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PII: S0039-9140(19)31120-8

DOI: <https://doi.org/10.1016/j.talanta.2019.120487>

Reference: TAL 120487

To appear in: *Talanta*

Received Date: 24 July 2019

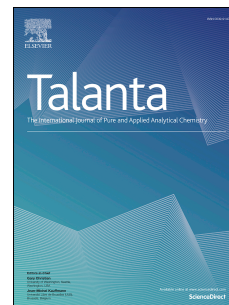
Revised Date: 16 October 2019

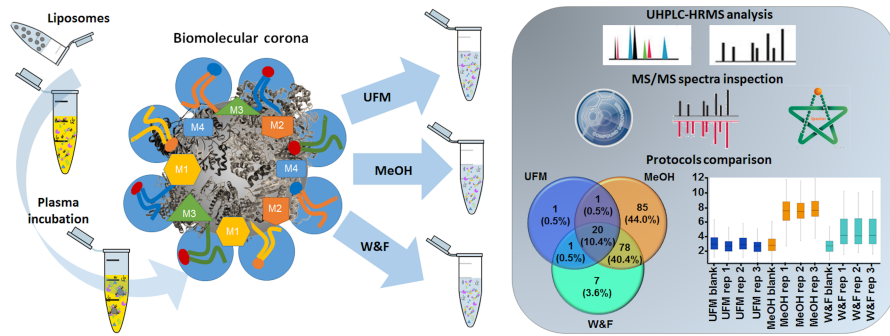
Accepted Date: 21 October 2019

Please cite this article as: G. La Barbera, A.L. Capriotti, G. Caracciolo, C. Cavaliere, A. Cerrato, C.M. Montone, S. Piovesana, D. Pozzi, E. Quagliarini, A. Laganà, A comprehensive analysis of liposomal biomolecular corona upon human plasma incubation: The evolution towards the lipid corona, *Talanta* (2019), doi: <https://doi.org/10.1016/j.talanta.2019.120487>.

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A comprehensive analysis of liposomal biomolecular corona upon human plasma incubation: the evolution towards the lipid corona

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Abbreviations

BMC biomolecular corona; DDA data dependent acquisition mode; DOTAP 1,2-dioleoyl-3-trimethylammonium-propane; FWHM full width at half-maximum; GC-MS gas chromatography coupled to mass spectrometry; HRMS high-resolution mass spectrometry; LC-MS liquid chromatography coupled to mass spectrometry; MeOH methanol; NCE normalized collision energy; NMR nuclear magnetic resonance; NPs nanoparticles; UFM ultrafiltration membrane; UHPLC ultra-high performance liquid chromatography; W&F Wessel & Flügge.

Abstract

When drug nanocarriers enter a physiological environment, their surface gets coated by a dynamic biomolecular corona (BMC) mainly constituted by proteins. Although a deep investigation has been performed on the composition of BMC in terms of proteins, scarce attention has been posed to low molecular weight metabolites present in human plasma. In this work, for the first time, the investigation of the BMC of liposomal nanoparticles (NPs) constituted by 1,2-dioleoyl-3-trimethylammonium-propane polar lipid has been carried out by an ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry based untargeted metabolomics approach. Compounds were tentatively identified based on matches with online databases and comparison of MS/MS spectra with available spectral libraries. Moreover, a comparison of three metabolite extraction strategies, including an ultrafiltration membrane based protocol, a methanol extraction based protocol, and Wessel & Flügge protocol, was performed. Methanol extraction procedure resulted in the widest metabolic coverage of liposomal NP BMC. A total of 193 metabolites has been tentatively identified, 166 of which belonged to the class of lipids including phospholipids, steroids, carnitines, fatty alcohols, diglycerides and fatty acids. The high abundance of lipids in the BMC can be explained by the adsorption of plasma lipoproteins onto liposome surface, confirming previous works on other kinds of NPs. Lipids are important bioactive molecules, which could impact NP circulation and uptake by cells. Extending the investigation of BMC beyond the protein corona and towards the “lipid corona” may be the keystone of a better understanding and control of NP fate in human body.

Keywords: liposome; corona; metabolomics; Lipidomics; high resolution mass spectrometry; lipoproteins

Introduction

In the recent years, a wide variety of drug nanocarriers have been synthesized and developed to address specific target cells, such as immune cells and cancer cells. However, when nanocarriers enter a physiological environment, their surface gets coated by a dynamic biomolecular corona (BMC) that modifies their physical-chemical properties. Nanoparticles (NPs) in contact with plasma or serum will immediately be covered by proteins forming the so-called protein corona, which provides them with a new identity referred to as “biological identity”. [1,2] The analytical characterization of protein corona has provided critical information about NP destiny in and impacts on organisms. [3–6] However, although a deep investigation has been performed on the composition of the BMC in terms of proteins, [2,7] scarce attention has been posed to many other biomolecules, such as metabolites and lipids, that may also be contained in the corona and that may play important biological roles, different from those of proteins. The wide range of small molecules present in the BMC may act as a substrate for enzymes altering the metabolism and possibly playing a role in cellular toxicity. Complete characterization of the NP BMC may be the key for investigating and predicting NP risk and destiny.

In the pioneering work of Hellstrand *et al.* [8] size exclusion chromatography, NMR, and enzymatic assays, showed that copolymer NPs bind cholesterol, triglycerides and phospholipids from human plasma. Due to these results, several following studies focused on the investigation of the lipid content of NP BMC. Incubation of different kinds of NPs such as copper oxide, titanium dioxide, zinc oxide, zirconium dioxide, carbon black NPs, [9] carbonate apatite NPs, [10]

amine-modified polystyrene NPs,[10] TiO₂ (anatase) NPs,[11] cellulose nanofibrils,[12] polystyrene NPs,[13] magnetic NPs,[14] and single-walled carbon nanotubes,[15] with different biological fluids was investigated. In these studies, lipid identification and quantitation by means of total glycerol assays,[11] cholesterol assays,[13,14] and liquid chromatography coupled to mass spectrometry (LC-MS)[12,14,15] was performed. Moreover, the investigation of BMC in terms of other low-molecular-weight metabolites was also carried out by LC-MS[10] and gas chromatography coupled to mass spectrometry (GC-MS).[9] Although most of these studies showed promising results, few of them exploited the most recent metabolomics and lipidomics untargeted approaches for the elucidation of the detailed composition of the BMC.[9,10,12,14,15] Furthermore, a deep investigation on the structure and identity of the detected compounds was rarely reported. Untargeted profiling methods allow to obtain the widest possible metabolic coverage, especially when performed by high-resolution mass spectrometry (HRMS) coupled to ultra-high performance (UHP)LC.[16–18] However, in order to achieve a reliable characterization of the whole BMC composition, the untargeted metabolomics and lipidomics approach requires a proper method development in terms of both chromatographic separation, mass-spectrometric detection, and in sample preparation.

In this work, we present a UHPLC-HRMS based untargeted metabolomics approach to analyze the low molecular weight compounds present in the BMC of liposomes constituted by 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) polar lipid. In particular, after the optimization of a proper chromatographic and mass spectrometric method, a comparison of three different metabolite and lipid extraction strategies, including an ultrafiltration membrane based protocol (UFM), a methanol extraction based protocol (MeOH) and Wessel & Flüge protocol (W&F),[19] was performed. Liposomes are considered to be among the most suitable NPs for

drug delivery [20] and several studies aimed at characterizing their synthetic and biological identity after in vitro and in vivo experiments, [21–24] mainly studying their protein corona. [25] To the best of our knowledge, this is the first investigation of liposomal NP BMC providing a detailed characterization of metabolites and lipids.

Materials and methods

Chemicals and materials

For LC-MS analysis: Optima LC-MS grade water was supplied by Thermo Fisher Scientific (Waltham, Massachusetts, USA); Ultra purity LC methanol was purchased from Romil Pure Chemistry (Pozzuoli, NA); formic acid was acquired from Sigma now Merck (St. Louis, MO, U.S.A.). For sample preparation: all chemicals, reagents, and organic solvents of the highest grade available were purchased from Sigma-Aldrich (St. Louis, MO, USA); Deionized water was prepared by an Arium 611 VF system from Sartorius (Göttingen, Germany); SPE C18 cartridges were Bond Elut (Varian, Palo Alto, CA, USA); DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL, USA), and used without further purification; Protein LoBind tubes were obtained from Eppendorf (Hamburg, Germany).

Liposome preparation

DOTAP liposomes were prepared according to standard protocols.[26] Briefly, 5 mg of DOTAP were dissolved in 100 μL of chloroform and then evaporated under vacuum for at least 24 h. Then the lipid films obtained were hydrated with 5 mL of the dissolving buffer, consisting in 10 mmol L^{-1} Tris-HCl (pH 7.4), 150 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA, to achieve the desired final lipid concentration (1 mg mL^{-1}). Unilamellar liposomes, with a hydrodynamic diameter of

78.8 ± 2.2 nm and with a zeta potential of 55.7 ± 1.8 mV, were produced by sonication to clarity. Unilamellar liposome hydrodynamic diameter and zeta potential were measured at 25 °C by a Zetasizer Nano ZS90 (Malvern, U.K.).

Liposome incubation with human plasma

The incubation procedure was conducted as previously described,[27] with some modifications. Five hundred microliters of liposome suspension (1 mg mL^{-1}) were added to 1 mL of human plasma (Sigma-Aldrich, catalog number P9523) in the dissolving buffer and incubated at 37 °C for 1 h to promote aggregation of the plasma biomolecules onto the surface of NPs. Afterwards, each vial was centrifuged for 10 min under $14,000\times g$ to precipitate the liposomes, and then the supernatant containing the free biomolecules was removed. The pellet was washed three times with 200 μL of washing buffer consisting of 10 mmol L^{-1} Tris-HCl (pH 7.4), 150 mmol L^{-1} NaCl, 1 mmol L^{-1} EDTA in order to remove the unbound biomolecules. The obtained pellets were air-dried at room temperature for 10 min. Liposome-BMC complexes were obtained with a hydrodynamic diameter of 156.5 ± 4.5 nm and with a zeta potential of -1.5 ± 0.5 mV.

Optimization of metabolite and lipid extraction method

Three extraction protocols were tested to identify the optimal extraction conditions for metabolite and lipid recovery from the NPs after incubation with human plasma. In protocol A (UFM) pellet was solubilized with 200 μL of 1% (w/v) sodium deoxycholate in 50 mmol L^{-1} Tris-HCl (pH 8.8). The resultant suspension was fractionated through an ultrafiltration membrane (UFM) having a 3,000 Da molecular weight cut-off. Membrane was initially rinsed twice with 500 μL of a buffer consisting of 1% (w/v) sodium deoxycholate in 50 mmol L^{-1} Tris-

HCl (pH 8.8) and 10% (v/v) MeOH, and once with 500 mL of deionized H₂O, centrifuging at 5,000xg for 30 min. The sample was diluted with a solution 90:10 H₂O:MeOH, transferred in Vivaspin® Sartorius device and spun at 5,000xg for 90 min at 20 °C. The filtered sample was reduced to 50 µL in the rotatory evaporator at 37 °C and then reconstituted with 150 µL H₂O:HCOOH 99.9:0.1 (v/v). In protocol B (MeOH) pellet was solubilized in 200 µL of MeOH and placed in an ultrasonic bath for 1 h at 37 °C, intermittently vortexing (5 min) every 15 min. The samples were centrifuged at 5,000xg for 5 min. The entire cycle was repeated three times. The three collected supernatant were collected and reduced firstly in a vacuum centrifuge, lately in the rotatory evaporator at 37 °C to a final volume of 50 µL and then reconstituted with 150 µL H₂O:HCOOH 99.9:0.1 (v/v). In protocol C (W&F), the pellet was solubilized with 200 µL of 1% (w/v) sodium deoxycholate in 50 mmol L⁻¹ Tris-HCl (pH 8.8) and placed in an ultrasonic bath for 30 min in order to promote pellet solubilisation. The solution was precipitated following the Wessel and Flügge protocol[19]. Briefly, 800 µL MeOH were added and the solution was vortexed for 1 min before the addition of 200 µL CHCl₃ and 600 µL water. After vortexing for one further minute, the sample was centrifuged for 5 min at 5000×g, obtaining a solution divided into two layers. The aqueous layer, containing metabolites and polar lipids, was collected. The organic layer was discarded. The protein pellet was washed twice with a solution MeOH:H₂O 80:20 in order to promote the desorption of small molecules adsorbed onto precipitated proteins. The washing solution was added to the aqueous layer, reduced to 50 µL in the rotatory evaporator at 37 °C and then reconstituted with 150 µL H₂O:HCOOH 99.9:0.1 (v/v).

All extraction protocols were performed in triplicate to ensure statistical significance of data. Moreover, for each protocol, one blank consisting in performing the experiment with solvents

only (solvent blank) and one blank consisting in performing the experiment with liposomes but without plasma incubation (liposome blank), were performed.

UHPLC-HRMS analysis of samples

All samples were analyzed by a Vanquish UHPLC system (Thermo Fisher Scientific) equipped with a binary pump H and an autosampler thermostated at 14 °C, coupled to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific) via a heated electrospray ionization (HESI) source. The separation was performed on one column Luna Omega Polar C18 (100 Å pore size) (Phenomenex, Torrance, CA, US) 100 mm × 2.1 mm i.d. with 1.6 µm particles size operated at 0.4 mL min⁻¹ and 40 °C (still air option). The samples were analyzed with H₂O:HCOOH 99.9:0.1 (v/v) as phase A and MeOH:HCOOH 99.9:0.1 (v/v) as phase B, using the following phase B linear gradient: 0 min (0.5% B), 22 min (98% B), followed by 5 min at 98% phase B and 3 min equilibration at 0.5% phase B. The total analysis time including column re-equilibration was 30 min. The ionization source tune parameters were the following: capillary temperature 275 °C in positive mode, 350 °C in negative mode; sheath gas 50 arbitrary units (a.u.) in positive mode, 20 a.u. in negative mode; auxiliary gas 15 a.u. in positive, 10 a.u. in negative mode; sweep gas in positive mode 3 a.u., in negative mode 0 a.u.; spray voltage 3.5 kV in positive mode, 2.8 kV in negative mode; auxiliary gas heater temperature 450 °C in positive mode, 350 °C in negative mode; S-lens RF level 55%. The Q Exactive mass spectrometer was operated in TOP 5 data dependent acquisition mode (DDA). For full-scan spectra acquisition, the resolving power was set at 70,000 (full width at half-maximum, FWHM), scan range 100–1000 *m/z*, automatic gain control target at 1×10⁶, maximum ion injection time at 200 ms, and isolation window width of 1.4 *m/z*. MS/MS fragmentation was performed by higher-energy collisional

dissociation at a stepped normalized collision energy (NCE) of 20, 40, 60 with a resolution FWHM of 17 500, automatic gain control target of 1×10^5 , dynamic exclusion of 3 s. The mass spectrometer was externally calibrated within a mass accuracy of 1 ppm every 2 days using the commercial Pierce positive and negative calibration solutions (Thermo Fisher Scientific). Raw MS/MS data files were acquired by Xcalibur software (version 3.1, Thermo Fisher Scientific). The three solvent blanks related to UFM, MeOH and W&F protocols were acquired in both positive and negative polarity mode in order to build an exclusion list. Later, the solvent blank, the liposome blank and the three experimental replicates of each protocol were acquired in both positive and negative polarity mode, after setting the corresponding exclusion list. Therefore, 10 chromatographic runs were performed for each sample and samples were run continuously over the time to minimize instrumental variability. The injection volume was 10 μ L.

Data analysis

All datasets from UHPLC-HRMS analysis were processed with Compound Discoverer 2.1 SP1 software (Thermo Scientific, San Jose, CA, USA). The solvent blank, the liposome blank and the three experimental replicates for each protocol were grouped, resulting in a total of three groups to be processed. The chosen untargeted metabolomics processing workflow included the following nodes: 1) select spectra node (filtering of the MS scan data from the raw files); 2) align retention time node (alignment of the raw data files belonging to the different samples to build a unique sample set); 3) detect unknown compound node (detection of the extracted ion chromatogram traces in MS scan for finding and grouping precursors, isotopes and adducts related to a compound); 4) group unknown compounds node (grouping chromatographic peaks belonging to the same feature and choosing the best MS and MS/MS spectra for the following

identification); 5) fill gaps node (filling missing peaks for subsequent statistical analysis); 6) mark background compounds node (subtracting the compounds in the blank samples); 7) predict composition node (associating a molecular formula to the detected features); 8) search Chemspider node (matching the detected compounds with the Chemspider database); 9) search mzCloud node (matching the MS/MS spectra of the detected compounds with the mzCloud library). The parameters that have been set for each node are reported in Table S1 in the Supporting Information. The selected workflow has produced a compound table listing all of the compounds detected across all the input files grouped by their molecular weight and retention time. Only compounds detected in all the three experimental replicates for each protocol were selected. For each compound the predicted chemical formula, the molecular weight, the ChemSpider results, the mzCloud results, the mzCloud best match and best similarity match, the area for each sample group calculated as medium, the coefficient of variance for each sample group, the group area ratio, the *P* value of group ratio calculated by *t* test and the Log₂ area fold change, were reported. Compounds showing at least one mzCloud match or mzCloud similarity match were selected for a deeper investigation: product ion data were manually checked to elaborate hypothetical fragmentation patterns and compare it with data in literature and in the Reference Library of mzCloud-Advanced mass spectral database (HighChem LLC, Slovakia). After a previous screening by Compound Discoverer, it was observed that a high number of tentatively identified compounds belonged to the class of polar lipids. Therefore, a software dedicated to lipid identification was used for further data analysis and structure elucidation.

All datasets from UHPLC-HRMS analysis were processed with Lipostar,[28] a commercially available software for lipidomics analysis. First, the platform Lipostar DB Manager was used to build a customized homemade database of lipid precursors and MS/MS theoretical ions. By

means of the Lipid Builder Tool several lipid polar heads were combined. In particular ceramide (Cer), monoglyceride (MG), diglyceride (DG), triglyceride (TG), glycerophosphate (PA), glycerophosphocholine (PC), glycerophosphoethanolamine (PE), glycerophosphoglycerol (PG), glycerophosphoinositol (PI), glycerophosphoserine (PS), sphingomyelin (SM) polar heads were chosen, representing the most common lipids in human plasma. All polar heads were combined with 1 or 2 acyl chains ranging from a length of C3 to C35 and from 0 to 9 double bonds. Typical fragmentation rules were also applied to generate the related MS/MS spectra. After building the customized database baseline and noise reduction, peak extraction, smoothing, signal-to-noise ratio filtering, deisotoping and deconvolution were performed as in the work of La Barbera *et al.*, [29] setting the parameters reported in Table S2 in the Supporting Information. Finally, database search of experimental MS and MS/MS spectra was performed with the following parameters for lipid identification: 5 ppm precursor ion mass tolerance and 10 ppm product ion mass tolerance. All lipid identifications based on both precursor and MS/MS were manually checked.

Results and discussion

Experimental set-up

The aim of this study was performing a metabolomics and lipidomics profiling of the BMC adsorbed onto liposomal NPs after incubation with human plasma. DOTAP liposome has been chosen as model system due to its well characterized size (pristine liposome 78.8 ± 2.2 nm, liposome after incubation with human plasma 156.5 ± 4.5 nm), charge (pristine liposome $55.7 \pm$

1.8 mV, liposome after incubation with human plasma -1.5 ± 0.5 mV) morphology and protein corona composition.[30] The protocol optimized in this work included a first part typical of a shotgun proteomics workflow in which liposomes were incubated with a biological fluid under physiological conditions (1 h at 37 °C).[31] A high excess of plasma, i.e. plasma:NPs ratio 2:1, was chosen for mimicking the physiological in vivo conditions.[32] Afterwards, the liposome-corona complexes were isolated by centrifugation. The supernatant was discarded while the pellet was treated for metabolite and lipid extraction. Particular attention had to be paid on the removal of proteins. Indeed, due to their high abundance in comparison with metabolites and lipids, proteins can be responsible for a high matrix effect and signal suppression. Based on these considerations, three protocols have been evaluated for protein removal and for metabolite and lipid extraction from liposomal BMC: i) UFM protocol consisting in filtering molecules with more than 3,000 Da molecular weight; ii) MeOH protocol consisting in a solid-liquid extraction of small metabolites; iii) W&F protocol[19]. The effect of the extraction on the pristine liposome was also taken into consideration. Indeed, during the extraction protocols, a hypothetical degradation of the liposomal NPs may occur giving rise to a series of lipid chain fragments, which may be easily confused with lipids endogenously present in plasma. In order to avoid misinterpretation of results, a liposome blank was performed in which liposome NPs, not incubated with human plasma, have been treated by the same extraction protocols as the liposome-BMC complexes. The signals detected in the liposome blank were subtracted by the samples during data analysis. On this regard, liposomal NPs composed of DOTAP lipid represented the best model for this preliminary study. Indeed, DOTAP is a synthetic lipid not naturally present in human plasma, which allowed to confirm that the subtraction of signals belonging to the liposome blank was effective and did not affect BMC analysis. Finally, a

solvent blank, consisting in performing the extraction protocols without neither liposomes nor human plasma, was performed in order to subtract also the background contaminant signals. All the samples were analyzed by a UHPLC-HRMS based untargeted metabolomics approach as explained in the following section.

Untargeted metabolomics workflow

Few research groups have used untargeted metabolomics or lipidomics approaches for the investigation of the BMC, [9,10,14,15] only one of them presenting a proper analytical method optimization.[12] In this study, before performing the analysis on the BMC, we optimized the chromatographic and mass spectrometric conditions on a sample of human plasma.

Human plasma was treated with MeOH in a 1:4 ratio for protein precipitation. The supernatant was dried under vacuum and resuspended with H₂O:HCOOH 99.9:0.1 (v/v). Several metabolites naturally present in human plasma and belonging to the most representative classes of metabolites were monitored under different chromatographic conditions. Indeed, due to their wide variety in terms of polarity and to the presence of several isomeric molecules, several column stationary phases and particle sizes were explored by using H₂O:HCOOH 99.9:0.1 (v/v) as phase A and MeOH:HCOOH 99.9:0.1 (v/v) as phase B, with the following phase B linear gradient: 0 min (1% B), 11 min (98% B), 16 min at (98% B), 20 min (1% B). Firstly, a fully porous Hypersilgold C18 column (2.1 x 15 mm, 3 µm particle size) was tested because often used in metabolomics studies. Hypersilgold C18 column is an endcapped, ultrapure, silica based column that allows to obtain a good peak symmetry reducing peak tailing.[33] Despite the good peak symmetry, the large chromatographic peak width of the detected metabolites (0.2 min) did not allow the separation of several isomers. Therefore, a column with a smaller particle size was

investigated, i.e. the core-shell Kinetex XB-C₁₈ (2.1 x 100 mm, 2.6 µm particle size). This column has some protective butyl side chains that allow better peak shape and enhance separation and retention of compounds compared to normal C₁₈ phases. [34] Despite a better isomer separation, this column showed not to be appropriate for the more polar metabolites, most of which were not sufficiently retained. To solve this issue, a core shell Accucore aQ C₁₈ column (2.1 x 100 mm, 2.6 µm particle size) was investigated, because its polar endcapped C₁₈ stationary phase should provide optimal retention of polar analytes.[33] However, despite the manufacturer's indications the retention of the most polar compounds was not showed to be enhanced. Finally, Luna Omega Polar C₁₈, a fully porous silica column (2.1 x 100 mm, 1.6 µm particle size) resulted in the best isomer separation, an enhanced retention of the most polar metabolites, and the narrowest peaks among the tested columns. In this column, while the C₁₈ chains provide hydrophobic interactions, a polar modified particle surface provides enhanced retention of the highly polar compounds, allowing a more balanced retention of polar and hydrophobic compounds.[34] Moreover, the small particle size allows to obtain very narrow chromatographic peaks, allowing a better isomer separation. A comparison of the extracted ion chromatograms of some of the most challenging metabolites detected in human plasma, obtained with the four columns, has been reported in Figure S1 in the Supporting information.

Once chosen the best column, several gradients starting from different percentages of phase B were tested, the gradient starting from 0.5% B giving the best result in terms of retention of the most polar compounds. Moreover, different percentages of HCOOH were tested in order to obtain the best separation of the monitored metabolites. Increasing the percentage of HCOOH up to 0.2% resulted in narrower peaks. However, because ion suppression was detected for some metabolites HCOOH 0.1% was kept as final condition. The final chosen conditions consisted in a

separation onto a column Luna Omega Polar C18 with H₂O:HCOOH 99.9:0.1 (v/v) as phase A and MeOH:HCOOH 99.9:0.1 (v/v) as phase B, using a gradient starting from 0.5% B for a total analysis time of 30 min.

After, the ionization parameters resulting in the best compromise for all the monitored metabolites in terms of signal intensity were chosen for the acquisition. Moreover, an acquisition mode was chosen based on the work of La Barbera *et al.*[17]. In this work, a data dependent acquisition (DDA) TOP 5 mode was developed, implying a first scan of the full mass spectra between 100 *m/z* and 1000 *m/z*, and a second scan in which the 5 most abundant ions (detected in the first scan) were selected and fragmented. The DDA method optimized by La Barbera *et al.*[17] allows the simultaneous acquisition of HRMS and MS/MS data for a high number of analytes, ensuring an efficient profiling and identification. Indeed, tentative metabolite annotation by the study of the *m/z* ratio only does not provide a single confident identification, requiring MS/MS experiments to ascertain structural information. However, not only the number of detected and fragmented metabolites has to be taken into account for a proper method development, but also the fragmentation parameters should be investigated. Firstly, a one step collision energy method was tested at either 20 or 40 or 60 or 80 NCE. However, due to the heterogeneity of metabolites in human plasma, choosing only one value of NCE did not allow to obtain an informative fragmentation spectrum for all the metabolites. A stepped collision energy method with 20, 40 and 60 NCE was chosen, allowing to obtain the needed diagnostic fragment ions for the whole range of monitored metabolites. Finally, also an exclusion list was included in the acquisition method. Indeed, low abundant metabolites may not be selected for fragmentation by the TOP 5 acquisition mode because of high abundant contaminants. In order to avoid this issue, after a previous injection of the solvent blank for each

protocol, the mass spectrometric method was modified excluding, from the fragmentation step, the ions deriving from the solvent blank. In addition, the ions detected in the solvent blank samples were used, in the following step of data analysis, for blank subtraction.

Metabolite and lipid identification

The optimization of the chromatographic and mass spectrometric method was followed by an accurate data analysis. Analysis of complex samples by modern separation methods in conjunction with HRMS can yield an enormous number of signals, often belonging to background interferences and contaminants, or to adducts and in-source fragments of real molecular entities. Thousands of signals need to be filtered and grouped into features characterized by a specific exact mass and retention time, thus being addressed to real metabolites. Finally, investigation and comparison of accurate masses, isotopic pattern, MS/MS spectra with databases and spectral libraries is required for the tentative identification of metabolites. Few studies on BMC showed the identification of metabolites and lipids by means of a proper HRMS based untargeted approach. In particular, in the two untargeted metabolomics works published by Pink *et al.* [9] and by Martel *et al.*, [10] over 200 and 350 metabolites, respectively, were tentatively identified based only on precursor ion match with databases. In the last work, 25 out of the 350 metabolites only were tentatively identified by MS/MS spectra comparison with spectral libraries and by reference standard comparison. In other three lipidomics works published by Raesch *et al.*, [14], Lee *et al.*, [12] and Kapralov *et al.* [15] a total of 277 lipids, 61 lipids and 108 lipids were detected, respectively. In the first case, lipids were monitored in Single Ion Monitoring (SIM) with a triple quadrupole, thus without providing any MS/MS match with spectral libraries. In the second case, fragmentation behavior of lipids was

investigated for their tentative identification but no details on fragment matches is reported. In the third case, both a triple quadrupole and a time-of-flight (TOF) mass spectrometer were used, without specifying which acquisition mode was used and without providing any detail on the MS/MS spectra. In contrast to the mentioned papers, in this work, two powerful and highly recommended software that perform identification by taking into consideration MS/MS fragmentation spectra were used, assuring a reliable tentative identification. Compound Discoverer determines compound elemental composition and searches multiple chemical databases for further annotation. After, it matches the experimental MS/MS data with mzCloud spectral library and in-house mzVault spectral libraries by sophisticated algorithms. Additionally, it can provide similarity search results by comparing MS/MS spectra of the whole set of compounds present in mzCloud regardless of whether the precursor ions of the reference compound and the unknown compounds match. Compound Discoverer was operated by setting up the parameters summarized in Table S1 in the Supporting Information. After the samples have been processed with Compound Discoverer, solvent and liposome blank were subtracted and metabolites were manually checked by inspecting the MS/MS fragmentation spectra. The results showed that the highest number of detected metabolites belonged to the class of lipids. Therefore, a deeper investigation for lipids only was performed with Lipostar (version 1.0.4), a lipidomics dedicated software.[28] Lipostar database can be customized by adding novel lipid structures not yet reported in the literature, containing both precursor ion masses, adducts, and product ion masses (generated by *in silico* fragmentation), allowing the possibility of operating a proper untargeted screening. Lipid identification confidence levels are provided through a stars-based classification system: four stars if both mandatory and recommended product ions are detected, together with at least one fatty acid chain; three stars if at least one mandatory product

ion is detected; two stars if at least one recommended product ion is detected; one star if neither mandatory nor recommended product ions are detected. After building a personalized database, samples were analyzed setting the parameters shown in Table S2 in the Supporting Information. Only lipids tentatively identified with 3 and 4 stars were considered and further manually inspected.

The whole set of metabolites and lipids tentatively identified by Compound Discoverer and Lipostar, respectively, was merged after normalizing the relative areas with a corrective factor due to the different peak integration algorithm available in the two software. Finally, based on the work of Schymanski *et al.*[35] and the slight modifications by La Barbera *et al.*,[16–18,29] different identification confidence levels were associated with the identified metabolites: level 1 for compounds identified by reference standard comparison; level 2 for compounds showing specific informative product ions; level 3 for compounds showing a fragmentation pattern typical of one class of molecules but with no detailed information. The molecular formula was reported for all level 2 identified compounds, whereas for some of the level 3 identified compounds any meaningful molecular formula was found although the fragmentation pattern gave reliable information on the chemical class of the metabolites. Concerning lipids, generally, the overall side-chain composition of each species (i.e., the total number of carbon atoms and double bonds) could be easily obtained from the detected m/z value allowing the assignment of a general identity to level 3 compounds. Only for level 2 compounds, the identity of the two fatty acid chains was specified. Several isomeric species have been detected and can be attributed both to the occupation of different positions on the glycerol backbone and to a different arrangement of the acyl chain carbon atoms. However, the position of the acyl chains on the glycerol backbone was not assessed. In Table S3 the metabolites and lipids identified in the three protocols are

reported together with their retention time, molecular formula and molecular weight. Further information on detected adducts, fragmentation spectra, identification confidence levels, area in the three experiments are provided in Table S4 in the Supporting Information. In conclusion, by means of the developed untargeted metabolomics approach, 193 molecules have been tentatively identified, 166 of which were classified as lipids including phospholipids, steroids, carnitines, fatty alcohols, diglycerides and fatty acids. This result is in agreement with previous findings showing a prevalence of lipid adsorption over other small metabolites on NP BMC.[10]

Evaluation of extraction methods

Many extraction methods have been already proposed in metabolomics studies on several biological fluids,[36] however, only few studies focused on the metabolite extraction from the BMC formed onto NPs, [9,10,12–14] and these have been carried out without a real optimization of the extraction conditions for both lipids and metabolites.

In this study, we compared three different metabolite and lipid extraction strategies in order to assess the best protocol for metabolite and lipid recovery from the liposomal NP BMC. Different aspects, namely the number and the peak areas of tentatively identified compounds, were evaluated in order to compare and select the best extraction method.

Among the total number of tentatively identified metabolites and lipids showed in Table S3, 23 were identified in the UFM protocol, 184 in the MeOH protocol and 106 in the W&F procedure. In particular, 85 compounds were exclusively identified by MeOH procedure, 7 unique compounds were identified by W&F procedure and only 1 was uniquely found by UFM method, while 20 were in common among the three data sets (Fig. 1a). For more details about the lists of unique metabolites and metabolites in common among the three protocols see Table S5 in the

Supporting Information. In summary, the MeOH method showed superior extraction yield over W&F and UFM protocols in terms of tentatively identified compounds. Also, the extraction methods were compared based on the relative areas of the tentatively identified compounds in the three protocols. The results are shown in Fig. 1b, where the Box-and-whisker plot calculated on the Log_{10} areas of metabolites tentatively identified in the liposome blank and in the three experimental replicates of UFM, MeOH and W&F protocols is shown. In summary, the median of the areas resulted to be higher in MeOH than in W&F protocol, whereas UFM method resulted in the lowest extraction yield, as demonstrated by the fact that the liposome blank and the three replicates showed similar results. Liposome blank areas have been reported in order to show that the tentatively identified lipids detected in the experimental replicates were not belonging to the lipid NPs used in this specific study, but rather derived from the liposome incubation with human plasma.

In conclusion, MeOH solubilization protocol resulted to be the best choice for the recovery of the highest number of compounds. However, delving more thoroughly the obtained data, a different trend between metabolites and lipids was highlighted. In Fig. 2 a Trend Chart showing the chromatographic peak areas, after normalization, for the tentatively identified compounds is reported for the three protocols. Peak areas for each compound was normalized by dividing it for the average of the peak areas calculated among the three protocols. As shown, the most part of compounds tentatively identified in the MeOH procedure resulted to belong to the class of lipids, whereas a more heterogeneous range of compounds, including both metabolites and lipids, resulted from the W&F protocol. No particular trend was observed for the UFM protocol, aside from the high abundance of indoxyl sulfate.

Finally, in Table S6 the top 25 most abundant metabolites and lipids tentatively identified in BMC of DOTAP NPs upon UFM, MeOH and W&F protocol are reported. Some of the most abundant metabolites were not identified because of inconsistencies between the calculated molecular formula and the fragmentation pattern or because not sufficiently diagnostic ion fragments were detected. However, for the UFM protocol the most abundant tentatively identified compounds were metabolites such as 17 α -hydroxyprogesterone, deoxycholecalciferol, phenylalanine and hydroxycholine. For the other two procedures, the most abundant molecules were lipids.

The differences in the extraction efficiency of the various methods may be closely related to the polarity and the water content of the extraction solvent. In W&F procedure the upper layer consisting of a solution of MeOH:H₂O 80:20 (v/v) was analyzed. The presence of water in the upper layer obtained by the W&F protocol results in a higher dielectric constant compared to the solution of MeOH only. The dielectric properties of the solvents used in the extraction process determine its ability to extract different compounds with different range of polarity. Therefore, using W&F procedure more polar compounds compared to lipids, e.g. citric acid, betaine which is a zwitterionic molecule, an amino acid like phenylalanine and different short chain organic acids were extracted. However, aside from few metabolites, the overall yields of MeOH protocol showed better results in terms of both tentatively identified metabolites and lipids. The combined extracted ion chromatogram of some of the metabolites and lipids tentatively identified in the liposomal BMC after extraction with MeOH protocol is reported in Figure S2 in the Supporting information.

Liposome biomolecular corona

After an extensive method development it was possible to perform a comprehensive characterization of liposomal NP BMC, resulting in the tentative identification of 166 lipids out of 193 total molecules. The high abundance of lipids detected in the BMC of DOTAP liposomes has been already reported for other kinds of NPs, and it has been justified by a lipoprotein mediated adsorption of lipids on NP surface.[8,14,37,38] Lipoproteins are complexes of apolipoproteins, phospholipids, triglycerides, and cholesterol, with a size and relative composition depending on the lipoprotein type.[39] Triglycerides and cholesterol esters are found in the core of lipoprotein particles and are surrounded by a monolayer of phospholipids and proteins. The most common class of proteins present in the lipoproteins is represented by apolipoproteins. However, during the last few years, proteomics studies have revealed that also proteins involved in complement system (e.g. vitronectin, clusterin, complement factors etc.), inflammation, protease inhibition, and wound healing (e.g. fibrinogen), among others, are included in lipoprotein particles.[40] Notably, in a previous work performed by our research group, the characterization of DOTAP liposome protein corona revealed that a wide range of proteins interacts with these particles, including apolipoproteins, coagulation factors, complement proteins and immune regulators.[30] Looking deeper into the results reported by Caracciolo *et al.*,[30] apolipoproteins resulted to be the most abundant class of proteins adsorbed onto DOTAP liposomal NPs accounting for the 13.7% (relative protein abundance-RPA) of the protein corona, followed by vitronectin (RPA 13.2%), and from fibrinogen (RPA 10.8%). Apolipoproteins, as well as vitronectin and fibrinogen, have already been shown to belong to lipoprotein NPs, suggesting that the absorption of lipids onto DOTAP liposome BMC, as well as onto other NPs previously investigated by other research groups, is mediated by lipoproteins. Whether this interaction occurs between intact lipoproteins and NP surface or after lipoprotein

disintegration has not been completely clarified. In some previous papers, the interaction of intact lipoproteins with NP surface has been reported.[8,14,37,38] However, in a recent work by Müller *et al.*,[13] the thermodynamic and morphological aspects of the interaction between different lipoproteins and polymeric NPs were investigated in more details. Based on this work, the lipoproteins disintegrate and cover the NP surface until saturation. Subsequently, the adsorption of additional intact lipoproteins may be detected. In this regard, some considerations can be done also for DOTAP liposome BMC, looking at the results of the different tested extraction protocols. As a matter of fact, in the UFM protocol, the pellet was solubilized with a solution of 1% sodium deoxycholate, a detergent effective in disrupting and dissociating many types of protein interaction. The subsequent mixture was fractionated with an UFM with a molecular weight cut-off of 3,000 Da, resulting effective in the retention of proteins with MW>5,000 Da. Although this protocol should have produced good results, cleaning-up the sample and reducing matrix effect, only 23 compounds were tentatively identified. It is likely that lipid adsorption on NPs is mediated by intact lipoproteins which, being characterized by high molecular weight, may have been removed by UFM resulting in the loss of the majority of the compounds. Whether the adsorption of lipoproteins occurs or not upon their disintegration might be dependent on the NP material, so that more hydrophilic surfaces could eventually hinder disintegration. NP surface could affect the mechanism of interaction and, consequently, the composition of the BMC upon NP interaction with biological fluids. Modulating NP surface could then result in a different BMC in terms of lipoproteins, proteins and lipids.

Liposomes are considered to be among the most suitable NPs for drug delivery for several factors, one of which is the possibility of modulating their physical-chemical properties by changing their lipid composition. The BMC can be modulated as a function of liposome lipid

composition, affecting liposome recognition by the biological machinery. NPs have not solved yet the issues related to the low efficient drug delivery, such as severe side effects and damage of healthy tissues. Recent studies have shown that just a minor fraction of the bound plasma proteins enhance liposome association with cancer cells, triggering efficient particle internalization[41]. On the other hand, some preliminary studies reported that carbon nanotubes covered by phospholipids are recognized and cleared by macrophages both *in vitro* and following inhalation *in vivo*[15,42], showing that the lipid component could play a crucial role for NP biorecognition. Based on these considerations, further studies on liposomes with different lipid composition should be carried out for a more extensive investigation of their BMC, especially focusing on either lipids or lipoproteins adsorption in addition to the extensively investigated protein adsorption.

Conclusion

In this study we investigated the formation of a BMC on the surface of liposomal NPs by a UHPLC-HRMS based untargeted metabolomics and lipidomics approach. Compounds were tentatively identified based on matches with available databases and comparison of MS/MS spectra with available spectral libraries. A total of 193 metabolites and lipids has been tentatively identified, 166 of which belong to the class of lipids including phospholipids, steroids, carnitines, fatty alcohols, diglycerides and fatty acids. Comparing the number and the relative abundances of the tentatively identified compounds among three tested protocols, MeOH extraction procedure resulted in the highest extraction yield followed by W&F and UFM protocols. Aside few polar metabolites, showing higher recoveries in W&F protocol, MeOH protocol provided the widest metabolic and lipid coverage of the BMC formed upon incubation of liposomal NPs with

human plasma. UFM procedure resulted to be not suitable for the extraction and analysis of BMC compounds, probably due to the removal of intact lipoproteins from NP surface. Indeed, recent findings showed a lipoprotein mediated interaction of lipids onto other NP surface.[8,14,37,38] Moreover, in a previous work by Caracciolo *et al.* [30] the investigation of DOTAP liposome protein corona showed a high abundance of apolipoproteins and other proteins commonly found in lipoproteins, supporting the hypothesis of a lipoprotein mediated interaction of lipid onto liposomal NPs as well. Lipids are important bioactive molecules which could impact NP circulation and uptake by cells, included endothelial cells and macrophages, which are responsible of NP removal from circulation [43]. Extending the investigation of BMC beyond the protein corona and towards the “lipid corona”, may be the keystone of a better understanding of NP “biological entity” and a better controlling of NP destiny in the body.

Conflict of Interest

The authors have declared no conflict of interest

References

- [1] G. Caracciolo, Liposome–protein corona in a physiological environment: Challenges and opportunities for targeted delivery of nanomedicines, *Nanomedicine Nanotechnology, Biol. Med.* 11 (2015) 543–557. doi:10.1016/J.NANO.2014.11.003.
- [2] M.P. Monopoli, C. Åberg, A. Salvati, K.A. Dawson, Biomolecular coronas provide the biological identity of nanosized materials, *Nat. Nanotechnol.* 7 (2012) 779–786. doi:10.1038/nnano.2012.207.
- [3] A.L. Capriotti, G. Caracciolo, C. Cavaliere, V. Colapicchioni, S. Piovesana, D. Pozzi, A. Laganà, Analytical Methods for Characterizing the Nanoparticle–Protein Corona, *Chromatographia*. 77 (2014) 755–769. doi:10.1007/s10337-014-2677-x.
- [4] M. Wang, O.J. R Gustafsson, E.H. Pilkington, ac Aleksandr Kakinen, I. Javed, A. Faridi,

- T.P. Davis, P. Chun Ke, Materials Chemistry B Materials for biology and medicine Nanoparticle-proteome in vitro and in vivo †, *J. Mater. Chem. B.* (2018). doi:10.1039/c8tb01634h.
- [5] T. Cedervall, I. Lynch, S. Lindman, T. Berggard, E. Thulin, H. Nilsson, K.A. Dawson, S. Linse, Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles, *Proc. Natl. Acad. Sci.* 104 (2007) 2050–2055. doi:10.1073/pnas.0608582104.
- [6] C.D. Walkey, J.B. Olsen, F. Song, R. Liu, H. Guo, D.W.H. Olsen, Y. Cohen, A. Emili, W.C.W. Chan, Protein corona fingerprinting predicts the cellular interaction of gold and silver nanoparticles, *ACS Nano.* 8 (2014) 2439–2455. doi:10.1021/nn406018q.
- [7] P.C. Ke, S. Lin, W.J. Parak, T.P. Davis, F. Caruso, A Decade of the Protein Corona, *ACS Nano.* 11 (2017) 11773–11776. doi:10.1021/acsnano.7b08008.
- [8] E. Hellstrand, I. Lynch, A. Andersson, T. Drakenberg, B. Dahlbäck, K.A. Dawson, S. Linse, T. Cedervall, Complete high-density lipoproteins in nanoparticle corona, *FEBS J.* 276 (2009) 3372–3381. doi:10.1111/j.1742-4658.2009.07062.x.
- [9] M. Pink, N. Verma, C. Kersch, S. Schmitz-Spanke, Identification and characterization of small organic compounds within the corona formed around engineered nanoparticles, *Environ. Sci. Nano.* 5 (2018) 1420–1427. doi:10.1039/C8EN00161H.
- [10] J. Martel, C.-Y. Wu, C.-Y. Hung, T.-Y. Wong, A.-J. Cheng, M.-L. Cheng, M.-S. Shiao, J.D. Young, Fatty acids and small organic compounds bind to mineralo-organic nanoparticles derived from human body fluids as revealed by metabolomic analysis, *Nanoscale.* 8 (2016) 5537–5545. doi:10.1039/C5NR08116E.
- [11] S.B. Gunnarsson, K. Bernfur, A. Mikkelsen, T. Cedervall, Analysis of nanoparticle biomolecule complexes, *Nanoscale.* 10 (2018) 4246–4257. doi:10.1039/C7NR08696B.
- [12] J.Y. Lee, H. Wang, G. Pyrgiotakis, G.M. DeLoid, Z. Zhang, J. Beltran-Huarac, P. Demokritou, W. Zhong, Analysis of lipid adsorption on nanoparticles by nanoflow liquid chromatography-tandem mass spectrometry, *Anal. Bioanal. Chem.* 410 (2018) 6155–6164. doi:10.1007/s00216-018-1145-0.
- [13] J. Müller, D. Prozeller, A. Ghazaryan, M. Kokkinopoulou, V. Mailänder, S. Morsbach, K. Landfester, Beyond the protein corona – lipids matter for biological response of nanocarriers, *Acta Biomater.* 71 (2018) 420–431. doi:10.1016/j.actbio.2018.02.036.
- [14] S.S. Raesch, S. Tenzer, W. Storck, A. Rurainski, D. Selzer, C.A. Ruge, J. Perez-Gil, U.F. Schaefer, C.-M. Lehr, Proteomic and Lipidomic Analysis of Nanoparticle Corona upon Contact with Lung Surfactant Reveals Differences in Protein, but Not Lipid Composition, *ACS Nano.* 9 (2015) 11872–11885. doi:10.1021/acsnano.5b04215.
- [15] A.A. Kapralov, W.H. Feng, A.A. Amoscato, N. Yanamala, K. Balasubramanian, D.E. Winnica, E.R. Kisin, G.P. Kotchey, P. Gou, L.J. Sparvero, P. Ray, R.K. Mallampalli, J. Klein-Seetharaman, B. Fadeel, A. Star, A.A. Shvedova, V.E. Kagan, Adsorption of surfactant lipids by single-walled carbon nanotubes in mouse lung upon pharyngeal aspiration, *ACS Nano.* 6 (2012) 4147–4156. doi:10.1021/nn300626q.
- [16] C.M. Montone, A.L. Capriotti, C. Cavaliere, G. La Barbera, S. Piovesana, R. Zenezini Chiozzi, A. Laganà, Peptidomic strategy for purification and identification of potential ACE-inhibitory and antioxidant peptides in *Tetrademus obliquus* microalgae, *Anal. Bioanal. Chem.* 410 (2018). doi:10.1007/s00216-018-0925-x.
- [17] G. La Barbera, A.L. Capriotti, C. Cavaliere, S. Piovesana, R. Samperi, R. Zenezini Chiozzi, A. Laganà, Comprehensive polyphenol profiling of a strawberry extract (*Fragaria*

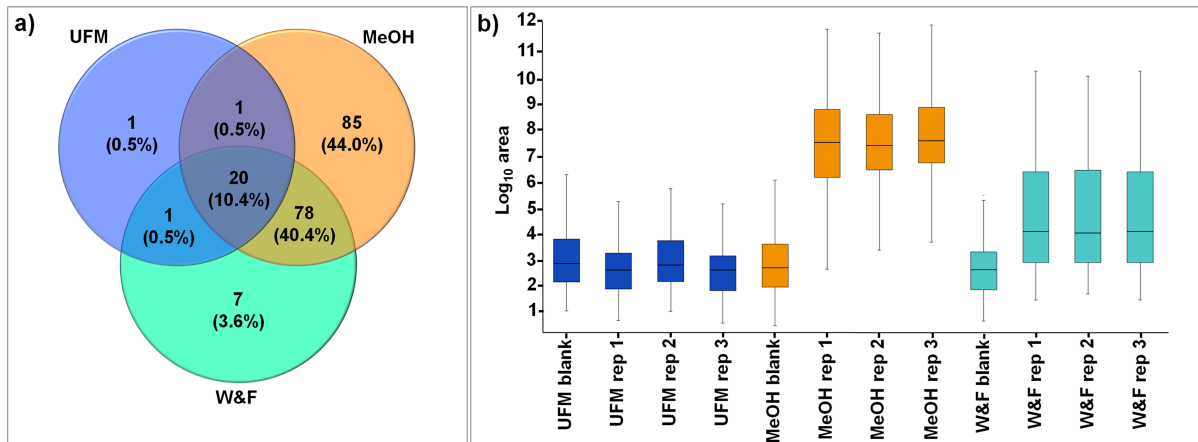
- × ananassa) by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry, *Anal. Bioanal. Chem.* 409 (2017) 2127–2142. doi:10.1007/s00216-016-0159-8.
- [18] A.L. Capriotti, C. Cavaliere, G. La Barbera, C.M. Montone, S. Piovesana, R. Zenezini Chiozzi, A. Laganà, Chromatographic column evaluation for the untargeted profiling of glucosinolates in cauliflower by means of ultra-high performance liquid chromatography coupled to high resolution mass spectrometry, *Talanta*. 179 (2018). doi:10.1016/j.talanta.2017.12.019.
- [19] D. Wessel, U.I. Flügge, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids, *Anal. Biochem.* 138 (1984) 141–143. doi:10.1016/0003-2697(84)90782-6.
- [20] G. Caracciolo, Clinically approved liposomal nanomedicines: lessons learned from the biomolecular corona, *Nanoscale*. 10 (2018) 4167–4172. doi:10.1039/C7NR07450F.
- [21] A.L. Capriotti, G. Caracciolo, C. Cavaliere, C. Crescenzi, D. Pozzi, A. Laganà, Shotgun proteomic analytical approach for studying proteins adsorbed onto liposome surface, *Anal. Bioanal. Chem.* 401 (2011) 1195–1202. doi:10.1007/s00216-011-5188-8.
- [22] A. Amici, G. Caracciolo, L. Digiaco, V. Gambini, C. Marchini, M. Tilio, A.L. Capriotti, V. Colapicchioni, R. Matassa, G. Familiari, S. Palchetti, D. Pozzi, M. Mahmoudi, A. Laganà, In vivo protein corona patterns of lipid nanoparticles, *RSC Adv.* 7 (2017) 1137–1145. doi:10.1039/C6RA25493D.
- [23] G. Caracciolo, D. Pozzi, A.L. Capriotti, C. Cavaliere, S. Piovesana, G. La Barbera, A. Amici, A. Laganà, The liposome–protein corona in mice and humans and its implications for in vivo delivery, *J. Mater. Chem. B*. 2 (2014) 7419–7428. doi:10.1039/C4TB01316F.
- [24] A.L. Capriotti, G. Caracciolo, C. Cavaliere, P. Foglia, D. Pozzi, R. Samperi, A. Laganà, Do plasma proteins distinguish between liposomes of varying charge density?, *J. Proteomics*. 75 (2012) 1924–1932. doi:10.1016/J.JPROT.2012.01.003.
- [25] D. Pozzi, G. Caracciolo, A.L. Capriotti, C. Cavaliere, G. La Barbera, T.J. Anchordoquy, A. Laganà, Surface chemistry and serum type both determine the nanoparticle-protein corona, *J. Proteomics*. 119 (2015). doi:10.1016/j.jprot.2015.02.009.
- [26] A.L. Capriotti, G. Caracciolo, G. Caruso, P. Foglia, D. Pozzi, R. Samperi, A. Laganà, Differential analysis of “protein corona” profile adsorbed onto different nonviral gene delivery systems, *Anal. Biochem.* 419 (2011) 180–189. doi:10.1016/J.AB.2011.08.003.
- [27] A.L. Capriotti, G. Caracciolo, G. Caruso, C. Cavaliere, D. Pozzi, R. Samperi, A. Laganà, Analysis of plasma protein adsorption onto DC-Chol-DOPE cationic liposomes by HPLC-CHIP coupled to a Q-TOF mass spectrometer, *Anal. Bioanal. Chem.* 398 (2010) 2895–2903. doi:10.1007/s00216-010-4104-y.
- [28] L. Goracci, S. Tortorella, P. Tiberi, R.M. Pellegrino, A. Di Veroli, A. Valeri, G. Cruciani, Lipostar, a Comprehensive Platform-Neutral Cheminformatics Tool for Lipidomics, *Anal. Chem.* 89 (2017) 6257–6264. doi:10.1021/acs.analchem.7b01259.
- [29] G. La Barbera, M. Antonelli, C. Cavaliere, G. Cruciani, L. Goracci, C.M. Montone, S. Piovesana, A. Laganà, A.L. Capriotti, Delving into the Polar Lipidome by Optimized Chromatographic Separation, High-Resolution Mass Spectrometry, and Comprehensive Identification with Lipostar: Microalgae as Case Study, *Anal. Chem.* 90 (2018) 12230–12238. doi:10.1021/acs.analchem.8b03482.
- [30] G. Caracciolo, D. Pozzi, A.L. Capriotti, C. Cavaliere, S. Piovesana, H. Amenitsch, A. Laganà, Lipid composition: a “key factor” for the rational manipulation of the liposome–

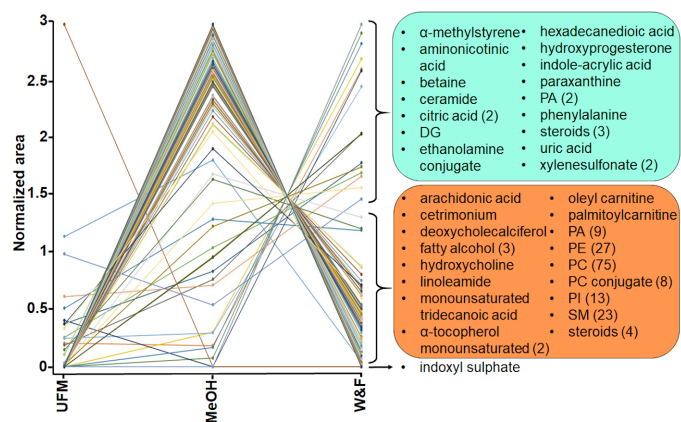
- protein corona by liposome design, *RSC Adv.* 5 (2015) 5967–5975. doi:10.1039/C4RA13335H.
- [31] M.C. Taira, N.S. Chiaramoni, K.M. Pecuch, S. Alonso-Romanowski, Stability of Liposomal Formulations in Physiological Conditions for Oral Drug Delivery, *Drug Deliv.* 11 (2004) 123–128. doi:10.1080/10717540490280769.
- [32] G. Caracciolo, D. Pozzi, A.L. Capriotti, C. Cavaliere, P. Foglia, H. Amenitsch, A. Laganà, Evolution of the Protein Corona of Lipid Gene Vectors as a Function of Plasma Concentration, *Langmuir.* 27 (2011) 15048–15053. doi:10.1021/la202912f.
- [33] www.thermoscientific.com.
- [34] www.phenomenex.com.
- [35] E.L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H.P. Singer, J. Hollender, Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence, *Environ. Sci. Technol.* 48 (2014) 2097–2098. doi:10.1021/es5002105.
- [36] R.-J. Raterink, P.W. Lindenburg, R.J. Vreeken, R. Ramautar, T. Hankemeier, Recent developments in sample-pretreatment techniques for mass spectrometry-based metabolomics, *TrAC Trends Anal. Chem.* 61 (2014) 157–167. doi:10.1016/J.TRAC.2014.06.003.
- [37] S. Lara, F. Alnasser, E. Polo, D. Garry, M.C. Lo Giudice, D.R. Hristov, L. Rocks, A. Salvati, Y. Yan, K.A. Dawson, Identification of Receptor Binding to the Biomolecular Corona of Nanoparticles, *ACS Nano.* 11 (2017) 1884–1893. doi:10.1021/acsnano.6b07933.
- [38] N.N. Nam, S.Y. Han, Formation of High-Density Lipoprotein (HDL) Coronas on Silica Nanoparticles Occurs by Adsorption of Intact HDL Particulates, *Bull. Korean Chem. Soc.* 37 (2016) 3–4. doi:10.1002/bkcs.10622.
- [39] M. Wang, M.R. Briggs, HDL: The Metabolism, Function, and Therapeutic Importance, *Chem. Rev.* 104 (2004) 119–138. doi:10.1021/cr020466v.
- [40] A. von Zychlinski, T. Kleffmann, Dissecting the proteome of lipoproteins: New biomarkers for cardiovascular diseases?, *Transl. Proteomics.* 7 (2015) 30–39. doi:10.1016/j.trprot.2014.12.001.
- [41] S. Palchetti, D. Caputo, L. Digiacomo, A.L. Capriotti, R. Coppola, D. Pozzi, G. Caracciolo, Protein Corona Fingerprints of Liposomes: New Opportunities for Targeted Drug Delivery and Early Detection in Pancreatic Cancer., *Pharmaceutics.* 11 (2019). doi:10.3390/pharmaceutics11010031.
- [42] N. V Konduru, Y.Y. Tyurina, W. Feng, L. V Basova, N.A. Belikova, H. Bayir, K. Clark, M. Rubin, D. Stolz, H. Vallhov, A. Scheynius, E. Witasz, B. Fadeel, P.D. Kichambare, A. Star, E.R. Kisin, A.R. Murray, A.A. Shvedova, V.E. Kagan, Phosphatidylserine targets single-walled carbon nanotubes to professional phagocytes in vitro and in vivo., *PLoS One.* 4 (2009) e4398. doi:10.1371/journal.pone.0004398.
- [43] J. Shannahan, The biocorona: a challenge for the biomedical application of nanoparticles, *Nanotechnol. Rev.* 6 (2017). doi:10.1515/ntrev-2016-0098.

Figure captions

Figure. 1 a) Venn diagram showing the overlap of identified lipids and metabolites among the UFM, MeOH and W&F protocols. b) Box-and-whisker plot calculated on the Log_{10} areas of metabolites and lipids identified in the liposome blank and in the three experimental replicates of UFM, MeOH and W&F protocols

Figure. 2 Trend Chart showing the chromatographic peak areas, after normalization, for all the identified compounds in the three protocols. The metabolites and lipids showing higher and lower area in W&F compared to MeOH protocol are highlighted in light blue and orange, respectively





Highlights:

- A comprehensive characterization of nanoparticle biomolecular corona is presented.
- An untargeted metabolomics and lipidomics approach has been developed.
- Three extraction protocols have been tested for extracting metabolites and lipids.
- The methanol extraction protocol resulted in the best results.
- A total of 193 metabolites has been identified, 166 of which were lipids.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: