading to the generation of nine files per sample. For each region of interest, the green channel (heparin) and the red channel (nanocarriers) were saved separately as tiff images.

Then, using PyCharm with the OpenCV package, we loaded and analysed the images. First, we created a mask based on the red channel to keep only the pixels having intensity above the background threshold. Then, we applied this mask to the corresponding green channel image to keep only the pixels where the red (NC) and the green (heparin) signals spatially overlap. Finally, the intensity of the all pixels present in the mask was summed up for the green channel (*Tot_mask_green*) and the red channel (*Tot_mask_red*). This gave us the total intensity of the overlapping pixels in the image. Additionally, we calculated the background intensity for the green channel in each images, named *Tot_bgd_green*. The output values displayed by the software were the number of pixels and the total intensity for the three *variables* defined above.

Finally, using Eq. S1, we calculated the intensity of the green image without background, named as *green_nobgd*. We calculated the ratio of the *green_nobgd* image over the red image intensity using Eq. S2, therefore, we obtained the intensity of the green pixel per red pixel. This value correlates to the amount of heparin per nanocarrier. As a quality control of the data extracted, we also calculated the ratio of the intensity of the red pixel in the mask per red pixel. Since, for one set of heparin concentration, we use the same liposomes, we should also find the same ratio for the red pixel.

$$Green_nobgd = Tot_mask_green - Tot_mask_green \quad (Eq. S1)$$

$$Ratio\left(\frac{Heparin}{NC}\right) = \frac{Green_nobgd}{Tot_mask_red} \quad (Eq. S2)$$

We analysed the three following conditions: AmBisome in HeLa cells, F5 in HeLa cells and F5 in RAW 264.7 cells. The data are summarized in Table S2 and Figure S10. It is important to note that RAW 264.7 cells internalize free heparin. Thus, we had to exclude the data from AmBisome® in RAW 264.7 cells as the background intensity coming for the free heparin in solution was too high to be reproducibly subtracted.

[Heparin] IU/mL	AmBisome		F5			
	HeLa		HeLa		RAW 264.7	
	Heparin/NC	Int. red pixel	Heparin/NC	Int. red pixel	Heparin/NC	Int. red pixel
0	0.19 (0.05)	8.0 (1.0)	0.35 (0.06)	9.6 (0.7)	0.40 (0.06)	9.3 (1.0)
0.5	0.35 (0.18)	8.0 (1.4)	1.84 (0.27)	9.3 (1.5)	1.36 (0.18)	9.3 (1.7)
1.0	0.43 (0.17)	7.6 (0.8)	2.98 (0.40)	9.7 (1.1)	2.52 (0.73)	10.0 (1.2)
4.0	0.55 (0.13)	7.8 (0.7)	3.14 (0.84)	12.7 (1.5)	2.88 (0.86)	11.8 (1.2)

Table S2. Overview of the amount of heparin per liposome based on the intensity of the green pixel per red pixel extracted from CLSM images of cells incubated with DiD-AmBisome® or DiD-F5 liposomes in the presence of FITC-Heparin and human serum. The values reported are the mean +/- standard deviation in parenthesis from 6 or 9 images.

2. Supplementary Figures:

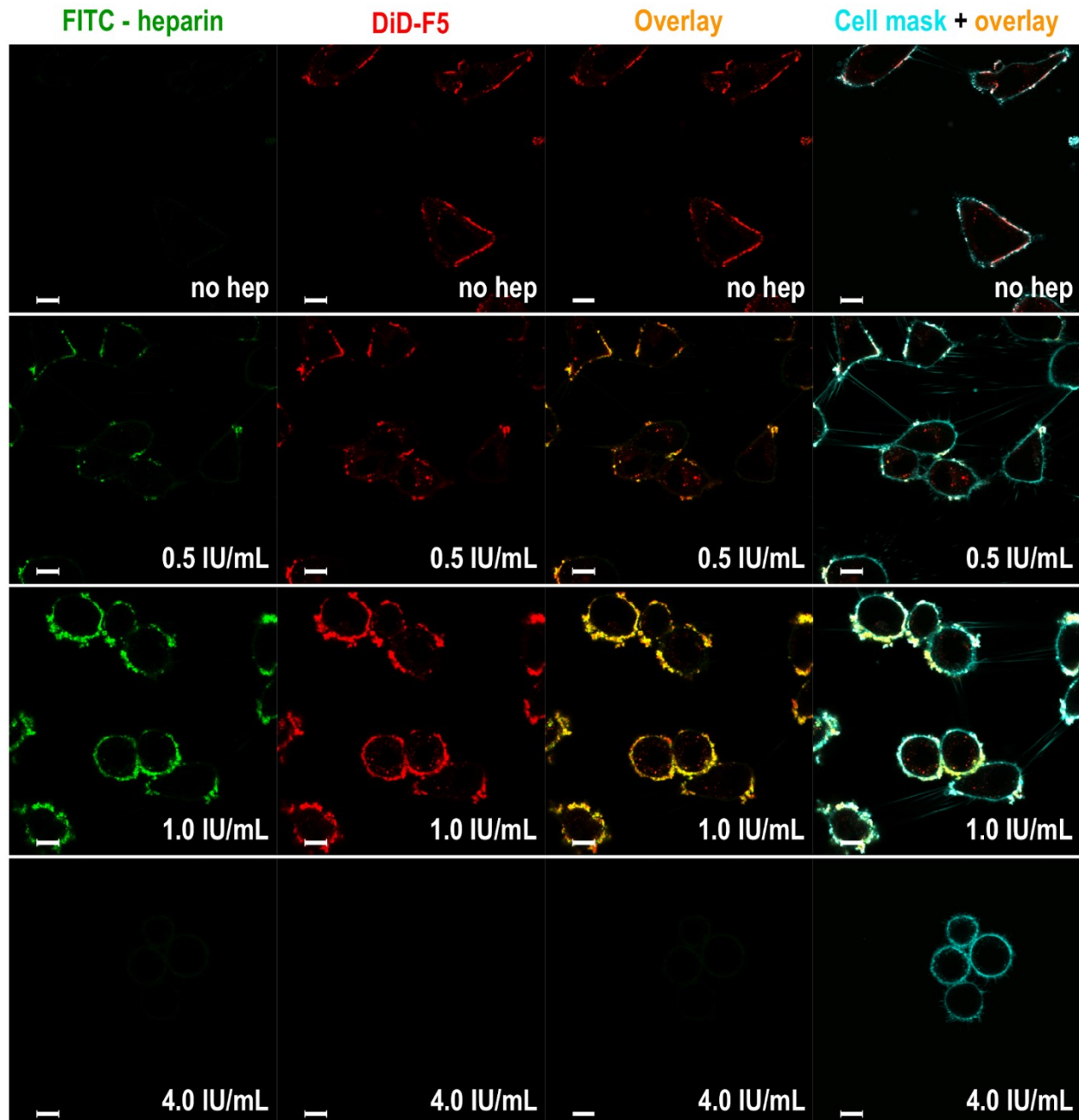


Figure S1. Imaging the interaction between F5 liposomes and HeLa cells in the presence of different concentration of heparin in 10% human serum after 3h incubation at 37 °C. In the first column, FITC-heparin is displayed in green; in the second column, DiD-labelled F5 liposomes are displayed in red; in the third column, the overlay of the heparin

and liposomes signal is displayed in orange; finally the last column represents the overlay of the first two columns and the cell membrane (light blue). The scale bar represents 10 μm .

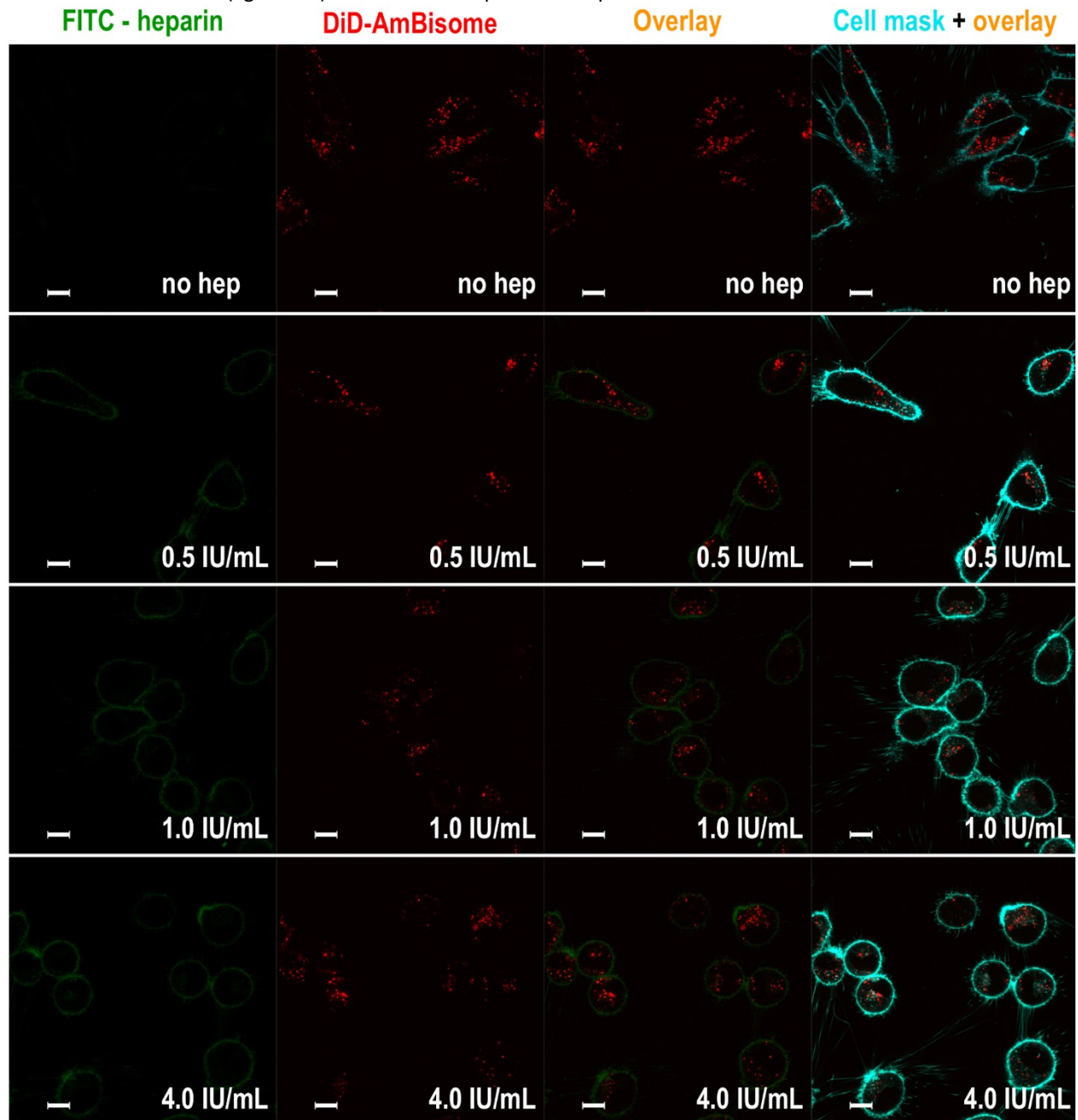


Figure S2. Visualizing the interaction between AmBisome® liposomes and HeLa cells in the presence of different concentration of heparin in 10% human serum after 3h incubation at 37 °C. In the first column, FITC-heparin is displayed in green; in the second column, DiD-labelled AmBisome® liposomes are displayed in red; in the third column, the overlay of the heparin and liposomes signal is displayed in orange; finally the last column represents the overlay of the first two columns and the cell membrane (light blue). The scale bar represents 10 μm .

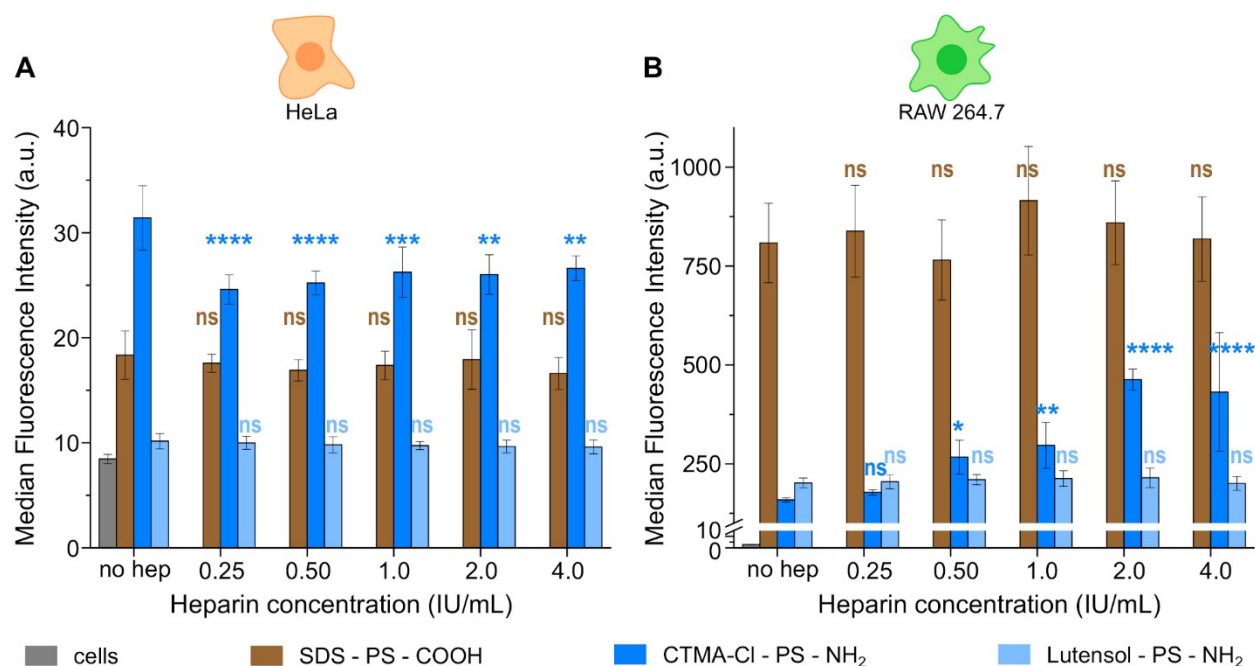


Figure S3. Quantification of the cellular uptake of polystyrene nanocarriers by HeLa cells (A) and RAW 264.7 macrophages (B) in presence of various concentration of heparin in 10% human serum after 3 h incubation at 37°C. The error bars represent the standard deviation between the mean median fluorescence intensity (n = 6). P-values are calculated by ANOVA One-Way using the “no heparin” condition as reference. P-values > 0.03 are considered non-significant (ns), p-values < 0.03 are represented by *, p-values < 0.002 are represented by **, p-values < 0.0002 are represented by ***, and p-values < 0.0001 are represented by ****.

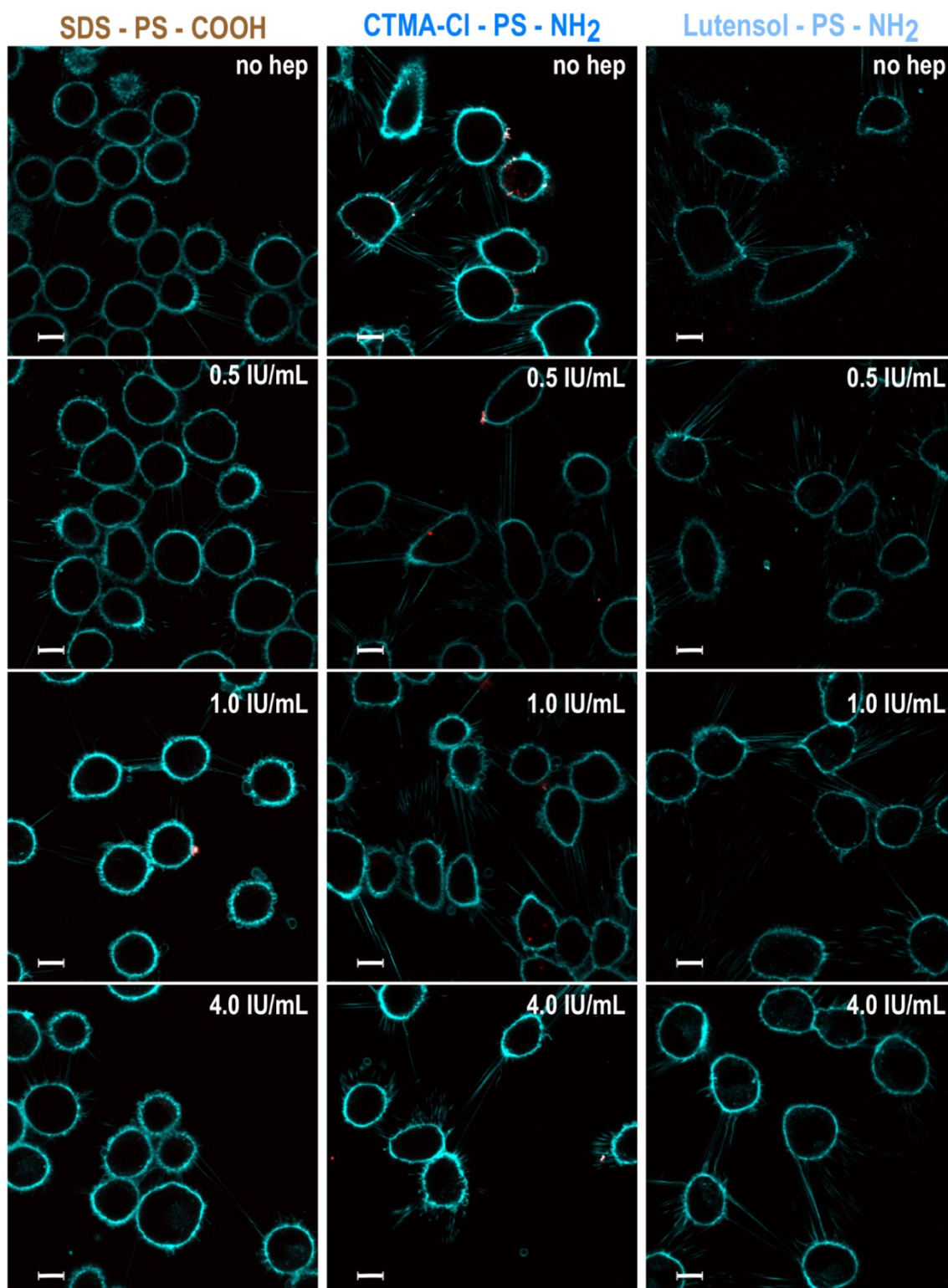


Figure S4. Visualizing the interaction between polystyrene nanocarriers and HeLa cells in the presence of different concentration of heparin in 10% human serum after 3h incubation at 37 °C. The nanocarriers are represented in red and the cell membrane in light blue. Each column represents a different nanocarrier, while the heparin concentration increases from the top row to the bottom row. The scale bar represents 10 μm .

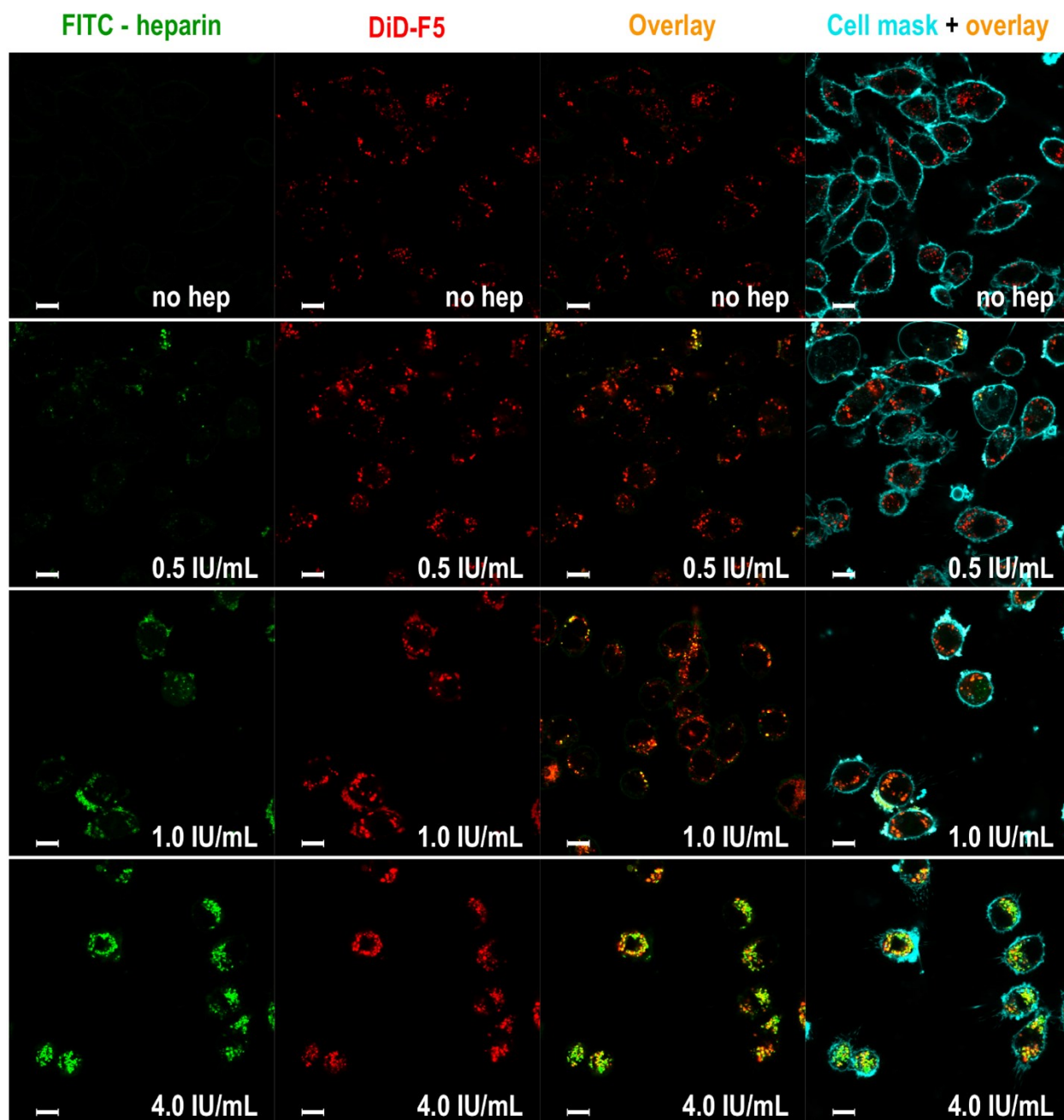


Figure S5. Visualizing the interaction between F5 liposomes and RAW 264.7 macrophages in the presence of different concentration of heparin in 10% human serum after 3h incubation at 37 °C. In the first column, FITC-heparin is displayed in green; in the second column, DiD-labelled F5 liposomes are displayed in red; in the third column, the overlay of the heparin and liposomes signal is displayed in orange; finally the last column represents the overlay of the first two columns and the cell membrane (light blue). The scale bar represents 10 μm .

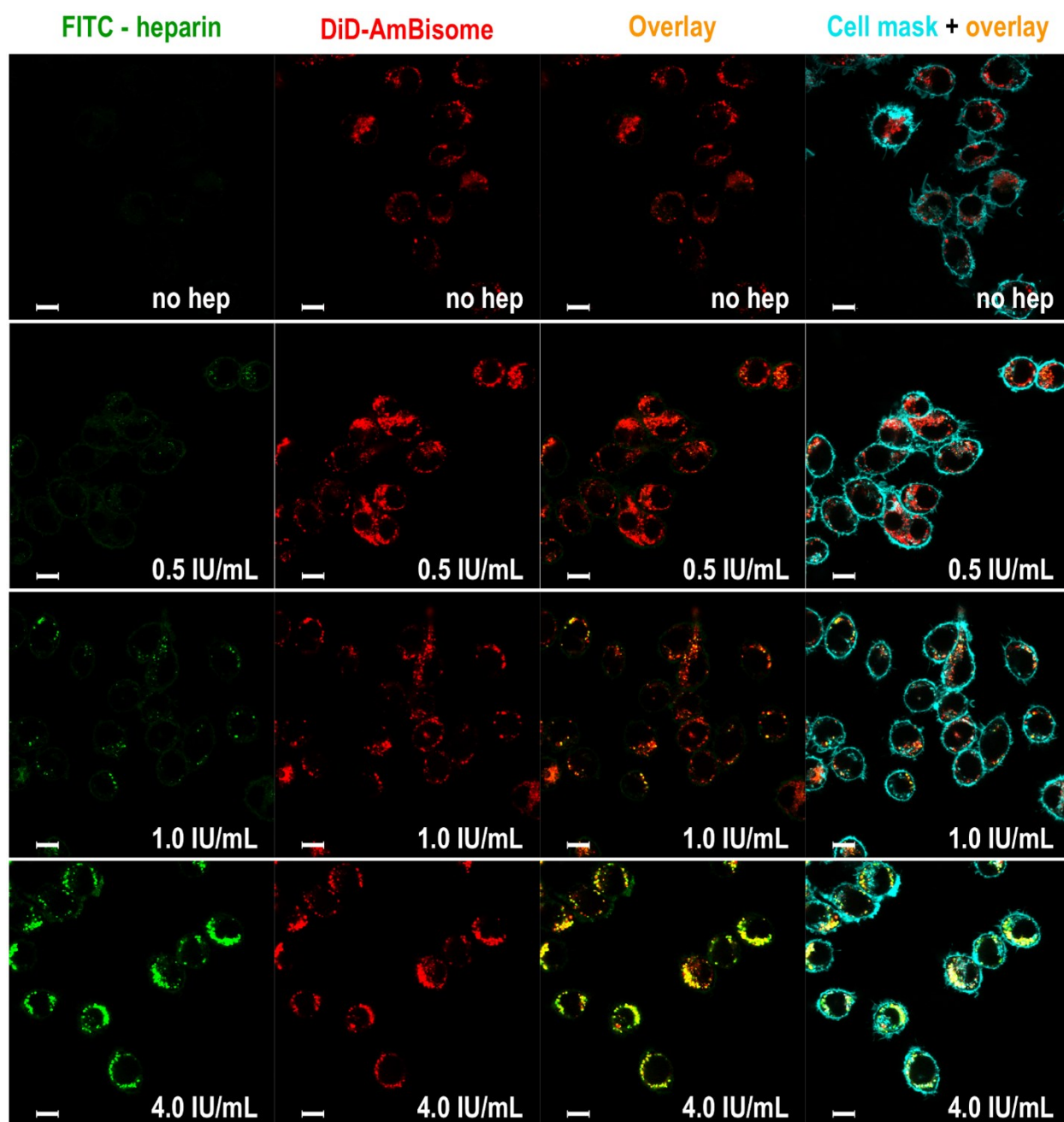


Figure S6. Visualizing the interaction between AmBisome® liposomes and RAW 264.7 macrophages in the presence of different concentration of heparin in 10% human serum after 3h incubation at 37 °C. In the first column, FITC-heparin is displayed in green; in the second column, DiD-labelled AmBisome® liposomes are displayed in red; in the third column, the overlay of the heparin and liposomes signal is displayed in orange; finally the last column represents the overlay of the first two columns and the cell membrane (light blue). The scale bar represents 10 μ m.

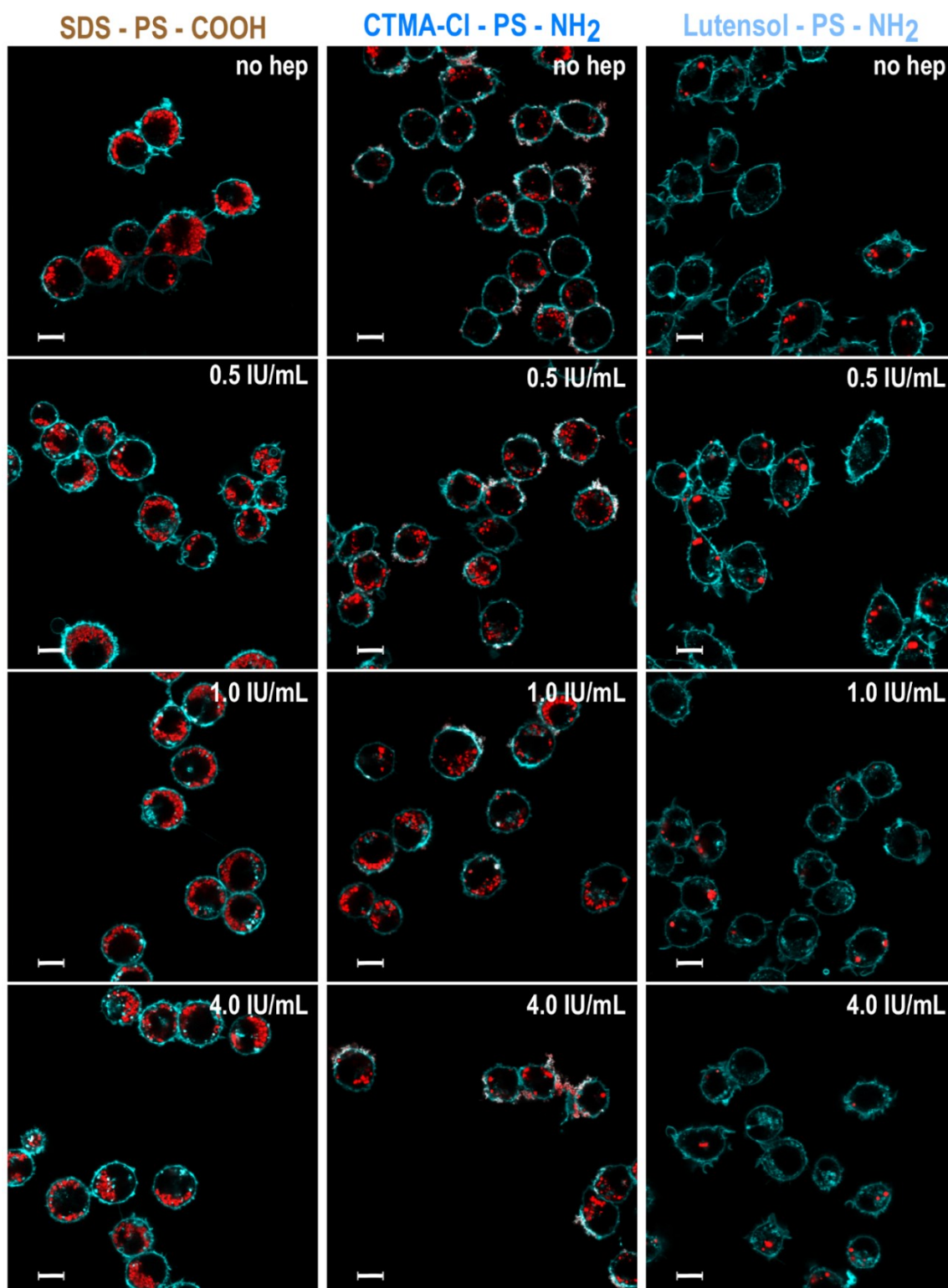


Figure S7. Visualizing the interaction between polystyrene nanocarriers and RAW 264.7 macrophages in the presence of different concentration of heparin in 10% human serum after 3h incubation at 37 °C. The nanocarriers are represented in red and the cell membrane in light blue. Each column represents a different nanocarrier, while the heparin concentration increases from the top row to the bottom row. The scale bar represents 10 μm.

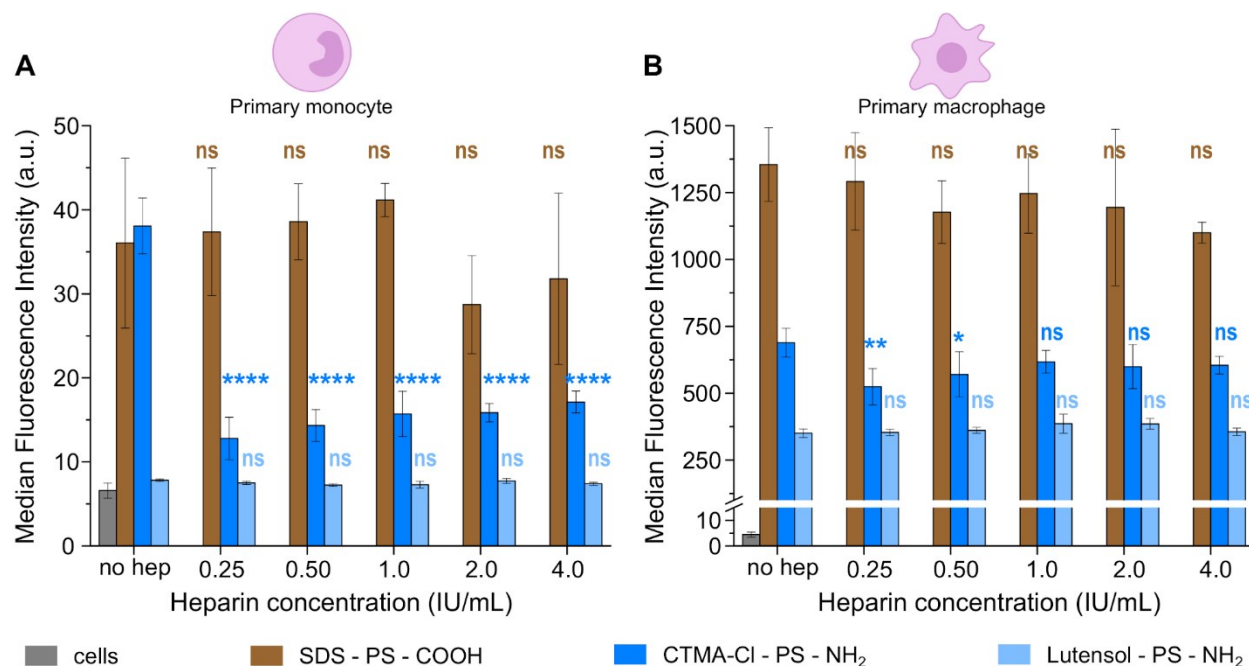


Figure S8. Quantification of the cellular uptake of polystyrene nanocarriers by primary monocytes (A) and primary macrophages (B) in presence of various concentration of heparin in 10% human serum after 3 h incubation at 37°C. The error bars represent the standard deviation between the mean median fluorescence intensity (n = 6). P-values are calculated by ANOVA One-Way using the “no heparin” condition as reference. P-values > 0.03 are considered non-significant (ns), p-values < 0.03 are represented by *, p-values < 0.002 are represented by **, p-values < 0.0002 are represented by ***, and p-values < 0.0001 are represented by ****.

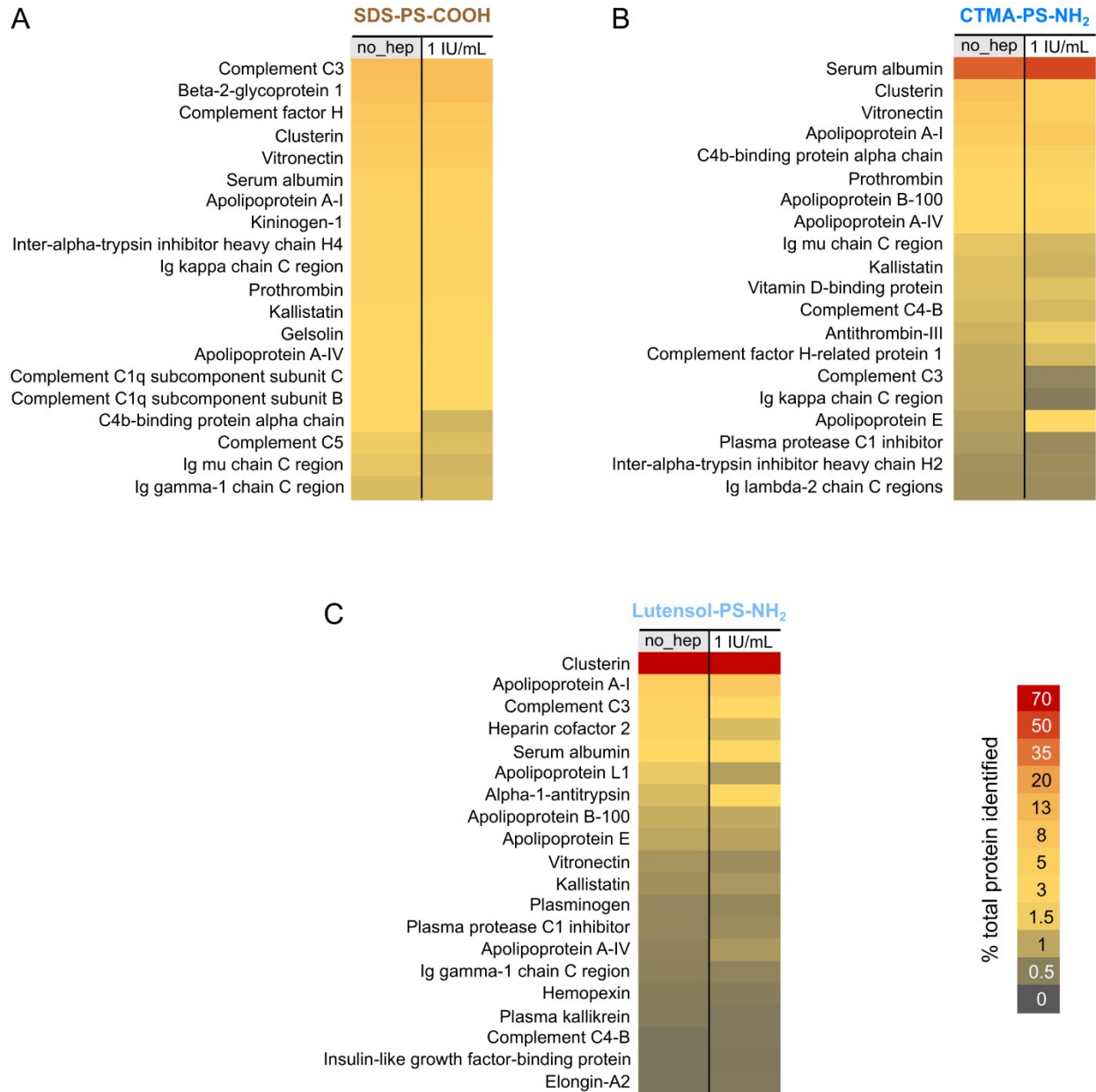


Figure S9. Proteomics analysis of the protein corona adsorbed on the polystyrene nanocarrier in the absence or presence of heparin. PS-COOH-SDS (A), CTMA-PS-NH₂ (B), and Lutensol-PS-NH₂ (C) were incubated for 1h at 37 with(out) heparin in 10% human serum. The proteins adsorbed on the surface were recovered and the protein mixture was analysed by LC-MS. The 20 most abundant proteins and their relative percent in the total corona were summarized in the heatmap.

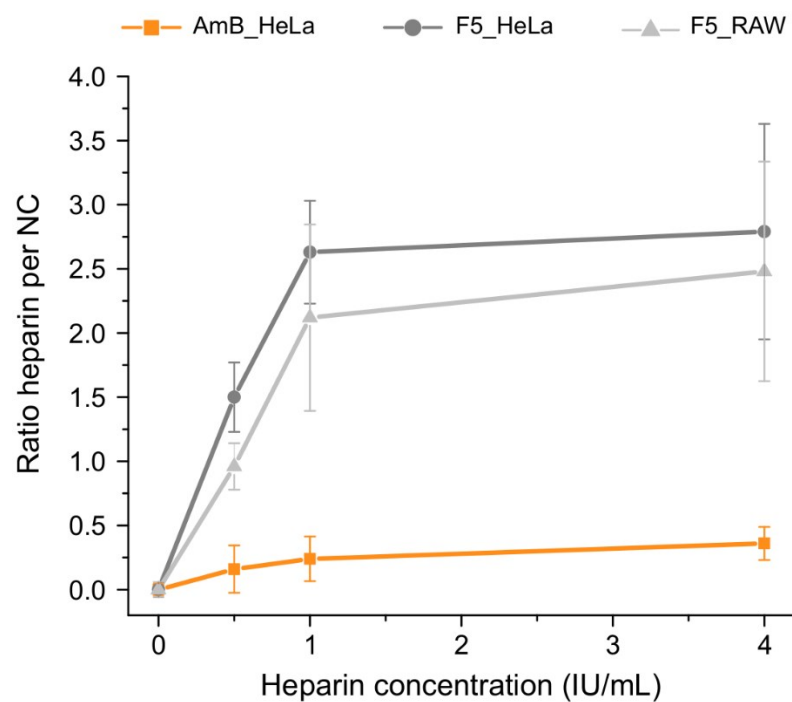


Figure S10. Estimation of the amount of heparin adsorbed on the nanocarrier. The liposomes (NC) were incubated with cells in the presence of heparin concentration ranging from 0.5 IU/mL to 4.0 IU/mL. The ratio of heparin per nanocarrier (NC) was estimated based on the intensity of the overlapping green and red pixels. The error bars represent the standard deviation of the mean value of at least 6 measurements.

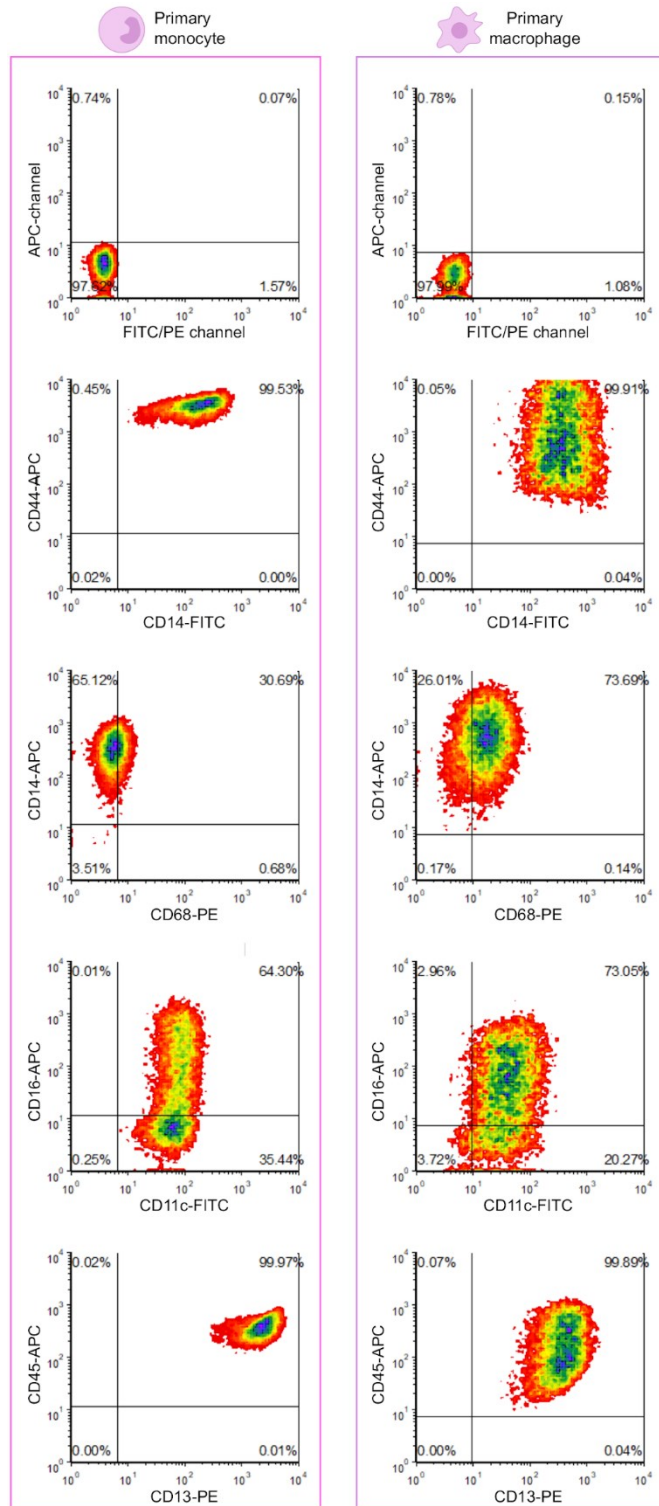


Figure S11. Verification of the cells population studied, the quadrants were positioned to have unlabelled cells constituting at least 95% of the bottom left quadrant. Primary monocytes expressed CD14 and CD44 to a high extent but CD68 to a lower extent. Primary macrophages expressed CD14, CD44, and CD68 to a high extent.