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The hard protein corona of stealth liposomes is sparse

Kasper Kristensen^{a,b,*}, Trine B. Engel^{a,b}, Allan Stensballe^c, Jens B. Simonsen^{a,b}, and Thomas L. Andresen^{a,b,*}

^aDepartment of Health Technology, DTU Health Tech, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

^bCenter for Nanomedicine and Theranostics, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

^cDepartment of Health Science and Technology, Aalborg University, 9220 Aalborg Ø, Denmark

*Corresponding authors at Department of Health Technology, DTU Health Tech, Technical University of Denmark, Produktionstorvet, Building 423, 2800 Kgs. Lyngby, Denmark. *E-mail addresses*: kakri@dtu.dk (K. Kristensen), tlan@dtu.dk (T. L. Andresen).

Abstract

The protein corona is widely recognized as a key concept in contemporary nanomedicine. In recent years, the interest in the protein corona has reached new heights as a number of reports have suggested that a comprehensive protein corona can form around nanomaterials that previously were thought to be resistant to protein binding. For example, PEGylated stealth liposomes were long thought to be protein repellent, but a number of recent studies have found that a significant protein corona forms around such liposomes in the bloodstream. Prompted by these surprising recent findings, we here present an extensive quantitative study of the binding of blood proteins to standard PEGylated stealth liposomes. To make the study relevant for targeted as well as non-targeted drug delivery systems, the liposomes were prepared both with and without a targeting antibody conjugated to their surface. The prepared liposomes were either incubated *in vitro* in fetal bovine serum or administered *in vivo* into the bloodstream of mice. Subsequently, the liposomes were recovered and analyzed using a variety of techniques. There was very little protein binding to the liposomes recovered after *in vitro* incubation. In contrast, there was more protein binding to the liposomes recovered after *in vivo* circulation, but a deeper analysis estimated that the bound proteins still only covered a very low fraction of the liposomal surface area. Both the liposomes recovered after *in vitro* incubation and the liposomes recovered after *in vivo* circulation completely retained their size and receptor targeting characteristics. Taken collectively, our results thus demonstrate that the hard protein corona of both non-targeted and antibody-targeted stealth

liposomes is sparse and does not affect the structure and function of the liposomes.

Keywords

- Drug delivery
- Nanomedicine
- Protein corona
- Liposomes
- Antibody targeting

1 Introduction

The protein corona has over the past decade emerged as a central concept in the field of nanomedicine [1, 2]. According to this concept, nanomaterials exposed to a biological environment readily adsorb a layer of proteins, which modify the surface properties and thereby the biological identity of the nanomaterials. Indeed, a plethora of studies have clearly demonstrated the implications of the protein corona on the behavior of nanomaterials [3–11].

Remarkably, there has in recent years been a number of studies reporting that a protein corona can even form around nanomaterials that previously were thought to be fairly resistant to protein binding [12]. As an important example of this, it was originally hypothesized that there is limited binding of blood proteins to polyethylene glycol-coated (PEGylated) stealth liposomes [13], which possibly are the most commonly used type of drug vehicle in contemporary nanomedicine. This was thought to minimize opsonization and capturing by the mononuclear phagocyte system, thereby conferring long-circulating properties on the liposomes [14, 15]. Recently, however, several studies have suggested that PEGylated stealth liposomes become covered with a protein corona upon exposure to blood [16–22]. It has even been proposed that such liposomes may attain their stealth properties because certain dysopsonizing blood proteins—like albumin—bind to the PEG chains and thereby mask the liposomes from the immune system [23].

In light of the discrepancy between the original hypothesis of little protein binding on the one side and the recent reports about a significant protein corona on the other side, we undertook a comprehensive study on the binding of blood proteins to PEGylated stealth liposomes. The liposomes used in our study were based on a lipid formulation consisting of DSPC, cholesterol, and DSPE-PEG2k. This lipid formulation—resembling that of the anticancer drug Doxil [24]—is

a standard choice for obtaining PEGylated stealth liposomes. To expand the relevance of our study to actively targeted drug delivery systems, the liposomes were prepared both with and without the antibody trastuzumab conjugated to the surface. Trastuzumab is a recombinant humanized monoclonal antibody, which specifically targets the human epidermal growth factor receptor 2 (HER2), a transmembrane glycoprotein receptor that is overexpressed in various types of cancer [25]. Of relevance, trastuzumab or fragments thereof have previously been conjugated to PEGylated liposomes to achieve targeted liposomal drug delivery systems [26–31].

Figure 1: Schematic illustration of the experimental protocol used in the present study. PEGylated stealth liposomes were prepared with or without the antibody trastuzumab conjugated to their surface. The liposomes were incubated *in vitro* in FBS or administered *in vivo* into the bloodstream of mice. After 1 h, the liposomes were recovered using size-exclusion chromatography. The concentration, size, and fluorescence properties of the recovered liposomes were evaluated using FCS. The concentration and identity of the proteins recovered together with the liposomes were evaluated using the micro-BCA assay, SDS-PAGE, and LC-MS/MS. The ability of the recovered liposomes to target the HER2-overexpressing cell line SKBR-3 was evaluated using flow cytometry. To have a positive control experiment with a type of nanoparticle known to bind extensive amounts of protein, we performed an *in vitro* set of experiments with polystyrene beads. To have a negative control experiment assessing the potential amount of unbound background proteins co-eluting with the liposomes on the size-exclusion chromatography column, we both performed an *in vitro* and *in vivo* set of experiments using blank buffer vehicle samples without any liposomes.

The experimental protocol of our study is summarized in Figure 1. In brief, the liposomes were first exposed to blood proteins, either through incubation *in vitro* in fetal bovine serum (FBS) or through circulation *in vivo* in the bloodstream of mice. The liposomes were then recovered using size-exclusion chromatography and analyzed using fluorescence correlation spectroscopy (FCS), micro bicinchoninic acid (micro-BCA) assay, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), liquid chromatography tandem mass spectrometry (LC-MS/MS), and flow cytometry. The overall purpose of this analysis was to assess the magnitude and composition of the hard protein corona of the liposomes, that is, to determine the amount and type

of blood proteins bound to the liposomes after the recovery step [1]. Additionally, the purpose was to evaluate the structural and functional integrity of the recovered liposomes. To make sure that the protocol produced complete and robust results, it included sets of both positive and negative control experiments.

Based on this experimental protocol, we detected very little protein binding to the liposomes recovered after *in vitro* incubation. In contrast, we detected some protein binding to the liposomes recovered after *in vivo* circulation, but a subsequent calculation implied that the bound proteins only covered a very low percentage of the surface area of the liposomes. Both the liposomes recovered after *in vitro* incubation and *in vivo* circulation completely preserved their size, fluorescence properties, and receptor targeting characteristics. Overall, our results thus demonstrate that the hard protein corona of PEGylated stealth liposomes—with or without antibodies conjugated to their surface—is sparse and does not affect the structure and targeting abilities of the liposomes.

2 Materials and methods

2.1 Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), ovine cholesterol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] ammonium salt (DSPE-PEG2k-maleimide) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] sodium salt (DSPE-PEG2k) was purchased from Lipoid (Ludwigshafen, Germany). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine labeled with Atto 488 (Atto 488 DPPE) was purchased from Atto-Tec (Siegen, Germany). Boric acid tablets, tert-butanol, ethylenediaminetetraacetic acid dipotassium salt (K₂ EDTA), phosphate-buffered saline (PBS) tablets, sterile PBS solution used for cell cultures, monosodium phosphate, disodium phosphate, glucose, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), Amicon Ultra-4 30 kDa and 100 kDa centrifugal filter units, penicillin-streptomycin solution, trypsin-EDTA solution, polystyrene latex beads, acetic acid, sodium deoxycholate, triethylammonium bicarbonate buffer, tris(2-carboxyethyl)phosphine hydrochloride solution, 2-chloroacetamide, ethyl acetate, and human serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Herceptin was

purchased from Roche (Basel, Switzerland). Alexa Fluor 488 hydrazide, 2-iminothiolane (Traut's reagent), acetonitrile, formic acid, micro-BCA assay kit, Pierce trypsin protease, trifluoroacetic acid, NuPAGE LDS sample buffer, PageRuler unstained protein ladder, MOPS SDS sample buffer, Pierce silver stain kit, McCoy's 5A modified medium, and Hanks' balanced salt solution (HBSS) with calcium and magnesium without phenol red were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ethanol 96% was purchased from VWR International (Radnor, PA, USA). Ethylenediaminetetraacetic acid disodium salt (Na_2 EDTA) was purchased from Merck (Darmstadt, Germany). Slurry for preparing Sepharose CL-4B columns was purchased from GE Healthcare (Little Chalfont, UK). Econo-Column glass chromatography columns (dimensions 1.5×20 cm and 1.5×50 cm) for size-exclusion chromatography were purchased from Bio-Rad (Hercules, CA, USA). Protein LoBind tubes were purchased from Eppendorf (Hamburg, Germany). Fetal bovine serum (FBS) was purchased from Biowest (Nuaille, France). Heat-inactivated FBS was prepared by incubating native FBS for 1 h at 56°C .

2.2 Experimental methods

The following section contains a description of some of the experimental methods used in the present study.

2.2.1 Fluorescence correlation spectroscopy (FCS)

FCS was used to determine the concentration, size, and fluorescence brightness of Atto 488-labeled liposomes in various samples. To investigate a given sample, 100 μL of the sample was transferred to a chamber well in an eight-well chambered coverslip (uncoated μ -slide 8 well, Ibidi, Martinsried, Germany). The given sample was left in the chamber well for ~ 5 min. The sample was then remixed in the chamber well by pipetting up and down ~ 10 times. Immediately after remixing, the eight-well chambered coverslip was placed on the microscope stage of the FCS setup and the sample was investigated by FCS. By this sample preparation approach, the coverslip was partially blocked with the liposomes in the sample, decreasing the adsorption rate of the liposomes to the coverslip during the FCS experiments and thereby ensuring stable photon count rates over the course of the experiments.

As described previously [32, 33], the FCS experiments were performed using a commercially available DCS-120 confocal scanning FLIM system (Becker & Hickl, Berlin,

Germany) connected to a Zeiss Axio Observer Z1 inverted microscope equipped with a C-Apochromat 40×/1.2 W Corr UV-VIS-IR water immersion objective (Carl Zeiss, Jena, Germany). The excitation source for the system was a 473 nm picosecond diode laser (BDL-473-SMC) operated at a pulse repetition rate of 50 MHz. After passing through a confocal pinhole with a diameter of 1.2 Airy units, a 485 nm longpass filter, and a 535/30 nm bandpass filter, the fluorescence emission was detected with an HPM-100-40 hybrid detector connected to an SPC-150 module. Lifetime gating was used to partially suppress background noise. SPCM software was used to calculate the experimental autocorrelation curves. The curves were subsequently exported to be fitted using MATLAB (The MathWorks, Natick, MA, USA). All samples were measured by positioning the laser focus ~ 100 μm above the top of the coverslip. The acquisition time for each FCS experiment was either 1 or 5 min, depending on the specific experiment. Calibration of the setup was done using solutions with Alexa Fluor 488 hydrazide. The effective detection volume was determined to be 1.3 fL (focus waist radius 300 nm) and the structural S -parameter (defined below) was determined to be 8.7. The S -parameter was fixed to this value when fitting the autocorrelation curves acquired in all subsequent experiments. Except for the curves used for determining the S -parameter, all autocorrelation curves were analyzed using unweighted least squares fitting. In a few rare instances, the autocorrelation curves were dominated by single bright events. These distorted curves were discarded in the data analysis.

The fluorescence emission photon count rates, F , recorded in the FCS experiments were used to determine the lipid concentrations, C_{lip} , of the investigated samples using that

$$C_{\text{lip}} = K(F - F_{\text{bg}}) \quad (1)$$

where F_{bg} is the background photon count rate—recorded from a blank sample only containing PBS—and K is a constant of proportionality determined from liposome samples of known lipid concentrations.

The autocorrelation curves recorded by FCS were fitted with the single-component autocorrelation function, $G(\tau)$, without triplet state contribution: [34]

$$G(\tau) = \frac{1}{N_{\text{meas}}} \left(1 + \frac{\tau}{\tau_D} \right)^{-1} \left(1 + \frac{\tau}{S^2 \tau_D} \right)^{-\frac{1}{2}} \quad (2)$$

where τ is the lag time, N_{meas} is the measured mean number of liposomes in the detection

volume, S is the ratio of the axial to radial dimensions of the detection volume, and τ_D is the characteristic translational diffusion time of the liposomes.

The mean number of liposomes in the detection volume, N , was calculated by the equation [35]

$$N = N_{\text{meas}} \frac{(F - F_{\text{bg}})^2}{F^2}. \quad (3)$$

The liposome fluorescence brightness, B , was then determined by the equation

$$B = \frac{F - F_{\text{bg}}}{N}. \quad (4)$$

The value of τ_D was related to the diffusion coefficient, D , of the liposomes by the equation [34]

$$D = \frac{\omega_0^2}{4\tau_D}. \quad (5)$$

where ω_0^2 is the waist radius of the detection volume. From the diffusion coefficient, the hydrodynamic diameter of the liposomes was then calculated using the Stokes-Einstein relation [36].

2.2.2 Micro bicinchoninic acid (micro-BCA) assay

The micro-BCA assay was used to measure the protein mass concentration of various samples. As the first step, 150 μL of the samples under investigation were transferred to a series of Protein LoBind tubes. Additionally, 150 μL of a number of standard samples with varying mass concentration of bovine serum albumin between 0–100 $\mu\text{g/mL}$ were also transferred to Protein LoBind tubes. Then, 150 μL working reagent was added to all tubes. The tubes were vortexed and placed for 1 h in a 60 $^{\circ}\text{C}$ water bath. After that, the tubes and their containing samples were allowed to cool at room temperature for a few minutes. Subsequently, 250 μL of each sample was transferred to a transparent 96-well microtiter plate to measure the absorbance at 570 nm by use of a Victor³ 1420 Multilabel Counter (PerkinElmer, Waltham, MA, USA). An absorbance-mass concentration standard curve was determined from the bovine serum albumin standard samples. Finally, the protein mass concentrations of the rest of the investigated samples were estimated by comparing their absorbances to the standard curve. If the protein mass concentration of a given

sample was $> 100 \mu\text{g/mL}$, it was further diluted in PBS and investigated again.

2.2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was used to gauge the protein content of various samples. As the first step, $37.5 \mu\text{L}$ of the samples under investigation were mixed with $12.5 \mu\text{L}$ $4\times$ NuPAGE LDS sample buffer in Protein LoBind tubes. The tubes were then incubated for 10 min in a 70°C water bath to denature the proteins in the samples. The tubes and their containing samples were subsequently allowed to cool at room temperature for a few minutes, and after that, $25 \mu\text{L}$ of each sample was loaded in a 10-well 1.0 mm NuPAGE 4-12% Bis-Tris protein gel (Thermo Fisher Scientific) mounted in an XCell SureLock Mini-Cell electrophoresis system (Thermo Fisher Scientific). One lane of each gel was loaded with 1 ng human serum albumin to provide a standard for estimating the amount of protein in the other lanes of the gel. Moreover, one lane of each gel was loaded with a PageRuler unstained protein ladder to provide a basis for estimating the molecular weights of the proteins in the other lanes of the gel. All gels were run for 45 min at 200 V in MOPS SDS running buffer and then stained using the Pierce silver stain kit following the manufacturer's guidelines. The gels were imaged using an ImageQuant LAS 4000 (GE Healthcare). The density of the individual bands were determined using ImageJ (<https://imagej.nih.gov/ij/>). The total mass of the serum/plasma proteins, m_{pro} , in a given lane was estimated using the equation

$$m_{\text{pro}} = m_1 \frac{\sum_i D_i}{D_1} \quad (6)$$

where i is the index of the individual serum/plasma protein bands in the lane, D_i is the density of the i th serum/plasma protein band, D_1 is the density of the albumin band in the standard lane on the same gel, and $m_1 = 1 \text{ ng}$ is the mass of the albumin loaded in the standard lane.

2.2.4 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was used to identify the proteins present in various samples. First, $200 \mu\text{L}$ of the samples under investigation were mixed with $200 \mu\text{L}$ lysis buffer (5% sodium deoxycholate, 50 mM triethylammonium bicarbonate, pH 8.5) in Protein LoBind tubes. The samples were then incubated at 95°C for 5 min for denaturation of the proteins. Subsequently, the samples were

transferred to a Microcon-10kDa centrifugal filter unit (Merck) and centrifuged at 14,000g until the solvent had flown through the filter. The filtrate was discarded, and 200 μ L 10 mM tris(2-carboxyethyl)phosphine, 50 mM 2-chloroacetamide in digestion buffer (0.5% sodium deoxycholate, 50 mM triethylammonium bicarbonate, pH 8.5) was added to the samples. The samples were then reduced and alkylated by incubating for 30 min at 37 °C. Next, the samples were centrifuged at 14,000g until the solvent had flown through the filter. The filtrate was discarded, and 200 μ L digestion buffer was added to the samples. The samples were again centrifuged at 14,000g until the solvent had flown through the filter. The inner spin filters were transferred to new collection tubes, and 50 μ L 20 μ g/mL Pierce trypsin protease in digestion buffer was added to the filters. The samples were vortexed and incubated for $\sim$ 12 h at 37 °C to digest the proteins. Then, the samples were centrifuged at 14,000g until the protein digest had flown through the filters. To ensure complete recovery of the protein digest, 100 μ L 50 mM triethylammonium bicarbonate, pH 8.5 buffer was added to the filters, and the filters were centrifuged at 14,000g until the buffer had flown through the filters. The spin filters were discarded, whereas the collection tubes with the filtrate containing the protein digests were saved. Next, 450 μ L ethyl acetate and 6 μ L trifluoroacetic acid were added to all tubes. The tubes were vortexed and centrifuged at 14,000g for 1 min to obtain phase separation. The bottom phase contained the peptides, whereas the top phase contained the sodium deoxycholate. The top phase was removed by pipette to get rid of the sodium deoxycholate. Additional 450 μ L ethyl acetate was added to all samples, and the samples were again vortexed and centrifuged at 14,000g for 1 min to obtain phase separation. The top phase was removed by pipette to ensure complete removal of the sodium deoxycholate from the samples. Each sample was then dried in a vacuum centrifuge and resuspended in 23 μ L 2% acetonitrile, 0.1% trifluoroacetic acid, 0.1% formic acid aqueous solution. The samples were vortexed, spun down using a minicentrifuge, and ultrasonicated for 2 min. Subsequently, the samples were spun down at 14,000g, and the supernatant loaded in a 96-well plate.

The samples were investigated in technical duplicate using a UPLC-nanoESI MS/MS setup consisting of a Dionex RSLC nanopump (Dionex RSLC 3500, Thermo Fisher Scientific) connected to a Q Exactive High-Field mass spectrometer (Thermo Fisher Scientific). The peptide samples were loaded onto a C18 reversed phase column (Dionex Acclaim PepMap RSLC C18, 2 μ m, 100 Å, 75 μ m $\times$ 2 cm) and separated using a C18 reversed phase column (Dionex Acclaim PepMap RSLC C18, 2 μ m, 100 Å, 75 μ m $\times$ 75 cm) at 60 °C with a flow rate of 300 nL/min. The

mobile phases were (A) water with 2% acetonitrile and 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The loading was done with 2 % B over 5 min. The separation was performed by a linear gradient from 10% B to 35% B over 35 min. A full MS scan in the mass range of m/z 375 to 1500 was acquired at a resolution of 120,000. In each cycle, the mass spectrometer triggered up to 20 MS/MS acquisitions of eluting ions based on highest signal intensity for fragmentation. The MS/MS scans were acquired with a dynamic mass range at a resolution of 15,000. The precursor ions were isolated using a quadrupole isolation window of m/z 1.4 and fragmented using higher-energy collision dissociation (HCD) with a normalized collision energy of 27. Fragmented ions were dynamically added to an exclusion list for 15 seconds.

All acquired MS scans were searched using default settings in MaxQuant/Andromeda 1.6.0.1 [37] against a customized version of *Bos taurus* Hereford reference proteome from UniProt (UP000009136; date stamp 16MAY2017) or *Mus musculus* C57BL/6J reference proteome database from UniProt (UP000000589; date stamp 16MAY2017). Both databases included the primary sequence of trastuzumab (<https://www.drugbank.ca/drugs/DB00072>). Standard settings were employed with carbamidomethyl (C) as a static modification and protein N-terminal acetylation, deamidation (NQ) and oxidation (M) as variable modifications.

The MaxQuant results were processed using Perseus 1.6.0.1 [38]. Two or more unique peptides in at least one group (control, non-targeted liposomes, targeted liposomes) were required for protein quantification.

For completeness, it should be mentioned that we by this experimental procedure detected small amounts of trastuzumab in samples without targeted liposomes. This observation was likely due to low carry-over (< 1%) from the samples with the targeted liposomes due to the very high concentration of the antibody in these samples. Alternatively, it could possibly also be that overlapping peptides of mouse and bovine antibodies in the samples were ascribed to trastuzumab. These effects did, however, not impact the overall interpretation of the LC-MS/MS data.

2.2.5 Cell cultures

SKBR-3 cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were grown in T-75 polystyrene tissue culture flasks with standard growth surfaces for adherent cells (Sarstedt, Nümbrecht, Germany) in 10 mL McCoy's 5A modified medium supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin

(supplemented McCoy's 5A medium) in a humidified 37 °C/5% CO₂ incubator.

The cells were passaged every ~3–4 days—typically after having grown to 70–90% confluence—using the following protocol: The cells in the T-75 flasks were washed with 10 mL PBS solution (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.1–7.5). Then, the cells were incubated for ~5 min with 2 mL trypsin-EDTA solution (0.5 g/L porcine trypsin and 0.2 g/L EDTA in HBBS with phenol red) in the humidified 37 °C/5% CO₂ incubator. Subsequently, the flasks were tapped a few times to loosen the cells, and 8 mL supplemented McCoy's 5A medium was added to halt the trypsinization. The cells were aspirated by pipetting, and some of the cells were transferred to a new T-75 flask together with more supplemented McCoy's 5A medium to obtain a final medium volume of 10 mL and a split ratio of ~1:2. Sometimes, to prepare cells for flow cytometry experiments, some of the cells were also transferred to Nunc Nunclon 12-well × 2 mL cell culture dishes (Thermo Fisher Scientific). In these cases, 1.5×10^6 cells in 1 mL supplemented McCoy's 5A medium (cell concentration determined by a Moxi Z mini automated cell counter, Orflo Technologies, Ketchum, ID, USA) were seeded in each well. The cells were allowed to grow and adhere to the wells for ~48 h and then used for flow cytometry experiments, as described below.

2.2.6 Flow cytometry

Flow cytometry was used to evaluate the association of Atto 488-labeled liposomes with SKBR-3 cells. The growth medium was removed from the wells in the Nunc Nunclon 12-well × 2 mL cell culture dishes. Then, ~1 mL PBS solution was added to all wells, and the dishes were incubated for 5 min in the humidified 37 °C/5% CO₂ incubator. The purpose of this step was to minimize the protein content present in the wells during the subsequent experiments. Next, the PBS solution was removed from all wells and 1 mL of the samples with the liposomes under investigation were added to the wells. The dishes were incubated for 30 min in the humidified 37 °C/5% CO₂ incubator. Subsequently, all wells were washed with 1 mL PBS solution. Then, 150 µL trypsin-EDTA solution diluted $\times 10$ in PBS solution was added to the wells, and the dishes were incubated for 2 min in the humidified 37 °C/5% CO₂ incubator. The dishes were gently tapped to detach the cells, and 1 mL HBBS with calcium and magnesium was added. The cells were aspirated by pipette and transferred to test tubes for flow cytometry (dimensions 12×75 mm, Beckman Coulter, Brea, CA, USA). The test tubes were centrifuged at 150g for 5 min. The

supernatant was removed, and 300 μ L HBBS with calcium and magnesium was added. The pellet was re-suspended with pipette, and the cells then investigated by flow cytometry.

The flow cytometry experiments were performed on an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The samples were excited at 488 nm, and the fluorescence emission was filtered using a 533/30 nm bandpass filter. A total of 40,000 events were recorded per sample. The data were analyzed using FlowJo software (FlowJo, Ashland, OR, USA). An overview of the gating strategy is given in Figure S2 in the Supplementary information.

2.3 Preparation and characterization of liposomes

Liposomes of the following molar lipid compositions were prepared: DSPC/cholesterol/DSPE-PEG2k (56.3:38.2:5.5) and DSPC/cholesterol/DSPE-PEG2k/DSPE-PEG2k-maleimide (56.3:38.2:5:0.5). Both formulations also included 0.05 mol% Atto 488 DPPE.

To prepare the liposomes, the lipids were first dissolved in tert-butanol/Milli-Q water (9:1). The solutions were heated to ~ 40 – 50 $^{\circ}$ C to ensure that the lipids were completely dissolved. Subsequently, the solutions were plunge frozen in liquid nitrogen. The solutions were then lyophilized overnight to remove the solvent. Next, the samples were hydrated in PBS (10 mM phosphate, 137 mM sodium chloride, 2.7 mM KCl, pH 7.4) with gentle vortexing every 5 min for a period of 30 min. At this point, the lipid concentration of the samples was ~ 50 mM. After hydration, the samples were extruded 21 times through a 100 nm polycarbonate filter (Whatman, GE Healthcare) using a mini-extruder (Avanti Polar Lipids) to form liposomes. Throughout the hydration and extrusion procedure, the samples were heated to at least 65 $^{\circ}$ C. After extrusion, the lipid concentration of the samples was ~ 40 mM.

The DSPE-PEG2k-maleimide-containing liposomes were functionalized with the antibody trastuzumab by the following protocol: We dissolved ~ 10 – 15 mg Herceptin powder—which is a drug containing trastuzumab—in 4 mL borate buffer (100 mM borate, 2 mM K_2 EDTA, prepared from boric acid and adjusted to pH 8 by NaOH solution). To separate trastuzumab from the excipients of Herceptin, the sample was transferred to an Amicon Ultra-4 30 kDa centrifugal filter unit and then centrifuged for 20 min at 4000g before the filtrate was discarded. To ensure effective removal of the excipients, three additional washing steps were performed, each time adding 4 mL borate buffer to the filter unit, centrifuging for 20 min at 4000g, and discarding the filtrate. After

the last centrifugation step, the mass concentration of trastuzumab in the solution remaining in the filter device sample reservoir was determined by recording the absorbance at 280 nm using a NanoDrop 2000c spectrophotometer (NanoDrop Products, Thermo Fisher Scientific) and assuming that the mass extinction coefficient of trastuzumab at 280 nm is $1.4 \text{ (mg/mL)}^{-1}\text{cm}^{-1}$, as estimated from the amino acid sequence and structure of the antibody [39]. A solution of 600 μL with 7.5 mg/mL of the purified trastuzumab in borate buffer was prepared in a Protein LoBind tube. Then, 8.5 μL 2 mg/mL Traut's reagent solution in borate buffer was added to the trastuzumab solution, followed by 1 h incubation at room temperature to allow Traut's reagent to thiolate primary amines on the surface of the antibody. Next, for the purpose of exchanging the borate buffer and removing unreacted Traut's reagent, the solution with the thiolated antibodies was transferred to an Amicon Ultra-4 30 kDa centrifugal filter unit, and PBS was added to a total volume of 4 mL. The filter unit was centrifuged for 20 min at 4000g and the filtrate discarded. To ensure complete buffer exchange and removal of unreacted Traut's reagent, additional 4 mL PBS was added to the filter unit, followed by centrifugation for another 20 min at 4000g. The mass concentration of the thiolated trastuzumab in the solution remaining in the filter device sample reservoir was determined from the absorbance at 280 nm, recorded using the NanoDrop 2000c spectrophotometer as described above. The degree of thiolation of trastuzumab was determined to be 0.9 ± 0.1 using Ellman's reagent. A volume of newly prepared thiolated trastuzumab solution, corresponding to 1.8 mg thiolated trastuzumab, was added to 840 μL newly prepared DSPE-PEG2k-maleimide-containing liposomes in a Protein LoBind tube. The air phase in the tube was replaced with N_2 , and the tube was incubated for 24 h at room temperature in the dark to allow the sulfhydryl groups on the surface of trastuzumab to couple to the maleimide groups on the surface of the liposomes. The liposomes were then separated from unbound antibody using a Sepharose CL-4B size-exclusion chromatography column (dimensions $1.5 \times 20 \text{ cm}$) eluted with PBS with a flow rate of $\sim 1 \text{ mL/min}$, and the column fractions were collected in Protein LoBind tubes. Finally, the liposome-containing fractions were pooled in an Amicon Ultra-4 100 kDa centrifugal filter unit and centrifuged at 2000g until the lipid concentration of the liposome solution was increased to $\sim 30\text{--}40 \text{ mM}$. Finalized samples with trastuzumab-functionalized liposomes were stored in Protein LoBind tubes.

Upon preparation, the liposomes—non-functionalized as well as antibody-functionalized liposomes—were characterized using a number of techniques: Inductively coupled plasma mass

spectrometry (ICP-MS, done on an iCAP Q ICP-MS, Thermo Fisher Scientific) was used to determine the phosphorus concentrations of the liposome solutions; for these experiments, the liposome samples were diluted $\times 17,000$ in 2% HCl, 10 ppb Ga solvent. Phospholipid concentrations were then estimated by subtracting the background of the PBS buffer (the background typically represented $\sim 30\%$ of the total amount of phosphorus), and the total molar lipid concentration was estimated by dividing the phospholipid concentrations by 0.618, thereby taking into account that cholesterol does not contain any phosphorus atoms. Dynamic light scattering (DLS, done on ZetaPALS, Brookhaven Instruments, Holtsville, NY, USA) was used to measure the size of the liposomes; for these experiments, the liposomes were diluted to 100 μM (concentration in terms of lipid) in PBS. Phase analysis light scattering (PALS, done on the ZetaPALS) was used to measure the zeta potential of the liposomes; for these experiments, the liposomes were diluted to 100 μM (concentration in terms of lipid) in a phosphate-glucose buffer (10 mM phosphate, 280 mM glucose, pH 7.4). FCS was used to measure the size and brightness of the liposomes; for these experiments, the liposomes were diluted to 500 μM (concentration in terms of lipid) in PBS, and FCS was performed as described above with an acquisition time of 5 min. The micro-BCA assay was used to measure the antibody concentration of the functionalized liposomes; for these experiments, the antibody-functionalized liposome stocks were diluted $\times 20\text{--}200$ in PBS, and the micro-BCA assay was performed to measure the protein mass concentration in the diluted liposome samples. To take into account that also the lipids can give a small contribution in the micro-BCA assay [40], we also performed a micro-BCA measurement on non-functionalized DSPE-PEG2k-maleimide-containing liposomes. The contribution of the lipids was subtracted to determine the true amount of antibody conjugated to the liposomes.

2.4 *In vitro* experiments

Samples of 550 μL with liposomes (2 mM lipid) in FBS/PBS (9:1) were prepared in Protein LoBind tubes. For reference, also blank samples with FBS/PBS (9:1) without any liposomes were prepared in Protein LoBind tubes. The reason that all samples contained 10% PBS was related to the fact that the liposomes were prepared in PBS. To be more specific, diluting the liposomes in FBS also meant adding a small amount of PBS; given this premise, we wanted to make sure that all samples contained the same amount of PBS to allow us to directly compare the results obtained with the different samples.

After preparation, each sample was incubated for 1 h in a 37 °C oven. Next, 500 µL of each sample was loaded onto a Sepharose CL-4B size-exclusion chromatography column (dimensions 1.5×50 cm) and eluted with PBS at a flow rate ~ 0.5 mL/min. After loading a given sample onto a column, we waited for 30 min. Then, 40 column fractions of 2 min were collected in Protein LoBind tubes.

2.5 *In vivo* experiments

All animal procedures were approved by The Danish Animal Welfare Council, the Danish Ministry of Justice. Six-week-old female Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and were given at least 11 days of adaption upon arrival.

The animal experiments were always initiated at ~ 8 a.m. to reduce experimental uncertainty related to daily variations in protein blood levels. The weight of the mice at the time of experimental initiation was 19 ± 1 g. The mice were injected in the tail vein with either 180 µL 20 mM liposome PBS solutions (concentration in terms of lipid) or 180 µL blank PBS solutions without any liposomes. One hour after injection, the mice were anesthetized using 4.5% sevoflurane and their blood was collected by cardiac puncture. The blood from each mouse (~ 200 – 500 µL) was transferred to a Protein LoBind tube containing 35 µL 170 mM Na₂ EDTA. Immediately after transferring the blood to a given Protein LoBind tube, the tube was inverted ten times to properly mix the blood and Na₂ EDTA solution. Subsequently, all tubes were centrifuged for 5 min at 1500g to pellet the blood cells. The plasma supernatants were then transferred to new Protein LoBind tubes, which were centrifuged again for 5 min at 1500g to ensure complete removal of blood cells. The plasma of four tubes from mice receiving the same injectant was pooled in a new Protein LoBind tube to ensure a sufficient sample volume for further sample treatment and analysis. Next, 500 µL of each of the pooled samples was loaded onto a Sepharose CL-4B size-exclusion chromatography column (dimensions 1.5×50 cm) and eluted with PBS at a flow rate ~ 0.5 mL/min. After loading a given sample onto a column, we waited for 30 min. Then, 40 column fractions of 2 min were collected in Protein LoBind tubes.

2.6 Experiments after size-exclusion chromatography

2.6.1 Liposome samples

After a given size-exclusion chromatography run, all of the collected column fractions were analyzed using the micro-BCA assay to determine the protein concentration. In addition, a subset of the collected fractions in the vicinity of the column void volume were investigated using FCS with 1 min acquisition time to determine the concentration of the liposomes. For the fractions investigated by FCS, the measured protein concentrations were corrected for the contribution of the liposomes using the following equation:

$$c_{\text{cor}} = c_{\text{meas}} - K_{\text{lip}} C_{\text{lip}} \quad (7)$$

where c_{cor} is the corrected protein mass concentration, c_{meas} is the measured protein mass concentration, C_{lip} is the molar lipid concentration, and K_{lip} represents the magnitude of the liposome contribution. This equation relied on the assumption that the contribution of the liposomes scaled linearly with their concentration, which was confirmed in separate control experiments on liposome samples of varying concentration. Based on these control experiments, the value of K_{lip} was determined for each batch of liposomes. Typically, K_{lip} was 1–2 $\mu\text{g/mL/mM}$ for the non-functionalized liposomes and ~ 20 $\mu\text{g/mL/mM}$ for the antibody-functionalized liposomes.

The void-volume fractions, identified as being the five fractions with the highest liposome concentration, were pooled for each size-exclusion chromatography run. The pooled samples were investigated by FCS using an acquisition time of 5 min to determine the concentration, hydrodynamic diameter, and fluorescence brightness of the liposomes. Samples with 50 μM of the liposomes (concentration in terms of lipid) from the pooled samples were then prepared in PBS, and the ability of the liposomes to associate with SKBR-3 cells was evaluated and compared to that of 50 μM pristine, non-treated liposomes using flow cytometry. The flow cytometry experiments were always done on the same day as the liposome had been exposed to serum/plasma.

Finally, the molecular weight and content of the proteins in the pooled samples were investigated using SDS-PAGE, and the identity of the proteins in the pooled samples was determined using LC-MS/MS.

2.6.2 Blank samples

After a given size-exclusion chromatography run, all of the collected column fractions were

analyzed using the micro-BCA assay to determine the protein concentration. The void-volume fractions—identified as being the five fractions with the highest protein concentration of an early peak eluting before the main serum/plasma protein peak—were pooled for each size-exclusion chromatography run. The molecular weight and content of the proteins in the pooled samples were investigated using SDS-PAGE, and the identity of the proteins in the pooled samples was determined using LC-MS/MS.

3 Results and discussion

3.1 Preparation and characterization of liposomes

Both non-targeted and targeted PEGylated stealth liposomes were formed by the common extrusion method, involving repeated extrusion of lipid suspensions through a polycarbonate filter with 100-nm pore sizes [41]. The targeted liposomes were functionalized with trastuzumab by the also-common thiol-maleimide coupling method, involving thiolation of trastuzumab and subsequent coupling of the free sulfhydryl groups on trastuzumab to maleimide groups on the distal ends of the PEG molecules on the surfaces of the liposomes [31]. To be applicable to FCS and flow cytometry experiments, all liposomes were fluorescently labeled by incorporating Atto 488 DPPE in their membranes. The lipid concentration of the prepared liposome stock samples was assessed using inductively coupled plasma mass spectrometry (ICP-MS). The trastuzumab conjugation level on the targeted liposomes was estimated using the micro-BCA assay to be 19 g trastuzumab per mol lipid. The composition of the prepared liposomes is summarized in Table 1.

Table 1: Lipid composition and trastuzumab conjugation level of the liposomes. The trastuzumab conjugation level is presented as the mean $\pm$ standard deviation of eight individual liposome batches.

Liposome type	Non-targeted	Targeted
DSPC (mol% lipid)	56.3	56.3
Cholesterol (mol% lipid)	38.2	38.2
DSPE-PEG2k (mol% lipid)	5.5	5.0
DSPE-PEG2k-maleimide (mol% lipid)	0	0.5

Atto 488 DPPE (mol% lipid)	0.05	0.05
Trastuzumab (g/mol lipid)	0	19 ± 5

The basic physicochemical characteristics of the prepared liposomes were evaluated using a number of techniques: The zeta potential of the liposomes was evaluated using phase analysis light scattering (PALS), the size of the liposomes was evaluated using both dynamic light scattering (DLS) and FCS, and the fluorescence brightness of the liposomes (fluorescence emission photon count rate per liposome) was evaluated using FCS. Further details on FCS as a method for studying liposomes are given in Figure S1 and the associated discussion in the Supplementary information. The physicochemical characteristics of the liposomes are summarized in Table 2. The zeta potential, size, and fluorescence brightness were similar for the non-targeted and targeted liposomes. Given the measured size of the liposomes, the average surface area per liposome was estimated to be 50,000 nm². Assuming the cross-sectional area of DSPC and DSPE-PEG2k to be 0.53 nm² and the cross-sectional area of cholesterol to be 0.22 nm² [42], we then estimated that the liposomes on average contained 250,000 lipids. Based on this and using that the molecular weight of trastuzumab is ~ 145 kDa [43], we estimated that the trastuzumab conjugation level of 19 g per mol lipid (Table 1) corresponded to 33 trastuzumab molecules per liposome.

Table 2: Physicochemical characteristics of the liposomes. The results are presented as the mean ± standard deviation of five individual non-targeted liposome batches and eight individual targeted liposome batches.

Liposome type	Non-targeted	Targeted
Zeta potential by PALS (mV)	-9 ± 2	-9 ± 2
Hydrodynamic diameter by DLS (nm)	123 ± 7	134 ± 9
Hydrodynamic diameter by FCS (nm)	121 ± 7	142 ± 17
Fluorescence brightness by FCS (kHz)	19 ± 6	24 ± 4

The ability of the prepared liposomes to interact with the HER2-positive cell line SKBR-3 was also evaluated. To minimize the impact of a potential protein corona, this experiment was done in a protein-free environment. More specifically, liposomes (50 or 100 µM lipid) dispersed in

PBS were incubated with SKBR-3 cells for 30 min at 37 °C. The cellular association of the liposomes was then evaluated using flow cytometry. Figure S2 in the Supplementary information shows the flow cytometry gating strategy, and Figure 2A shows the mean fluorescence intensity (MFI) values measured in the experiment. There was no detectable association of the non-targeted liposomes to the cells. In contrast, the targeted liposomes clearly associated with the cells with the MFI increasing as a function of the liposome concentration. To confirm the role of trastuzumab in the association of the targeted liposomes with the SKBR-3 cells, the same experiment was repeated, but this time with 100 µg/mL free trastuzumab in the buffer. Figure 2B shows the measured MFI values. The free trastuzumab completely abolished the binding of the targeted liposomes to the cells. This demonstrated that the free trastuzumab blocked the receptors mediating the cellular association of the targeted liposomes. Thus, it was clear that the association of the targeted liposomes with the SKBR-3 cells was induced by the trastuzumab molecules conjugated to the surface of the liposomes. In other words, the prepared liposomes overall possessed the cellular interaction characteristics that we expected them to have.

Figure 2: Association of liposomes to the HER2-positive cell line SKBR-3. Samples containing 50 or 100 µM liposomes (concentration in terms of lipid) in PBS with or without 100 µg/mL free trastuzumab were prepared and incubated with the cells for 30 min at 37 °C. For reference, blank PBS samples without liposomes and with or without 100 µg/mL free trastuzumab were also incubated with the cells. The fluorescence intensity of the cells was then measured using flow cytometry. (A) MFI values for samples without free trastuzumab. (B) MFI values for samples with free trastuzumab. In panel A, the data are the average of three separate experiments, and in panel B, the data are the average of two–three separate experiments. In both panels, the error bars represent the standard deviations.

3.2 The liposome recovery method

Before commencing our studies on blood protein binding to the prepared liposomes, we needed to choose a method for recovering the liposomes after exposure to blood serum/plasma. We chose to work with size-exclusion chromatography as this is a common method for separating nanomaterials from unbound blood proteins [2]. More specifically, we chose to work with Sepharose CL-4B as this column material previously has been used to recover liposomes from

blood serum/plasma [16, 17, 44, 45]. As an initial test experiment, we compared the ability of a 20-cm and a 50-cm long Sepharose CL-4B column to separate liposomes from unbound serum proteins. This experiment demonstrated that the 50-cm column provided a better separation of the liposomes from unbound serum proteins than the 20-cm column—see Figure S3 in the Supplementary information. Consequently, we chose to use the 50-cm column in all subsequent experiments.

3.3 Liposomes recovered after *in vitro* incubation in fetal bovine serum

Having prepared the liposomes and chosen a recovery method, we were ready for our actual experiments. To begin with, we wanted to study liposomes incubated *in vitro* in FBS, as this is the standard type of serum used in most cell culture experiments. For these experiments, we prepared samples containing 2 mM liposome (concentration in terms of lipid) and 90% FBS. For comparison, we also prepared blank samples with 10% PBS and 90% FBS without any liposomes. After 1 h incubation at 37 °C, 500 μ L of each of the prepared samples was loaded onto a Sepharose CL-4B column eluted with PBS at a flow rate of $\sim$ 0.5 mL/min, and 2-min column fractions were collected.

The lipid concentration of the collected column fractions was measured using FCS—or to be more accurate using the fluorescence emission photon count rates of the FCS recordings assuming that the count rates were linearly proportional to the lipid concentration. The protein concentration of the collected column fractions was measured using the micro-BCA assay. It should be mentioned that not only the serum proteins but also the liposomes gave a contribution to the protein levels determined by the micro-BCA assay. This liposome contribution was particularly pronounced for the targeted liposomes due to the antibodies conjugated to their surface. However, for all liposomes, also the lipids gave rise to a small false protein signal in the micro-BCA assay [40]. Consequently, the protein concentrations determined by the micro-BCA assay were corrected for the contribution of the liposomes—that is, the contribution of the antibodies and the lipids—thus only representing the contribution of the serum.

Figure 3A,B shows some examples of elution profiles recorded for liposome-containing samples, and Figure 3C shows an example of an elution profile recorded for a blank control sample. To obtain a basic understanding of the recorded elution profiles, we performed a few calculations. First, for the elution profiles with the liposome-containing samples, we calculated the

total amount of lipid eluting in the void volumes by summing the lipid amounts of the individual void-volume fractions determined using FCS. Figure 3D shows the results of this calculation. Both for the non-targeted and targeted liposomes, $\sim 0.8 \pm 0.1$ mol lipid eluted in the void volumes, corresponding to $\sim 80\%$ of the amount loaded onto the column. Accordingly, it was clear that the bulk of the liposomes loaded onto the columns eluted in the void volumes.

Figure 3: Liposomes recovered after *in vitro* incubation in FBS. Samples containing liposomes (2 mM lipid) in 90% FBS were prepared. For reference, blank samples with 90% FBS without any liposomes were also prepared. All of the samples were incubated for 1 h at 37 °C. Then, 500 μ L of each sample was loaded onto a 1.5 $\times$ 50-cm Sepharose CL-4B size-exclusion chromatography column eluted with ~ 0.5 mL/min PBS, and 2-min column fractions were collected. The lipid and protein concentration of the column fractions was determined using FCS and the micro-BCA assay, respectively. (A-C) Examples of size-exclusion chromatography elution profiles for samples with non-targeted liposomes (A) or targeted liposomes (B) or without any liposomes (C). The void volumes are highlighted with a grey area. (D) Total amount of liposome (in terms of lipid) eluting in the void volumes, calculated by summing the lipid amount of the individual void-volume fractions determined by FCS. (E) Total amount of serum protein eluting in the void volumes, calculated by summing the serum protein amount of the individual void-volume fractions determined by the micro-BCA assay. The individual void-volume fractions were pooled and further investigated using a number of techniques: (F) Protein profile of pooled void-volume samples determined by non-reducing SDS-PAGE; for comparison, a sample with pristine targeted liposomes, which had not been exposed to FBS, was also investigated. (G) The ten serum proteins in the pooled void-volume samples with the highest LFQ values in LC-MS/MS. (H,I) Hydrodynamic diameter (H) and fluorescence brightness (I) of the liposomes in the pooled void-volume samples determined by FCS; for comparison, the diameters and brightnesses of pristine liposomes of the same batches were also measured. (J) MFI values of SKBR-3 cells treated for 30 min at 37 °C with 50 μ M liposomes (concentration in terms of lipid) from the pooled void-volume samples measured using flow cytometry; for comparison, the MFI values of cells treated with 50 μ M pristine liposomes of the same batches were also measured. In panels D, E, and G-J, the data are the average of two separate experiments. The reported error bars/experimental uncertainties represent the standard deviations.

For all recorded elution profiles, we also calculated the total amount of serum proteins eluting in the void volumes by summing the serum protein amounts of the individual void-volume fractions determined using the micro-BCA assay. Figure 3E shows the results of this calculation. The total amount of serum protein in the void volume was similar for all samples, independent of whether the investigated samples contained liposomes or not. Thus, based on the micro-BCA assay, it was not possible to detect binding of serum proteins to the liposomes above the serum background. In other words, a significant amount of the serum proteins co-eluting with the liposomes were likely not bound to the liposomes but rather incorporated into particles from the serum, such as lipoproteins, extracellular vesicles, or protein aggregates [46, 47].

We next wanted to investigate whether serum protein binding to the liposomes could be detected with another technique than the micro-BCA assay. Consequently, the individual void-volume fractions were pooled for each size-exclusion chromatography run, and the pooled samples were investigated using non-reducing SDS-PAGE. The gels were run and subsequently stained using a highly sensitive silver staining method with a band detection limit of ~ 0.5 – 1 ng—see Figure S4 in the Supplementary information. According to the micro-BCA results, each gel lane was loaded with ~ 10 – 20 ng of serum proteins. We would, therefore, only be able to detect a given serum protein if it represented ≥ 5 – 10% of the total amount of serum protein in the loaded samples, in turn implying that we would only observe a low number of serum protein bands on the gels. Figure 3F shows an example of the imaged SDS-PAGE lanes. At first sight, it was clear that the sample with the targeted liposomes displayed a number of strong bands > 100 kDa and some fainter bands at ~ 80 kDa and ~ 25 kDa. However, these bands were not ascribed to any serum proteins but rather to the antibody conjugated to the liposomes. Indeed, pristine targeted liposomes, which had not been exposed to any serum, displayed the same band pattern. The reason that the antibody did not display one single band at ~ 145 kDa but instead a peculiar pattern of bands may be due to antibody fragmentation, for example, because disulfide bond scrambling may occur in non-reducing SDS-PAGE [48–50]. In our case, the disulfide bond scrambling effect may have been enhanced because the antibodies were thiolated as part of the process of conjugating them to the liposomes. Additionally, the migration properties of some of the antibodies and fragments thereof may have been further altered as they were covalently coupled to DSPE-PEG2k. In any case, besides the bands related to the antibody, only a low number of very faint bands were

visible on the gels; that is, in agreement with our expectation, the pooled void-volume samples only displayed a low signal from serum proteins. Thus, it was not possible to demonstrate any convincing association of serum proteins to the liposomes using SDS-PAGE.

We next used yet another technique to investigate whether serum protein binding to the liposomes could be detected. More specifically, the pooled void-volume samples were evaluated using LC-MS/MS. From this evaluation, we determined the label-free quantification (LFQ) values, representing the relative abundances of the proteins in the samples. Figure 3G shows the ten most abundant serum proteins identified in the samples. The liposome samples contained more apolipoprotein A-I and apolipoprotein E than the blank samples. This observation imply that these apolipoproteins—either in free form or incorporated into lipoproteins—associated with the liposomes. On the other hand, the liposome samples and the blank samples generally contained the same proteins, supporting the idea that most of the serum proteins co-eluting with the liposomes were, in fact, not bound to the liposomes but rather incorporated into serum particles in the background.

Having studied the proteins co-eluting with the liposomes in the void volumes using three different techniques, we next turned our attention to the properties of the recovered liposomes themselves. For this purpose, we evaluated the liposomes in the pooled void-volume samples using FCS. Figure 3H,I shows the size and fluorescence brightness, respectively, of the recovered liposomes, and for comparison, also the size and fluorescence brightness of pristine liposomes of the same liposome batches. Neither the size nor the fluorescence brightness of any of the liposomes changed significantly after exposure to FBS, providing a good indication that FBS did not perturb the structure of the liposomes. (Incidentally, the fact that the fluorescence properties of the liposomes did not change after exposure to FBS evidenced that it was, indeed, valid to assume that the fluorescence emission photon count rates recorded in the FCS experiments were linearly proportional to the lipid concentration.)

To cast further light on the properties of the recovered liposomes, we also evaluated their interaction with SKBR-3 cells. For these experiments, the liposomes in the pooled void-volume samples were further diluted in PBS to a lipid concentration of 50 μ M and incubated with the SKBR-3 cells for 30 min at 37 °C. The cellular association was then evaluated using flow cytometry. Figure 3J shows the measured MFI values, and for comparison, also the MFI values of cells treated with 50 μ M pristine liposomes of the same liposome batches. The recovered

liposomes displayed the same association to the SKBR-3 cells as the pristine liposomes; that is, exposure to FBS did not compromise the stealth abilities of the non-targeted liposomes nor the HER2 targeting abilities of the targeted liposomes. It is worth mentioning that the fairly large size of the error bars for the MFI values in the experiments with the targeted liposomes primarily was ascribed to variations in the targeting abilities of individual liposome batches as well as variations in HER2 expression levels on the cell membranes and metabolic activity of the cells between individual experimental days. If the MFI values measured for cells treated with the recovered liposomes were normalized to the MFI values of cells treated with pristine liposomes of the same batch measured on the same day, the size of the error bars decreased significantly—see Figure S5 in the Supplementary information—further emphasizing that the liposomes retained their targeting abilities after exposure to FBS.

It should be mentioned that many cell culture experiments are not conducted with native FBS but rather with heat-inactivated FBS. This heat inactivation could, in principle, induce denaturation and/or unfolding of some of the serum proteins, altering their propensity for binding liposomes [51]. Therefore, we also performed a complete set of experiments to investigate liposomes incubated in heat-inactivated FBS. Figure S6 in the Supplementary information shows the results of these experiments. All in all, the results obtained for the liposomes treated with the heat-inactivated FBS were similar to the results obtained for the liposomes treated with native FBS. In other words, heat inactivation of the serum did not lead to any detectable differences in the liposome binding affinity of the serum proteins.

Given that we did not detect any significant protein binding to the liposomes recovered after incubation in FBS, we next performed a set of positive control experiments with a type of nanoparticle known to have a high protein binding capacity. We chose to perform these experiments using solid polystyrene nanobeads as there are several reports saying that an extensive protein corona can form around such beads [4, 52–54]. The results of the experiments, shown in Figure S7 in the Supplementary information, confirmed these reports, demonstrating that the amount of protein binding to the polystyrene beads was much higher than the amount of protein binding to the liposomes. In other words, our experimental protocol was applicable for studying protein binding to nanoparticles, but the protein binding to the liposomes was just so low that it was difficult to detect.

3.4 Liposomes recovered after *in vivo* passage in mice

The *in vitro* experiments described above were performed under quiescent conditions mimicking the environment of a typical cell culture experiment. However, liposomes circulating in the bloodstream are subject to a completely different dynamic situation and shear stress. One could well imagine that the protein binding to the liposomes would be very different between an *in vitro* and an *in vivo* situation, as suggested by several previous studies [16, 55].

Accordingly, we next wanted to gauge the protein binding to liposomes after passage in the bloodstream of an *in vivo* system. Consequently, we chose to investigate liposomes recovered after circulation in the bloodstream of mice, as this animal model is commonly used for *in vivo* studies with liposomes. The mice were injected with 180 μ L 20 mM liposome sample (concentration in terms of lipid), yielding a plasma concentration of ~ 4 mM lipid, estimated using that the average weight of the mice was 19 g and assuming a blood volume of 72 mL/kg mouse and a hematocrit level of 50% [56]. We also injected some mice with 180 μ L of a blank PBS sample. After 1 h, the blood of the mice was recovered by cardiac puncture, and the blood cells were pelleted by centrifugation. Since the plasma volumes recovered from the individual mice were too small for the further experiments, a number of pooled plasma samples were prepared using the plasma from four mice treated with the same injectant. Then, the samples were separated using size-exclusion chromatography as in the *in vitro* study with liposomes.

We estimated the lipid and protein concentration of the collected column fractions using FCS and the micro-BCA assay, respectively. Again, protein concentrations measured using the micro-BCA were corrected for the liposome contribution—that is, the contribution of the lipids and the conjugated antibodies—thus only representing the plasma. Figure 4A,B shows some examples of elution profiles recorded for liposome-containing samples, and Figure 4C shows an example of an elution profile recorded for a blank control sample.

Figure 4: Liposomes recovered after *in vivo* passage in mice. Mice were injected with 180 μ L of 20 mM liposome samples (concentration in terms of lipids). For reference, some mice were injected with 180 μ L blank samples without any liposomes. After 1 h, the blood from the mice was recovered *via* cardiac puncture and the blood cells were pelleted by centrifugation. Then, 500 μ L of the resulting plasma samples were loaded onto a 1.5 $\times$ 50-cm Sepharose CL-4B size-exclusion chromatography column eluted with ~ 0.5 mL/min PBS, and 2-min column fractions were

collected. The lipid and protein concentration of the column fractions was determined using FCS and the micro-BCA assay, respectively. (A-C) Examples of size-exclusion chromatography elution profiles for samples with non-targeted liposomes (A) or targeted liposomes (B) or without any liposomes (C). The void volumes are highlighted with a grey area. (D) Total amount of liposome (in terms of lipid) eluting in the void volumes, calculated by summing the lipid amount of the individual void-volume fractions determined by FCS. (E) Total amount of serum protein eluting in the void volumes, calculated by summing the serum protein amount of the individual void-volume fractions determined by the micro-BCA assay. The individual void-volume fractions were pooled and further investigated using a number of techniques: (F) Protein profile of pooled void-volume samples determined by non-reducing SDS-PAGE; for comparison, a sample with pristine targeted liposomes, which had not been exposed to mouse blood, was also investigated. (G) The ten plasma proteins in the pooled void-volume samples with the highest LFQ values in LC-MS/MS. (H,I) Hydrodynamic diameter (H) and fluorescence brightness (I) of the liposomes in the pooled void-volume samples determined by FCS; for comparison, the diameters and brightnesses of pristine liposomes of the same batches were also measured. (J) MFI values of SKBR-3 cells treated for 30 min at 37 °C with 50 μ M liposomes (concentration in terms of lipid) from the pooled void-volume samples measured using flow cytometry; for comparison, the MFI values of cells treated with 50 μ M pristine liposomes of the same batches were also measured. In panels D, E, and G-J, the data are the average of three separate experiments. The reported error bars/experimental uncertainties represent the standard deviations.

For the elution profiles of the liposome-containing samples, we calculated the total amount of lipid eluting in the void volume by summing the lipid amounts of the individual void-volume fractions determined using FCS. Figure 4D shows the results of this calculation. For the non-targeted liposomes, 1.7 ± 0.2 μ mol lipid eluted in the void volume, whereas for the targeted liposomes, 0.8 ± 0.2 μ mol lipid eluted in the void volume. The difference between the amount of lipid eluting for the two types of liposomes was related to the fact that the non-targeted liposomes had a longer circulation time than the targeted liposomes in the bloodstream of the mice, possibly because the Fc region of trastuzumab was recognized by various phagocytic immune cells [57]. As a consequence, the lipid concentration of the recovered plasma was 3.7 ± 0.1 mM in the experiments with the non-targeted liposomes and 1.9 ± 0.4 mM in the experiments with the

targeted liposomes, as determined using FCS. Taking this into account, the amount of lipid eluting in the void volumes corresponded to $\sim 90\%$ of the amount loaded onto the column for the non-targeted liposomes and $\sim 80\%$ for the targeted liposomes.

For all recorded elution profiles, we also calculated the total amount of plasma proteins eluting in the void volumes by summing the plasma protein amounts of the individual void-volume fractions determined using the micro-BCA assay. Figure 4E shows the results of this calculation. Considering the size of the error bars, there were no significant differences between the amount of plasma protein eluting in the void volumes for the liposome-containing samples compared to the blank samples. Thus—like in the *in vitro* experiment—the micro-BCA assay did not reveal any protein binding to the liposomes. Along the same lines, we also used the Bradford assay to investigate the total amount of plasma protein in the void volumes, but the results of this experiment—shown in Figure S8 in the Supplementary information—did not reveal any protein binding to the liposomes either. Thus, we concluded that a significant amount of unbound plasma particles co-eluted with the liposomes in the void volumes. Incidentally, this conclusion was further corroborated by another observation from the elution profiles of the liposome samples—such as those shown in Figure 4A,B—namely that the liposome peak generally eluted slightly before the plasma protein peak in the void volumes.

Next, we again turned our attention to SDS-PAGE as an alternative technique for detecting protein binding to the liposomes. More specifically, we pooled the individual void-volume fractions of each size-exclusion chromatography run and investigated the pooled samples using non-reducing SDS-PAGE. According to our micro-BCA assay results, we were able to load ~ 100 ng of plasma proteins onto each gel lane; therefore, we expected to observe a significant number of bands. Figure 4F shows an example of the imaged SDS-PAGE lanes. The blank sample did—contrary to our expectation—not display any clear bands, except for one diffuse band > 200 kDa. This possibly indicated that the proteins in this sample were irreversibly bound in large and complex particles/aggregates, not allowing for well-defined migration patterns or effective staining. In contrast, the liposome samples displayed a significant number of bands: The sample with the targeted liposomes displayed a number of strong bands > 100 kDa and some fainter bands at ~ 80 kDa and ~ 25 kDa, which again were ascribed to the antibody conjugated to the liposomes, but in addition, the samples with both the non-targeted and targeted liposomes displayed a number of plasma protein bands, which—because they were not found in the blank

sample—were identified as being plasma proteins bound to the liposomes. The most prominent of these bands was found at ~ 55 kDa. The density of the detected plasma protein bands corresponded to 20 ± 1 ng protein per lane for the samples with the non-targeted liposomes and 12 ± 2 ng protein per lane for the samples with the targeted liposomes. Thus, the plasma proteins detected in the liposome samples only corresponded to ~ 10 – 20% of the total amount of plasma protein that according to the micro-BCA assay was loaded onto each lane, supporting the conclusion that the majority of the plasma proteins co-eluting with the liposomes in the void volumes on the size-exclusion chromatography columns were not bound to the liposomes but rather incorporated into background plasma particles not detectable by the SDS-PAGE method.

We next investigated the pooled void-volume samples using LC-MS/MS. Figure 4G shows the ten most abundant plasma proteins identified in the samples. The by far most abundant protein in the liposome samples was found to be serum albumin, but in the blank samples, this protein was not even among the ten most abundant proteins. This suggested that serum albumin was the plasma protein with the highest binding to the liposomes. Incidentally, serum albumin displays migration properties corresponding to a protein of ~ 55 kDa in non-reducing SDS-PAGE [58]—see also Figure S4—further supporting the notion that this was the plasma protein with the highest binding to the liposomes. It was, however, not possible to unambiguously determine the identity of any other proteins bound to the liposomes. In fact, the liposome samples and the blank samples contained many of the same proteins, further strengthening the conclusion that most of the plasma proteins co-eluting with the liposomes in the void volumes on the size-exclusion chromatography columns were not bound to the liposomes but rather incorporated into plasma particles in the background. In particular, both the liposome samples and the blank samples contained a lot of apolipoproteins, suggesting that some of the plasma background particles were lipoproteins.

Having considered the protein binding to the liposomes, we again also wanted to focus on the properties of the recovered liposomes themselves. Thus, we evaluated the size and fluorescence brightness of the liposomes in the pooled void-volume samples using FCS. As shown in Figure 4H,I, neither the size nor the fluorescence brightness of the liposomes changed upon *in vivo* passage. We also evaluated the ability of the liposomes in the pooled void-volume samples to interact with SKBR-3 cells. As shown in Figure 4J, the non-targeted liposomes completely retained their stealth properties and the targeted liposomes completely retained their HER2 targeting abilities after *in vivo* passage, see also Figure S5. In other words, the liposomes overall

preserved their structural and functional characteristics after circulation in the bloodstream of the mice.

3.5 The nature of the protein corona of the recovered liposomes

Protein binding to liposomes recovered after exposure to blood serum or plasma is often quantified *via* the so-called protein binding value [13, 55], representing the protein mass bound per mol lipid. Accordingly, we next asked ourselves what we could say about the protein binding value of our liposomes.

We first considered the liposomes recovered after *in vitro* incubation in FBS: From the FCS and micro-BCA data in Figure 3D,E, we estimated a serum protein/lipid ratio in the void volume of ~ 5 g protein per mol lipid for both the non-targeted and targeted liposomes. However, as explained above, much of the protein in the void volume was incorporated into unbound particles in the background. Accordingly, we concluded that the protein binding value was < 5 g protein per mol lipid in these experiments. In reality, it could well be that the protein binding value was much lower than this value, but from our data, it was not possible to judge whether this was the case.

We then went on to consider the liposomes recovered after *in vivo* passage in the bloodstream of mice: From the FCS and micro-BCA data in Figure 4D,E, we calculated a plasma protein/lipid ratio in the void volume of 12 g protein per mol lipid for the non-targeted liposomes and 17 g protein per mol lipid for the targeted liposomes. Again, however, much of the protein in the void volume was not bound to the liposomes but rather incorporated into unbound particles in the background. Thus, we concluded that the protein binding value was < 12 g protein per mol lipid for the non-targeted liposomes and < 17 g protein per mol lipid for the targeted liposomes.

To further narrow down the protein binding value of the liposomes recovered after *in vivo* passage, we also considered the imaged SDS-PAGE gels, of which one example is shown in Figure 4F. As mentioned above, 20 ng and 12 ng plasma protein was detected in the gel lanes with non-targeted and targeted liposomes, respectively. Given the FCS data in Figure 4D, we calculated that 6.6 nmol and 3.2 nmol lipid was loaded onto the gel lanes with the non-targeted and targeted liposomes, respectively, and based on this, we estimated a plasma protein/lipid ratio of 3 g protein per mol lipid for the lanes with non-targeted liposomes and 4 g protein per mol lipid for the lanes with targeted liposomes. Since all of the plasma proteins detected in SDS-PAGE was bound to the

liposomes, we were overall able to conclude that the protein binding value was in the range of 3–12 g protein per mol lipid for the non-targeted liposomes and 4–17 g protein per mol lipid for the targeted liposomes. However, it should again be emphasized that the upper range of these intervals likely overestimate the protein binding value due to the unbound background plasma particles co-eluting with the liposomes in the void volumes.

To interpret the meaning of the above protein binding values, we performed a calculation assuming that all of the bound proteins had a molecular weight of 70 kDa—which is a typical molecular weight for blood proteins—and that each liposome was comprised of 250,000 lipids—as estimated above. By these assumptions, we estimated that < 18 proteins bound to both the non-targeted and targeted liposomes recovered after *in vitro* incubation, between 11–44 proteins bound to the non-targeted liposomes recovered after *in vivo* passage, and between 14–61 proteins bound to the targeted liposomes recovered after *in vivo* passage. In other words, it was clear that a fairly low number of proteins were bound per liposome.

We also wanted to estimate the protein surface coverage on the surface of the liposomes. For this calculation, we used that a globular 70 kDa protein typically has a radius of 2.7 nm [59], corresponding to a cross-sectional area of 23 nm². Given the measured protein/lipid ratios and assuming the cross-sectional area of DSPC and DSPE-PEG2k to be 0.53 nm² and the cross-sectional area of cholesterol to be 0.22 nm² [42], we then estimated < 0.8% protein surface coverage for both the non-targeted and targeted liposomes recovered after *in vitro* incubation, between 0.5–1.9% protein surface coverage for the non-targeted liposomes recovered after *in vivo* passage, and between 0.6–2.7% protein surface coverage for the targeted liposomes recovered after *in vivo* passage. Table 3 summarizes the results of the above calculation. The results of the calculation would change slightly if we assumed different protein sizes and/or partial unfolding of the bound proteins, but the overall conclusion was solid: the surface of the recovered liposomes was only sparsely covered with protein.

Table 3: Magnitude of the protein corona of the recovered liposomes. The protein binding value was estimated using data from the micro-BCA assay, FCS, and SDS-PAGE. The proteins bound per liposome was estimated by assuming that the mass of all proteins was 70 kDa and that each liposome was comprised of 250,000 lipids. The protein surface coverage was estimated assuming the cross-sectional area of the bound proteins to be 23 nm², the cross-sectional area of DSPC and

DSPE-PEG2k to be 0.53 nm^2 , and the cross-sectional area of cholesterol to be 0.22 nm^2 [42].

	<i>In vitro</i> in FBS		<i>In vivo</i> in mice	
Liposome type	Non-targeted	Targeted	Non-targeted	Targeted
Protein binding value				
(g protein/mol lipid)	< 5	< 5	3–12	4–17
Proteins per liposome	< 18	< 18	11–44	14–61
Protein surface coverage (%)	< 0.8	< 0.8	0.5–1.9	0.6–2.7

For the liposomes recovered after *in vivo* passage, it was clear that the primary component of the protein corona was the most abundant protein in blood plasma, namely serum albumin. This indicates that protein binding to the liposomes was non-specific, that is, protein binding to the lipid membranes did not occur due to any specific structural motifs of the proteins. Again, however, it should be emphasized that there was only sparse protein binding to the liposomes. In other words, even though serum albumin was the dominant protein in the corona for the liposomes recovered after *in vivo* passage, only ~ 8 –10 albumin molecules were bound per liposome according to our SDS-PAGE results.

The notion of sparse protein binding was further corroborated by the observation that the recovered liposomes completely retained their size, fluorescence properties, and cellular interaction abilities. In other words, there was not enough protein binding to induce any detectable structural or functional perturbations of the liposomes.

The observation that the bound proteins did not compromise the specific receptor targeting abilities of the targeted liposomes was particularly interesting: several previous studies have been concerned with the impact of the protein corona on the targeting abilities of ligand-targeted solid nanoparticles [60], but when it comes to ligand-targeted liposomes—in spite of their high clinical relevance—there are only a few published studies concerned with the effect of the protein corona on their targeting abilities [16, 61, 62]. These studies even reached somewhat contrasting conclusions, implying that more studies on the topic are highly warranted. Accordingly, the findings of the present study are of high relevance as they clearly demonstrate that the proteins bound to the PEGylated stealth liposomes recovered after exposure to blood serum/plasma does not lead to any steric shielding of antibodies conjugated to the liposomal surfaces.

For completeness, it should be mentioned that the present study—in the same way as

previous studies on the protein corona of PEGylated stealth liposomes [13, 16–19, 44, 45, 63]—included a liposome recovery step. By this step, we were able to study the tightly bound proteins—commonly referred to as the hard protein corona [1]. However, we were not able to study more loosely bound proteins—commonly referred to as the soft protein corona [1]—as these proteins would have been washed away during the recovery step. In principle, we can not rule out that a comprehensive soft protein corona existed around our liposomes. On the other hand, several pieces of information indicate that this is not the case. Firstly, we performed an additional experiment to study the association of the liposomes to SKBR-3 cells in cell medium supplemented with 10% heat-inactivated FBS. The results of this experiment, shown in Figure S9 in the Supplementary information, demonstrate that the non-targeted liposomes did still not associate with the cells under these conditions, whereas the targeted liposomes largely retained their targeting abilities. In other words, if a soft protein corona was formed by the FBS, it was not sufficiently extensive that it induced much change in the receptor-interaction properties of the liposomes. Secondly, we recently studied the binding of human serum albumin to PEGylated phosphatidylcholine-based liposomes using FCS [32]. This method does not require a separation step and thereby allows for studying both tightly and loosely bound proteins [64], but nonetheless, we only detected sparse protein binding to the liposomes. Likewise, no other researchers have to our knowledge been able to demonstrate any clear indications of a soft protein corona around PEGylated stealth liposomes. Consequently, the existence of a soft corona around such liposomes remains speculative.

3.6 Methodological aspects of studying the hard protein corona of liposomes

Some previous studies have—in agreement with our results—reported a protein binding value of ~ 5 – 15 g protein per mol lipid for PEGylated stealth liposomes [13, 44]. However, several recent studies have reported a somewhat higher protein binding value of ~ 20 – 40 g protein per mol lipid [45, 63] or a much higher protein binding value > 100 g protein per mol lipid [16, 17] or even > 1000 g protein per mol lipid [22]. Yet others have reported protein binding without determining the protein binding value [18, 21]. Altogether, these results have lead to significant confusion about the properties of the protein corona around PEGylated stealth liposomes. It cannot be ruled out that these varying results have to do with subtle differences in the liposome formulation procedures, incubation conditions, serum or plasma sources, or animal models between the

individual studies [65]. On the other other hand, the varying results also stress the necessity for understanding the impact of the chosen methodology on the obtained results.

In the present study, we did a methodological observation worth highlighting: Using size-exclusion chromatography as the method for recovering liposomes from blood serum or plasma—perhaps the most commonly used recovery method [16, 17, 22, 44, 45, 63]—we found that some blood particles, such as lipoproteins and protein aggregates, were sufficiently big that they were co-recovered with the liposomes. These proteinaceous particles gave a significant contribution to various methods commonly used for evaluating the protein corona, for example, the micro-BCA assay and LC-MS/MS. If great care is not taken, researchers using size-exclusion chromatography as the recovery method could thus by mistake ascribe unbound proteins in the background to the corona. Incidentally, it should be noted that other methods commonly used for recovering liposomes from blood serum or plasma—such as spin column methods [13, 55] and centrifugation [18, 66, 67]—may potentially be prone to the same issues; that is, from a theoretical point of view, certain blood particles are of a size and/or density that they will be co-recovered with the liposomes [46]. Thus, it may be that researchers applying these recovery methods also have to take the background into account when interpreting their results.

4 Conclusions

There is currently some uncertainty about the magnitude of the protein corona forming around PEGylated stealth liposomes circulating in the bloodstream. However, the data contained in the present paper unambiguously and robustly point to the conclusion that the hard protein corona on such liposomes recovered after exposure to blood serum or plasma is sparse, that is, only a very small fraction of the liposomal surface area is covered with protein. In agreement with this, the bound proteins do not impact the structure of the liposomes, nor do they compromise the specific receptor targeting abilities of the liposomes if the liposomes are functionalized with an antibody.

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References

- [1] M. P. Monopoli, C. Åberg, A. Salvati, K. A. Dawson, Biomolecular coronas provide the biological identity of nanosized materials, *Nat. Nanotechnol.* 7 (2012) 779–786.
- [2] D. Docter, D. Westmeier, M. Markiewicz, S. Stolte, S. K. Knauer, R. H. Stauber, The nanoparticle biomolecule corona: Lessons learned – challenge accepted?, *Chem. Soc. Rev.* 44 (2015) 6094–6121.
- [3] A. Salvati, A. S. Pitek, M. P. Monopoli, K. Prapainop, F. B. Bombelli, D. R. Hristov, P. M. Kelly, C. Åberg, E. Mahon, K. A. Dawson, Transferrin-functionalized nanoparticles lose their targeting capabilities when a biomolecule corona adsorbs on the surface, *Nat. Nanotechnol.* 8 (2013) 137–143.
- [4] S. Tenzer, D. Docter, J. Kuharev, A. Musyanovych, V. Fetz, R. Hecht, F. Schlenk, D. Fischer, K. Kiouptsi, C. Reinhardt, K. Landfester, H. Schild, M. Maskos, S. K. Knauer, R. H. Stauber, Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology, *Nat. Nanotechnol.* 8 (2013) 772–781.
- [5] C. D. Walkey, J. B. Olsen, F. Song, R. Liu, H. Guo, D. W. H. Olsen, Y. Cohen, A. Emili, W. C. W. Chan, Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles, *ACS Nano* 8 (2014) 2439–2455.
- [6] G. M. Mortimer, N. J. Butcher, A. W. Musumeci, Z. J. Deng, D. J. Martin, R. F. Minchin, Cryptic epitopes of albumin determine mononuclear phagocyte system clearance of nanomaterials, *ACS Nano* 8 (2014) 3357–3366.
- [7] K. Saha, M. Rahimi, M. Yazdani, S. T. Kim, D. F. Moyano, S. Hou, R. Das, R. Mout, F.

- Rezaee, M. Mahmoudi, V. M. Rotello, Regulation of macrophage recognition through the interplay of nanoparticle surface functionality and protein corona, *ACS Nano* 10 (2016) 4421–4430.
- [8] N. Bertrand, P. Grenier, M. Mahmoudi, E. M. Lima, E. A. Appel, F. Dormont, J.-M. Lim, R. Karnik, R. Langer, O. C. Farokhzad, Mechanistic understanding of *in vivo* protein corona formation on polymeric nanoparticles and impact on pharmacokinetics, *Nat. Commun.* 8 (2017) 777.
- [9] F. Chen, G. Wang, J. I. Griffin, B. Brennen, N. K. Banda, V. M. Holers, D. S. Backos, L. Wu, S. M. Moghimi, D. Simberg, Complement proteins bind to nanoparticle protein corona and undergo dynamic exchange *in vivo*, *Nat. Nanotechnol.* 12 (2017) 387–393.
- [10] S. Lara, F. Alhasser, E. Polo, D. Garry, M. C. Lo Giudice, D. R. Hristov, L. Rocks, A. Salvati, Y. Yan, K. A. Dawson, Identification of receptor binding to the biomolecular corona of nanoparticles, *ACS Nano* 11 (2017) 1884–1893.
- [11] S. Lara, A. Perez-Potti, L. M. Herda, L. Adumeau, K. A. Dawson, Y. Yan, Differential recognition of nanoparticle protein corona and modified low-density lipoprotein by macrophage receptor with collagenous structure, *ACS Nano* 12 (2018) 4930–4937.
- [12] S. Schöttler, G. Becker, S. Winzen, T. Steinbach, K. Mohr, K. Landfester, V. Mailänder, F. R. Wurm, Protein adsorption is required for stealth effect of poly(ethylene glycol)- and poly(phosphoester)-coated nanocarriers, *Nat. Nanotechnol.* 11 (2016) 372–377.
- [13] S. C. Semple, A. Chonn, P. R. Cullis, Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour *in vivo*, *Adv. Drug Deliv. Rev.* 32 (1998) 3–17.
- [14] H. M. Patel, Serum opsonins and liposomes: Their interaction and opsonophagocytosis, *Crit. Rev. Ther. Drug Carrier Syst.* 9 (1992) 39–90.
- [15] M. L. Immordino, F. Dosio, L. Cattel, Stealth liposomes: Review of the basic science, rationale, and clinical applications, existing and potential, *Int. J. Nanomed.* 1 (2006) 297–315.
- [16] M. Hadjidemetriou, Z. Al-Ahmady, M. Mazza, R. F. Collins, K. Dawson, K. Kostarelos, *In vivo* biomolecule corona around blood-circulating, clinically used and antibody-targeted lipid bilayer nanoscale vesicles, *ACS Nano* 9 (2015) 8142–8156.
- [17] M. Hadjidemetriou, Z. Al-Ahmady, K. Kostarelos, Time-evolution of *in vivo* protein

- corona onto blood-circulating PEGylated liposomal doxorubicin (DOXIL) nanoparticles, *Nanoscale* 8 (2016) 6948–6957.
- [18] M. Papi, D. Caputo, V. Palmieri, R. Coppola, S. Palchetti, F. Bugli, C. Martini, L. Digiacomo, D. Pozzi, G. Caracciolo, Clinically approved PEGylated nanoparticles are covered by a protein corona that boosts the uptake by cancer cells, *Nanoscale* 9 (2017) 10327–10334.
- [19] Z. S. Al-Ahmady, M. Hadjidemetriou, J. Gubbins, K. Kostarelos, Formation of protein corona *in vivo* affects drug release from temperature-sensitive liposomes, *J. Control. Release* 276 (2018) 157–167.
- [20] G. Caracciolo, Clinically approved liposomal nanomedicines: Lessons learned from the biomolecular corona, *Nanoscale* 10 (2018) 4167–4172.
- [21] G. Caracciolo, S. Palchetti, L. Digiacomo, R. Z. Chiozzi, A. L. Capriotti, H. Amenitsch, P. M. Tentori, V. Palmieri, M. Papi, F. Cardarelli, D. Pozzi, A. Laganà, Human biomolecular corona of liposomal doxorubicin: The overlooked factor in anticancer drug delivery, *ACS Appl. Mater. Interfaces* 10 (2018) 22951–22962.
- [22] M. Hadjidemetriou, S. McAdam, G. Garner, C. Thackeray, D. Knight, D. Smith, Z. Al-Ahmady, M. Mazza, J. Rogan, A. Clamp, K. Kostarelos, The human *in vivo* biomolecule corona onto PEGylated liposomes: A proof-of-concept clinical study, *Adv. Mater.* 31 (2019) 1803335.
- [23] X. Yan, G. L. Scherphof, J. A. A. M. Kamps, Liposome opsonization, *J. Liposome Res.* 15 (2005) 109–139.
- [24] Y. Barenholz, Doxil – the first FDA-approved nano-drug: Lessons learned, *J. Control. Release* 160 (2012) 117–134.
- [25] W. Tai, R. Mahato, K. Cheng, The role of HER2 in cancer therapy and targeted drug delivery, *J. Control. Release* 146 (2010) 264–275.
- [26] J. W. Park, K. Hong, D. B. Kirpotin, G. Colbern, R. Shalaby, J. Baselga, Y. Shao, U. B. Nielsen, J. D. Marks, D. Moore, D. Papahadjopoulos, C. C. Benz, Anti-HER2 immunoliposomes: Enhanced efficacy attributable to targeted delivery, *Clin. Cancer Res.* 8 (2002) 1172–1181.
- [27] D. B. Kirpotin, D. C. Drummond, Y. Shao, M. R. Shalaby, K. Hong, U. B. Nielsen, J. D. Marks, C. C. Benz, J. W. Park, Antibody targeting of long-circulating lipidic nanoparticles

- does not increase tumor localization but does increase internalization in animal models, *Cancer Res.* 66 (2006) 6732–6740.
- [28] T. Yang, M.-K. Choi, F.-D. Cui, J. S. Kim, S.-J. Chung, C.-K. Shim, D.-D. Kim, Preparation and evaluation of paclitaxel-loaded PEGylated immunoliposome, *J. Control. Release* 120 (2007) 169–177.
- [29] E. Barrajon-Catalan, M. P. Menendez-Gutiérrez, A. Falco, A. Carrato, M. Saceda, V. Micol, Selective death of human breast cancer cells by lytic immunoliposomes: Correlation with their HER2 expression level, *Cancer Lett.* 290 (2010) 192–203.
- [30] J. O. Eloy, R. Petrilli, D. L. Chesca, F. P. Saggioro, R. J. Lee, J. M. Marchetti, Anti-HER2 immunoliposomes for co-delivery of paclitaxel and rapamycin for breast cancer therapy, *Eur. J. Pharm. Biopharm.* 115 (2017) 159–167.
- [31] J. O. Eloy, R. Petrilli, L. N. F. Trevizan, M. Chorilli, Immunoliposomes: A review on functionalization strategies and targets for drug delivery, *Colloids Surf. B Biointerfaces* 159 (2017) 454–467.
- [32] K. Kristensen, A. J. Urquhart, E. Thormann, T. L. Andresen, Binding of human serum albumin to PEGylated liposomes: Insights into binding numbers and dynamics by fluorescence correlation spectroscopy, *Nanoscale* 8 (2016) 19726–19736.
- [33] K. Kristensen, J. R. Henriksen, T. L. Andresen, Quantification of leakage from large unilamellar lipid vesicles by fluorescence correlation spectroscopy, *Biochim. Biophys. Acta* 1838 (2014) 2994–3002.
- [34] O. Krichinsky, G. Bonnet, Fluorescence correlation spectroscopy: The technique and its applications, *Rep. Prog. Phys.* 65 (2002) 251–297.
- [35] S. T. Hess, W. W. Webb, Focal volume optics and experimental artifacts in confocal fluorescence correlation spectroscopy, *Biophys. J.* 83 (2002) 2300–2317.
- [36] J. Ries, P. Schille, Fluorescence correlation spectroscopy, *Bioessays* 34 (2012) 361–368.
- [37] J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, *Nat. Biotechnol.* 26 (2008) 1367–1372.
- [38] S. Tyanova, T. Temu, P. Sinitcyn, A. Carlson, M. Y. Hein, T. Geiger, M. Mann, J. Cox, The Perseus computational platform for comprehensive analysis of (prote)omics data, *Nat. Methods* 13 (2016) 731–740.

- [39] S. C. Gill, P. H. von Hippel, Calculation of protein extinction coefficients from amino acid sequence data, *Anal. Biochem.* 182 (1989) 319–326.
- [40] R. J. Kessler, D. D. Fanestil, Interference by lipids in the determination of protein using bicinchoninic acid, *Anal. Biochem.* 159 (1986) 138–142.
- [41] T. M. Allen, P. R. Cullis, Liposomal drug delivery systems: From concept to clinical applications, *Adv. Drug Deliv. Rev.* 65 (2013) 36–48.
- [42] Y. Wang, P. Gkeka, J. E. Fuchs, K. R. Liedl, Z. Cournia, DPPC-cholesterol phase diagram using coarse-grained molecular dynamics simulations, *Biochim. Biophys. Acta* 1858 (2016) 2846–2857.
- [43] D. Nebija, C. R. Noe, E. Urban, B. Lachmann, Quality control and stability studies with the monoclonal antibody, trastuzumab: Application of 1D- vs. 2D-gel electrophoresis, *Int. J. Mol. Sci.* 15 (2014) 6399–6411.
- [44] S. A. Johnstone, D. Masin, L. Mayer, M. B. Bally, Surface-associated serum proteins inhibit the uptake of phosphatidylserine and poly(ethylene glycol) liposomes by mouse macrophages, *Biochim. Biophys. Acta* 1513 (2001) 25–37.
- [45] N. Dos Santos, C. Allen, A.-M. Doppen, M. Anantha, K. A. K. Cox, R. C. Gallagher, G. Karlsson, K. Edwards, G. Kenner, L. Samuels, M. S. Webb, M. B. Bally, Influence of poly(ethylene glycol) grafting density and polymer length on liposomes: Relating plasma circulation lifetimes to protein binding, *Biochim. Biophys. Acta* 1768 (2007) 1367–77, 1367–1377.
- [46] J. B. Simonsen, What are we looking at? extracellular vesicles, lipoproteins, or both?, *Circ. Res.* 121 (2017) 920–922.
- [47] F. A. W. Coumans, A. R. Brisson, E. I. Buzas, F. Dignat-George, E. E. E. Drees, S. El-Andaloussi, C. Emanuelli, A. Gasecka, A. Hendrix, A. F. Hill, R. Lacroix, Y. Lee, T. G. van Leeuwen, N. Mackman, I. Mäger, J. P. Nolan, E. van der Pol, D. M. Pegtel, S. Sahoo, P. R. M. Siljander, G. Sturk, O. de Wever, R. Nieuwland, Methodological guidelines to study extracellular vesicles, *Circ. Res.* 120 (2017) 1632–1648.
- [48] H. Liu, G. Gaza-Bulsecu, C. Chumsae, A. Newby-Kew, Characterization of lower molecular weight artifact bands of recombinant monoclonal IgG1 antibodies on non-reducing SDS-PAGE, *Biotechnol. Lett.* 29 (2007) 1611–1622.
- [49] L. Zhang, C. P. Chou, M. Moo-Young, Disulfide bond formation and its impact on the

- biological activity and stability of recombinant therapeutic proteins produced by *Escherichia coli* expression system, *Biotechnol. Adv.* 29 (2011) 923–929.
- [50] Z. C. Zhu, Y. Chen, M. S. Ackerman, B. Wang, W. Wu, B. Li, L. Obenauer-Kutner, R. Zhao, L. Tao, P. M. Ihnat, J. Liu, R. B. Gandhi, B. Qiu, Investigation of monoclonal antibody fragmentation artifacts in non-reducing SDS-PAGE, *J. Pharm. Biomed. Anal.* 83 (2013) 89–95.
- [51] A. Lesniak, A. Campbell, M. P. Monopoli, I. Lynch, A. Salvati, K. A. Dawson, Serum heat inactivation affects protein corona composition and nanoparticle uptake, *Biomaterials* 31 (2010) 9511–9518.
- [52] M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, K. A. Dawson, Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts, *Proc. Natl. Acad. Sci. USA* 105 (2008) 14265–14270.
- [53] M. P. Monopoli, D. Walczyk, A. Campbell, G. Elia, I. Lynch, F. B. Bombelli, K. A. Dawson, Physical–chemical aspects of protein corona: Relevance to *in vitro* and *in vivo* biological impacts of nanoparticles, *J. Am. Chem. Soc.* 133 (2011) 2525–2534.
- [54] M. Kokkinopoulou, J. Simon, K. Landfester, V. Mailänder, I. Lieberwirth, Visualization of the protein corona: Towards a biomolecular understanding of nanoparticle-cell-interactions, *Nanoscale* 9 (2017) 8858–8870.
- [55] A. Chonn, S. C. Semple, P. R. Cullis, Association of blood proteins with large unilamellar liposomes *in vivo*. relation to circulation lifetimes, *J. Biol. Chem.* 267 (1992) 18759–18765.
- [56] K.-H. Diehl, R. Hull, D. Morton, R. Pfister, Y. Rabemampianina, D. Smith, J.-M. Vidal, C. van de Vorstenbosch, A good practice guide to the administration of substances and removal of blood, including routes and volumes, *J. Appl. Toxicol.* 21 (2001) 15–23.
- [57] S. Sofou, G. Sgouros, Antibody-targeted liposomes in cancer therapy and imaging, *Expert Opin. Drug Deliv.* 5 (2008) 189–204.
- [58] D. Nguyen, S. Oliver, N. N. M. Adnan, C. Herbert, C. Boyer, Polymer-protein hybrid scaffolds as carriers for CORM-3: Platforms for the delivery of carbon monoxide (CO), *RSC Adv.* 6 (2016) 92975–92980.
- [59] H. P. Erickson, Size and shape of protein molecules at the nanometer level determined by sedimentation, gel filtration, and electron microscopy, *Biol. Proced. Online* 11 (1) (2009)

- 32–51.
- [60] W. Xiao, H. Gao, The impact of protein corona on the behavior and targeting capability of nanoparticle-based delivery system, *Int. J. Pharm.* 552 (2018) 328–339.
 - [61] H. Shmeeda, D. Tzemach, L. Mak, A. Gabizon, Her2-targeted pegylated liposomal doxorubicin: Retention of target-specific binding and cytotoxicity after *in vivo* passage, *J. Control. Release* 136 (2009) 155–160.
 - [62] E. Markoutsas, K. Papadisa, A. D. Giannou, M. Spella, A. Cagnotto, M. Salmona, G. T. Stathopoulos, S. G. Antimisiaris, Mono and dually decorated nanoliposomes for brain targeting, *in vitro* and *in vivo* studies, *Pharm. Res.* 31 (2014) 1275–1289.
 - [63] T. Ishida, M. Ichihara, X. Wang, K. Yamamoto, J. Kimura, E. Majima, H. Kiwada, Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes, *J. Control. Release* 112 (2006) 15–25.
 - [64] S. Milani, F. B. Bombelli, A. S. Pitek, K. A. Dawson, J. Rädler, Reversible *versus* irreversible binding of transferrin to polystyrene nanoparticles: Soft and hard corona, *ACS Nano* 6 (2012) 2532–2541.
 - [65] P. Jain, R. S. Pawar, R. S. Pandey, J. Madan, S. Pawar, P. K. Lakshmi, M. S. Sudheesh, In-vitro in-vivo correlation (ivive) in nanomedicine: Is protein corona the missing link?, *Biotechnol. Adv.* 35 (2017) 889–904.
 - [66] A. Amici, G. Caracciolo, L. Digiacomo, V. Gambini, C. Marchini, M. Tilio, A. L. Capriotti, V. Colapicchioni, R. Matassa, G. Familiari, S. Palchetti, D. Pozzi, M. Mahmoudi, A. Laganà, *In vivo* protein corona patterns of lipid nanoparticles, *RSC Adv.* 7 (2017) 1137–1145.
 - [67] M. Lundqvist, C. Augustsson, M. Lilja, K. Lundkvist, B. Dahlbäck, S. Linse, T. Cedervall, The nanoparticle protein corona formed in human blood or human blood fractions, *PLoS ONE* 12 (2017) e0175871.

Graphical abstract

Figure 1

Figure 2

Figure 3

Figure 4