

# Analysis of plasma protein adsorption onto DC-Chol-DOPE cationic liposomes by HPLC-CHIP coupled to a Q-TOF mass spectrometer

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**Abstract** Plasma protein adsorption is regarded as a key factor in the *in vivo* organ distribution of intravenously administered drug carriers, and strongly depends on vector surface characteristics. The present study aimed to characterize the “protein corona” adsorbed onto DC-Chol-DOPE cationic liposomes. This system was chosen because it is one of the most efficient and widely used non-viral formulations *in vitro* and a potential candidate for *in vivo* transfection of genetic material. After incubation of human plasma with cationic liposomes, nanoparticle–protein complex was separated from plasma by centrifugation. An integrated approach based on protein separation by one-dimensional 12% polyacrylamide gel electrophoresis followed by the automated HPLC-Chip technology coupled to a high-resolution mass spectrometer was employed for protein corona characterization. Thirty gel lanes, approximately 2 mm, were cut, digested and analyzed by HPLC-MS/MS. Fifty-eight human plasma proteins adsorbed onto DC-Chol-DOPE cationic liposomes were identified. The knowledge of the interactions of proteins with liposomes can be exploited for future controlled design of colloidal drug carriers and possibly in the controlled creation of biocompatible surfaces of other devices that come into contact with proteins in body fluids.

**Keywords** Cationic liposomes · HPLC-Chip · Mass spectrometry · Nanoparticles · Plasma · Protein corona

## Introduction

In modern molecular medicine, a front of very promising research is gene therapy, a procedure that transfers genetic material to prevent or cure a disease. Safe, efficient and specific delivery of therapeutic genes remains an important bottleneck in the development of gene therapy [1, 2]. Ideally, an efficient site-specific gene delivery system should be stable, biocompatible, nontoxic, cost effective, and capable of transferring exogenous highly anionic genetic materials in tissue specific sites. The limitations of viral vectors, in particular, their relatively small capacity for therapeutic DNA, safety concerns, and difficulty in targeting to specific cell types, have led to evaluation and development of alternative vectors based on synthetic, non-viral delivery systems. The last three decades have seen tremendous developments in various non-viral gene delivery systems like cationic liposomes, surfactant vesicles, and polymers. The latter became popular after the introduction of a second generation of cationic polymers, i.e., polyethylenimines by Behr and co-workers [3], including also polyallylamine, poly-L-lysine, chitosan, etc. [4–6]. A major drawback of cationic polymer–DNA complexes (polyplexes) is their significantly low efficiency in the presence of serum.

Complexes between cationic lipids and nucleic acids (lipoplexes) are typically prepared by simple mixing of preformed cationic liposomes and DNA in an aqueous solution. Electrostatic interactions between the positive charges of the cationic lipid headgroups and the phosphate DNA backbones are the main driving force of the lipoplex

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formation. A major goal of gene therapy is to obtain targeted vectors for transferring genes efficiently into specific cell types. Lipoplexes for such medical applications are frequently given via parenteral administration. As with any foreign material, the body mounts a biological response to an administered nanovector. In particular, lipoplexes, upon contact with biological matrices such as plasma, are immediately coated by proteins, leading to a “protein corona” [7–9]. The nature of the proteins that interact with specific lipoplex formulations depends on the particle size as well as their surface characteristics [10].

Recently, the binding of plasma proteins to nanoparticles has been identified as a critical step in determining their fate *in vivo* [11, 12]. Moreover, plasma proteins play an important role in recognizing foreign bodies that enter circulation. The macrophages of the mononuclear phagocytic system have the ability to remove unprotected nanoparticles from the bloodstream within seconds of intravenous administration, rendering them ineffective as site-specific drug delivery devices [13]. These macrophages, which are typically Kupffer cells, or macrophages of the liver, cannot directly identify the nanoparticles themselves, but rather recognize specific opsonin proteins bound to the surface of the particles [14]. Several methods of camouflaging or masking nanoparticles have been developed, which allow them to temporarily bypass recognition by the mononuclear phagocytic system and increase their blood circulation half-life [13, 15, 16]. Nanoparticle–protein interactions are important for understanding nanoparticle circulation, clearance rates, blood half-life, stability, immunogenicity and organ biodistribution. Improving the functionality of nanoparticles in the biological environment is a priority in order to fully realize their biomedical value. This is possible by studying nanoparticle–protein interactions.

The present study aimed to gain information about protein adsorption on 3-[*N,N,N*-dimethylaminoethane]-carbamoyl] cholesterol (DC-Chol)-dioleoylphosphatidylethanolamine (DOPE) cationic liposomes. This system was chosen because it is one of the most efficient and widely used non-viral formulations *in vitro* [17, 18], and a potential candidate for *in vivo* transfection [19].

Nowadays, high-resolution mass spectrometry (MS) coupled to nano-high-performance liquid chromatography (HPLC) systems is the technique of choice in protein characterization, particularly in analyzing small amounts of biological samples. For this purpose we used an integrated approach based on protein separation by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) followed by the automated HPLC-Chip technology coupled with a quadrupole-time of flight (Q-TOF) mass spectrometer to identify the adsorbed proteins on DC-Chol-DOPE cationic liposomes.

## Experimental

### Chemicals and reagents

Ethylenediaminetetraacetic acid (EDTA), tris (hydroxymethyl)aminomethane (TRIS), sodium chloride (NaCl), polyacrylamide, 1,4 dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ), sodium dodecyl sulfate (SDS), 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). Modified porcine trypsin, sequencing grade, was commercialized by Promega (Madison, WI, USA).

### Cationic liposomes preparation

The cationic lipid 3-[*N,N,N*-dimethylaminoethane]-carbamoyl] cholesterol (DC-Chol) and the neutral ‘helper’ lipid dioleoylphosphatidylethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purifications. The liposome solution was prepared by solving appropriate amount of DC-Chol and DOPE in  $\text{CHCl}_3$ , so that the molar ratio of neutral lipid in the bilayer was  $\Phi=0.5$  (neutral lipid/total lipid). The solvent was evaporated under a stream of nitrogen and then under vacuum for 12 h. Multilamellar vesicles were formed by hydrating the dry lipid film with  $10 \text{ mmolL}^{-1}$  Tris-HCl (pH 7.4),  $150 \text{ mmolL}^{-1}$  NaCl and  $1 \text{ mmolL}^{-1}$  EDTA; the final lipid concentration was  $1 \text{ mg mL}^{-1}$ . The obtained liposome solution was sonicated to clarity and stored at  $30 \text{ }^\circ\text{C}$  for 48 h to achieve full hydration.

### Human plasma samples

Samples of human whole blood were obtained from Department of Experimental Medicine (Sapienza University of Rome) by venipuncture of ten healthy volunteers aged 20–40 years, by means of BD™ P100 Blood Collection System (Franklin Lakes, NJ, USA) with  $\text{K}_2\text{EDTA}$  anticoagulant and protease inhibitors cocktail. Human plasma was prepared in the usual way; finally, plasma from donors was pooled, split into  $100 \text{ }\mu\text{L}$  aliquots, and stored at  $-80 \text{ }^\circ\text{C}$  in labeled Protein LoBind tubes, until further use. For analysis, the aliquots were thawed at  $4 \text{ }^\circ\text{C}$  and then allowed to warm at room temperature.

### Cationic liposomes incubation with human plasma

Plasma protein binding to nanoparticles was studied by incubating  $100 \text{ }\mu\text{L}$  of cationic liposome suspension

(1 mg mL<sup>-1</sup>) in 10 mmol L<sup>-1</sup> Tris-HCl (pH 7.4), 150 mmol L<sup>-1</sup> NaCl and 1 mmol L<sup>-1</sup> EDTA with 100 μL of plasma in an ice bath for 1 h. Thereafter, the incubation was carried on at 25 °C for 1 h to promote aggregation. The sample was centrifuged at 15 kRCF for 10 min to pellet the liposome-protein complexes. The pellet was washed with 250 μL of 10 mmol L<sup>-1</sup> Tris-HCl (pH 7.4), 150 mmol L<sup>-1</sup> NaCl and 1 mmol L<sup>-1</sup> EDTA, using a vortex mixer, transferred into a new Protein LoBind tube, and centrifuged again to pellet the liposome-protein complexes; this procedure was repeated twice. The tubes were changed after each washing step to minimize contamination of plasma proteins bound to the tubes. A plasma aliquot not incubated with liposomes was subjected to the same procedure as control to verify the absence of protein precipitation.

#### Protein separation by 1D-PAGE

Proteins were separated by 1D-PAGE according to the original protocol by Laemmli [20] with some modifications. Proteins were desorbed from liposomes by adding SDS-PAGE sample buffer (62.5 mmol L<sup>-1</sup> Tris-HCl, pH 6.8; 2% w/v SDS; 10% v/v glycerol; 2.5% v/v β-mercaptoethanol; 0.0025% w/v bromophenol blue) to the pellet and boiling the solution. A 4% polyacrylamide gel was used as stacking gel, and a 12% polyacrylamide gel as separating gel. Electrophoresis was carried out at constant current (30 mA gel<sup>-1</sup>) in electrophoresis buffer, until the dye front reached the lower end of the gel (1.5 h). Proteins were fixed on the gels with H<sub>2</sub>O/CH<sub>3</sub>OH/CH<sub>3</sub>COOH (50:45:5, v/v/v) for 1 h under gentle agitation. Coomassie PhastGel Blue R-350 was used to stain the gels under agitation, according to the manufacturer's manual (GE Healthcare). All the experiments were repeated five times to verify the reproducibility of the liposome-protein complex pellet sizes, general pattern, and band intensities on the 1D gels.

#### In-gel digestion

Thirty gel lanes, approximately 2 mm, were cut with a manual microtome into consecutive slices from the pre-frozen, stained gel. The protocol for in-gel tryptic digestion was based on that developed by Shevchenko et al. [21], with some modifications. The procedure was optimized for polyacrylamide gels of a 1 mm thickness. The Coomassie-stained slices were destained into Protein LoBind tubes; each gel piece was rinsed once for 30 min with 100 μL of 25 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub>/CH<sub>3</sub>CN (95:5, v/v), and twice for 30 min with 100 μL of 25 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub>/CH<sub>3</sub>CN (50:50, v/v), then the gel pieces were dehydrated for 10 min with 200 μL of CH<sub>3</sub>CN. Thereafter the solvent was removed and the gel slices dried in a vacuum centrifuge. The proteins were reduced for 1 h at 56 °C with a volume

of 10 mmol L<sup>-1</sup> DTT in 100 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> sufficient to cover the gel pieces. Then, the DTT was replaced with the same volume of 55 mmol L<sup>-1</sup> IAA in 100 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> to alkylate the proteins, and the gel pieces were incubated for 45 min at room temperature in the dark with occasional vortexing. Finally, the gel pieces were washed with 100 μL of 100 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> for 10 min, dehydrated by addition of CH<sub>3</sub>CN, and swelled by rehydration in 100 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub>, and shrunk again by addition of the roughly same volume of CH<sub>3</sub>CN. The liquid phase was removed, and the gel pieces were completely dried in a vacuum centrifuge. The enzymatic digestion was therefore performed at 37 °C, overnight, by adding a digestion buffer containing 15 ng μL<sup>-1</sup> of trypsin in 50 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub>. After 45 min, the supernatant was removed and replaced with a volume of 50 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> sufficient to cover the gel pieces and keep it wet during enzymatic cleavage. The resulting peptides were extracted in several steps; the supernatant of trypsin hydrolysis was transferred to a new Protein LoBind tube and the gel slices were extracted with 25 μL of 50 mmol L<sup>-1</sup> NH<sub>4</sub>HCO<sub>3</sub> and twice with 25 μL of CH<sub>3</sub>CN/H<sub>2</sub>O/HCOOH (50:45:5, v/v/v). For each extraction, the gel slices were incubated for 15 min at room temperature while shaking. The supernatants of each extraction were pooled with the trypsin digest supernatant and dried in a vacuum centrifuge. The peptides were resuspended in 25 μL of loading buffer with 0.1% (v/v) HCOOH prior to LC-MS/MS analysis. A “control” piece of gel was cut from a blank region and processed in parallel with the sample.

#### LC-MS conditions

LC was performed using an Agilent series 1200 instrument (Agilent Technologies, Waldbronn, Germany) consisting of a nanopump with degasser, and a capillary pump connected to a thermostatted microwell-plate autosampler. An Agilent G4240 ChipCube was used as the chip interface to the mass spectrometer. The following components were integrated onto the customized HPLC-Chip G4240-63001 (Agilent Technologies, Santa Clara, CA, USA): a 500-nL enrichment column and a 75 μm × 150 mm separation column, both packed with reversed phase ZORBAX 80 SB-C18, 5 μm particle size; and a nanospray emitter (50 μm i.d.).

For fractionation of peptides, phase A was H<sub>2</sub>O:CH<sub>3</sub>CN 95:5 (v/v) and phase B was CH<sub>3</sub>CN:H<sub>2</sub>O 95:5 (v/v), both containing 0.1% (v/v) HCOOH, set to a flow rate of 300 nL min<sup>-1</sup>. The initial composition of the LC mobile phase was 5% B, linearly increased to 45% over 50 min; afterwards, B was increased to 90% over 10 min, and kept constant for 5 min to rinse the column. Finally, the B content was restored to 5% over 5 min, and the column re-equilibrated for 10 min.

The capillary pump was operated at a flow rate of 4  $\mu\text{Lmin}^{-1}$ , using as mobile phases  $\text{H}_2\text{O}$  (C) and  $\text{CH}_3\text{CN}$  (D), both containing 0.1% (v/v)  $\text{HCOOH}$ . Enrichment of the peptide mixture prior to gradient start was performed with 0% D. A 2  $\mu\text{L}$  aliquot of sample was loaded onto the Chip device by the autosampler. The intelligent sample loading feature of the HPLC-Chip ChemStation menu was used, which allowed sample loading onto the enrichment column during the pre-run time. After sample enrichment, the rotary valve interface was switched to the load position and then elution gradient started, at the same time an elution gradient with the capillary pump was done to rinse the enrichment column.

The mass spectrometer was an Agilent 6520 Q-TOF equipped with an electrospray ionization (ESI) source. The Agilent Mass Hunter ChemStation software (version B03.00) was used for data acquisition and processing.

Ionization and mass spectrometric conditions were optimized for the analytes by infusing, at 600  $\text{nLmin}^{-1}$ , a 100  $\text{pg}\mu\text{L}^{-1}$  standard peptide mixture in  $\text{H}_2\text{O}/\text{CH}_3\text{CN}$  90:10 (v/v) containing 0.1% (v/v)  $\text{HCOOH}$ , using a MS calibration and diagnostic Chip (G4240-61001, Agilent Technologies).

Daily mass calibration and resolution adjustments of TOF were performed automatically (software managed) utilizing the Agilent tuning mix.

The ESI source was operated in positive ion mode by applying a potential of 1,820 V to the capillary; the fragmentor voltage was 175 V, and the skimmer voltage was 65 V. Nitrogen was used as a drying gas (5  $\text{Lmin}^{-1}$ , heated at 325  $^\circ\text{C}$ ) and collision gas; air was used for ChipCube applications and set to 1.5  $\text{Lmin}^{-1}$ .

Mass spectra were acquired in a data-dependent manner, automatically switching between MS ( $m/z$  range 100–2000) and MS/MS ( $m/z$  range 50–2000). The three most intense multiprotonated molecules (charge preference: 2>3) above the threshold of 200 counts  $\text{s}^{-1}$  were selected for collision-induced fragmentation, using a collision energy corresponding to 3.6 V per 100  $m/z$  units. Per selected precursor, two MS/MS spectra could be generated using the applied 10 s dynamic exclusion window.

All the samples were analyzed in triplicate.

## Data analysis

Deisotoped peak lists (\*.mzData.xml) for database searching were generated from the raw data using Agilent Masshunter software (B01.03) by applying the algorithm “Find by Auto MS/MS” (positive MS/MS TIC threshold 1,000 counts, mass match tolerance 0.05  $m/z$ ). The in-house software “mzData Union—Customize” was used to merge the mzData files obtained and to remove optional information (further details are reported in [Electronic Supplementary Material](#)).

Significance threshold was set to  $p < 0.05$ .

Mascot software (version 2.2.04, Matrix Science, London, UK) was used for database searching against all human proteins from the Swissprot database (version 57.7). Precursor mass tolerance for database searching was set to 5 ppm, fragment mass tolerance was 0.3 Da, and trypsin specificity was applied with a maximum of one missed cleavage. Carbamidomethyl-cysteine was used as a fixed modification, whereas oxidation of methionine, and N-terminal Gln to pyroGlu conversion were selected as variable modifications. Identified proteins with the main data provided by the Mascot search engine are reported in Table 1.

## Results and discussion

### Separation of nanoparticle–protein complex from plasma

Recent studies have shown that the effective unit of interest in cell–nanoparticle interaction is not the nanoparticle per se, but the nanoparticle and its associated protein corona deriving from plasma or other body fluids, which together form the nanoparticle–protein complex [7–25]. Many methods are commonly used to study nanoparticle–protein interactions [7, 26–31]. Centrifugation is, to date, the preferred method for isolating nanoparticle–protein, although the results may be influenced by washing conditions and centrifugation time [29, 31]. In fact, high abundance proteins in plasma may be identified as associated with the nanoparticle because of insufficient washing. Furthermore, after a long centrifugation time, sedimentation of large proteins, formation of protein aggregates, and co-precipitation may further complicate the picture [31]. Nevertheless, there are only few papers reporting other methods for protein isolation from nanoparticles, such as gel filtration, magnetic separation, and microfiltration [29, 31].

Here we are interested in the proteins strongly adsorbed onto liposomes, so-called “hard corona” [7, 29], whose biological macromolecules have a high affinity for the liposome surface. To achieve this we used stringent washing conditions, after centrifuging the plasma incubated with liposomes to obtain the pellet of the liposome–protein complex. It was washed vigorously three times in 250  $\mu\text{L}$  of 10  $\text{mmolL}^{-1}$  Tris–HCl, pH 7.4, 150  $\text{mmolL}^{-1}$  NaCl and 1  $\text{mmolL}^{-1}$  EDTA, using a vortex mixer to remove all unbound proteins. We also used a short centrifugation time, just 2 min, to avoid sedimentation of large proteins, formation of protein aggregates, and co-precipitation. The large number of proteins identified (see Table 1) as components of “protein corona” suggests that most proteins remained bound to liposomes despite three vigorous washes.

**Table 1** Proteins identified by one-dimensional gel electrophoresis followed by HPLC-Chip ESI-Q-TOF MS/MS analysis

Protein name	UniProt Protein ID	Molecular mass	Mowse Score	Unique peptides	Queries matched	Sequence coverage (%)	Subcellular location
Actin cytoplasmic 1	P60709 ACTB_HUMAN	42,052	1,712	21	83	63	Cytoplasm > cytoskeleton
Alpha-2-antiplasmin	P08697 A2AP_HUMAN	54,873	94	4	4	9	Secreted
Alpha actinin 1	P12814 ACTN1_HUMAN	103,563	407	18	26	25	Cytoplasm > cytoskeleton. cytoplasm > myofibril > sarcomere > Z-disk
Apolipoprotein A-I	P02647 APOA1_HUMAN	30,759	557	17	27	63	Secreted
Apolipoprotein B-100	P04114 APOB_HUMAN	516,666	280	17	20	4	Secreted
Apolipoprotein C-II	P02655 APOC2_HUMAN	11,277	448	6	16	56	Secreted
Apolipoprotein C-III	P02656 APOC3_HUMAN	10,845	1,124	3	37	34	Secreted
Apolipoprotein D	P05090 APOD_HUMAN	21,547	1,200	10	79	38	Secreted
Apolipoprotein F	Q13790 APOF_HUMAN	33,727	173	1	5	5	Secreted
Apolipoprotein(a)	P08519 APOA_HUMAN	514,737	64	2	3	0.5	–
C4b-binding protein alpha chain	P04003 C4BPA_HUMAN	69,042	677	25	41	46	Secreted
Coagulation factor XIII A chain	P00488 F13A_HUMAN	83,728	62	4	4	6	Cytoplasm Secreted Note: Secreted into the blood plasma
Cofilin-1	P23528 COF1_HUMAN	18,719	261	6	7	48	Nucleus matrix. Cytoplasm > cytoskeleton
Complement C3	P01024 CO3_HUMAN	188,569	503	35	40	21	Secreted
Complement C4-A	P0C0L4 CO4A_HUMAN	194,247	242	11	13	5	Secreted
C-reactive protein	P02741 CRP_HUMAN	25,194	122	6	12	27	Secreted
Dermcidin	P81605 DCD_HUMAN	11,391	171	4	14	30	Secreted
Fibrinogen beta chain	P02675 FIBB_HUMAN	56,577	111	8	12	14	Secreted
Fibrinogen gamma chain	P02679 FIBG_HUMAN	52,106	101	7	10	19	Secreted
Filamin-A	P21333 FLNA_HUMAN	283,301	730	32	50	16	Cytoplasm > cell cortex. Cytoplasm > cytoskeleton
Fructose-bisphosphate aldolase A	P04075 ALDOA_HUMAN	39,851	88	5	6	16	–
Gelsolin	P06396 GELS_HUMAN	86,043	183	15	17	17	Isoform 1: Secreted
Glyceraldehyde-3-phosphate dehydrogenase	P04406 G3P_HUMAN	36,201	264	8	14	25	Cytoplasm. Cytoplasm > perinuclear region. Membrane
Hemoglobin subunit alpha	HBA_HUMAN	15,305	576	11	42	88	–
Hemoglobin subunit beta	P68871 HBB_HUMAN	16,102	1,265	12	60	86	–
Hyaluronan-binding protein 2	Q14520 HABP2_HUMAN	64,740	1,471	22	122	34	Secreted
Ig gamma-1 chain C region	P01857 IGHG1_HUMAN	36,596	187	4	17	28	Secreted
Ig gamma-2 chain C region	P01859 IGHG2_HUMAN	36,505	153	3	11	18	–
Ig kappa chain C region	P01834 IGKC_HUMAN	11,773	870	5	29	80	Extracellular region
Ig kappa chain V-II region RPMI 6410	P06310 KV206_HUMAN	14,811	156	2	4	15	–
Ig kappa chain V-III region SIE	P01620 KV302_HUMAN	11,882	118	3	6	51	Extracellular region
Ig lambda chain C regions	P01842 LAC_HUMAN	11,401	602	6	17	81	–
Ig mu chain C region	P01871 IGHM_HUMAN	49,960	53	5	5	9	Isoform 1: Secreted
Integrin alpha-IIb	P08514 ITA2B_HUMAN	114,446	602	18	25	21	Membrane > Single-pass type I membrane protein



**Table 1** (continued)

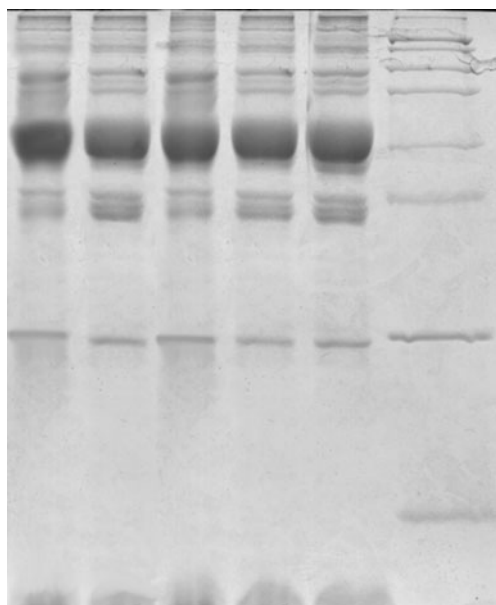
Protein name	UniProt Protein ID	Molecular mass	Mowse Score	Unique peptides	Queries matched	Sequence coverage (%)	Subcellular location
Integrin beta-3	P05106 ITB3_HUMAN	90,194	63	4	5	5	Membrane; Single-pass type I membrane protein
Inter-alpha-trypsin inhibitor heavy chain H1	P19827 ITIH1_HUMAN	101,782	651	11	25	13	Secreted
Inter-alpha-trypsin inhibitor heavy chain H2	P19823 ITIH2_HUMAN	106,853	801	19	69	20	Secreted
Inter-alpha-trypsin inhibitor heavy chain H3	Q06033 ITIH3_HUMAN	100,072	347	13	19	15	Secreted
L-lactate dehydrogenase B chain	P07195 LDHB_HUMAN	36,900	85	6	6	19	Cytoplasm
Platelet basic protein	P02775 CXCL7_HUMAN	14,171	104	5	11	38	Secreted
Platelet factor 4	P02776 PLF4_HUMAN	11,123	197	4	17	35	Secreted
Platelet glycoprotein Ib beta chain	P13224 GP1BB_HUMAN	22,274	74	4	9	19	Membrane; Single-pass type I membrane protein.
Profilin-1	P07737 PROF1_HUMAN	15,216	305	5	11	47	Cytoplasm > cytoskeleton
Protein AMBP	P02760 AMBP_HUMAN	39,886	451	5	17	14	Secreted
Prothrombin (Coagulation factor II)	P00734 THRB_HUMAN	71,475	1,740	27	83	49	Secreted > extracellular space
Ras-related protein Rap-1b	P61224 RAP1B_HUMAN	21,040	665	8	22	45	Cell membrane. Cytoplasm > cytosol
Secreted phosphoprotein 24	Q13103 SPP24_HUMAN	24,607	122	4	7	20	Secreted
Serum albumin	P02768 ALBU_HUMAN	71,317	1,630	34	83	51	Secreted
Superoxide dismutase [Mn], mitochondrial	P04179 SODM_HUMAN	24,878	81	3	5	19	Mitochondrion matrix
Talin-1	Q9Y490 TLN1_HUMAN	271,766	986	38	50	17	Cell projection > ruffle membrane; Peripheral membrane protein; Cytoplasmic side
Thrombospondin-1	P07996 TSP1_HUMAN	133,291	343	17	32	15	–
Tropomyosin alpha-4 chain	P67936 TPM4_HUMAN	28,619	300	13	26	44	Cytoplasm > cytoskeleton
Tubulin alpha-1B chain	P68363 TBA1B_HUMAN	50,804	70	4	5	16	–
Vinculin	P18206 VINC_HUMAN	124,292	242	20	30	19	–
Vitamin K-dependent protein C	P04070 PROC_HUMAN	53,406	117	4	7	8	–
Vitamin K-dependent protein S	P07225 PROS_HUMAN	77,127	269	15	28	21	Secreted
Vitronectin (Serum-spreading factor)	P04004 VTNC_HUMAN	55,069	5,170	23	280	41	Secreted > extracellular space
14-3-3 protein zeta/delta	P63104 1433Z_HUMAN	27,899	390	12	19	56	–

### Protein separation by 1D gel electrophoresis

In literature, a common method for separating and identifying the plasma proteins bound to nanoparticles is the two-dimensional (2D)-PAGE separation followed by comparison to a known 2D master map of human plasma proteins [31]. Alternatively, gel filtration with size-exclusion or affinity chromatography is employed for protein separation.

In the present work, we preferred to use 1D-PAGE rather than 2D-PAGE. Although 1D-PAGE resolution is limited

because proteins are resolved in one dimension according only to their molecular weight, it is suitable for proteins with unusual physicochemical properties, like hydrophobic proteins, proteins with very high molecular mass, and proteins with very high or low isoelectric points, that are normally excluded from 2D-PAGE analysis. As shown in Fig. 1, reporting the obtained 1D-PAGE, there are many proteins with high molecular mass, and the employment of 1D-PAGE instead of 2D-PAGE allowed the determination of proteins such as Apolipoprotein B-100, Apolipoprotein(A), Complement C3, and Complement C4-A (see Table 1).



**Fig. 1** One-dimensional 12% polyacrylamide gel electrophoresis obtained by analyzing five samples of plasma proteins desorbed from cationic liposomes

Low molecular weight proteins were also identified (Table 1), although they were not visible in the gel (Fig. 1) due to the system used for staining. Even if the PhastGel Blue R-350 used to stain the gel is more sensitive than the commercially available R-150 and R-250, it can detect most proteins at 50–100 ng/band.

#### Protein identification

In addition, to develop a reproducible analytical method for the separation of complex nanoparticle–proteins, the main goal of this study was to identify plasma proteins adsorbed onto liposome surfaces. In fact, an important task for complete development and full use of liposomes as drug delivery is to understand the nature of “protein corona” that affects their fate in the body.

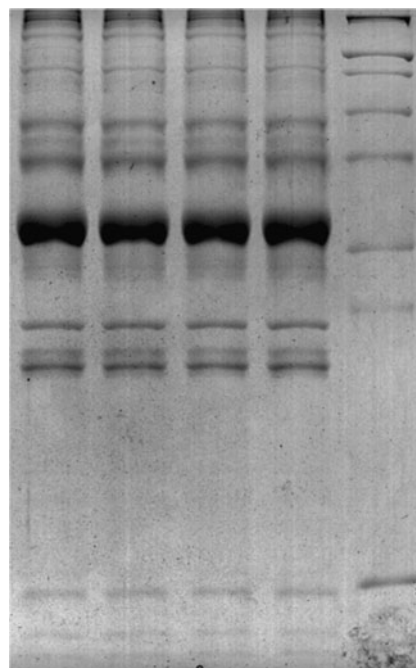
The employment of the HPLC-Chip technology coupled to a high-resolution Q-TOF mass spectrometer permitted the analysis of very small amounts of sample with high efficiency and sensitivity.

In a first attempt, data deriving from the single LC-MS/MS runs relative to the 30 gel fractions were processed separately. However, since some proteins could be spread in more than one band, by combining data with the in-house software mzData Union—Customize, increased protein sequence coverage was obtained. See also [Electronic Supplementary Material](#) for further details.

As reported in Table 1, 58 proteins have been identified by HPLC-Chip/ESI-MS/MS, according to criteria reported in [Electronic Supplementary Material](#). Protein identification probability ranged between 98.9% and 100% for all

proteins but two. Although these two proteins were identified only by one or two unique peptides, respectively, the relative MS/MS spectra allowed their unequivocal identification, as can be seen from [Supplementary Material Figure S1–S3](#).

The identified proteins include HSA, various immunoglobulins (Igs), APOs, fibrinogen, proteins of the complement pathways, and other proteins. Indeed, Aggarwal et al. [31] reported that in literature, though some proteins were specific to certain types of nanoparticles depending on their structure, the proteins that were identified on virtually all nanoparticles were mainly HSA, IgG, fibrinogen, and APOs. In fact, it has been observed that nanoparticles in contact with biological fluids may initially bind the highly abundant plasma proteins, which are then replaced by higher affinity proteins [29]. In the present experiment, the large amount of Igs adsorbed onto liposomes (as can be seen in Fig. 2, reporting a better resolved 10% 1D-PAGE obtained by the analysis of four samples of plasma proteins desorbed from cationic liposomes) is indeed correlated to their abundance in plasma. Conversely, there is a very low amount of HSA adsorbed onto liposomes (Fig. 2) related to its abundance in plasma (35 mgmL<sup>-1</sup>). On the other hand, among the proteins identified as adsorbed onto liposomes, there are also proteins, such as Coagulation factor IX, whose normal concentration in plasma is about 100 times lower than that of other ones (e.g. fibrinogen, vitronectin, APO A–I). This suggests that the link between liposomes and proteins mostly depends on their particular affinity for



**Fig. 2** One-dimensional 10% polyacrylamide gel electrophoresis obtained by analyzing four samples of plasma proteins desorbed from cationic liposomes

liposome surfaces, as demonstrated also by the large amount of different APOs identified.

The Igs adsorbed onto liposomes can act as an opsonin and promote phagocytosis of nanoparticles by macrophages or other phagocytic cells such as hepatic Kupffer cells [32]. In addition, Igs are involved in many processes, and they can activate the complement system, leading to an inflammatory response. In plasma, Igs can interact with the Fc $\alpha$  receptor and promote the release of pro-inflammatory cytokines [33]. APOs have been reported as one of the major plasma proteins adsorbed by particles with hydrophobic surfaces [34]. This group of proteins is involved in transporting lipids and cholesterol in the blood and therefore most likely to affect the intracellular trafficking, fate and transport of nanoparticles in the body. The fibrinogen, a glycoprotein involved in coagulation, is a plasma protein that is commonly found associated with various nanoparticles [7]. It binds to foreign surfaces and promotes attachment of immune cells such as monocytes, macrophages and neutrophils. The liposomes also bind most proteins related to the complement pathways, a vital part of the innate immune response, suggesting that liposomes are active in the complement pathways. Many of the identified proteins, such as actin, alpha actinin, integrins, vinculin, cofilin, filamin, gelsolin, and talin, are involved in cell signaling and junctions. In addition to these proteins, a number of minor proteins were observed bound to the liposomes.

These results show that the protein profile adsorbed onto DC-Chol-DOPE comprises several classes of proteins, many of them having quite distinct biological roles, as already reported by other authors [35].

## Conclusions

The current work is, to our knowledge, the first study to identify the proteins that bind to DC-Chol-DOPE cationic liposomes. In this work, we show that centrifugation, with careful choice of washing and centrifugation time, followed by 1D-PAGE separation and HPLC-Chip ESI-MS/MS characterization, may be applied for proteomic analysis of “protein corona”. Because protein binding onto liposomes can be critical in determining the extent of interaction with cells and tissues *in vivo*, understanding how and why plasma proteins are adsorbed to these particles may be important for elucidating their biological responses. Moreover, since the *in vivo* fate of intravenously administered liposomes is strongly influenced by interactions with blood proteins, enrichment of certain proteins might be able to direct the particles to specific target cells or tissues. The knowledge of the behavior of the proteins absorbed and the subsequent body distribution would be an undeniable

advantage for the development of carriers for site-specific drug delivery. Data from experiments as presented in this study, with 58 proteins identified, could serve as a basis for the establishment of these correlations.

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