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Tuning Liposome Composition to Modulate the Corona Forming in Human Serum and Uptake by Cells

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ABSTRACT

Nano-sized objects such as liposomes are modified by adsorption of biomolecules in biological fluids. The resulting corona critically changes nanoparticle behavior at cellular level. A better control of corona composition could allow to modulate uptake by cells. Within this context, in this work, liposomes of different charge were prepared by mixing negatively charged and zwitterionic lipids to different ratios. The series obtained was used as a model system with tailored surface properties to modulate corona composition and determine the effects on liposome interactions with cells. Uptake efficiency and uptake kinetics of the different liposomes were determined by flow cytometry and fluorescence imaging. Particular care was taken in optimizing the methods to isolate the corona forming in human serum to prevent liposome agglomeration and to exclude residual free proteins which could confuse the results. Thanks to the optimized methods, mass spectrometry of replicate corona isolations showed excellent reproducibility and this allowed semi-quantitative analysis to determine for each formulation the most abundant proteins in the corona. The results showed that by changing the

fraction of zwitterionic and charged lipids in the bilayer, the amount and identity of the most abundant proteins adsorbed from serum differed. Interestingly, the formulations also showed very different uptake kinetics. Similar approaches can be used to tune lipid composition in a systematic way in order to obtain formulations with the desired corona and cell uptake behavior.

Keywords

Liposomes, nanoparticle corona, human serum, cellular uptake

1. Introduction

Nanomaterials have been widely applied to engineer novel delivery platforms for drugs, genes and therapies to treat various diseases [1,2]. Among them, liposomes, vesicles enclosed by a lipid bilayer, are undoubtedly among the most clinically established drug delivery systems in nanomedicine [3,4]. Since the introduction on the market in 1995 of the first liposomal formulation, Doxil, nowadays, several liposomal formulations have been approved for routine clinical use [4–6]. Thanks to the unique self-assembled structure and controllable synthetic identity, liposomes can be designed with varied size, charge and surface properties to enable efficient passive or active drug delivery, and overall their biocompatibility and biodegradability make liposomes a versatile tool to load both hydrophilic or hydrophobic agents for diagnosis and therapy [3,6].

Once introduced into biological fluids, nanoscale objects such as liposomes adsorb numerous proteins and biomolecules on their surface forming a layer known as “protein corona” [7,8]. This layer affects nanoparticle charge, size and surface properties and confers to nanomaterials new biological properties [9,10], which affect the following nanoparticle performance, such as distribution, toxicity, cellular internalization and final fate [9,11–13]. Several studies have

described the corona forming on liposomes [14–19]. For instance, Caracciolo et al. showed that lipid composition can be manipulated in order to affect corona composition [14] and other works showed how the corona changes once liposomes are applied *in vivo* [15].

Additionally, different efforts have been made to correlate corona composition with nanoparticle uptake efficiency by cells. Similar studies allow to identify corona proteins or protein patterns correlating to higher or lower uptake by cells and/or different fate *in vivo*. For instance, Ritz et al. showed that apoA4 or apoC3 in the corona of polystyrene nanoparticles correlated with lower uptake by cells [20]. Using liposomes, Bigdeli et al. used quantitative structure–activity relationship (QSAR) in order to connect different physico-chemical properties of the particles, including their corona, with uptake and viability in cells [16]. Lazarovits et al., instead, used supervised learning and mass spectrometry of corona composition to predict the *in vivo* fate of nanoparticles [13].

Because of the difficulty in predicting and controlling corona formation, strategies such as poly(ethylene glycol) (PEG) modification have been used to make carriers “stealth”, and try to avoid protein corona formation [21,22]. However, PEGylation cannot completely suppress protein adsorption, and recent studies suggested that the stealth properties of PEG are actually conferred by a specific protein, *i.e.* clusterin, which adsorbs in the corona of PEGylated nanocarriers [23]. At the same time, it is also emerging that corona proteins can be actively recognized by cell receptors, thus the corona-nanoparticle complexes constitute the real biological unit interacting with cells [24,25]. For instance, it has been shown that the proteins adsorbed on 100 nm silica nanoparticles can present functional epitopes from low-density lipoprotein and immunoglobulin G, which allow specific recognition by cell receptors [24]. Prapainop et al. also reported that misfolding of corona proteins adsorbing on a small molecule modified quantum dot led to cell specific receptor-mediated internalization [26]. In light of similar observations, it has been proposed that rather than trying to limit its formation, the

protein corona could be exploited to enable novel opportunities for the design of targeted nanomedicines, as well as for the discovery of biomarkers [25,27–29].

Overall, whether exploring strategies to control corona formation or to exploit its biological properties for novel applications, it is clear that a comprehensive and accurate characterization of the corona, including the factors affecting its composition and the resulting corona effects on the nanomaterial outcomes at organism and cell levels is essential.

Within this context, in this work, we chose liposome as a nanomedicine model to prepare a series of nanosized carriers with tailored surface properties. While in many examples in literature, liposomes of different composition and their coronas were compared [14,16,18], here a liposome series of tailored surface properties was prepared by mixing common charged and zwitterionic lipids in different ratios in a systematic way, and, in this way, to tune the resulting coronas in human serum. Then, the stability of the different liposomes in biological conditions was tested in order to select stable formulations and in this way avoid confusing results for the corona forming on agglomerates. Liposome uptake kinetics were obtained by flow cytometry, using HeLa cells as a common cell model, in order to determine the effect of lipid composition and resulting corona in human serum on uptake efficiency by cells. Particular efforts were spent in the optimization of the methods for corona isolation in order to recover well-dispersed liposome-corona complexes and to avoid contamination of residual free proteins from serum after corona isolation, which could confuse the results. Thus, SDS-PAGE and protein identification by mass spectrometry were used to characterize the corona forming on the different liposomes. Particular attention was paid in repeating corona isolation and identification by mass spectrometry multiple times for each formulation in order to confirm reproducibility with the optimized isolation methods and to obtain robust values of relative protein abundance to allow statistical analysis. Thanks to this and the optimized methods, differences in protein composition were analyzed and for each formulation, the most enriched

proteins determined. This allowed us to connect the abundance of specific proteins in the corona forming on the different liposomes with the uptake kinetics in cells.

2. Materials and methods

2.1 Preparation of liposomes

The zwitterionic lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), cationic lipids 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3 β -[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC CHOL), anionic lipid 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG) and cholesterol were purchased from Avanti Polar Lipids. To prepare different liposomal formulations, 10 mg lipid mixtures at different molar ratios were prepared and dissolved in chloroform. The composition of all formulations tested is given in Supplementary Table S1. Then, the solvent was evaporated using a nitrogen stream and then by incubation under vacuum overnight. The lipid films were hydrated with 1 mL 25 mM sulforhodamine B (SRB) in PBS at room temperature and vortexed to produce fluorescently labeled multilamellar liposomes. Small unilamellar liposomes were obtained by performing 8 freeze-thaw cycles followed by extrusion 21 times through a 0.1 μ m polycarbonate membrane using the Avanti Mini-Extruder (Avanti Polar Lipids). Residual free SRB was removed by centrifugation with a Zeba Spin Desalting Column, 7K MWCO (Thermo Fisher Scientific). The obtained unilamellar liposomes were stored at 4 °C and were used only for up to 1 month after preparation.

2.2 Protein corona formation and isolation

In order to allow corona formation, 0.5 mg/mL liposomes were mixed with fetal bovine serum (FBS) (Gibco Thermo Fisher Scientific) or human serum from pooled donors (TCS Biosciences Ltd) at a final concentration of 40 mg/mL and were incubated in a Thermo-Shaker (Grant

Instruments Ltd) at 37 °C, 250 rpm. After 60 min incubation, corona-coated liposomes were separated from the excess serum proteins by size exclusion chromatography using a Sepharose CL-4B (Sigma-Aldrich) column (15 × 1.5 cm) pre-balanced with PBS. Fractions of 0.5 mL eluent were collected up to a total volume of 15 mL (30 fractions) and their absorption at 280 nm and 565 nm were measured using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) in order to determine, respectively, the protein and SRB elution profiles. The fractions containing SRB-labelled corona-coated liposomes (roughly fractions 7-10) were pooled together and concentrated using a Vivaspin 6 centrifugal concentrator (10K MWCO, Sartorius) by centrifugation at 1600 g at 4 °C until the final volume of the solution was less than 0.2 mL.

In order to prepare particle-depleted human serum by removing larger objects eluting in the same fractions as the liposomes, 1 mL human serum was injected in the column and fractions were collected as described above. Then, the fractions corresponding to larger particles were discarded and all other fractions (roughly from 11 to 30) were pooled together and the total protein content determined using a Bio-Rad *DC* protein assay (Bio-Rad Laboratories, Inc.) as described below. The particle-depleted serum was then used to prepare liposome-corona complexes. Given that the corona composition changes when the ratio between the total nanoparticle surface area and protein content is varied [30], in order to apply the same conditions as for the isolation in full FBS or full HS, 0.075 µg/mL liposomes were dispersed in 6 mg/mL serum. Then, the isolation of corona-coated liposomes was performed as described above.

2.3 Size and zeta potential measurements

Size distribution and zeta potential of the liposomes dispersed in various media were measured using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., USA). Samples at a final concentration of 50 µg/mL were prepared by dilution of the liposome stock solution in

different media, including MilliQ water, PBS, and MEM cell culture medium supplemented with 10% v/v FBS (roughly corresponding to 4 mg/mL) (cMEM) or with 4 mg/mL human serum (hsMEM) and measured just after dispersion. For size measurements, each sample was measured 3 times at 20 °C with automatic setting for the measurement duration. To measure the zeta potential, disposable folded capillary cells were used and 3 measurements per sample were recorded at 20 °C with automatic setting for the measurement duration and monomodal analysis. The size distribution and zeta potential of the corona-coated liposomes after the isolation procedure were also determined in the same way.

2.4 Protein assay and lipid assay

To compare the protein-binding capacity of the different liposome formulations, the protein/lipid ratio (μg of protein/ μmol of lipid) was calculated by determining the amount of proteins and lipids in the corona-coated liposomes after isolation from free proteins and spin-concentration. The amount of proteins in the corona was quantified using the Bio-Rad *DC* protein assay (Bio-Rad Laboratories, Inc.) using dilutions of bovine serum albumin (BSA) at known concentrations as a standard. Briefly, standard solutions containing 0.1 mg/mL to 3.2 mg/mL BSA were prepared, then 5 μL sample or standards were mixed with the working reagent. After 15 min, the absorbance at 650 nm was read using a ThermoMAX microplate reader (Molecular Devices, LLC). Then, the protein concentration was calculated according to the BSA standard curve. The lipid concentration was determined via the Stewart assay. For this, a ferrothiocyanate reagent was prepared by dissolving 27.0 mg ferric chloride hexahydrate (Sigma Aldrich) and 30.4 mg ammonium thiocyanate (Sigma Aldrich) in 1 mL Milli-Q water. Then, 20 μL liposome samples or lipid standards (from 0 mg/mL to 1 mg/mL) were mixed with 1 mL chloroform and 1 mL ferrothiocyanate reagent and vortexed for 60 s followed by centrifugation at 300 g for 10 min. 900 μL of the organic phase was transferred to a quartz cuvette, and the absorbance at 470 nm was measured on a Unicam UV500 Spectrophotometer

(Unicam Instruments). Lipid concentrations were calculated according to a standard curve obtained with the same lipid from samples at known concentrations. The protein/lipid ratios were determined. The average and standard deviation of the results obtained in 3 independent experiments was calculated.

2.5 SDS-PAGE electrophoresis

In order to separate the isolated corona proteins, corona-coated liposomes corresponding to 0.05 μmol lipid were mixed with 4 $\times$ loading buffer (containing 200 mM Tris-HCl, 400 mM DTT, 8% SDS, 0.4% bromophenol blue and 40% glycerol) to a final volume of 40 μL and heated for 5 min at 95 $^{\circ}\text{C}$. 20 μg human serum was also loaded as control using the same procedure. Samples were then loaded onto a 10% polyacrylamide gel and run for 1 h at 120 V at room temperature. The gels were stained by using a solution containing 0.1% Coomassie blue R-250 in a water–methanol–glacial acetic acid (5:4:1) mixture with gentle agitation, followed by destaining in hot ultrapure water. Images were captured using a ChemiDoc XRS (Bio-Rad).

2.6 Protein digestion and peptide desalting

Mass spectrometry was used to identify the corona proteins isolated on the liposomes after dispersion in particle-depleted human serum, and –as a reference – the proteins in full human serum. Protein digestion was performed as described in literature [31]. Briefly, after corona formation and isolation, samples containing 10 μg corona proteins and –as a control - the same amount of human serum proteins were resuspended in 40 μL of 8 M urea in 50 mM NH_4HCO_3 (Sigma-Aldrich) and incubated for 30 min at room temperature. The protein solutions were then reduced by adding 2 μL 200 mM DL-dithiothreitol (DTT) (Sigma-Aldrich) and incubation for 30 min at room temperature, followed by alkylation with 8 μL 200 mM iodoacetamide (Sigma-Aldrich) for 30 min. 8 μL 200 mM DTT was added, and the solution was kept at 56 $^{\circ}\text{C}$ for 30 min. The sample solution was then diluted with 50 mM NH_4HCO_3 to a final urea concentration of 1 M, and 2 μg trypsin (Promega Corporation) was added.

After overnight digestion with trypsin at 37 °C, the reaction was stopped by addition of trifluoroacetic acid (TFA) to 0.4% (v/v) final concentration. The solutions were dried in an Eppendorf centrifugal vacuum concentrator (Sigma-Aldrich) and resuspended in 40 µL 0.1% TFA. The digested samples were desalted and lipids were removed using a C18 ZipTip (Merck Millipore). Briefly, tips were washed with acetonitrile (ACN) 3 times and equilibrated with 0.1% TFA. Samples were then loaded onto tips, followed by washing the tips with 0.1% TFA. Peptides were then eluted with 100 µL of 0.1% TFA/50% ACN (50:50, v/v), and the solvent was removed using a centrifugal vacuum concentrator. The dried peptides were dissolved in 10 µL 0.1% HCOOH (v/v) for LC/MS analysis.

2.7 Shotgun LC/MS-MS analysis, data processing and bioinformatics analysis

Tryptic peptides were analyzed with an UltiMate 3000 RSLC UHPLC system (Dionex) linked to an Orbitrap Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) performing in a data-dependent acquisition (DDA) mode, and the obtained raw data were analysed with PEAKS Studio software (version 8.5) [32] using the SwissProt human database (20197 entries; downloaded on July 27th, 2016). The detailed protocol is included in Supplementary materials.

A relative quantitation of the identified proteins was performed by determining the ion peak area (Area), which was the sum of the areas of all unique peptides mapping to the protein group. For each identified protein, the protein Area was normalized by the protein mass and expressed as the relative protein abundance according to the following equation:

$$\text{Area}_x = [(\text{Area}/\text{Mw})_x / \sum_{i=1}^n (\text{Area}/\text{Mw})_i] \times 100 \quad (1)$$

Based on the calculated Area value, data were further analyzed and visualized with an in-house developed script in R (version 3.4.3) and R Studio (version 1. 0.143). The quantitative Area values were subjected to median scale normalization [33].

In order to determine the most abundant proteins in the corona, the Rank Product method [34] was applied to rank the identified proteins as follows: in Fig. 6A and Supplementary Fig. S7 to rank the most abundant corona proteins identified on all of the 3 different formulations (3 liposomes with 3 replicate isolations each, thus 9 corona samples in total) and in Supplementary Figs. S8A-C instead to rank the corona proteins of each liposome (in 3 replicate isolations), thus the corona proteins of DOPC liposomes in Supplementary Fig. S8A, DOPC/G liposomes in Fig. S8B and DOPG liposomes in Fig. S8C. The detailed protocol is included in Supplementary materials.

2.8 Cell culture

HeLa cells (ATCC CCL-2) were cultured in MEM (Gibco Thermo fisher Scientific) supplemented with 10% v/v FBS (roughly corresponding to 4 mg/mL proteins) (complete cell culture medium, cMEM) in a humidified atmosphere containing 5% CO₂ at 37 °C. Cells were passaged two to three times a week and used for up to maximum 20 passages. Cells were tested against mycoplasma monthly to exclude mycoplasma contamination.

2.9 Liposome uptake by flow cytometry

Uptake of the different liposomes in different media by HeLa cells was determined by flow cytometry. HeLa cells were seeded at a density of 5×10^4 cells/well in a 24-well plate and incubated in cMEM for 24 h. Cells were then washed with serum free medium three times and incubated with 50 µg/mL liposome dispersions prepared by dilution of the liposome stock in to cMEM or hsMEM. After exposure to the liposome, HeLa cells were washed once with cMEM and twice with PBS in order to remove excess liposomes outside the cells and harvested by incubation for 5 minutes with 0.05% trypsin-EDTA at 37 °C. Cells were then centrifuged at 300 g for 5 minutes, resuspended in PBS and measured immediately using a BD FACSAarray (BD Biosciences) with a 532 nm laser. Gates were set in the forward and side scattering double scatter plots to exclude dead cells and cell doublets. For each condition 2 samples were prepared

and 2×10^4 cells were recorded for each sample. Data were analyzed with FlowJo software (FlowJo, LLC), and the average of the median fluorescence intensity and standard deviation over the replicates calculated.

In order to measure potential uptake in energy depleted cells, cells were incubated with 5 mg/mL sodium azide (Merck). The detailed protocol is given in Supplementary materials.

2.10 Fluorescence imaging

For fluorescence microscopy, 1.5×10^5 cells were seeded in 35 mm dishes with a 170 μ m thick glass bottom. To visualize the lysosomes, cells were incubated for 30 min with 100 nM LysoTracker Deep Red in cMEM and nuclei stained by incubation with 1 μ g/mL Hoechst 33342 Solution in cMEM for 5 min (both from Thermo Fisher Scientific). Cells were imaged using a Leica TCS SP8 fluorescence confocal microscope (Leica Microsystems) or a DeltaVision Elite (GE Healthcare Life Science). ImageJ software (<http://www.fiji.sc>) was used for image processing and brightness and contrast were adjusted using the same setting for all samples in order to allow better visualization. The detailed protocols are included in Supplementary materials.

2.11 Statistical analysis

One way ANOVA analysis was used to test the difference of each identified corona protein across DOPC, DOPC/G and DOPG liposomes. After Benjamini-Hochberg correction for multiple testing, the calculated p values were considered to be significant for p value ≤ 0.01 .

All cellular uptake data are displayed as the average and standard deviation over 2 replicates of the median cells fluorescence obtained by flow cytometry.

3. Results and discussion

3.1 Liposome formulation and characterization

Liposome series were prepared by gradually mixing lipids of different charge in different ratio. All the liposome formulations tested are listed in Supplementary Table S1. Sulforhodamine B (SRB) was chosen as a hydrophilic dye to label all the different formulations and in this way quantify their uptake and localization inside cells. This was preferred to using a lipid dye, because polar molecules such as SRB, once loaded in the inner aqueous volume of liposomes are unable to cross the liposome and cell membrane by simple diffusion. Additionally, similar hydrophilic labels cannot easily transfer from the liposome into cell membranes, as observed with several hydrophobic dyes [35]. Fluorescence measurements showed that all liposomes encapsulated comparable amounts of SRB, with the DOPC liposomes usually showing slightly higher encapsulation efficiency. Cholesterol was added in order to improve liposome stability in biological conditions: exposure to biological media such as serum is known to affect bilayer stability, especially at higher protein concentration, as used here. This could lead to leakage of the delivered drugs or –in our case – the fluorescent label SRB from the inner volume, which could confuse uptake quantification [36].

In order to allow corona formation, a medium supplemented with human serum was used as a more relevant source of serum when studying uptake in human cells, as in this case. Additional results for liposomes dispersed in a standard cell culture medium supplemented with fetal bovine serum (FBS) were also included as a reference for comparison.

All liposomes were characterized by dynamic light scattering (DLS) and ζ -potential measurements after extrusion and after dispersion in relevant biological fluids, including cell culture medium (MEM) supplemented with roughly 4 mg/mL of either fetal bovine serum (standard 10% FBS complete cell culture medium, cMEM) or human serum (here referred to as hsMEM). After preparation, all formulations showed narrow size distributions with very low polydispersity indexes in the range of 0.1-0.3, confirming that preparation by extrusion allowed

us to obtain liposomes with excellent homogenous properties (Fig. 1A). Once introduced into biological medium supplemented with serum, for the zwitterionic and anionic liposomes homogeneous dispersions could be obtained in both cMEM and hsMEM (Fig. 1A). In some cases, a small peak around 10 nm was visible from the excess free proteins in solution. For all formulations, the zeta potential in PBS reflected the different lipid composition, but once introduced in medium with serum all converged towards neutrality, independently of the original surface charge, as expected as a consequence of protein adsorption on the surface and corona formation (Fig. 1B and Supplementary Fig. S1) [9]. Additionally, in both cell culture media, the dispersions of the optimized liposome formulations remained stable for up to 24 h under biological conditions (37 °C, 5% CO₂ as used for experiments with cells) (Fig. 1C-1D). The cationic liposomes instead showed strong aggregation in both biological media (Supplementary Fig. S1). It is known that bridging flocculation can occur between negatively charged proteins and positively charged nanoparticles [37]. Hence, in order to avoid confusing results for the corona forming on agglomerates, only the series with the zwitterionic DOPC and anionic DOPG lipids mixed in different ratios was used for further studies.

3.2 Uptake efficiency by cells and uptake kinetics

In order to investigate cellular uptake behavior of the liposome series, HeLa cells were selected as a standard cell model commonly applied for similar liposome uptake and corona studies [16,38]. As a first step, flow cytometry was used to measure the median fluorescence intensity of cells after exposure to liposomes for different times. Because of their comparable fluorescence, liposomes were exposed to the same total amount of lipids, obtained as described in the Materials and methods. Uptake kinetics were determined for both liposomes dispersed in hsMEM and cMEM, and also liposomes dispersed in higher concentration of human serum (Fig. 2A and Supplementary Fig. S2A and B, respectively).

When comparing uptake efficiency for the different formulations (Fig. 2A), the zwitterionic DOPC liposomes showed lower uptake efficiency by cells, and their uptake increased with increasing exposure time. On the contrary, the kinetics of uptake of the anionic DOPC/G and DOPG liposomes differed strongly, with much higher uptake levels in the first few hours (with the fully anionic DOPG liposomes showing the highest efficiency), followed by a progressive decrease in the average cell fluorescence, converging to similar levels for the two formulations over 24 hours. Zwitterionic objects are known to bind less to cells in comparisons to charged particles, thus it is generally observed that increasing particle charge can improve their uptake efficiency. For instance, Lee et al. [39] found that the endocytosis of anionic liposomes including phosphatidylglycerol (PG), phosphatidylserine (PS) or phosphatidic acid (PA) was faster and more efficient than for zwitterionic liposomes. Allen et al. also observed that for several liposomes (e.g. liposomes containing PS or various gangliosides) the uptake efficiency in bone marrow macrophages *in vitro* was positively correlated with the clearance by the reticuloendothelial system observed *in vivo* [40]. However, as mentioned, once liposomes were introduced to medium with serum, the resulting corona-nanoparticle complexes, all converged to equivalent zeta potential values, regardless of the starting charge (Fig. 1B) [9]. This is an important consequence of corona formation, still often overlooked, that should be kept in mind when tuning nanoparticle charge in the attempt of improving uptake efficiency. Given the similar zeta potential of the different formulations once dispersed in medium with serum, the different uptake efficiency in the first hours of exposure is likely due to differences in affinities and binding capacity between cell membrane components and liposomes, possibly mediated by specific differences in corona composition.

It is interesting to note also that the uptake of liposomes was lower in medium with human serum than with bovine serum (as observed when comparing the results in Supplementary Fig. 2A and Fig. 2A), and uptake levels decreased further when increasing human serum

concentration in the medium (Supplementary Fig S2B). It is known that protein concentration and also protein source both have significant implications on nanoparticle uptake. Kim et al. [41] for instance showed that increasing serum concentration decreased uptake of polystyrene nanoparticles, and Schöttler et al. [42] showed that dispersion in human serum or plasma reduced the uptake of amino-modified polystyrene nanoparticle dramatically compared the uptake levels in medium with bovine serum.

In order to determine whether liposome uptake was energy dependent, cells were exposed to the metabolic inhibitor sodium azide, commonly used to deplete cell energy (Figs. 2 B-D for liposome dispersions in hsMEM and Supplementary Figs. S2 for dispersions in cMEM or higher human serum concentration). The results showed that exposure to sodium azide lowered strongly uptake levels, which indicated that liposome uptake was energy dependent. This suggested that liposomes were internalized by cells as intact nanoparticles via some active mechanism of endocytosis, and not by a passive mechanism of direct fusion with the cell membrane. Transport inhibitors and other methods are required in order to determine the mechanism of uptake for each formulation, as we analyzed in detail in a separate study (Yang et al, in preparation).

In order to confirm that the measured cell fluorescence was due to the active uptake of SRB encapsulated in the liposomes, as opposed to the uptake of residual free SRB or SRB leaking from the liposomes, additional controls for liposome stability were performed (Supplementary Fig S3). First, size exclusion chromatography (SEC) was used to separate eventual free dye leaking from the liposome. This confirmed excellent removal of residual free SRB after liposome preparation and no leaking in PBS (Supplementary Fig.S3A). The same was repeated after exposure to human serum (40 mg/ml) to test liposome stability and eventual leakage after corona formation. SEC showed that for DOPC/G and DOPC liposomes most SRB remained

encapsulated in the liposomes, while dye leakage was observed for DOPG liposomes (Supplementary Fig. S3B), possibly contributing to its peculiar uptake kinetics.

However, as shown in Supplementary Fig. S3C, the cellular uptake of the same amount of free SRB was much lower, and particularly in comparison to DOPG and DOPC/G liposomes. Additionally, exposure to sodium azide led to only 25% uptake reduction for free SRB (as opposed to 50% for DOPC and 70-80% for DOPC/G and DOPG liposomes), possibly also because of compromised cell membrane permeability in the energy depleted cells (see Supplementary Fig. S3 for details). Altogether, the lower uptake efficiency of free SRB and the smaller effect of sodium azide confirmed that the fluorescence measured in cells exposed to the liposomes was primarily resulting from the uptake of SRB encapsulated in the liposomes. On the contrary, when a lipid dye was used to label the liposomes, almost no reduction of uptake was observed in the presence of sodium azide (Supplementary Fig. S3D). This possibly resulted from the transfer of the lipid dye from the liposomes to the cell membrane [35,39]. Because of this observation, to exclude similar effects which could confuse uptake results, liposomes labelled with SRB were chosen for the study.

Next, fluorescence microscopy was used to determine the final intracellular location of the liposomes after 3 h exposure (Fig. 3 for experiments in hsMEM and Supplementary Fig. S4A-C for comparable experiments in cMEM). Imaging indicated that the DOPC/G and DOPG liposomes in cMEM or hsMEM colocalized with intracellular vesicles stained by LysoTracker, confirming that these liposomes were efficiently trafficked along the endosomal pathway by cells and mostly accumulated in the perinuclear area in the lysosomes. In agreement with the lower uptake levels observed by flow cytometry (Fig. 2A), when using the same microscopy setting almost no signal from the DOPC liposomes could be detected (Fig. 3 and Supplementary Figs. S4A-C). However, by increasing gain settings, uptake and colocalization with LysoTracker were observed also in cells exposed to the DOPC liposomes (Supplementary Fig.

S4E), confirming internalization and trafficking along the endo-lysosomal pathway also for this formulation. Interestingly, in comparison, cells exposed to the same amount of free SRB for the same time showed almost no signal, and no clear intracellular SRB could be detected even when imaging cells with increased gain (Supplementary Figs. S4D and F). In line with the flow cytometry results with free SRB, this confirmed once more that the SRB signal detected in cells exposed to the liposomes comes primarily from the active delivery of SRB encapsulated in the liposomes, rather than free dye released prior to or during uptake.

In summary, all the liposomes were internalized by cells as intact nanoparticles via energy-dependent pathways, and trafficked along the endo-lysosomal pathway, delivering into cells higher levels of SRB than what obtained in cells exposed to the same amount of free dye (much higher in the case of the DOPG and DOPC/G liposomes). However, uptake efficiency and kinetics varied strongly for the negatively-charged and zwitterionic formulations, likely as a result of specific interactions of corona proteins with cells.

3.3 Isolation and characterization of protein corona coated liposomes

As a next step, in order to connect the observed differences in cellular uptake behavior with potential differences in corona composition, corona-coated liposomes were isolated from the excess serum to allow identification and quantification of protein corona composition [43,44]. As a first step, liposomes dispersed in medium with FBS were used for optimization of the isolation procedure.

Although centrifugation is often used for nanoparticle-corona isolation, this method is not best suited for nanomaterials of smaller size or with low density such as liposomes, as sedimentation is difficult and using higher centrifugal forces could result in strong agglomeration which could affect corona composition. For our formulations, in fact, centrifugation led to strong

agglomeration (Supplementary Fig. S5) [7,9]. Hence, in order to avoid to study the corona forming on agglomerates, additional efforts were spent in the optimization of other methods to allow isolation of well dispersed corona-coated liposomes. This was achieved by isolation via size exclusion chromatography (SEC). As depicted in Fig. 4A, the corona proteins are carried by the liposomes and elute in earlier fractions than free serum. Thus, the absorbance at 280 nm and the fluorescence of SRB were monitored to determine the fractions in which the fluorescently labelled corona-coated liposomes eluted. Comparison of the elution profiles of liposomes alone, serum alone and their mixture confirmed successful isolation of corona-coated liposomes (Fig. 4B). The fractions containing liposomes were pooled together and concentrated by membrane ultrafiltration. DLS measurements confirmed isolation of well-dispersed corona-coated liposomes, with a small increase in size distribution as a consequence of corona formation, and overall still very low polydispersity (Fig. 4C).

The optimized procedure for corona isolation was then used for samples dispersed in human serum. However, different from FBS, when using human serum, we observed that some components eluted out in the same fractions as the liposomes (Supplementary Figs. S6A-B). DLS results confirmed that these fractions contained particles with sizes comparable to the liposomes (between 100-200 nm), and SDS-PAGE images showed that they also contained many proteins (Supplementary Figs. S6C-D). Thus, human serum contains protein particles which have similar size as the liposomes. The same was observed also with human plasma and when using other sources of human serum pooled from donors (data not shown). In agreement with our results, Caby et al. [45] observed that exosomes-like particles are present in plasma samples of healthy donors, with sizes around 100 nm and containing tetraspanin molecules and other known exosomes enriched proteins. It is likely that these objects also included protein aggregates formed after freezing the serum for storage. The presence of these aggregates and/or particles in the same fractions in which liposomes elute can contaminate the isolated corona-

coated liposomes and their proteins can confuse corona protein identification. Similar issue were reported by Kristensen et al. [46]. Different approaches can be followed to address this issue, also depending on the purpose of the study [29,46,47]. In our case, in order to avoid contamination of corona proteins by exosomes or protein aggregates in the serum, SEC was used to first remove particles and aggregates from the serum in the fractions corresponding to the liposomes. All other serum fractions were pooled together (excluding those containing particles) and SEC was performed again to confirm efficient particle removal (Fig. 5A). This will be referred to as particle-depleted human serum (particle-depleted HS). Although the removal of a (very small) fraction of proteins and protein aggregates is likely to affect the final corona composition, for the purpose of this study this approach was preferred in order to ensure that after liposome isolation the proteins identified only belonged to their corona, and no contamination of other protein particles or aggregates was present. Then, liposomes were incubated 1 h with the particle-depleted HS and finally the corona-coated liposomes were isolated by SEC (Fig. 5B). We note that most SRB eluted in the same fractions as the liposomes, confirming stability of DOPC also after incubation in serum). DLS measurements after corona isolation showed a small shift to larger sizes which confirmed corona formation on the liposomes (Fig. 5C). Additionally, dispersions with very narrow size distributions were obtained, indicating that the liposome remained intact and highly homogeneous after corona formation and isolation.

In order to qualitatively compare corona properties for the three different liposomes, the protein binding capacity (PBC) defined as the amount of absorbed proteins (μg) per micromole of lipid was evaluated, and gel electrophoresis (SDS-PAGE) was performed to separate the corona proteins (see Materials and methods for details). As shown in Fig. 5D, DOPC and DOPC/G liposomes showed comparable PBC, while the PBC of DOPG liposomes was significantly higher. This indicated that much more proteins adsorbed on the DOPG liposome surface once

introduced in a biological environment in comparison to the other formulations. Thus, addition of increasing amount of the zwitterionic DOPC lipid resulted in liposomes with lower zeta potential (Fig. 1B) and in turns this resulted in lower protein binding (Fig. 5D), as well as lower uptake efficiency in cells (Fig. 2A).

SDS-PAGE was then used to separate the corona recovered from the same amount of liposomes. Full serum and the particle-depleted HS were also loaded as a control, showing no major differences in the bands detected (Fig. 5E). Instead, the corona proteins identified on the three liposomes differed strongly, mainly showing differences in their intensities (Fig. 5E). Indeed, in agreement with the PBC evaluation, higher band intensities were observed for liposomes with increasing percentage of the charged DOPG lipid in their formulation. In general, liposomes with a charged surface tend to have stronger protein adsorption than the ones with neutral surface [48], which is consistent with our results. Zwitterionic modifications are in fact used as alternative to PEGylation to reduce protein binding [49] and usually zwitterionic particles also show lower cellular adhesion [49], while liposomes possessing charged headgroups have been found to activate the complement more efficiently and be cleared faster [50], especially the one with cationic lipids [48]. Our results are in agreement with these observations.

3.4 Identification of corona proteins and comparative analysis of corona composition

The next step was to identify the recovered corona proteins and analyze differences in corona composition for the different liposome formulations, in order to connect the corona composition to the observed differences in uptake behavior on cells. For this, a quantitative characterization was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS). In order to obtain similar total ion current (TIC) and similar dynamic concentration range for each sample, for each of the liposomes, the same amount of corona proteins were injected for LC-MS/MS

analysis. The relative protein abundance of the identified corona proteins was calculated with eqn (1) as described in the Materials and methods. Usually, the spectral counts normalized by the protein molecular weight are used to calculate a relative protein abundance and compare corona composition in different samples [15,16,30]. Here instead, the ion peak area was used for a more accurate intensity-based quantification of the protein abundance [11,51]. It has been shown that the total protein approach (TPA) as obtained in single stage MS quantification is a robust measurement of protein concentration [52]. In order to compare the quantities of the different proteins identified within a corona sample, the raw quantitative protein values (peak areas) were first normalized by the protein mass, and then, in order to correct for differences in the amount of sample injected, by the sum of all normalized protein areas in the same sample. Thus the relative protein abundance is expressed as a percentage in respect to the total amount of proteins identified in each corona (see Materials and methods and Supplementary Information for more details).

The top 20 most abundant common proteins identified on all the 3 liposomal formulations in three independent corona preparations and isolations are shown in Fig. 6A. Additionally, in Supplementary Fig. S7 their abundance in the full human serum is also included as a reference for comparison (the complete list of identified proteins is provided in Supplementary Materials). The relative protein abundance of each protein was calculated with eqn(1) and the proteins were ranked as described in the Methods. It is important to notice the high reproducibility of corona composition in triplicate experiments, confirming that the optimized procedure allowed us to isolate corona proteins in a robust and reproducible manner. This also allowed us to compare the relative protein abundance for the different formulations and apply statistical analysis to determine the most statistically different proteins among the liposomes and the serum.

The comparison of the relative protein abundance of the top 20 corona proteins with their abundance in the full serum, as shown in Supplementary Fig. S7, confirmed that –as well

established in the field- corona formation led to specific and strong enrichment of proteins on the liposomes [7–10]. As an example of this, the most abundant protein in serum, albumin, which alone constituted roughly 60% of the total proteins identified in serum, was not the most abundant protein in the corona (its abundance ranged between 5 and 7% for the different formulations, with higher values in the case of the zwitterionic liposomes). On the contrary, low abundance proteins were highly enriched. For instance APOC1, which had around 0.15% abundance in full serum, was strongly enriched on the DOPC/G liposomes, where alone it constituted roughly 31% of the total amount of proteins in the corona.

To better analyze how liposome formulation affected corona composition, Fig. 6A shows a similar analysis to determine the 20 most abundant proteins identified in all the 3 liposomes (without including the full serum). Additionally, in Supplementary Figs. S8A-C proteins were ranked based on the rank product of each formulation to show the 20 most abundant proteins in the DOPC, DOPC/G, and DOPG liposomes, respectively, together with their abundance in the other formulations, as well as in the serum, for comparison. The results clearly showed that the identity and relative abundance of the proteins adsorbed on the different liposomes varied strongly, confirming that tuning liposome formulation by mixing neutral and charged lipids in different ratio allowed to enrich corona of very different composition, and –as a result of that – to obtain formulations with very different uptake kinetics in cells (Fig. 2).

The ranking of top 20 abundant proteins (Fig. 6A) showed – as expected - enrichment of lipoproteins, immunoglobulins and other proteins on all samples, as commonly observed in similar corona studies [14–16]. Several apolipoproteins including APOA1, APOA2, APOC1, APOC2 and APOE were included in the top 20 list, but their amount on three liposomes was different. To evaluate the effect of lipid composition on corona formation, a Venn diagram was used to show the common and unique corona proteins identified on the three liposomes (Fig. 6B). A first important observation is that the number of corona proteins identified on DOPG

liposome and DOPC/G liposome (281 and 258 proteins, respectively) was much higher than for DOPC liposome (186 proteins). Interestingly, almost all of the proteins identified on DOPC liposomes were in common with the other formulations (170 proteins out of 186 total proteins identified), whereas by adding increasing amounts of the negatively charged DOPG lipid, the number of unique corona proteins on the liposomes increased from 4 unique proteins for DOPC, to 35 and up to 60 in the case of DOPC/G and DOPG respectively. Thus, adding increasing amounts of the negatively charged lipid had a strong influence on corona formation, and not only increased the total amount of absorbed proteins (Fig. 5C) but also protein variety and the presence of unique proteins.

A Z-score heat map allowed to have a closer look at the most statistically different proteins adsorbed on the different formulations (Fig. 6C, p value ≤ 0.01). From this graph and the list of the top 20 most abundant proteins in each formulation (Supplementary Figs. S8A-C) some interesting conclusions can be drawn. First, immunoglobulins were among the most abundant proteins on DOPC liposomes (Supplementary Fig. S8A). Additionally, DOPC showed enrichment of several unique lipoproteins including APOB, APOM, APOD and APOC3 which had higher Z-score on the DOPC liposomes in comparison to the other formulations, although their relative abundance in the DOPC corona was not very high (Supplementary Fig. S8A). Enriched proteins distinguishing the corona of a specific formulation may have strong biological impact even if their relative abundance is low. Similarly, the complement proteins C4BPA and C4BPB had higher Z-score on the DOPC liposomes. On the opposite side, on the anionic DOPG liposomes, a different set of lipoproteins was enriched, including for instance APOC1, APOH and APOE (the first two highly abundant also on the mixed DOPC/G liposomes). Additionally, it was interesting to note that several of the proteins most statistically different on DOPG liposomes (Fig. 6C), such APOE, VTNC and THRB were proteins that – based on their isoelectric point (pI) [53]– have a negative charge in a physiological environment.

This suggested that corona composition cannot be predicted solely based on the particle surface charge [9]. A classification of the identified corona proteins based on their pI is included in Supplementary Fig. S9. Despite the variation of protein pI distribution among the different formulations, for all liposomes more than 50% of the corona proteins had a $pI < 7.4$ thus a negative charge in physiological environment. This may contribute to the so-called ‘normalization’ effect of the corona in serum, where most nanomaterials, regardless of their initial charge, tend to similar (low and slightly negative) zeta potential upon corona formation, as indeed we showed also for these liposomes (Fig. 1B) [9].

As a final step, we tried to connect uptake efficiency and corona composition. In agreement with our results, a strong positive correlation has been reported between the overall serum protein binding capacity of nanomaterials and their cellular uptake *in vitro* [54] and *in vivo* [55]. Thus, in our case, with the addition of the anionic DOPG lipid, liposomes with increased amount and larger variety of absorbed proteins were obtained, and they also showed higher uptake rate in HeLa cells, especially in the first hours of exposure. It is important to stress that all formulations had comparable zeta potential once dispersed in serum, as a consequence of protein adsorption and corona formation, regardless of the very different charge and zeta potential in pristine conditions (Fig. 1B). We have recently shown that when different coronas form on silica nanoparticles and DOPG liposomes, nanoparticles are recognized by different cell receptors and cells use different mechanisms for their uptake[38]. Similarly, Digiacomo et al. showed that the mechanism of internalization of liposomes was changed from macropinocytosis to clathrin-mediated endocytosis after corona formation [56]. Therefore, in the current study, the very different uptake kinetics of DOPC, DOPC/G and DOPG liposomes were likely a result of specific interactions of their different corona components with cell receptors and activation of different uptake mechanisms.

Additionally, Ritz et al. correlated the enrichment of corona proteins on polystyrene nanoparticles with their cellular uptake [20], and they reported that a higher relative amount of THRB, VNTIII, VTN, ITIH4, PLF4 and APOH correlated with a higher cellular uptake, while a negative correlation was observed between APOA4, APOC3, ALBU and CO3 and uptake. In our case, immunoglobulins, albumin and the lipoproteins APOC1, APOA1 and APOA2 were present in all of the coronas, but in different abundance for the different formulations. In agreement with the results by Ritz et al., APOC3 was most abundant and most statistically different in the corona of DOPC liposomes for which the lower uptake was observed, while THRB, VTNC, PLF4 and APOH were most abundant and enriched on the DOPG liposomes, which had the highest cellular uptake efficiency. In line with these results, given the higher enrichment of APOC2 and APOM on the DOPC liposomes, it may be interesting to determine whether the presence of these proteins in the corona leads to lower uptake by cells, as demonstrated for APOC3. Similarly, the specific enrichment of APOE on the DOPG liposomes may explain the higher uptake of these liposomes. It was previously reported that PG lipids promote interactions with the low-density lipoprotein (LDL) receptor and also that APOE in the corona or APOE functionalized nanoparticles may enable access to the brain via the blood brain barrier [57–59]. Based on these observations, it would be interesting to explore whether DOPG liposomes could be exploited as a strategy to target the LDL receptor or promote transcytosis to the brain.

4. Conclusions

It is nowadays well established that nano-sized materials including nanomedicines once applied in a biological environment are modified by adsorption of a layer of biomolecules resulting in a corona which strongly affects the behavior on cells [7–10]. Growing evidence has indicated

that this layer can be recognized by specific cell receptors, opening up interesting possibilities of tuning corona composition in order to affect nanomedicine uptake and behavior on cells [24,25,38]. Within this framework here we have prepared and optimized a liposomes series of tailored surface properties by mixing zwitterionic DOPC and anionic DOPG lipids in different ratios. All liposomes gave homogeneous dispersions in serum and remained stable up to 24 h in cell culture conditions. Additionally, they were internalized by cells as intact nanoparticles via energy dependent mechanisms and trafficked along the endo-lysosomal pathway towards the lysosomes. However the different formulations showed very different uptake efficiency and kinetics: by adding increasing amounts of the negative lipid DOPG, liposomes with higher serum protein binding capacity were obtained, and – in line with other similar results - the protein binding capacity had strong positive correlation with uptake efficiency by cells [49,50]. Thus we optimized procedures for corona isolation to avoid contamination of serum particles and aggregates and we have identified the proteins recovered on the different liposomes by mass spectrometry. Thanks to the optimized methods, replicate experiments showed excellent reproducibility in the isolated corona proteins. Additionally, ion peak area and single stage MS quantification were used, instead of commonly applied spectral counting, for a more accurate intensity-based quantification of the protein abundance and thanks to the replicate isolations and mass spectrometry data, statistical analysis was applied to compare the corona composition on the different formulations. The amount and identity of the proteins adsorbed varied strongly, as also the observed uptake efficiency and uptake kinetics by cells. Given the comparable zeta potential of the liposomes in cell media following corona formation, it is likely that the observed differences in uptake behavior on cells were due to specific interactions with corona proteins and activation of different mechanisms of uptake, as opposed to simple charge differences. Thus, overall, we showed that by mixing lipids to different ratios, liposome series of tailored surface properties can be prepared to gradually tune corona composition in a systematic way. Tuning

corona composition allowed us to obtain liposomes with very different uptake behavior on cells. Thus, formulations can be optimized to achieve the desired uptake efficiency and kinetic profiles as required for specific applications. Additionally, careful controls combining size measurements and size exclusion chromatography were included to exclude the presence of particle agglomeration during corona isolation and eventual residual free proteins which could confuse the results.

At a broader level, further studies are required in order to determine how uptake efficiency by cells correlates with liposome outcomes *in vivo* [40]. Additionally, using similar approaches as we showed here to measure uptake by macrophages, further insights on effects of corona composition on clearance by the immune system could be obtained. Thus, novel strategies to prolong liposome circulation time *in vivo* could also be discovered [60].

Overall, the correlation of corona composition and uptake by cells (or clearance by macrophages) can be used as a novel tool to identify proteins which correlate with higher and/or lower uptake by cells [16,20]. Recent studies are indicating that the corona may interact with cell receptors via patterns or novel epitopes forming after corona formation, and not only by interactions of individual proteins with their specific receptors. Thus the simple identity of the proteins in the corona may not capture all possible interactions with cell receptors and, similarly, patterns of corona proteins together may be responsible of nanoparticle outcomes at organism level [13,61]. Nevertheless, by studying the specific role of individual proteins identified in the corona in mediating interactions with their corresponding cell receptors and promoting or blocking nanoparticle uptake, additional strategies to control nanomedicine outcomes on cells may be developed, including for reducing their clearance [20,23].

Clearly, more work is needed to be able to predict corona formation and to predict uptake behavior based on corona composition and corona pattern. Overall, a deeper understanding of the complex modifications nano-sized materials encounter in biological environments and their

consequences on their outcomes at cell and organism level is needed to guide the design of successful nanomedicines. Approaches such as those presented can be used to optimize formulations with desired properties for specific applications.

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Disclosures

The authors declare no competing financial interests.

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Figure 1. Physicochemical characterization of liposomes. (A) Size distribution by intensity (diameter, nm) of liposomes in different media. 50 $\mu\text{g/mL}$ liposomes in PBS, cMEM and hsMEM were characterized by DLS as described in Materials and methods. (B) Zeta potential of liposome dispersions in different media. 50 $\mu\text{g/mL}$ liposomes were incubated in PBS, cMEM and hsMEM and their zeta potential was measured as described in Materials and methods. The results are the average and standard deviation over 3 measurements. (C-D) Liposome stability in cMEM (C) and hsMEM (D). 50 $\mu\text{g/mL}$ liposomes were dispersed in cMEM (C) or hsMEM

(D) and their size distribution was measured by DLS just after dispersion (0 h) or after 1 h and 24 h incubation at 37 °C 5% CO₂.

Figure 2. Uptake of liposomes in hsMEM by HeLa cells. (A) Uptake kinetics of liposomes in hsMEM. HeLa cells were exposed to 50 µg/mL DOPC, DOPC/G and DOPG liposomes in hsMEM, and after different exposure times, cells were collected for flow cytometry measurement as described in Materials and methods. (B-D) Uptake of DOPC (B), DOPC/G (C) and DOPG (D) liposomes in energy depleted cells. Briefly, after 30 min pre-incubation with 5 mg/mL sodium azide, HeLa cells were exposed to 50 µg/mL liposome in standard hsMEM or hsMEM containing 5 mg/mL sodium azide, followed by cell fluorescence measurement by flow cytometry (see Materials and methods for details).. In all panels, the results are the average and standard deviation over 2 replicates of the cell fluorescence intensity obtained by flow cytometry (error bars are included in all graphs but in some cases are not visible because very small).

Figure 3. Fluorescence microscopy images of HeLa cells exposed to liposomes in hsMEM. Briefly, HeLa cells were exposed for 3 h to 50 $\mu\text{g/mL}$ SRB-labelled DOPC, DOPC/G or DOPG liposomes and imaged using the same settings to allow comparison of the uptake levels by cells in hsMEM. Blue: Hoechst stained nuclei. Red: SRB stained liposomes or free SRB. Green: LysoTracker stained lysosomes (see Materials and methods for details and Supplementary Fig. S3 for images at different settings to confirm uptake also for DOPC liposomes). Scale bar: 10 μm .

Figure 4. Isolation and characterization of liposome-corona complexes. (A) Scheme of corona isolation by size exclusion chromatography (SEC) (see Materials and methods for details). (B) Elution profiles of different samples following SEC. Briefly, the corona-complexes forming on 1 mL 0.5 mg/mL DOPC liposome in 40 mg/mL FBS (orange) were isolated by SEC as described in Materials and methods. The same amounts of FBS (green) and liposomes (blue) alone were also loaded in the column for comparison. Then, fractions were collected and their absorbance at 280 nm measured to obtain the protein elution profile. (C) Size distribution by intensity of liposome dispersions in PBS and the corresponding corona-coated liposomes in FBS after corona isolation. The size distributions were obtained by DLS immediately after the corona fractions were pooled together and concentrated as described in the Materials and methods.

Figure 5. Preparation of particle-depleted human serum (particle-depleted HS) and isolation of corona-coated liposomes. (A) Elution profiles of human serum after depletion of larger particles by SEC. Briefly, SEC was used to remove larger particles from human serum as described in the Materials and methods, then 1 mL particle-depleted HS was loaded again into a Sepharose CL-4B column and the protein elution profile was obtained. (B) Elution profile of DOPC liposomes after 1 h incubation with particle-depleted HS. Briefly, 0.075 mg/mL liposomes were mixed with 6 mg/mL particle-depleted HS and the obtained corona-coated liposomes were separated by SEC and the elution profiles at 280 and 565 nm determined as described in Materials and methods. (C) DLS size distributions by intensity of DOPC, DOPC/G and DOPG

liposomes in PBS and the corresponding corona-coated liposomes in particle-depleted HS after corona isolation. (D) Comparison of the amount of protein absorbed on DOPC, DOPC/G and DOPG liposomes after corona isolation. The protein binding capacity (PBC) was defined as the amount of absorbed proteins (μg) per micromole of lipid. The results are the average and standard deviation from three independent corona isolation experiments. (E) SDS-PAGE image of corona proteins recovered from DOPC, DOPC/G and DOPG liposomes in particle-depleted HS. The same amount of lipids was loaded in each lane ($0.05\ \mu\text{mol}$), together with $20\ \mu\text{g}$ full human serum and particle-depleted HS as controls.

Figure 6. Analysis of the protein corona on different liposomal formulations. (A) Analysis of the top 20 most abundant proteins in the different liposomes. For each liposome, 3 replicate corona isolations and identifications have been performed and the relative abundance of each of the top 20 proteins is indicated (calculated using eqn (1) and ranked as described in the Materials and methods). For each protein, the Area value obtained in each replicate sample is specified, while the balloon area shows the relative percentage of the median scale normalized protein abundance and the colors from black to red correspond to the p-values of the one way

ANOVA analysis. (B) Venn diagram of the corona proteins identified on the 3 liposomes. (C) Z-score heat map of the most statistically different proteins in the corona forming on the 3 liposomes (p value ≤ 0.01), calculated as described in the Materials and methods.

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