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TRENDS

Liposome protein corona characterization as a new approach in nanomedicine

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Abstract

This trends article describes the analytical approaches for the in-depth characterization of the protein corona on liposome nanoparticles. In particular, examples since 2014 are summarized according to the analytical approach. Traditional protein corona characterizations from in vitro static experiments are provided along with the newly introduced experimental setups for characterization of the protein corona by in vitro dynamic and in vivo studies. Additionally, a special attention is also devoted to the need for introduction of new experimental workflows for characterization of a much wider array of biomolecules. In the most recent years, an extension of the protein corona concept to the biomolecular corona was introduced, and the analytical targets are no longer restricted to proteins, but to other important biomolecules as well, as they can potentially affect the biodistribution and interaction of nanoparticles with the biological systems. The few recent examples in this field are discussed for the characterization of metabolites and lipids in the biomolecular corona with examples, also extending the discussion from liposome to other types of nanoparticles. A final discussion is provided on the potential key role of the most recent omics approaches in the study of the nano-bio interface, with an overview on top-down proteomics, which allows a better elucidation of proteoforms, and on lipidomics and metabolomics, which allow a comprehensive untargeted characterization of lipids and metabolites, respectively.

Keywords Liposomes · Biomolecular corona · Protein corona · Proteomics · Metabolomics · Lipidomics

Introduction

Liposomes are promising vectors in drug delivery due to their capability of delivering therapeutics via membrane fusion, with concomitant release of liposome encapsulated cargo, rather than endocytotic pathways [1]. Many formulations have been developed and studied in the last two decades; nevertheless, only few of them reached the clinical trial stage and even fewer clinical practices. Despite the huge preclinical investigation, the success rate of liposomes in clinical trials is limited, mainly due to the complexity of the nano-bio interface. As previously demonstrated for inorganic nanoparticles by Dawson and coworkers [2–4], in a biological system, upon contact with any biological fluid, liposomes lose their synthetic identity due to adsorption of biomolecules on their surface, especially proteins [5, 6]. Several factors are known to affect protein binding on liposomes, which include the surface chemistry, topography, curvature, charge, protein affinity, and protein binding sites [7]. Nevertheless, the classification of liposomes based on their physico-chemical features is not sufficient to predict the protein corona formation. Environmental factors deeply affect the protein corona formation, and they include temperature, protein concentration, nature of the medium (polarity, ionic strength, and free energy of the solvent), incubation time, protein source (e.g., serum vs. plasma), and organism (e.g., mouse vs. human plasma) [7]. The protein corona has a dynamic nature, which includes protein-protein interactions, and variations over time are quantitative rather than qualitative [8]. It is generally accepted that the formation of the protein corona provides the synthetic nanoparticles with a new biological identity. The type of proteins, their relative abundance, and conformation in the protein corona are now believed to affect blood circulation time, biodistribution, cellular uptake, and intracellular localization of nanoparticles [8]. The resulting protein corona was

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demonstrated to significantly affect the physiological and therapeutic outcome, as it mediates recognition by the immune system and macrophage activation [9], with subsequent removal of the nanovector form circulation [10].

The protein corona effect is, nevertheless, very complex and far from being completely elucidated. The protein corona formation has been shown to interfere with targeting strategies and hinder the interaction of a targeting protein with receptors [11], but at the same time, this interaction also depends on the protein affinity. In the case of targeting with antibodies, for which affinity is very high, the targeting specificity can be largely conserved even in a complex biological milieu, as human serum [12]. Moreover, in a recent report, it was suggested that the hindering effects of the protein corona could be avoided using nanoparticles with antibodies adsorbed on them. Compared to covalently bound antibodies, this strategy allowed targeting also in 100% serum or plasma [13]. In this context, not only the protein composition is important to evaluate a response in vivo, but also protein structural features have been demonstrated to affect the interaction of nanoparticles with cells. For instance, differential centrifugal sedimentation, immunogold labels, and subsequent image processing have been used to identify the spatial location of proteins, their functional motifs, and their binding sites [14]. The study of the orientation and functionality of key proteins in the corona sheds light on the mechanisms that mediate most of the early biological interactions, thus allowing better elucidation of protein residual biological function and interaction with cellular receptors [15]. For instance, this approach indicated that only ~3.5% of proteins grafted on the surface of inorganic nanoparticles have a favorable orientation for recognition by cellular receptors [16].

There are two main approaches to cope with the protein corona formation. One strategy relies on preserving the surface functionality of liposomes in vivo by reducing the protein corona formation. The goal was mainly achieved by surface coating of the nanovector with hydrophilic polymers, in particular polyethylene glycol (PEG), which can reduce protein adsorption [17].

The second strategy relies on better describing the protein corona composition and guides the protein corona formation by tuning the liposome formulations, in order to control protein adsorption and exploit the protein corona for targeting [7]. By this approach, the perspective on the protein corona is dramatically changed from an obstacle, which hindered targeting by hiding the targeting moiety, to an opportunity, by means of which targeting can be achieved by adsorbing proteins which are then specifically recognized by cells. In either case, the key role of the protein corona is becoming increasingly clear, as the final success is strictly dependent on the protein corona composition [18]. At the same time, the importance of the characterization of the protein corona is not only limited to delivery of therapeutic agents, but it also finds application in biomarker discovery and personalized medicine.

Regardless of specific application, the investigation techniques which can be employed for characterization of the protein corona do not significantly change. The techniques can be divided into two main groups, i.e., direct or indirect approaches (Fig. 1) [19]. In the indirect approaches, which are the most commonly exploited, the protein corona is investigated by measuring changes in the physico-chemical properties of the nanoparticle-protein complexes, such as size, charge, density, mass, absorbance, and fluorescence. However, the direct approaches allow to obtain much more information. Standard biochemical protein quantification assays (i.e., Bradford and bicinchoninic acid assays) can be used for total protein quantitation. For a qualitative description of the protein corona composition, the most exploited approach is based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), also coupled with in-gel digestion and mass spectrometry (MS) protein identification of selected bands and quantitation [20]. Alternatively, direct shotgun proteomic workflows with in-solution digestion are used and coupled with chromatography for a comprehensive, both qualitative and quantitative, characterization of the protein corona composition [21]. These techniques provide information about the identity of the adsorbed proteins, but not on their structure [19].

In this context, this trends article aims at describing the most recent approaches in the characterization of nanoparticle biomolecular corona, significant for nanomedicine applications. A special emphasis will be devoted to the recently introduced need for characterization of protein corona in in vivo studies and personalized medicine. Finally, the recent extension of the protein corona concept to the biomolecular corona concept will also be described, with selected examples, when necessary also from studies involving nanoparticles different from liposomes. The coverage is not meant to be exhaustive, but to provide an overview of the current research in the field as well as possible future developments and trends.

In vitro static studies for the characterization of the protein corona

Most of the studies on liposome protein corona in the last 4 years still rely on the in vitro investigation of proteins under static conditions. In this context, most works provide a detailed qualitative characterization of the protein corona composition by exploiting the typical shotgun proteomic workflows (Table 1). The approach of these works has not significantly changed from previous reports [19, 21]. In

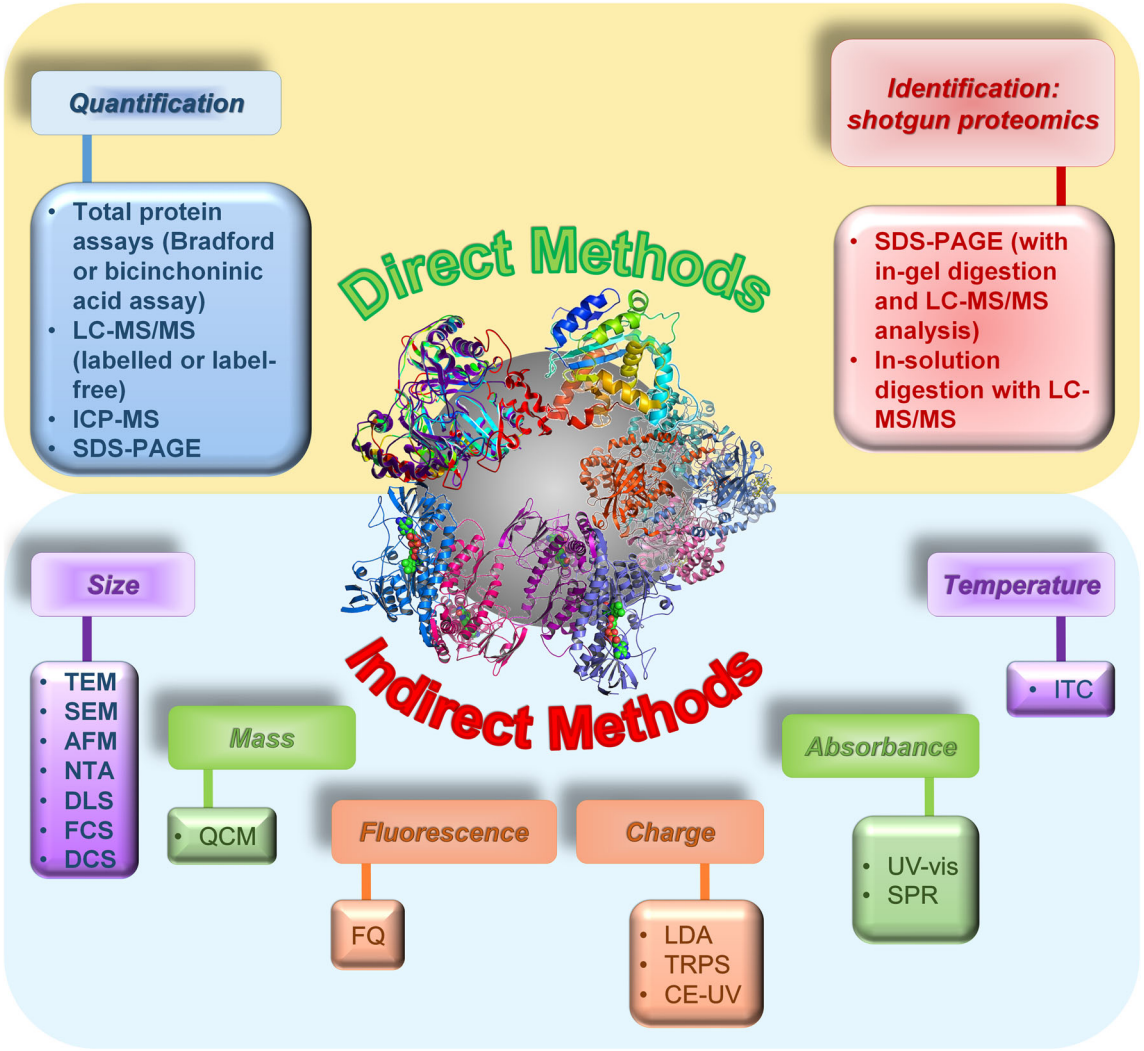

Fig. 1 Graphical overview of the analytical approaches most frequently employed for the investigation of the protein corona of nanoparticles. ICP-MS, inductively coupled plasma mass spectrometry; TEM, transmission electron microscopy; SEM, scanning electron microscopy; AFM, atomic force microscopy; NTA, nanoparticle tracking analysis; DLS, dynamic light scattering; FCS, fluorescence correlation spectroscopy; DCS, differential centrifugal sedimentation; QCM, quartz crystal microbalance; FQ, fluorescence quenching; LDA, laser Doppler anemometry; TRPS, tunable resistive pulse sensing; CE, capillary electrophoresis; SPR, surface plasmon resonant; ITC, isothermal titration calorimetry

shotgun proteomic works, liposomes are incubated with a biological fluid (human or animal plasma or serum is the most used ones) with a dilution ratio of 1:1 which is typically under physiological conditions (1 h at 37 °C) (Table 1). Afterwards, the liposome-protein corona complexes are isolated, usually by centrifugation, but other techniques are sometimes exploited, in particular size exclusion chromatography (SEC). The excessive media components are then removed by washing. By this approach, only the hard corona can be isolated, as the soft corona, which is the result of second adsorption by protein-protein interactions, is removed in the procedure. As recently observed, this workflow has an intrinsic weakness, as weakly bound proteins are lost and the full corona may not be characterized [7]. Most studies still provide only a qualitative composition of the protein corona, even though a detailed quantitative study is fundamental to predict the biological impact.

In vitro dynamic studies for the characterization of the protein corona

One major drawback of in vitro static studies is that they are performed under slight agitation rather than under a continuous flow. This limitation has emerged in the field in the last years and prompted the development of different experimental conditions for protein corona formation to mimic the dynamics of blood flow. In fact, blood moves at very different speeds in the body, from few micrometers per second (capillaries) up to 60 cm s^{-1} (ascending aorta). The main consequence of flowing is a shear

Table 1 Protein corona investigations (since 2014 until writing of this trends article) ordered according to liposome formulation (with related lipid molar ratio in brackets), the type of study, experimental conditions, and characterization approach

Liposome formulation	Study type	Biological medium	Sample preparation	Separation and detection	Protein identification	Ref
DSPC/charged lipid/cholesterol (4:1:2) Charged lipids: DSPG, DPPS, or DPTAP	In vitro (static)	Fetal calf serum or complement component 1q	Incubation (1 h at 37 °C); centrifugation; 3 washings; concentration by MWCO 1000 kDa	SDS-PAGE; western blot	–	[22]
HSPC/cholesterol/mPE-G_{2000}-DSPE/peptides-PE-G_{3400}-DSPE (52:43:3:2) or HSPC/cholesterol/DOTAP (52:43:5)	In vitro (static) and in vivo	Mouse serum (in vitro experiments) or blood sampled after 1 h or 4 h of administration in mice (in vivo experiments)	Serum preparation (in vivo experiments only); centrifugation; 2 washings (in vitro experiments only); SDS-PAGE; in-gel tryptic digestion	Nano-HPLC-MS/MS (in vitro); western blot (anti-mouse IgM antibody) (in vivo)	Yes (in vitro only)	[23]
Doxoves and the plain control	In vitro (static)	Commercial human plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	SDS-PAGE; nano-HPLC-MS/MS	Yes	[24]
Temozolomide-loaded cationic liposomes (DOTAP/DC-Chol/DOPC/D-OPE liposomes, 1:1 neutral/total lipid molar ratio)	In vitro (static)	Human plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[25]
Doxorubicin-loaded traditional or lysolipid-containing temperature-sensitive liposomes	In vivo	Blood sampled after 10 min of administration in mice	Plasma preparation; SEC (Sepharose CL-4B), MWCO (10,000 Da then 1,000,000 Da); 3 washings; SDS-PAGE; in-gel tryptic digestion	Nano-HPLC-MS/MS	Yes	[26]
DOTAP/DOPC/DC-Chol/DOPE (1:1:1:1) and DOTAP/DOPC/DC-Chol/D-OPE-PEG (1:1:1:0.7:0.3) liposomes	In vitro (static and dynamic)	Fetal calf serum	Incubation (5 min or 90 min at 25 °C); 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[27]
DSPC/Chol/$mPEG_{2000}$-DSPE (215:143:1) liposomes	In vitro (static)	Human serum	Incubation (1 h at 37 °C); centrifugation; 3 washings	SDS-PAGE	–	[28]
Egg phosphatidylcholine/-phosphatidylinositol--methotrexate-diglyceride/-melphalan-diglyceride (8:1:1)	In vitro (static)	Human plasma	Incubation (15 min at 37 °C); delipidation	SDS-PAGE; western blot	–	[29]
DC-Chol/DOTAP/DOPC/DOPE (1:1:1:1)	In vitro (static)	Human plasma	Incubation (15 min at 37 °C); 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[30]
AmBisome® and Doxil®	In vitro (static)	Mouse serum	Incubation (5 min at 37 °C)	HPLC on polymer-coated silica monolithic columns; DAD, fluorescence detector (ex. 480 nm, em. 575 nm)	–	[31]
	In vivo			Nano-HPLC-MS/MS	Yes	[32]

Table 1 (continued)

Liposome formulation	Study type	Biological medium	Sample preparation	Separation and detection	Protein identification	Ref
DPPC/DOPG/DSPC/Chol (5:3:1:1) liposomes and leukosomes		Blood sampled after 10 min or 1 h of administration in mice	Plasma preparation; centrifugation; 3 washings; SDS-PAGE; in-gel tryptic digestion			
Doxil®	In vivo	Blood sampled after 10 min, 1 h, or 3 h of administration in mice	Plasma preparation; SEC (Sepharose CL-4B); membrane ultrafiltration (10,000 MWCO; 1,000,000 MWCO); 3 washings; SDS-PAGE; in-gel tryptic digestion	Nano-HPLC-MS/MS	Yes	[33]
DPPC/DOPC/Chol (6:3:1)	In vitro (static)	Mouse plasma	Incubation (1 h at 37 °C); SDS-PAGE; in-gel tryptic digestion	Nano-HPLC-MS/MS	Yes	[34]
AmBisome® (100 nm) liposomes	In vitro (static) and in vivo	Mouse blood (in vitro experiments) or blood sampled after 10 min of administration in mice (in vivo experiments)	Incubation (10 min at 37 °C, in vitro) or plasma preparation (in vivo); centrifugation; exclusion chromatography (Sepharose CL-2B); MWCO (10,000 Da then 10,000 Da); 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[35]
7 liposomal formulations	In vitro (static)	Human plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[36]
DOTAP/DOPC/DC-Chol/DOPE (1:1:1:1), DOTAP/DOPC/DC-Chol/D-OPE/DOPE-PEG (1:1:1:0.7:0.3)	In vitro (static)	Human plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[17]
DOTAP/DOPE-PEG_{1000} (0.9:0.1), DOTAP/DSPC/Chol (0.3:0.5:0.2)	In vitro (static)	Human or mouse plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[37]
DOTAP/DOPC/DC-Chol/DOPE (1:1:1:1)	In vitro (static and dynamic)	Fetal calf serum	Incubation (1.5 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[38]
DOTAP, DOTAP/DOPE, DOTAP/Chol, DC-Chol, DC-Chol/DOPE, DC-Chol/Chol unilamellar cationic liposomes (neutral lipid/total lipid = 0.5)	In vitro (static)	Human plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[39]
Sphingosine/Chol/DSPC (3:2:5), DOTAP/Chol/DPPC (3:2:5), DOTAP/Chol/DSPC (3:2:5), DOTAP/Chol/PC, (3:2:5), Chol/PC (2:8)	In vitro (static)	Human or mouse plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[40]
				Nano-HPLC-MS/MS	Yes	[41]

Table 1 (continued)

Liposome formulation	Study type	Biological medium	Sample preparation	Separation and detection	Protein identification	Ref
HSPC/Chol (59.6:40.4), HSPC/Chol/DSPE-PEG_{2000} (56.3:38.2:5.5), targeted HSPC/Chol/DSPE-PEG_{2000}/-Ab (56.3:38.2:5.5)	In vitro (static) and in vivo	Blood sampled after 10 min of administration in mice (in vivo experiments) or mouse plasma (in vitro experiments)	Plasma preparation; SEC (Sepharose CL-4B); membrane ultrafiltration (MWCO 10,000 Da then MWCO 1,000,000 Da); 3 washings; SDS-PAGE; in-gel tryptic digestion			
DOTAP/DOPC/DC-Chol/D-OPE/DOPE-PEG (1:1:1:0.70.3)	In vitro (static and dynamic)	Fetal calf serum	Incubation (1.5 h at 25 °C); centrifugation; 3 washings; in-solution tryptic digestion	Nano-HPLC-MS/MS	Yes	[42]

DSPC 1,2-distearoyl-*sn*-glycero-3-phosphocholine, *DSPG* 1,2-distearoyl-*sn*-glycero-3-phosphoglycerol, *DPPS* 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine, *DPTAP* 1,2-dipalmitoyl-3-trimethylammonium-propane, *MWCO* molecular weight cutoff, *HSPC* hydrogenated soy phosphatidylcholine, *DSPE* 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, *DOTAP* 1,2-dioleoyl-3-trimethylammonium-propane, *DC-Chol* 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl]cholesterol, *DOPC* 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, *DOPE* 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, *Chol* cholesterol, *DAD* diode array detector, *DPPC* 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, *DOPG* 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, *PC* 1,2-diarachidoyl-*sn*-glycero-3-phosphocholine

stress on the nanoparticles and a continuous change in the surrounding environment, which result in a change of nearby biomolecules. To tackle this issue, new experimental setups have been proposed for the investigation of the protein corona, by exploiting a wide range of microfluidic approaches [43] (Table 1). Another issue to be considered is the incubation time. It has already been recognized as a fundamental parameter affecting the corona composition in static experiments, as the equilibrium needs to be established to obtain a stable corona composition. Still, the use of long incubation times (typically 1 h) is inconsistent with the in vivo application, because most nanoparticles have a much shorter in vivo blood residency, thus experiencing less incubation time, and are exposed to different microenvironments [43]. While on the one hand the problem of exposure to different microenvironments was never addressed, yet, on the other hand, incubation time was extensively studied. In a study by Palchetti et al. [27], multicomponent liposomes, both plain or PEGylated (Table 1), were incubated under both conventional static and dynamic in vitro conditions for 5 min or 1.5 h, and results were compared. While the dynamic conditions did not significantly affect the size and zeta potential of nanoparticle-protein complexes, significant differences were found by the proteomic experiments. The analytical workflow was basically unmodified for dynamic experiments and included purification and isolation of the hard corona by centrifugation. The total protein quantitative assay and the SDS-PAGE analysis indicated a large increase of protein binding under dynamic conditions and over time. Shotgun proteomics by in-solution tryptic digestion and nano high-performance liquid chromatography (nano-HPLC) coupled to high-resolution MS analysis was also performed for qualitative protein identification and label-free quantitation. The incubation time affected the protein corona composition both qualitatively and quantitatively, and the percentage of common protein identifications between the two tested times was particularly lower for the dynamic conditions. Differences induced by the dynamic flow can be significant from an applicative perspective, as specific classes known to affect nanoparticle circulation and targeting can vary depending on the incubation conditions. For instance, another proteomic comparative study on PEGylated liposomes indicated that the protein corona in dynamic in vitro conditions had larger amounts of apolipoproteins and acute-phase proteins than the protein corona formed under static conditions [38].

In vivo studies for the characterization of the protein corona

Most of the works on the protein corona characterization found in the current literature come from in vitro experiments, basically under static conditions, which, as it is now apparent, cannot accurately represent the complexity of a living organism. If discrepancies are found between static and dynamic

protein corona characterizations, even larger differences emerge from the few studies in which the protein corona was obtained in vivo. One of the main obstacles in studying the protein corona from in vivo models that is provided by the difficulty in separating the protein corona complexes with the nanoparticles after systemic administration and by the difficulty in recovering them is sufficient amount for analysis [10]. Up to now, only few papers investigated the protein corona in vivo, exclusively in rodents as animal models (for a recent overview of such works, we refer the reader to a very recent review on the topic in [44]). From an experimental perspective, these works all share the same approach (Fig. 2). The nanoparticles are intravenously administrated in mice, then blood is withdrawn, usually after 10 min. Longer administration times have also been tested and indicated a significant effect on the corona composition, with fluctuations [33], and an increase of lower molecular weight proteins over time, in particular proteins involved in coagulation [32] (Table 1). Plasma is then prepared by centrifugation and recovered. At this point, the protein corona-nanoparticle complexes need to be purified and separated from unbound plasma proteins (Fig. 2). While inorganic magnetic nanoparticles can be recovered by magnetic decantation, for all other nanoparticle types, SEC is the technique of choice [44], with only few exceptions where traditional centrifugation is used [23, 32]. In the case of liposomes, the protocol developed for doxorubicin encapsulation is used, and the protein complexes are concentrated and purified from large unbound proteins by ultrafiltration on molecular weight cutoff (MWCO) membranes (10,000 Da in the first step, 1,000,000 Da in the second step). In most works, the subsequent protocol for protein analysis is the same to that used for in vitro experiments, in which the recovered complexes are washed to obtain the hard corona and then proteins were analyzed by shotgun proteomics either after SDS-PAGE with in-gel tryptic digestion [26] or after in-solution tryptic digestion [35].

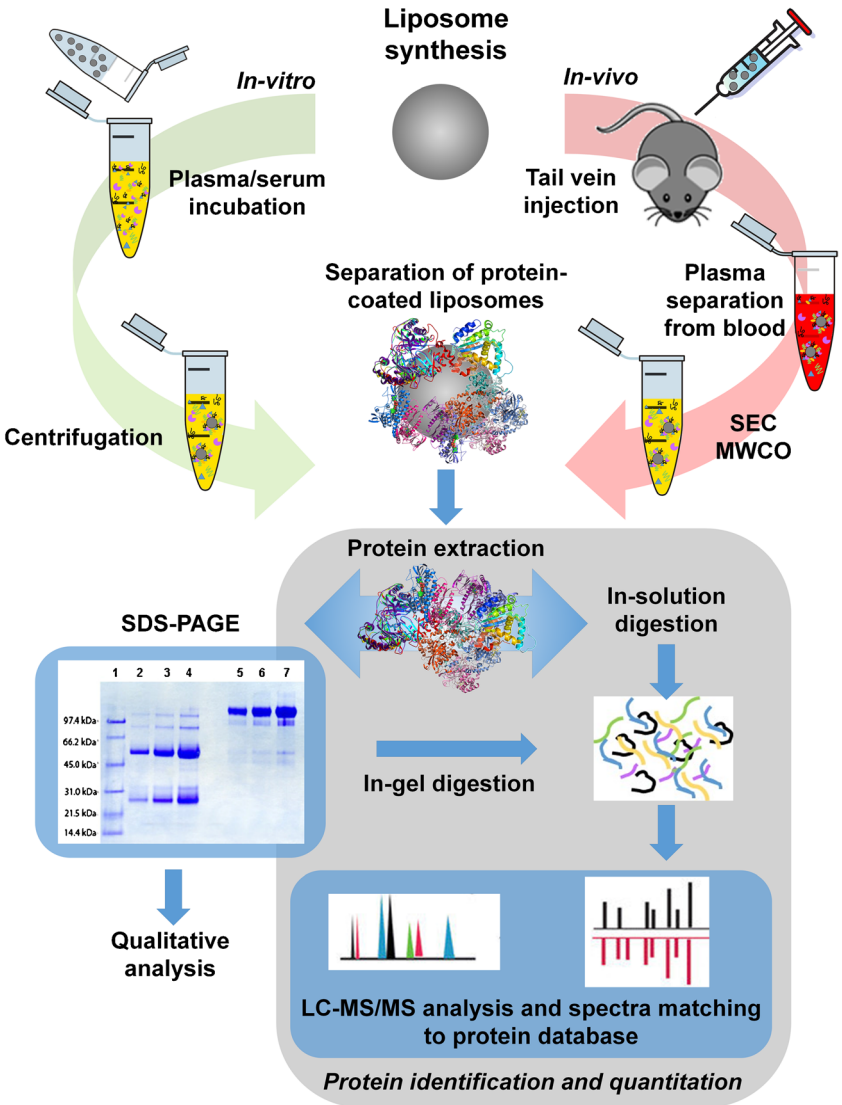

Fig. 2 Typical analytical workflow for proteomic analysis of the protein corona in in vitro (green, left side) and in vivo (red, right side) experiments

Regardless of the specific proteomic workflow, results indicated a poor correlation between in vitro and in vivo protein corona characterizations and the in vivo ones [43, 44]. For example, under in vivo and static in vitro conditions, significant differences were found not only in drug release by temperature-sensitive liposomes [26] but also in the qualitative composition of their protein coronas [35, 41], suggesting that a more complex protein corona is formed under in vivo conditions.

Enrichment of disease biomarkers: the protein corona in personalized medicine

As previously stated, the protein corona composition is strictly dependent on the protein source, which, in turn, depends also on the different health conditions (i.e., health vs. disease and type of disease). In fact, the composition of plasma evolves during the disease and disease progress. In addition, other factors have been suggested to affect the plasma composition, which are strictly dependent on the specific person, and include age, gender, and ethnicity [43]. Of course, such dependence on specific individual conditions affects the protein corona composition and, consequently, the nano-bio interactions including efficacy, toxicity, and eventual biological fate. However, the protein corona formation can be conveniently exploited also to investigate disease-specific circulating biomarkers, for instance in case of cancer, as the case of the secretome. In this context, the protein corona gains a new significance, as it becomes an analytical enrichment system for biomarkers and, at the same, a means for the development of tailor-made delivery systems, which indeed falls in the scope of personalized medicine. Differences in the protein corona have been investigated by the traditional in vitro approach under static conditions using size and zeta potential analysis along with SDS-PAGE and principal component analysis. In this case, though particularly informative, shotgun proteomics with HPLC tandem mass spectrometry (MS/MS) would not be affordable, as it is extremely expensive and time consuming [45].

From the protein corona to the biomolecular corona

As previously explained, the protein corona has evolved from the in vitro studies to in vivo much recent studies. Nevertheless, this is not the only evolution. The characterization of biomolecules different from proteins is also emerging in the field and would benefit from the development of the most recent untargeted strategies of metabolomics and lipidomics. In fact, different types of biomolecules, such as lipids, sugars, nucleic acids, hormones, and metabolites in general, are attracting the attention of the scientific community in the study of a more general biomolecular corona adsorbed on nanoparticles (Fig. 3) [46].

For instance, lipids are important bioactive molecules, which could impact nanoparticle circulation and uptake by cells, and included macrophages and endothelial cells, which are responsible for nanoparticle removal from the circulation, by interaction with scavenger receptors [53]. On a general discussion, also extending consideration to nanoparticles different from liposomes, the investigation of the biomolecular corona rather than the protein corona alone is still limited in the scientific community; nevertheless, few reports further support the potential key role of different biomolecules (Table 2). For instance, in a pioneer work on lipid characterization in the biomolecular corona, it was demonstrated that copolymer nanoparticles bind cholesterol, triglycerides, and phospholipids from the human plasma. The study was performed using SEC for sample fractionation while identification was achieved by nuclear magnetic resonance (NMR) and total lipid enzymatic determination by commercial kits. The lipid and protein binding patterns corresponded closely with the composition of high-density lipoproteins [47]. In a recent study on polystyrene nanoparticles, results from the thermodynamic aspects of the interactions with lipoproteins indicated the adsorption of not only apolipoproteins but also lipids. Significant amounts of cholesterol were detected in the biomolecular corona on the surface of the nanoparticles even after washing and centrifugation, i.e., the protocol for isolation of the hard corona. The results were obtained after incubation with human plasma, and cholesterol was quantified by a colorimetric commercial assay and investigated by isothermal titration calorimetry and transmission electron microscopy (TEM) imaging. The proposed dynamics involved an initial disintegration of the lipoproteins and coverage of the nanoparticle surfaces until saturation, with additional adsorption of intact lipoproteins in some cases [48]. In this regard, the investigation of the biomolecular corona would benefit from the application of the most recent metabolomic technologies and improvement of the available quantitative analysis in comprehensive molecular characterizations.

Lipidomic characterization has recently emerged as a powerful tool in lipid investigation of nanoparticle interaction with native porcine surfactant as a model. Polymeric nanoparticles with different surface chemistries were incubated with native porcine under conventional in vitro conditions, with washing to isolate the hard corona. Then, phospholipids were extracted by liquid-liquid extraction (hexane/ammonium formate 1%, 50:40:10 (*v*/*v*/*v*), four times and once with chloroform/methanol, 2:1. (*v*/*v*); Table 2). The lipid extract was separated by normal-phase HPLC and analyzed by triple-quadrupole MS. From the comparison to the protein corona qualitative and quantitative characterization, the lipid profile appeared less discriminant, as results showed a conserved lipid composition in the coronas of all investigated nanoparticles regardless of

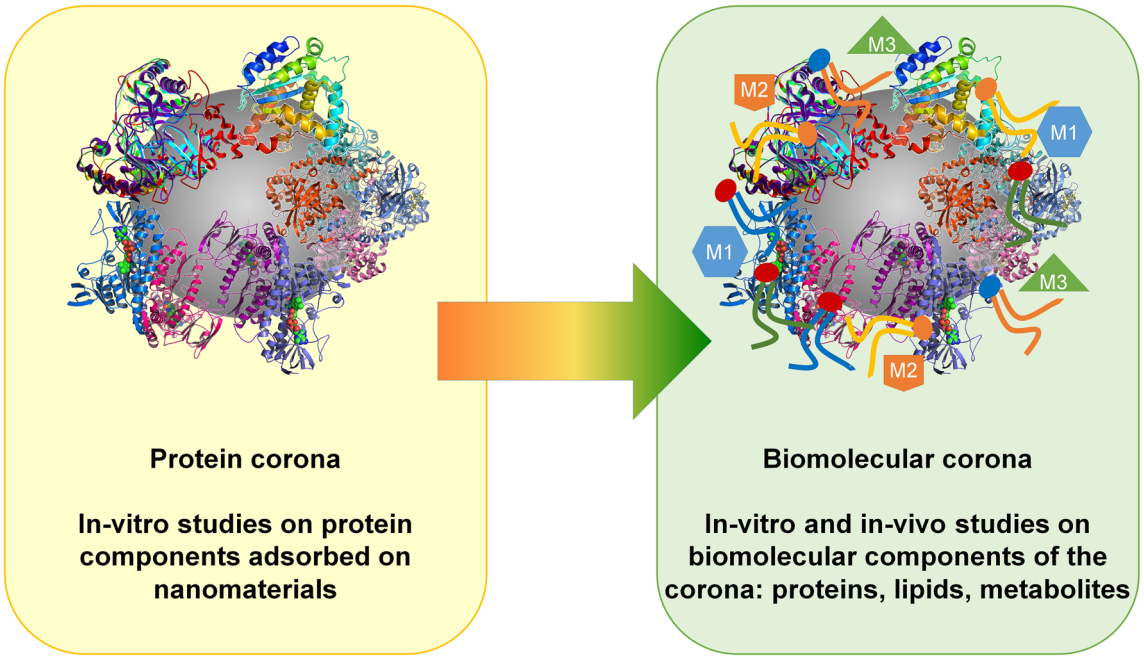

Fig. 3 Graphical representation of the changes in the concept of the corona in the nano-bio interface: moving from a protein corona to a more complex biomolecular corona, made up not only of proteins but also of other potentially significant molecules, in particular lipids and metabolites

their surface properties, whereas marked differences were reported for the protein corona [49].

A similar protocol was recently optimized for characterization of the lipid components in the biomolecular corona. The method was developed on cellulose nanofibrils and then extended to polystyrene and TiO_2 nanoparticles. Model systems of liposomes incubated with cellulose nanofibrils were used to optimize the extraction procedure, by comparison of six versions of the Fölch method. Samples were finally analyzed by reversed phase (RP) capillary HPLC coupled to MS/MS. The best protocol for lipid extraction, chosen based on recovery experiments with standard lipids, was a simple two-step liquid-liquid extraction with chloroform and evaporation under reduced pressure (Table 2). The method was finally applied to the characterization of the lipid components in the biomolecular corona also in serum under conventional in vitro conditions [50].

Apart from the investigation of lipids, a more general metabolomic approach was also used to screen for additional small organic molecules. There are only few studies in this field mainly on inorganic nanoparticles. For instance, carbonate apatite nanoparticles were used to investigate the interaction with metabolites after incubation with cell culture medium containing body fluids. An untargeted metabolomic approach, based on RP ultrahigh-performance liquid chromatography and quadruple time-of-flight MS/MS, was used to identify the organic compounds that bind to mineral nanoparticles formed in human body fluids (serum, plasma, saliva, and urine; Table 2). The results indicated that several organic molecules are part of the bimolecular corona, including fatty acids, glycerophospholipids, amino acids, sugars, and amides [51]. Similar experimental conditions were also used for the characterization of the organic biomolecular corona on different inorganic nanoparticles. The particles were suspended in two different biological fluids (cell culture medium and simulated lung fluid) for 24 h, and the complexes were recovered by the usual centrifugation and washing protocols. In this case, analytes were derivatized for gas chromatographic analysis coupled to MS and identified using an untargeted metabolomic approach (Table 2). More than 200 small organic compounds were detected and constituted a unique "organic fingerprint" which allowed distinguishing between the utilized nanoparticles, their surface chemistry, and the biological fluids based on analyte chemical classes and abundance [52].

Though lacking a comprehensive metabolomic investigation, metabolites were recently demonstrated to affect the protein corona composition in a function of a specific disease condition. In particular, in a recent study, the effect of glucose and cholesterol was considered, as they are model metabolites for diabetes and hypercholesterolemia. Molecular dynamic simulations were employed to probe their influence on the interaction between fibrinogen and polystyrene nanoparticles. Substantial changes on the binding site were thus identified, which, in turn, indicate that metabolites can substantially determine the immune triggering potency of nanoparticles, which is thus disease dependent and fundamental for yielding safe clinical applications. The results are particularly relevant as the biomolecular corona can play an underestimated important role in personalized medicine [54].

Table 2 Biomolecular corona investigation ordered according to the type of nanoparticles, the type of study, experimental conditions, and characterization approach

Nanoparticle type	Study type	Biological medium	Sample preparation	Separation	Detection	Biomolecular corona identification	Ref
N-Isopropylacrylamide/*N*-*t*-butylacrylamide copolymer nanoparticles	Lipid classes composition	Plasma	Incubation (1 h at 0 °C, 30 min at 23 °C); centrifugation; 3 washings	SEC (total lipid only); extraction with $CHCl_3$ (NMR only)	NMR	Total triglycerides, phospholipids, and cholesterol quantitation by commercial assays; NMR	[47]
Polystyrene nanoparticles	Interaction of lipoproteins	Lipoproteins isolated from the human plasma	Incubation (1 h at 37 °C); centrifugation; 3 washings	–	Isothermal titration calorimetry; TEM	Cholesterol quantitation by commercial assay	[48]
Magnetic nanoparticles, 3 functionalization (poly(D,L-lactide-*co*-glycolic acid), phosphatidylcholine, and PEG_{5000})	Lipidomic analysis (phospholipids)	Native porcine lung surfactant	Incubation (1 h at 37 °C); magnetic separation; 3 washings	Liquid-liquid extraction: isopropanol/hexane/-ammonium formate 1% (50:40:10 *v*/*v*/*v*, 4 times); chloroform/methanol (2:1 *v*/*v*, once) Normal-phase chromatography (diol-HILIC column, 150 × 2.1 mm)	Triple-quadrupole MS (selected ion monitoring)	Cholesterol quantitation by commercial assay; phospholipid quantitation against an external standard curve	[49]
Cellulose nanofibrils, polystyrene, TiO_2 nanoparticles	Lipidomic analysis	Lipid vesicles; serum	Incubation (30 min); centrifugation; 2 washings	Liquid-liquid extraction: chloroform (2 times); RP chromatography (C18, 75 μm × 60 mm)	MS/MS (data-dependent mode)	Yes	[50]
Carbonate apatite, amine-modified polystyrene nanoparticles	Untargeted metabolomics	Serum, plasma, saliva, urine	Incubation (overnight); centrifugation; 2 washings	Acid dissolution of nanoparticles; evaporation; RP chromatography (C18)	Quadrupole time-of-flight MS/MS	Yes (confirmation by chemical standards or database)	[51]
CuO, TiO_2, ZnO, ZrO_2, and carbon black nanoparticles	Untargeted metabolomics	Cell culture medium, simulated lung fluid	Incubation (24 h); centrifugation; 3 washings; derivatization	Gas chromatography (5% phenyl–95% dimethylpolysiloxane, 0.25-μm film, 30 m × 0.25 mm)	Quadrupole MS	Yes (database)	[52]

HILIC hydrophilic interaction liquid chromatography, *NMR* nuclear magnetic resonance, *RP* reversed phase

Outlook

The need for further knowledge on the protein corona is mainly connected with the need of more realistic conditions for formation of protein corona complexes, which resemble the entrance way into the body, and distribution from dynamic body fluids to individual organs and microenvironments, as recently indicated in several review articles [15].

Nevertheless, improvements in analytical workflows would also be valuable and desirable in this field, to apply up-to-date analytical strategies to an important topic, as the bio-nano interface. From this point of view, little evolution can be observed in recent papers with respect to previous reports, as discussed in the previous chapters. Traditional bottom-up shotgun proteomics is the main technique for individual protein corona characterization. While improvements have been done in proteomic technologies, the analytical strategies for the characterization of the protein corona have completely lagged behind.

Advancement in the protein corona characterization

Top-down proteomics has been completely overlooked up to now, but it is mature enough for application for sample complexities of a typical protein corona sample and beyond. Top-down proteomics has been applied to complex samples, such as cell lysates, for high-throughput characterization of intact proteins [55] of hundreds of proteins and proteoforms. Intact protein analysis would provide more insight into protein complexity, allowing the investigation of proteoforms, i.e., the different forms of proteins produced from the genome with a variety of sequence variations, splice isoforms, and myriad post-translational modifications, which are currently indicated as the next step in proteomic analysis [56]. By top-down proteomics, not only protein complexity would be better elucidated, but also improvements in quantitative analysis would most likely be provided. Protein quantitation by bottom-up approaches, in either labeled or label-free strategies, is based on estimating the total amount of a given protein using only a limited subset of derived peptides. On the contrary, top-down approaches allow quantitation of the entire protein, as well as the individual proteoforms, which would give additional insight and information on the protein corona composition. This would be particularly relevant, as proteoform-level abundance information can have important biological consequences, because different proteoforms often vary dramatically with respect to function [57]. A larger coverage can be obtained by using fractionation strategies on intact proteins, such as SEC, for better characterization of low and high molecular weight proteins [58], or HILIC, the latter particularly suitable for post-translational modifications analysis, such as glycosylation, acetylation, and methylation. In this regard, the analysis of protein post-translational modifications in the protein corona characterization has been nearly completely ignored but would potentially provide additional knowledge on the interaction with cells. For instance, protein glycosylation was investigated for silica nanoparticles and indicated a key role of glycans in the interaction with cells, as the removal of glycans from the protein corona enhanced cell uptake, resulting in the generation of a pro-inflammatory milieu by macrophages [59]. The coupling with electrophoretic approaches would also represent a valuable strategy to improve the proteome coverage by top-down proteomics: for instance, capillary zone electrophoresis was recently exploited for deep top-down proteomics of *Escherichia coli* in an orthogonal multidimensional separation platform that coupled SEC and RP HPLC-based protein prefractionation to capillary zone electrophoresis coupled to MS/MS. Fractionation is particularly interesting, as it allows the use of common high-resolution MS instrumentation based on hybrid Orbitrap without a need of the most high-performance Tribrid Orbitrap or Fourier transform ion cyclotron resonance mass spectrometers [60]. Also, traditional gel electrophoresis can be a key part of top-down proteomics. The use of either one-dimensional or two-dimensional gel electrophoresis can provide essential information, from protein identity to sample heterogeneity, ligand binding, substrate turnover, structural topology, and dynamics of assembly [61].

Advancement in the biomolecular corona characterization

The analytical stagnation in the study of the bio-nano interface does not only apply to the protein corona. The possible pivotal role of other organic molecules has been suggested by too few reports. Again, also in this field, powerful analytical techniques are maturing for the comprehensive characterization of small organic molecules (metabolomics) or lipids (lipidomics). In the case of metabolomics, difficulties persist and need to be tackled, which include the confident identification of metabolites, improvement of a dynamic range of metabolite identification (which is currently restricted to high or medium abundant compounds), improvement of the quantitative analysis in untargeted studies, and finally, the development of miniaturized analytical workflows suitable for investigation of minute sample amounts [62], as the ones of biomolecular corona. A similar situation is found for lipidomics [63], but lipid identification in this case is facilitated by bioinformatics and chemical class less heterogeneity [64]. In this regard, significant information may be gathered, for instance, in the study of oxidized lipids, which could better elucidate issues connected with nanoparticle toxicity.

Thus, although the importance of proteins and, in small part, of biomolecules in the nanoparticle corona has been long demonstrated, further efforts are needed to increase knowledge in this regard.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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