Supplementary Information

Functionalization of liposomes with hydrophilic polymers results in macrophage uptake independent of the protein corona

Claudia Weber‡, [a], Matthias Voigt‡, [b], Johanna Simon[a, c], Ann-Kathrin Danner[d], Holger Frey[d], Volker Mailänder [c,a], Mark Helm[b], Svenja Morsbach, [a] and Katharina Landfester[a]*

‡ authors contributed equally

* corresponding author

[a] Max Planck Institute for Polymer Research, Ackermannweg 10, 55128 Mainz, Germany

[b] Institute of Pharmacy and Biochemistry, Johannes Gutenberg-University, Staudingerweg 5, 55128 Mainz, Germany

[c] Department of Dermatology, University Medical Center of the Johannes Gutenberg-University Mainz, Langenbeckstrasse 1, 55131 Mainz, Germany

[d] Institute of Organic Chemistry, Johannes Gutenberg-University, Duesbergweg 10-14, 55128 Mainz, Germany

1. Experimental: Liquid chromatography – Mass spectrometry (LC-MS)

In solution digestion and LC-MS analysis were performed as previously described[1,2]. Briefly, proteins were precipitated using Proteo Extract protein precipitation kit (Merck Millipore, Germany) according the manufacturer's instruction. The isolated protein pellet was resuspended with 0.1% RapiGest SF (50 mM ammonium bicarbonate solution, Waters, USA) and incubated at 80 °C for 15 min. Dithiothreitol (Sigma-Aldrich, USA, 5 mM, 45 min, 56 °C) and Iodoacetamide (Sigma-Aldrich, USA, 15 mM, 1 h) were added to the protein solution. Proteins were digested with trypsin with a 1:50 ratio (enzyme:protein). Hydrochloric acid (2 µL, Sigma-Aldrich, USA) was added to stop the digestion the next day. Peptide samples were diluted with 0.1% formic acid (Thermo Fisher, USA) and 50 fmol μL^{-1} Hi3 E.coli Standard (Waters, USA) for absolute quantification. A Synapt G2 Si mass spectrometer coupled to NanoACQUITY with a C18 analytical reversed-phase column (1.7 µm, 75 µm x 150 mm) and a C18 nanoACQUITY Trap Column (5 µm, 180 µm x

20 mm, both Waters, USA) were used for proteomic measurements. Mobile phase A 0.1% (v/v) formic water and mobile phase B of 0.1% (v/v) formic acetonitrile (both Biosolve, Netherlands) were used. A flow rate of 0.3 µL min^{-1} over a gradient from 2% to 40% from mobile phase A to B was applied. Glu-Fibrinopeptide and Leucine Enkephaline (both Sigma-Aldrich, USA) served as reference component infused at a flow rate of 0.5 µL min^{-1}. Electrospray ionization (ESI) was performed with a NanoLockSpray in positive ion mode and data-independent acquisition (MSe) experiments were carried out.

Peptides and proteins were identified by Progenesis QI software. The following criteria were set for analysis: energy (120 counts), high energy (25 counts) and peptide intensity (750 counts) were set and continuum LC-MS data was post acquisition lock mass corrected. A protein false discovery rate of 4% for all samples was applied. Peptides were searched against a human reviewed database from Uniprot. The data base was spiked with the sequence of Hi3 E.coli standard and porcine trypsin. The final criteria were chosen: 1 peptide = 3 fragments; 1 protein = 2 peptides and 5 fragments. The absolute amount (in fmol) of each protein was generated based on the Top/Hi3 approach[41]. Relative amounts of each protein were calculated based on the total amount of all identified proteins.

1. Schottler, S.; Becker, G.; winzen, S.; Steinbach, T.; Mohr, K.; Landfester, K.; Mailander, V.; Wurm, F.R.; Protein adsorption is required for stealth effect of poly(ethylene gylcol)- and poly(phosphoester)-coated nanocarriers. *Nat Nanotechnol* **2016**; *11(4),* 372-377.

2. Hofmann, D.; Tenzer, S.; Bannwarth, M.B.; Messerschmidt, C.; Glaser, S.F.; Schild, H.; Landfester, K.; Mailänder, V.; Mass Spectrometry and Imaging Analysis of Nanoparticle-

Containing Vesicles Provide a Mechanistic Insight into Cellular Trafficking. ACS Nano **2014**; *8(10),* 10077-10088.

2. Synthesis and characterization of dialkyl-based hyperbranched polyglycerol amphiphiles (*hb*PG)

2.1 Materials and Methods

2.1.1 Reagents

All chemicals were obtained from S*igma Aldrich, Acros Organics, Fisher Scientific* or *TCI Europe* unless mentioned otherwise. Deuterated solvents (pyridine-d_5) were purchased from *Deutero GmbH*.

2.1.2 Instrumentation

NMR spectra were measured on a Bruker Avance II spectrometer operated at 400 MHz (5 mm BBFO smart probe and SampleXPress 60 auto sampler) at 296 K. Pyridine-d_5 was used as solvent. The NMR spectra were referenced internally to the respective signals of the deuterated solvent. Analysis of all spectra was carried out using the software MestReNova version 9.0. SEC measurements were performed in dimethylformamide (DMF) with 0.25 g L^{-1} lithium bromide on an Agilent 1100 Series equipped with PSS HEMA 300/100/40 column, RI and UV detector (275 nm). Monodisperse linear PEG standards from *Polymer Standard Service GmbH* (PSS) were

used for calibration and the RI signal is displayed for the SEC trace. Analysis was performed, applying the software PSS WinGPC Unity.

2.2 Synthesis of BisHD-*hb*PG

The macroinitiator BisHD-*lin*PG_{24} (0.225 g, 0.098 mmol, 1 eq.) was placed in a Schlenk flask, dissolved in benzene (2 mL) and dried for 16 h in vacuo. Afterwards, BisHD-*lin*PG_{24} was again dissolved in benzene (2 mL) and cesium hydroxide (0.041 g, 0.25 mmol, 2.6 eq. (equates to a degree of deprotonation of 10 % of hydroxyl groups)) was added. The solution was stirred at 80 °C for 2 h to allow the formation of the alkoxide. Afterwards, the alkoxide was dried for 16 h under reduced pressure. The macroinitiator was dissolved in *N*-methyl-2-pyrrolidone (NMP) and a solution of glycidol (0.064 mL, 0.973 mmol, 10 eq.) (5 %) in NMP was added to the initiator solution over a time period of 6 h at 100 °C. Subsequently, the solvent was removed under reduced pressure and the crude product was precipitated twice in cold diethyl ether. The solvent was removed under reduced pressure and the product was dried *in vacuo*. Yield: 82 %.

1H NMR, COSY (400 MHz, pyridine-d5, δ): 6.04 (s, 38H, hydroxyl groups of hbPG), 4.36-3.51 (m, 160H, polyether backbone, glycidol H) 1.70-1.61 (m, 6H, -OCH2CH2-(BisHD)), 1.45-1.26 (m, 54H, -OCH2CH2(CH2)15CH3(BisHD)), 0.90-0.87 (m, 6H, -O(CH2)17CH3(BisHD)).

2.3 Characterization Data

Table S1.1. Properties of the synthesized BisHD-*hb*PG$_{30}$.

#	Composition	M_n^{th}	M_n^{NMR}	$M_n^{SEC, a}$	$M_w/M_n^{SEC, a}$
		g mol^{-1}	g mol^{-1}	g mol^{-1}	
1	BisHD-*hb*PG$_{30}$	3500	2760	2100	1.30

[a] obtained from SEC measurement in DMF using PEG standards.

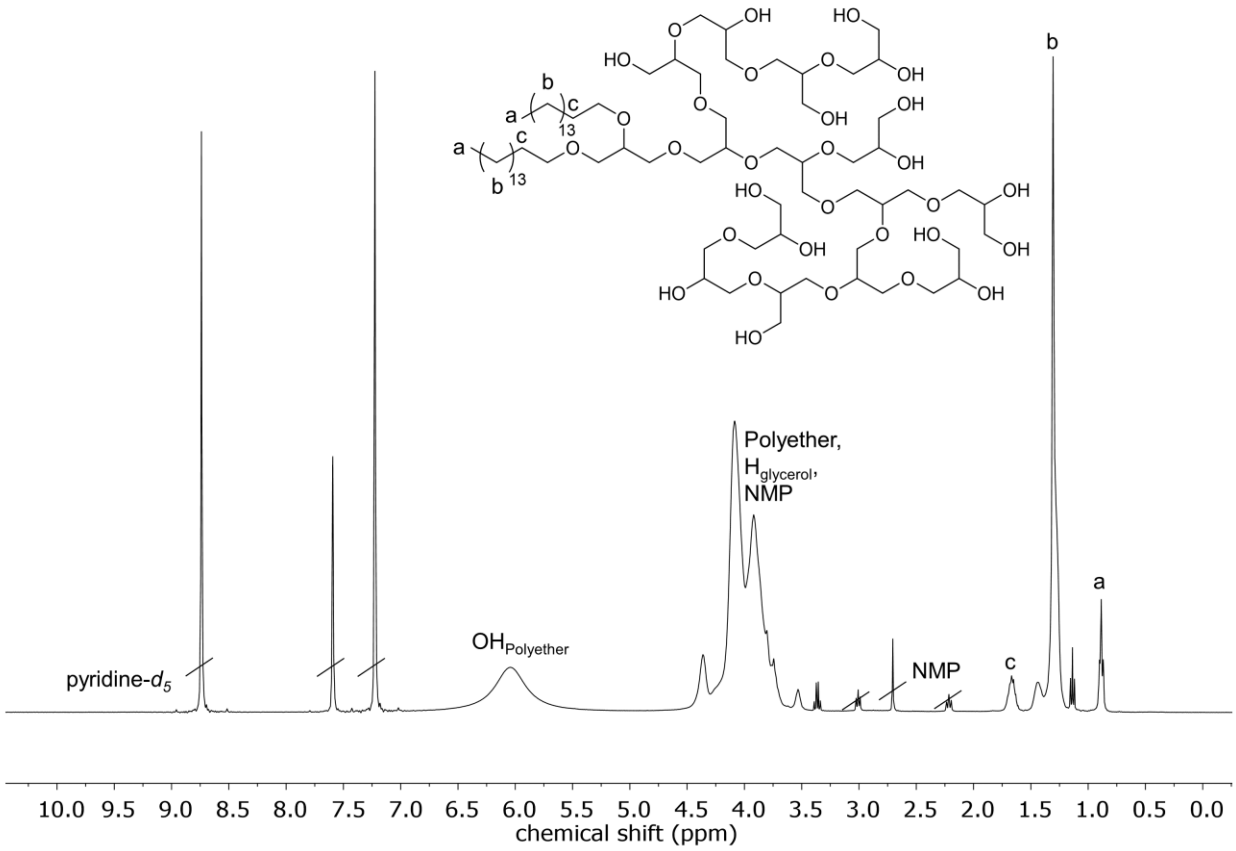

Figure S2.1. ^{1}H NMR (pyridine-d_5, 400 MHz) of BisOD-*hb*PG$_{30}$.

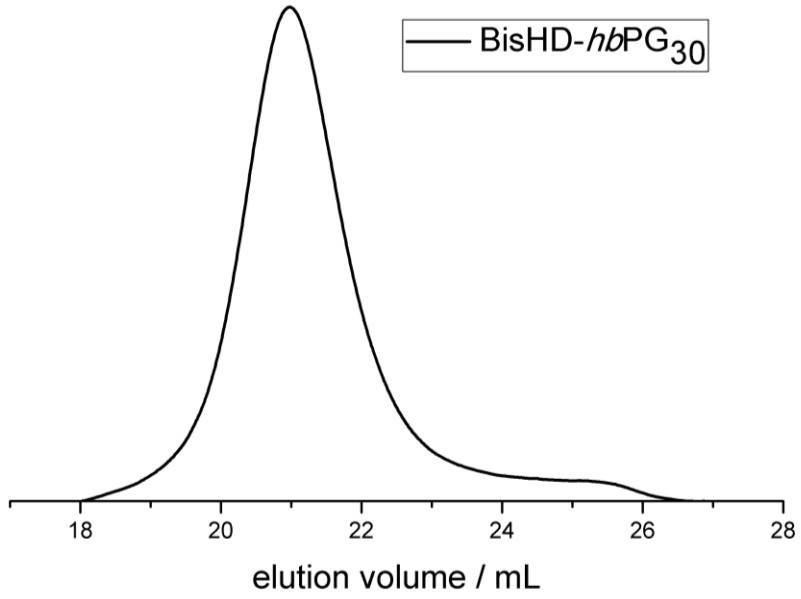

Figure S2.2. SEC trace (DMF, PEG standard, RI signal) of BisOD-*hb*PG_{30}.

3. Protein quantification

$$LOD = x + 3 \cdot s$$

$$LOQ = x + 10 \cdot s$$

LOD: limit of detection

LOQ: limit of quantification

x: mean value of 10 blank measurements

s: standard deviation of 10 blank measurements

Applied on the Pierce Assay for protein quantification of the protein corona the limits in adsorption are:

$$LOD = 0.0752$$

$$LOQ = 0.0953$$

After AF4:

L-un: LOD < measured absorbance 0.0827 < LOQ

L-PEG: LOD < measured absorbance 0.0778 < LOQ

L-*hb*PG: LOD < measured absorbance 0.0769 < LOQ

After centrifugation:

L-un: measured absorbance 0.0663 < LOD < LOQ

L-PEG: measured absorbance 0.0647 < LOD < LOQ

L-*hb*PG: measured absorbance 0.0692 < LOD < LOQ

4. TEM micrographs of liposomes after centrifugation

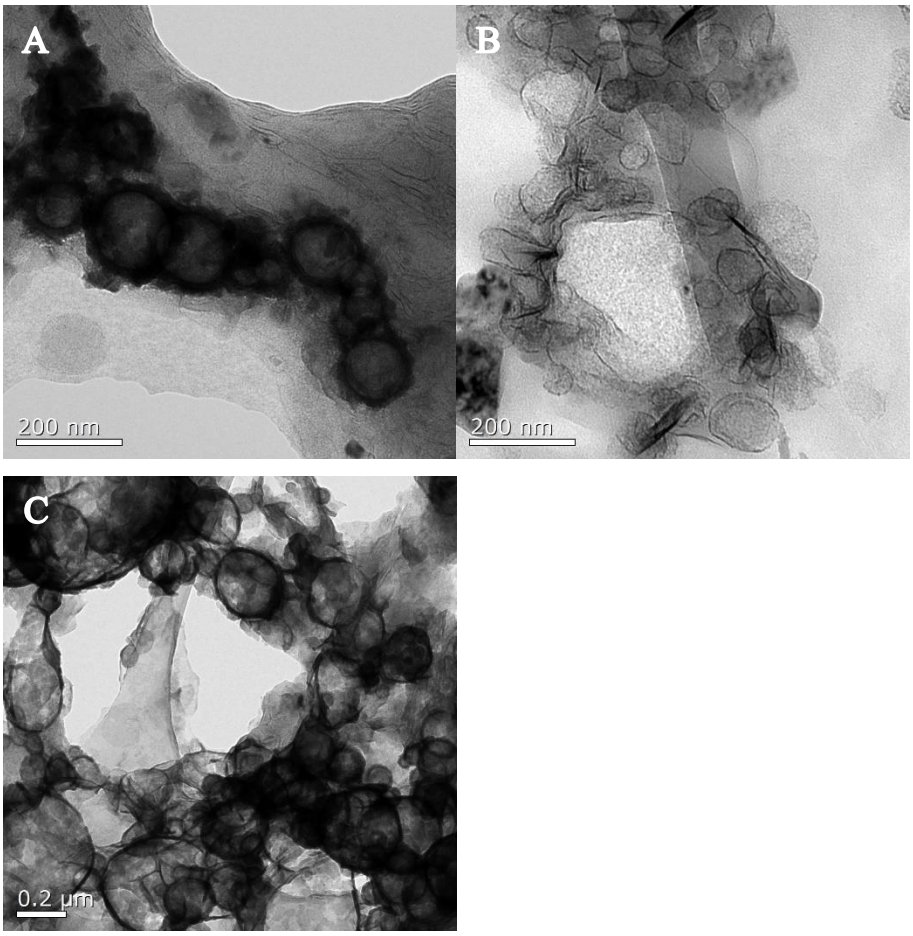

Figure S2. TEM micrographs of A) L-un, B) L-PEG and C) L-*hb*PG. The samples were centrifuged one time for 1 h at 20 000 g to check their stability during the centrifugation process. On all three micrographs intact liposomes are visible after the treatment. This confirms the high stability of the used liposome formulation and their suitability for centrifugation.

5. Additional cell experiments at higher liposome concentration for cLSM experiments

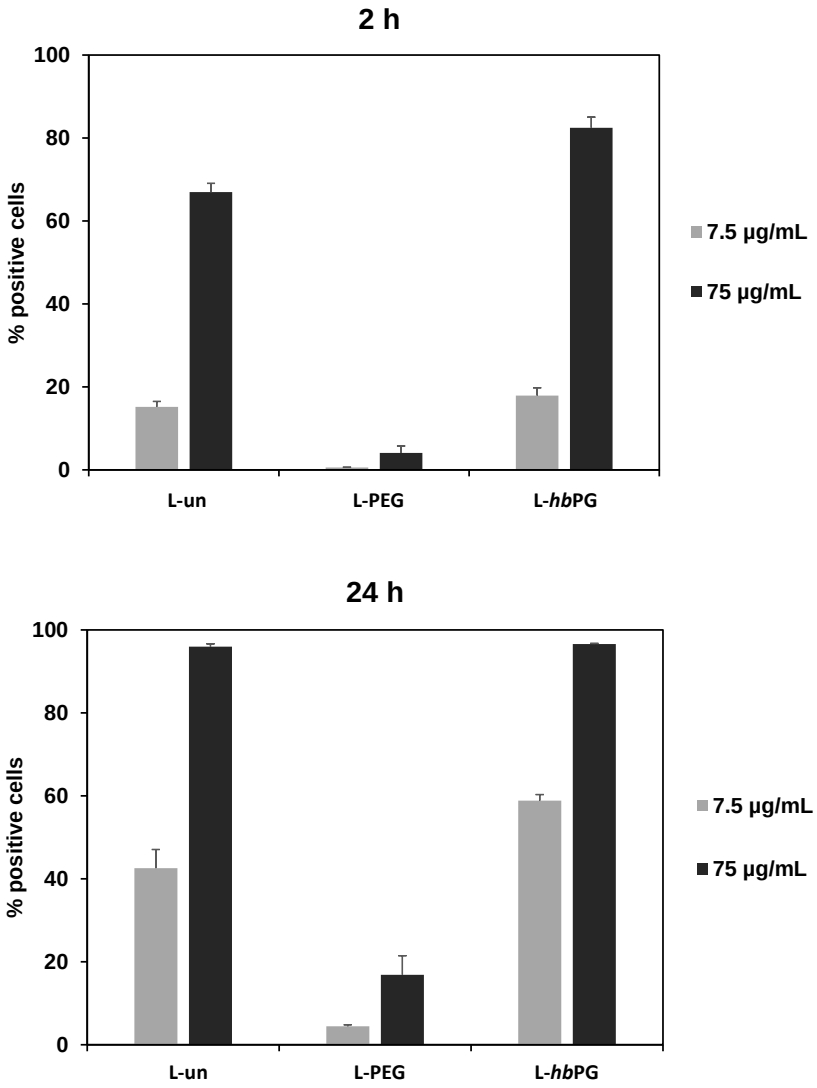

Figure S3. Cellular uptake of differently functionalized liposomes in different liposome concentrations into RAW cells. The overall trend of the cell uptake is the same for different concentrations.