Proteomic fingerprinting of *protein corona* formed on PEGylated multi-walled carbon nanotubes

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ABSTRACT

In Nanomedicine, carbon-based nanomaterials, like Carbon Nanotubes (CNT), are considered potential candidates as drug delivery systems. *In vivo* adsorption of physiological proteins onto carbon nanotubes, through noncovalent interactions, forms a *protein corona* or *bio corona*, able to influence biological properties and biocompatibility of CNT. This study aimed to explore the composition of *protein corona* formed onto PEGylated Multi-Walled Carbon Nanotubes (MWCNT-PEG5k), after their incubation in human plasma. Plasma proteins were sequentially eluted in different conditions by using both native and denaturant buffers, useful to characterize *soft* and *hard corona*. Proteomic methods and mass spectrometry analysis have identified proteins in *soft corona*, involved in the regulation of immune response and in the CNT transport, and biomolecules in *hard corona* with a role in the maintenance of host homeostasis. These promising results have demonstrated the potential of PEGylated Multi-Walled Carbon Nanotubes as future candidates for drug delivery.

1. Introduction

Carbon nanotubes (CNT) are a rather new category of nanomaterials that have received great attention in the biomedical field for their high aspect ratio, high surface areas and nanosized stability [1–3]. Their chemical features favor the loading of different molecules along the nanotubes surface. Further, the polyaromatic structure of their surface allows the supramolecular binding of aromatic compounds by π-π stacking interactions [4,5]. It has been demonstrated that biological and bioactive species, such as proteins, can be conjugated with CNT [6–8]. Despite the great potentiality of carbon nanotubes as drug delivery systems, their specific and targeted functionalization is a prerequisite to prepare biocompatible CNT with low cytotoxicity and good drug delivery performance [9–12].

The surface functionalization of CNT, by chemically attach of organic functional groups or organic compounds (polymer), will aid carbon nanotubes to become biocompatible, improving their solubility in physiological solutions and their selective binding to biological targets [10–13].

Polymers, like polyethylene glycol (PEG), are known for their high biocompatibility, their dispersibility in aqueous solution and their low toxicity *in vitro* and *in vivo*, making them some of most efficient surface enhancers for CNT [4,14–17]. The functionalization of CNT (*f*-CNT) with PEG contributes also to increase the circulation time of new drug delivery system in the bloodstream, by reducing the interactions with opsonins and inhibiting the recruitment of macrophages for the elimination of nanotubes [18]. PEG, bound on the surface of MWCNT, enhance π-π interactions with aromatic molecules, present in drug molecules commonly used in chemotherapy such us Doxorubicin (DOX) and Paclitaxel (PTX) [4,16].

PEG–CNT, conjugated with PTX, have shown low toxicity in different cancer cells (HeLa and MCF-7) with insignificant effects on cells proliferation rate, becoming a promising drug delivery system for cancer therapeutics [19].

Considering the huge applications of *f*-CNT in clinical, therapeutic and diagnostic fields, it is necessary to understand the nature and dynamics of interaction between *f*-CNT and proteins present in biological fluids like Human Plasma (HP).

Physiological proteins on the surface of *f*-CNT form a layer called "*protein corona*" or *"bio-corona"*, able to modify the biological identity of the nanomaterials, controlling the biocompatibility of nanostructures. The structure and the composition of *"protein corona"* depend on

Abbreviations: MWCNTs, Multi Walled Carbon Nanotubes; PEG 5 kDa, methoxypolyethylene glycol amine 5000 Da; MWCNTs-PEG5k, MWCNTs surface functionalized with PEG 5 kDa.

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physical–chemical properties of nanomaterial (shape, size, functionalizing groups, and charge), on features of physiological environment (such us blood, cytoplasm, extracellular matrix) and on exposure time [20].

The layer of *"bio-corona"* could be divided into *hard corona*, characterized by proteins that directly interact with CNT surface, and into *soft corona*, composed by proteins that weakly interact with biomolecules of the *hard corona* and biological fluids.

Protein corona is dynamic because its composition normally changes based on affinity for proteins during the different phases of CNT administration (e.g. injection, stay in bloodstream): the different physiological environment (e.g. biological fluids vs. tissues) modifies the compatibility of CNT surface for biomolecules, causing a gradual substitution and modification of *bio corona,* as stated by "Vroman Effect" [21].

The interactions between proteins and nanomaterials surface may change the secondary and tertiary structures of biomolecules and such conformational changes may consequently play an important role in the activation of immune response. For this reason, the investigation of both *hard* and *soft corona* is required in order to elucidate the biocompatibility of carbon nanotubes. In addition, the composition of *protein corona* depends on the types of interactions (e.g. π-π stacking, hydrophobic interactions), that occur between proteins and chemical groups used to functionalized CNT surface. Therefore, the functionalization is strictly related with both CNT stability and biocompatibility, required in future drug delivery systems.

Until now, one promising polymer, used to functionalize CNT surface, is the polyethylene glycol (PEG) because it is able to control the interactions with physiological proteins, favoring binding with selected biomolecules [22,23]. The possibility to control the composition of protein corona may be useful to target drugs up to specific sites of pathologies [24,25].

The aim of our research is to characterize the *bio-corona* formed after incubation in human plasma of sample of multi-walled carbon nanotubes (MWCNT) functionalized with methoxypolyethylene glycol amine 5000 Da (PEG 5 kDa). The choice of PEG-functionalized nanotubes was due to results reported in recent literature that confirm such polymer as a promising functionalization for several dugs delivery system, despite few results were reported about the specific PEGylation of multi-walled carbon nanotubes [22,23]. In addition in our previous research, simple chemical modifications of MWCNTs surface, like carboxylation or introduction of quaternary ammonium groups, did not play a crucial role in the specificity of protein corona, despite the protein layer on functionalized carbon nanotubes was formed by biomolecules, able to promote the biocompatibility and the bio-distribution of nanoparticles [26]. In the presented work, we have tried to distinguish *soft* from *hard corona* by sequential elutions with specific buffers and to investigate proteins molecular functions in order to elucidate the CNT affinity for human plasma proteins. The biocompatibility of PEGylated nanotubes (MWCNTs-PEG5k) was investigated by a proteomic approach based on monodimensional electrophoresis (SDS-PAGE) for protein separation and on mass spectrometry analysis (nLC-MS/MS) for protein identification.

2. Material and methods

2.1. Multi-walled Carbon Nanotubes (MWCNT) preparation

The Multi-walled Carbon Nanotubes (MWCNT) were prepared by the typical Chemical Vapour Deposition (CVD) protocol using ethylene, as carbon source, and they were characterized by TEM microscopy, Thermo Gravimetric Analysis (TGA), Inductively Coupled Plasma (ICP) analysis and X-ray photoelectron spectroscopy (XPS), as reported in hour previous work [26,27].

The MWCNT synthetized were sonicated in an ultrasonic bath (260 W, 2 L) for 2 h in order to obtain an average lengths of 1–2 μm.

2.2. MWCNT carboxylation

The MWCNT were oxidized in order to obtain carboxylic acid functions (corresponding MWCNT-COOH) on their surface using the "Piranha" solution. Particularly, 120 mL of H_2SO_4 97% were slowly added to 30 mL of 30% H_2O_2 by cooling in an ice bath. 1 g of pristine MWCNT was dispersed in the solution thus obtained. The resulting suspension was sonicated in an ultrasound bath (260 W, 2 L) for 2 min, heated and stirred for 7 h at 70 °C. The suspension was filtered, washed with hot water (until neutral pH value of the washing water) and dried at 90 °C overnight. Chemical-physical properties of pristine carbon nanotubes were reported in Supplementary Table 1A.

2.3. MWCNT-COOH functionalization with methoxypolyethylene glycol amine 5000 (PEG 5 kDa)

40 mg of MWCNT-COOH was dispersed in 10 mL of anhydrous THF, then 100 mg of PEG 5 kDa, 40 mg of DCC and 12 mg of DMAP were added (Supplementary Table 1B). The obtained suspension was sonicated in an ultrasound bath (260 W, 2 L) for 2 min and then left under vigorous stirring at room temperature for 6 days. The nanotubes were filtered and then were washed with 10 mL of hot DMF and 10 mL with hot methanol both for 5 times. Finally, the MWCNT-PEG5k were dried at 90 °C overnight (Supplementary Fig. 1).

To prepare the suspension of MWCNT-PEG5k, 10 mg of nanotubes were dispersed in 2 mL of a solution of H_2O/ethanol 8:2 and the resulting mixtures were sonicated 5 min in order to obtain homogeneous dispersions. Then, the aliquots were withdrawn and treated as follow.

2.4. MWCNT-PEG5k incubation in human plasma sample

60 μl of MWCNT-PEG5k suspension were incubated with 1940 μl of human plasma solution (provided by the San Raffaele Hospital, Milan) for 4 h under stirring at controlled temperature of 37 °C (Fig. 1A). After incubation, Pirce ® Centrifuge Column 0.8 mL (Thermo Scientific) were used to separate MWCNTs-PEG5k from plasma proteins by vacuum filtration. The proteins were eluted with 80 μl of eluent solution at room temperature, collecting proteins by centrifugation (4000 rpm × 1 min) using the same columns.

For this experiment, 5 different eluent solutions were used: 30 mM Tris-HCl pH 7.4; 1 M of NaCl; TUC (2 M Urea, 7 M Thiourea, 3% CHAPS); 4% SDS (w/v) and 20 mM DTT (w/v); Hydro Organic solution (6% acetonitrile, 12% *iso*-propanol, 2% NH_3, 80% H_2O). All five eluents were sequentially used on the same centrifuge column, following the order shown above.

2.5. SDS-PAGE analysis

For one-dimensional (SDS-PAGE) electrophoresis analyses, 0.25 μl of untreated human plasma and different volume of eluates were firstly mixed with Laemmli buffer without β-mercaptoethanol at room temperature and secondly loaded onto SDS-PAGE gel. In detail, the aliquots of eluates were 6 μl for Tris-HCl, 6 μl for NaCl, 3 μl for TUC, 3 μl for SDS-DTT and 6 μl for Hydro Organic. Each sample was run in triplicate by performing three biological SDS-PAGE replicates.

The SDS-PAGE gel was composed by a 4% polyacrylamide stacking gel (125 mM Tris–HCl, pH 6.8, 0.1%, m/v, SDS) and a 12% resolving polyacrylamide gel (in 375 mM Tris–HCl, pH 8.8, 0.1%, m/v, SDS buffer). A Tris-glycine buffer at pH 8.3 (with 0.1% SDS, m/v) was employed to fill the cathode, whereas a Tris buffer at pH 8.8 was used in the anode. Electrophoresis was performed with three steps with increasing voltage: first step was set at 50 V for 20 min, second step at 100 V for 40 min and third step at 150 V until the dye front reached the bottom of the gel.

Staining and distaining of triplicate SDS-PAGE gels were performed with Colloidal Coomassie Blue and 7% (v/v) acetic acid in water,

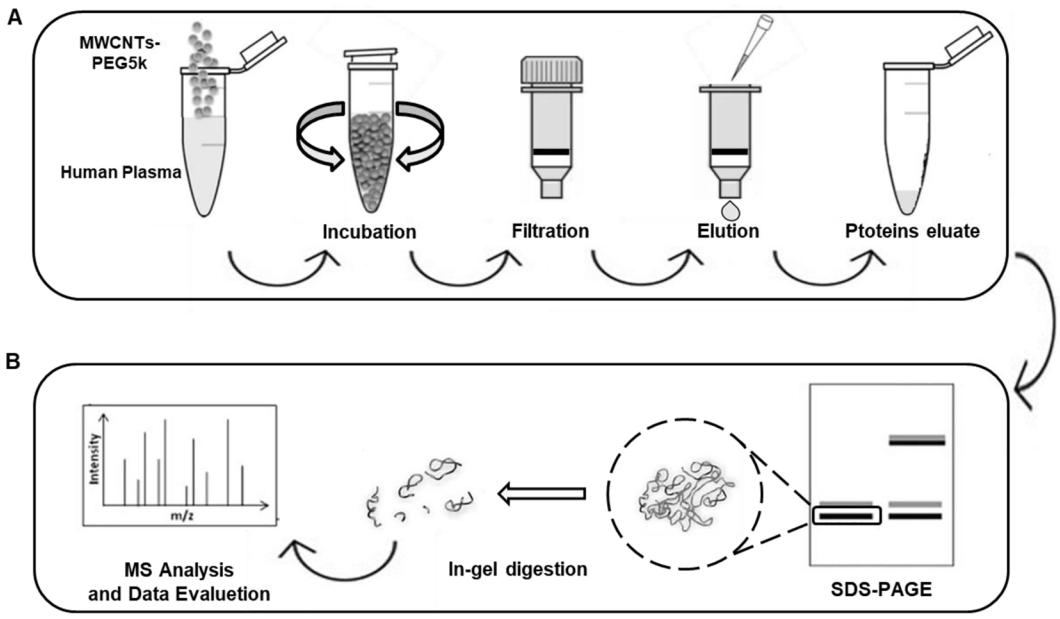

Fig. 1. Experimental workflow. (A) Schematic steps of the incubation protocol. (B) Eluates treatment starting from SDS-PAGE to MS analysis.

respectively [28]. Finally, the gel was scanned with a VersaDoc imaging system (Bio-Rad) (Fig. 2).

2.6. Mass spectrometry and data analysis

After acquisition of gels images, all protein bands (from three biological SDS-PAGE replicates) were firstly selected and finally excided to perform mass spectrometry analysis (Fig. 1B and Supplementary Fig. 2).

Gel's bands were subjected to washing steps with 50 mM Ammonium Bicarbonate (AmBic) and Acetonitrile (ACN), at 56 °C under stirring, in order to remove all colloidal Blue Coomassie.

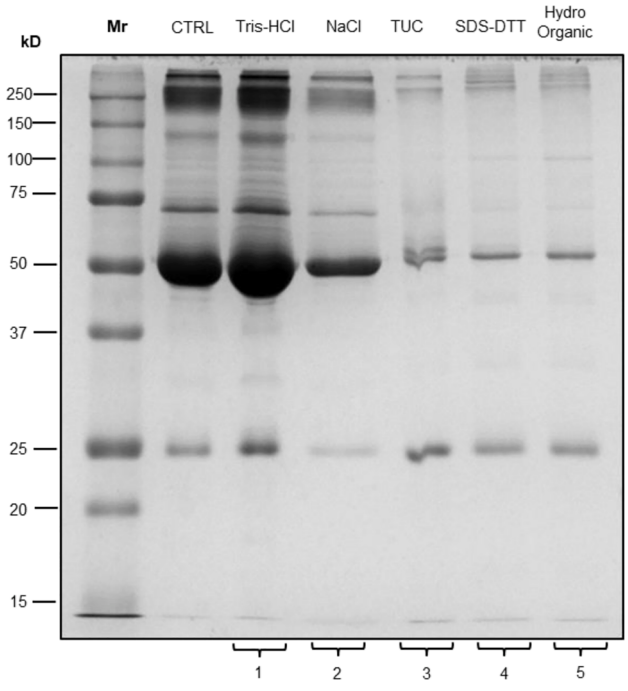

Fig. 2. SDS-PAGE containing protein profiles of different eluates: Tris-HCl (6 μl), NaCl (6 μl), TUC (3 μl), SDS-DTT (3 μl) and Hydro Organic (6 μl). All eluates were collected after incubation of 60 μl MWCNT-PEG5k with 1.94 mL human plasma at 37 °C. CTRL: 0.25 μl of human plasma (untreated sample).

Afterwards, the gel slices were reduced and alkylated with 1.5 mg/ml DTT (in 50 mM AmBic) at 56 °C and 10 mg/ml iodoacetamide (in 50 mM AmBic) at room temperature, respectively. Finally, proteins were digested with 0.02 μg/μl trypsin (in 25 mM AmBic) at 37 °C overnight. The tryptic mixtures were acidified with formic acid (FA) up to a final concentration of 10% [28].

The peptide mixtures need to be cleaned and concentrated, for these reasons it was necessary to employ an extraction with Stage Tips containing reverse phase C_{18} ("GELoader" pipette tip C_{18} material, Thermo Scientific), using the known protocol present in literature [29].

8 μl of tryptic digested samples were injected on a reversed-phase trap column (Acclaim PepMap100, C18, 100 Å, 5 μm, 100 μm ID × 2 cm length, Thermo Scientific) for peptide clean-up and preconcentration. After clean-up the trap column was placed in series with a fused silica reverse-phase column (picoFrit column, C18 HALO, 90 Å, 75 μm ID, 2.7 μm, 10.5 cm length, New Objective). A nanochromatography system (UltiMate 3000 RSLCnano System, Thermo Scientific) delivered a constant flow rate of 300 nl/min. The separating gradient ramped linearly from 4% buffer A (2% ACN and 0.1% FA in water) to 96% buffer B (2% water and 0.1% FA in ACN) in 60 min. The eluting peptides were on-line sprayed in a LTQ XL mass spectrometer (Thermo Scientific). Full mass spectra were acquired in the linear ion trap in the mass range *m/z* 350 to *m/z* 1800 Da. The 5 most intense ions were automatically selected and fragmented in the ion trap. Target ions already selected for fragmentation were dynamically excluded for 30 s.

The MS data were analyzed by the Mascot search engine (Version 2.3.01), using the Proteome Discoverer software (v. 1.2.0 Thermo) and consulting specific UniProtKB/Swiss-Prot protein database (www.uniprot.org). In detail, taxonomy *Homo sapiens* (1188582 sequences, 344,584,622 residues) was selected for human plasma. A preliminary subtraction of common contaminants was performed using definite *Contaminants* database (262 sequence, 133,770 residues). Carbamidomethylation of cysteine residues was set as fixed modification, while oxidation of methionine residues as variable modifications; one missed cleavages were allowed to trypsin; peptide mass tolerance was set to 1 Da, fragment mass tolerance to 0.8 Da, ion source cut-off of 20 and significance threshold of $p < 0.01$ (Supplementary Table 2).

For data validation, all tryptic digestions of excised bands, from all triplicates of each eluate, were analysed and the complete lists of identifications were reported in Supplementary Table 3. For each type of eluate, all MS identifications were compared and only the proteins,

present and recognized in all triplicates, were considered as reliable. Supplementary Table 4 has reported the lists of validated proteins, identified in all five eluates. Finally, validated proteins were classified by molecular function using Gene Ontology (GO) analysis (https://www.ebi.ac.uk/QuickGO).

3. Results

3.1. MWCNT-PEG5k preparation

The MWCNT, synthetized by CVD protocol, were characterized by similar dimensions, purity and presence of catalyst (Fe and Al trapped inside the nanotubes) as those reported in our previous work and in the literature [27,30,31].

The Thermo Gravimetric Analysis (TGA) of the oxidized nanotubes (Supplementary Fig. 3A) showed a loss of mass of about 4.4% w/w between 200 and 450 °C, related with the loss of the carboxylic groups (−COOH) on the CNTs surface. This value correspond to about one −COOH every 120 C atoms. This data was in agreement with XPS analysis. After the functionalization with PEG 5 kDa (MWCNT-PEG5k), the TGA curve (Supplementary Fig. 3B) showed a loss of mass of about 25.8% between 300 and 350 °C. This latter is related to the thermal release of the PEG substituent initially covalently bond to the nanotube surface. Considering the mass of the all PEG substituent, it results that about 10% of the carboxylic groups have been functionalized.

3.2. Incubation of MWCNs-PEG5k with human plasma

Fig. 2 depicted the SDS-PAGE profiles of control (untreated human plasma) and of five eluates collected after each sequential elution. Despite the similarity between control and first elution, the number and the intensity of bands of other eluates were quite different, probably due to a reduction of protein content.

nLC-MS/MS analysis (Supplementary Table 3) and data validation (Supplementary Table 4) were performed to compare all identifications. Fig. 3A reported numbers of validated proteins, identified for each eluent. The data validation, derived by three technical replicates, was necessary in order to increase the reliability of analysis [32].

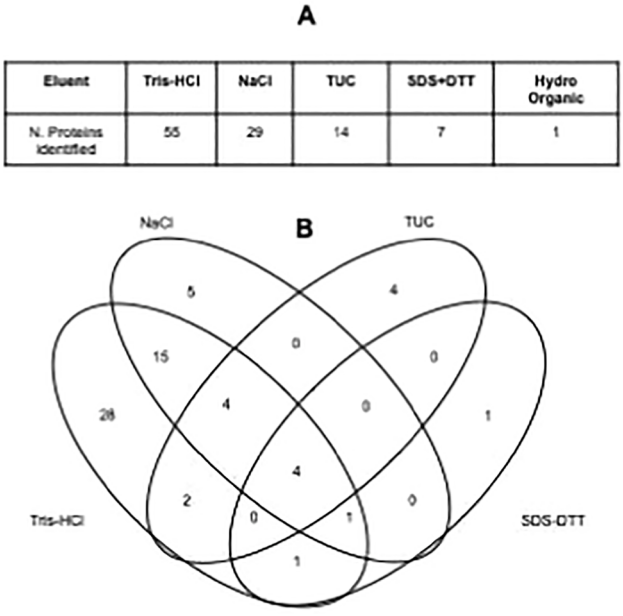

Fig. 3. Number of proteins identified after MS/MS analysis. (A) Identifications of validated human plasma proteins in each eluates. (B) Venn diagram compared proteins eluted by five solutions (Tris-HCl, NaCl, TUC, SDS-DTT and Hydro Organic).

The Venn diagram in Fig. 3B compared proteins, identified for all eluents: 43% was specifically eluted by Tris-HCl, ~8% by NaCl, 6% by TUC and ~2% by SDS-DTT.

The Venn diagram did not report proteins eluted by hydro organic solution because the only one identification (Accession Number: Q6N030) was present also in SDS-DTT eluate.

Gene Ontology (GO) analysis was carried out on molecular functions in order to investigate a possible correlation between the type of eluent and the *protein corona* in terms of biological activities. As reported in Table 1, the molecular functions of proteins, exclusively identified in TUC, were quite different from those found in Tris-HCL, NaCl, SDS-DTT. In detail, in TUC only one molecular function, *GO:0008201 Heparin binding*, was in common with others, while there were many common GO IDs belonging to Tris-HCl, NaCl and SDS-DTT and two belonging to SDS-DTT and to NaCl like *GO:0003823 Antigen binding* and *GO:0034987 Immunoglobulin receptor binding* (Table 2).

All used eluents can be divided into two distinct categories: native solutions (Tris-HCl and NaCl) and denaturant ones (SDS-DTT, TUC and Hydro Organic), considering in the last case the possibility to misfolded proteins, enhancing their detach from *bio-corona*. Moreover, native and denaturant buffers could be useful both to separately characterize components of *soft* and *hard corona,* respectively, and to investigate the possible role of molecular functions in the interaction with nanotubes. Venn diagram in Fig. 4A compared 60 proteins of native eluents with 17 of denaturant ones: 48 proteins were specifically eluted in native condition, while 5 in denaturant condition.

Furthermore, the corresponding GO analysis has revealed different molecular functions, referred to specific identifications and reported in

Table 1
Gene Ontology analysis on molecular functions concerning the unique proteins eluted by each specific buffer.

GO IDs	*Name*
Tris-HCl	
GO:0005515	Protein binding
GO:0019899	Enzyme binding
GO:0004252	Serine-type endopeptidase activity
GO:0051087	Chaperone binding
GO:0004867	Serine-type endopeptidase inhibitor activity
GO:0005509	Calcium ion binding
GO:0051787	Misfolded protein binding
GO:0008233	Peptidase activity
GO:0008236	Serine-type peptidase activity
GO:0016787	Hydrolase activity
GO:0001540	Amyloid-beta binding
GO:0008201	Heparin binding
GO:0008289	Lipid binding
GO:0031210	Phosphatidylcholine binding
NaCl	
GO:0005201	Extracellular matrix structural constituent
GO:0005515	Protein binding
GO:0003823	Antigen binding
GO:0005102	Signaling receptor binding
GO:0005198	Structural molecule activity
GO:0004857	Enzyme inhibitor activity
GO:0004867	Serine-type endopeptidase inhibitor activity
GO:0005179	Hormone activity
GO:0034987	Immunoglobulin receptor binding
GO:0046872	Metal ion binding
GO:0050839	Cell adhesion molecule binding
TUC	
GO:0005044	Scavenger receptor activity
GO:0005518	Collagen binding
GO:0008201	Heparin binding
GO:0030247	Polysaccharide binding
GO:0042802	Identical protein binding
GO:0050840	Extracellular matrix binding
SDS-DTT	
GO:0003823	Antigen binding
GO:0034987	Immunoglobulin receptor binding

Table 2
Comparison between the molecular functions of specific proteins identified in different eluents.

GO IDs	*Name*	*Tris-HCl*	*NaCl*	*TUC*	*SDS-DTT*
GO:0005515	Protein binding	X	X		
GO:0019899	Enzyme binding	X			
GO:0004252	Serine-type endopeptidase activity	X			
GO:0051087	Chaperone binding	X			
GO:0004867	Serine-type endopeptidase inhibitor activity	X	X		
GO:0005509	Calcium ion binding	X			
GO:0051787	Misfolded protein binding	X			
GO:0008233	Peptidase activity	X			
GO:0008236	Serine-type peptidase activity	X			
GO:0016787	Hydrolase activity	X			
GO:0001540	Amyloid-beta binding	X			
GO:0008201	Heparin binding	X		X	
GO:0008289	Lipid binding	X			
GO:0031210	Phosphatidylcholine binding	X			
GO:0005201	Extracellular matrix structural constituent		X		
GO:0003823	Antigen binding		X		X
GO:0005102	Signaling receptor binding		X		
GO:0005198	Structural molecule activity		X		
GO:0004857	Enzyme inhibitor activity		X		
GO:0005179	Hormone activity		X		
GO:0034987	Immunoglobulin receptor binding		X		X
GO:0046872	Metal ion binding		X		
GO:0050839	Cell adhesion molecule binding		X		
GO:0005044	Scavenger receptor activity			X	
GO:0005518	Collagen binding			X	
GO:0030247	Polysaccharide binding			X	
GO:0042802	Identical protein binding			X	
GO:0050840	Extracellular matrix binding			X	

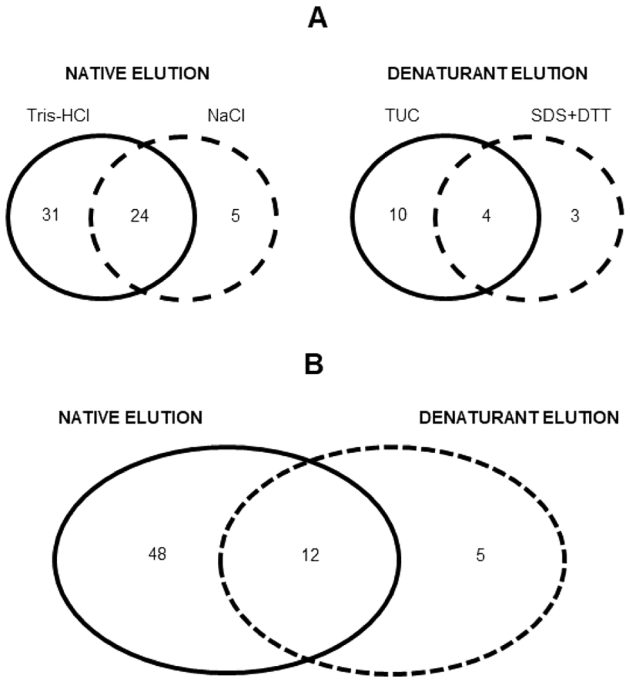

Fig. 4. Comparison native elution vs denaturant elution. (A) Venn diagrams highlighted all proteins eluted in native (Tris-HCl, NaCl) and denaturant (TUC, SDS-DTT and Hydro Organic) conditions, respectively. (B) Venn diagram compared proteins released in native and denaturant conditions.

Table 3
Gene Ontology on molecular functions of proteins recovered in native and denaturant conditions.

Go IDs	*Name*
Proteins eluted by native buffer	
GO:0005515	Protein binding
GO:0019899	Enzyme binding
GO:0004252	Serine-type endopeptidase activity
GO:0003823	Antigen binding
GO:0005201	Extracellular matrix structural constituent
GO:0004867	Serine-type endopeptidase inhibitor activity
GO:0008233	Peptidase activity
GO:0051087	Chaperone binding
GO:0005102	Signaling receptor binding
GO:0005509	Calcium ion binding
GO:0008236	Serine-type peptidase activity
GO:0016787	Hydrolase activity
GO:0008289	Lipid binding
Proteins eluted by denaturant buffer	
GO:0005044	Scavenger receptor activity
GO:0005518	Collagen binding
GO:0008201	Heparin binding
GO:0030247	Polysaccharide binding
GO:0042802	Identical protein binding
GO:0050840	Extracellular matrix binding
Proteins recovered by both eluents	
GO:0048306	Calcium-dependent protein binding
GO:0004867	Serine-type endopeptidase inhibitor activity
GO:0005515	Protein binding
GO:0008289	Lipid binding
GO:0017127	Cholesterol transporter activity
GO:0002020	Protease binding
GO:0004866	Endopeptidase inhibitor activity
GO:0005102	Signaling receptor binding
GO:0005319	Lipid transporter activity
GO:0030414	Peptidase inhibitor activity
GO:0071813	Lipoprotein particle binding

Fig. 4B (Table 3).

4. Discussion

For this research project PEGylated multi-walled carbon nanotubes (MWCNT-PEG5k) were chosen for their possible biocompatibility with biological fluids and for their potential to become drug delivery systems, as reported in literature [33,34]. In particular, biocompatibility seemed to be related with the composition and the size of *protein corona,* formed on CNT surface after injection *in vivo*.

The idea to perform a sequential elution of *protein corona*, using both native (Tris-HCl and NaCl) and denaturant solutions (SDS-DTT, TUC and Hydro Organic), aimed to characterize proteins belonging to *soft* and *hard corona,* respectively. In fact while Tris-HCl and NaCl were able to break protein–protein interactions present in *soft corona,* the denaturant reagents, like SDS and Urea present in denaturant buffers, were involved in the breaching of π-π bonds and other chemical bonds performed between physiological proteins and CNT surface or PEG functional branch. In SDS-PAGE, the first elution, performed by Tris-HCl solution, showed a protein profile similar in bands number to the control (human plasma untreated sample) but different in bands intensity, due to capacity of eluent to break most of weak interactions protein–protein in *soft corona*. Furthermore, the concentration of proteins in the first sample has demonstrated the capacity of MWCNT-PEG5k to interact with human plasma proteins [29]. The other native eluent (NaCl solution) was characterized by lightener bands than Tris-HCl one, confirming that majority of proteins belonging to *soft corona* were previously detached. The protein profiles of three denaturant buffers (TUC, SDS-DTT and Hydro Organic) were characterized by a lower number and less intense bands than Tris-HCl. Despite this, specific protein profiles proved that protein denaturation may contribute to disrupt interactions

between proteins and CNT surface, typical of *hard corona*: such data led to speculate the possible presence of a thinner layer of *hard corona* than that of s*oft corona.*

This hypothesis seemed to be confirmed by results of mass spectrometry analysis: 43% of proteins were identified in Tris-HCl, ~8% in NaCl, 6% in TUC and ~2% in SDS-DTT. Salts in the first two eluents (Tris-HCl and NaCl) were probably able to overcome Van der Walls and electrostatic forces, occurred between proteins *soft corona* and they are able to elute a quite high number of plasma proteins. Instead, denaturant reagents, in TUC and SDS-DTT, may break π-π interactions, formed between proteins and carbon nanotubes surface, releasing proteins belonging to inner layer of *bio corona*, also called *hard corona*.

A deeper characterization of *protein corona* was performed by GO analysis, focused on biological functions of biomolecules (Table 1). In Tris-HCl eluate, the major molecular functions belonged to the *protein binding* category (such as Enzyme binding, Chaperone binding, Calcium ion binding, Misfolded protein binding, Amyloid-beta binding, Heparin binding, Lipid binding, Phosphatidylcholine binding) and to *catalytic activity* (e.g. Serine-type endopeptidase activity, Peptidase activity and Hydrolase activity). In NaCl eluent, proteins were characterized by similar molecular functions, like *protein binding activity*, and by specific GO categories like *regulatory* and *immunological activity* (e.g. Antigen binding, Signaling receptor binding and Immunoglobulin receptor binding). GO analysis has revealed both similar and unique proteins eluted by different solutions: these data may confirm the abundance of *soft corona* and the eluent's specificity, able to detach different biomolecules.

Such functional peculiarity was emphasized in TUC eluate, formed by proteins involved in cell adhesion, in the diffusion of specific organic compounds in blood and tissues and scavenger processes. Whereas in SDS-DTT eluent, the unique identified protein was involved in the processes of the immune system, defined as *Antigen binding* and *Immunoglobulin receptor binding* previously detected also in NaCl eluent.

The investigation of biological activities aimed to find a sort of correlation between the composition of *bio corona* and the affinity for CNT. In literature, it was demonstrated that composition of *protein corona* depends by physiological environments and by chemical functionalization of CNT surface and that properties of *protein corona* influence the biocompatibility of nanostructures used as new potential drug delivery systems [3–5,20–22]. In this context, the composition of *protein corona*, divided into *soft* and *hard bio corona*, was investigated exploring the biological functions of proteins through a Gene Ontology analysis. The majority (74%) of bounded proteins were detached by native elution, while only ~8% has required stronger conditions to be released. Such result may reflect the possibility to form a thicker layer of *soft corona* than the *hard* one.

In *soft corona* ~20% of 48 unique proteins were immunoglobulins, categorized as *GO:0005515 protein binding* and *GO:0003823 antigen binding*. Their finding was quite expected, considering the high amount of immunoglobulins in human plasma, but it could be also related to CNTs the biocompatibility considering their possibility to trig the immune response. Besides, the majority of proteins eluted in native conditions belonged to a class of proteins, called Acute-Phase Proteins (APP), whose plasma concentrations increased (positive acute-phase proteins) or decreased (negative acute-phase proteins) during the inflammation [35]. Examples of APP, identified in native eluates, were Apolipoproteins, Fibrinogen, Ceruloplasmin, Prothrombin, Alpha 1-antichymotrypsin, Plasminogen, coagulation factors, Antithrombin and Transferrin. The presence of APPs on the *soft corona* could balance the presence of immunoglobulins, avoiding the recognition of MWCNT-PEG5k as external agent. In particular, apolipoproteins, identified also in denaturant eluates, have a fundamental role in the interaction with hydrophobic lipids, adsorbed in intestine or present in the bloodstream: they are structural components, together with phospholipids, of lipoprotein particles, involved in lipids transport. Apolipoproteins may act as ligands for receptors and as co-factors for enzymes, controlling the movement in bloodstream and the delivery of target [36]. Furthermore, in literature it was demonstrated that the most abundant apolipoproteins, like apoA-IV, apoB-100, apoE and apoA-I also identified in our samples, could favor the translocation of nanoparticles through the blood brain barrier (BBB) [22,37]. For this reason, it was hypothesized that nanoparticles with adsorbed or covalently attached apolipoproteins may mimic lipoprotein particles, masking CNT and promoting their recognition as safe nano-system.

The five proteins, identified only in denaturant eluates, were involved in cell adhesion (*GO:0050840 Extracellular matrix binding*), in the binding of extracellular matrix proteins such as collagen and heparin (*GO:0005518* and *GO:0008201*) and in the interaction with specific receptors (*GO:0005044*). Considering this last GO, a recent study has declared that proteins with Scavenger receptor activity may have a crucial role in maintenance of host homeostasis, in regulation of tumor behavior and host immune responses to cancer [38]. In detail, Vitronectin, a protein belonged to this GO category and identified in our denaturant samples, may act as inhibitor of the membrane-damaging effect during the cytolytic complement pathway [39,40].

Our research was inspired by an increasing interest, recently expressed in literature, on the biological potential of coronation of nanomaterials, but in the same time, it has represented a starting point for a deep preliminary characterization of *bio-corona*, formed during incubation of PEGylated MWCNTs in human plasma [41,42]. The use of specific buffers (native vs. denaturant) gave the possibility to separately investigate the composition of both *soft* and *hard corona*, highlighting their role in the nano-structure biocompatibility.

In *soft bio-corona* the presence of immunoglobulins may suggest a possible control of immune response in the recognition of PEGylated nanotubes as safe nano-systems, potentially used in drug delivery. This hypothesis could be proved by the identification of proteins, such as apolipoproteins, able to mask carbon nanotubes and to probably promote their permanence and their transport *in vivo*. Also the composition of *hard corona* has suggested a role in the maintenance of host homeostasis.

Despite our findings were preliminary data because they require an experimental validation, they have suggested that in future PEGylated carbon nanotubes could be considered as potential candidates as drug delivery systems in nanomedicine.

5. Conclusion

The data of our research have demonstrated that MWCNTs-PEG5k, incubated with human plasma, formed a *protein corona*, distinguishable in *soft* and *hard* layer. The *soft corona*, the most dynamic layer, was characterized by a balanced presence of both immunoglobulins and apolipoproteins, suggesting the possible difficult recognition of MWCNT-PEG5k as external agent. Many proteins, belonging to APPs and Scavenger receptors, were specifically identified in *hard corona*, confirming the biocompatibility of PEGylated nanostructures and their possibility to maintain host's homeostasis.

CRediT authorship contribution statement

Maria Nicoletti: Methodology, Investigation, Validation, Writing - original draft. **Cristian Gambarotti:** Resources, Investigation, Writing - review & editing. **Elisa Fasoli:** Conceptualization, Methodology, Validation, Writing - review & editing.

Declaration of Competing Interest

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2020.122504.

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