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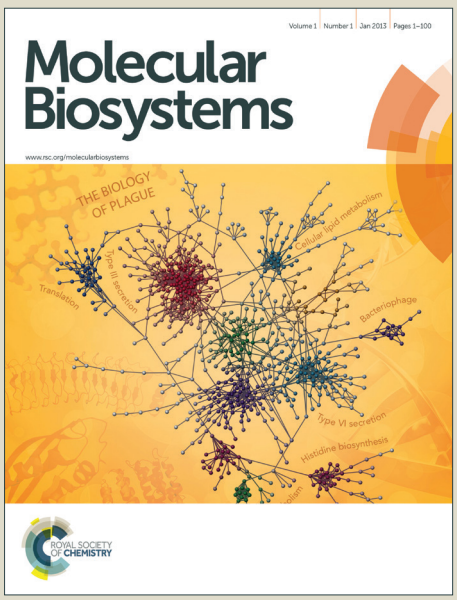

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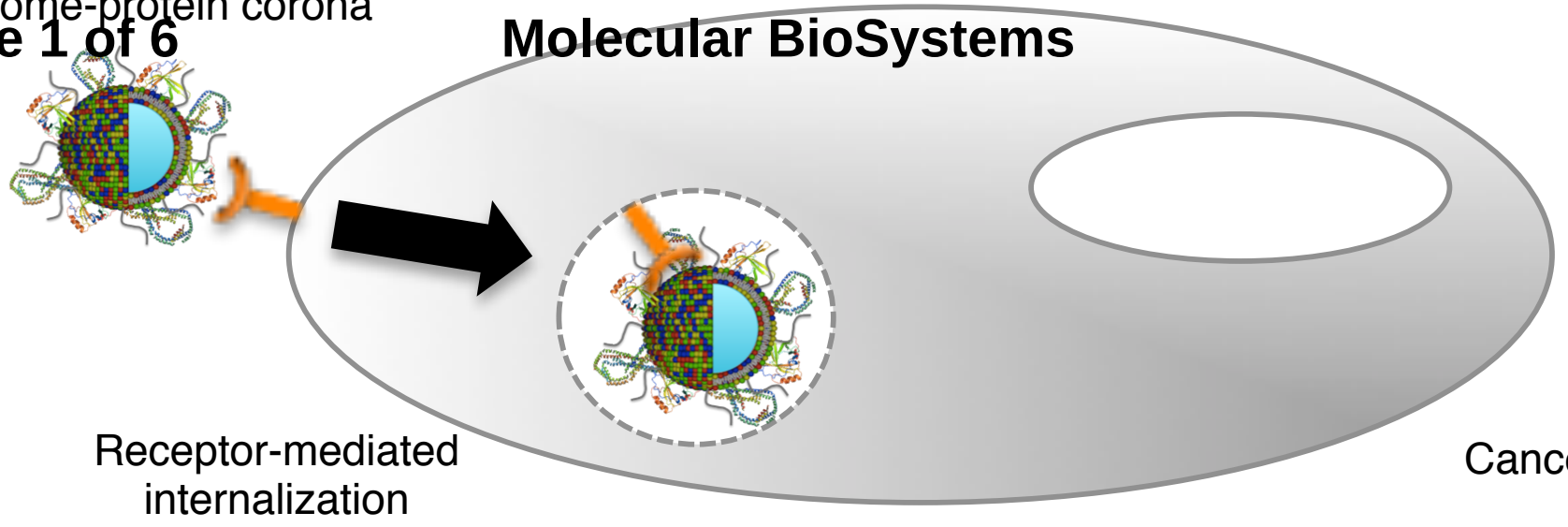

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A proteomics-based methodology to investigate the protein corona effect for targeted drug delivery

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Here we introduce a proteomics methodology based on nanoliquid-cromatography tandem mass spectrometry (nanoLC/MS-MS) to investigate the "protein corona effect for targeted drug delivery", an innovative strategy, which exploits the "protein corona" that forms around nanoparticles in a physiological environment to target cells.

Introduction

Over the past few years, there has been extensive interest in developing nanoparticles (NPs) for the delivery of drugs, proteins, peptides and nucleic acids [1-3]. Among the plethora of existing NPs, liposomes are particularly attracting due to their efficiency and biocompatibility [4]. When liposomes are injected into biological fluid (e.g. human plasma (HP)), several biomolecules, especially proteins, bind to the liposome surface [5, 6]. Quickly the identity of the pristine liposome is lost, and the layer of adsorbed proteins, usually referred to as the "protein corona" [7-13], is the bio-entity actually "seen" by target cells. Moreover, since coverage of liposomes with polyethylene glycol (PEG) cannot completely prevent formation of the corona [14], some authors have suggested exploiting it for targeted drug delivery [15-17]. Basically, proteins engaged from the blood could let the nanoparticle to interact with specific receptors expressed on the plasma membrane of target cells. In a recent paper [17], we have provided the first proof of concept of the "protein corona effect for targeted drug delivery" in vitro. It has been shown that lipid/DNA particles, which in human plasma (HP) become covered by a vitronectin-rich protein corona, are efficiently uptaken in cancer cells expressing high levels of the $\alpha_v\beta_3$ integrin, the vitronectin receptor distributed in the target cell plasma membrane. Accurate quantification of the protein corona composition coupled to knowledge of the receptors over-expressed at the plasma membrane of target cells, could therefore be used to predict the interaction of nanoparticle-protein corona with target cells in a physiological environment [18, 19]. The plasma membrane is rich in proteins that operate crucial functions for the cells, interacting with both cellular and extracellular components, structures and signaling molecules. Moreover, plasma membrane proteins contain more than two-thirds of the known protein targets for existing drugs [20]. In principle, endocytosed particles would take cell receptors inside the cell, thus altering the protein profile of the plasma membrane [21]. Estimating this change in the protein profile, it could be proved that if a long-standing corona with relevant receptor-binding sites stays associated with the cells long enough, they can activate the cell's uptake machinery by endocytosis. To provide insights into such corona-mediated cellular uptake, quantitative analytical techniques are necessary. Among them, nanoliquid-cromatography tandem mass spectrometry (nanoLC–MS/MS) has several advantages in the analysis of plasma membrane components. First, it requires only low amounts of any given protein for MS analyses, so is suitable for scarcer proteins. Second, digestion of enriched membranes can be performed in the presence of trace amounts of ionic detergents, which, along with the use of reducing and alkylating reagents, allows for improved trypsin cleavage leading to higher sequence coverage for a given protein.

In this work, we introduce a proteomics-based methodology to validate the "protein corona effect for targeted drug delivery". To this end, we first applied (nanoLC-MS/MS) to identify the proteins in the corona of multicomponent (MC) liposome-HP complexes. Subsequently, nanoLC-MS/MS was used to quantify the proteins of plasma membrane of human prostate cancer PC3 cells, before and after interaction with liposome-HP complexes.

By comparing differentially expressed proteins we concluded that the "protein corona" forming around MC liposomes induces a switch from a not specific to a receptor-mediated uptake mechanism in prostate cancer PC3 cells.

To our knowledge, this is the first study that focused on how the expression of plasma membrane proteins changes after interaction with nanoparticles using proteomics-based techniques. The results obtained provide a platform for future investigations that will allow us to understand liposome-HP interactions with protein of plasma membrane and if the "protein corona" forming around nanoparticles after interaction with bodily fluid induces a receptor-mediated uptake and could be potentially used for active targeting.

Results and discussion

After introduction in the bloodstream, liposomes are instantly surrounded by high concentrations of free protein driven either by a potential energy gradient, or just by diffusion. Recently, it has been shown that, while PEGylation of liposomes can diminish the total amount of bound proteins, it does not alter the 'adsorbome'[14]. This result has clarified that PEGylated liposomes are not actually "stealth" in nature, but they are just surrounded by a layer of proteins thinner than that formed around their unPEGylated counterpart. Thus, it has been recently suggested that the "corona" forming around PEGylated liposomes could be exploited to target cancer cells by proteins engaged from the blood. Such "natural functionalization" could let the nanoparticle to interact with specific receptors over-expressed on the plasma membrane of target cells. Endocytosed particles would bring cell receptors inside the cell, thus potentially altering the protein profile of the plasma membrane. To test this suggestion, a detailed identification and quantification of the corona proteins is mandatory. The protein corona of PEGylated MC liposome-HP complexes was fully characterized by nanoLC-MS/MS. We identified 237 proteins (Table S1). The 35 most-abundant corona proteins are listed in Table 1. Calculation of the RPAs showed that Igs (RPA=17.2%), complement proteins (RPA=14.2%) and apolipoproteins (RPA=11.5%) are the most abundant classes. While both Igs and complement proteins are opsonins, apolipoproteins are considered dysopsonins because they prolong the circulation time of nanoparticles in the blood. Apolipoproteins are the main component of very low-density (VLDL), low-density (LDL) and high-density lipoproteins (HDL), which are known to target specific cell receptors located in the extracellular leaflets of both caveolae and clathrin coated pits. For instance, the LDL receptor is a cell-surface receptor that recognizes apoE (RPA=1.95%) and apoB100 (RPA=0.81%) and is present in clathrin-coated pits [22]. Scavenger receptor (SR)-BI mediates the selective uptake of HDL, which contains several types of apolipoproteins including apo-AI, II, IV, apo-CI, II, and III, apo-D, and apo-E. In PC3 cells, SR-BI is very abundantly expressed and is associated with caveolae. Cell surface binding of apo-AI (RPA=1.66%) is also regulated by Caveolin-1 (Cav-1), a structural protein required for the formation of caveolae [23]. Table 1 also shows that the corona of PEGylated liposome-HP complexes is rich in fibrinogen (RPA=2.8%) and vitronectin (RPA=1%). The integrin family is the most common class of cell receptors for both fibrinogen and vitronectin [24].

	Identified Protein	RPA	s.d.
1	Ig kappa chain C region	5.79	0.48
2	Complement C3	5.21	0.30
3	Ig lambda-2 chain C regions	3.42	0.18
4	Apolipoprotein C-III	3.14	0.14
5	Complement C1q subcomponent subunit A	2.58	0.39
6	Serum albumin	2.46	0.30
7	Ig mu chain C region	2.40	0.10
8	Apolipoprotein E	1.95	0.15
9	Complement C1q subcomponent subunit B	1.79	0.10
10	Complement C4-B	1.69	0.04
11	Apolipoprotein A-I	1.66	0.16
12	Apolipoprotein A-II	1.47	0.07
13	Apolipoprotein C-II	1.31	0.25
14	Alpha-1-antitrypsin	1.22	0.09
15	Hemoglobin subunit alpha	1.22	0.21
16	Hemoglobin subunit beta	1.19	0.05
17	Complement C1q subcomponent subunit C	1.17	0.06
18	Apolipoprotein A-IV OS=Homo sapiens	1.17	0.14
19	Ig gamma-1 chain C region	1.12	0.08
20	Ig kappa chain V-III region HAH	1.10	0.08
21	Fibrinogen beta chain	1.07	0.02
22	Ig gamma-2 chain C region	1.02	0.16
23	Actin, cytoplasmic 1	0.99	0.04
24	Fibrinogen gamma chain	0.98	0.08
25	C4b-binding protein alpha chain	0.96	0.06
26	Vitronectin	0.90	0.03
27	Thymosin beta-4	0.89	0.15
28	Clusterin	0.84	0.02
29	Fibrinogen alpha chain	0.83	0.12
30	Ig kappa chain V-IV region	0.82	0.06
31	Haptoglobin-related protein	0.81	0.01
32	Apolipoprotein B-100	0.81	0.02
33	Immunoglobulin lambda-like polypeptide 5	0.80	0.08
34	Complement factor H	0.79	0.05
35	Ig heavy chain V-III region GAL	0.78	0.10

Table 1. The 35 most-abundant corona proteins identified in the protein corona of PEGylated multicomponent liposomes following 1 h incubation with human plasma. The error associated to the calculated relative protein abundance (RPA) is the standard deviation (s.d.) between nine experimental replicates (three technical replicates of three different experimental samples).

Given the composition of the protein corona, we asked whether treatment of PC3 cells with liposome-protein complexes could affect the expression of proteins located in membrane compartments involved in endocytosis. As a first step, proteins from the plasma membranes of PC3 cells after interaction with liposome-HP complexes were identified and quantified. As a control, we also quantified plasma proteins from the plasma membranes of PC3 cells both untreated and treated with bare PEGylated liposomes (i.e. in the absence of the protein corona).

The Venn diagram of Figure 1 shows the number of common and unique proteins. We identified 259 plasma proteins from untreated PC3 cells, 244 proteins in the plasma membrane from PC3 cells after interaction with liposomes and 336 proteins in the plasma membrane from PC3 cells after interaction with liposome-HP complexes.

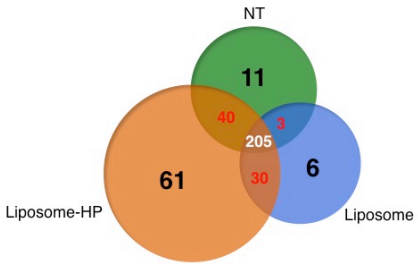

Figure 1. Venn diagram reporting the number of proteins identified on the plasma membrane of PC3 cells: not treated (NT, green) and treated with either PEGylated multicomponent liposomes (blue) or PEGylated multicomponent liposome-HP complexes (orange). 205 proteins were found in common, while 11, 6 and 61 proteins were unique.

The overlap between the three conditions is 205 proteins, while 11, 6 and 61 proteins were unique in the plasma membrane from untreated PC3 cells, in the plasma membrane from PC3 cells after interaction with liposomes and in the plasma membrane from PC3 cells after interaction with liposome-HP complexes respectively. Clearly, the highest number of proteins revealed on the plasma membrane of PC3 cells treated with liposome-HP complexes are due to the protein corona forming around liposomes after interaction with HP.

Figure 2 shows the RPA of plasma proteins located in different membrane compartments for the untreated PC3 cells (green bars), and for PC3 cells treated with bare PEGylated MC liposomes (blue bars) and MC liposomes-HP complexes (orange bars). Treatment of PC3 cells with bare liposomes did not alter the protein profile significantly.

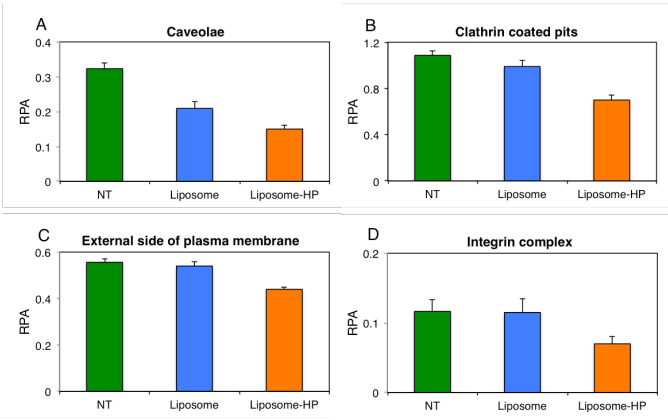

Figure 2. Relative protein abundance (RPA) of proteins located in different compartments of plasma membrane: caveolae (panel A), clathrin coated pits (panel B), external side of plasma membrane (panel C) and integrin complex (panel D). Untreated PC3 cells (green), PC3 cells treated with either PEGylated multicomponent liposomes (blue) or PEGylated multicomponent liposome-HP complexes (orange). RPA values are mean from nine replicates ± standard deviation. Significance was $p < 0.01$ for all samples.

The only exception is represented by the expression of caveolar proteins. This result is in good agreement with recent findings showing that caveolae-mediated endocytosis is a preferred internalization pathway of lipid systems. On the other side, we found a significant decrease in the RPAs of proteins found in caveolae (panel A), clathrin coated pits (panel B), external side of plasma membrane (panel C) and in the expression level of integrins (panel D). As above explained many proteins found in the corona of PEGylated MC liposomes are known to interact with cell receptors located in the extracellular leaflets of both caveolae and clathrin coated pits (Table 1). Collectively, our data seem to suggest that the "protein corona" induces a receptor-mediated liposome uptake in PC3 cells. Further example is given by results of Figure 2 panel D showing the RPA of membrane proteins associated with the integrin complex. It does not change form untreated PC3 cells to PC3 cells treated with liposomes, while a clear reduction is observed in PC3 cells treated with liposome-HP complexes. nanoLC-MS/MS has revealed that the corona of PEGylated liposome-HP complexes is also abundant in fibrinogen and the integrin family is the most common fibrinogen receptor. Therefore, the "protein corona" forming around liposomes after interaction with bodily fluid induces a receptor-mediated uptake and can be used for active targeting.

Experimental

Cationic liposomes preparation

1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and (3β-[N-(N′,N′-dimethylaminoethane)-carbamoyl])-cholesterol (DC-Chol), dioleoylphosphocholine (DOPC), dioleoylphosphatidylethanolamine (DOPE) and DOPE-polyethyleneglycol (PEG) 2000 were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Liposomes were prepared in accordance with standard procedures [25] according with this molar ratio DOTAP:DOPC:DC-Chol:DOPE:DOPE-PEG2k (1:1:1:0.7:0.3).

Human Plasma

Human whole blood was obtained by venipuncture of ten healthy volunteers aged 20–40 years and treated according with the institutional bioethics code as elsewhere reported [26]. When used, aliquots were thawed at 4°C and then left to warm at room temperature.

Cell line

Human prostate cancer (PC3) cell line, derived from human bone prostate cancer metastasis, was purchased from ATCC (Manassas, VA, USA). PC3 cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/mL penicillinstreptomycin, 1 mM sodium pyruvate, 10 mM hepes, 1.5 mg/L sodium bicarbonate, and 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA).

Plasma membrane isolation

Liposomes were mixed with HP (1:1 v/v) and were incubated at 37 °C for 60 min. PC3 cells were seeded on 150 mm dishes and after 24 hours treated with liposomes and with liposome-HP complexes for 3 hours. Cells were washed twice with cold PBS, harvested in 3 mL of Buffer A (250 mM sucrose, 20 mM tricine, 1 mM EDTA, protease inhibitor, pH 7.8) and then centrifuged at 1,000 x g for 5 minutes at 4°C. The resulting pellet was re-suspended in 1 mL of Buffer A and broken by 50 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,000 x g for 10 minutes at 4°C. The resulting supernatant was stored on ice, whereas the pellet was re-suspended in 1 mL of Buffer A, homogenized and centrifuged at 1,000 x g for 10 minutes at 4°C. The two supernatants were collected together, layered on the top of 23 mL of 30% Percoll (Sigma-Aldrich) in Buffer A in 39 mL polycarbonate bottle and centrifuged at 84,000 x g for 30 minutes at 4°C. The pellet containing plasma membrane fraction was re-suspended and centrifuged at 105,000 x g for 30 minutes to remove Percoll. The resulting plasma membrane pellet was suspended in 100 uL of Buffer A.

Proteomics experiments

In solution digestion and desalting were performed as elsewhere reported [26]. Tryptic peptides were analyzed by a Dionex Ultimate 3000 (Sunnyvale, CA, USA) nanoLC system connected to the hybrid mass spectrometer LTQ Orbitrap XL (Thermo Fisher Scientific Bremen, Germany), equipped with a nanoelectrospray ion source. The experimental details are described in ref [27]. All samples were analyzed in triplicate to assess the variation due to the experimental procedure and to increase the number of identified proteins. Finally, Xcalibur (v.2.07, ThermoFisher Scientific) raw data files were submitted to Proteome Discover (1.2 version, Thermo Scientific) for database search using Mascot (version 2.3.2 Matrix Science). Data were searched against SwissProt database (57.15 version, 20266 sequences) using the decoy search option of Mascot. For peptide and protein validation, the peptide probability was set to minimum 95%, whereas the protein probability was set at 99%, as calculated by the Protein and Peptide Prophet algorithms implemented into Scaffold (version Scaffold_4.0.5, Proteome Software Inc., Portland, OR). Protein identifications were accepted if at least two unique peptides were present; proteins that contained shared peptides and could not be differentiated were grouped together. With these parameters, the resulting false discovery rate (FDR) for peptides and proteins was < 0.1% with no decoy identifications. For protein quantitative analysis, Scaffold software allows the normalization of the spectral countings (normalized spectral countings, NSCs) and offers various statistical tests to identify significant abundance differences in two or more categories. The mean value of NSCs from nine experimental replicates (three technical replicates of three different experimental samples) was calculated for each protein and then normalized to the protein molecular weight (MWNSC) to obtain the relative protein abundance (RPA) of each identified protein [12]. The error associated to the calculated RPAs was calculated as the standard deviation between the nine replicates.

Conclusions

In this study, we introduced a proteomics-based methodology to investigate the effect of the protein corona on the uptake of liposomes in cancer cells. Combining quantitative assessment of protein corona with differential evaluation of plasma protein profiles before and after liposome administration to PC3 cancer cells, we concluded that binding of proteins to the liposome surface results in a switch from a caveolae-mediated entry pathway to a receptor-mediated internalization. We expect this methodology will be used as an analysis to further validate the "protein corona effect for targeted drug delivery". This will also make possible prediction of the cellular internalization mechanism of liposomes in a physiological environment.

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Notes and references

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Electronic Supplementary Information (ESI) available: The full list of all the plasma proteins adhering on PEGylated MC liposome-HP as identified by NanoLC-MS/MS (Table S1); The full list of all identified peptides with the protein identification probability, the percentage of sequence coverage, the number of total and unique peptides, the peptide sequence, peptide identification probability and the peptide charge (Table S2). See DOI: 10.1039/c000000x/

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