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Differential analysis of "protein corona" profile adsorbed onto different nonviral gene delivery systems

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ABSTRACT

A shotgun proteomics approach was used to characterize and compare the proteins that lead to the formation of a rich "protein corona" adsorbed onto the surfaces of cationic liposomes (CLs), lipoplexes, and lipid/polycation/DNA (LPD) complexes, when they come into contact with plasma. After separation of the nanoparticle–protein complex from plasma, the protein mixture was digested, and peptides were analyzed by nanoliquid chromatography–Orbitrap LTQ-XL mass spectrometry. The number of proteins bound to lipoplexes was double that of those identified in the corona of CLs (208 vs 105), while 77 proteins were common to both coronas. The number of proteins bound to the surface of the LPD complexes (158, 133 of which are common to lipoplexes) is intermediate between those found in the protein corona of both CLs and lipoplexes. About half of them were found in the protein corona of CLs. By overlapping the three formulations, it can be seen that only 12 proteins are peculiar to LPD complexes. These results may help in designing gene delivery systems capable of binding the minimum possible quantity of proteins that influence transfection negatively, binding selectively proteins capable of helping in steering *in vivo* the vector toward the target, and obtaining more efficient and effective gene therapy.

One of the most important requirements in gene therapy is the development of safe and efficient gene delivery systems. Retroviruses, adenoviruses, and adeno-associated viruses are viral vectors that have shown a high transfection efficiency and are used in many clinical trials, but may be dangerous for routine clinical use [1]. In the past two decades, because of the advantages of their nonimmunogenicity, convenience of handling, and high capability for the delivery of genetic materials [2,3], nonviral vectors have attracted a growing interest in the scientific community. Cationic liposomes (CLs)[1] have been extensively studied as nonviral vectors since the first lipofection was reported in 1987 [4]. One of the most common nonviral gene delivery vectors is the DNA–cationic lipid complex (lipoplex). Much work has been done, with different experimental techniques, toward determining the lipoplex microstructure and stability, as well as on its transfection efficiency [5–14]. The cationic lipids used as transfection agents are indeed readily and easily metabolized by the target tissues [15–17]. One critical element for efficient gene delivery is the lipid composition of cationic liposomes, and many quaternary ammonium surfactants have been tried, including compounds with alkyl, ether, and ester bonds. Monocationic lipids are widely used to make most cationic carriers [17–19], and 1,2-dio-leoyl-3-trimethylammonium-propane (DOTAP), a double chain quaternary ammonium surfactant, is the most popular cationic lipid used in lipoplex formation. When cationic lipids are used as carriers of nucleic acids (RNA, DNA), periodic multilayer structures with DNA chains adsorbed between lipid membranes are often formed [7,20]. This multilayer structure offers protection from DNA degradation inside the cell; on the other hand, the DNA is not properly released from endosomal compartments. Recently, this problem was overcome by developing the lipid/polycation/DNA (LPD) complex, where the plasmid DNA (pDNA) is condensed with a polycation and encapsulated by a lipid envelope [21]. It has been shown that the gene transfer mediated by the LPD complex works better than the conventional gene transfer using liposomes for delivering a gene into the liver [22]. Medical administration of these gene delivery vectors is frequently carried out by parenteral injection. Therefore, on exposure to biological media, the administered gene delivery vectors are immediately covered by plasma proteins that lead to the formation of a rich "protein corona" [23–25]. Recent studies have shown that the binding of plasma

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[1] *Abbreviations used:* CLs, liposomes; CT, calf thymus; DOTAP, 1,2-dio-leoyl-3-trimethylammonium-propane; DTT, 1,4-dithiothreitol; IAA, iodoacetamide; LPD, lipid/polycation/DNA; MPS, mononuclear phagocytic system; SUVs, small unilamellar liposomes; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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proteins to nanoparticles, such as gene delivery vectors, is a critical step in determining their fate in vivo [26,27]. Moreover, plasma proteins play an important role in the identification of foreign bodies in the bloodstream. Macrophages of the mononuclear phagocytic system (MPS) remove unprotected nanoparticles from the bloodstream within seconds of intravenous administration, thus rendering them ineffective as site-specific drug delivery devices [28]. Several methods have been developed to camouflage or mask nanoparticles, allowing them to temporarily bypass recognition by the MPS and increase their blood circulation half-life [28–30]. From these examples it is clear that nanoparticle–protein interactions are important for understanding the circulation, clearance rates, blood half-life, stability, immunogenicity, and organ biodistribution of nanoparticles. Moreover, in order to fully realize the biomedical value of the gene delivery vectors it is important to improve their functionality in the biological environment by studying the nanoparticle–protein interactions.

In the present study, we compare the binding of human plasma proteins onto the surface of DOTAP CLs, DOTAP/DNA lipoplexes, and DOTAP/protamine/DNA complexes. For this purpose, we have employed a shotgun proteomics approach based on centrifugation for separating the nanoparticle–protein complex, followed by "in-solution" proteolytic digestion of the whole protein mixture, and determination of the resulting peptides by nano-high-performance liquid chromatography (nano-HPLC) coupled with a high-resolution Orbitrap LTQ-XL mass spectrometer.

To the authors' best knowledge, this is the first study using this approach to characterize the protein corona of cationic liposomes, lipoplexes, and lipid/polycation/DNA complexes. We found that these nanoparticles are capable of binding different plasma protein categories with important biological functions, such as lipoproteins, immunoglobulins, acute-phase proteins, proteins which play an essential role in protein synthesis, proteins strongly related to cellular activity, and proteins involved in complement pathways and coagulation. These results could help in designing gene delivery systems capable of binding selectively certain proteins rather than others, and of steering their biodistribution *in vivo* so as to obtain a more efficient and effective gene therapy.

Materials and methods

Reagents and chemicals

DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL), and used without further purification. Calf thymus (CT) Na–DNA and protamine sulfate salt (P) from salmon (MW = 5.1 kDa) were purchased from Sigma–Aldrich (St. Louis, MO). Tris(hydroxymethyl)aminomethane (Tris), sodium chloride, polyacrylamide ethylenediaminetetraacetic acid (EDTA), iodoacetamide (IAA), 1,4-dithiothreitol (DTT), ammonium bicarbonate, and Coomassie PhastGel Blue R-350 were purchased from GE Healthcare (Amersham Biosciences, Uppsala, Sweden). All organic solvents were the highest grade available from Carlo Erba Reagents (Milan, Italy). Ultrapure water was produced from distilled water by a Milli-Q system (Millipore Corporation, Billerica, MA, USA). Protein LoBind tubes were obtained from Eppendorf (Hamburg, Germany). Porcine trypsin (modified, sequencing grade) was commercialized by Promega (Madison, WI, USA).

Human plasma collection, preparation, and storage

Human whole blood was obtained from the Experimental Medicine Department (Sapienza University of Rome) by venipuncture of healthy volunteers aged 20–40 years, by means of a BD P100 Blood Collection System (Franklin Lakes, NJ, USA), with protease inhibitors cocktail and K_2EDTA anticoagulant. Each tube was turned upside down 10 times to ensure mixing of blood with the K_2EDTA and, immediately after sediment formation, centrifuged for 10 min at 1000*g* for 5 min to pellet the blood cells. After verifying the absence of hemolysis, the supernatant plasma was removed and pooled to reduce the overall subject-to-subject variation and to reduce differences between individuals. The pooled plasma was split into 200-μL aliquots, and stored at −80 °C in labeled Protein LoBind tubes to ensure plasma stability during storage. When plasma was used for experiments, aliquots were thawed at 4 °C and then allowed to warm at room temperature (RT).

Liposomes

DOTAP CLs were prepared according to standard protocols [31]. Briefly, 5 mg of DOTAP was 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 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}). Small unilamellar liposomes (SUVs), with mean diameter around 100 nm, were produced by sonication to clarity.

Lipoplexes

Self-assembled DOTAP/DNA lipoplexes, at a single lipid/DNA volume ratio (R_v) (R_v = DOTAP/DNA (vol/vol) = 1) were obtained by mixing 20 μL of the 1 mg mL^{-1} CT DNA solution to 100 μL of CLs SUVs dispersion. At this volume ratio, lipoplexes exhibited the lowest colloidal dimensions (about 200 nm, data not reported) and were positively charged (about 50 mV, data not reported) [32].

LPD complexes

Negatively charged P/DNA microspheres were prepared at a protamine/DNA weight ratio (R_w) of 0.5 [32]. LPD complexes were prepared by mixing P/DNA microspheres with DOTAP SUVs at a single lipid/DNA volume ratio (R_v = DOTAP/DNA (vol/vol) = 1). At this volume ratio, LPD systems exhibited the lowest colloidal dimensions (about 200 nm, data not reported), and were positively charged (about 45 mV, data not reported).

Incubation of CLs, lipoplexes, and LPD complexes with plasma and centrifugation

The incubation procedure was conducted as previously described [32], with minor modifications. Two hundred microliters of CL suspension (1 mg mL^{-1}) was added to 200 μL of plasma in the dissolving buffer and incubated at 37 °C for 1 h to promote aggregation of the plasma proteins onto the surface of nanoparticles. The same procedure was used for lipoplexes and LPD complexes. The samples were centrifuged at 15,000*g* for 10 min to pellet the nanoparticle–protein complexes. The pellet was washed three times with 250 μL of the dissolving buffer, using a vortex mixer, and then the sample was transferred into a new Protein LoBind tube and centrifuged again to pellet the nanoparticle–protein complexes. The tubes were changed after each washing step to minimize contamination from plasma proteins bound to the tube walls, and plasma without nanoparticles was used as a control to ensure there was no protein precipitation.

In-solution trypsin digestion

The enzymatic digestion procedure was conducted as previously described [32]. Briefly, the nanoparticle–protein complexes were resuspended in 40 μL of 8 mol L^{-1} urea solution in