Exploiting the "protein corona effect" for targeted drug and gene delivery

G. Caracciolo*, D. Pozzi*, A. L. Capriotti**, C. Cavaliere**, F. Cardarelli***, F. Salomone***, A. Laganà**

*Department of Molecular Medicine, "Sapienza" University of Rome, Viale Regina Elena 324, 00161, Rome, Italy
**Department of Chemistry, "Sapienza" University of Rome, P.le Aldo Moro 5, 00185, Rome, Italy
***Center for Nanotechnology Innovation @NEST, Istituto Italiano di Tecnologia, Piazza San Silvestro 12, 56127 Pisa, Italy

ABSTRACT

When nanoparticles (NPs) are exposed to biological fluids they adsorb biomolecules (mainly proteins) to form a protein corona. We have applied nanoliquid-cromatography mass spectroscopy (NanoLC-MS/MS) to quantitatively determine the proteins associated with several lipid NP formulations after incubation with human plasma (HP). By NanoLC-MS/MS we identified vitronectin as the most promising protein corona component for active targeting. To exploit the "protein corona effect" we investigated the interactions of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)/DNA cationic liposome/DNA complexes (lipoplexes) with target cells. By applying two-color fluorescence laser scanning confocal microscopy (LSCM) we demonstrate that vitronectin directs a receptor-mediated uptake of lipoplexes into target cells. This experiment sets the basis for a rational exploitation of the protein corona for targeted drug and gene delivery.

Keywords: protein corona, drug delivery, targeting, cell uptake, lipids

1 INTRODUCTION

Lipid nanoparticles (NPs) are core-shell-type nanocarriers with a drug core coated by single or multiple layers of lipids that constitute the shell [1]. Lipid NPs have emerged as a robust delivery platform which combines high loading of multiple therapeutic agents with controllable particle size and biocompatibility. However, lipid NPs have limitations from the viewpoint of physical and chemical stability. When administered in vivo, lipid NPs are covered by a rich protein layer, known as "protein corona" that changes the size, aggregation state, and interfacial properties of the NP, giving it a biological identity that is different from its original synthetic identity in term of size, curvature, shape, charge, hydrophobicity, surface chemistry, etc. [2,3]. Despite adverse implications (e.g. the opsonization process), favorable properties can be exploited such as the possibility to target cells via proteins associated to the NP-corona [4]. This novel targeting strategy needs the following requirements to work: (i) quantitative determination of the "protein corona" composition; (ii) identification of the proteins with the highest affinity for NP surfaces of different compositions or characteristics; (iii) understanding which plasma proteins effectively deliver NPs to which location (i.e. target cells over-expressing suitable receptors). Identification of the lipid NP-binding proteins has been greatly facilitated by recent advances in proteomics and mass spectrometry [5]. Here we have applied nanoliquid-cromatography mass spectroscopy (NanoLC-MS/MS) to identify the proteins associated with the corona of several lipid NP formulations after incubation with human plasma (HP). Since the surface properties of lipid NPs are largely determined by their lipid envelope [1], cationic liposomes (CLs) are commonly used for this purpose. CLs used in the present study were made of those lipid species that are more frequently used for drug and gene delivery purposes such as the cationic lipids 1,2-dioleoyl-3- trimethylammonium-propane (DOTAP) and 3β-[N-(*N',N'*-dimethylaminoethane)-carbamoyl]-cholesterol (DC-Chol) and the zwitterionic helper lipids dioleoylphosphatidylethanolamine (DOPE) and Cholesterol. Both non-specific and specific effects, which contribute to protein adhering to CLs, were identified. Finally the "protein corona effect for targeted drug delivery" was exploited by targeting cells that over-express receptors for the most abundant proteins found in the NP-corona.

2 MATERIALS AND METHODS

2.1 Chemicals, human plasma and cells

All chemicals were from Sigma Aldrich (St. Luis, MO, USA) unless otherwise stated. Organic solvents were from Carlo Erba Reagents (Milan, Italy). The sequencing grade modified trypsin was from Promega (Madison, WI, USA). Ultrapure water (resistivity 18.2 MΩ cm) was obtained by an Arium water purification system (Sartorius, Florence, Italy). Solid phase extraction (SPE) C18 cartridges were BOND ELUT (Varian, Palo Alto, CA, USA). Sample of human whole blood were obtained by venipuncture of ten healthy volunteers aged 20–40 years. HP was pooled and stored as described elsewhere [6]. For analysis, HP aliquots

were thawed at 4 °C and then allowed to warm at room temperature. Human embryonic kidney 293 (HEK293) cells were purchased from American Type Culture Collection (ATCC) and were grown in Dulbecco's modified medium (DMEM) supplemented with 10% of Fetal Bovine Serum at 37°C and in 5% CO_2. Plasmid transfection was performed 24 h before the experiment with 1 μg of DNA and lipofectamine reagent (Invitrogen, Carlsbad CA) according to the manufacturer's instruction.

2.2 Cationic liposomes

All the lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. DOTAP, DOTAP-DOPE, DOTAP-Cholesterol, DC-Chol, DC-Chol-DOPE and DC-Chol-Cholesterol unilamellar CLs (1 mg/mL) were prepared according to standard protocols [1]. All liposome dispersions were prepared keeping constant the moles of lipids.

2.3 Proteomics

After 1 hour incubation with HP, the samples were centrifuged to pellet the particle/protein complexes. The pellet was resuspended in the saline buffer, transferred into a new vial, and centrifuged again; this procedure was repeated three times. NanoLC-MS/MS experiments were performed by a Dionex Ultimate 3000 NanoLC (Dionex, Sunnyvale, CA, USA), equipped with a degasser and a thermostatted microwell-plate autosampler. Raw data files, obtained from Xcalibur software, were submitted to Proteome Discoverer (1.2 version, Thermo Scientific) for database search using Mascot (version 2.3.2 Matrix Science). Data were searched against human entries in the SwissProt protein database (57.15 version, 20266 sequences) selecting the built-in decoy option. Trypsin was specified as the proteolytic enzyme with up to two missed cleavages. Carbamidomethylation of cysteine and oxidation of methionine were set as fixed and variable modification, respectively. The monoisotopic mass tolerance for precursor ions and fragmentation ions were set to 10 ppm and 0.8 Da, respectively. To validate protein identifications derived from MS/MS sequencing results, the Mascot output files (.dat) were submitted in the commercial software Scaffold (v3.1.2, Proteome Software, Portland, Oregon, USA; http://www. proteomesoftware.com/). Scaffold tool to integrate Mascot identification results with X!Tandem search engine results (performed in automatic with the same parameters settled for Mascot) was used. Only protein identification based on mass spectra correlating to at least two unique tryptic peptides were considered; minimum peptide identification probability was set at 95 %, whereas protein identification probability was set at 99 %. For protein quantitative analysis, Scaffold software allows the normalization of the spectral countings (SCs) (normalized SCs, NSCs) and offers various statistical tests to identify significant abundance differences in two or more categories. The mean value of NSCs obtained in the three experimental replicates for each protein was further normalized to the protein molecular weight in kDa (MW) and expressed as the relative protein quantity (RPA) by applying the following equation:

$$RPA_k = \frac{(NSC/MW)_k}{\sum_{i=1}^{n}(NSC/MW)_i} \times 100 \qquad (1)$$

This correction takes into account the protein size and evaluates the actual contribution of each protein reflecting its relative protein abundance (RPA) in the corona [3].

2.4 Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) experiments were performed using a Leica TCS SP5 inverted confocal microscope (Leica Microsystems AG, Wetzlar, Germany), interfaced with an Ar laser for excitation at 458, 476, 488, and 514 nm, and with a He-Ne laser for excitation at 543 and 633 nm. Glass-bottom petri dishes containing transfected cells were mounted in a thermostated chamber (Leica Microsystems) and viewed with a 40 × 1.25 numerical aperture oil immersion objective (Leica Microsystems). Live cell imaging was always performed at 37 °C. Confocal images were collected using 0.65–10 kw/cm^2 excitation power at the sample and monitoring the emission by means of the Acousto-Optical Beam Splitter (AOBS)-based built-in detectors of the confocal microscopes.

3 RESULTS AND DISCUSSION

3.1 Protein identification

The RPA of all the proteins identified in the coronas of the six lipid formulations was calculated by Eq. 1. The most abundant plasma proteins adhering to DOTAP- and DC-Chol-containing CLs are reported in Table 1 and Table 2 respectively. For clarity, we restricted to classes of proteins and single proteins with RPA≥1 in at least one corona. To elucidate the role played by each lipid species on the corona formation, we calculated the ratio between the RPAs of every single protein in each couple of formulations, R. As a first step, we used R to compare the coronas of DOTAP–DOPE and DOTAP–cholesterol CLs with that of pure DOTAP ones (Fig. 1, top and bottom panel respectively). Remarkably, fibrinogen, prothrombin, vitamin K, and vitronectin coalesced on the same region of R (R≤0.5). This result suggests that substituting cationic DOTAP with neutral lipids, being indifferently DOPE or cholesterol, decrease the affinity of those proteins for the vesicle surface. Since fibrinogen, prothrombin, vitamin K, and vitronectin are negatively charged proteins at physiological pH, this finding is most likely to indicate that substituting

DOTAP molecules with neutral lipids decreases the net charge of the lipid vesicle making it less attractive for electrostatic binding of negatively charged proteins.

	RPA(%)		
	DOTAP	**DOTAP-DOPE**	**DOTAP-Cholesterol**
Apolipoprotein	13.7	29.6	17.6
Clusterin	2.3	3.7	0.9
Complement	4.8	2.5	7.4
Fibrinogen	10.8	2.9	5.9
Ig	6.6	6.2	11.1
Prothrombin	5.1	1.1	0.8
Serum Albumin	4.5	8.6	4.1
Vitamin-k	4.2	0.7	0.9
Vitronectin	13.3	8.9	2.7

Table 1: Relative protein abundance of the most abundant plasma proteins adhering to DOTAP-containing CLs after 1h incubation with HP as identified by nanoLC-MS/MS.

	RPA(%)		
	DC-Chol	**DC-Chol-DOPE**	**DC-Chol-Cholesterol**
Apolipoprotein	8.9	25.2	10.9
Clusterin	0.7	1.2	1.6
Complement	9.4	5.2	7.2
Fibrinogen	7.7	4.6	8.4
Ig	16.8	9.9	16.8
Prothrombin	4.1	0.7	3.0
Serum Albumin	4.7	5.5	4.6
Vitamin-k	6.1	0.4	3.9
Vitronectin	2.9	1.8	2.5

Table 2: The most abundant plasma proteins adhering to DOTAP-containing CLs after 1h incubation with HP as identified by nanoLC-MS/MS.

Fig. 1 clealry shows that DOPE promotes the adsorption of apolipoproteins and serum albumin (R > 1; Fig. 1, top panel), while cholesterol seems to induce the preferential binding of immunoglobulins (Ig) and complement proteins (R > 1; Fig. 1, bottom panel). Remarkably, when we compared the coronas of DC-Chol–DOPE and DC-Chol–cholesterol CLs with that of DC-Chol CLs by R, very similar results were obtained (not shown for space consideration). However, interactions other than electrostatic seem to be relevant for the corona formation. Indeed, the protein adsorption profiles of DOTAP and DC-Chol CLs were found to be largely different from each other. While DOTAP induces the preferential adsorption of vitronectin, DC-Chol promotes the binding of Ig and complement proteins. Furthermore, one more consideration regarding neutral lipids can be made. We notice that, when mixed with cationic lipids, DOPE modifies the protein adsorption profiles of DOTAP and DC-Chol CLs similarly, whereas the effect of Cholesterol is somewhat more complex.

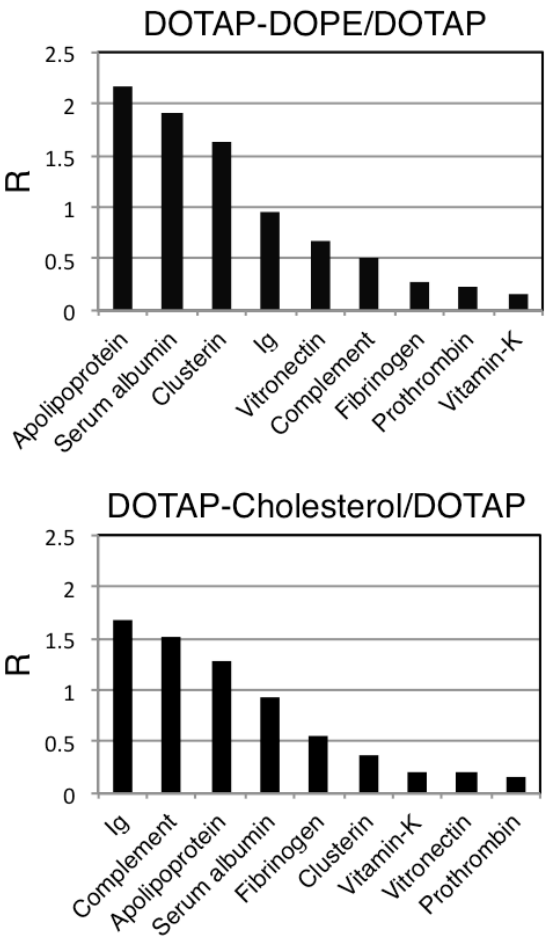

Figure 1: (top panel) The RPAs of the most abundant proteins found in the corona of DOTAP-DOPE and DOTAP CLs are compared by their relative ratio, R. (bottom panel) The RPAs of the most abundant proteins found in the corona of DOTAP-cholesterol and DOTAP CLs are compared by their relative ratio, R. RPAs were calculated by Eq. 1.

When mixed with DOTAP, Cholesterol strongly reduces the affinity of the lipid surface for vitronectin and vitamin-k. On the other side, in mixture with DC-Chl, Cholesterol has a minor effect, if any, in the protein profile. In summary our findings have provided the following body of evidence: i) non-specific interactions, which contribute to protein bio-conjugation, are mainly electrostatic; ii) plasma proteins bind specifically to functional chemical groups onto the lipid surface; iii) the affinity of each lipid species for plasma proteins is affected by the presence of other species in the lipid bilayer. Evidently, all these observations should be carefully considered to design lipid NPs for drug

delivery in vivo. Finally, we exploited the "protein corona effect for targeted delivery". To this end, the widely used DOTAP/DNA lipoplex system was chosen [1]. The presence of DNA at the lipid surface may affect the protein bioconiugation [7]. Thus, the protein corona of DOTAP/DNA lipoplexes was assessed quantitatevily by nanoLC-MS/MS (data not reported). After 1 h incubation with HP, vitronectin was found to be the most abundant protein in the corona (RPA > 31%). Vitronectin is a 75 kDa glycoprotein consisting of 459 amino acid residues. It contains an RGD (45-47) sequence, which is a binding site for membrane-bound integrins, e.g., the vitronectin receptor ($\alpha_v\beta_3$ integrins). To determine if vitronectin receptor could be responsible for the protein-mediated binding of DOTAP-DNA-protein complexes, we monitored the localization of Cy-3 labeled lipoplexes (red) in HEK-293 cells expressing GFP-conjugated integrins (green) using CLSM. We found that fluorescently labeled DOTAP/DNA lipoplexes exhibit minimal, if any, colocalization with GFP-conjugated integrin (Figure 2 top panel). These results demonstrate that the bare lipoplexes do not bind to vitronectin receptors before being internalized. On the other hand, DOTAP/DNA-protein complexes are mainly taken up by a vitronectin receptor-mediated mechanism (Figure 2, bottom panel). These results show that vitronectin receptors are necessary for the binding and subsequent uptake of the lipoplex-protein complexes.

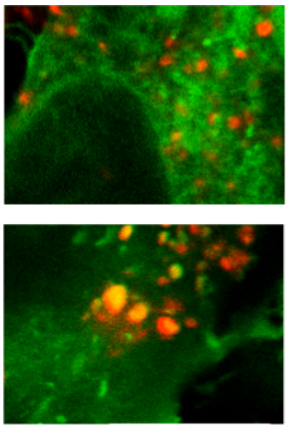

Figure 2: (Top panel) Representative confocal image of HEK cells expressing GFP-conjugated $\alpha_v\beta_3$ integrin treated with fluorescently labeled DOTAP/DNA lipoplexes (red). No clear evidence of fluorescence signals colocalization was found. (Bottom panel) Representative confocal image of HEK cells expressing GFP-conjugated $\alpha_v\beta_3$integrin treated with fluorescently labeled DOTAP/DNA-protein complexes (red). Large colocalization reveals that, in the presence of protein corona, DOTAP/DNA complexes enter HEK cells expressing $\alpha_v\beta_3$ integrin by a receptor-mediated mechanism.

In summary, NanoLC-MS/MS has allowed us to identify general principles regulating bio-nanointeractions between lipid NP and HP proteins. In one case vitronectin was identified as one of the most promising protein corona components for active targeting. We found that, in the presence of a vitronectin-rich protein corona, lipoplexes enter cells by a vitronectin receptor-mediated mechanism. In contemplating the possibility of using the bio–nano interface to effect the targeting, proteins other than vitronectin could be relevant. Among them, apolipoproteins were definitely the most abundant class of HP proteins associated with DC-Chol–DOPE CLs (Table 2). A protein corona rich in apolipoproteins may affect the NP interaction with cells, since lipoprotein complexes play a key role in the cellular processes of cholesterol metabolism. There are several receptors for apolipoprotein complexes on the cell surfaces, to which NPs with surface-decorated apolipoproteins can bind. For instance, apolipoproteins can promote interaction with low-density lipoprotein (LDL) receptors, resulting in transport across the blood-brain barrier. Experiments aimed at exploiting the protein corona effect to target cells over-expressing LDL receptors are currently under investigation in our laboratories.

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