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The Human In Vivo Biomolecule Corona onto PEGylated Liposomes: A Proof-of-Concept Clinical Study

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The self-assembled layered adsorption of proteins onto nanoparticle (NP) surfaces, once in contact with biological fluids, is termed the "protein corona" and it is gradually seen as a determinant factor for the overall biological behavior of NPs. Here, the previously unreported in vivo protein corona formed in human systemic circulation is described. The human-derived protein corona formed onto PEGylated doxorubicin-encapsulated liposomes (Caelyx) is thoroughly characterized following the recovery of liposomes from the blood circulation of ovarian carcinoma patients. In agreement with previous investigations in mice, the in vivo corona is found to be molecularly richer in comparison to its counterpart ex vivo corona. The intravenously infused liposomes are able to scavenge the blood pool and surface-capture low-molecular-weight, low-abundance plasma proteins that cannot be detected by conventional plasma proteomic analysis. This study describes the previously elusive or postulated formation of protein corona around nanoparticles in vivo in humans and illustrates that it can potentially be used as a novel tool to analyze the blood circulation proteome.

The surface identity of nanoparticles (NPs) is modified once injected in the bloodstream because of their spontaneous interaction with a wide range of surrounding proteins and other biomolecules.[1] The spontaneous and layered adsorption of proteins onto NPs has been termed the "protein corona."[2] Even though surface modification of NPs with hydrophilic polymers (e.g., PEGylation) results in diminution of protein adsorption onto NP surfaces, the complete elimination of protein corona formation has not been achieved so far by such functionalization strategies.[3–7] During the last decade protein corona research has gained popularity with a flurry of attempts made to molecularly characterize corona profiles after the ex vivo incubation of NPs with biofluids (mainly plasma), and more recently, in vivo (in rodents).[4–7]

The biological effects arising from the presence of biomolecules onto NP surfaces can be both unfavorable and advantageous to their biomedical applications.[1,8] It is now reasonably established that the protein corona defines the NP surface characteristics and forms the interface between NPs and biological systems, also referred by some to as the "bio-nano" interface. The presence of the protein corona mediates the interaction of NPs with cells and has been shown to significantly impact their cytotoxicity, cellular internalization, and targeting capability.[9,10]

Among a plethora of nanoscale drug delivery systems, liposomes (phospholipid-based vesicles) are considered to be the most clinically established nanomedicine constructs.[11] The substantial reduction in the cardiotoxicity of doxorubicin upon encapsulation within PEGylated liposomes led to the approval of the first nanoscale anticancer agent (Doxil, Caelyx) by the FDA in 1995 for the treatment of AIDS-related Kaposi Sarcoma and in 1999 for the treatment of recurrent ovarian carcinoma.[12,13] This liposomal construct shows superiority to free doxorubicin owing to its prolonged blood circulation time, the stable retention of the drug in the interior of liposomes while in blood circulation, leading to an overall improved toxicity profile and enhanced tumor accumulation.[12,14]

Despite the clinical track record of liposomes for more than 20 years, the role that protein corona plays in liposomal pharmacology has been barely studied. To date, only a few studies

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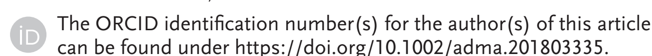
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describe the molecular composition of the protein corona formed around NPs in vivo.[4–6,15] In the vast majority of investigations, NPs are characterized after their ex vivo interaction with plasma proteins (mouse or human), extrapolating on the erroneous assumption that corona fingerprints formed under such a static biological setting can reflect the dynamics and molecular richness of the in vivo milieu.[1]

In our previous studies, we developed a robust protocol to retrieve and purify NPs from blood to investigate the in vivo protein corona formation and its evolution onto clinically used liposomes after their intravenous administration (tail vein) in rodents. These studies revealed that the molecular composition of the in vivo protein corona cannot be sufficiently predicted by the ex vivo plasma incubation of NPs.[4–6] Blood flow dynamics, the interaction with blood circulating cells, and immune responses triggered after NP administration are some of the factors that cannot sufficiently be simulated by ex vivo studies and could explain the differences observed between the ex vivo and in vivo formed protein coronas.[1] Although in vivo models shed light on our understanding of the self-assembly mechanism of the protein corona formation under more biologically relevant settings, extrapolation of data from mice to humans should be made with extreme caution.[16] Nanoparticle-based systems that work efficiently in rodent disease models often fail to offer similar efficacy in humans.[17] Yet, the formation of a protein corona around blood-circulating nanoparticles in humans has not been experimentally described at all, let alone studied as a potential factor to explain such discrepancies between preclinical and clinical achievements.

In the present study, we attempted to investigate and characterize the in vivo protein corona formation in humans. PEGylated, doxorubicin-encapsulated liposomes (Caelyx) were infused intravenously in six patients with platinum-resistant recurrent ovarian carcinoma and subsequently recovered from the blood circulation immediately on completion of their first cycle of Caelyx treatment. Liposomes were isolated from blood components along with their formed protein corona that was quantitatively and qualitatively characterized using a battery of techniques. The data indicated that an in vivo human protein corona forming around intravenously infused liposomal nanoparticles can be reproducibly identified and molecularly described. The human in vivo protein corona was rich in multiple low-molecular-weight and low-abundance plasma proteins that could not be detected by conventional plasma proteomic analysis, which revealed the potential utilization of the biomolecule corona as a tool to address the issue of the high dynamic range of plasma proteome.

Physicochemical Characterization of Liposomes and Human In Vivo Corona-Coated Liposomes: To characterize the in vivo formed, human-derived protein corona, PEGylated doxorubicin-encapsulated liposomes were recovered from the blood circulation of ovarian carcinoma patients ($n = 6$) at the end of their first cycle of intravenous Caelyx infusion (at a dose of 40 mg m^{-2}), for platinum-resistant disease (**Figure 1**A). Patient clinical and basic blood analysis characteristics are summarized in Table S1 (Supporting Information). The physicochemical properties of the commercially available PEGylated doxorubicin-encapsulated liposomes (Caelyx) were investigated by dynamic light scattering (DLS), ζ-potential measurements, and negative stain transmission electron microscopy (TEM) before and after their intravenous infusion in patients and are summarized in Figure 1B,C. Liposomes had a mean hydrodynamic diameter of 82.6 nm, a negative surface charge of −33.8 mV, and displayed low polydispersity values (<0.1) representing a narrow size distribution (Figure 1B). TEM imaging showed well-dispersed, drug-encapsulated round-shaped vesicles, with their size correlating that of DLS measurements (Figure 1C).

Immediately after the completion of the intravenous infusion of Caelyx (that lasted for ≈90 min), blood (≈10 mL) was collected and plasma was prepared without delay by centrifugation. A two-step purification protocol (size exclusion chromatography and membrane ultrafiltration) was then employed for the isolation of liposome–corona complexes and the complete elimination of unbound proteins, as we have previously described (Figure S1, Supporting Information).[4,5] It should be noted that although the protein corona is often described as a multilayered structure consisting of an inner layer of tightly bound proteins ("hard corona") and an outer dynamically bound layer of proteins ("soft corona"), current purification protocols might disturb loosely bound biomolecules. Therefore, the existence and the biological relevance of the "soft corona" remain unclear and we can only refer to the analytically accessible protein corona.[18]

Dynamic light scattering measurements of corona-coated liposomes demonstrated that their size distribution broadened (Figure 1B). Formation of the corona is often associated with an increase in the mean nanoparticle diameter since layers of proteins are adhered onto the NP surface. In the case of soft nanomaterials, however, the adsorption of proteins could also lead to a reduction in their mean diameter due to osmotic "shrinkage." This has been previously reported[4,5,19] and also observed here in the case of human in vivo and ex vivo corona-coated liposomes (Figure 1B; Figure S2, Supporting Information). In addition, our data showed that the mean surface charge of liposomes remained negative after their interaction with plasma proteins (Figure 1B; Figure S2, Supporting Information) in agreement with previous studies proposing that negatively charged NPs do not exclusively interact with positively charged proteins, as electrostatic interactions are not the only driving force at the NP–protein interface.[20]

Moreover, TEM imaging revealed well-dispersed and corona-coated liposomes that retained an intact structure after recovery and purification (Figure 1C). Although protein corona is usually illustrated as a dense layer covering the entire surface of a nanoparticle, this has not been experimentally confirmed. In agreement with our previously reported observations in rodents,[4,5] the human in vivo protein corona did not appear to coat entirely the liposome surface. Cryo-EM imaging of the recovered liposomes further confirmed the presence of a protein corona adsorbing around the doxorubicin-encapsulated (Caelyx) liposomes, but without fully covering their surface (Figure 1C). Similarly, Kokkinopoulou et al. have recently described the protein corona formed around polystyrene NPs as an undefined and unfolded network surrounding the NP surface.[21] Moreover, small vesicular structures surrounding the liposome surface could be observed by TEM in Figure 1C. We hypothesize that these could be either osmotically shrunk liposomes or blood-circulating extracellular vesicles adsorbed

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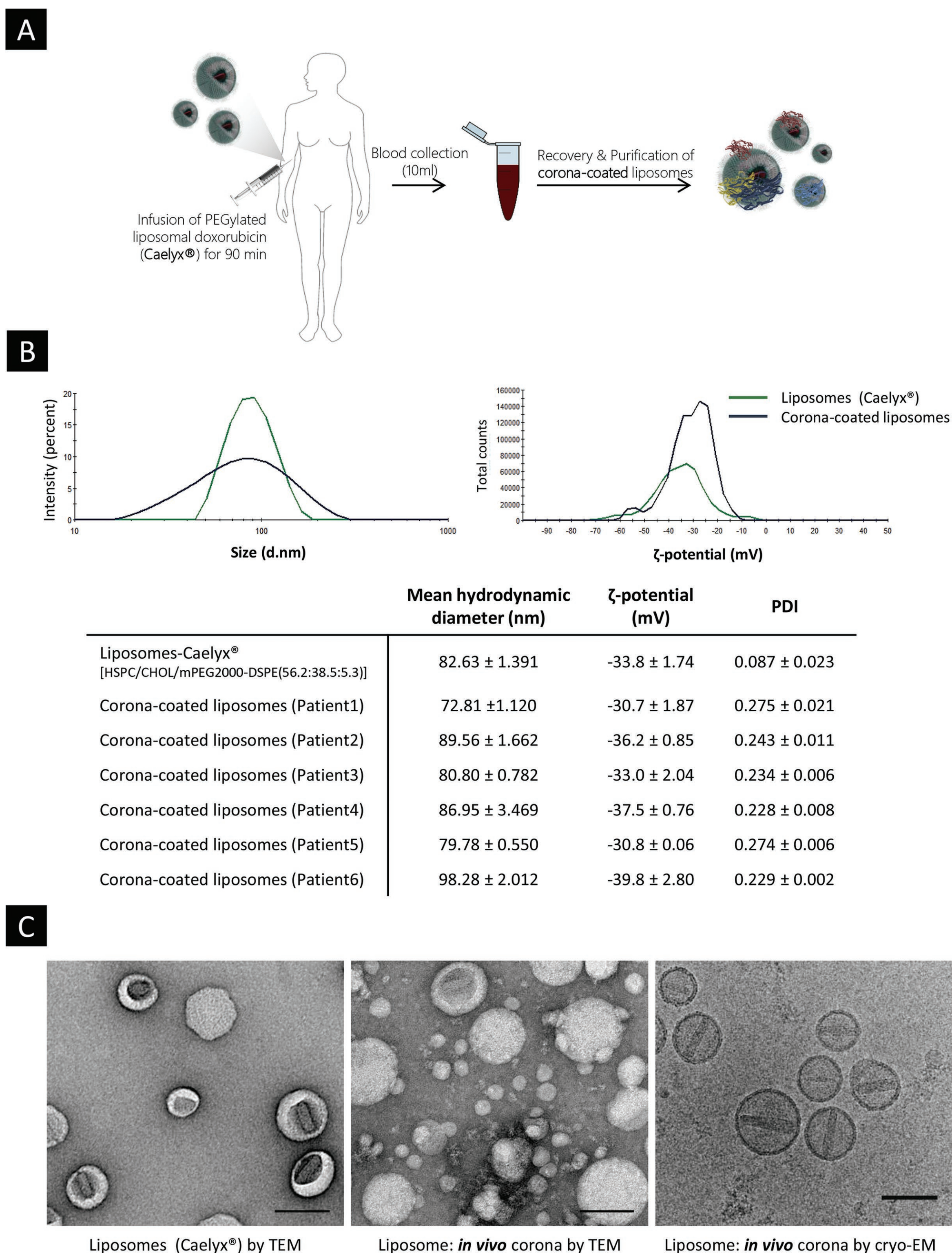

	Mean hydrodynamic diameter (nm)	ζ-potential (mV)	PDI
Liposomes-Caelyx® [HSPC/CHOL/mPEG2000-DSPE(56.2:38.5:5.3)]	82.63 ± 1.391	-33.8 ± 1.74	0.087 ± 0.023
Corona-coated liposomes (Patient1)	72.81 ±1.120	-30.7 ± 1.87	0.275 ± 0.021
Corona-coated liposomes (Patient2)	89.56 ± 1.662	-36.2 ± 0.85	0.243 ± 0.011
Corona-coated liposomes (Patient3)	80.80 ± 0.782	-33.0 ± 2.04	0.234 ± 0.006
Corona-coated liposomes (Patient4)	86.95 ± 3.469	-37.5 ± 0.76	0.228 ± 0.008
Corona-coated liposomes (Patient5)	79.78 ± 0.550	-30.8 ± 0.06	0.274 ± 0.006
Corona-coated liposomes (Patient6)	98.28 ± 2.012	-39.8 ± 2.80	0.229 ± 0.002

Figure 1. The effect of protein corona formation on the physicochemical characteristics and morphology of liposomes. A) Schematic description of the experimental design. PEGylated, doxorubicin-encapsulated liposomes (Caelyx) were infused intravenously in patients with recurrent ovarian carcinoma and subsequently recovered from the blood circulation immediately on completion of their first-ever cycle of Caelyx treatment. Corona-coated liposomes were isolated from blood components and protein corona was quantitatively and qualitatively characterized. B) The table shows the mean diameter (nm), ζ-potential (mV), and polydispersity index (PDI) values of bare and corona-coated liposomes recovered from the blood circulation of six adenocarcinoma patients. Representative size and ζ-potential distributions of corona-coated liposomes recovered from Patient 1 are also shown. C) Negative stain TEM and Cryo-EM imaging of liposomes after their IV injection and recovery from ovarian carcinoma patients. All scale bars are 100 nm.

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onto the surface of liposomes. Based on these observations, further studies will be needed to explore the adherence of other molecules or vesicular structures onto the surface of blood-circulating NPs.

Characterization of the Human In Vivo Protein Corona: Previous antiopsonization studies have emphasized that the total amount of protein adhered onto NPs can be used to predict the NP blood circulation time.[22–24] Even though the overall protein adsorption is moderated by the PEG chains present onto the NP surface, it cannot be fully suppressed.[3–5] In agreement with these previous findings, the present study demonstrated that PEGylated nanoscale surfaces are not entirely inert and interact with plasma proteins upon intravenous administration in humans.

To investigate the total amount of protein adsorbed, we calculated the protein binding ability (Pb), defined as the amount of protein associated with each µmole of lipid. As shown in **Figure 2**A, the average Pb value observed was 1532 µg of protein per µmole of lipid, that is more than 10 times higher than what was obtained for the same liposome composition (produced in the laboratory) after injection in healthy CD-1 mice.[5] Whether this is a result of mouse-to-human differences and/or due to the neoplastic disease present at an advanced stage in the patients that participated in this study is difficult to conclude and will require further investigation. Ex vivo incubations of Caelyx liposomes with plasma samples, obtained from the same patients before Caelyx infusion, were performed as a control. As shown in Figure S3 (Supporting Information), a significantly lower average Pb value was observed which confirms our previous data suggesting that the ex vivo incubation of NPs with human plasma samples cannot predict the formation of protein corona under realistic in vivo conditions.[4]

The manner in which proteins adsorb and pack onto the NP surface is highly dependent on their physicochemical properties and especially their size, shape, and functionalization. However, concrete relationships between the nanomaterial synthetic identity and their ensuing biological identity in physiological environments remain vague and unpredictable.[1] Distinct proteins could be either enriched or displayed weak affinity for the NP surface depending on the balance between their rates of association (K_{on}) and dissociation (K_{off}).[25] It has previously been shown by us in vivo[5] and others in vitro[20,26] that the protein corona is a temporally dynamic entity. In complex biofluids, such as blood, proteins present at high concentrations are characterized by high K_{on} values and therefore have high possibility to interact with the surface of NPs. However, these proteins might be replaced by other molecules of lower abundance, but of higher binding energy (characterized by low K_{off} values). Our previous time evolution studies in rodents revealed that a molecularly rich in vivo protein corona was formed around PEGylated liposomal doxorubicin (Caelyx) as early as 10 min postinjection. Even though the total amount of protein adsorbed and the identity of the corona proteins did not significantly change, the abundance of each protein fluctuated over time, indicating that competitive exchange processes were taking place. Interestingly, liposomes were coated by a complex mixture of low-molecular-weight (MW) proteins at all different time points of investigation.[5]

To examine whether our previous observation in rodents applies also under the in vivo conditions in humans, we comprehensively identified the protein molecules that self-assembled to form the corona around the intravenously infused liposomes by mass spectrometry. Surface-bound proteins were classified according to their molecular weight. As illustrated in Figure 2B, plasma proteins with MW < 80 accounted for almost 80% of the protein coronas formed. It is possible that the low MW proteins identified (Figure 2B) have high affinity and interact directly with the surface of PEGylated liposomes and/or they are trapped between other corona-carrier proteins that are adhered to the NP surface.

To further understand the protein composition of the in vivo human corona, the average value (n = 6 patients) of relative protein abundance (RPA) for each identified protein was calculated. Figure 2C summarizes the 20 most abundant proteins associated with the surface of recovered Caelyx liposomes for all patients. The most abundant corona protein was full-length cDNA clone CS0DD006YL02 (with accession number Q86TT1; SwissProt database). To the best of our knowledge, this protein has not been previously reported to associate with the surface of liposomes or any other type of nanoparticle after their incubation in full plasma. It has been only previously shown to interact with maltose-functionalized PEGylated hybrid magnetic NPs after their incubation with fractionated human plasma sample.[27] The 20 most abundant proteins identified in each patient and their respective RPA values are also shown in Table S2 (Supporting Information) to further illustrate the consistency of the above observation. The fact that the most abundant corona proteins were common between the six patients illustrates the consistency of liposome–protein interactions. Out of 445 corona proteins identified, 122 were repeatedly detected in all patients (Figure S4; Table S5, Supporting Information).

In agreement with our previous investigations of the liposomal protein coronas in mice,[5] immunoglobulins, lipoproteins, and complement proteins were the most abundant classes of proteins, contributing to 28%, 9%, and 4% of the total protein content respectively (Figure 2D; Tables S2 and S3, Supporting Information). The presence of opsonins (such as immunoglobulins), known to activate the mononuclear phagocytic system (MPS), can favor recognition and clearance of the blood-circulating PEGylated liposomes used in this study. However, the PEG-mediated inhibition of interactions between nanoparticles and circulating blood cells has been proposed to explain their long circulation time.[28] As the molecular identification of the human corona suggests, lipoproteins were the second most abundant class of proteins, proposed to have dysopsonic activity (i.e., favor long blood circulation), possibly via competitive binding for the liposomal surface with opsonic proteins.[29] The high affinity and adherence of lipoproteins to the lipid surface of Caelyx NPs observed was not surprising considering that blood circulating lipoproteins are involved in lipid and cholesterol transport and metabolism.

Intravenously infused, doxorubicin-encapsulated PEGylated liposomes have also been shown to interact with the complement system, in some cases triggering a transient and mostly mild hypersensitive reactions known as C activation-related pseudoallergy (CARPA).[30,31] Despite the presence of several key complement cascade proteins in the liposomal corona involved in the classical (complement C1s and C1qb, C4b binding protein) alternative (complement factor h, complement C3) and in lectin (mannan-binding lectin serine protease) pathways of

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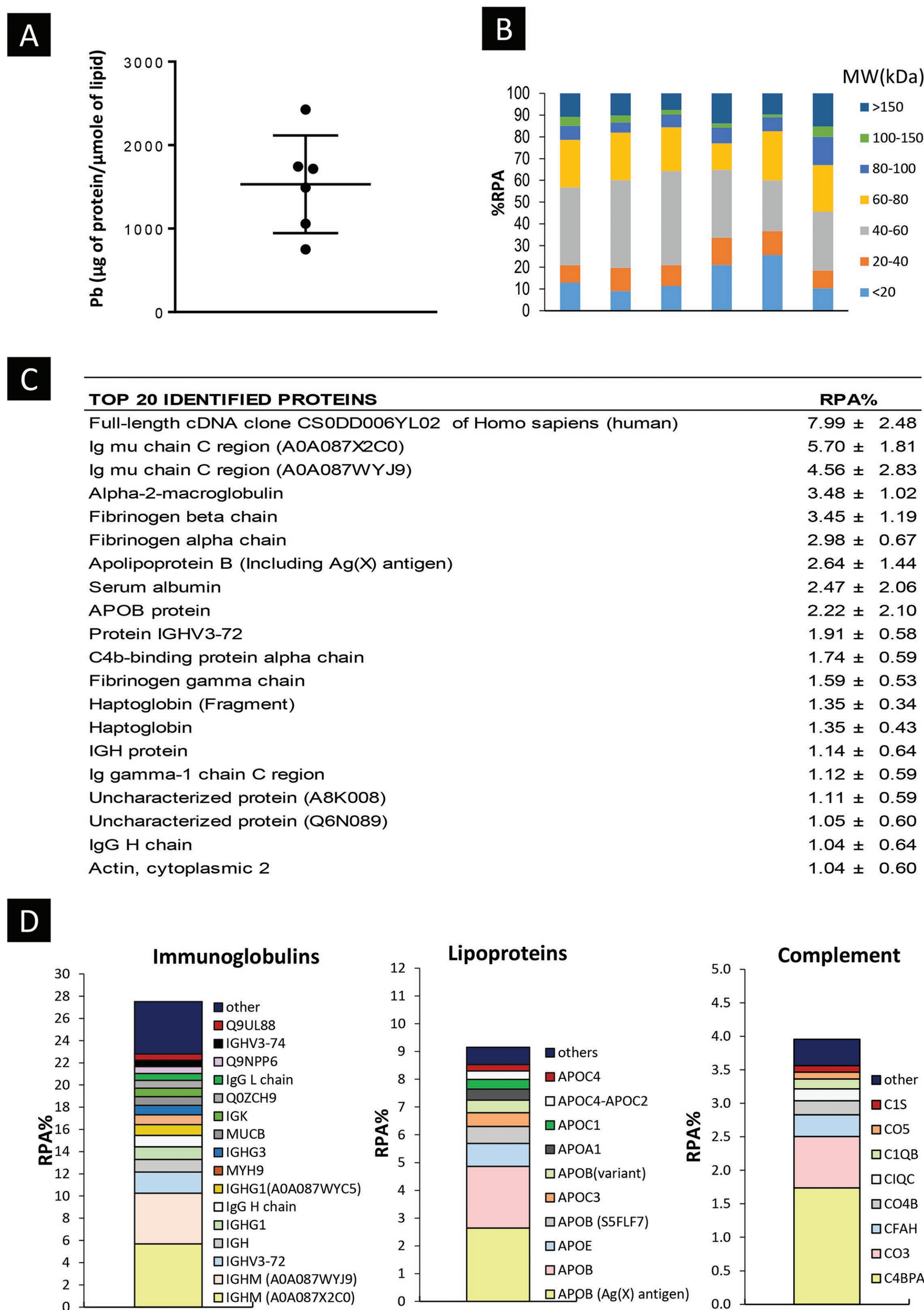

TOP 20 IDENTIFIED PROTEINS	RPA%
Full-length cDNA clone CS0DD006YL02 of Homo sapiens (human)	7.99 ± 2.48
Ig mu chain C region (A0A087X2C0)	5.70 ± 1.81
Ig mu chain C region (A0A087WYJ9)	4.56 ± 2.83
Alpha-2-macroglobulin	3.48 ± 1.02
Fibrinogen beta chain	3.45 ± 1.19
Fibrinogen alpha chain	2.98 ± 0.67
Apolipoprotein B (Including Ag(X) antigen)	2.64 ± 1.44
Serum albumin	2.47 ± 2.06
APOB protein	2.22 ± 2.10
Protein IGHV3-72	1.91 ± 0.58
C4b-binding protein alpha chain	1.74 ± 0.59
Fibrinogen gamma chain	1.59 ± 0.53
Haptoglobin (Fragment)	1.35 ± 0.34
Haptoglobin	1.35 ± 0.43
IGH protein	1.14 ± 0.64
Ig gamma-1 chain C region	1.12 ± 0.59
Uncharacterized protein (A8K008)	1.11 ± 0.59
Uncharacterized protein (Q6N089)	1.05 ± 0.60
IgG H chain	1.04 ± 0.64
Actin, cytoplasmic 2	1.04 ± 0.60

Figure 2. Characterization of in vivo protein corona: A) the total amount of proteins adsorbed in vivo onto liposomes recovered from the blood circulation of ovarian carcinoma patients. Pb values (µg of protein μM^{-1} lipid) represent the mean and standard deviation from six patients; B) classification of the corona proteins identified according to their molecular mass; C) most abundant proteins (top 20) identified in the protein corona of PEGylated doxorubicin-encapsulated liposomes by LC–MS/MS. Relative protein abundance values represent the average and standard deviation from six ovarian carcinoma patients; D) the relative percentage of immunoglobulins, lipoproteins, and complement proteins identified in the protein corona. The percentage of relative protein abundance (%RPA) for each protein class represents the average from six ovarian carcinoma patients.

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activation (Figure 2D; Table S3, Supporting Information), none of the patients in our study experienced a clinically symptomatic reaction to Caelyx infusion.

Patient-by-Patient Blood-Circulation Proteome Analysis: Blood predominantly contains high abundance, high MW proteins, such as albumin and immunoglobulins, that hamper the detection of the lower MW blood proteomic fractions.[32] There is an urgent need for tools to facilitate the discovery of new and, more importantly, combinations of protein molecule panels to improve early cancer diagnosis, evaluate disease progression, and monitor response to the treatment. Robust high-throughput proteomic discovery platforms that will enable the identification of blood-buried molecules are of immediate clinical importance.

The fact that albumin "masking" was largely eliminated from the in vivo human-derived corona prompted us to investigate further whether liposomes interacted with plasma proteins that could not be directly detected by plasma proteomic analysis. Proteins associated with liposomes in vivo were separated by SDS-PAGE and visualized by Imperial Protein stain, as illustrated in **Figure 3**A. Contrary to plasma control, the distinct bands of corona proteins, even at the low MW region, confirmed the ability of liposomes to surface-capture low-abundance proteins minimizing the "noise" from highly abundant proteins (Figure 3A).

Low-molecular-weight proteins are easily cleared from blood circulation (mainly through proteolytic plasma clearance), which limits their detection by conventional plasma proteomic analysis.[33–35] The only way a small molecule can remain in the blood circulation for longer periods is to adhere to a long-circulating high abundance protein, such as albumin.[34,35] The adherence of smaller MW biomolecules onto the surface of NPs once injected in the bloodstream could significantly increase their blood circulation and allow their successful "enrichment" for detection. Therefore, the characterization of the purified corona proteins can be employed as a "fractionation" plasma tool that addresses the signal-to-noise challenge.

The nanoparticle-mediated capture of low MW proteins from biofluids has been so far attempted exclusively ex vivo. For instance, Nanotrap technology developed by Liotta and co-workers uses core–shell hydrogel nanoparticles as protein harvesters. This technology is based on the NP-mediated size and charge-dependent fractionation of complex biofluids, such as plasma and urine, prior to proteomic analysis. The porous outer shell of *N*-isopropylacrylamide (NIPAm) NPs blocks the entry of high MW proteins, while the internal core contains covalently attached chemical affinity baits that capture low MW proteins.[36,37]

To compare the human corona proteins with the plasma proteome, we analyzed plasma samples obtained from the same patients before the infusion of Caelyx. The Venn diagram in Figure 3B illustrates the number of common and unique proteins between the liposomal corona and plasma as identified by mass spectrometry. A significantly higher total number of proteins was detected in the corona samples in comparison to the number of proteins identified when plasma samples were analyzed (Figure 3B). In addition, the most abundant plasma proteins were not the predominant corona proteins, as depicted in Figure 3C and Tables S3 and S4 (Supporting Information). Strikingly, the most abundant corona protein (full-length cDNA clone, CS0DD006YL02), which contributed to 8% of the total protein content, was not detected in any of the plasma control samples (Figure 3C; Table S4, Supporting Information). Although, full-length cDNA clone CS0DD006YL02 has been previously identified by mass spectrometry analysis of arachnoid cyst fluid,[38] peritoneal effluent[39] and saliva,[40] current proteomic approaches fail to detect this low-abundance protein in complex mixtures like plasma.

The above findings prompted us to further investigate the molecular composition of the ex vivo protein corona after the incubation of Caelyx liposomes with plasma samples obtained from the same ovarian carcinoma patients before Caelyx infusion. In agreement with our previous data in rodents, a more complex molecular fingerprint was detected for the in vivo protein corona in comparison to its counterpart ex vivo corona (Figure S5, Supporting Information). Despite the fact that the cDNA clone CS0DD006YL02 protein was not detected by mass spectrometry in any of the control plasma samples, it was identified as the most abundant protein of both the in vivo and ex vivo formed protein coronas (Figure 2A; Table S6, Supporting Information). Control investigations of the ex vivo corona formed onto Caelyx liposomes upon incubation with plasma samples from healthy volunteers (Table S6, Supporting Information) were also performed. The cDNA clone CS0DD006YL02 protein was found to be the fifth most abundant protein in the control cohort suggesting that tumorigenesis may affect the dynamics of corona formation. Overall, the above data provide an initial evidence that the liposome protein corona results in an "enriched" sampling of the blood proteome which renders the need for much more work on the biomarker discovery front necessary, but beyond the scope of this study.

Previously unreported experimental evidence that a biomolecule corona self-assembles around nanoparticles in humans while in their blood circulation has been offered in the present study. The successful recovery and purification of corona-coated lipid bilayer vesicles from the blood circulation of ovarian carcinoma patients allowed the proteomic analysis of the human in vivo protein corona. We demonstrated that the clinically used liposomal nanoparticles interact and can be stably coated with a complex mixture of plasma proteins, including low MW and low-abundance molecules otherwise "masked" under the overwhelming signal of highly abundant proteins (such as albumin and immunoglobulins). The corona-based elimination of this "masking" effect enabled the uncovering of multiple protein molecules that could not be detected by plasma sample analysis performed in comparison. This work is thought to act as the impetus for many future studies needed to improve our further understanding of how the human in vivo biomolecule corona can affect the overall clinical performance of NPs, but also provide the technology springboard to allow the clinical exploitation of protein corona fingerprinting as a novel tool to comprehensively analyze the blood circulation proteome

Experimental Section

Ethical Approvals: This project was reviewed and approved by the Manchester Cancer Research Centre Biobank Sample Access Committee and all sample collection was conducted under the MCRC Biobank Research Tissue Bank Ethics (ref.: 07/H1003/161 + 5). Ovarian carcinoma patients signed informed consent documents before participation in the study.

Blood Sample Collection: Caelyx is indicated for the treatment of advanced ovarian cancer in women who have failed a first-line platinum-based

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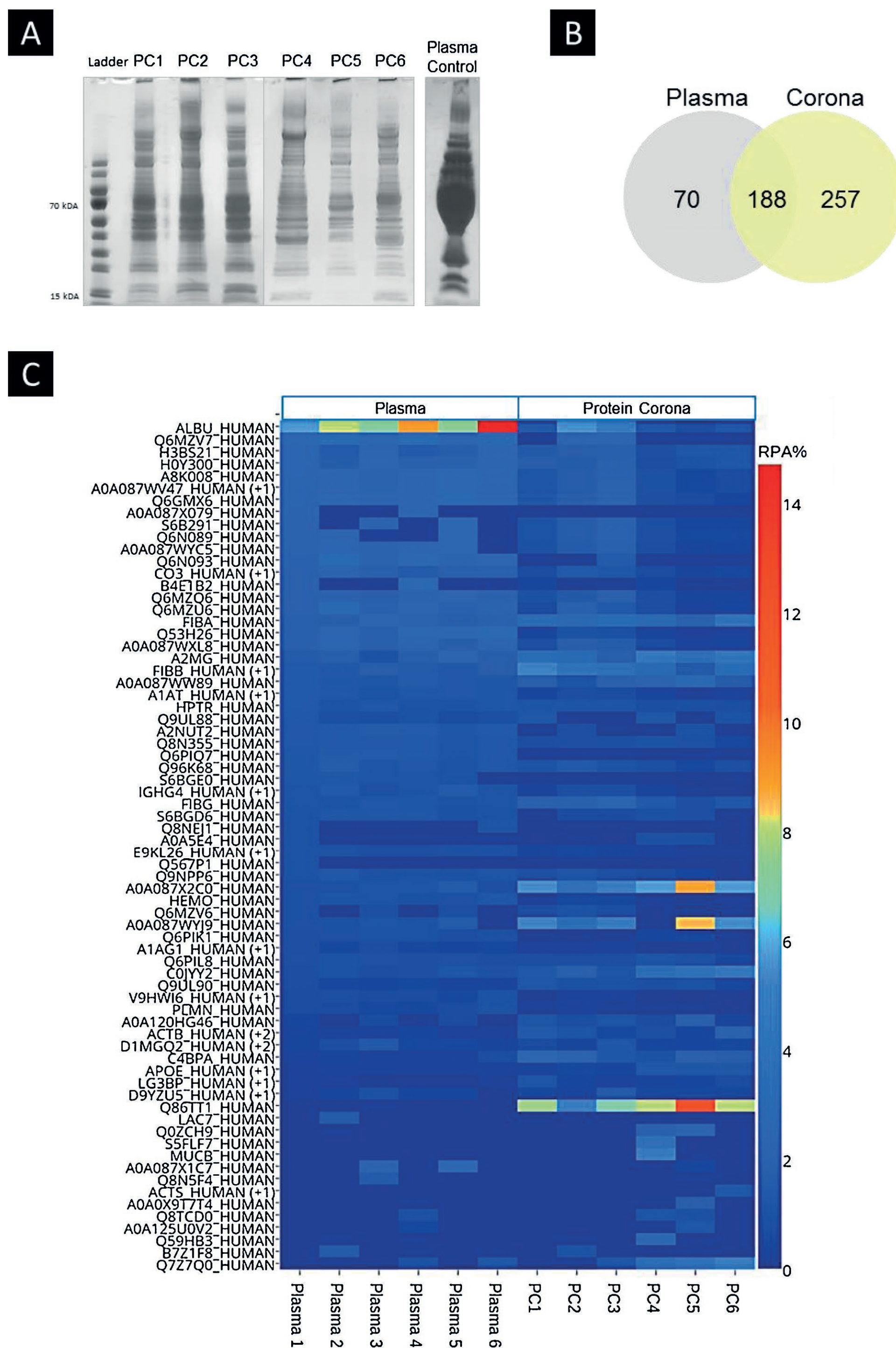

Figure 3. Blood-circulation proteome analysis: A) imperial stained SDS-PAGE gel of corona proteins associated with liposomes in the blood circulation of six ovarian carcinoma patients and plasma control; B) Venn diagram reports the number of unique and common proteins between corona and plasma proteins and their respective overlaps as identified by LC–MS/MS. Proteins were identified in at least one of the six plasma and/or corona samples; C) heatmap of RPA (%) of corona and plasma proteins, as identified by LC–MS/MS. Only proteins with RPA > 1% on at least one of the samples are shown. Protein rows are sorted according to the RPA% values (from highest to lowest) of the first sample (plasma, patient 1). The full list of proteins identified and their respective accession numbers are shown in Tables S3 and S4 (Supporting Information).

chemotherapy. Patients receive in total six cycles of Caelyx with cycle frequency 28 days. Eligible cases for this study included women with recurrent ovarian cancer commencing single agent Caelyx for the first time, at a dose of 40 mg m^{-2}. Caelyx contains 2 mg mL^{-1} doxorubicin hydrochloride encapsulated in a PEGylated liposomal formulation (16 mg lipid content). Based on established pharmacokinetic data for

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the clinically used PEGylated liposomes encapsulating doxorubicin, the time of blood collection (after the completion of infusion that lasted for ≈90 min), the majority of injected liposomal nanoparticles are still in blood circulation.[14,41]

Paired plasma samples (before and immediately after cycle 1 infusion) were collected into commercially available anticoagulant-treated tubes (K2 EDTA BD Vacutainer). Plasma was then prepared by inverting 10 times the collection tubes to ensure mixing of blood with EDTA and subsequent centrifugation for 12 min at 1300 RCF at 4 °C. Following centrifugation supernatant was immediately collected into labeled Protein LoBind Eppendorf Tubes and samples were maintained on ice while handling.

Separation of Corona-Coated Liposomes from Unbound and Weakly Bound Proteins: Corona-coated liposomes were separated form excess plasma proteins by size exclusion chromatography followed by membrane ultrafiltration as we have previously described.[4,5]

Ex Vivo *Protein Corona Formation*: To investigate the ex vivo protein corona, Caelyx liposomes were incubated with plasma samples obtained from the same six patients prior to Caelyx infusion and from healthy donors. Considering the impact of the anticoagulant agent on the formation of protein corona,[42] the same protocol was followed for the preparation of plasma samples, as described above for the in vivo experiment (K2 EDTA BD Vacutainer tubes, centrifugation for 12 min at 1300 RCF at 4 °C). The ex vivo protein corona was allowed to form using the same liposome concentration (0.15×10^{-3} M) as that extracted in 1 mL of plasma from intravenously injected patients. Liposomes were incubated with plasma for 90 min at 37 °C in orbital shaker at 250 rpm. The purification of the ex vivo corona-coated liposomes was performed as described above for the in vivo recovered liposomes.

Size and Zeta Potential Measurements Using Dynamic Light Scattering: Liposome size and surface charge were measured using Zetasizer Nano ZS (Malvern, Instruments, UK).

Transmission Electron Microscopy: Liposomes were stained by uranyl acetate solution 1% and visualized with transmission electron microscopy (FEI Tecnai 12 BioTwin) before and after their in vivo interaction with plasma proteins. Samples were diluted to 0.5×10^{-3} M lipid concentration and carbon Film Mesh Copper Grids (CF400-Cu, Electron Microscopy Science) were used.

Cryo-Electron Microscopy: EM grids of liposomes were prepared in an FEI Vitrobot using 3 µL of sample absorbed to freshly glow-discharged R 3.5/1 Quantifoil grids. Grids were blotted for 4–5 s in a 95% humidity chamber before plunge freezing into liquid ethane. Data were imaged on a Tecnai T20 (FEI) electron microscope operating at 200 keV with a Gatan 626 cryo stage. Images were recorded on a 4K Gatan Ultrascan CCD camera under low-dose conditions between 0.5 and 2.0 µm defocus and at 3.8 Å per pixel and had a maximum electron dose of <25 electrons $Å^{-2}$.

SDS-PAGE Electrophoresis: Proteins associated with 0.025×10^{-6} M of liposomes were loaded onto a 4–20% NOVEX Tris-Glycine Protein Gel (ThermoFisher Scientific). The gel was run until the proteins neared the end of the gel (25-40 min at 225 V). Staining was performed with Imperial Gel Staining reagent (Sigma Life Science).

Quantification of Adsorbed Proteins: Proteins associated with recovered liposomes were quantified by BCA Protein assay kit according to manufacturer's instructions. To make sure that liposomes in solution do not interfere with the absorbance at 562 nm, we measured the absorbance of corona-coated liposomes in HEPES buffered saline and subtract it from the total absorbance, measured when corona-coated liposomes were mixed with the BCA reagent. Lipid concentration was quantified by Stewart assay and Pb values (µg of protein μM^{-1} lipid) were then calculated.

Mass Spectrometry: In-gel digestion of corona (40 µg) and plasma (5 µL) proteins was performed prior to LC–MS/MS analysis, as we have previously described.[4–6] Digested samples were analyzed by LC–MS/MS using an UltiMate 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA) coupled to a Q Exactive Hybrid Quadrupole-Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer. Data produced were searched using Mascot (Matrix Science UK), against the SwissProt_2016_04 database with taxonomy of [human] selected.

The Scaffold software (version 4.3.2, Proteome Software Inc.) was used for relative protein quantification based on spectral counting. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified peptides. Semiquantitative assessment of the protein amounts was conducted using normalized spectral countings as previously described.[4–6] Heatmaps of relative protein abundance values were prepared using Plotly 2.0 software.

Statistical Analysis: Statistical analysis of the data was performed using GraphPad Prism software. One-way analysis of variance (ANOVA) was used and p values <0.05 were considered significant.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomolecule corona, Doxil, nanomedicine, ovarian cancer

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